Leak current, even with gigaohm seals, can cause misinterpretation of stem cell-derived cardiomyocyte action potential recordings

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Running title: Seal leak misleads iPSC-CM AP interpretation

Key points

- Human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) are an essential tool in the study of cardiac arrhythmia mechanisms.
- Their immature and heterogeneous action potential phenotype complicates the interpretation of experimental data, and has slowed their acceptance in industry and academia.
- We suggest that a leak current caused by an imperfect pipette-membrane seal during single-cell patch-clamp experiments is partly responsible for inducing this phenotype.
- Using in vitro experiments and computational modelling, we show that this seal-leak current affects iPSC-CM AP morphology, even under ‘ideal’ experimental conditions.
- Based on these findings, we make recommendations that should be considered when interpreting, analysing and fitting iPSC-CM data.

Abstract

Human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) have become an essential tool to study arrhythmia mechanisms. Much of the foundational work on these cells, and the computational models built from the resultant data, has overlooked the contribution of seal-leak current on the immature and heterogeneous phenotype that has come to define these cells. Here, we use in silico and in vitro studies to demonstrate how seal-leak current depolarises action potentials (APs), substantially affecting their morphology, even with seal resistances (Rseal) above 1 GΩ. We show that compensation of this leak current is difficult due to challenges with recording accurate measures of Rseal during an experiment. Using simulation, we show that Rseal measures: 1) change during an experiment, invalidating the use of pre-rupture values, and 2) are polluted by the presence of transmembrane currents at every voltage. Finally, we posit the background sodium current in baseline iPSC-CM models imitates the effects of seal-leak current and is increased to a level that masks the effects of seal-leak current on iPSC-CMs. Based on these findings, we make three recommendations to improve iPSC-CM AP data acquisition, interpretation, and model-building. Taking these recommendations into account will improve our understanding of iPSC-CM physiology and the descriptive ability of models built from such data.
1 Introduction

Human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) are a renewable and cost-effective model for studying cardiac arrhythmia mechanisms in human cells. Patient-specific cells can be used to investigate genetic disease mechanisms (Han et al., 2014), drug cardiotoxicity (Mathur et al., 2015), and inter-patient variability (Blinova et al., 2019). Computational approaches have even been developed to translate experimental results from iPSC-CMs to make predictions in adult cardiomyocytes (Jæger et al., 2021).

Progress in many of these areas, however, has been slowed by the immature phenotype and cell-to-cell heterogeneity of iPSC-CMs (Jonsson et al., 2012; Goversen et al., 2018a). Investigating the source of this variability and its biological implications is important as we come to use iPSC-CMs (and mechanistic models describing their behaviour) for drug safety assessment (Mirams et al., 2016). Recently, Horváth et al. (2018) showed that these limitations can be attributed, at least in part, to the presence of leak current ($I_{\text{leak}}$) during patch-clamp experiments. $I_{\text{leak}}$ is an experimental artefact caused by an imperfect seal between the electrode pipette tip and cell membrane (Figure 1). Compared to adult cardiomyocytes, typical iPSC-CMs are smaller (leading to a lower membrane capacitance $C_m < 100\text{pF}$) and have fewer ion channels. Combined, this makes membrane potential recordings in iPSC-CMs particularly susceptible to imperfect seals. We believe the effects of $I_{\text{leak}}$ on the interpretation of iPSC-CM action potential (AP) data has been overlooked by the field, including ourselves (Lei et al., 2017; Clark et al., 2022). Such data have been used in numerous studies to investigate cell-line specific characteristics, and have formed the basis for widely-used iPSC-CM computational models (Paci et al., 2013; Koivumäki et al., 2018; Kernik et al., 2019).

In this study, through in vitro experiments and computational modelling we show that $I_{\text{leak}}$ affects iPSC-CM AP morphology, even under ‘ideal’ experimental conditions. We show that seal resistance ($R_{\text{seal}}$) cannot be easily compensated because it cannot be accurately measured during an experiment. Additionally, we posit that the background sodium current ($I_{\text{bNa}}$) in iPSC-CM models may be overestimated and mimic the effects of leak on AP morphology. Ultimately, we argue that leak current should be considered when interpreting, analysing, and fitting iPSC-CM AP data.

Figure 1: Presence of $I_{\text{leak}}$ has undesirable effects on AP morphology and muddles data interpretation. Leak current is an undesirable artefact in patch-clamp experiments. It flows through the seal formed between the pipette tip and cell membrane, and has a magnitude that is inversely proportional to the size of the seal resistance. This artefact affects AP morphology, with greater deviations from baseline (indicated by the arrow) as membrane resistance ($R_m$) increases and/or $R_{\text{seal}}$ decreases.

2 Results

2.1 Leak affects AP morphology even at seal resistances above 1 GΩ

To investigate the effects of leak current on AP morphology, we added a leak equation to the Kernik (Kernik et al., 2019) and Paci (Paci et al., 2013) models. Knowing that leak acts as a depolarising current in iPSC-CM studies, and lacking information about specific charge carriers, we modelled $I_{\text{leak}}$ as having a reversal potential...
of zero (Ahrens-Nicklas and Christini, 2009; Fabbri et al., 2020):

\[ I_{\text{leak}} = \frac{1}{R_{\text{seal}}} V = g_{\text{seal}} V, \]  

where \( R_{\text{seal}} \) is the seal resistance and \( V \) denotes the membrane potential. The inverse of \( R_{\text{seal}} \) is a conductance, \( g_{\text{seal}} \), and will be used throughout this study. Note that more complicated equations for leak current (non-linear, and/or with a non-zero reversal potential) may be required in experiments where \( \text{CaF}_2 \) seal enhancer is used (Lei et al., 2021).

The effect of \( I_{\text{leak}} \) on the evolution of \( V \) was modelled as:

\[ \frac{dV}{dt} = -\frac{1}{C_m} (I_{\text{ion}} + I_{\text{leak}}), \]  

where \( I_{\text{ion}} \) represents the sum of transmembrane currents and \( C_m \) is the membrane capacitance.

We used these models to simulate AP recordings with \( g_{\text{seal}} \) set to values between 0.1 nS and 1 nS (i.e., \( R_{\text{seal}} \) between 10 GΩ and 1 GΩ). The results show that \( I_{\text{leak}} \) substantially alters AP morphology, even when \( g_{\text{seal}} < 1 \text{nS} \), equivalent to \( R_{\text{seal}} \geq 1 \text{GΩ} \) (Figure 2). In simulations with either model, an increase in \( g_{\text{seal}} \) causes depolarisation of the minimum potential (MP), as \( I_{\text{leak}} \) is inward at negative potentials. Increased leak also causes a substantial reduction in the maximum upstroke velocity, \( dV/dt_{\text{max}} \), likely due to an incomplete recovery of sodium channels at these depolarised MPs. Interestingly, \( I_{\text{leak}} \) effects on the action potential duration at 90% repolarisation (APD₉₀) differ for the two models — increased \( g_{\text{seal}} \) causes AP shortening in the Kernik model and AP prolongation in the Paci model. These differences are likely due to differences in the relative size of \( I_{\text{leak}} \) compared to the other repolarising currents during phases one and two of the AP. There are also differences in the effect of \( g_{\text{seal}} \) on cycle length (CL): In the Kernik model, increases in \( g_{\text{seal}} \) lead to a decrease in CL. The Paci model shows more complex dynamics — increases in \( g_{\text{seal}} \) initially lead to prolongation, but then shortening as \( g_{\text{seal}} \) approaches 1 nS.
Figure 2: Effect of seal on Kernik and Paci APs. Simulations from the Kernik+leak (A) and Paci+leak (B) models, each with capacitance set to 98.7 pF (i.e., Paci baseline value), and $g_{\text{seal}}$ set to values from 0.1 nS to 1 nS (i.e., from 10 GΩ down to 1 GΩ). The dashed red trace shows a baseline (leak-free) simulation. Four AP morphology metrics for the Kernik (C) and Paci (D) models are plotted against $g_{\text{seal}}$ (displayed on log-scaled x-axis): minimum potential (MP), maximum upstroke velocity ($dV/dt_{\text{max}}$), action potential duration at 90% repolarisation (APD$_{90}$), and cycle length (CL). Grey boxes denote the metrics from the two Kernik simulations that did not produce full APs.

Given these model predictions, it appears likely $R_{\text{seal}}$ can alter AP morphology, even at values above 1 GΩ (i.e., below 1 nS). This finding points to the importance of recording accurate measures of $R_{\text{seal}}$, and the need for a strategy to address $I_{\text{leak}}$ effects during experiments. In the following sections, using in silico and in vitro data, we show the challenges of devising such a strategy and how, under certain conditions, it may be impossible to determine $R_{\text{seal}}$.

2.2 $R_{\text{seal}}$ is not stable

Unlike voltage-clamp recordings, the effects of $I_{\text{leak}}$ on AP morphology (measured in current clamp mode) cannot be corrected in post-processing. Current-clamp leak compensation requires the real-time injection of a current that opposes $I_{\text{leak}}$ at every instant during an action potential. This can be achieved using dynamic clamp, but requires a reliable approximation of $R_{\text{seal}}$ to recover the leak-free phenotype.

$R_{\text{seal}}$ is typically estimated before gaining access to a cell. It can be calculated using a small test pulse in voltage-clamp mode (HEKA Elektronik GmbH, 2016):

$$R_{\text{seal}} = \frac{\Delta V_{\text{cmd}}}{\Delta I_{\text{out}}}$$  \hspace{1cm} (3)

Here, $\Delta V_{cmd}$ is the applied voltage step and $\Delta I_{out}$ is the difference in recorded current before and during the step. Once access is gained to a cell it can be difficult to estimate $R_{seal}$, as the measured input resistance ($R_{in}$) depends on both $R_m$ (membrane resistance) and $R_{seal}$ (Equation 4, Figure 3):

$$\frac{1}{R_{in}} = \frac{1}{R_m} + \frac{1}{R_{seal}}.$$  \hspace{1cm} (4)

Figure 3: $R_{seal}$ cannot be measured directly once access is gained. Once access is gained, we can only measure the combined resistance $R_{in}$, which is equal to the parallel resistances of $R_{seal}$ and $R_m$ (Equation 4). The presence of $R_m$ introduces uncertainty when $R_{in}$ is used to approximate $R_{seal}$, making it difficult to accurately correct for leak current effects. For simplicity, we have omitted other elements of this patch-clamp diagram (e.g., series resistance, capacitance, etc.).

Since $R_{seal}$ is difficult to determine during experiments with iPSC-CMs, it is tempting to measure the value before gaining access and assume it remains unchanged for the duration of an experiment. To investigate this, we considered in vitro $R_{in}$ measures taken at two times during iPSC-CM experiments. $R_{in}$ was measured with 5 mV steps from a holding potential of 0 mV (i.e., the leak reversal potential) before and after acquiring current clamp data. Due to the large range of $R_{in}$ measures (0.182 GΩ to 52 GΩ), we chose to display the distribution of this data as $1/R_{in}$, or $g_{in}$ (Figure 4A). The data are skewed, with a mean of 1.43 nS ($R_{in}$=0.70 GΩ) and median of 1.19 nS ($R_{in}$=0.84 GΩ).

The relative change in $g_{in}$ from the first to the second time point was calculated, and is plotted against the time elapsed between $g_{in}$ measurements in Figure 4B. The mean increase in $g_{in}$ was +46% (with a standard deviation of 155%) and the median increase was +18%. Because positive and negative changes cancel each other out in these statistics, we also inspected the absolute change, where we found a mean of 67% (a standard deviation of 147%) and a median of 25%.

The data in Figure 4B illustrate that $R_{in}$ measurements often change over time. If we assume $R_m$ is stable during experiments, this change in $R_{in}$ should be be attributed to $R_{seal}$, and suggests that the average cell’s $I_{leak}$ increases over time. These findings demonstrate that pre-rupture $R_{seal}$ measures cannot be taken as ground truth throughout an experiment. As a result, it becomes desirable to find accurate measures of $R_{seal}$ and $R_m$ after access is gained.
Figure 4: **R\textsubscript{in} changes during iPSC-CM experiments.** A, Distribution of initial $g\textsubscript{in}$ measurements from iPSC-CMs acquired with a +5 mV step from 0 mV ($n=39$). B, The percentage change in $g\textsubscript{in}$ plotted against the time elapsed between $g\textsubscript{in}$ measurements. Data is shown for all cells where two $g\textsubscript{in}$ measures were available ($n=38$). The interval between measurements ranged from 1 to 10 minutes. Time was recorded to the nearest minute, leading to the appearance of banding in the ∆Time measure.

### 2.3 R\textsubscript{in} is not a good approximation of R\textsubscript{seal} at any holding potential

In Figure 4 we showed $R\textsubscript{in}$ measurements from a holding potential of 0 mV. A holding potential of $-80$ mV is a common choice for approximating $R\textsubscript{seal}$ with $R\textsubscript{in}$ measures. At this potential, sodium, calcium, and several potassium currents are expected to be largely inactive, but contributions from both $I\textsubscript{K1}$ and $I\textsubscript{f}$ must still be considered.

We recently showed that $I\textsubscript{f}$ is present in at least some of the iPSC-CMs used in this study (Clark et al., 2022). $I\textsubscript{f}$ is also present in both the Kernik and Paci models, and we found the dynamics of the Kernik $I\textsubscript{f}$ model to be quite similar to the *in vitro* data in this study (Figure 5A-B). Figure 5A shows a typical cell’s response to an $I\textsubscript{f}$-activating hyperpolarising step before and after treatment with quinine, at a concentration expected to lead to 32% $I\textsubscript{f}$ block (this data is taken from a section of a larger protocol — see Figure 6A of Clark et al., 2022). A change in total current of 2 A/F is observed after holding near $-120$ mV for 1 s (Figure 5A). Simulations using the Kernik model with 32% block of $I\textsubscript{f}$ show a similar directional change, but only a 1 A/F shift in $I\textsubscript{ion}$ (Figure 5B). Given that most currents, besides $I\textsubscript{K1}$ and $I\textsubscript{f}$, are not active at $-120$ mV, and quinine does not block $I\textsubscript{K1}$ at the concentration used in the study, we assume the 2 A/F change is due entirely to $I\textsubscript{f}$ block. Following from this assumption, we can say the cell’s $I\textsubscript{f}$ conductance per unit capacitance is approximately twice as large as in the baseline Kernik model.

To illustrate the effect of $I\textsubscript{f}$ on leak calculations, we compared simulations from Kernik+leak models with $R\textsubscript{seal} = 1 \text{ G}\Omega$ and with $g\textsubscript{f}$ set to either the Kernik baseline value ($g\textsubscript{f} = 0.0435 \text{nS/pF}$) or twice its baseline value ($g\textsubscript{f} = 0.087 \text{nS/pF}$) (Figure 5C). To highlight that $I\textsubscript{f}$ effects on $R\textsubscript{in}$ are largely independent of $I\textsubscript{K1}$, we also reduced the $g\textsubscript{K1}$ in these models to 10% of its original value. The calculated $R\textsubscript{in}$ values for these models at $-80$ mV are 1.25 GΩ for $g\textsubscript{f}=0.0435 \text{nS/pF}$ (baseline) and 0.96 GΩ for $g\textsubscript{f}=0.087 \text{nS/pF}$ ($2 \times$ baseline) — in other words a +25% and −4% error in $R\textsubscript{seal}$ prediction (Figure 5C). These simulations show that, at $-80$ mV: 1) $I\textsubscript{f}$ contributes to $I\textsubscript{out}$ and affects measures of $I\textsubscript{leak}$, and 2) $R\textsubscript{seal}$ may be over- or under-estimated depending on the value of $g\textsubscript{f}$.

We then calculated $R\textsubscript{in}$ values at multiple holding potentials between $-90$ and $+30$ mV to determine whether we could find a potential where $R\textsubscript{in}$ is close to $R\textsubscript{seal}$, thereby minimising the prediction error (Figure 5D). The model predicts that 10 mV ($R\textsubscript{in}=0.99 \text{G}\Omega$) minimises the error in our approximation of $R\textsubscript{seal}$. This makes intuitive sense, as these potentials overlap with the large $R\textsubscript{in}$ plateau phase of the AP. This does not however, mean that...
The $R_{in}$ measurements at 10 mV will always produce the best estimate of $R_{seal}$. Instead, it indicates the size of $I_{ion}$ does not change much when taking a 5 mV step from this potential. There is, however, a considerable amount of total current present, making this $R_{seal}$ prediction sensitive to variations in the predominant ionic currents at this potential. Moreover, $I_{leak}$ will be small and therefore more difficult to measure as 10 mV is close to the leak reversal potential (0 mV). It is also worth noting that the complex voltage- and time-dependent behaviour of transmembrane currents make $R_{in}$ measures sensitive to both the duration and size of the voltage step (see e.g. the supplement to Clerx et al., 2021). In summary, it is difficult to find a holding potential where $R_{seal}$ can be measured without contamination from any transmembrane currents (i.e., where $I_{leak} = I_{out}$).

Taken together, these findings provide evidence to the claim that $R_{seal}$ cannot be reliably measured in iPSC-CMs once access is gained.

Figure 5: **Ignoring the presence of $I_f$ makes it impossible to accurately measure $R_{seal}$ after gaining access.** A, Voltage clamp data acquired from an iPSC-CM before and after treatment with quinine, which is expected to block 32% of $I_f$ at the concentration used. B, Kernik model response at baseline and with 32% block of $I_f$. C, Kernik+leak voltage clamp simulations conducted with $R_{seal}=1 \, \Omega$, $g_{K1}$ reduced by 90%, and $g_l$ set to 0.0435 nS/pF (solid line) or 0.087 nS/pF (dashed line). A voltage step from $-80 \, \text{mV}$ to $-75 \, \text{mV}$ was applied, as is commonly used to estimate $R_{in}$. This $R_{in}$ value is sometimes used to approximate $R_{seal}$ when the holding potential is near $-80 \, \text{mV}$. The amplifier-measured ($I_{out}$), total transmembrane ($I_{ion}$), and leak currents ($I_{leak}$) are displayed. The red arrow (top) indicates the change in $I_{out}$ caused by the different $g_l$. The $R_{in}$ values calculated based on $\Delta I_{out}$ are 1.25 $\, \Omega$ and 0.96 $\, \Omega$ for the 0.0435 nS/pF and 0.087 nS/pF simulations, respectively. D, $R_{in}$ values are plotted against holding potential for Kernik+leak models with $R_{seal} = 1 \, \Omega$ and $g_l=0.0435$ nS/pF or $g_l=0.087$ nS/pF. The red dotted line shows the true simulated $R_{seal}$ value of 1 $\, \Omega$.

Next, we compared the effect of $I_f$ on $R_m$, and the error in assuming $R_{seal} \approx R_{in}$, at both a 0 mV and $-80 \, \text{mV}$ holding potential. At 0 mV the Kernik+leak model is not sensitive to changes in $g_l$, as $I_f$ is largely non-conductive.
(Figure 6A). However, due to an increased relative contribution of inward currents at 0 mV, the Kernik+leak model predicts an $R_{\text{in}}$ with a large overestimation of $R_{\text{seal}}$ (Figure 6B). This error increases as the true value of $R_{\text{seal}}$ increases. Figure 6B also illustrates the sensitivity of the model to variations in $g_f$ at $-80$ mV, with the $0.087$ nS/pF model producing a small underestimate of $R_{\text{seal}}$ while the $0.0435$ nS/pF model overestimates $R_{\text{seal}}$; these errors increase as $R_{\text{seal}}$ increases. The improved prediction accuracy of the $0.087$ nS/pF model at $-80$ mV is a coincidental side-effect of doubling $g_f$: with a different distribution of ion current densities or a larger baseline $g_f$ value, the same doubling could just as easily worsen $R_{\text{seal}}$ predictions. For example, the $R_{\text{in}}$ of an iPSC-CM with a large $I_{K1}$ current may slightly underestimate $R_{\text{seal}}$ at $-80$ mV — doubling $g_f$ in this case would result in a greater underestimation, increasing the error of the estimate.

![Figure 6: $R_{\text{in}}$ predictions of $R_{\text{seal}}$ are overestimated at the reversal potential for leak current.](image)

A. The current response ($I_{\text{out}}$) for Kernik+leak models with a 1GΩ seal and $g_f$ of $0.0435$ nS/pF (solid line) or $0.087$ nS/pF (dashed line) to a 50 ms $+5$ mV voltage clamp step from 0 mV (top) or $-80$ mV (bottom). B. Effect of $R_{\text{seal}}$ on $R_{\text{in}}$ measures for models with $g_f$ set to $0.0435$ (solid) or $0.087$ (dashed) nS/pF. $R_{\text{in}}$ was calculated with Equation 3. The $+5$ mV voltage steps were taken from either 0 or $-80$ mV. The $R_{\text{seal}} = R_{\text{in}}$ line (red dotted) is provided as a reference for when $R_{\text{in}}$ correctly predicts $R_{\text{seal}}$. The 0 mV lines are overlapping, illustrating that $R_{\text{in}}$ is not sensitive to $g_f$ at this voltage.

### 2.4 $C_m$ and $R_{\text{in}}(0 \text{ mV})$ correlate with minimum potential

The iPSC-CMs used in this study displayed a heterogeneous phenotype (Figure 7), producing both spontaneously firing ($n=27$) and non-firing ($n=12$) current clamp recordings. Figure 7A shows three cells with very different baseline current-clamp recordings: non-firing and depolarised (grey), spontaneously firing with a short AP (black), and spontaneously firing with a long AP (blue). Non-firing cells (MP = $-42 \pm 8$ mV) and cells with spontaneously-firing APs were depolarised (MP = $-55 \pm 7$ mV) — the spontaneously-firing cells also had a shorter AP duration ($APD_{90} = 133 \pm 73$ ms) (Figure 7B) relative to adult cardiomyocytes (O’Hara et al., 2011) and iPSC-CM models (Kernik et al., 2019; Paci et al., 2013).

In this section, we use linear regression analyses to determine if there is a correlation between $g_{\text{in}}/C_m$ and AP biomarkers. The values of each cell’s $g_{\text{in}}$ and $C_m$ are shown in Figure 7C. $I_{\text{leak}}$’s effect on AP morphology is expected to scale directly with $g_{\text{in}}$ and inversely with $C_m$. This is because $g_{\text{in}}$, even if a poor estimate, is expected to correlate with $g_{\text{seal}}$ (Figure 6B). Additionally, a given $g_{\text{leak}}$ will cause a smaller contribution in larger cells (i.e., cells with larger $C_m$), because the ionic currents are expected to scale with the size of the cell.
Figure 7: Cells appeared phenotypically heterogeneous, with uncorrelated variation in $g_{in}$ and $C_m$. A, Current clamp recordings from three cells show phenotypic heterogeneity: non-spontaneous (grey), spontaneous AP with short APD (black), and spontaneous AP with long APD (blue). B, MP and APD$_{90}$ for spontaneously beating cells ($n=27$). Note the broken $x$-axis which just allows us to display an outlying data point. C, The relationship between $C_m$ and $g_{in}$ for all cells ($n=39$).

Four AP biomarkers (MP, APD$_{90}$, CL, and dV/dt$_{max}$) were compared to $g_{in}/C_m$ (Figure 8). The MPs of spontaneously firing ($R=0.47$, $p<.05$) and non-firing ($R=0.76$, $p<.05$) cells are positively correlated with $g_{in}/C_m$ (Figure 8A). This finding is in agreement with our in silico studies showing that increasing $g_{in}$ will depolarise the cell (Figure 2). The other three biomarkers failed at least one of the assumptions that is required when conducting a linear regression analysis (see Methods). There are no obvious trends when comparing $g_{in}/C_m$ to CL or dV/dt$_{max}$. The APD$_{90}$ plot, however, indicates there may be some AP shortening as $g_{in}/C_m$ increases. Due to undersampling and a lack of linearity, we cannot make any claims of significance between these two measures. Leak simulations with the models, though correlated, did not predict a linear relationship between $g_{seal}$ and these biomarkers (Figure 2C-D). However, the MP vs. $g_{in}/C_m$ relationship passes all tests of linear regression assumptions and trends in the same direction as the Kernik and Paci simulations in Figure 2.

In summary, we found that a higher $g_{in}/C_m$ (indicating greater $I_{leak}$ contribution) correlated with more depolarised MPs. This supports the idea that $I_{leak}$ affects AP shape and cell-to-cell variability in the iPSC-CMs used in this study.
Figure 8: **Relationship between \( g_{\text{in}}/C_m \) and AP biomarkers.** A, \( g_{\text{in}}/C_m \) plotted against MP. Spontaneously firing cells are denoted as black points and non-firing cells as yellow squares. Linear regression fits to data from spontaneous (black dashed, \( R = 0.47, \ p < 0.05 \)) and non-firing (yellow dotted, \( R = 0.76, \ p < 0.05 \)) cells are overlaid on the plot. No statistically significant relationship was found between \( g_{\text{in}}/C_m \) and APD\(_{90} \) (B), CL (C), or \( dV/dt_{\text{max}} \) (D). 

2.5 **Fitting background currents in iPSC-CM models can absorb and imitate I\(_{\text{leak}}\)**

iPSC-CM models contain linear background currents (sodium and calcium) that differ from I\(_{\text{leak}}\) only in terms of reversal potentials and the ions they conduct. However, their presence and their magnitudes have not been experimentally investigated in iPSC-CMs (see Discussion). Here, we show that the background currents in existing iPSC-CM models can imitate \( I_{\text{leak}} \), and we hypothesise that the contribution of \( I_{\text{leak}} \) may erroneously be ascribed to background currents when models are fit to experimental data.

We used a genetic algorithm (GA) to study the potential of background currents to imitate leak effects (see Methods). We fit the baseline Kernik model to a Kernik+leak model with \( R_{\text{seal}} = 5 \ \text{G}\Omega \) (Figure 9), allowing only the background sodium (\( g_{\text{bNa}} \)) and background calcium (\( g_{\text{bCa}} \)) conductances to vary. These currents were selected because they were incorporated into the Kernik model without independent iPSC-CM experimentation or validation. The best fit individual had an increased \( g_{\text{bNa}} \) (\( \times 7.0 \)), while \( g_{\text{bCa}} \) (\( \times 1.0 \)) did not change much relative to the baseline model (Figure 9A). While not a perfect match, the best-fit trace reproduced qualitative features of the baseline+leak trace, showing a depolarised MP and a smaller amplitude (Figure 9B). This indicates that increased \( I_{\text{bNa}} \) can affect the AP in a fashion similar to \( I_{\text{leak}} \).

We then compared the background current IV curves of the fit model to the original baseline+leak model (Figure 9C-D). The IV curves of \( I_{\text{bNa}} \) (\( E_{\text{Na}} = +79 \ \text{mV} \)) and \( I_{\text{bCa}} \) (\( E_{\text{Ca}} = +112 \ \text{mV} \)) are negative at all tested voltages (-90 to +60 mV), while \( I_{\text{leak}} \) reverses at 0 mV (Figure 9C). The best-fit model \( I_{\text{bNa}} \) conducts a much larger
negative (i.e., depolarising) current at all tested voltages when compared to \( I_{\text{leak}} \).

We also investigated composite IV curves for: 1) \( I_{\text{bNa}} + I_{\text{bCa}} + I_{\text{leak}} \) from the original baseline+leak model, 2) fitted \( I_{\text{bNa}} + I_{\text{bCa}} \) from the best fit model, and 3) \( I_{\text{bNa}} + I_{\text{bCa}} \) from the original baseline model (Figure 9D). The fitted background IV curve (red) is negatively shifted, relative to the original baseline model (grey), as the \( I_{\text{bNa}} \) component conducts large depolarising currents, mimicking the effects of \( I_{\text{leak}} \) at large negative potentials. Despite good AP agreement (Figure 9B), the divergence in the Kernik+leak and fitted model IV curves illustrates that the depolarising effects of \( I_{\text{leak}} \) and \( I_{\text{bNa}} \) at negative voltages are most likely responsible for the morphological agreement seen throughout the cycle length (Figure 9D).

![Figure 9](image_url)

**Figure 9:** A simulated example of how leak can be absorbed into background currents: Kernik baseline model fit to Kernik+leak model. The \( I_{\text{bNa}} \) and \( I_{\text{bCa}} \) conductances \((g_{\text{bNa}}, g_{\text{bCa}})\) of the baseline Kernik model were fit to a Kernik+leak model (i.e., original+leak) with \( R_{\text{seal}} \) set to 5 GΩ. A GA with a population size of 150 individuals and 20 generations was used to fit the model. A, The conductances for all individuals (grey) and the best fit individual (red square) from the last generation. B, Traces from the original baseline Kernik+leak model with a 5 GΩ seal (black), the best fit model from the last generation (red dashed), and the original baseline Kernik model (grey dotted). C, IV curves for baseline \( I_{\text{bNa}}, I_{\text{bCa}}, \) and \( I_{\text{leak}} \), and for fitted \( I_{\text{bNa}} \) and \( I_{\text{bCa}} \). D, IV curves for: 1) \( I_{\text{bNa}} + I_{\text{bCa}} + I_{\text{leak}} \) from the original+leak model, 2) Fitted \( I_{\text{bNa}} + I_{\text{bCa}} \) from the fitted Kernik model, and 3) \( I_{\text{bNa}} + I_{\text{bCa}} \) from the original baseline Kernik model.

In this section, we have shown that \( I_{\text{bNa}} \) can affect AP morphology in a similar way to \( I_{\text{leak}} \). Here, the Kernik model was used as our ground truth, but was constructed using iPSC-CM data that may have been polluted by leak current. Unless \( I_{\text{leak}} \) is explicitly handled, either by experimental real-time dynamic clamp leak correction.
or in the mathematical model itself at the time of its construction, mathematical iPSC-CM models may absorb the effects of $I_{\text{leak}}$ by erroneously increasing background currents.

3 Discussion

Leak current is a common and unavoidable experimental artefact that affects patch-clamp recordings. In this study, using both model predictions and experimental data, we show that leak current: 1) affects iPSC-CM AP morphology; 2) can vary during experiments; 3) cannot be accurately estimated after access is gained to an iPSC-CM; and 4) may be absorbed by linear equations for background currents when iPSC-CM models are fit to experimental AP data. During iPSC-CM current-clamp studies, leak consideration often starts with a pre-rupture seal measurement (with a 1 GΩ threshold) and is ignored if the seal appears to remain stable throughout the study. Here, we argue leak effects should be quantitatively scrutinised at all points during the acquisition, analysis and fitting of experimental data. Furthermore, we believe cell-to-cell variation in seal resistance contributes to observed iPSC-CM AP heterogeneity — often attributed nearly entirely to variations in ionic current densities.

3.1 Leak affects AP morphology

Leak is known to affect the shape of AP morphology in small cardiac cells. Simulations in chick embryonic cells (with model $C_m = 25.5 \, \text{pF}$) have shown that leak current will substantially depolarise the MP and shorten the CL, even with large $R_{\text{seal}}$ values (5 GΩ, Krogh-Madsen et al., 2005). More recently, Horváth et al. (2018) showed that in vitro iPSC-CMs were depolarised during single-cell experiments, but not when cells were clustered. These results indicate that iPSC-CMs are not inherently depolarised, but may be affected by the increased influence of leak current in isolated cells with a low capacitance.

Our in vitro and in silico findings support this conclusion and strengthen the argument that iPSC-CM AP morphology is strongly affected by leak current.

iPSC-CMs have long been defined by their immature and heterogeneous phenotype (Ma et al., 2011; Doss et al., 2012). Over the years, optimisations of the differentiation and dissociation processes have improved cell maturity and consistency, but issues remain (Herron et al., 2016). Such shortcomings of the cells have often been attributed to variations in ionic current conductances and a reduced $I_{K1}$ density (Ma et al., 2011; Doss et al., 2012). However, even iPSC-CMs with large $I_{K1}$ have displayed depolarised MPs and large cell-to-cell variability (Horváth et al., 2018; Feyen et al., 2020). In addition to ionic current densities, we suggest that variations in leak current play a critical role in both the heterogeneity and apparent immaturity (characterised by depolarised MPs) of these cells.

3.2 Predicting $R_{\text{seal}}$ during experiments

Useful implementation of a leak compensation current requires accurate measures of $R_{\text{seal}}$ throughout an experiment. $R_{\text{seal}}$ can be well-approximated prior to gaining access, but after perforation (or rupture) the presence of membrane currents make it impossible to obtain an accurate measurement (Figure 5). This is problematic due to the tendency of $R_{\text{seal}}$ to change over the course of an experiment (Figure 4).

To address these difficulties, we believe it may be feasible to use the pre-rupture $R_{\text{seal}}$ and post-rupture $R_{\text{in}}$ measures to calculate estimates of $R_{\text{seal}}$ during an experiment. This approach would require an accurate measure of $R_{\text{in}}$ just after access is gained. Using $R_{\text{seal}}$ and the initial $R_{\text{in}}$, it is possible to calculate $R_{\text{in}}$ (Figure 3). An estimate of $R_{\text{seal}}$ could then be made at any time during the experiment, assuming the calculated $R_{\text{in}}$ stays constant, by re-measuring $R_{\text{in}}$ and using Equation 4. This approach relies on two major assumptions: 1) the perforation/rupture step does not affect the seal; and 2) a protocol or procedure exists that can be used prior to each measurement of $R_{\text{in}}$ to ensure that the contribution of $R_{\text{in}}$ is consistent. We cannot say for certain that these assumptions will always be valid. However, we believe that recording frequent $R_{\text{in}}$ measurements, estimating $R_{\text{seal}}$, and scrutinising changes are important steps for the correct interpretation of iPSC-CM current clamp data.
3.3 Correcting for $R_{\text{seal}}$ during experiments

We believe these $R_{\text{seal}}$ estimates should be used in a dynamic clamp leak compensation setup to address the limitations caused by a depolarised and variable MP. The approach works by injecting simulated currents into a cell in a real-time continuous loop during current clamp experiments (Ortega et al., 2018). $I_{\text{K1}}$ dynamic clamp has been used on iPSC-CMs to attain quiescence at a MP below $-70\,\text{mV}$ so the cells can be paced at a desired frequency (Meijer van Putten et al., 2015; Goversen et al., 2018b; Li et al., 2021; Clark et al., 2022). A dynamically clamped leak-compensation current has also been implemented and used in manual patch-clamp studies with neonatal mouse cardiomyocytes (Ahrens-Nicklas and Christini, 2009), demonstrating the potential of using such an approach with small cardiomyocytes. The effects of leak and the ability of leak compensation to recover adult cardiomyocyte behaviour has also been demonstrated in an in silico study (Fabbri et al., 2020). Together, these investigations demonstrate the potential of dynamic clamp as an experimental tool to simultaneously address shortcomings of the cells (i.e., $I_{\text{K1}}$ density) and experimental setup (i.e., $I_{\text{leak}}$). This technique has the potential to improve the descriptive ability of iPSC-CMs when used in biophysical and drug investigations.

Inaccuracies in these estimates, however, will remain, resulting in the potential to under- or overcompensate. Undercompensation, while an improvement over no compensation, will still result in a depolarised MP and shortened AP duration relative to its true value. Overcompensation will hyperpolarise the MP relative to its true value, but also prolong phases 1 and 2 of the AP. This is because leak compensation is an inward current at positive voltages. Due to the prolongation caused by overcompensation, we believe undercompensation is preferable. We suggest injecting a fraction of the full compensatory current to mitigate the risk of underestimating $R_{\text{seal}}$. The Nanion Dynamite® sets the leak percent compensation to 70%, which seems reasonable (Becker et al., 2020). Further investigation is needed to provide advice on how to choose this value in all circumstances.

3.4 Background currents absorb leak effects

Ion-specific background currents in the Kernik and Paci iPSC-CM models were taken from the ten Tusscher et al. (2004) model. These currents can trace their roots to the seminal work of Luo and Rudy (1994). The currents were included in the Luo and Rudy (1994) and ten Tusscher et al. (2004) models to help to maintain physiologically realistic intracellular concentrations. In the ten Tusscher et al. (2004) model, these currents helped to produce $[\text{Na}^+]_i$ frequency changes in line with in silico cardiac simulations (Boyett and Fedida, 1988), and equilibrium concentrations within the ranges from an in vitro study with human cardiomyocytes (Pieske et al., 2002).

Direct measurements of $I_{\text{bCa}}$ and $I_{\text{bNa}}$ in iPSC-CMs have not been reported. The Kernik and Paci iPSC-CM models both adopted the ventricular ten Tusscher et al. (2004) formulation for $I_{\text{bCa}}$ and $I_{\text{bNa}}$, and then set the conductances of these currents by comparing model predictions of the AP with in vitro measurements in iPSC-CMs. We posit that $I_{\text{bNa}}$ is overestimated and compensates for the absence of leak current, a source of discrepancy between these models and reality. We expect inclusion of leak when constructing iPSC-CM models to reduce background sodium and result in a more realistic model of in vitro single-cell iPSC-CMs.

3.5 Modelling experimental artefacts

While the effects of experimental artefacts in single-cell studies are well-established, consideration of them while building ion channel and action potential models has been limited (Whittaker et al., 2020). In silico studies investigating series resistance effects on voltage clamp recordings have been done in fast-activating currents, such as $I_{\text{Na}}$ and $I_{\text{K}}$ (Ebihara and Johnson, 1980; Montnach et al., 2021), but to our knowledge artefact equations have not been included in the calibration process for widely-used models of these currents — although the $I_{\text{Na}}$ model by Ebihara and Johnson was incorporated directly into the widely copied $I_{\text{Na}}$ model by Luo and Rudy (1994). Recently, Lei et al. (2020) demonstrated that coupling experimental artefact equations with an $I_{\text{Kr}}$ mechanistic model improved predictions. These studies show that experimental artefact equations can improve the descriptive ability of electrophysiological models. As such, we believe experimental artefacts should be explicitly taken into...
account at the modelling phase, and not ignored simply because a pre-determined minimum threshold is reached (e.g., 1 GΩ). Based on the findings of our study, we believe cardiomyocyte models, and especially iPSC-CM models, should explicitly include leak currents when fitting to experimental current clamp data.

### 3.6 Recommendations

The results in this manuscript provide important insights for experimentalists and modellers alike. We developed the following recommendations based on our findings:

1. **Experimental:** $R_{\text{seal}}$ should be recorded before gaining access to a cell, and $R_{\text{in}}$ should be measured frequently during an experiment. It is important to measure $R_{\text{in}}$ from a voltage that provides a consistent measure of $R_{\text{m}}$, such that any changes in $R_{\text{in}}$ can be attributed to changes in $R_{\text{seal}}$. If these measures of $R_{\text{in}}$ do not vary, this may be indicative of a stable $R_{\text{seal}}$.

2. **Experimental:** Dynamic injection of a leak compensation current can help the cell recover its native MP and produce an AP with little contribution from $I_{\text{leak}}$. Because $R_{\text{seal}}$ is difficult to measure during experiments, and to avoid overcompensation, we advise injecting a current that compensates for a fraction (e.g., 70%) of the estimated $I_{\text{leak}}$. Additionally, the $R_{\text{seal}}$ and $R_{\text{in}}$ measures should be reported along with iPSC-CM data.

3. **Modelling:** Inclusion of the $I_{\text{leak}}$ equation will improve the descriptive ability of iPSC-CM models. While this equation may not always improve fits to AP data, it will take into account an important current affecting iPSC-CM recordings.

### 3.7 Limitations and future directions

This study has several limitations that should be considered during future investigations that may be affected by $I_{\text{leak}}$. First and foremost, when gathering these data for a previous study we did not follow our own recommendation of recording the exact value of $R_{\text{seal}}$ before gaining access and then measuring $R_{\text{in}}$ just after perforation. In the future, we hope to use these two values to predict $R_{\text{seal}}$ at multiple timepoints during an experiment, as outlined in Section 3.2. Second, we only conducted these experiments in one cell line. While our results appear similar to data from other labs (e.g., Horváth et al., 2018), it would be useful to conduct this study on multiple cell lines in the same lab. Third, we did not attempt dynamic injection of a leak compensation current — in future work we would like to investigate this as an approach to reducing cell-to-cell heterogeneity. Finally, the iPSC-CM models have innumerable differences from the cells used in this study, which is evident when comparing AP morphologies of *in vitro* cells (Figure 7A) to *in silico* models (Figure 2). However, agreement that we did see between simulations and our *in vitro* data demonstrate the potential of improving the descriptive ability of iPSC-CM models by including a leak current.

### 3.8 Conclusion

In this study, we demonstrate that leak current affects iPSC-CM AP morphology, even at seal resistances above 1 GΩ, and contributes to the heterogeneity that characterises these cells. Using both *in vitro* and *in silico* data, we showed the challenges of estimating $R_{\text{seal}}$ after gaining access to a cell and that $R_{\text{seal}}$ is subject to change during the course of an experiment. We also posit that background sodium current in iPSC-CM models may be responsible for masking leak effects in *in vitro* data. Based on these results, we made three recommendations that should be considered by anyone who collects, analyses, or fits iPSC-CM AP data.

### 4 Methods

All data, code and models can be downloaded from [https://github.com/Christini-Lab/iPSC-leak-artifact](https://github.com/Christini-Lab/iPSC-leak-artifact).
4.1 Modelled concentrations

$\text{I}_{\text{leak}}$ in the baseline Kernik model destabilises intracellular concentrations and causes a slow and continuous decrease in $[\text{K}^+]_i$. To address this, we fixed the Kernik $[\text{K}^+]_i$ to its steady state value. This was not required for the Paci model, which already did not allow $[\text{K}^+]_i$ to change. We also fixed the Kernik and Paci $[\text{Na}^+]_i$ to their baseline steady state values (taken after 1000 s of spontaneous current clamp simulation using the published model initial values as starting points). We did not fix the intracellular calcium concentration, because it is less affected by the pipette solution during perforated patch-clamp experiments with amphotericin B as only monovalent ions can diffuse through the pores.

4.2 Genetic algorithm

A GA was used to fit the Kernik model to the Kernik+leak model (with $R_{\text{seal}}=5 \Omega$) by minimising a point-by-point squared difference objective function:

$$E_{\text{in}}(\theta) = \sum_{t=0}^{1000} (V_{\text{target}}(t) - V_{\text{individual}}(t, \theta))^2,$$

where $\theta$ is a vector containing the varied conductance parameters, $V_{\text{target}}(t)$ is the target membrane potential at time $t$, and $V_{\text{individual}}(t, \theta)$ is the current individual’s membrane potential at time $t$ as a function of $\theta$.

The GA had a population size of 150 individuals and was run for 20 generations. The initial population parameter values were selected from a log uniform distribution between 0.1 and 10 times their baseline values. Individuals in a new generation were created by mating two individuals from the previous generation — two selected parent individuals from the previous generation had a 90% chance of mating. If they did not mate, they would continue to the next generation without swapping parameters. If they mated, there was a 20% chance of swapping each of their parameter values. As such, each time two individuals mated, they would produce two child individuals consisting of the parent parameter values. Each individual in a new generation had a 90% chance of being mutated. If an individual was mutated, there was a 20% chance each parameter would be changed. To mutate a parameter, a new value was selected from a normal distribution centred around the current value, with a standard deviation equal to 10% of the current value.

The Kernik+leak target and each individual were run for 100 s before comparison. The third-to-last AP was identified from each, and traces were aligned by the $dV/dt_{\text{max}}$ of these APs. Traces were compared from 200 ms before the $dV/dt_{\text{max}}$ to 800 ms after it. The code for this GA can be found on the project GitHub page.

4.3 Linear regression

A linear least-squares regression was used to compare $g_{\text{in}}/C_m$ to AP biomarkers. MP was the only biomarker that did not violate any linear regression assumptions when compared to these independent variables. Tests of these assumptions can be found on the project GitHub repository.

4.4 Software and simulations

Simulations were performed in Myokit v1.33.7 (Clerx et al., 2016). The genetic algorithm was developed in Python and made use of the DEAP library v1.2.2 (Fortin et al., 2012). Additional analysis was done in Python using NumPy v1.21.6 and SciPy v1.7.3 (Virtanen et al., 2020).

4.5 iPSC-CM cell culture

Frozen vials of iPSC-CMs were obtained from Joseph C. Wu, MD, PhD at the Stanford Cardiovascular Institute Biobank. The iPSC-CM line was derived from an African American female donor and the differentiation was approved by the Stanford University Human Subjects Research Institutional Review Board. 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.

4.6 Electrophysiological setup

Perforated patch-clamp experiments were conducted following the protocol described in Clark et al. (2022). Borosilicate glass pipettes were pulled to a resistance of 2-4 MΩ using a flaming/brown micropipette puller (Model P-1000; Sutter Instrument, Novato, CA). Pipette tips were first dipped into intracellular solution containing 10 mM NaCl, 130 mM KCl, 1 mM MgCl₂, 10 mM CaCl₂, 5.5 mM dextrose, 10 mM HEPES. Pipettes were then backfilled with intracellular solution with 0.44 mM amphotericin B, a perforating agent. Amphotericin B allows only monovalent ions to pass through the cell membrane, so a high intrapipette calcium concentration was included to induce cell death in the case of an unintended rupture. Coverslips containing iPSC-CMs were placed in the bath and constantly perfused with an extracellular solution at 35-37°C containing 137 mM NaCl, 5.4 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 10 mM dextrose, and 10 mM HEPES.

Patch-clamp measurements were made at a 10 kHz sampling frequency by an amplifier with the low-pass filter set to 5 kHz (Model 2400; A-M Systems, Sequim, WA), and was controlled by the Real Time eXperiment Interface (RTXI; http://rtxi.org). After immersing a pipette into the extracellular solution, voltage was set to zero — any remaining offset in the recordings is assumed to be equal to the liquid junction potential of ~2.8 mV. After contact was made with a cell and a seal of > 300 MΩ was formed, the perforating agent slowly decreased the access resistance to the cell (usually 10–15 minutes). A series resistance of 9–50 MΩ was maintained for all experiments. After gaining access, Rₘ at 0 mV was measured before and after acquiring AP data.
References


Additional information

Competing interests

The authors declare that they have no competing interests.

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