Decoding Ca^{2+} signals as a non-electrophysiological method for assessing drug toxicity in stem-cell derived cardiomyocytes

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Abstract

We describe a new method to profile the Ca^{2+} signalling fingerprint of stem-cell-derived cardiomyocytes (CM) under drug-naïve and drug-exposed conditions. This method, termed SALVO, takes into account 1) the quantification of very low amplitude Ca^{2+} fluxes that occur *between* large Ca^{2+} spikes, that in the absence of any effect on Ca^{2+} spikes *per se*, may have a profound impact on cell phenotype, and 2) the statistical assessment of the variability in the amplitude and temporal components of Ca^{2+} spike trains. In a previous study, SALVO identified the potential cardiovascular (CV) risk of drugs that had no measurable impact on cellular electrophysiological profile and also yielded a toxicity score that exhibited a better correlation with human QT prolongation liabilities than other conventional electrophysiological readouts.

This chapter sets out a step-by-step protocol for establishing phenotypically-consistent CM populations and our method for performing the Ca^{2+} signalling analysis in which the output data are reconciled with phenotypic endpoints (e.g. cytotoxicity and susceptibility to apoptosis). We present a worked example using two drugs that disrupt Ca^{2+} signalling via different mechanisms; drug A causes the cessation of spontaneous Ca^{2+} oscillation via progressive attenuation in Ca^{2+} spike amplitude whereas drug B elicits different periodic behaviour. Both drugs have comparable effects on the frequency of Ca^{2+} spikes, but SALVO analysis discriminates their actions. An extensive set of notes is included that will help the reader reproduce our methods with high fidelity.

1. Introduction

Cardiovascular (CV) drug development needs have led to enhanced screening systems to improve early-stage assessments of potential hazard.[1, 2] The widespread adoption of human stem cell-derived cardiomyocytes (CM) – a term encompassing both embryonic stem cell-derived CM (ES-CM) and induced pluripotent stem cell-derived CMs (iPSC-CM) – into the drug screening toolkit goes some way to recreating the cell-to-cell interactions that modulate the behaviour of individual CM *in vivo,* and addresses many of the problems associated with non-human cell-based assays.[1, 3-5] However, these cells will only become a truly useful resource in the drug-screening landscape if they are harnessed to new methodologies that move beyond conventional electrophysiological (EP) assessments that sometimes fail to adequately assess the potential hazards of drugs in the clinical setting.[1, 2, 6, 7] In one notorious example, EP measurements during FDA-mandated screening appeared not to identify the CV risk associated with rofecoxib (Vioxx™), a drug that was withdrawn because of unacceptable CV hazard[8] and subject to a $5.8 billion dollar lawsuit.[9] The comprehensive *in vitro* proarrhythmia assay (CIPA) initiative aims to address these issues.[10-12]

We have tackled the problem from a different perspective that complements and extends the current armoury of electrophysiology-based methods. Calcium (Ca^{2+}) signals modulate almost every biological process[13-15] and the normal synchronization of Ca^{2+} signalling within and between cells in a population is collectively determined by multiple coupled oscillators acting across multiple scales.[15-22] Given the intricacies of these processes, it is perhaps not surprising that disrupted Ca^{2+} signalling is a good index of phenotypic deterioration and drug-induced cytotoxicity in CM.[6, 23] Consequently, the detailed assessment of spatiotemporal organization of Ca^{2+} signals under normal and drug-exposed conditions is valuable. Although methods do exist for investigating complex phenomena in Ca^{2+} signalling data[17, 24, 25] these tend to focus on very specific aspects of cell behaviour rather than provide a 'holistic' evaluation of the patterns of cellular Ca^{2+} signals.

Our approach is to profile the Ca^{2+} signalling fingerprint of CM under drug-naïve and drug-exposed conditions.[18, 26, 27] This in itself is not especially new because assessments of the characteristics of super-threshold Ca^{2+} signals (e.g. the frequency and amplitude of large Ca^{2+} 'transients' or 'spikes') are commonly employed. However, the novelty of our approach comes from 1) the quantification of very low amplitude Ca^{2+} fluxes that occur *between* these Ca^{2+} spikes, that in the absence of any effect on Ca^{2+} spikes *per se* may have a profound impact on cell phenotype (e.g. susceptibility to cell death)[15, 28-30], and

2) the statistical determination of the variability in the amplitude and temporal components of Ca^{2+} spike events. In order to integrate the analysis of Ca^{2+} spikes with information that is encoded by signals occurring *between* these spike events (i.e. in inter-spike intervals), we developed SALVO (an acronym of **s**ynchronization, **a**mplitude, **l**ength and **v**ariability of **o**scillation), a software program that enables the detailed quantification of the spatiotemporal patterning of Ca^{2+} signals in CM.[31]

In a proof-of-concept study using a small panel of drugs, this approach accurately identified those drugs with established pro-arrhythmogenic liability[23] and confirmed that disrupted cellular Ca^{2+} signalling *in vitro* is a hallmark feature of drugs with known CV risk in humans. Importantly, this study also validated the use of SALVO to 1) identify the potential CV risk of valdecoxib, an analog of rofecoxib (Vioxx™) and a drug which had no measureable impact on cellular EP profile, and 2) generate drug toxicity scores that exhibit better correlation with QT prolongation liabilities in humans than other conventional electrophysiological readouts (e.g. cardiac action potential duration (APD_{90}) or measurements of cellular impedance).[7, 32-34]

This chapter sets out our protocols for establishing phenotypically consistent CM populations, and the systematized approach we take to performing drug-toxicity assessments utilizing a detailed SALVO-based analysis of super- and sub-threshold cellular Ca^{2+} signals. We also include an extensive set of notes ('tips-and-tricks') that will enable the reader to reproduce our methods with high fidelity.

2. Materials

Cardiomyocytes

The protocols described in this chapter relate specifically to post-differentiated CM from human ESC and IPS; optimised methods for these differentiation processes have been described previously (e.g. [35-38]). Typically, these protocols do not yield CM exclusively. For example, H7- stem cell-derived Cytiva™ (GE Healthcare), which are produced via a monolayer differentiation protocol adapted from Xu and colleagues[35], are supplied as a heterogeneous cell population in which CM account for approximately half of the cells, while fibroblasts and other non-CM cell types comprise the remainder.[23] In our experience, the proportion of cardiac troponin-T (cTnT)-positive CM remains constant in culture, suggesting a stable population of differentiated CMs and the absence of proliferative or differentiation-competent non-CM

cells (Figure 1B). Acknowledging this issue, in this chapter we use the term CM to refer to all cells in stem-cell derived populations (Note 1).

Matrigel (Corning) is diluted 1:1 (v/v) in Knockout DMEM (KO-DMEM, ThermoFisher Scientific), and is snap frozen on dry ice prior to storage at -80ºC. This solution is further diluted to 1:30 (v/v) in ice-cold KO-DMEM immediately prior to use. We have found that other adhesion matrices (e.g. fibronectin or laminin), presumably because of different adhesion capacities, tend to inhibit axial realignment of CMs on the culture surface (Figure 1B and C) (Note 2).

Silicon gaskets

Reusable silicon gaskets (diameter 0.3 mm and depth 1 mm, CultureWell™ MultiWell inserts [Grace BioLabs]) are purchased in 10 x 5 configurations and then cut into 2x2 squares (9.5mm x 9.5mm) with a razor blade (Figure 1A).

Solutions, buffers and media

- RPMI medium

Containing 1x serum-free B27 supplement.[39, 40] The concentration of free Ca^{2+} ($[Ca^{2+}]$), from the dissociation of calcium nitrate, $Ca(NO_3)_2$, is approximately 0.4mM (Note 3). RPMI/B27 is stored at 4°C for up to two weeks and is always pre-warmed to 37ºC prior to addition to cells (Note 4). The formulation of RPMI is available at the link provided in the References section[41]

- Leibovitz L-15 medium

This medium is buffered by base amino acids and phosphate, rather than sodium bicarbonate, having been formulated to maintain pH7.4 in non-CO_2 environments. Free $[Ca^{2+}]$ is approximately 1.3mM. We find that Leibovitz L-15 provides a better means of maintaining pH levels throughout the protocol (Figure 2) than the use of a bicarbonate-buffered medium (e.g. RPMI) in conjunction with 5% CO_2 delivered via environmental control of the microscope chamber. To avoid attenuating the fluorescent signal intensity from the Ca^{2+} dye, we use the formulation without phenol red. The full formulation of this medium is available via the link provided in the References section[42]

- Krebs-Ringer-Hepes-buffered solution (KRH)

We sometimes use this solution for Ca^{2+} imaging; it contains (in mM): NaCl (120), HEPES (25), KCl (4.8), $CaCl_2$ (1.3), KH_2PO_4 (1.2), $MgSO_4$ (1.2), glucose (5.5) with pH adjusted to 7.4.

Equipment for Ca^{2+} imaging

We routinely use a Leica 6000 DMRBE microscope fitted with a resonant-scanning confocal microscope (SP5, Leica Microsystems) that limits photobleaching during relatively prolonged imaging cycles (Note 5). We have also validated the method using low-level excitation illumination on a camera-based imaging system (e.g. Hamamatsu Orca attached to a Zeiss Axiovert microscope) and, over shorter imaging periods, on a high-content screening platform (IN Cell Analyzer 2200, GE Healthcare).

3. Methods

3.1 Recovery from freezing and plating

For the majority of users, post-differentiated CM will be obtained from commercial suppliers or via collaboration, and will be frozen in medium containing a cryoprotectant (e.g. DMSO). This sub-section describes our protocols for minimizing the variability of the resultant CM on thawing and plating.

1. Silicon gaskets (2x2 wells) are sterilised by autoclaving, wetted in 70% (v/v) ethanol and pressed to a 14mm diameter glass-bottomed coverslip in individual plastic chambers (CellVis) (Figure 1A) (Note 6). Chambers strongly adhere to the glass via evaporation and are left to dry completely for 1 h at RT. When completely dry, chambers are chilled at -20°C for 1 h, and then pre-chilled Matrigel/KO-DMEM solution (1:30 (v/v)) is added to each well of the gasket (15 µl) such that it typically forms a domed meniscus. A 'pocket' of KO-DMEM (250 µl) is pipetted at the periphery of each chamber to preserve the relative humidity of the chamber and prevent evaporation of the Matrigel solution from the gasket. Chambers are stored for 12-15h at 4°C until use (Step 4). After this time, the Matrigel should have formed a homogeneously dispersed phase across the glass surface which is clearly visible under a light microscope.

2. Cryovials containing CM in a medium/DMSO cryoprotectant are transferred from storage (typically in the vapour phase of liquid N_2) to dry-ice for 2-3 minutes (Note 7) before being brought up to 37 °C using a hotblock or water bath.

3. During step 2 and immediately as the last ice crystals disappear, CM in the freezing solution are taken up into 5ml syringe fitted with a wide-bore filling tube already containing 2ml of pre-warmed RPMI/B27 (Note 8). The resulting cell suspension, in which no attempt is made to mix the DMSO-containing freezing solution with the RPMI/B27 in the syringe, is carefully layered at the bottom of a pre-warmed 15ml conical-bottomed centrifuge tube (Note 9). Pre-warmed RPMI/B27 (8ml) is added to the suspension dropwise over the course of 3 minutes (Note 10). Cell suspension is centrifuged at 400 x g for 4 min and the supernatant containing non-intact cells / ruptured membranes is removed (Note 9). Cells are gently resuspended in RPMI/B27 (3-5ml).

4. A small amount of this CM suspension (≈ 50 µl) is removed, mixed with an equal volume of Trypan Blue solution and incubated at RT for 1 min. This CM/Trypan Blue solution is applied to the counting chamber of a haemocytometer, and the number of viable cells (i.e those that are not stained blue) is determined (Note 11). The efficiency of plating (i.e. the proportion of viable cells that will adhere to the culture surface and exhibit functional competency post-plating) must also be taken into account. We empirically determine the plating efficiency on a batch-to-batch basis; the protocol described here assumes a typical plating efficiency of approximately 60-70%. Following an assessment of viability, the CM suspension is subsequently re-centrifuged (400 x g, 4 min) and the supernatant is decanted. The resulting pellet is resuspended very gently using a 1000 µl pipette tip to a density of 1,000 viable cells per µl (corrected for plating efficiency) in pre-warmed RPMI/B27.

5. Matrigel-treated glass surfaces from step 1 are warmed to 37°C for 1 h in advance of CM plating, and the Matrigel solution is removed from the gaskets < 1min prior to seeding (Note 12). We have empirically determined the optimal seeding density as 2,500 viable CM per mm^2 (adjusted for plating efficiency) (Note 13). From the cell suspension in step 4, 15 µl (15,000 CM) is seeded in a single well of the silicon gasket (7 mm^2 surface area) (Note 14), and the KO-DMEM 'pocket' from step 1, which should still be present, is retained. Chambers are transferred to 37 °C in a humidified 5% CO_2 environment. CM are allowed to

adhere to the coverslip for 2 h before pre-warmed RPMI/B27 is added (2 ml) (Note 15). RPMI/B27 medium is exchanged every 48 h and cells are allowed to mature 'on plate' for 4 to 7 days, at which time the CM will exhibit well-ordered spontaneous Ca^{2+} oscillations (Note 16).

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3.2 Ca^{2+} imaging

1. The excitation and emission profiles, signal-to-noise characteristics, kinetics and Ca^{2+}-binding affinity of Fluo-4[43] make it our fluorescent Ca^{2+}-indicator dye of choice for these studies (Note 17). Cells are incubated under a meniscus (100 μl) of RPMI/B27 containing Fluo-4 AM (5 μM, Molecular Probes) diluted from a freshly prepared stock (1 mM dissolved in DMSO) for 30 min at 37°C in a humidified 5 % CO_2 environment. These conditions result in homogeneous loading of cell cytoplasm and nuclei, but not the intracellular organelles (Note 18).

2. Ca^{2+}-dye loaded CM are transferred to KRH solution or Leibovitz medium (1.9 ml, i.e. to a final volume of 2 ml) (Notes 19 and 20). CM are allowed to equilibrate in KRH or Leibovitz L-15 for 20 min at 37 ºC prior to Ca^{2+} imaging (Figure 2). Regions of a single CM-containing silicon-gasket well are selected at random, and imaging is performed using resonant-scanning confocal microscopy (e.g. SP5, Leica Microsystems) or a camera-based system (e.g. Orca Flash 4.0, Hamamatsu). All Ca^{2+} imaging is performed using a standardised protocol (Figure 2) that aims to reduce inter-experimental variability – using 63X or 20X objectives with images acquired at 512 x 512 or 1024 x 1024 pixels, respectively (Notes 21, 22, 23, 28).

3.3. Determination of cell viability and apoptosis

Immediately following imaging CM viability, cytotoxicity and the extent of apoptosis are determined using Promega's ApoToxGlo triplex assay according to the manufacturer's instructions. Following the ApoToxGlo assay, we also visualise apoptotic nuclei in the same populations using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method according to the manufacturer's instructions (DeadEnd, Promega) (Note 24).

We also investigate latent toxicity, which we define as a measureable effect on cell phenotype over a prolonged period of time, in the absence of any increase in cytotoxicity over the standard 30 min imaging protocol (Note 25). In parallel experiments, CM are subject to medium exchange and are incubated for a further 6 h in RPMI with or without B27 supplement and with or without drug (Notes 26 and 27) (Figure 2).

3.4 Data extraction and processing

Numeric data are extracted from an image series using Leica's image-acquisition software (Leica LAS-AF) or FiJI image analysis tools.[44] We employ two methods for extracting data. For imaging using a 63X objective, we assign regions of interest (ROI, 50 μm^2) where each ROI corresponds to a single CM within the population (Figure 4). We usually assign up to 20 ROIs per field of view (FOV). For Ca^{2+} imaging confluent populations using a 20X objective, we use a regional analysis approach based on dividing the FOV into grids (16, 25, 49 etc.)[6] (Figure 4) (Note 28).

SALVO, a Python-based computer program, is used to profile the amplitude and temporal organization of Ca^{2+} signalling[23, 31]. Ca^{2+}-spike signal maxima and minima and inter-spike periods are detected using SALVO's auto-detection algorithms (based on spline detect or threshold detection methods), or by manual assignation of the start, peak and end of each Ca^{2+} spike. SALVO outputs are archived and mined using a linked SQL-based database system (DB Miner).

We cannot cover all aspects of SALVO functionality (e.g. statistical analysis of parametric variation) in this chapter; more detailed information can be obtained from the corresponding author (CG). For the purposes of this chapter, we focus on a nine-parameter description of Ca^{2+} signaling which is given in Figure 3B and C. A brief explanation of each of the terms:

- Five of these selected outputs parameterise the Ca^{2+} spikes: *frequency, amplitude, duration, area, rate of decay*.
- *Inter-transient noise (ITN)* is a novel descriptor of very low amplitude Ca^{2+} fluxes during inter-spike periods, and is the cumulative signal variability (SV) in a given trace. The calculation of the SV is described in detail elsewhere.[23, 45]

- *Temporal heterogeneity index (THI)* and *amplitude heterogeneity index (AHI)* are statistical measurements of the variability in the duration of inter-spike intervals and Ca^{2+} spike signal intensity maxima, respectively. THI and AHI will be zero in trains of Ca^{2+} spikes in which the amplitude and temporal regimentation of sequential Ca^{2+} spikes are perfect, and will increase as the Ca^{2+} spikes become more disordered.[23]
- *Synchronization* is the calculation of the extent of intercellular Ca^{2+} spike synchronization (Figure 3C).

The data extraction and analytical processes are schematized in Figure 4. Multiparametric SALVO outputs are amenable to incorporation into a single toxicity score (e.g. Figure 5 in [23]) or may be subjected to hierarchical cluster analysis (HCA) to discriminate drug-induced effects (e.g. Figure 6 in [23]), but we do not cover these downstream applications in this chapter.

In the given example both drugs A and B produced marked disruption of Ca^{2+} signalling, but the modes of perturbation were qualitatively different: drug A caused the cessation of spontaneous Ca^{2+} oscillation via the progressive attenuation in Ca^{2+} spike amplitude, whereas drug B elicited different periodic behaviour (Figure 3A). Despite these different modes of Ca^{2+} disruption, quantification of Ca^{2+} spike frequency revealed no differences between drugs A and B with both drugs producing values similar to those obtained under control (no drug) conditions (Figure 4). Clearly this highlights a real limitation of any method of Ca^{2+} spike analysis that does not take account of the specific temporal distribution of the Ca^{2+} spikes. To this end, our novel indices of Ca^{2+} signal organization, namely ITN, AHI and THI, discriminate the different effects of these two drugs (Figure 4).

4. Notes

Note 1. It is conceivable that contaminant non-CMs influence A) the development of CM functional competency, and B) the measured effect of drugs on CMs (e.g. EC_{20} values). We previously used a FACS-based strategy to enrich CMs, but although this improved some of functional properties of the CM it generally resulted in greater phenotypic variability across the population (see Supplementary Figure 6 in

[23]). We also investigated the extent of cell division in these 'non-enriched'/heterogeneous cell populations. In the three years that we have been using Cytiva™ CM we have found no evidence of cell division. In contrast, when using CM differentiated from patient fibroblasts using the method of Burridge and colleagues[37] we do observe cell division although this is rare (see Supplementary Movie 8 in [6]). Even then, we have only ever observed cell division on gelatin matrices, possibly indicating a role for the culture matrix (i.e. 'adhesiveness') in this phenomenon.

Note 2. The axial alignment of cells (Figure 1B and C) is associated with the intracellular redistribution of cardiac TnT into well-organized striatal arrangements, and is a characteristic typical of CM on days 4 to 7 post-seeding (Figure 1D). In this form, CM exhibit spontaneous and caffeine-dependent sarcoplasmic reticulum (SR) Ca^{2+} release and are responsive to electrical stimulation at frequencies that exceed the endogenous Ca^{2+} spike frequency. Although these features suggest the relative maturation of the SR maturation[46], cTnT striation is not a good marker of CM functional maturity.[6, 23, 47-49]

Note 3. This sub-physiological external [Ca^{2+}] is sufficient to support spontaneous contractility, but helps maintain the cells in a relatively 'quiet' state and avoids over-loading the SR which can be a consequence of longer-term culture in medium where [Ca^{2+}] is higher (i.e. ≈ 1.3 mM).

Note 4. CM are extremely sensitive to temperature. We pre-warm the media to 39°C so that it is ≈ 37 °C at the point of addition.

Note 5. On this system operating in uni-directional mode, a scan configuration of 512 x 512 pixels takes 71 ms. The acquisition of an image every 100ms intervals (10Hz) thus gives a period of ≈ 30 ms free from illumination. Under these conditions, the background drift (decay) in fluorescence signal intensity due to photobleaching is typically < 5% across the time series.

Note 6. The dimensions of the 'wells' in the silicon gaskets are 3 mm x 1 mm (diameter and depth, respectively) creating a surface area for CM attachment of 7 mm^2. This is comparable to that of a single well of a 384-well plate, and very similar to the dimensions of the hemispherical front lens of a standard microscope objective. However, unlike in 384-well format, we find that the high value of 'CM-to-medium' in

these chambers (≈ 100 µl medium per 1,000 cells) facilitates the dilution of some para- and autocrine factors that can interfere with cellular synchronization. The silicon gaskets survive multiple rounds of autoclaving but their adhesion to glass is reduced beyond 8 cycles.

Note 7. Warming cells directly from liquid N_2 to 37 °C without this intermediate step on dry ice results in a much greater incidence of cell bursting and a poor recovery of intact cells.

Note 8. We avoid the use of pipettes at this stage and typically use a syringe fitted with a 4mm diameter filling tube to avoid sheering CM.

Note 9. The use of a 'tall' tube, in which the cell pellet is separated from the surface of the 10 ml of solution by ≈ 8 cm, facilitates the separation of the ruptured/non-intact cells from the intact CM intended for seeding. Post-centrifugation the supernatant solution is removed from the top i.e. "chased down" the tube towards the CM pellet.

Note 10. In our hands, the very slow dilution of the DMSO-containing cryopreservation medium to an eventual ratio of 1:10 (v/v) with RPMI/B27 is essential to avoid 'media shock'- a process by which rapid dilution of the DMSO-containing freezing medium damages CM. We don't fully understand the reasons for this.

Note 11. Since non-intact/ruptured cells should be removed in Section 3.1 step 3, CM viability at this stage should exceed 90%.

Note 12. The Matrigel-treated glass surface should not be allowed to dry out and CM should be seeded as soon as possible after the Matrigel is removed. We typically follow a cycle of removing approximately 90% of the solution from 6 chambers (using an automatic pipette set to 14 µl), followed by seeding into these 6 chambers and so on. It is also important that this step be done as cleanly as possible, since any wetting of the gasket surface will lead to the inability to form a stable meniscus described in Step 5.

Note 13. This is the initial seeding density that results in homogeneous dispersion of CM across the culture surface. Subsequent 'self-organization' and alignment of the CMs (see Figure 1B and C), especially the dynamic movement of some CM on a gelatin matrix (see Supplementary Movies 6 and 7 in [6]), means that the distribution of cells on the surface can be very heterogeneous after a few days in culture.

Note 14. Although 'low-density' plating of cells (i.e. 500 - 1,200 cells per mm^2) has been reported to drive a phenotype consistent with electrical remodelling and cellular hypertrophy[50] we find no measureable effect on the Ca^{2+} handling behaviour of the cells or on the extent of intercellular synchronization at cell densities between 200 and 2000 cells per mm^2. CM are only seeded into one gasket because we use the chambers as 'single use' i.e. to investigate one drug concentration (Figure 2).

Note 15. We do not remove the solution from the gaskets prior to 'topping-up' with RPMI/B27. For reasons that we do not fully understand, the development of functional competency in the CMs is adversely affected if the few non-adherent cells (which are usually spherical in shape and/or granular in appearance) are removed after 2 h.

Note 16. We have described the progressive reduction in spontaneous activity in CM with continued time in culture which may correlate with an increased maturation of the cellular Ca^{2+} handling machinery.[6, 23] In our view, the most 'adult-like' phenotype will correspond to the absence of spontaneous Ca^{2+} oscillations, but full responsiveness of CM to external electrical stimulation (i.e. 'pacing'). This will involve CM culture beyond 7 days and is not covered in this chapter.

Note 17. We have used several Ca^{2+} dyes including Fluo-3, Fluo-5 and Fluo-8 that work just as well. However, each dye will produce a different 'fingerprint' of Ca^{2+} signalling that precludes inter-dye comparisons. For this reason, it is best to define which Ca^{2+} reporter dye works best in a particular experimental configuration, and then perform all experiments with the selected dye. Since SALVO analysis depends on recording low amplitude Ca^{2+} fluxes, good definition of basal (inter-spike / inter-transient) Ca^{2+} signals is required. We have used some Ca^{2+} dyes that are not good for this purpose (e.g. Ca^{2+}-orange and Ca^{2+}-crimson).

Note 18. Many researchers use DMSO containing pluronic F127, a poloaxamer non-ionic surfactant, to dissolve Fluo-4 AM. We find that good quality, dessicated-stored DMSO (preferably supplied in low volumes glass vials (1-5ml)) renders the addition of pluronic F127 unnecessary. We also routinely use Fluo-4 that has been packaged in aliquots of 50 µg and one of these vials will typically be used within the course of a week's experiments. The accumulation of Fluo-4 into intracellular organelles will be seen between 60 and 90 min post-loading; during experiments we co-ordinate the timings of loading to negate to this limitation. All CM are imaged within 1 h of Fluo-4 loading.

Note 19. Up to this point, CM have been maintained in culture for several days and then loaded with Fluo-4 in RPMI/B27 containing low [Ca^{2+}] (≈ 0.4 mM) (Note 3). The transfer of CM to KRH solution or Leibovitz L-15 medium raises [Ca^{2+}] to a physiologically-relevant level (≈ 1.3 mM) and reproducibly results in spontaneous physical contraction and Ca^{2+} oscillation at frequencies > 0.3 Hz in > 90% of CM populations after 3-5 mins.

Note 20. For those drugs with relatively low water solubility (e.g. maximum solubility in water of 10 mM) at pH7.4 and at 23 °C, or those first require solubilisation in DMSO or methanol, we use KRH. Otherwise Leibovitz L-15 is our medium of choice. Neither medium/solution has any effect on the biological activity of any drug we have investigated to date.

Note 21. In this protocol the maximum experimental window is limited to 30 min to avoid the intracellular accumulation of Fluo-4 inside organelles that makes data interpretation difficult (Note 18). The use of genetically encoded Ca^{2+} indicators[51] for longer term studies of Ca^{2+} signalling may avoid this problem. In the study of Lewis et al., drug-induced perturbation in Ca^{2+} signalling was evident after just 3 min.[23]

Note 22. Accurate deconstruction of Ca^{2+} signals (Figure 3B) requires image sampling at rates between 5 and 10 times greater than the frequency of Ca^{2+} spikes. For example, a Ca^{2+} spike frequency of 1Hz would require image acquisition between 5 and 10 Hz (e.g. every 100 or 200 ms).

Note 23. Sometimes the extent of physical contractility makes imaging difficult. For example, a CM cluster may move between ROIs on a grid (Figure 4). In these instances, we have used blebbistatin (5 μM), an agent that inhibits myofilament shortening but preserves normal Ca^{2+} cycling.[23, 52] In our experience, blebbistatin has a small but measurable effect on CM Ca^{2+} signalling, and the impact of this agent must be taken into account when interpreting the effects of subsequent drug additions on blebbistatin-immobilised CMs.

Note 24. Although there is usually an excellent correlation between caspase 3/7 activation and the full induction of apoptosis, we have (rarely) found that data obtained from caspase 3/7 activation assays do not correlate entirely with the extent of apoptosis 'classically' determined by DNA fragmentation assays. Anecdotally, we sometimes observe that caspase-activation-dependent signals are disproportionally higher than the number of apoptotic nuclei identified by TUNEL. This may point to reversibility in the caspase-activation dependence of apoptosis as others have reported in other cell types.[53-55] To negate this issue, we routinely perform both caspase-activation (ApoToxGlo) and TUNEL (DeadEnd) assays on the same CM population.

Note 25. An investigation of latent toxicity should be a component of CIPA.

Note 26. We have not observed any different concentration-dependent effects of a drug depending on whether the B27 supplement was included in the medium or not during this 6 h incubation. However, it is possible that some drugs do interact with the components of B27 thereby reducing their effective free concentration. To address this, parallel experiments in the absence of the B27 supplement are done.

Note 27. This 6 h incubation in the presence of drug allows the exploration of the more chronic aspects of toxicity. The rationale for performing parallel investigations in the *absence* of drug comes from reports of 'cell memory' in which cell populations can apparently recover from noxious stimuli only to exhibit marked dysfunction at a later time point.[15, 56]

Note 28. To avoid artefact, the calculation of ITN requires the analysis of signal intensity from > 500 pixels. Using a 63X objective, the FOV corresponds to 21,025 μm^2 (145 x 145 μm). At 512 x 512 pixels

(262,144 pixels), user-defined ROIs of 50 μm^2 (white boxes; 63X panels) correspond to 625 pixels. In this configuration, the number of pixels per μm^2 is 12. Using a 20X objective, the FOV corresponds to 207,936 μm^2 (456 x 456 µm). In these data acquired at 1024x1024 pixel resolution (1,048,576 pixels) each region of the grid is $1/25^{th}$ of the FOV and will comprise 8,317 μm^2 (41,943 pixels). In this format, the number of pixels per μm^2 is 5.

Note 29. CM are very sensitive to mechanical deformation and even the addition of drug-containing medium can provoke altered Ca^{2+} signalling. To avoid this, any additions to the chamber are performed well away from the silicon gasket/glass culture surface, and the addition of a typical volume of 100 µl ensures good mixing of the drug.

Note 30. Confocal microscopy generates images of discrete planes of depth (z-sections) and those cells that exhibit very different morphologies (e.g sphericity) lying above the plane of the adherent monolayer do not contribute to the Ca^{2+}-dependent fluorescent signal. Imaging modalities that capture fluorescent signals throughout the entire depth of the sample (e.g. non-confocal imaging) will not differentiate signals coming from *bona fide* CM that are adherent to the attachment matrix, and those cells which lie above this plane.

Note 31. To avoid artefact from CM that exhibit atypical behaviour under baseline conditions, we apply inclusion criteria; typically frequency > 0.3Hz, AHI < 0.2, baseline drift < 5 % over 30 s, signal-to-noise > 2. These thresholds typically result in the inclusion of > 90% of all data.

Note 32. In those traces in which a concentration of drug produces a cessation of Ca^{2+} spikes (e.g. drug A at t = 16, Figure 3A) it is not possible to perform SALVO analysis. In these instances it is obvious that these drug concentrations / time points have catastrophic effects.

5. Conclusion

The methods described here, which provide an integrative analysis of Ca^{2+} spike characteristics and low amplitude inter-spike Ca^{2+} fluxes, comprehensively profile CM Ca^{2+} signalling. However, with some work-

up, the system could be configured for 'multiplexing' spectrally-separated ion-sensitive fluorescent probes, or combined with electrophysiological platforms.[57] In addition to enabling enhanced drug safety/toxicology screening in CM, SALVO could be applied to profiling drug toxicity in other cells (e.g. hepatocytes) and to the analysis of other cellular signals that change on different timescales (e.g. pH).

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Figure legends

Figure 1. CM plating and formation of syncytia

(A) A silicon gasket, containing four wells each with a surface area each of 7mm^2, on a glass bottomed chamber.

(B) CM between days 4 and 7 post-plating should be confluent and clear directional alignment (arrows).

(C) In some instances, CM form highly tensile 'strings' that contract in a unidirectional manner.

(D) CM alignment is associated with cTNT striation (red). Nuclei are counter-stained with DAPI (blue). From [23]

Figure 2. Experimental protocol

In the initial step (a), CMs are transferred to 1.9 ml of pre-warmed KRH or Leibovitz L-15 for 20 min prior to imaging baseline Ca^{2+} signals ('Basal', 30 s) (Notes 19 and 20). Stock solutions of all drugs in either KRH or Leibovitz L-15 are added to the chamber in a bolus (100 µl; i.e. a 1:20 (v/v) dilution in the chamber) (Note 29). For the 6 h incubation after imaging at t = 30 min, CM are transferred to RPMI with or without B27 and with or without the drug under test (Notes 26 and 27).

Figure 3. Quantifying different modes of Ca^{2+} signalling perturbation.

(A) Ca^{2+} oscillations in single CM under basal (drug naïve) conditions and following the addition of media only (control) or Drugs A and B as per the protocol described in Figure 2. Ca^{2+} signals immediately

following control- or drug-addition (t=0) and subsequently at 4-, 8-, and 16 min are shown. These data are analysed in Figure 4.

(B) The deconstruction of Ca^{2+} spikes, and inter-spike intervals, into eight parameters that describe intracellular Ca^{2+} signalling. *, the descriptions calculate data for individual spikes which are then averaged across the Ca^{2+} spike train. Adapted from Lewis *et al.*[23]

(C) The extent of intercellular synchronization is calculated using a matrix which quantifies the temporal co-incidence of Ca^{2+} spikes across CM. In the given example, using four CM (1-4) with four Ca^{2+} spikes in each CM (a,b,c,n), the intercellular synchronization is 33.3%. Reproduced with permission from Lewis *et al.*[23]

Figure 4. Scheme for data processing and visualisation.

* Notes 28 and 30

** Note 31

The upper panel shows typical FOVs obtained from brightfield (top) or Fluo-4-dependent fluorescence (bottom) imaging of CM using 63X or 20X objectives. For those images acquired using a 63X objective, the white boxes represent 50 μm^2 ROI, whereas for images obtained using a 20X objective the FOV is divided into grids (see Note 28).

The lower panel shows the visualisation of SALVO analysis corresponding to the Ca^{2+} oscillation traces shown in Figure 3A. Since these traces are from single cells, the extent of intercellular synchronization is not given. The absence of Ca^{2+} spikes at t=16 with drug A (Figure 3A) precludes SALVO analysis, so only data from t = 0, 4 and 8 are shown (Note 32).

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