Chapter 10

Pharmacogenomic Screening of Drug Candidates using Patient-Specific hiPSC-Derived Cardiomyocyte High-Throughput Calcium Imaging

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Abstract

Calcium imaging is an invaluable technique to detect and characterize calcium flux in cells. The use of calcium dye provides information on the concentration and spatial distribution of calcium. Calcium imaging is a well-established technique to assess the calcium-induced calcium release mechanism in cardiomyocytes. It can also be used to characterize mutations in genes crucial for this mechanism that frequently causes arrhythmia. Here we describe a high-throughput methodology of calcium imaging that records individual calcium transients in more than 10,000 human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) in less than 30 min.

Key words hiPSC-derived cardiomyocytes, Drug screening, Calcium imaging, Arrhythmia, High-throughput

1 Introduction

Calcium is a ubiquitous intracellular messenger that passes through ion channels, pumps, and exchangers to create a calcium gradient. This gradient is crucial to cellular calcium signaling. Calcium signaling occurs in excitable cells such as cardiomyocytes as well as non-excitable cells such as endothelial cells and is involved in many pathways contributing to motility, proliferation, differentiation, and apoptosis [1]. Calcium signaling can be observed as an intracellular calcium transient which is described as a "wave" or by a localized increased concentration known as a spark.

Excitation-contraction coupling (ECC) is the major mechanism that converts electrical excitation to cardiomyocyte contraction [2]. Calcium enters the cell through L-type calcium channels, increasing the intracellular concentration of calcium locally at the submembrane. This leads to ryanodine receptor activation on the sarcoplasmic reticulum (SR) and results in massive calcium release

from the SR to the cytosol. This mechanism is called calcium-induced calcium release (CICR) and can be observed in cells as a calcium transient [3]. Localized release of calcium through the SR can be observed as a spark. The increase in total intracellular calcium signals the cell to contract, relax, and cycle calcium out of the cytosol to both the extracellular space and the SR. Capturing this mechanism through calcium imaging has generated significant progress in understanding the contribution of calcium homeostasis dysfunction in arrhythmias. Calcium imaging remains an essential tool to study clinical phenotypes involving calcium dysfunction.

Calcium signaling was first observed in living cells, specifically muscle fibers, in 1967 [4] and CICR was first imaged in 1980 [5]. The development of confocal microscopy has led to significant progress in calcium imaging, including the first recording of calcium sparks in cardiomyocytes in 1993 [6]. These analyses are performed by recording signals from one cell at a time, which is low throughput and not compatible with drug screens that require a high number of cell recordings and various drugs and concentrations. Recent high-throughput systems such as the FLIPR assay have been used to facilitate drug screenings but are only able to capture the whole cardiac flux generated by thousands of cells in an individual well. Here we describe a protocol that supports high-throughput experiments and provides single cell recording and analysis. Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are a powerful in vitro tool to study patient-specific clinical phenotypes and utilize in drug screening. Here we describe a high-throughput method for calcium imaging at the single cell level using patient-specific hiPSC-CMs to understand disease mechanisms and identify new treatments for patients.

2   Materials

2.1   Cell Preparation

1. 96- or 384-well black cell culture microplates, polystyrene, F-bottom, µClear (Greiner).

2. Matrigel: Growth factor-reduced Matrigel diluted 1:800 in Dulbecco's Modified Eagle Media (DMEM, Corning).

3. RBAI media for hiPSC-CMs: RPMI 1640 (Corning), 500 µg/ml fatty acid-free albumin (GenDEPOT), 200 µg/ml L-ascorbic acid 2-phosphate (Wako), 5 µg/mL *E. coli*-derived recombinant human insulin (Gibco). Supplemented with 10% Cosmic Calf Serum for cell plating.

4. hiPSC-CMs: Generated using typical protocols, on d16 of differentiation, dissociated.

2.2   Recording Ca²⁺ Transients

1. Dye loading solution: Hanks' Balanced Salt Solution (HBSS, Corning), 20 mM HEPES (1 M stock, Corning), 0.04%

Pluronic F-127 (5% stock in water, Sigma), 2.5 mM probenecid (500 mM stock, water-soluble, AAT Bioquest), 2 drops/10 mL NucBlue Live ReadyProbes Reagent (Invitrogen), 1 µM Cal-520 AM (1 mM stock in DMSO, AAT Bioquest).

2. Imaging solution: FluoroBrite DMEM (Gibco), 2.5 mM probenecid.

3. Vala Sciences IC200-Kinetic Imager Cytometer (KIC).

4. CyteSeer Scan software.

2.3 Analysis

1. CyteSeer Analysis software.

2. Microsoft Excel.

3. GraphPad Prism.

3 Methods

3.1 Cell Preparation

1. Coat a 96- or 384-well black cell culture plate with diluted Matrigel (100 µL or 25 µL, respectively, per well).

2. Seed dissociated d16 hiPSC-CMs into a 96-well black cell culture plate (7.5×10^4 cells per well) or 384-well black cell culture plate (2.5×10^4 cells per well) in RBAI media supplemented with 10% CCS for 48 h. Keep cells at 37 °C, 5% CO_2.

3. Change media with RBAI without CCS every other day until drug addition. Add drug to hiPSC-CMs at the pre-determined dose and timepoint.

4. On the day of the experiment (typically d30), take 10 mL of HBSS supplemented with HEPES and add 80 µL of 5% Pluronic F-127, two drops of NucBlue Live, 50 µL of 500 mM probenecid, and 10 µL of 1 mM Cal-520 AM. Keep protected from light. Aspirate media and add 100 µL of the dye loading solution per well of a 96-well or 25 µL per well of a 384-well and incubate 1 h at 37 °C, 5% CO_2. After an hour of incubation, aspirate media and add 100 µL of the imaging solution to each well of a 96 well (*see* **Notes 1** and **2**).

3.2 Scan

1. Open CyteSeer Scan software, click "Eject" on the main screen, and firmly load the plate with lid into the holder. Press OK to close the drawer (*see* **Note 3**).

2. Click "Setup Scan" on the main menu to create a new configuration file (skip to **step 3** if a configuration file has already been created).

   (a) File and Well Plate Settings.

      Create a name for the protocol to use for future experiments by entering a name in the "Configuration Name" field. Click "Eject Plate" if the plate has not yet been loaded. Use the "Well Plate Type" drop-down menu

to select a 96- or 384-well plate layout (*see* **Note 4**). Once the well plate type is loaded, select the wells to be scanned on the plate map and select the field of view(s) within the intrawell arrangement map (*see* **Note 5**). The current objective should be 20×. This field will default to the objective that is currently installed in the machine. This cannot be edited once the protocol is saved so a separate protocol needs to be created to run the same protocol with a different objective (*see* **Note 6**).

(b) Focus Settings.

For the focus method select "Image Contrast and Surface Tracking." For the fluorochrome, select "DAPI" (or appropriate channel for the nuclear stain). The automatic focus works better with the nuclear staining rather than the calcium flux (*see* **Note 7**). To begin, use 100 ms time exposure and 50% intensity and adjust as needed (*see* **Note 8**). The "Focus Position" section is useful for manually focusing and exploring wells. The Z step size can be changed from a more fine or coarse focus by adjusting between 100 μm, 10 μm, 1 μm, and 0.1 μm steps. The "Find Surface" button will locate the cells making it easier to manually focus. The focus position scale can be moved from 0 to 20,000. Be careful when scrolling manually up and down because the objective can knock the plate out of the holder. Use both the plate map and well plate position to move around plate and each well to determine which areas to image. Click "Advanced Settings." Set focus stack settings to 20.0 range (μm), 1.0 step size (μm), lower cutoff of 100, and upper cutoff of 300. Adjust these settings accordingly depending on the autofocus range needed and the type of plate. Click "Image Trail Focus" to adjust different z positions and z step sizes as well as lower and upper cutoffs to see what works best for the focus of cells in a specific well. Once these parameters are established, click "Full Image Trial" to set the autofocus for the run. Once the configuration file is established, perform this step last when setting up a scan. Once the full image trial is complete, click "Done" to exit out of this screen and return to the focus setting screen (*see* **Note 9**). Click the light bulb icon to check the focus of the nuclear stain and switch to the FITC channel to ensure the calcium staining is also focused.

(c) Channel Acquisition Settings.

Establish the channel acquisition settings by using the "+" symbol in top right corner to add channels or the "x" symbol to delete channels. Two channels will need to be established with the following settings as a starting point and adjusted as needed:

Nuclear Channel – Channel Name: Nuclear Channel. Acquisition Type: Single Image. Fluorochrome: DAPI. Exposure: 100 ms. Intensity: 50%. Offset: 0 μm

Calcium Channel – Channel Name: Calcium Channel. Acquisition Type: Kinetic Series. Fluorochrome: FITC. Exposure: 10 ms (this provides the fastest capture rate possible for this machine). Intensity: 100%. Offset: −0.1 μm. Within the Advanced Settings: KIC Acquisition Length: 10 s. Frame Rate: 99.75

  (d) Scan Summary.

Click the paper and pen icon to save the configuration, close out of the configuration setup, and return to the main menu. Once the scan configuration is saved, this template can be used for future experiments instead of repeating this process each time.

3. Click "Scan Editor" on the main screen (use this once a scan configuration is established).

4. Go to "File," then "Load Scan Configuration," and select the desired scan configuration. Navigate through the protocol by clicking on the sections in the menu on the left.

  (a) Well Plate: Select the well plate type as well as the wells and areas within the wells to be scanned. The objective set for this scan configuration cannot be changed (*see* **Note 10**).

  (b) Focus: Perform a full trial focus using the nuclear channel before each plate is scanned.

  (c) Acquisition: Keep exposure and intensity settings the same between different scans to be able to compare experiments more accurately.

  (d) At the end, click "Save and Scan Time" to go to the scan window.

5. Select scan properties window.

  (a) Scan Configuration File: "File Path" and "Configuration Name" will be the file path and configuration loaded through the scan editor.

  (b) Data Destination: Select where to save the data collected during the scan (*see* **Notes 11** and **12**).

  (c) Check the boxes next to "File Set" and "Scan Selection Set" to make the "Scan Now" button active and click it.

  (d) The scan will begin. The images can be viewed as they are acquired in real time. It is important to ensure that both channels are in focus. Otherwise, exit out of the scan, try refocusing in the scan editor, and start the scan again. If this does not fix the focus problem, make sure the plate is sitting properly in the holder.

3.3 Analysis

Open the CyteSeer Analysis software. Configure the "Source Folder" to the scan file to be analyzed. If this is the first analysis since opening the software and of this experiment, the "Result" folder will be set to "<Automatically Create Results Folder>" by default. Select IC200 (normal) for the "Image Naming Convention" and then select "Plate." Use the algorithm called "Cardiac Calcium Time Series." Below are the different parameters to configure when setting up an analysis to run.

1. Defining Masking Parameters: In the "Assign Channels" column, check the boxes next to the "Nuclear Channel" and the "Calcium Channel." The "Nuclear Channel" corresponds to the DAPI filter used to detect the nuclear stain and the "Calcium Channel" corresponds to the FITC filter used to detect the intracellular calcium transients. To adjust the masking of the cells, modify the "Sensitivity" and "Size Value." The sensitivity may vary from 25% to 400% and the size value from 3 to 30 (*see* **Note 13**). Go to the "Setup Run" tab next. All the wells scanned are listed here. To determine the masking parameters, select only one or two wells. Click "Run" and then "OK" on the following "Configure Algorithm" window (*see* **Note 14**). After the run is complete, the software will generate a "Results Folder" that contains the masking results. To check the quality of the masking, go to "View" in the menu and select "Image Viewer." Select a well to examine from the dropdown menu. Open the "Image Viewer Set Colors" and select "Cell Mask" and "Cytoplasm" in the "Edge" column as well as the color for each mask. Zoom in or out on the image to better view the masking for each channel.

2. Running Analysis: Once the appropriate mask for each channel is determined, the calcium transient analysis can be run. On the "Assign Channels" tab, enter the chosen masking parameters. Then, go to the "Setup Run" tab and select the wells to be analyzed. Click "Run." The same "Configure Algorithm" window will open. Check the box "Make Measurements" to obtain the complete data rather than just the mask information. Check or uncheck the "Reanalyze Data Tables" box. In both cases, the program will reanalyze. For the "Starting Time Point," drag the cursor to 0, and for the "Ending Time Point," drag the cursor to the last point. Calculate the "msec Per Frame" value. This value is directly linked to the parameters set when the plate was scanned so this value must be calculated for each individual run. The default value is 33.333 and the software does not automatically calculate it (*see* **Note 15**). If there is significant photobleaching, check the box "Subtract Exponential Baseline." With this setting, the software will correct for photobleaching (*see* **Note 16**). The default value for the "Smoothing Radius" is 2 frames. The default value for "Peak

Noise Threshold" is 0.1 (*see* **Note 17**). Check the box "Detect EADs" to include this parameter in the results. Check "Accept Only Full Peaks" (*see* **Note 18**). The default value for the "Max Baseline Asymmetry" is 0.1 (*see* **Note 19**). Always check "Trim Peaks Before Upswing." Do not check "Align the Peaks Before Averaging."

3. Exporting Results: Once the analysis is complete, the data need to be exported. If data are exported after closing the analysis software, be sure the "Source" and "Result" folders are set to the file path of the analysis to be exported.

   (a) Individual Cells in a Well

   On the main screen, go to the "View" menu and select "Data." This menu will have a "Well Data" tab where the individual cell data per well can be viewed. Select a well to display the time series of individual cell transients and scan the results for quality.

   Display Time Series (Traces): To view the whole trace of the recording, select "Trimmed Cell on Cell Mask Average Pixel Intensity Function of Time" within the measurement list. This option corresponds to the normalized data. Every datapoint is divided by the minimum of the fluorescence (F_{min}) and then subtracted by 1 (formula: $\frac{F}{F_{min}} - 1$). To view the first peak of each cell, select "Pk#1 Cell on Cytoplasm Average Pixel Intensity Function of Time." Click "Export" to export CSV file of these traces and plot in a program of choice.

   Display Table (Values): Click on the "All Measurement" drop-down menu and select "Means." Click the "Uncheck All" button. Click all measurements (25 total) that begin with "Cell on Cytoplasm Average Pixel Intensity" and "Mean of Pk #1 Cell on Cytoplasm Average Pixel Intensity" (*see* **Note 20**). Click "Update." Click "Export" to export a CSV file.

   (b) Well Average

   The "Data" menu also has a "Well Plate Data" tab which provides average well data.

   Display Time Series (Traces): To view the whole trace of the recording, select "Average of Trimmed Cell on Cell Mask Average Pixel Intensity Function of Time" within the measurement list (*see* **Note 21**). In the "Measurement" list, select "Average of Pk#1 Cell on Cytoplasm Average Pixel Intensity Function of Time." This will display the average of the first peak of each well (*see* **Note 22**). Click "Export" to export CSV file of these traces and plot in a program of choice (*see* Fig. 1).
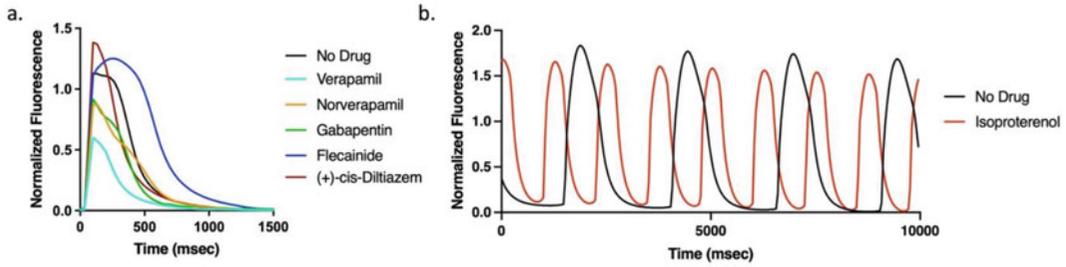
Fig. 1 Intracellular Ca^{2+} transients on Vala KIC. (**a**) Well average calcium transient traces of control hiPSC-CMs +/− drug treatment. Cells were treated for 72 h with media (control) or verapamil (10 μM), norverapamil (30 μM), gabapentin (30 μM), flecainide (30 μM), and (+)-cis-diltiazem (30 μM). (**b**) Whole trace of recording of 10 s of control hiPSC-CMs treated or not with isoproterenol (1 μM)

> *Display Table (Values)*: Click on the "All Measurements" drop-down menu and select "Means." Click the "Uncheck All" button. Click all measurements (25 total) that begin with "Cell on Cytoplasm Average Pixel Intensity" and "Mean of Pk #1 Cell on Cytoplasm Average Pixel Intensity" (*see* **Note 20**). Click "Update." Click "Export" to export a CSV file.

4   Notes

1. Do not forget the nuclear dye. The CyteSeer software algorithm will not be able to detect the cells without the nuclear staining. If the nuclear dye is not present at the time of the scan, simply prepare the imaging solution with two drops of Nuc-Blue (*see* Subheading 3.1, **step 4**) and replace the media in the plate with this solution. The nuclei will be stained within 10 min. If the plate has been read, the only option is to analyze the data manually with ImageJ but this is time consuming for a full 96-well plate.

2. The imaging solution can be pre-warmed for 10 min in a water bath before adding it to the plate and/or the plate can be incubated for 10 min after adding the imaging solution in the IC200-KIC incubator at 37 °C, 5% CO$_2$, during the scan setup.

3. The built-in IC200-KIC incubator should be turned on at the time of dye loading to allow the incubator to reach the set temperature. The CO$_2$ can be turned on at the time of the scan.

4. The IC200-KIC is compatible with most brand and size of plate. New plate-specific templates can be created by following the machine manual.

5. The wells and location within the wells to be scanned can be changed at a later point in the protocol if needed.

6. This machine is compatible with various objectives including $10\times$, $20\times$, and $40\times$ objectives. To change the objective, go to main screen and click "Setting." From the settings dialog screen, select "Change Objective" and select the desired objective. Once this objective is manually installed, select "OK." The desired objective is now ready to use.

7. Other channels include DAPI, FITC, TRITC, Cy5, and Brightfield.

8. Intensity needs to be set to greater than 1 in order to visualize the cells. Otherwise, the screen will remain black until this setting is increased.

9. If this fails, the nuclear dye might not have been added, the wrong channel is selected, or the intensity is set to 0. To check for dye, click "Find Plate Surface" on the focus settings screen and manually look for the nuclear stain. Another method is to try the full image trial using the calcium dye channel but this may fail due to the continuous flux causing the machine to not focus properly.

10. If the configuration requires an objective that is not currently installed in the machine, an error message will notify the users and the objective will need to be changed before running the protocol.

11. Create a new folder and give it a descriptive name. The file name automatically generated is based on the configuration file name and date and there are no fields to enter descriptive information for each run.

12. Based on the numbers of wells and fields selected, the estimated space required will be calculated, and if the file is too large, a warning message will appear saying "unable to start scan: space required exceeds free space." In order to start the run, the estimated space required must be less than the space available in the location where the file will be stored. For example, to scan 10 s at 10 ms exposure for a 96-well plate, ~1 TB is required. Therefore, it is important to have large amounts of storage available on the computer collecting the scan data.

13. To find an appropriate mask for the "Nuclear Channel," begin with a size value of 3 and the sensitivity set to 100%. For the nuclear mask, if there are two masks for a single nucleus, the size value of the "Nuclear Channel" is too low. Increase this value until there is a single mask for each nucleus. On the contrary, if there is one mask for two nuclei, the size value of the "Nuclear Channel" is too high. By adjusting the sensitivity
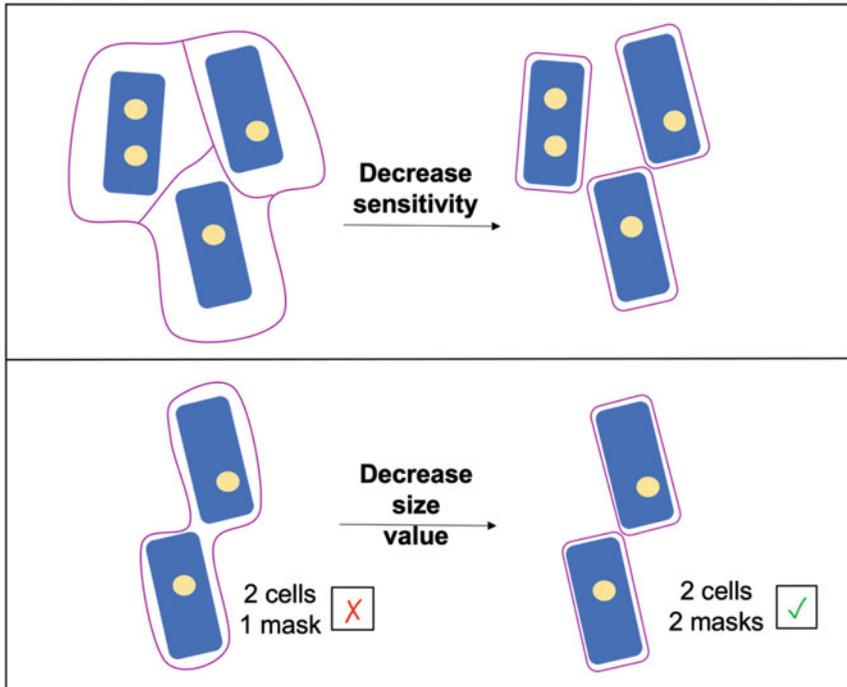
Fig. 2 Schema representing how sensitivity and size value affect the masking of the cells

of the "Nuclear Channel," the size of the mask will change. Therefore, if the current mask does not distinguish two different nuclei, lower the sensitivity value. To find an appropriate mask for the "Calcium Channel," begin with a size value of 30 and the sensitivity set to 100%. Adjust these parameters as needed, following the above guidelines. For example, if the mask line is too far from the cell border, decrease the sensitivity value (see Fig. 2).

14. It can take several runs to test different masking parameters. Each run can take 5 min or more. To decrease the run time, first, uncheck all the boxes on the "Configure Algorithm" window. Second, choose 0 as a starting time point and 5–10 as the ending time point since this will be sufficient to determine the "Nuclear Channel" and "Calcium Channel" mask.

15. Failure to calculate this value for each scan will result in data that contains incorrect time course information and will require the analysis to be run again with the correct value. To calculate this value, divide the duration of the recording (e.g., 10,000 ms) by the number of frames (e.g., 249 frames). In this example, the value would be 40.16 ms so 40.16 would be entered into the field instead of 33.3333. There are two methods to determine the number of frames:
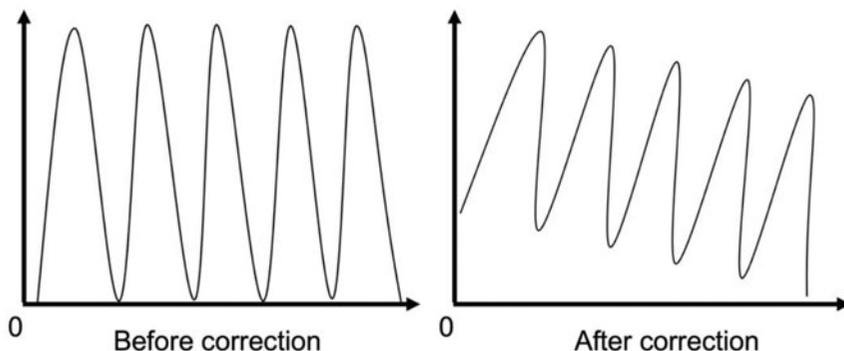
Fig. 3 Example of overcorrection. Whole trace of recording before and after correcting by selecting "Subtract Exponential Baseline" when no photobleaching was present during the experiment

(a) In the "Configure Algorithm," move the cursor to the end in the time point section of the window. The number between the brackets after "Ending Time Point" is the number of frames for this run.

(b) Go to the results folder stored on the computer, open the "Scan" folder, and within that folder, open any well folder. Then, open the folder named "Calcium." The number of pictures in this folder is the number of frames.

16. If "Subtract Exponential Baseline" is selected but there is no photobleaching, the fluorescence at t_0 will be greater than 0 (*see* Fig. 3). If this occurs, re-analyze without checking this box.

17. If the detection of the number of peaks is not accurate, the "Peak Noise Threshold" may need to be changed.

18. If the recording started when the cells were in the middle of calcium transient, the first peak will be incomplete. If the "Accept Only Full Peaks" is checked, the software will not count the peak selected in red (*see* Fig. 4).

19. When the value is 0.1, the software will accept a 10% difference between the baseline before the peak and after the peak. If the difference is higher than 10%, the software might count the two peaks as one or consider the next peak as an EAD. If the value of the "Max Baseline Asymmetry" is 0.1 and the blue arrow represents more than 10% of the red arrow, the software will count the last two peaks as one peak as if the third peak was an EAD after the second peak (see Fig. 5).

20. Here is a list of measurements we routinely use for each experiment: Max Downstroke Velocity, Max Upstroke Velocity, Peak Value, CTD10, CTD25, CTD40, CTD50, CTD70, CTD75, and Time from 75% to 25%. Relative velocity sigma provides information about noise.
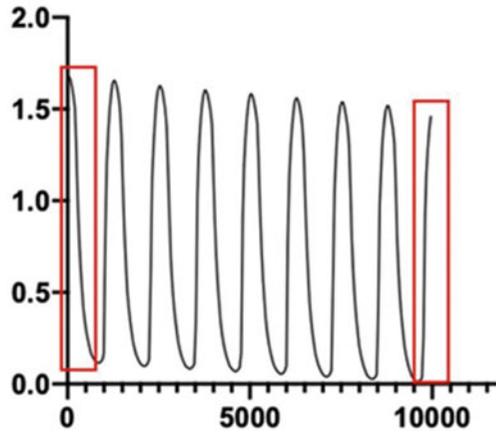
Fig. 4 Whole trace of recording where the first and last peak are incomplete
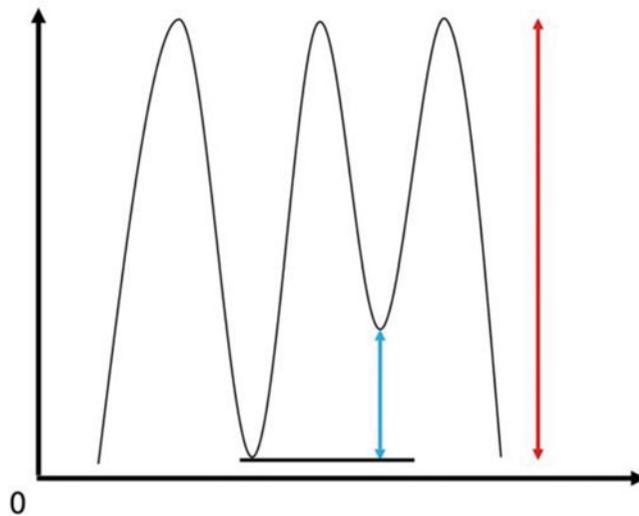

Fig. 5 Baseline asymmetry detection

21. The average whole trace displays the average recordings of all the cells in a well over the full duration of the recording. If the cells are synchronously fluxing calcium, the individual trace for each cell will align to one trace. If the cells are not synchronously fluxing calcium, the individual trace for each cell will not align and appear disorganized. This will not correspond to any relevant calcium information.

22. It is important to check that the parameter selected in the "Configure Algorithm" window for the run analysis is showing the correct results. The detection of the first peak can be incorrect because the first two peaks can be detected as one (*see* **Note 17**). In that case, screen each well by eye and verify that there is only one peak. If the first peak has a small second

bump attached to it, it is possible that the software correctly detected the first peak for some cells but incorrectly detected two peaks instead of one for other cells. The average will display an "abnormal" peak with a second bump that can be incorrectly interpreted as a phenotype. To ensure this is not the case, look through the traces of the first peak at the individual cell level to confirm that there is only one peak. If the software has detected two or more peaks instead of one, re-run the analysis with new parameters.

Acknowledgement

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