Assessment of pro-arrhythmic effects using Pluricyte® Cardiomyocytes on the Axion BioSystems Maestro™ MEA system

Version 1.2

CONTENTS

GETTING STARTED

MSDS

A Material Safety Data Sheet (MSDS) for Pluricyte® Cardiomyocytes is available online at www.pluriomics.com/safety.

Technical support and training

Our scientists are ready to help you with any questions you may have regarding this application note or our Pluricyte® Cardiomyocytes. In addition, in-lab training is available upon request. For further information please visit our website www.pluriomics.com, or contact us directly by e-mail (support@pluriomics.com).

1. INTRODUCTION

Pluricyte® Cardiomyocytes are highly suitable for Axion Maestro MEA assays

Pluricyte® Cardiomyocytes are fully functional human induced pluripotent stem cell (hiPSC) derived ventricular cardiomyocytes that are particularly suitable for electrophysiology-based multi-electrode array (MEA) assays for predictive safety pharmacology, toxicity testing and efficacy screening in early drug discovery. The combination of Pluricyte® Cardiomyocytes and the Axion Maestro MEA system enables detailed electrophysiological detection of potential cardiotoxic/proarrhythmic effects of test compounds at 48- and 96-well plate formats. Pluricyte® Cardiomyocytes' well-pronounced depolarization and repolarization peaks permits easy detection of electrophysiological parameters (e.g. depolarization/repolarization peak amplitudes, beat rate, field potential duration) and facilitate efficient data analysis and interpretation of studies performed with the Axion Maestro MEA system.

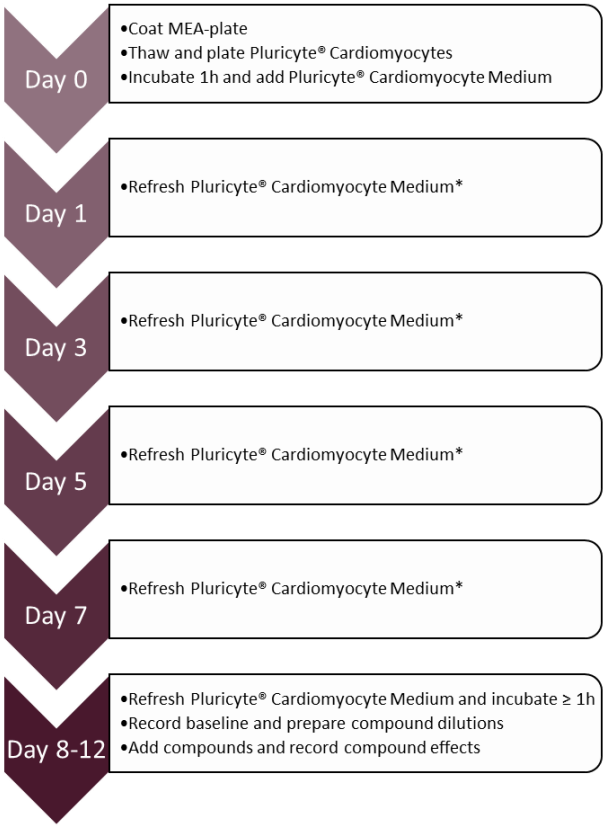
Pluricyte® Cardiomyocytes strengths and characteristics

Pluricyte® Cardiomyocytes exhibit a relatively high level of maturity, when compared to other human stem cell-derived cardiomyocytes and present the following unique characteristics:

- High purity of ventricular cardiomyocytes
- Low resting membrane potentials (~-78 mV)
- Fast upstroke velocities and action potential amplitudes
- Organized sarcomeric structures
- Monolayer field potential contains well-pronounced depolarization and repolarization peaks, enabling easy detection of field potential durations in MEA assays

This application note describes our recommendations for the analysis of Pluricyte® Cardiomyocytes electrophysiology by MEA measurements using the Maestro MEA system (Axion Biosystems, Atlanta, GA, USA) on **48- and 96-well plate formats**. In addition, a case study describing the assessment of the effects of a set of pro-arrhythmic compounds in Pluricyte® Cardiomyocytes, showing the expected pharmacological responses is provided in this document. Pluricyte® Cardiomyocytes, cultured in Pluricyte® Cardiomyocyte Medium, in combination with the Axion Maestro system provide a highly relevant *in vitro* assay platform to study the cardiac safety profile of compounds during drug development.

2. WORKFLOW



* Optional: in order to monitor the condition of the Pluricyte® Cardiomyocyte monolayer it is advised to perform daily measurements (≥ 1h after refreshment).

IMPORTANT RECOMMENDATIONS

- Carefully follow the thawing and seeding instructions, this step is essential for optimal cell survival and attachment to the plate (**Section 4.2 & 4.5**).
- We strongly recommend to use fibronectin as coating substrate for the MEA plates. Other types of coatings may reduce the signal and/or impact the condition of the cells.
- Always refresh the Pluricyte® Cardiomyocyte Medium of the cells the day after seeding the cells (**Section 4.3 & 4.6**). Subsequently, refresh the medium of the cells every 2 days, or 3 days when refreshing at Friday and Monday to prevent weekend work.
- First contractions of Pluricyte® Cardiomyocytes appear between 24-48 hours post-thawing. It will take 3-4 days before the cells have formed an electrically coupled monolayer. Stable beating monolayers can be observed 7-8 days post-thawing. The optimal time window to perform electrophysiology-based assays with Pluricyte® Cardiomyocytes is between 8-12 days after plating the cardiomyocytes.
- Turn on and warm the Maestro MEA system to 37˚C before each measurement, this can take up to 10 minutes.

3. EQUIPMENT, MATERIALS AND REAGENTS

Table 3.1: Equipment

Equipment	Manufacturer
Axion Maestro™ MEA system + AxIS software	Axion Biosystems
Class 2 laminar flow cabinet	Various
Incubator at 37˚C, with 5% CO_2 and humidified air	Various
P20, P200 and P1000 pipettes	Various
12-channel Multichannel pipette (or adjustable pipette)	Various
Hemocytometer	Various

Table 3.2: Materials

Materials	Manufacturer	Catalog number
Classic MEA 48 plate	Axion Biosystems	M768-KAP-48
Classic MEA 96 plate	Axion Biosystems	M768-KAP-96
Sterile disposable 5ml pipettes	Various	
Sterile disposable 10ml pipettes	Various	
Sterile disposable 25ml pipettes	Various	
Sterile Eppendorf tubes	Various	
Sterile 50ml conical tubes	Various	
Sterile 20µl Filter pipette tips	Various	
Sterile 200µl Filter pipette tips	Various	
Sterile 1000µl Filter pipette tips	Various	
Sterile multichannel reservoirs	Various	

Table 3.3: Reagents

Reagents	Manufacturer	Catalog number
Fibronectin (1mg/ml)	Sigma	F1141
1x DPBS +Ca^{2+}+Mg^{2+}	e.g. Life technologies	Gibco 14040
Pluricyte® Cardiomyocyte Medium	Pluriomics	MR035-100ml
Pluricyte® Cardiomyocytes	Pluriomics	PCMi-1031-1

4. METHOD

4.1 Coating the Maestro Classic MEA 48 plate

The MEA plate is coated with fibronectin on the day of plating the Pluricyte® Cardiomyocytes (≥ 3h before thawing of the cells).

Note: The volumes used below are calculated for one Classic MEA 48 plate. For plating more than one 48-well MEA plate, multiply the volumes used by the number of MEA-plates needed.
For 96-well MEA plate see section 4.4.

1. Dilute 20µl of the fibronectin solution in 380µl sterile D-PBS (incl. Ca^{2+} and Mg^{2+}) in an Eppendorf tube to get a 50µg/ml fibronectin coating solution. Mix the solution carefully. *Note: Fibronectin is susceptible to shear stress, do not vortex or spin the solution, and avoid harsh pipetting.*
2. Carefully pipette a droplet of 8µl of the 50µg/ml fibronectin coating solution to the center of each well of the MEA plate covering the electrodes. See **Figure 4.1** for correct droplet placement. *Note: Avoid touching the bottom of the plate with the pipette tips.*
   Add 3ml PBS to the grooves on both sides of the MEA plate to avoid evaporation of the coating solution (**see Figure 4.3**).
3. Incubate the MEA plate at 37°C, 5% CO_2 for 3 hours.
   Note: Do not let the fibronectin coating dry out, this will cause irreversible loss of matrix properties.

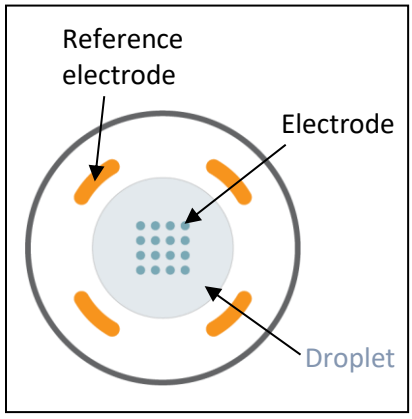


Figure 4.1. Droplet placement.
Graphical representation of a well on a Maestro Classic MEA 48 plate. The coating or cell droplet (light blue area) should be placed in the center of the well covering the electrodes, while avoiding the reference electrodes.

4.2 Thawing Pluricyte® Cardiomyocytes and seeding onto the Maestro Classic MEA 48 plate

This part of the protocol describes the thawing and directly plating of Pluricyte® Cardiomyocytes onto the Maestro MEA plate. Complete the following steps of the thawing procedure in a time-efficient manner to facilitate optimal viability and performance.

4. Warm 25ml Pluricyte® Cardiomyocyte Medium to room temperature.
5. Take 1 vial of Pluricyte® Cardiomyocytes per 48-well MEA plate from LN_2 storage.
   Note: The volumes used below are calculated for one Pluricyte® Cardiomyocytes vial to be plated on one 48-well MEA plate. For plating more than one 48-well MEA plate, multiply the number of vials and the volumes used by the number of MEA plates needed. We recommend to thaw maximum 3 vials per operator at a time.
6. Thaw the vial in an incubator at 37˚C for 4 minutes.
7. Gently transfer the contents of the vial (300µl) to a 50ml tube using a P1000 pipette.
8. Rinse the vial with 1ml Pluricyte® Cardiomyocyte Medium (pre-warmed to room temperature) and add this drop-wise to the 50ml tube, 1 drop every 5 seconds using a P1000 pipette.
9. Add 4.7ml Pluricyte® Cardiomyocyte Medium drop-wise to the 50ml tube, 1 drop every 2 seconds using a 5ml pipette.
   Note: the total volume of the cell suspension is now 6ml.
10. Take a 20µl sample of the homogenous cell suspension and add to a micro centrifuge tube.
11. Spin down the cell suspension for 3 minutes at 250xg.
12. Aspirate the medium and gently resuspend the cells in 200µl Pluricyte® Cardiomyocyte Medium.
13. Determine the total cell number and cell viability as follows:

    *We highly recommend to perform the cell counting manually using a hemocytometer. For instance, by using the Fuchs Rosenthal Counting Chamber (**Figure 4.2**):*

    a. Add 20µl Trypan blue solution to the 20µl cell sample (collected in step 10), mix carefully.
    b. Add 20µl of the Trypan blue/cell suspension mix to the counting chamber.
    *c. Calculate the total number of cells according to **equation 1**.*

14. Calculate the dilution factor to reach 30,000 cells/8µl and add Pluricyte® Cardiomyocyte Medium to the cell suspension accordingly.
15. Carefully aspirate the coating solution from the MEA plate.
16. Place an 8µl droplet of 30k cell suspension to the center of each well, covering the electrodes. See **Figure 4.1** for correct droplet placement.
17. Incubate the MEA plate with the seeded Pluricyte® Cardiomyocytes at 37°C, 5% CO_2 for 1 hour.
18. Add 16ml Pluricyte® Cardiomyocyte Medium to a 50 ml tube and incubate at 37°C, 5% CO_2 for 20 minutes.
19. After 1 hour, gently add 150µl of pre-warmed (37°C) Pluricyte® Cardiomyocyte Medium to the side of each well, while moving the pipette in a circular motion following the contour of the well, thereby surrounding the droplet of cells with medium.
    Note: adding the medium too quickly will dislodge the adhered cardiomyocytes.
20. Gently add another 150µl Pluricyte® Cardiomyocyte Medium to the side of each well.

21. Incubate the MEA plate at 37°C, 5% CO_2.

Equation 1 Cell counting

Count 4 #2 squares according to **Figure 4.2**

Viable cells: ___+___+___+___=_____ (#vc)

Non-viable (blue) cells: ___+___+___+___=_____(#nvc)

_____/4 x 2 x 5000 =_____cells/ml
[#vc]

_____=_____(= cells in total)
[# of cells/ml] [total volume after step 9]

Viability = ____: (_____+_____) x 100 =_____%
[#vc] [#vc] [#nvc]

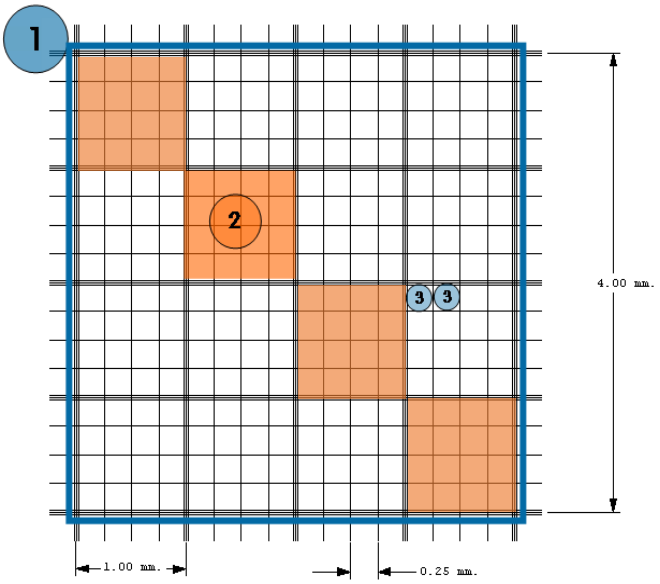


Figure 4.2. Lay-out of a Fuchs Rosenthal Counting chamber.

4.3 Maintenance of the Pluricyte® Cardiomyocytes in the Maestro Classic MEA 48 plate

It is crucial to always refresh the Pluricyte® Cardiomyocyte Medium of the cells one day after seeding the cells (day 1), and subsequently every 2 days (see workflow in section 2).

22. Pipette 16ml Pluricyte® Cardiomyocyte Medium into a sterile 50ml conical tube and incubate the tube at 37°C, 5% CO_2 for at least 20 min.
23. Transfer the MEA plate from the incubator to the flow cabinet.
24. Add the warm medium to a multichannel reservoir.
25. Aspirate the medium from each well or remove the medium using a multichannel pipette, avoid disturbing the cell monolayer.
    *Note: **Figure 4.3** explains how to replace the medium using a 12-channel pipette. Alternatively, a pipette with adjustable tip spacing can be used.*
26. Add 300µl medium per well using a multichannel pipette. Avoid disturbing the cell monolayer by gently pipetting to the side of each well.
27. Incubate the MEA plate at 37°C, 5% CO_2.
28. Maintain the cardiomyocytes for 8-12 days, refreshing the medium every 2-3 days.

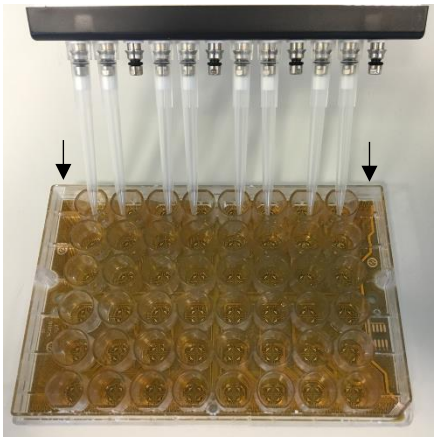


Figure 4.3. Pipetting method for Maestro Classic MEA 48 plate. Medium in Maestro Classic MEA 48 plate can be replaced using a 12-channel pipette, when the 3rd, 6th, 9th and 12th pipette tips are removed. Black arrows indicate grooves of the plate (for addition of PBS to avoid evaporation in the wells).
Note: to avoid contamination, remove pipette tips from the box using a pipette and avoid touching the pipette tips using your hands.

For data acquisition move to section 4.7.

4.4 Coating the Maestro Classic MEA 96 plate

The MEA plate is coated with fibronectin on the day of plating the Pluricyte® Cardiomyocytes (≥ 3h before thawing of the cells).

Note: The volumes used below are calculated for one Maestro Classic MEA 96 plate. For plating more than one 96-well MEA plate, multiply the volumes used by the number of MEA-plates needed.

1. Dilute 25µl of the fibronectin solution in 475µl sterile D-PBS (incl. Ca^{2+} and Mg^{2+}) in an Eppendorf tube to get a 50µg/ml fibronectin coating solution. Mix the solution carefully. *Note: Fibronectin is susceptible to shear stress, do not vortex or spin the solution, and avoid harsh pipetting.*
2. Carefully pipette a droplet of 5µl of the 50µg/ml fibronectin coating solution to the center of each well of the MEA plate covering the electrodes. See **Figure 4.4** for correct droplet placement. *Note: Avoid touching the bottom of the plate with the pipette tips.*
   Add 3ml PBS to the grooves on both sides of the MEA plate to avoid evaporation of the coating solution (**see Figure 4.3**).
3. Incubate the MEA plate at 37°C, 5% CO_2 for 3 hours.
   Note: Do not let the fibronectin coating dry out, this will cause irreversible loss of matrix properties.

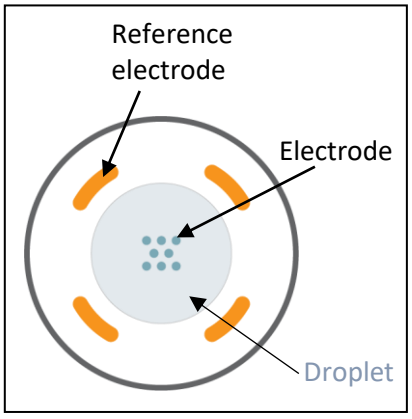


Figure 4.4. Droplet placement.
Graphical representation of a well on a Maestro Classic MEA 96 plate. The coating or cell droplet (light blue area) should be placed in the center of the well covering the electrodes, while avoiding the reference electrodes.

4.5 Thawing Pluricyte® Cardiomyocytes and seeding onto the Maestro Classic MEA 96 plate

This part of the protocol describes the thawing and directly plating of Pluricyte® Cardiomyocytes onto the Maestro MEA plate. Complete the following steps of the thawing procedure in a time-efficient manner to facilitate optimal viability and performance.

4. Warm 25ml Pluricyte® Cardiomyocyte Medium to room temperature.
5. Take 2 vial of Pluricyte® Cardiomyocytes per 96-well MEA plate from LN_2 storage.
   Note: The volumes used below are calculated for two Pluricyte® Cardiomyocyte vials to be plated on one Maestro Classic MEA 96 plate. For plating more than one Maestro Classic MEA 96 plate, multiply the number of vials and the volumes used by the number of Maestro Classic MEA 96 plates needed. We recommend to thaw maximum 3 vials per operator at a time.
6. Thaw the vials in an incubator at 37˚C for 4 minutes.
7. Gently transfer the contents of both vials (2x 300µl) to a 50ml tube using a P1000 pipette.
8. Rinse the vials with 1ml Pluricyte® Cardiomyocyte Medium (pre-warmed to room temperature) and add this drop-wise to the 50ml tube, 1 drop every 5 seconds using a P1000 pipette.
9. Add another 1ml Pluricyte® Cardiomyocyte Medium drop-wise to the 50ml tube, 1 drop every 5 seconds using a P1000 pipette.
10. Add 9.4ml Pluricyte® Cardiomyocyte Medium drop-wise to the 50ml tube, 1 drop every 2 seconds using a 10ml pipette.
    Note: the total volume of the cell suspension is now 12ml.
11. Take a 20µl sample of the homogenous cell suspension and add to a micro centrifuge tube.
12. Spin down the cell suspension for 3 minutes at 250xg.
13. Aspirate the medium and gently resuspend the cells in 200µl Pluricyte® Cardiomyocyte Medium.
14. Determine the total cell number and cell viability as follows:
    *We highly recommend to perform the cell counting manually using a hemocytometer. For instance, by using the Fuchs Rosenthal Counting Chamber (**Figure 4.2**):*
    d. Add 20µl Trypan blue solution to the 20µl cell sample (collected in step 11), mix carefully.
    e. Add 20µl of the Trypan blue/cell suspension mix to the counting chamber.
    *f. Calculate the total number of cells according to **equation 1**.*

15. Calculate the dilution factor to reach 20,000 cells/5µl and add Pluricyte® Cardiomyocyte Medium to the cell suspension accordingly.
16. Carefully aspirate the coating solution from the MEA plate.
17. Place a 5µl droplet of 20k cell suspension to the center of each well, covering the electrodes. See **Figure 4.4** for correct droplet placement.
18. Incubate the MEA plate with the seeded Pluricyte® Cardiomyocytes at 37°C, 5% CO_2 for 1 hour.
19. Add 10ml Pluricyte® Cardiomyocyte Medium to a 50ml tube and incubate at 37°C, 5% CO_2 for 20 minutes.
20. After 1 hour, gently add 100µl of pre-warmed (37°C) Pluricyte® Cardiomyocyte Medium to the side of each well using a multichannel pipette.
    Note: adding the medium too quickly will dislodge the adhered cardiomyocytes.
21. Incubate the MEA plate at 37°C, 5% CO_2.

4.6 Maintenance of the Pluricyte® Cardiomyocytes in Maestro Classic MEA 96 plate

It is crucial to always refresh the Pluricyte® Cardiomyocyte Medium of the cells one day after seeding the cells (day 1), and subsequently every 2 days (see workflow in section 2).

22. Pipette 10ml Pluricyte® Cardiomyocyte Medium into a sterile 50ml conical tube and incubate the tube at 37°C, 5% CO_2 for at least 20 min.
23. Transfer the MEA plate from the incubator to the flow cabinet.
24. Add the warm medium to a multichannel reservoir.
25. Aspirate the medium from each well or remove the medium using a multichannel pipette, avoid disturbing the cell monolayer.
26. Add 100µl medium per well using a multichannel pipette. Avoid disturbing the cell monolayer by gently pipetting to the side of each well.
27. Incubate the MEA plate at 37°C, 5% CO_2.
28. Maintain the cardiomyocytes for 8-12 days, refreshing the medium every 2-3 days.

4.7 Data acquisition during maintenance

In order to monitor the condition of the Pluricyte® Cardiomyocyte monolayer, it is advised to perform daily measurements during the maintenance. See the AxIS User Guide for specific instructions on using the AxIS software for data acquisition and analysis. First contractions of Pluricyte® Cardiomyocytes appear between 24-48 hours post-thawing. It will take 3-4 days before the cells have formed an electrically coupled monolayer. Stable beating monolayers can be observed 7-8 days post-thawing.

29. Turn on and warm the Maestro MEA system to 37˚C before each measurement.
30. Place the MEA plate in the Maestro MEA system (optional: place the lid and turn on the gas cylinder for carbogen administration). Monitor the activity of the Pluricyte® Cardiomyocytes on the MEA plate from day 1 using the Maestro MEA system.
    Note: Wait ≥1 hour after medium refreshments before measurement.

4.8 Compound assays

The optimal time window to perform electrophysiology-based assays with Pluricyte® Cardiomyocytes is between 8-12 days after plating the cardiomyocytes. Two different protocols for drug testing are outlined in paragraph 4.5.1 and 4.5.2 below.

4.8.1 Studying acute drug effects

To study acute drug effects, we recommend to dilute test compounds in Pluricyte® Cardiomyocyte Medium at ≥10x the desired final concentration and to add the compound in a volume of maximum 10% of the total medium volume of the well (e.g. 30µl in a total volume of 300µl for Maestro Classic MEA 48 plate or 10µl in a total volume of 100µl for a Maestro Classic MEA 96 plate). We recommend not to use DMSO concentrations above 0.1%.

1. Replace the medium in the Maestro Classic MEA plate according to section 4.3 (for the Maestro Classic MEA 48 plate) or 4.6 (for the Maestro Classic MEA 96 plate) at least 1 hour before the compound assay and return the plate back to the incubator.
2. Prepare the test compounds in Pluricyte® Cardiomyocyte Medium at ≥10x the desired final concentration in a normal tissue culture plate and place this "compound-plate" in an incubator at 37˚C, 5% CO_2 for at least 10 min.
3. Turn on and warm the Maestro MEA system to 37˚C.
4. Transfer the Maestro Classic MEA plate and the compound plate from the incubator to the flow cabinet.
5. From each well of the Maestro Classic MEA plate, remove the chosen volume (e.g. 30µl in a total volume of 300µl for the Maestro Classic MEA 48 plate or 10µl in a total volume of 100µl for the Maestro Classic MEA 96 plate).
6. Gently mix the Pluricyte® Cardiomyocyte Medium containing the compounds in the compound-plate by pipetting up and down 3 times to ensure a homogeneous solution. Pipette the chosen volume from the compound plate (e.g. 30µl or 10µl), and add to the Maestro Classic MEA plate.
7. Place the Maestro Classic MEA plate into the Maestro MEA system immediately following compound addition and start measurements. **Figure 4.5** provides an example of data acquisition for acute studies.

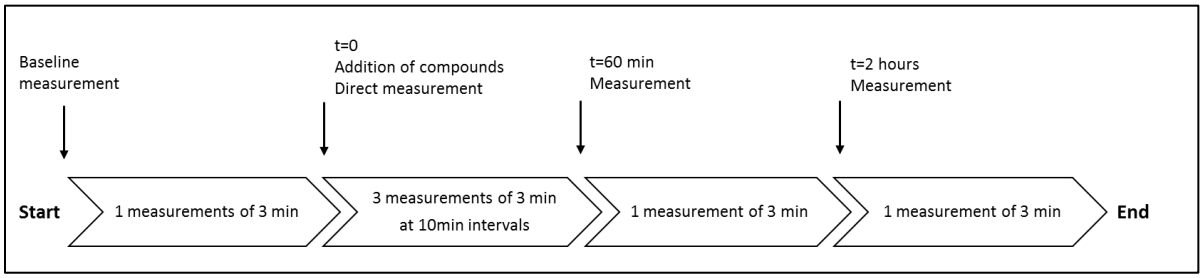


Figure 4.5 Example of a measurement scheme for studying acute drug effects.

4.8.2 Studying long term drug effects

For a long term study, we recommend to dilute the test compounds in Pluricyte® Cardiomyocyte Medium to the desired final concentration and completely replace the medium in the plate. We recommend to wait at least 30-60 minutes before performing the first measurement to avoid measuring the effects of medium change. We recommend to not use DMSO concentrations above 0.1%.

1. For a test in a Maestro Classic MEA 48 plate; prepare each test compound in the desired final concentration in 350µl Pluricyte® Cardiomyocyte Medium per well in a normal 48-well tissue culture plate and place this "compound-plate" in an incubator at 37˚C, 5% CO_2 for 30 minutes.

2. For a test in a Maestro Classic MEA 96 plate; prepare each test compound in the desired final concentration in 125µl Pluricyte® Cardiomyocyte Medium per well in a normal 96-well tissue culture plate and place this "compound-plate" in an incubator at 37˚C, 5% CO_2 for 30 minutes.
3. Transfer the Maestro Classic MEA plate and the compound plate from the incubator to the flow cabinet.
4. Aspirate the medium from the Maestro Classic MEA plate.
5. Add 300µl/well from the compound plate to the side of each well to a Maestro Classic MEA 48 plate using a multichannel pipette (see **Figure 4.3** for the pipetting method) or add 100µl/well from the compound plate to the side of each well to a Maestro Classic MEA 96 plate using a multichannel pipette.
6. Incubate the Maestro Classic MEA plate at 37˚C, 5% CO_2 for at least 30 minutes to avoid measuring the effects of the medium change.
7. Turn on and warm the Maestro MEA system to 37˚C.
8. Place the Maestro Classic MEA plate in the Maestro MEA system and perform a recording, return the plate to the incubator in between measurements. **Figure 4.6** provides an example of data acquisition for long term studies.

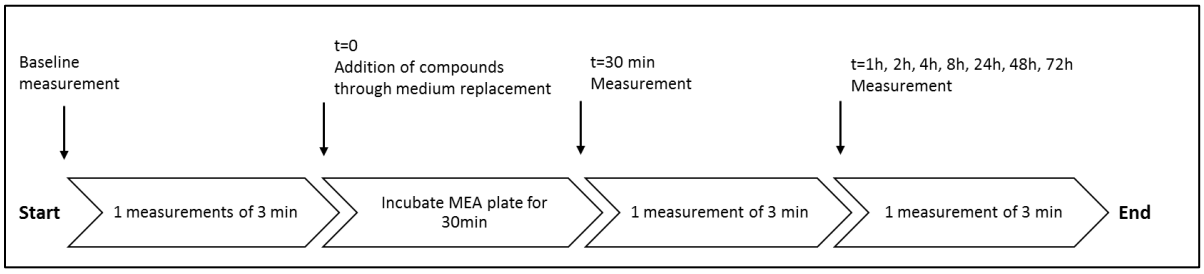


Figure 4.6 Example of a measurement scheme for studying long term drug effects.

4.9 Data analysis

9. Analyze the acquired data using the AxIS software.

   Note: See the AxIS User Guide for specific instructions on using the AxIS software for data acquisition and analysis.
10. Open a recording or a batch process to analyze several recordings simultaneously.
11. Load the *Cardiac Offline Spontaneous* configuration.
12. Double click on the cardiac beat detector to change the settings (see **Figure 4.7** for recommended settings). *Note: The settings may need to be adjusted to analyze compound effects, as the waveforms may change in shape and duration upon compound addition.*

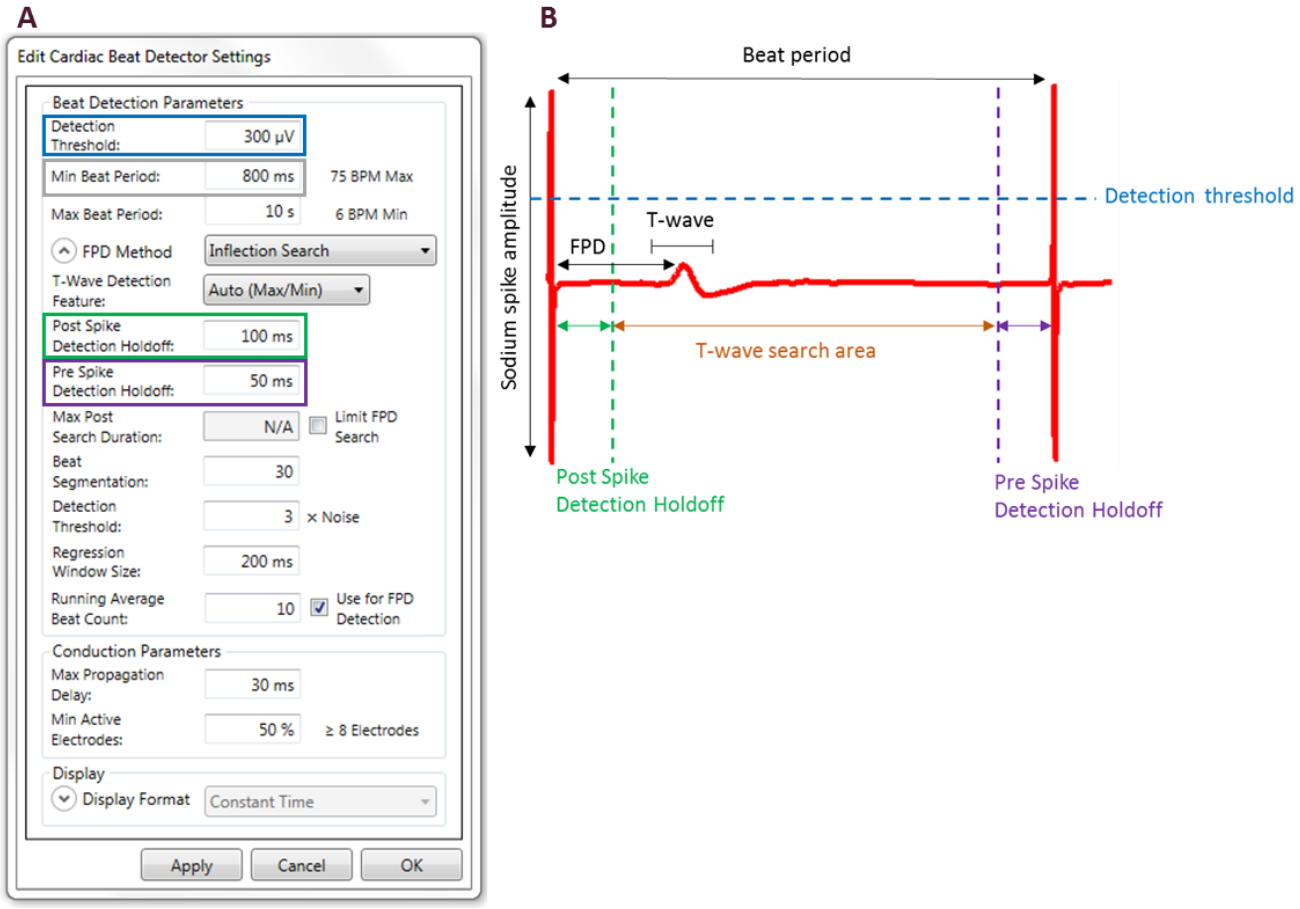


Figure 4.7. Recommended Cardiac Beat Detector Settings for Pluricyte® Cardiomyocytes. **A**: Cardiac Beat Detector settings editor, showing the recommended settings for Pluricyte® Cardiomyocytes. The Detection threshold (in blue) can be adjusted to the spike amplitude of the signals. Ideally, the detection threshold is set lower than the sodium spike amplitude but higher than the "T-wave" amplitude (see **B**). We recommend to set a minimum beat period (in grey) that is longer than the field potential duration (FPD) to prevent the "T-wave" from being recognized as a sodium spike. The "Inflection Search" in combination with the "T-wave" detection feature "Auto (Max/Min)" generally leads to the best results for determining the FPD. Post and Pre Spike Detection Holdoff settings are optimized for Pluricyte® Cardiomyocytes (in green and purple respectively).

5. CASE STUDY: ASSESSMENT OF PRO-ARRHYTHMIC EFFECTS USING PLURICYTE® CARDIOMYOCYTES ON THE AXION MAESTRO MEA SYSTEM

Pluricyte® Cardiomyocytes Field Potential measured using the Axion Maestro MEA system

The Axion Maestro MEA system records the extracellular field potential of cardiomyocytes in real-time using proprietary microelectrode array (MEA) technology. The majority of cardiac side effects, like torsade de pointe (TdP) and ventricular fibrillation, are associated with drugs interfering with the function of ion channels such as the human Ether-à-go-go-Related Gene (hERG) K^+-channels, the Nav1.5 Na^+-channels or the Ca^{2+}-channels. Through the MEA measurements, the Axion Maestro MEA system captures changes in the extracellular field potential of Pluricyte® Cardiomyocytes, generated by the electrophysiological processes across the cell membrane. Drugs affecting different ion channels can consequently be studied using the MEA measurement. **Figure 5.1** depicts a typical waveform of the extracellular field potential signal of Pluricyte® Cardiomyocytes obtained using the Axion Maestro MEA system. Indicated are the depolarization phase, characterized by the robust sodium spike, during which an influx of sodium occurs (I_{Na}), the plateau phase, during which an influx of Calcium ($I_{Ca\text{-}L}$) occurs, and the repolarization phase, during which an efflux of potassium occurs (I_{Kr}/I_{Ks}), characterized by the clear repolarization peak. Parameter measurements that can be analyzed using the AxIS software may include the sodium spike amplitude, the beat period (time period between two successive sodium spikes, a parameter from which the beat rate can be derived as follows: $\text{beat rate (BPM)} = \frac{60}{beat\ period\ (s)}$) and the field potential duration (the time period between the depolarization and repolarization peaks), as depicted in **Figure 5.1.**

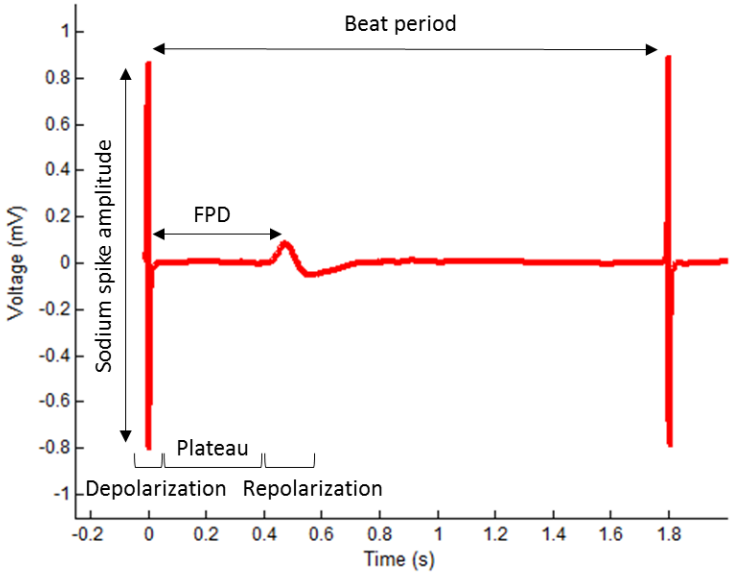


Figure 5.1. Typical waveform of the extracellular field potential signal of Pluricyte® Cardiomyocytes obtained using the Axion Maestro MEA system. The sodium spike amplitude, field potential duration (FPD), the beat period (time period between two successive sodium spikes), the depolarization and repolarization phases are indicated.
[Image was generated using the Axion Cardiac Data Plotting Tool, version 1.2.1].

5.1 Experimental design to study acute drug effects

To assess the effects of a set of pro-arrhythmic compounds, Pluricyte® Cardiomyocytes were cultured on Maestro Classic MEA 48 plate in Pluricyte® Cardiomyocyte Medium for 9 days. The set of pro-arrhythmic drugs (**Table 5.1**) was dissolved in DMSO at a concentration of 10mM and then diluted in Pluricyte® Cardiomyocyte Medium in 10-fold serial dilutions. The Pluricyte® Cardiomyocytes were then treated with this set of pre-diluted pro-arrhythmic drugs in a cumulative dose response experiment (**Figure 5.2**). Acute-drug effects were directly measured using the Axion Maestro MEA system. Compound concentrations were increased by 3-fold (**Figure 5.2.1**) for each separate recording step (**Figure 5.2.2**). The data were analyzed using Axion Integrated Studio (version 2.1.1.16) to determine the compound effects on beat period, field potential duration, and spike amplitude.

Table 5.1. List of pro-arrhythmic drugs and their expected effects on hiPSC-derived cardiomyocytes.

Drug class	Drug	Expected effects on hiPSC-cardiomyocytes electrophysiology
hERG channel blocker (I_{Kr})	E4031	Delays repolarization phase by blocking the hERG channel, resulting in prolonged FPD and ultimately arrhythmias
hERG channel blocker (I_{Kr})	Dofetilide	Delays repolarization phase by blocking the hERG channel resulting in prolonged FPD and ultimately arrhythmias
Sodium channel blocker (I_{Na})	Mexiletine	Reduces sodium spike amplitude by blocking sodium channels. Higher concentrations also block potassium channels resulting in an increased FPD
Sodium channel blocker (I_{Na})	Flecainide	Reduces sodium spike amplitude by blocking sodium channels. Higher concentrations also block potassium channels resulting in an increased FPD
Calcium channel blocker ($I_{Ca,L}$)	Nifedipine	Decreases FPD by blocking Ca^{2+} channels
Calcium channel blocker ($I_{Ca,L}$)	Diltiazem	Decreases FPD by blocking Ca^{2+} channels
β-adrenergic receptor agonist	Isoproterenol	Increases beat rate by activating β-adrenergic receptors resulting in decreased FPD

Figure 5.2. Cumulative Dose-Response Experiment.

Compounds	t=0	t=30min	t=60min	t=90min	t=120min
E4031	3nM	10nM	30nM	100nM	--
Dofetilide	3nM	10nM	30nM	100nM	--
Mexiletine	300nM	1µM	3µM	10µM	30µM
Flecainide	300nM	1µM	3µM	10µM	30µM
Nifedipine	3nM	10nM	30nM	100nM	300nM
Diltiazem	30nM	100nM	300nM	1µM	3µM
Isoproterenol	300pM	1nM	3nM	10nM	30nM

Figure 5.2.1. Compound concentrations tested

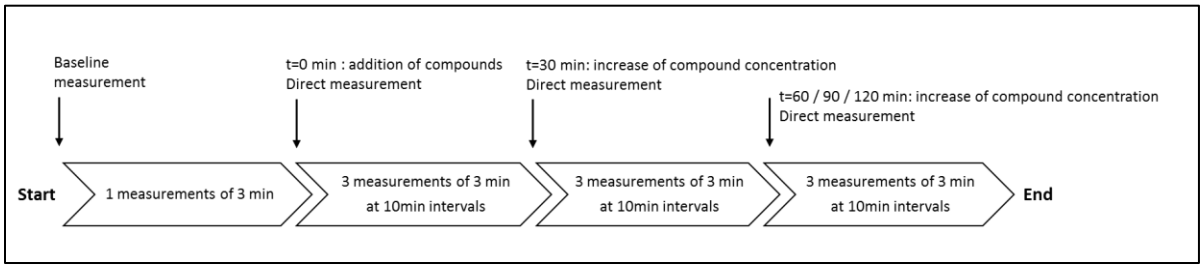


Figure 5.2.2. Measurement Scheme

5.2 Results

hERG potassium channel blockers block the rapid component of the delayed rectifier outward potassium current (I_{kr}), thereby delaying the repolarization phase. This results in an increase in field potential duration and flattening of the repolarization peak. At higher concentrations, blocking of the hERG channel may lead to TdP-like arrhythmias.[4] **Figure 5.3** shows this prolongation of the field potential duration (FPD) of Pluricyte® Cardiomyocytes induced by hERG potassium channel blockers E4031 and dofetilide. Furthermore, TdP-like arrhythmias were frequently observed at high concentrations of E4031 and dofetilide.

Calcium channel blockers affect the plateau phase between the depolarization and repolarization phase, resulting in a shortening of the field potential duration.[3] As shown in **Figure 5.4**, L-type calcium channel blockers nifedipine and diltiazem shorten the field potential duration of Pluricyte® Cardiomyocytes in a concentration-dependent manner.

Sodium channel blockers affect the depolarization phase of the field potential by blocking sodium channels (I_{Na}), resulting in a decrease in sodium spike amplitude.[8] **Figure 5.5** shows that sodium channel blockers mexiletine and flecainide indeed decrease the sodium spike amplitude in Pluricyte® Cardiomyocytes in a concentration-dependent manner. Both mexiletine and flecainide also block hERG (I_{Kr}) potassium channels,[5] shown here by an increase in field potential duration.

Isoproterenol is a β-adrenergic receptor agonist; activation of this receptor results in an increased beat rate (decreased beat period) and consequently a reduction in field potential duration.[6] **Figure 5.6** shows that isoproterenol has a concentration-dependent effect on the beat rate of Pluricyte® Cardiomyocytes, as well as on the absolute field potential duration.

Figure 5.7 provides an overview of the different cardioactive compounds and their effects on beat period, field potential duration and sodium spike amplitude.

Concluding Remarks

In this case study, we assessed the effects of a set of pro-arrhythmic compounds on Pluricyte® Cardiomyocytes electrophysiology by MEA measurements using the Axion Maestro MEA system (Axion Biosystems, Atlanta, GA, USA). Pluricyte® Cardiomyocytes are exceptionally well-suited for implementation in safety pharmacology screening assays due to their unique strengths and characteristics (**Section 1**). Pluricyte® Cardiomyocytes, cultured in Pluricyte® Cardiomyocyte Medium, showed expected pharmacological responses in a reproducible manner, which could be readily detected with the Axion Maestro MEA system. The combination of Pluricyte® Cardiomyocytes with the Axion Maestro MEA platform provides a highly relevant *in vitro* assay to study the cardiac safety profile of compounds at an early stage of drug development.

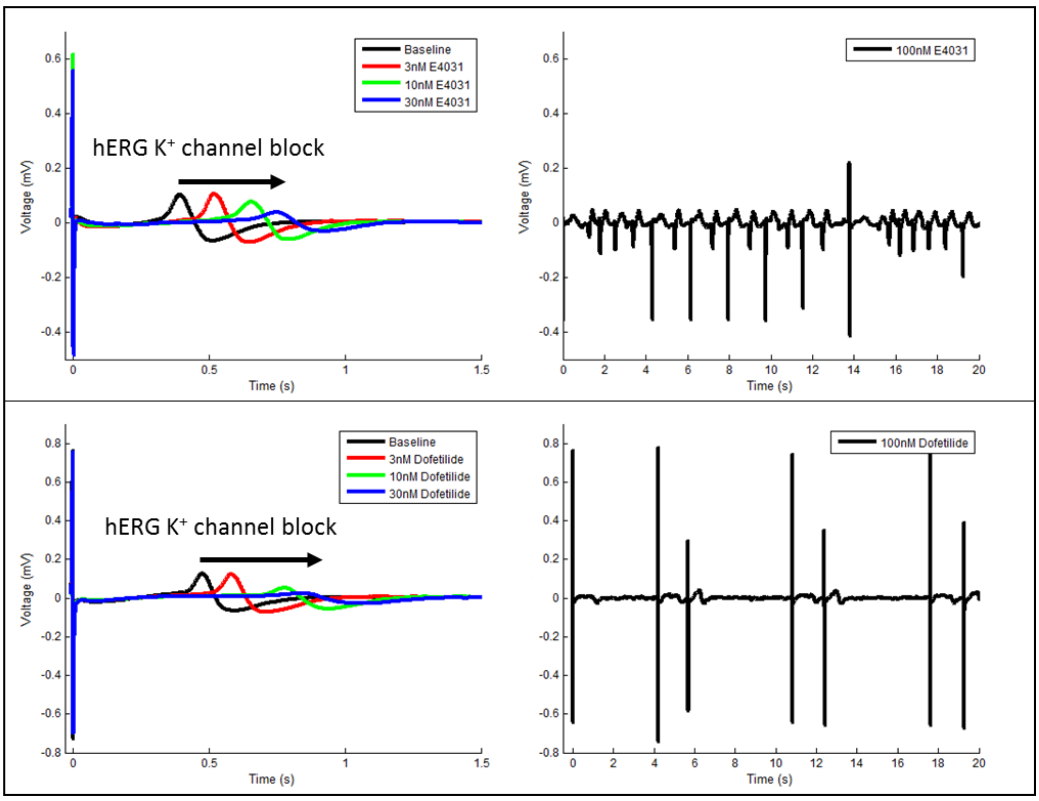


Figure 5.3. hERG channel blockers.hERG potassium channel blockers E4031 (top panel) and dofetilide (bottom panel) increase the field potential duration and cause flattening of the repolarization peak of Pluricyte® Cardiomyocytes, as shown here in an overlay of averaged waveforms. Despite flattening of the peak, Axis software could accurately detect the repolarization of Pluricyte® Cardiomyocytes even in the presence of high concentration hERG channel blockers. TdP-like arrhythmias were observed at high concentrations for both E4031 and dofetilide. [Images were generated using the Axion Cardiac Data Plotting Tool, version 1.2.1]

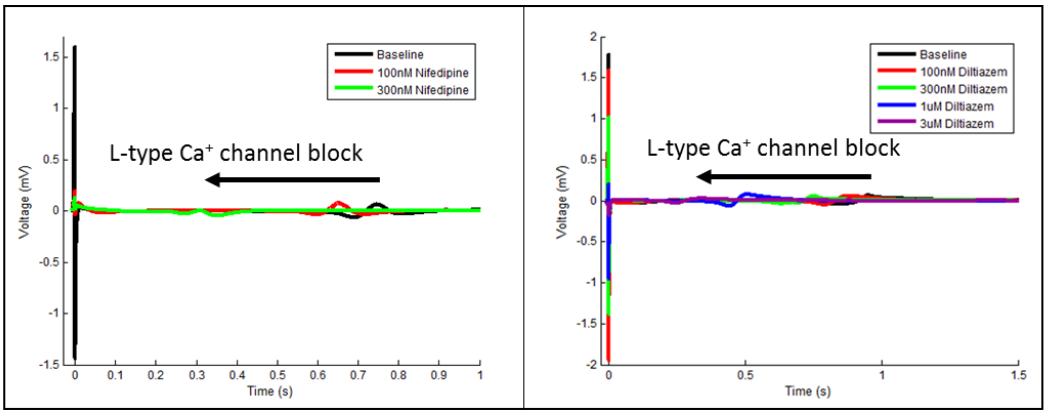
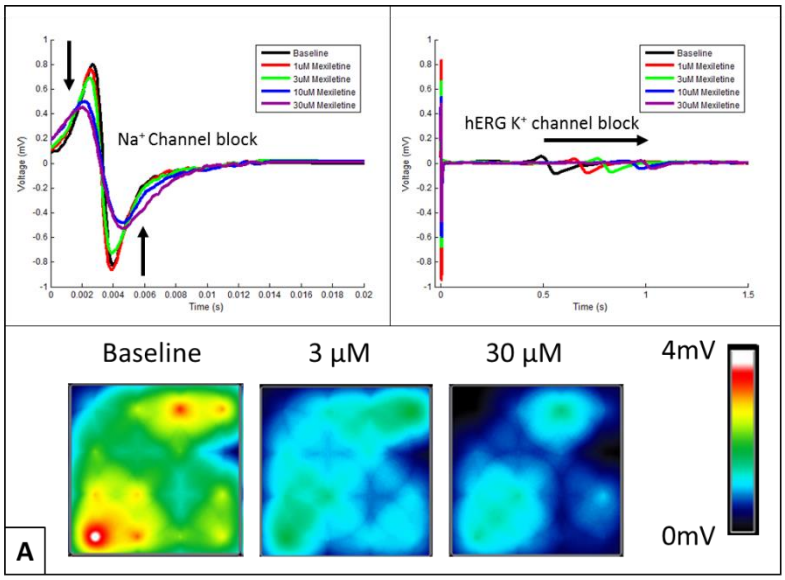


Figure 5.4. Ca^{2+} channel blockers. Calcium channel blockers nifedipine (left panel) and diltiazem (right panel) reduce the field potential duration of Pluricyte® Cardiomyocytes, as shown here in an overlay of averaged waveforms. [Images were generated using the Axion Cardiac Data Plotting Tool, version 1.2.1]



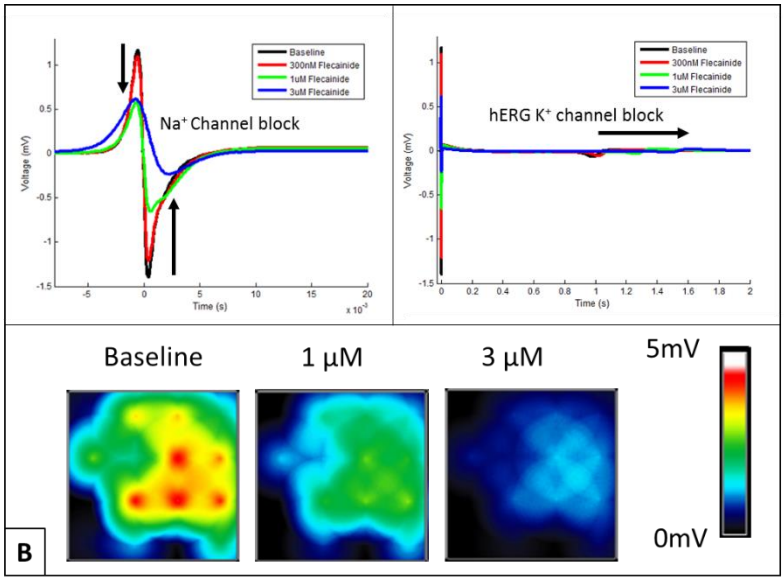


Figure 5.5. Na$^+$-channel blockers mexiletine and flecainide.

By blocking the Nav1.5 Na$^+$ -channels, mexiletine (A) and flecainide (B) reduce the amplitude of the sodium spike of Pluricyte® Cardiomyocytes, shown here in an overlay of the sodium spike (top left panels), and in heat plots depicting the sodium spike amplitude (bottom panels). Both mexiletine and flecainide also block hERG K$^+$-channels resulting in a prolongation of the field potential duration and flattening of the repolarization peak (top right panels).

Top panels show overlays of 10 averaged waveforms for each condition.

[Images were generated using the Axion Cardiac Data Plotting Tool, version 1.2.1 and Axion Integrated Studio, version 2.1.1.16]

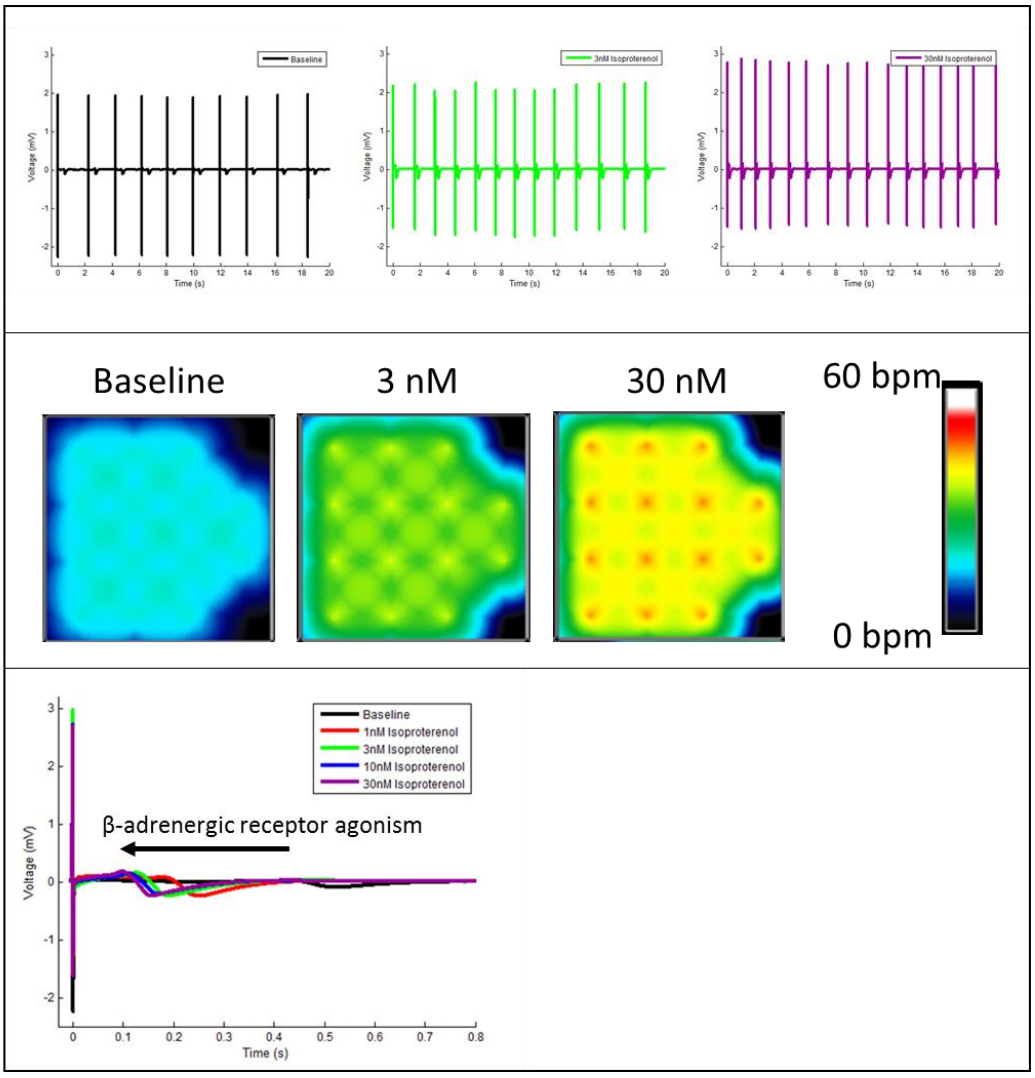


Figure 5.6. β-adrenergic receptor agonist.Isoproterenol activates the β-adrenergic receptor, resulting in an increase in beat rate in Pluricyte® Cardiomyocytes, shown here by an increase in spike frequency in a 20 seconds measurement (top panel), and in heat plots depicting beat rate in beats per minute (bpm) (the reciprocal of the beat period, middle panel). In addition, isoproterenol shortens the field potential duration of Pluricyte® Cardiomyocytes, as shown in an overlay of averaged waveforms (bottom panel). [Images were generated using the Axion Cardiac Data Plotting Tool, version 1.2.1 and Axion integrated studio, version 2.1.1.16]

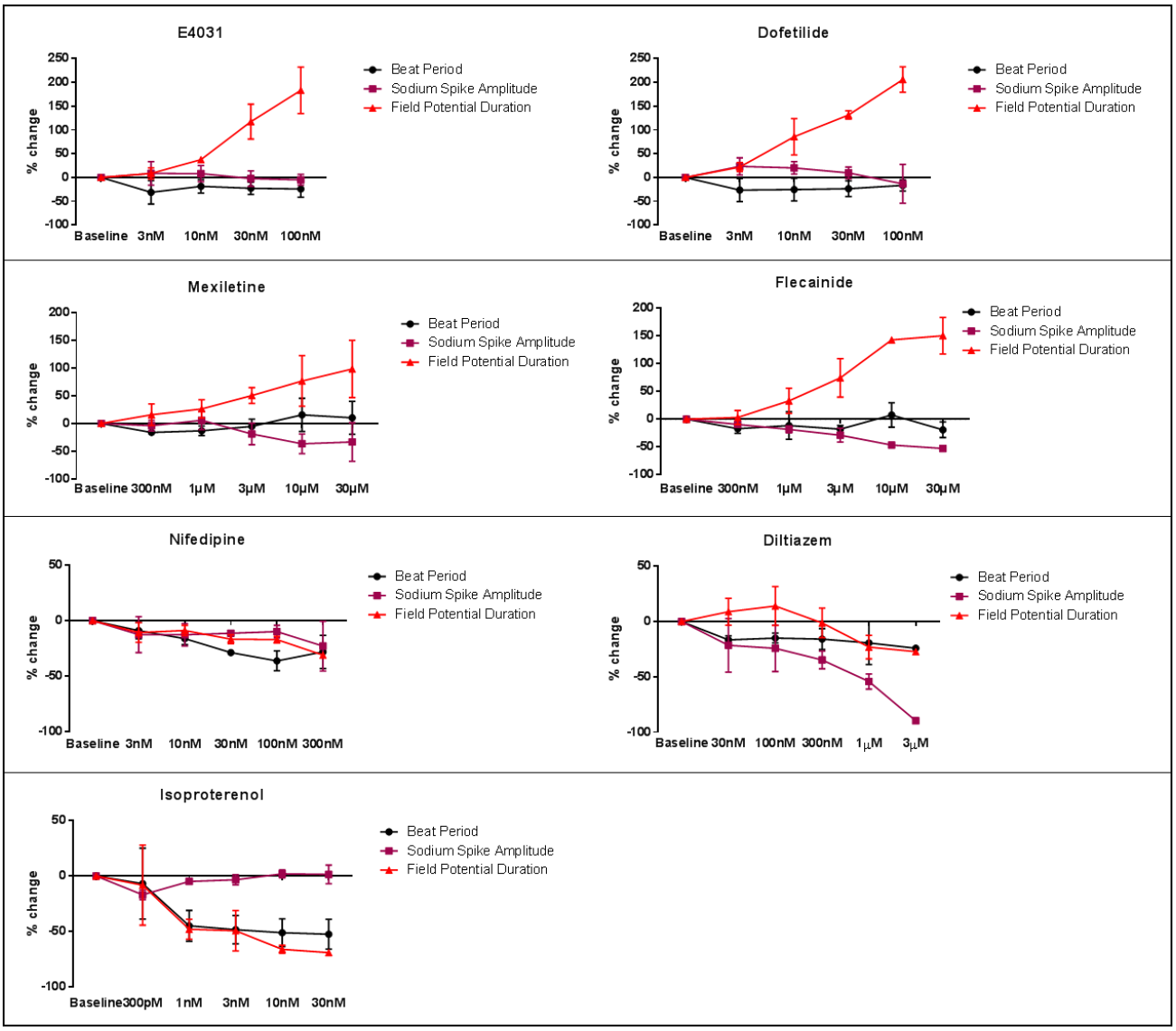


Figure 5.7. Overview of effects of cardioactive compounds on Pluricyte® Cardiomyocytes. Overview of the different cardioactive compounds and their effects on beat period (displays the time period between two successive sodium spikes), spike amplitude, and field potential duration of Pluricyte® Cardiomyocytes (time between the detected repolarization peak and the preceding sodium spike, Figure 5.1). Data are expressed as percentage of change when compared to the baseline. Mean ± SD, N= 3 wells for each condition. [Graphs were generated using GraphPad Prism version 6.07]

6. REFERENCES

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