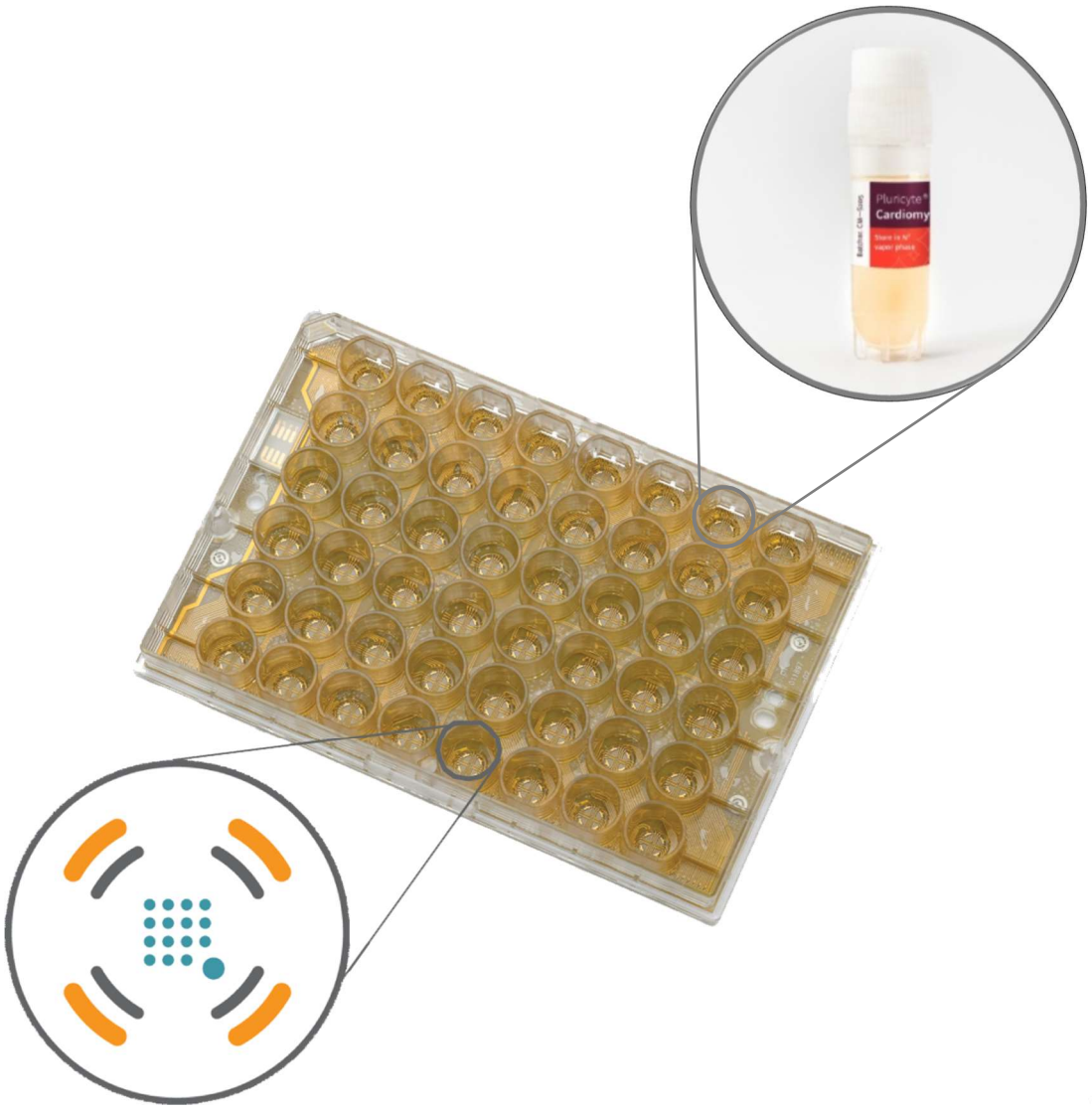
Cardiac safety assessment during electrical pacing using Pluricyte® Cardiomyocytes in combination with Axion BioSystems Maestro E-Stim+ MEA technology



Version 1.0

Contents

GETTING STARTED

Please make sure to read the entire Pluricyte® Cardiomyocyte Manual (available online at www.pluriomics.com/support/application-protocols) carefully before you start to thaw the cells.

Our application note for standard Axion Maestro MEA plates ("Assessment of pro-arrhythmic effects using Pluricyte® Cardiomyocytes on the Axion Maestro system") is available online at www.pluriomics.com /support/application-protocols.

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 microelectrode array (MEA) assays for predictive safety pharmacology, toxicity testing and efficacy screening in early drug discovery and development. Pluricyte® Cardiomyocytes' well-pronounced depolarization and repolarization peaks permit the 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.
The combination of Pluricyte® Cardiomyocytes with the Axion Maestro MEA system enables detailed electrophysiological detection of cardioactive/proarrhythmic effects of test compounds at 48- or 96-well plate formats.

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 high action potential amplitudes
- Organized sarcomere structures
- Monolayer field potential containing well-pronounced depolarization and repolarization peaks that enable easy detection of field potential durations in MEA assays
- Relatively low spontaneous beat rate (20-30 BPM), making the cardiomyocytes highly suitable for pacing applications: a broad window of pacing rates can be applied

Benefits of pacing in Pluricyte® Cardiomyocyte Maestro assays

Axion BioSystems (Axion BioSystems, Atlanta, GA, USA) developed a new pacing system, the Maestro E-Stim+ Classic MEA plate, that delivers high-quality MEA results with superior stimulation capacity. Pluricyte® Cardiomyocytes readily adapt to electrical stimulation ('pacing') applied through Axion Maestro E-Stim+ MEA technology. Controlling the beat rate by pacing provides several advantages and enables the user to do a more precise and more in-depth analysis of cardioactive/proarrhythmic compound effects. These advantages include:

- Assessment of cardiac safety at user-defined beat rates
- Detection of beat rate dependent effects (use-dependence)
- Increased physiological relevance
- Reduced well-to-well variability (**Figure 1A**)
- Reduced assay-to-assay variability (**Figure 1B**)

This application note describes the function of electrical stimulation in Pluricyte® Cardiomyocytes by using Maestro E-Stim+ plates in combination with the Maestro MEA system. In addition, a case study describing the assessment of pro-arrhythmic compound effects in stimulated Pluricyte® Cardiomyocytes is provided.

Electrical stimulation of Pluricyte® Cardiomyocytes provides a reproducible and relevant *in vitro* assay to record the field potential signal under a stable and defined beat rate to study cardiac safety profile of novel drug candidates.

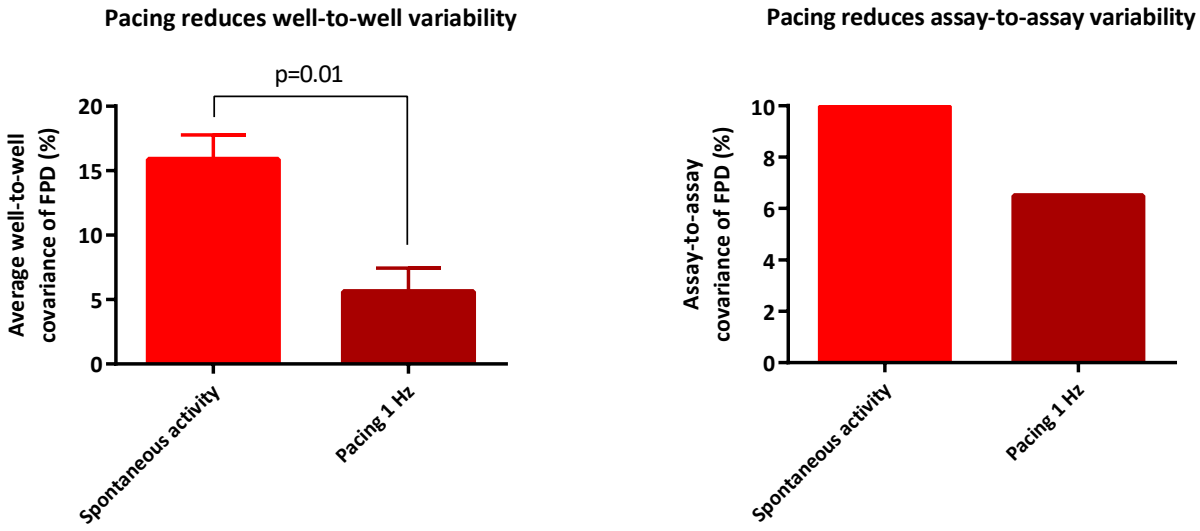


Figure 1. Reduction of well-to-well and assay-to-assay variability in FPD during pacing compared to spontaneous activity in the same wells. Since many physiological processes are coupled to the beat-rate, synchronization of beat rate is expected to reduce variability. These results show that well-to-well and assay-to-assay variability of the field potential duration (FPD) in spontaneous beating cells is only 10-15% but can be even further minimized by synchronization of the beat rate with pacing. **(A)** Average well-to-well covariance during spontaneous activity and pacing (1Hz) in 4 plates. Well-to-well covariance was calculated as the percentage of well-to-well SD compared to the average FPD for each experiment. A two-tailed non-paired t-test was performed to calculate significance (p=0.01) **(B)** Assay-to-assay covariance of the FPD, calculated as assay-to-assay SD of 4 independent experiments divided by the average FPD.

2. CHARACTERISTICS OF PLURICYTE® CARDIOMYOCYTES DURING ELECTRICAL STIMULATION

Pluricyte® Cardiomyocytes quickly adapt their physiology to new pacing rates applied through the MEA system (**Figure 2.1A**). In response to an increased pacing rate the FPD shortens until a steady state is reached, which occurs after about 2 minutes of pacing (**Figure 2.1B**). The combination of Pluricyte® Cardiomyocytes and the E-Stim+ plates in the Axion Maestro MEA system results in reproducible and well-pronounced signals after application of built-in artefact removal algorithms (**Figure 2.1C**).

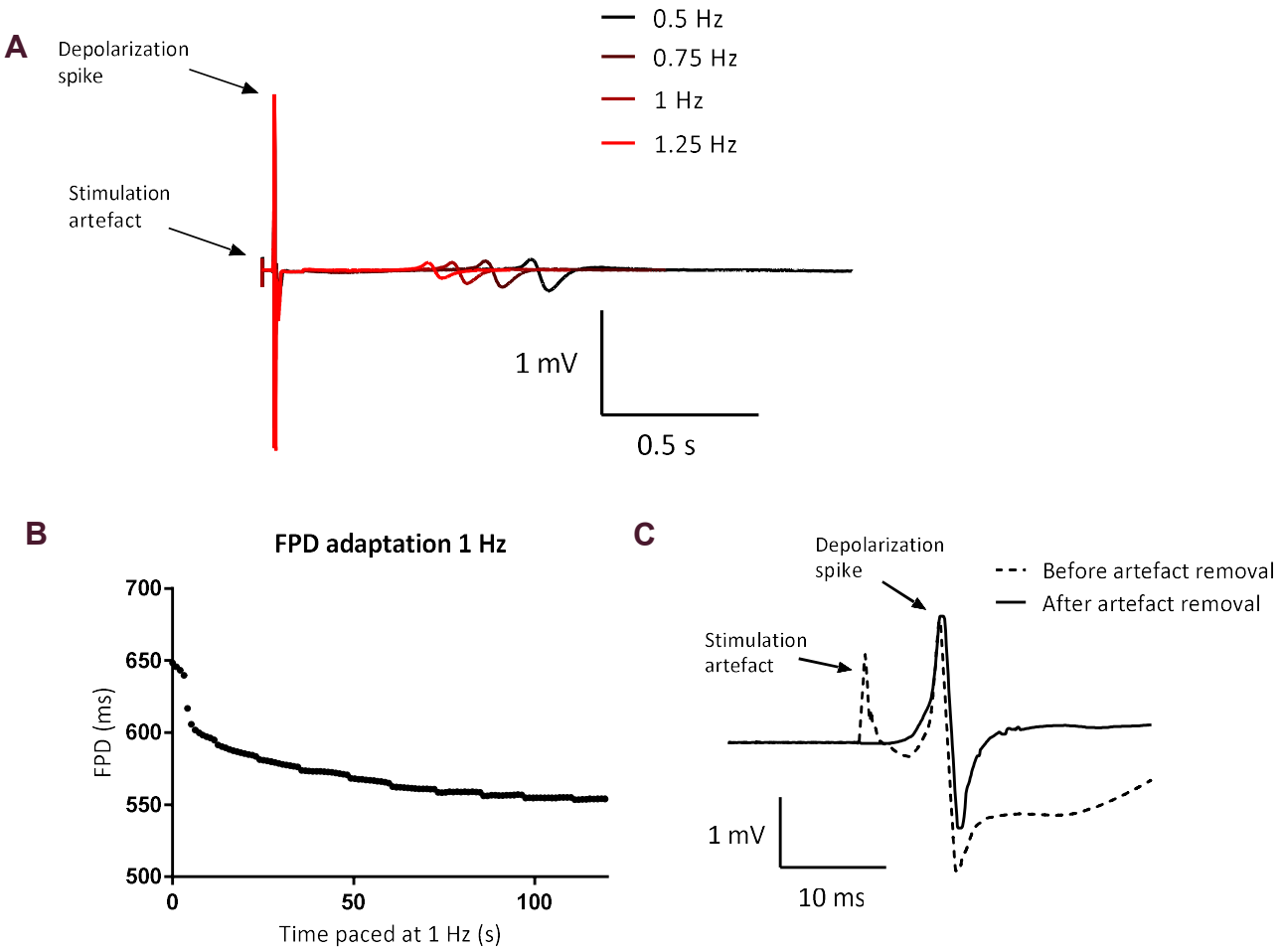
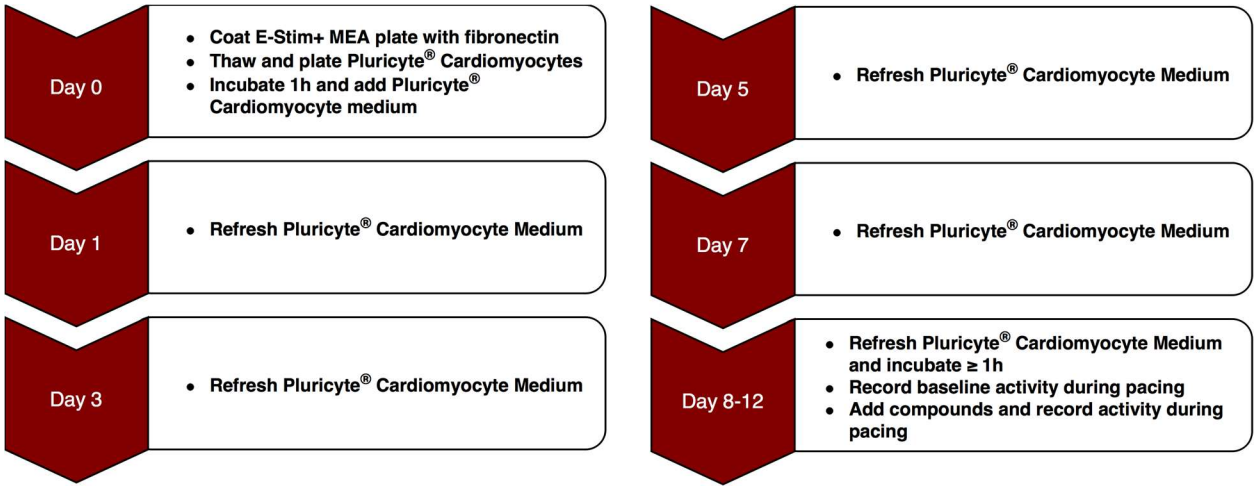


Figure 2.1. Adaptation of Pluricyte® Cardiomyocyte field potential during electrical pacing using the Axion Maestro MEA system. Due to the robust field potential signals of Pluricyte® Cardiomyocytes **(A)** and reliable artefact removal in the AxIS software **(C)**, the signals are of high quality for robust detection of depolarization and repolarization peaks. Pluricyte® Cardiomyocytes adapt their electrophysiology to several pacing rates defined by the user. When the pacing protocol is initiated, the cardiomyocytes need about 2 minutes to adapt to the new beat rate **(B)**. The artefact removal function in the AxIS software removes most of the signal disturbance caused by the applied electrical stimulus **(C)**.

3. WORK FLOW



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

IMPORTANT RECOMMENDATIONS

- Good attachment of the Pluricyte® Cardiomyocytes to the stimulation electrode is essential for optimal electrical stimulus delivery (see **Section 5.1**). To ensure good stimulation electrode coverage:
  - Carefully follow the thawing, seeding and maintenance instructions described in **Section 5.2**.
  - Apply the coating and cell suspension in droplets centered towards the stimulation electrode (**Figure 5.1**).
  - Be extra careful when adding the medium 1 hour after seeding of cell droplets. Apply the medium as slowly as possible and avoid pipetting near the droplet to avoid dislodging of adhered Pluricyte® Cardiomyocytes.
- We strongly recommend to use the fibronectin specified in table 4.3 as coating substrate for the MEA plates. Other types of coatings may reduce the signal and/or affect the condition of the cells.
- Always refresh the Pluricyte® Cardiomyocyte Medium (PCM) of the cells the day after seeding the cells. Subsequently, refresh the PCM of the cells every 2 days. The Pluricyte® Cardiomyocytes could be refreshed on Friday afternoon and Monday morning 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. Stably 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.
- Switch on and warm the Maestro MEA system to 37˚C before each measurement, this can take up to 10 minutes.
- Always pace Pluricyte® Cardiomyocytes for two minutes at the desired rate prior to start of the recording. This allows the cardiomyocytes to adapt to the new beat rate.

4. EQUIPMENT, MATERIALS AND REAGENTS

Table 4.1: Equipment

Equipment	Manufacturer
Axion BioSystems Maestro system + AxIS software (version 2.4 or later)	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 4.2: Materials

Materials	Manufacturer	Catalog number
E-Stim+ Classic MEA 48 plate	Axion BioSystems	M768-KAP-48S
Sterile disposable 5 mL pipettes	Various	
Sterile disposable 10 mL pipettes	Various	
Sterile disposable 25 mL pipettes	Various	
Sterile Eppendorf tubes	Various	
Sterile 50 mL conical tubes	Various	
Sterile 20 µL Filter pipette tips	Various	
Sterile 200 µL Filter pipette tips	Various	
Sterile 1000 µL Filter pipette tips	Various	
Sterile multichannel reservoirs	Various	

Table 4.3: Reagents

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

5. METHOD

Critical note: Good attachment of Pluricyte® Cardiomyocytes onto the surface of the dedicated stimulation electrode is essential for electrical stimulus delivery to the cardiomyocyte monolayer. To ensure good coverage of the stimulation electrode, the droplet of fibronectin coating, as well as the cell suspension droplet, should be centered towards the dedicated stimulation electrode (**Figure 5.1)**. Additionally, the first medium addition after plating should always be performed very gently in order to prevent dislodging the cells from the stimulation electrode.

5.1 Coating the Maestro E-Stim+ Classic MEA 48 plate

The E-Stim+ 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 48-well E-Stim+ MEA plate. For coating more than one plate, multiply the volumes used by the number of plates needed.

1. Dilute 20 µl of the fibronectin solution in 380 µL sterile D-PBS (incl. Ca^{2+} and Mg^{2+}) in an Eppendorf tube to get a 50 µg/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. Per well: carefully pipette a droplet of 8 µL of the 50 µg/mL fibronectin coating solution and place it centered towards the stimulation electrode (see **Figure 5.1**).
   Note: Avoid touching the bottom of the plate with the pipette tips.
3. Add 3 mL PBS to the grooves on both sides of the MEA plate to avoid evaporation of the coating solution (see **Figure 5.3**).
4. Incubate the MEA plate at 37°C, 5% CO_2 for 3 hours.
   Note: Do not to let the fibronectin coating dry out. This will cause irreversible loss of matrix properties.

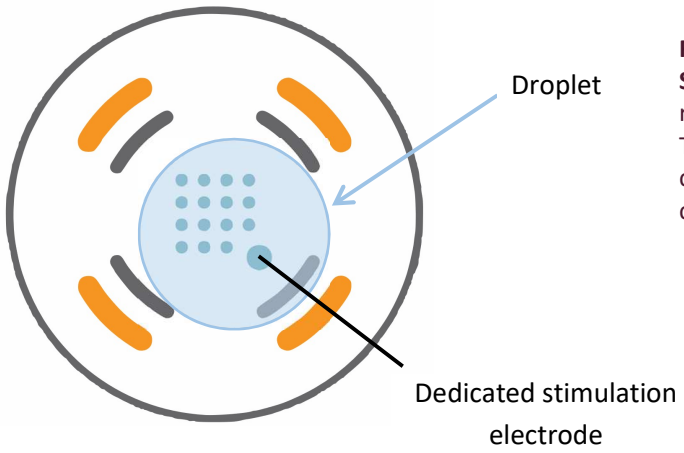


Figure 5.1. Coating and cell droplet placement in E-Stim+ Classic MEA 48 plates. Graphical representation of a well on an E-Stim+ MEA 48 plate. The coating or cell droplet (blue area) should be centered towards the stimulation electrode and also cover the remaining microelectrodes.

5.2 Thawing Pluricyte® Cardiomyocytes and seeding onto the Maestro E-Stim+ Classic MEA 48 plate

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

5. Warm 25 mL Pluricyte® Cardiomyocyte Medium to room temperature.
6. Take 1 vial of Pluricyte® Cardiomyocytes per E-Stim+ Classic MEA 48 plate from LN_2 storage.
   Note: The volumes used below are calculated for one vial of Pluricyte® Cardiomyocytes to be plated on one E-Stim+ Classic MEA 48 plate. For plating more than one plate, multiply the number of vials and the volumes used by the number of plates needed. We recommend to thaw maximum 3 vials per operator at a time.
7. Thaw the vial in an incubator at 37˚C for 4 minutes.
8. Gently transfer the contents of the vial (300 µL) to a 50 mL tube using a P1000 pipette.
9. Rinse the vial with 1 mL Pluricyte® Cardiomyocyte Medium (pre-warmed to room temperature, see step 5) and add this drop-wise to the 50 mL tube, 1 drop every 5 seconds using a P1000 pipette.
10. Add 4.7 mL PCM drop-wise to the 50 mL tube, 1 drop every 2 seconds using a 5 mL pipette.
    Note: The total volume of the cell suspension is now 6 mL.
11. Take a 20 µL 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 200 µL 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 5.2**):*

    a. Add 20 µL Trypan blue solution to the 20µl cell sample (collected in step 11), mix carefully.
    b. Add 20 µL of the Trypan blue/cell suspension mix to the counting chamber.
    *c. Calculate the total number of cells according to **equation 1**.*

    Equation 1: Cell Counting

    Count 4 #2 squares according to **Figure 5.2**.

    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]

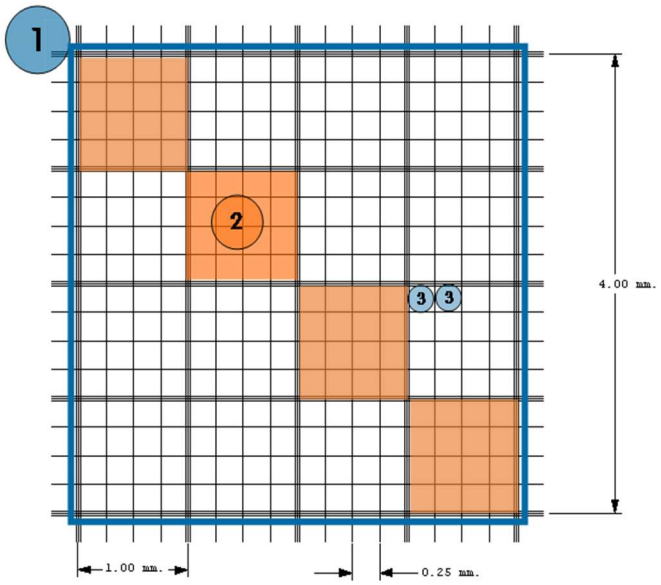


Figure 5.2. Lay-out of a Fuchs Rosenthal Counting chamber.

15. Calculate the dilution factor to reach 30,000 cells/8 µL and add Pluricyte® Cardiomyocyte Medium to the cell suspension accordingly.
16. Carefully aspirate the coating solution from the plate.
17. Place an 8 µL droplet of 30,000 cells in suspension centered towards the stimulation electrode. See **Figure 5.1** for correct droplet placement.
18. Incubate the E-Stim+ MEA plate with the seeded Pluricyte® Cardiomyocytes at 37°C, 5% CO_2 for 1 hour.
19. Add 16 mL Pluricyte® Cardiomyocyte Medium to a 50 mL tube and incubate at 37°C, 5% CO_2 for 20 minutes.
20. After 1 hour, gently add 150 µL 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.
21. Gently add another 150 µL Pluricyte® Cardiomyocyte Medium to the side of each well using a multi-channel pipette (see **Figure 5.3**). Incubate the E-Stim+ MEA plate at 37°C, 5% CO_2.

5.3 Maintenance of the Pluricyte® Cardiomyocytes in the Maestro E-Stim+ 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 3).

22. Pipette 16 mL Pluricyte® Cardiomyocyte Medium into a sterile 50 mL conical tube and incubate the tube at 37°C, 5% CO_2 for at least 20 minutes.
23. Transfer the E-Stim+ Classic 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 5.3** explains how to replace the medium using a 12-channel pipette. Alternatively, a pipette with adjustable tip spacing can be used.*
26. Add 300 µL 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 days.

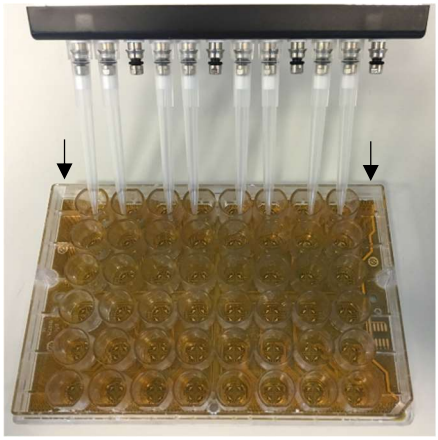


Figure 5.3. Pipetting method for E-Stim+ Classic 48 MEA plate. Medium in E-Stim+ Classic 48 MEA plates can be replaced using a 12-channel pipette, when the 3rd, 6th, 9th and 12th pipette tips are removed. Black arrows indicate grooves of the plate (for addition of PBS to avoid evaporation in the wells).

5.4 Data acquisition during maintenance

In order to monitor the condition of the Pluricyte® Cardiomyocyte monolayer, we recommend 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 system to 37˚C before each measurement.
30. Place the MEA plate in the Maestro system (optional: place the lid and turn on the gas cylinder for carbogen administration). Monitor the spontaneous activity of the Pluricyte® Cardiomyocytes on the MEA plate from day 1 using the Maestro system.
    Note: Wait ≥1 hour after medium refreshments before measurement.

5.5 Stimulation and compound assays

The optimal time window to perform electrophysiology-based pacing assays with Pluricyte® Cardiomyocytes is between 8-12 days after plating the cardiomyocytes. Our recommendations for finding the right stimulation settings and studying drug effects during pacing are described in the sections below.

5.5.1 Setting up the stimulation protocol

31. Replace the medium in the E-Stim+ Classic MEA 48 plate according to **section 5.3** at least 2 hours before the compound assay.
32. Measure one sweep of spontaneous activity to monitor the field potentials and determine the spontaneous beat rate of the cells (in Pluricyte® Cardiomyocytes this is usually 0.3 – 0.5 Hz). The pacing rate should always exceed the spontaneous beat rate in order to achieve successful capture of the desired rate.
33. Pace the cells for 1 minute at 0.75 Hz at our recommended settings shown in **Figure 5.4**: **Cardiac Pacing Stimulation Duration: 0.7 ms, Current: 40 µA, Voltage Amplitude: 800 mV.** Make sure to enable artefact removal by setting the configuration to '**Cardiac Real-Time, Electrically Paced**'). For more specific details on stimulation settings, see the AxIS user manual (version 2.4 or later). After stimulation starts, the cells need to adapt to the new beat rate. Therefore, it is recommended to wait with recording until 2 minutes after the start of stimulation. After 2 minutes the cells will reach a steady state with a stable FPD (see **Figure 2.1B**).
34. Determine whether the pacing was successfully captured by studying the field potential signals (see **Figure 5.5**). Successful pacing has been achieved when:
    a. Every spike (depolarization peak) in the signal is followed by a clear repolarization peak. This peak is expected to follow within 400-1200 ms after depolarization, depending on the stimulation rate.
    b. The spikes occur at the exact same rate as the pacing rate.

c. The amplitude of consecutive spikes is consistent within each electrode channel.

If successful pacing was not achieved, see **Table 5.1 for troubleshooting.**

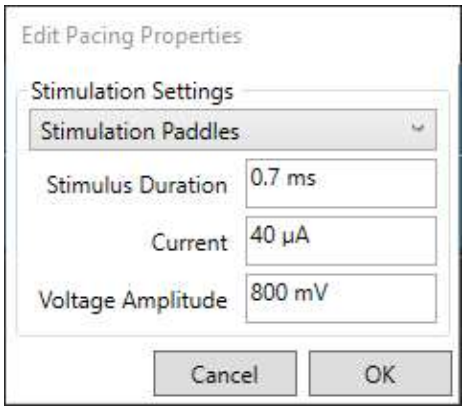


Figure 5.4. Stimulation settings for Pluricyte® Cardiomyocytes on E-Stim+ plates.

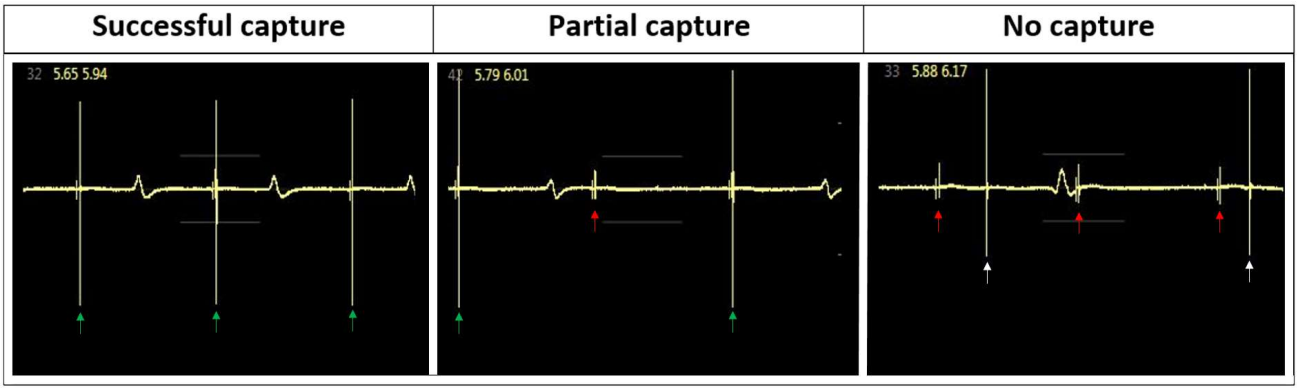


Figure 5.5. Examples of field potential signals when Pluricyte® Cardiomyocytes either successfully, partially or did not capture the electrical stimuli. Green arrows represent instances of stimulation in which a depolarization spike was elicited. Red arrows indicate when a stimulus was applied but no depolarization spike was induced, resulting in a stimulation artefact in the signal. White arrows represent spontaneous depolarization spikes (independent of the electrical stimuli).

Table 5.1. Troubleshooting if successful pacing is not achieved

Situation	Observation	Possible causes	Solution
No capture or partial capture	**No capture:** Spontaneous activity occurs independent from stimulation. (See **Figure 5.5**) **Partial capture:** Alternation of pacing artefacts and depolarization spikes. The depolarization spikes are larger than the artefacts and followed by a repolarization peak (see **Figure 5.5**)	No cell coverage on stimulation electrode	Place the coating and cell droplet on the stimulus electrode (Section 5). Refresh medium carefully
		Stimulus settings too low	Increase stimulus duration or current (steps of 10 µA or 0.1 ms)
		Compound effect	Record result; change pacing rate if desired
		Pacing rate too high (only in case of partial capture)	Decrease the pacing rate (usually 0.5-1.25 Hz is possible)
Spontaneous beating rate exceeds pacing rate	Irregular occurrence of spontaneous beats next to the stimulated beats	Spontaneous beat rate is faster than beat rate	Increase pacing rate
Too strong artefacts	Field potential signals are too distorted to analyze depolarization spikes and repolarization peaks	Stimulus settings too high	Decrease stimulus duration or current (steps of 10 µA or 0.1 ms)

5.5.2 Studying drug effects during stimulation

To study acute drug effects during stimulation, 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. 30 µL in a total volume of 300 µL). We recommend not to use final DMSO concentrations above 0.1%.

35. Replace the medium in the E-Stim+ MEA plate according to **section 5.3** at least 2 hours before the compound assay.
36. Prepare the test compounds in Pluricyte® Cardiomyocyte Medium at ≥10x the desired final concentration in a regular 48-well tissue culture plate and place this "compound plate" in an incubator at 37˚C, 5% CO_2 for at least 10 min.
37. Turn on and warm the Maestro system to 37˚C.
38. Transfer the E-Stim+ MEA plate from the incubator to Maestro system (37˚C) and perform a baseline measurement by recording the field potential under the desired stimulation (for stimulation settings, see **section 5.5.1**).

39. Transfer the E-Stim+ MEA plate and the compound plate to the flow cabinet.
40. From each well of the plate, remove the chosen volume (e.g. 30 µL from the total volume of 300 µl).
41. Pipette the chosen volume from the compound plate (e.g. 30 µL), and add to the E-Stim+ MEA plate.
42. Place the E-Stim+ MEA plate into the Axion Maestro system immediately following compound addition and start stimulation and recording according to the experimental design. **Figures 5.6** and **5.7** provide examples of experimental designs for data acquisition to study acute compound effects during pacing.
    Note: Pacing can be performed at a rate defined by the user, within the range of 0.5 Hz to 1.25 Hz. Using a single rate (e.g. 1 Hz) is recommended.

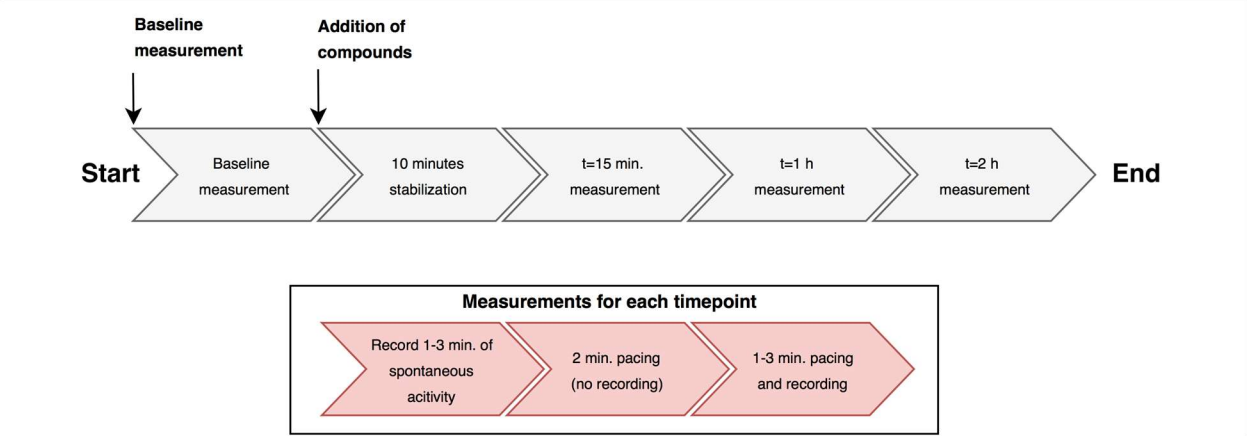


Figure 5.6. Example of an experimental design of a single dose compound assay. After compound addition, the E-Stim+ MEA plate is placed into the Maestro system (37°C, connected to carbogen). For each time point (e.g. directly after addition, 1 hour or several hours after addition) first record 1-3 minutes of spontaneous activity, followed by 2 minutes of pacing at the desired frequency (without recording) and finally record for 1-3 minutes to capture the drug effects of paced Pluricyte® Cardiomyocytes.

Although a single dosing design enables analysis of effects over a longer range of time (**Figure 5.6**), cumulative studies can also be performed in order to increase the amount of information obtained per well. **Figure 5.7** provides an example of an experimental design for cumulative compound additions (red arrow). Additionally, if interested in the effects of a drug at different beat rates, the experimental design can be adjusted to include measurements of pacing at several rates. **Figure 5.7** also illustrates how to incorporate several pacing rates (for example 0.5-1.5 Hz) in the measurement scheme in order to determine use-dependent effects of drugs (green arrows).

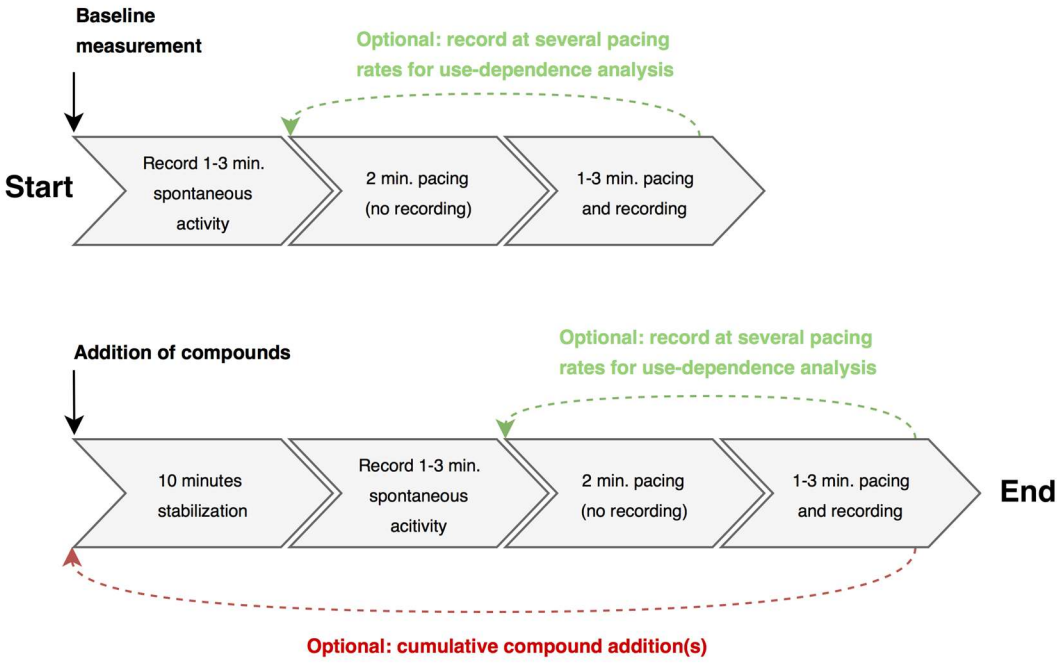


Figure 5.7. Example of an experimental design for the assessment of use-dependence of drug effects (green arrows) whether or not during a cumulative compound assay (red arrows). This experimental outline gives options to implement different pacing rates as well as cumulative compound additions to the assessment.

5.6 Data analysis of electrical stimulation assays

43. 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.

44. Open a recording or a batch process to analyze several recordings simultaneously.
45. Load the *Cardiac Offline Electrically Paced* configuration.
46. Double click on the cardiac beat detector to adjust the settings (see **Figure 5.8** for recommended settings).

Note 1: Double check that the detection threshold for depolarization peaks ("sodium spikes") does not result in detection of stimulation artefacts or repolarization peaks. Usually, depolarization spike amplitudes are high enough to use a threshold of about 500-600 µV.

Note 2: The settings may need to be adjusted to analyze compound effects, as the field potential may change upon compound addition.

Note 3: Capture of the pacing stimulus could be lost due to ion-channel effects of compounds. Make sure to exclude data where the beat period is different from the stimulation period.

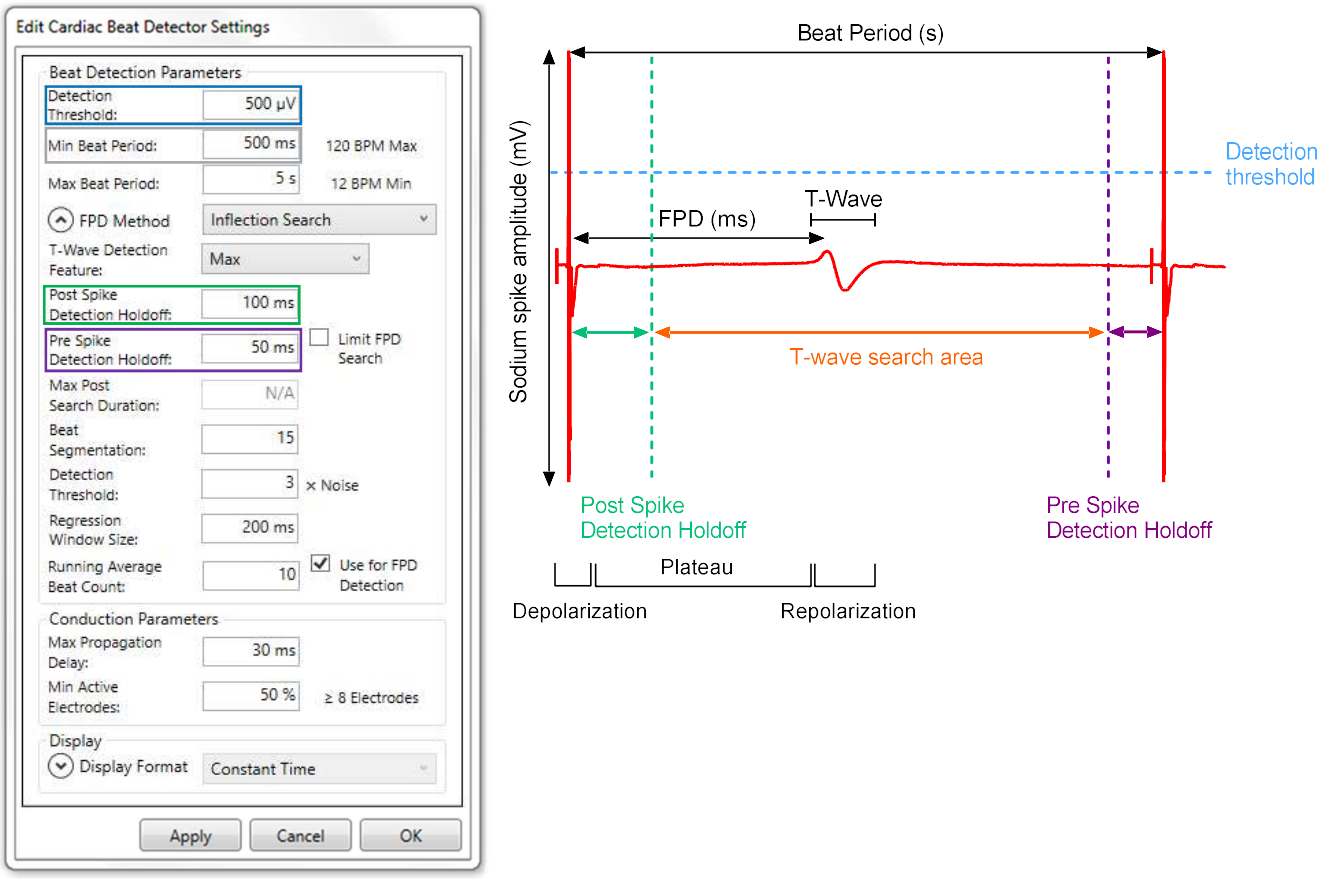


Figure 5.8. Recommended Cardiac Beat Detector Settings for Pluricyte® Cardiomyocytes in combination with E-Stim+ MEA plates. The right panel shows a typical waveform of the extracellular field potential signal of Pluricyte® Cardiomyocytes obtained during pacing in the Axion Maestro system at 0.5 Hz. Indicated are the depolarization phase, characterized by the robust sodium spike, during which an influx of sodium occurs (INa), the plateau phase, during which an influx of Calcium (Ica,L) occurs, and the repolarization phase, during which an efflux of potassium occurs (IKr/IKs), characterized by the clear "T-wave".

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 stimulation artefact and "T-wave amplitude". The Inflection Search in combination with the "T-wave" detection feature "Max" generally leads to the best results for determining the field potential duration (FPD). Post- and Pre-Spike Detection Holdoff settings (in green and purple respectively) are optimized for Pluricyte® Cardiomyocytes.

6. CASE STUDY: ASSESSMENT OF PRO-ARRHYTHMIC EFFECTS USING PLURICYTE® CARDIOMYOCYTES DURING ELECTRICAL STIMULATION IN MAESTRO E-STIM+ MEA PLATES

Pluricyte® Cardiomyocytes field potential measurements during stimulation using Maestro E-Stim+ MEA technology

The Axion BioSystems Maestro system enables the recording of Pluricyte cardiomyocyte extracellular field potentials in real-time using proprietary microelectrode array (MEA) technology. Combined with Maestro E-Stim+ Classic 48 MEA plates, which have a dedicated pacing electrode in each well, the field potential of cardiomyocytes can be measured during pacing.

In the pacing assay described here ventricular cardiomyocytes adapt to beat rates applied by external stimuli, resembling the *in vivo* situation where the ventricles rely on external input from pacemaker cells. The low spontaneous beating rates of Pluricyte® Cardiomyocytes enables pacing at a wide range of beating rates, allowing the user to pick a beating rate that is relevant for the application. Additionally, pacing of Pluricyte® Cardiomyocytes increases performance of cardiac safety assays by eliminating any variability caused by variation in beat rate. Another advantage of pacing is that (reverse) use-dependence of drugs can be tested. This phenomenon, described as a different magnitude of effect at different beat rates is observed with several types of ion channel blockers. Compounds in which a higher beat rate increases their effect, are known as use-dependent compounds. Use-dependence is typically observed in sodium channel blockers[1]. On the other hand, hERG channel blockers are typically more effective at lower beat rates, which is referred to as reverse use-dependence[2]. Reverse use-dependence is one of the pillars of TRIaD (Triangulation, Reverse use-dependence, Instability, and Dispersion) of ventricular repolarization. It is believed that augmentation of TRIaD provides the proarrhythmic substrate, individual parameters are thus of interest for the assessment of the pro-arrhythmic potential of a compound[3].

Figure 5.8 depicts a typical waveform of the extracellular field potential signal of Pluricyte® Cardiomyocytes obtained while pacing in the Axion Maestro system. Parameter measurements that can be analyzed using the AxIS software may include the depolarization amplitude, the beat period and the field potential duration (the time period between the depolarization and repolarization peaks), as depicted in **Figure 5.8.** Furthermore, analysis may also include the number of wells in which capture of the pacing stimulus is lost, for example due to compound effects on excitability or conductivity.

6.1 Experimental design to study acute drug effects during electrical stimulation

Pluricyte® Cardiomyocytes were cultured on Maestro E-Stim+ Classic MEA 48 plates in Pluricyte® Cardiomyocyte Medium for 8 days. The set of pro-arrhythmic drugs (**Table 6.1**) was dissolved in DMSO at a concentration of 10 mM and then diluted in Pluricyte® Cardiomyocyte Medium in serial dilutions. The Pluricyte® Cardiomyocytes were then treated with this set of pre-diluted pro-arrhythmic drugs in a cumulative dose response experiment (**Table 6.2**). Acute-drug effects were measured while pacing at different rates using the Maestro system. Compound concentrations were increased by 2-fold (**Table 6.2**)

for each separate recording step. After each compound addition, the cardiomyocytes were paced during steady state at several rates (**Table 6.3**). The data were analyzed using AxIS (version 2.4.1) to determine the compound effects on the field potential duration and spike amplitude and to determine the capture of the stimulus at each of the pacing rates.

Table 6.1. 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 Dofetilide	Reverse use-dependent delay of the repolarization phase by blocking the hERG channel, resulting in prolonged FPD and ultimately arrhythmias
Sodium channel blocker (I_{Na})	TTX	Use-dependent decrease of the depolarization amplitude by blocking sodium channels
Calcium channel blocker ($I_{Ca,L}$)	Diltiazem	Shortening of the FPD by blocking calcium influx through L-type calcium channels
Slow delayed rectifier channel blocker (I_{Ks})	JnJ303	Small FPD prolongation, without occurrence of arrhythmias at higher concentrations

Table 6.2. Final compound concentrations used

Compounds	t=0*	t=30 min.	t=60 min.	t=90 min.	t=120 min.	t=150 min.
E4031	0 nM	1 nM	2 nM	4 nM	8 nM	16 nM
Dofetilide	0 nM	1 nM	2 nM	4 nM	8 nM	16 nM
JnJ303	0 nM	50 nM	100 nM	200 nM	400 nM	800 nM
TTX	0 nM	750 nM	1500 nM	3000 nM	6000 nM	12000 nM
Diltiazem	0 nM	100 nM	200 nM	400 nM	800 nM	1600 nM
DMSO	0%	0.01%	0.02%	0.04%	0.08%	0.16%

t=0 is start of recording, after stabilization (see **Table 6.3)*

Table 6.3. Measurement and pacing schedule for each compound addition

Time after compound addition	Activity
0-10 minutes	Wait for field potential signals to stabilize
11-12 minutes	1 minute recording of spontaneous activity
12-30 minutes	Record activity during steady state at various pacing rates ranging from 0.5 Hz to 1.75 Hz

6.2 Results

Analysis of the different field potential parameters before and after application of compounds showed that Pluricyte® Cardiomyocytes show expected results to several ion channel blockers during pacing. **Figures 6.1** and **6.2** provide an overview of the different cardioactive compound effects on average field potential traces (**Figure 6.1A-E**) as well as on field potential duration, sodium spike amplitude and stimulus capture during pacing at 1 Hz (**Figure 6.2A-E**). Additionally, analysis of compound effects at different pacing rates revealed that hERG blockers and sodium channel blockers exhibited, as expected, reverse use-dependence and use-dependence, respectively (**Figure 6.3**).

hERG potassium channel (I_{Kr}) 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 Torsade de Pointe (TdP)-like arrhythmias[4]. **Figures 6.1A-B** and **6.2A-B** show prolongation of the field potential duration (FPD) of Pluricyte® Cardiomyocytes induced by hERG potassium channel blockers E4031 and dofetilide. Additionally, analysis of the FPD at different pacing rates after treatment with E4031 showed that the inverse relationship between FPD and beat rate was reverse use-dependent (**Figure 6.3A**), a phenomenon that is well known to occur during *in vivo* hERG channel blockade[2]. For examples of TdP-like arrhythmias caused by hERG channel blockers at higher concentrations than presented here, please refer to our application note for standard Axion BioSystems Maestro MEA plates (www.pluriomics.com/support /application-protocols/).

Calcium channel ($I_{Ca,L}$) blockers affect the plateau phase between the depolarization and repolarization phase, resulting in a shortening of the field potential duration[5]. As shown in **Figures 6.1C** and **6.2C**, the L-type calcium channel blocker diltiazem shortens the field potential duration of Pluricyte® Cardiomyocytes during pacing at 1 Hz in a concentration-dependent manner.

K_v7.1 channel (I_{Ks}) blockers block the slow component of the delayed rectifier outward potassium current (I_{Ks}), resulting in a delay of the repolarization phase. In human cardiomyocytes this current is known to be relatively small compared to the I_{Kr} current[6], resulting in less severe FPD prolongations. Also, blockade of this channel does not result in TdP-like arrhythmias in human cardiomyocytes. As expected, the Kv7.1 channel blocker JnJ303 induced a small but robust prolongation of the FPD during pacing at 1 Hz (**Figures 6.1D** and **6.2D**). Furthermore, no TdP-like arrhythmias were observed after application of this blocker.

Sodium channel (I_{Na}) blockers affect the depolarization phase of the field potential, resulting in a decrease in sodium spike amplitude[7]. The effect of sodium channel blockers on the Na_v1.5 channel is known to be use-dependent[1]. **Figures 6.1E** and **6.2E** show that sodium channel blocker TTX indeed decreases the sodium spike amplitude in Pluricyte® Cardiomyocytes in a concentration-dependent manner. Additionally, the loss of excitability due to sodium channel blockade was noticed as a loss of stimulus capture. At

concentrations higher than 6 µM, stimulation was not possible at all. Furthermore, analysis of the reduction in sodium spike amplitude at different beat rates showed that the effect of TTX exhibits use-dependence: the reduction in sodium spike amplitude tended to be larger at higher beat rates (**Figure 6.3B**).

Concluding Remarks

Pluricyte® Cardiomyocytes in combination with Axion Maestro E-Stim+ MEA technology are highly suitable for next generation of cardiac safety screening assays. Using this technology, Pluricyte® Cardiomyocytes readily adapt their electrophysiology to various pacing rates and still exhibit their unique strengths and characteristics including field potential signals containing well-pronounced depolarization and repolarization peaks.

Pluricyte® Cardiomyocytes, while electrically paced using specialized E-Stim+ MEA plates, showed the expected pharmacological responses, including (reverse) use-dependent drug effects in a reproducible manner.

The combination of Pluricyte® Cardiomyocytes with E-Stim+ MEA technology for the Maestro system provides a highly relevant *in vitro* assay to study cardiac safety profiles of compounds under stable and defined pacing rates at an early stage of drug development. Compared to assays using spontaneous beating cardiomyocytes, this next generation assay provides several advantages: increased biological relevance, reduction of variability and detection of (reverse) use-dependence.

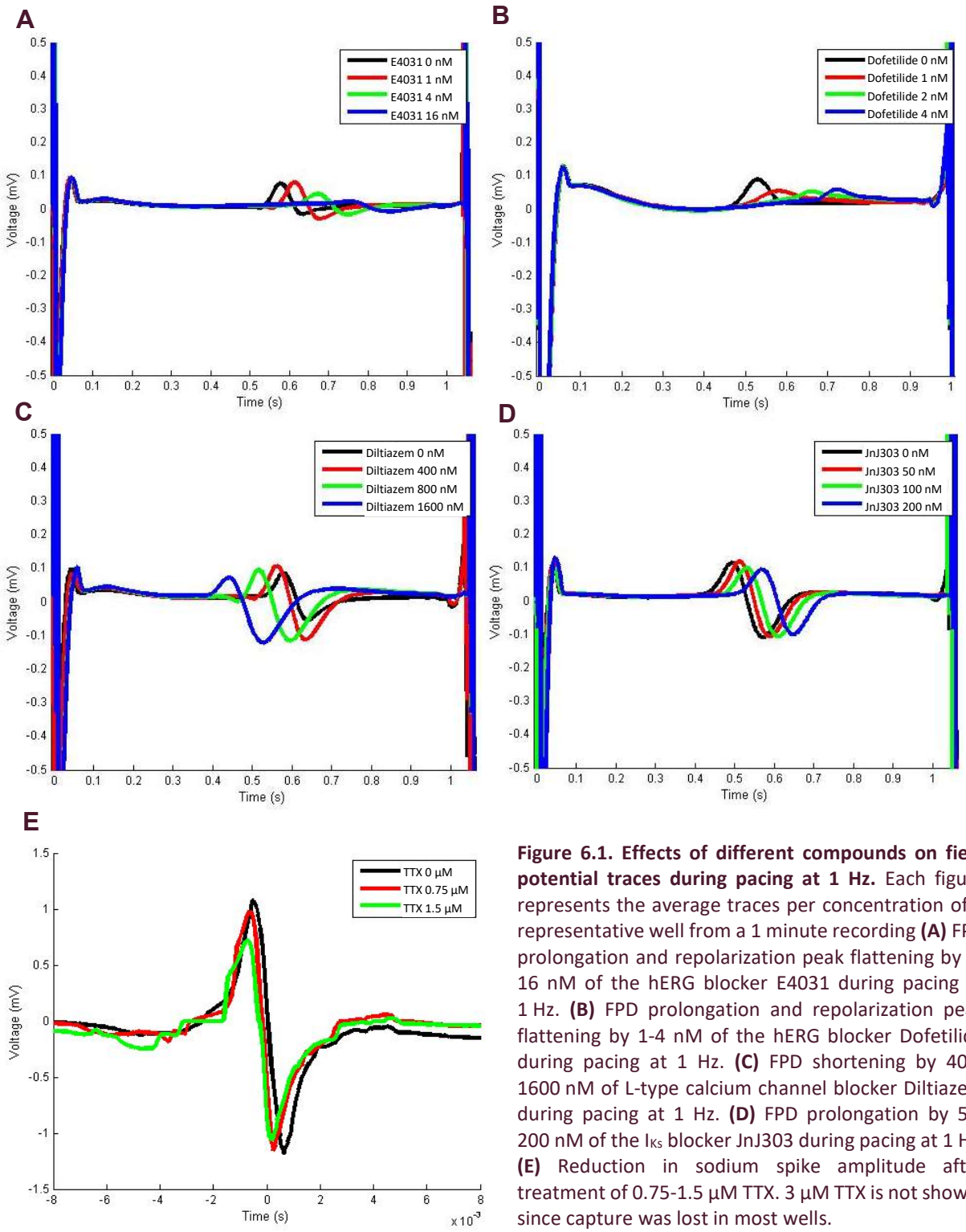


Figure 6.1. Effects of different compounds on field potential traces during pacing at 1 Hz. Each figure represents the average traces per concentration of 1 representative well from a 1 minute recording **(A)** FPD prolongation and repolarization peak flattening by 1-16 nM of the hERG blocker E4031 during pacing at 1 Hz. **(B)** FPD prolongation and repolarization peak flattening by 1-4 nM of the hERG blocker Dofetilide during pacing at 1 Hz. **(C)** FPD shortening by 400-1600 nM of L-type calcium channel blocker Diltiazem during pacing at 1 Hz. **(D)** FPD prolongation by 50-200 nM of the I_{Ks} blocker JnJ303 during pacing at 1 Hz. **(E)** Reduction in sodium spike amplitude after treatment of 0.75-1.5 µM TTX. 3 µM TTX is not shown, since capture was lost in most wells.

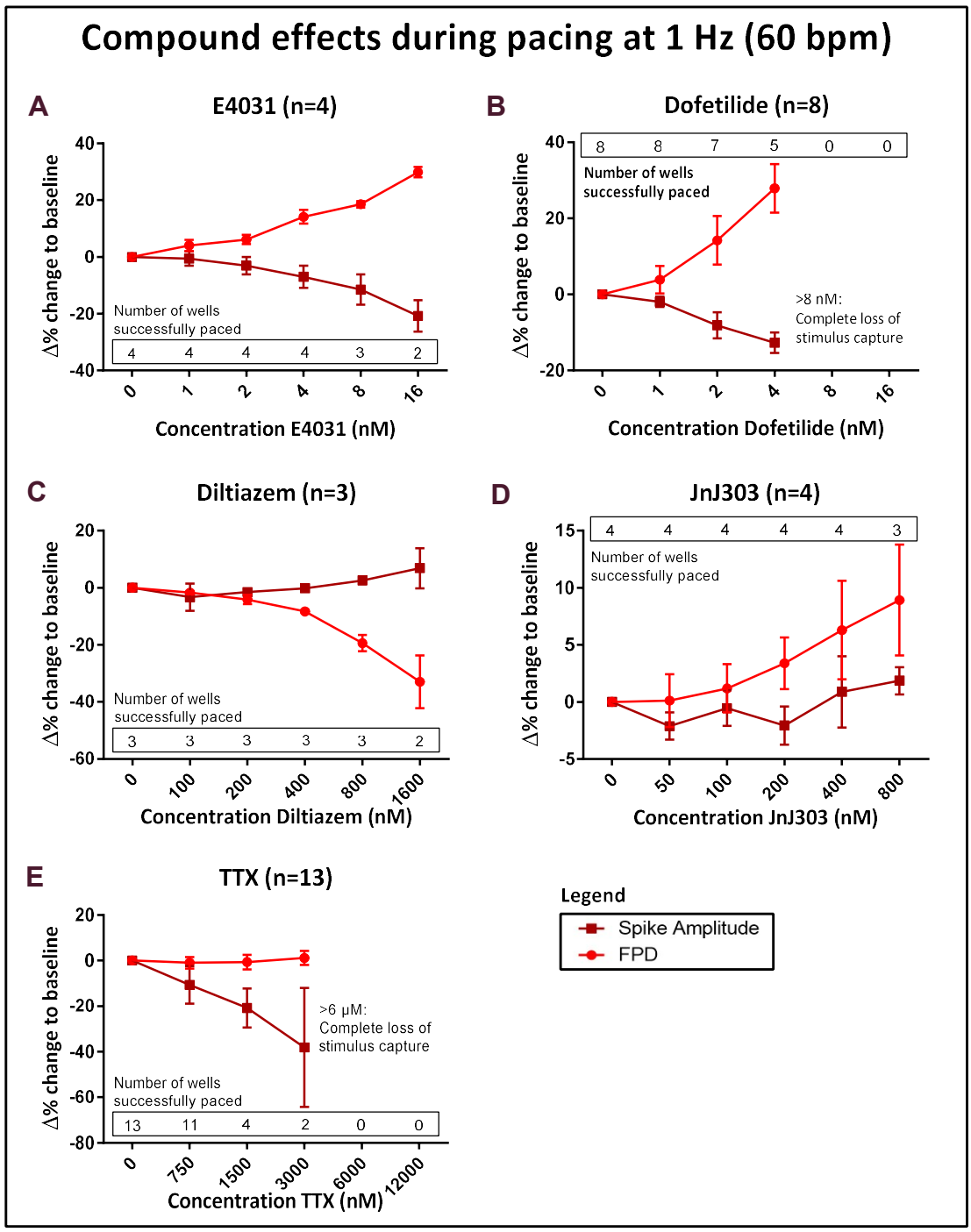


Figure 6.2. Effects of different compounds on field potential parameters during pacing at 1 Hz. Y-values are plotted as Δ% change, meaning that the values are normalized to the control wells by subtracting the average % change in DMSO-treated control wells. Error bars represent standard deviations. **(A)** FPD prolongation by 1-16 nM of hERG blocker E4031 during pacing at 1 Hz. **(B)** FPD prolongation by 1-4 nM of hERG blocker Dofetilide during pacing at 1 Hz. At concentrations of 8 nM or higher, stimulation at 1 Hz was no longer possible. **(C)** FPD shortening by 400-1600 nM of L-type calcium channel blocker Diltiazem during pacing at 1 Hz. **(D)** Tendency of FPD prolongation by 50-200 nM of I_{Ks} blocker JnJ303 during pacing at 1 Hz. **(E)** Reduction in spike amplitude and stimulus capture after treatment of 750-3000 nM TTX. At concentrations of 6 µM or higher, stimulation at 1 Hz was no longer possible. For TTX, experiment was repeated and data was pooled to compensate for the reduction of stimulus capture typically seen after sodium channel blockade.

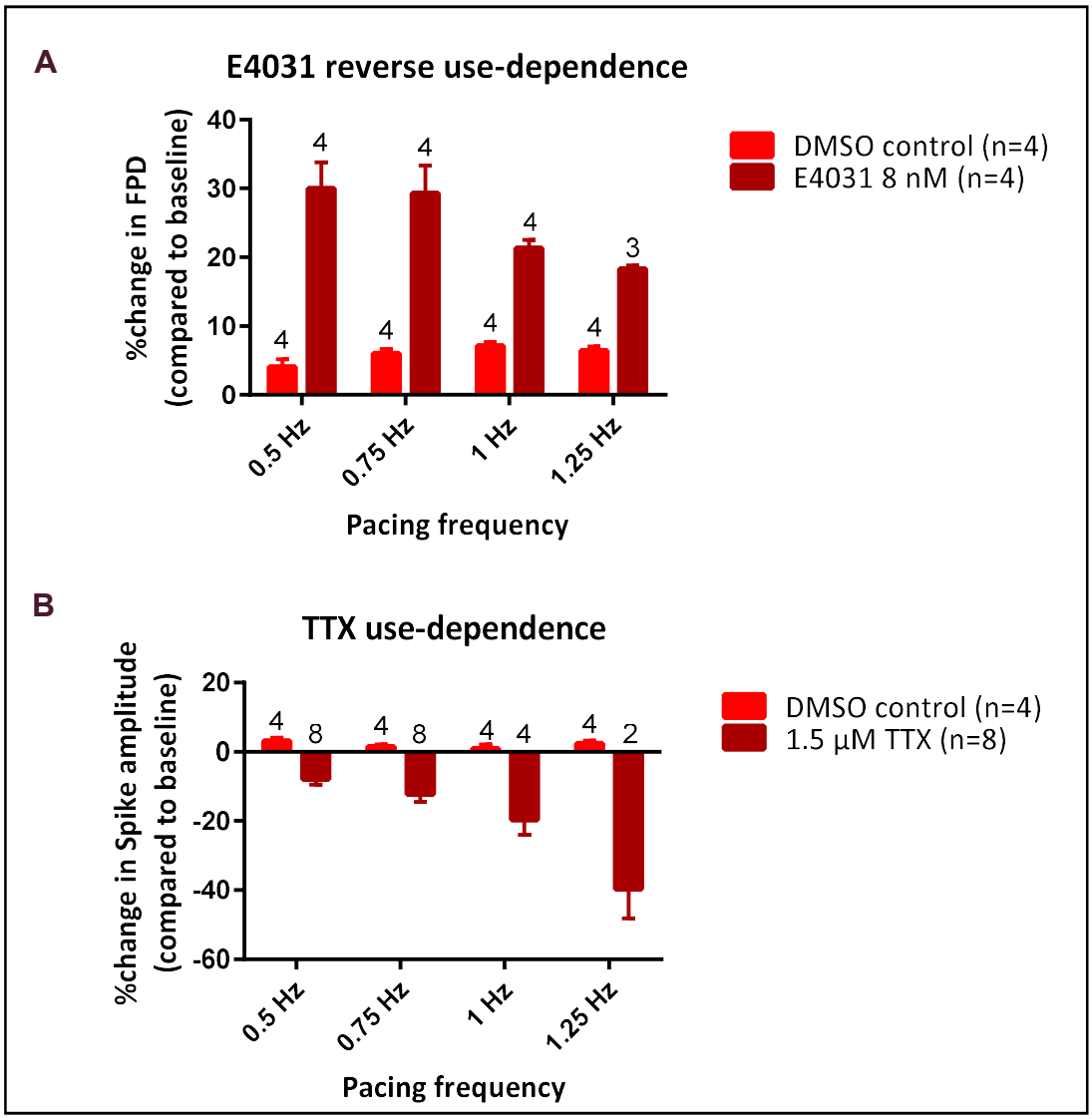


Figure 6.3. Effects of 8 nM E4031 and 1.5 µM TTX at different beat rates. Error bars depict standard error of the mean, number on top of bars depict number of wells successfully paced. **(A)** Reverse use-dependent effects of hERG channel blocker E4031 on the field potential duration of Pluricyte® Cardiomyocytes paced at 0.5-1.25 Hz. **(B)** Use-dependence of sodium channel blocker TTX on the spike amplitude of Pluricyte® Cardiomyocytes paced at 0.5-1.25 Hz. Note that TTX treatment also results in loss of capture in a considerate amount of wells, due to lower opening probability of the sodium channels.

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