



The most sensitive microelectrode array system
for *in vitro* extracellular electrophysiology

MED64 Application Note

Human Stem Cell-derived Cardiomyocytes

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Contents

1. Introduction	1
1-1. Acknowledgement	1
1-2. Disclaimer	2
2. Pre-coating the MED Probes and plating hSC-CMs	2
2-1. Coating the MED Probes	2
2-2. Collecting hSC-CMs from the tissue culture plate	2
2-3. Plating dissociated hSC-CMs onto the MED Probes	3
2-4. Collecting and plating hSC-CM embryoid bodies or clusters onto the MED Probes	4
2-5. Pre-coating the MED Probes, and preparation and plating of Collectis hES-CMC™	5
Coating the MED Probes	5
Plating of hES-CMC™	5
2-5. Cleaning the used MED Probes	7
Trypsin-EDTA- collagenase treatment method.....	7
Bleaching method	8
3. Data acquisition	8
3-1. Setting up the microenvironmental conditions	8
1) Use of the CO ₂ incubator	8
2) Use of the MED Heated Connector	8
3-2. Data acquisition	9
Available Mobius workflow templates	10
Recommended acquisition settings	10
4. Data analysis	11
4-1. Beat frequency analysis	11
Instantaneous beat frequency analysis.....	11
Graphing chronotropic drug effects	12
4-2. Field potential duration analysis	14

FPD analyses available in Mobius QT software	15
Exporting extracted waveforms	17
4-3. Amplitude and slope analysis	19
5. Pacing hSC-CMs	20
5-1. Suppressing stimulus artifacts.....	20
1) Use of platinum wire reference electrodes.....	20
2) Applying bi-polar stimulation	20
5-2. Acquiring paced activity	21
6. References	22

1. Introduction

Human stem cell-derived cardiomyocytes (hSC-CMs) are the subject of intense study in both the pharmaceutical industry and academic institutions. The MED64 has become a popular platform for modeling human hereditary cardiomyopathies and drug screening, since hSC-CMs possess the same genetic background as primary human CMs and their pharmacology is presumably very similar.

The MED64 only requires culture of the hSC-CMs on the MED Probe to extract basic electrophysiological parameters such as beat frequency and field potential duration (FPD). In addition, chronotropic drug effects can be measured thanks to the non-invasive nature of the MED Probe. Nonetheless, the MED64 can pace the hSC-CMs using any of the microelectrodes to study frequency- or use-dependent drug effects.

The goal of this application note is to describe how to set up experiments with human stem cell-derived cardiomyocytes (hSC-CMs), acquire relevant data, and extract the data for presentation or publication. This material has been prepared by scientists with expertise in stem cell biology and cardiovascular pharmacology. A complete protocol for plating, culturing, and carrying out experiments on hSC-CMs has been prepared based on the users' experience.

1-1. Acknowledgement

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1-2. Disclaimer

This application note is a summary of information shared by MED64 users and is to be considered marketing material. These methods have been developed, tested, and verified in the course of projects published in peer-reviewed literature. However, Alpha MED Scientific does not guarantee that the information written in this document is correct and is free from all liabilities. Please refer to the scientific literature for further insight on these techniques, as well as the MED64 and Mobius manuals for detailed instructions on use of the MED64 System.

2. Pre-coating the MED Probes and plating hSC-CMs

These protocols were developed for coating and plating a MED Probe with 4 x 16 - electrode grids (MED-P5004A). However, the method can be scaled up for MED Probes with 2 x 32 - electrode grids (e.g. MED-P5D15A) or a single 64 electrode grid (e.g. MED-P515A).

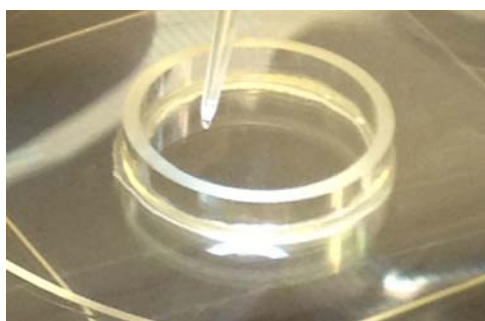
CAUTION:

Avoid contact with the electrodes in all of following procedures as they are extremely fragile.

2-1. Coating the MED Probes

1. Sterilize the MED Probe by dipping in a beaker containing 70% ethanol. Allow it to dry or aspirate the ethanol in a sterile hood.
2. Reconstitute and store matrigel (BD Biosciences Cat # 354277) according to the manufacturer's instructions. Aliquot/store at -20°C and thaw just before use.
3. Place a 2 μ l bead of matrigel over the electrode grids within the MED Probe. (See Figure. 1)
4. Place the MED Probe in a sterile 10 cm diameter tissue culture dish with a lint-free laboratory tissue (kimwipe - Kimtech Science Brand) around it and dampen the tissue with appx 1-2 ml sterile water. The exact volume of sterile water is not critical. Place the kimwipe so as to allow observation of the MED Probe under the microscope.
5. Incubate the matrigel-coated MED Probe for at least 30 minutes.

Longer incubation times are acceptable as long as the matrigel does not evaporate.



A.



B.

Figure. 1. Coating the MED Probe. **A.** Note the 2 μ l bead of matrigel on the pipette tip. **B.** Matrigel coating over 3 of the 4 electrode grids (MED-P5004A, 4 x 16-electrode grids).

2-2. Collecting hSC-CM from the tissue culture plate

These protocols were developed using a 2D monolayer differentiation method that produces appx 2 million cardiomyocytes per well in a 6 well tissue culture plate. For optimal cell density on the MED Probes use 2 wells.

1. Aspirate the growth media from the wells containing the hSC-CMs to be tested.
2. Rinse the hSC-CMs with 1 ml of PBS (Invitrogen, Cat # 14190) and aspirate the PBS.
3. Add 1 ml of TrypLE (Invitrogen, Cat # 12563) to each well and place in the incubator for appx 10 minutes. Incubation times may vary. Optimize the incubation time to your culture conditions by observing the monolayer after 3 minutes every minute or so. Once the cardiomyocytes start dissociating into single cells, gentle taps to the side of the plate should dislodge them.
4. Detach the cardiomyocytes by gently rinsing with the TrypLE 3 - 5 times over the surface of the plate using a 1 ml pipettor. Avoid introducing air bubbles during rinsing.
5. Add 2 ml of growth media containing serum to inactivate the TrypLE.
6. Transfer the TrypLE/growth medium suspension into a 15 ml conical tube.
7. Rinse each well with an additional 2 ml of growth medium 3 times and add the rinse to the same 15 ml tube.
8. Pellet the hSC-CMs using a tabletop centrifuge at 180 x g for 5 minutes (at room temperature).
9. Aspirate the supernatant, being careful not to disturb the hSC-CM pellet. Resuspend the cell pellet in 0.5 μ l of growth medium by gently pipetting.
10. Count the hSC-CMs to confirm a final concentration of 8,000-10,000 viable hSC-CMs/ μ l.
11. If necessary, pellet the hSC-CMs again using the tabletop centrifuge at 180 x g for 5 minutes and resuspend them to a final concentration of 8,000-10,000 hSC-CMs/ μ l.

2-3. Plating dissociated hSC-CM onto the MED Probes

Note: *For the initial plating the growth medium should contain at least 20% serum to promote adherence to the MED Probe (as much as 50% can be used). Add the media placing the pipette tip on the outside wall of the MED Probe, evenly distributing it and stopping before reaching one of the hSC-CM droplets. Then slowly add media until the droplet is reached. If the hSC-CMs are not dislodged, add more until reaching a volume of 1 ml. Otherwise, wait a prudent amount of time before adding more media.*

1. Remove the matrigel-coated MED Probe from incubator and carefully aspirate the matrigel from the microelectrode grids. Avoid contact with the electrodes as they are extremely fragile.
2. Place 2 μ l beads of the hSC-CMs suspension (approximately 16,000-20,000 viable cardiomyocytes) over the microelectrode grids on the MED Probe (Fig .2).
 - The MED Probe has 4 reference electrodes surrounding 64 recording electrodes. These need to be free of cells. Avoid contact with the reference electrodes as well as they are also easily damaged.

2. Pre-coating the MED Probes and plating hSC-CMs

3. Allow the plated hSC-CMs to settle and adhere to the bottom of the MED Probe in a sterile incubator at 37°C for 3-4 hrs.
4. Gently add 1ml of growth media with serum, using a 200 μ l pipettor under microscopic observation taking care not to dislodge the adhered hSC-CMs. Before adding the media, tap the MED Probe gently to confirm attachment of the hSC-CMs.
5. Incubate overnight and add an additional 1ml of media the next day. Replace half the media daily. Serum-free media may be used after the hSC-CMs are firmly adhered.

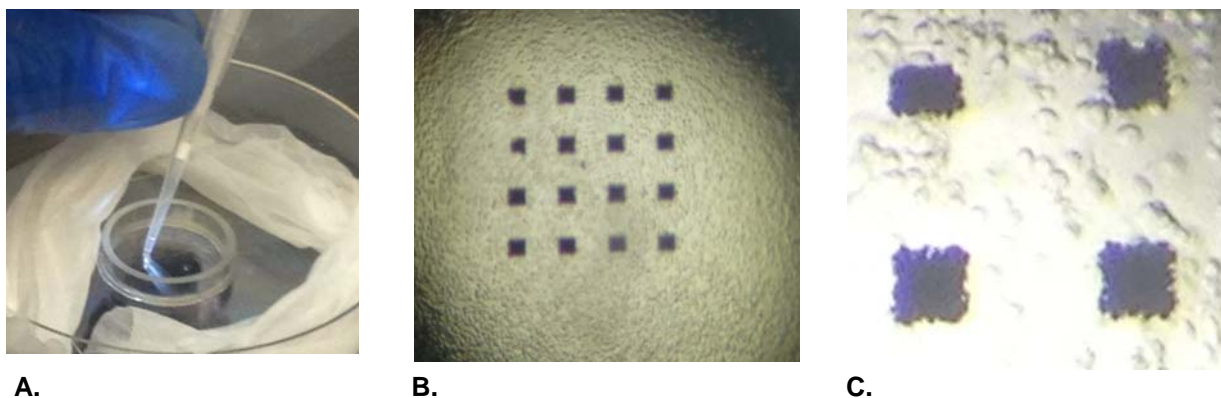


Figure 2. Plating dissociated hSC-CM onto the MED Probe. **A.** A 2 μ l bead of the cardiomyocyte suspension is pipetted onto each of the four electrode grids. **B.** Low- magnification view of the 2 μ l cardiomyocyte bead on a 4 x 4 microelectrode grid in the MED-P5004A Probe. **C.** High-magnification view of dissociated hSC-CMs settling around individual electrodes.

2-4. Collecting and plating hSC-CM embryoid bodies or clusters onto the MED Probes

1. Place a dry MED Probe (MED-P515A) in a sterile 10 cm diameter tissue culture dish and fill with 1 ml of matrigel.
2. Incubate the matrigel-coated MED Probe for at least 30 minutes. Longer incubation times are acceptable as long as the matrigel does not evaporate.
3. Remove the matrigel-coated MED Probe from the incubator, aspirate the matrigel, and add appx 1 ml of growth media.

Note: For the initial plating the growth medium should contain at least 20% serum (as much as 50% can be used) to promote adherence to the MED Probe.

4. Place a Pyrex® 10 x 10 mm cloning cylinder (Cat # 3166-10) around the electrode grid to contain the embryoid bodies after plating.
5. Pick the embryoid bodies or clusters using a 10 μ l pipettor (cut the pipette tip to a bore size that will allow aspiration) and pipette the embryoid bodies or clusters within the cloning cylinder.
Note: Multiple embryoid bodies or clusters can be plated but they may couple electrically over time.
6. Using a Bunsen burner and forceps, make two 45 degree bends at the thin portion of a glass Pasteur pipette. Pull the end so the point fits into the cloning cylinder and gently push the embryoid bodies or clusters onto the microelectrode grid.

7. Incubate overnight and add an additional 1ml of media the next day. Replace half the media daily. Serum-free media may be used after the hSC-CMs are firmly adhered.

2-5. Pre-coating the MED Probes, and preparation and plating of Cellectis hES-CMC™

These protocols were developed for coating and plating a MED Probe with a single 64 electrode grid (e.g. MED-P515A) using hES-CMC™ (CMC-202-CLUS, Cellectis AB, Sweden) in the original 3D structure format, supplied in low binding 96 well plates.

The hES-CMC™ should be removed from the low-binding transport plates as soon as possible after arrival, and transferred to gelatin coated tissue culture plates (according to the manufacturer's instructions). This will promote growth of fibroblasts and ensure attachment of the CMC to the platform surface. It usually takes 4-7 days to achieve adequate attachment.

CAUTION:

Avoid contact with the electrodes in all of following procedures as they are extremely fragile. These procedures should be performed under aseptic conditions as much as possible.

Coating the MED Probes

1. Prepare plating medium, which consists of the maintenance medium with 20% serum instead of 10% serum.
2. Sterilize the MED Probe by dipping in a beaker containing 70% ethanol. Allow it to dry or aspirate the ethanol in a sterile hood.
3. Place the MED Probe in a 10 cm diameter Petri dish.
4. Place a droplet of fibronectin (human, Sigma Aldrich, cat # F0895), at a concentration of 32 µg/ml, over the MED Probe electrode grid and cover with the Petri dish lid.
5. Place the MED Probe in a humidified incubator at 37 °C for at least 30 minutes.
6. Remove the MED probe from the incubator and place in a sterile hood.
7. Add 500 µl of plating medium to the MED Probe to cover the entire chamber surface.

Plating of hES-CMC™

Manipulation of the CMCs is best performed using a stereomicroscope.

1. Prepare and pre-heat maintenance medium according to the manufacturer's instructions.
2. Cut around the CMC with a scalpel (as recommended in the manufacturer's instructions) so that a "skirt" or "collar" of outgrowing cells accompanies the detached CMC.
3. The CMC with attached outgrowth can then be placed on to the electrode region of the MED Probe, using a 200 µl pipette tip.

2. Pre-coating the MED Probes and plating hSC-CMs

4. Remove enough plating medium from the MED Probe chamber so that the CMC is barely covered by liquid. This will vary depending on the CMC size.
5. The CMC can then be gently nudged into place being careful not to touch the electrodes as they are extremely fragile.
6. To prevent evaporation, place a smaller tissue culture dish with pre-warmed sterile water in the Petri dish alongside the MED Probe.
7. Replace the Petri dish lid and very gently move the MED Probe to a humidified incubator (37°C, 95% CO₂).
8. Leave the plated MED Probe undisturbed for at least 24 hours. Make sure that the chamber does not dry out.
9. Confirm attachment of the CMC by tapping the MED Probe gently. If the CMC is not attached incubate for another 24 hours.
10. Fill the chamber to a volume of 1 ml with culture medium. Be very gently so as not to dislodge the CMC as the attachment at this stage is extremely fragile.
11. Replace half the volume of medium every two to three days.
12. Serum-free media may be used after the CMC is firmly attached.

2-6. Cleaning the used MED Probes

The MED Probe's electrical characteristics are best during the first use. High quality signals can be recorded and effective stimulation is possible with the MED64 System's MED Probes thanks to the electrodes' characteristics, the lowest impedance in a commercially available microelectrode array. The electrodes' impedance will increase with repeated use of the MED Probes due to damage in handling and/or residual cellular debris and coating materials. However, the MED Probes can be re-used if they are handled and cleaned very carefully. The following are protocols MED64 users recommend:

CAUTION:

ALWAYS avoid contact with the surface of the MED Probe to preserve the microelectrodes and insulation layer.

Trypsin-EDTA - collagenase treatment method

1. Pipette 0.5 mM Trypsin-EDTA (GIBCO, Cat # 25300-054) into the MED Probe chamber and incubate for 1 hour.
2. Rinse the chamber with PBS 3 times.
3. Dissolve collagenase type I (Sigma, Cat # C0130) in PBS at a concentration of 20 unit/ml.
4. Pour the collagenase solution into the chamber and incubate for 1 hour at 37°C.
5. Discard the used collagenase solution and rinse the MED Probe with double distilled water (DDW) at least 3 times.
6. Dry the MED Probe in a clean area.
7. Store the MED Probe (according to instructions) in a beaker with DDW in a darkened area. (Please refer to the MED Probe product manual.)

Bleaching method

1. Carefully pour or pipette 1 ml of Bleach (Clorox®) into the MED Probe and leave it for about 15-30 seconds.
2. Aspirate and repeat step 1 three times. If the MED Probe is not clean after this, apply 2 more rinses and a longer exposure. (usually 1-2 minutes is enough, but as long as 15 minutes is acceptable.)

CAUTION:

Avoid exposing the MED Probe to Bleach over 20 minutes.

3. Rinse the chamber 5 times with double distilled water (DDW).
4. Allow it to dry or aspirate in a sterile hood.
5. Store the MED Probe (according to instructions) in a beaker with DDW in a darkened area. (Please refer to the MED Probe product manual.)

3. Data acquisition

The cultured hSC-CMs start beating spontaneously within a day or so, but beating becomes consistent and rhythmic usually 3-7 days after plating. Although spontaneous beating is considered a sign of immaturity, drug effects on beating frequency can be examined.

3-1. Setting up the microenvironmental conditions

35.8-36.5°C is the recommended temperature for recording from hSC-CMs. Beat frequency is very sensitive to temperature. Beat rate can increase dramatically as temperature rises above 37°C.

Changes in pH can also cause fluctuations in beat frequency. Changes in pH can be caused by changes in CO₂ concentration in the air. Thus, **it is very important to let the hSC-CM sit in an environment where the CO₂ concentration is stable**. Two methods are recommended to achieve stability of temperature and the CO₂ concentration.

1) Use of CO₂ incubator

Place the MED Connector (MED-C03) inside a CO₂ incubator. Please note that incubators can introduce noise. Please refer to page 29-36 in the MED64 Handbook (vol1) to avoid noise introduced by incubators. Particularly, recordings can be compromised by noise introduced by the rapid temperature increases that occur as they power on. Wait until the desired temperature is stable before starting acquisitions (it could take several hours depending on the incubator).

Place the drug aliquot to be used for your experiment in a heated water bath to minimize the temperature changes caused by drug applications. Administer the drugs quickly as opening the incubator door allows the temperature to cool down and the CO₂ concentration to change (which can change beat rate). When the incubator door is opened, note and consider waiting until the stability in desired temperature and the CO₂ concentration is achieved before re-starting acquisitions (this could take longer than drug effect).

2) Use of the MED Heated Connector

The MED Heated Connector (MED-CP02H) warms up the MED Probe chamber bottom. The use of the MED Connector Cover (MED-CC01) is recommended to maintain the desired temperature and CO₂ concentration (to avoid the pH changes) around the MED Probe. Provide gas mixtures (the mix ratio depends on composition of your culture medium) through the "gas port" on the top of the Connector Cover. Bubble the gas through a beaker containing distilled water to maintain a humidified environment.

In order to achieve stability for the temperature and the CO₂ concentration, follow the instructions below.

1. Turn on the ThermoClampTM-1 (controller for the MED Heated Connector) connected to the MED Heated Connector at least 30 minutes before starting acquisition.
2. If/When the set-temperature is changed, wait for the temperature measured by the ThermoClampTM-1 controller to stabilize at the new temperature. It could take 5-30 minutes or more.
3. Do NOT place the MED Heated Connector in an environment where temperature changes frequently, for example in the proximity of an air conditioner.

4. For drug application:

- Warm up the drugs to the same temperature or a few degrees higher than the bath media prior to administration.
- Use the lid for drug application. Open and close it quickly.
- Once the lid is opened, wait for 5 minutes or more until the temperature and CO₂ concentration stabilize.

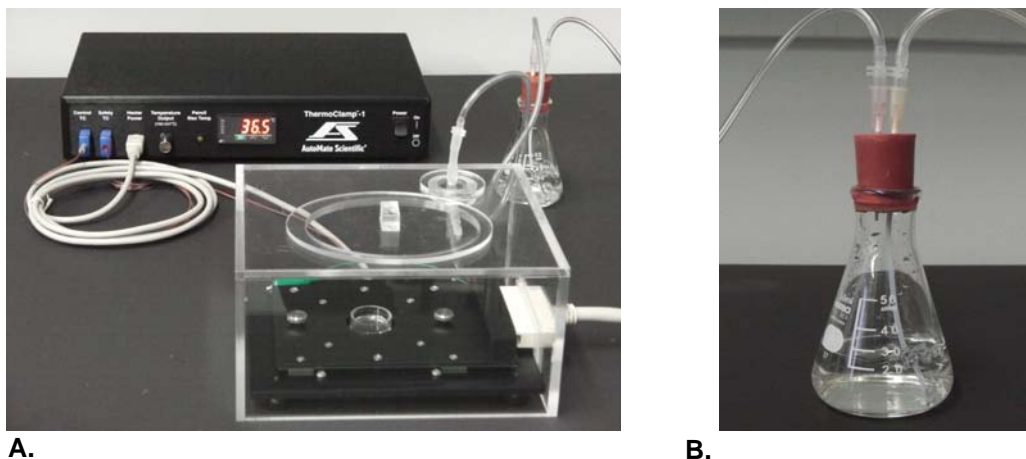


Figure 3. Equipment for microenvironmental control. **A.** Complete experimental set up with the MED Heated Connector/ThermoClamp™-1 controller and MED Connector Cover. **B.** Bubble the gas through a beaker containing distilled water to maintain a humidified environment.

3-2. Data acquisition

The MED Probe has 64 recording electrodes as well as 4 reference electrodes. The differences between the field potential acquired at the recording electrodes and the potential at the reference electrodes are measured by the MED64 System. Acquired signals are sent to the MED64 Head Amplifier through the MED Connector/MED Heated Connector. The raw signals are amplified by x10 with the Head Amplifier, and then amplified further and digitized with the MED64 Main Amplifier. We highly recommend reading the "Product manual" for each component, as well as the "MED64 Handbook", and "Mobius Tutorial" before using the MED64 System.

Required MED64 System Components

- 1) MED Probe
- 2) MED Connector (MED-C03) / MED Heated Connector (MED-CP02H) *1
- 3) MED64 Head Amplifier (MED-A64HE1)
- 4) MED64 Main Amplifier (MED-A64MD1)
- 5) Acquisition PC
- 6) Mobius software (Mobius QT Package or Mobius QT and EP package)
 - *1. MED Heated Connector requires the ThermoClamp™-1 controller.

Mount the MED Probe on to the MED Connector/MED Heated Connector.

CAUTION:

Clean the terminals on the outer portion of the MED Probe with a Kimwipe soaked in ethanol before mounting the MED Probe. Salt sediments can damage the contact pins on the MED (Heated) Connector.

Available Mobius workflow template

Data can be acquired immediately using the available Mobius workflow templates enumerated below (please refer to page 83, Chapter 4: Mobius QT on the Mobius Tutorial):

- Beat_recording: For recording and real-time analysis of beat frequencies and interspike intervals (ISI).
- QT_recording: For recording and real-time analysis of field potentials obtained from spontaneous beating.
- Pacing_recording: For recording of paced responses and real-time FPD analysis.

Recommended acquisition settings

The following parameters are recommended to perform data acquisition for FPD analysis:

Input Range (Maximum input signal level):	2.9 mV
Low cut freq (High pass filter) :	1 Hz
High cut freq (Low pass filter):	1000 Hz

These are guidelines that can be changed depending on the signal amplitude and/or purpose of the study. Particularly change the Input Range to 5.0 mV when 10 kHz is selected for the low pass filter. Please refer to page 88 in the Mobius Tutorial for more details.

4. Data analysis

The MED64 can easily record field potentials from beating hSC-CMs. Mobius QT has a variety of built-in analyses for studying different parameters. Analyses can be exported for post-processing using other software packages.

4-1. Beat frequency analysis

Mobius QT's built-in "Beat_recording" and "Beat_frequency_analysis" workflow templates can extract beat frequency and interspike interval (ISI) both during and post-acquisition, respectively. Please refer to page 98 and 99 in the Mobius Tutorial for detailed instructions.

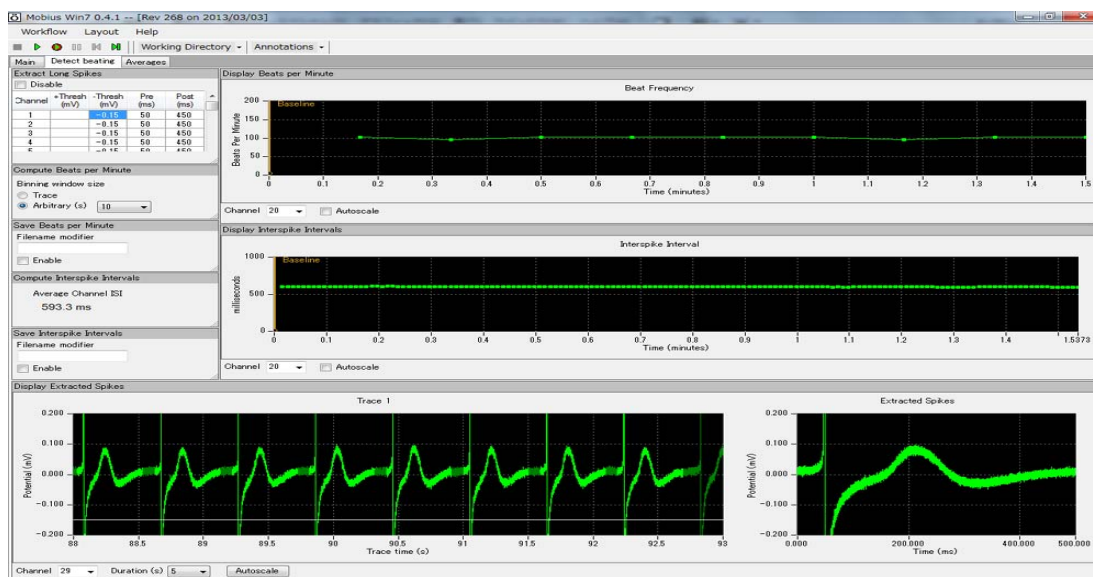


Figure 4. Screen shot of Mobius QT's built-in workflow for beat frequency analysis. Cardiac signals that surpass user-determined thresholds are extracted. Beat frequencies and ISI can be saved as ASCII files. Beat frequencies and interspike intervals (ISI) are computed and graphed.

Instantaneous beat frequency analysis

The beat frequency and ISI data can be exported as ASCII files using the aforementioned workflow templates. Instantaneous beat frequency can be calculated dividing 1000 by the Interspike Interval (in milliseconds) and multiplying the result by 60, as seen in the function dialogue box $[fx=(1000/ISI)*60]$ in the Figure. 5.

The calculated instantaneous beat frequency data can easily be graphed in Excel or any data analysis software to make a figure showing the timecourse of a drug effect on beating frequency.

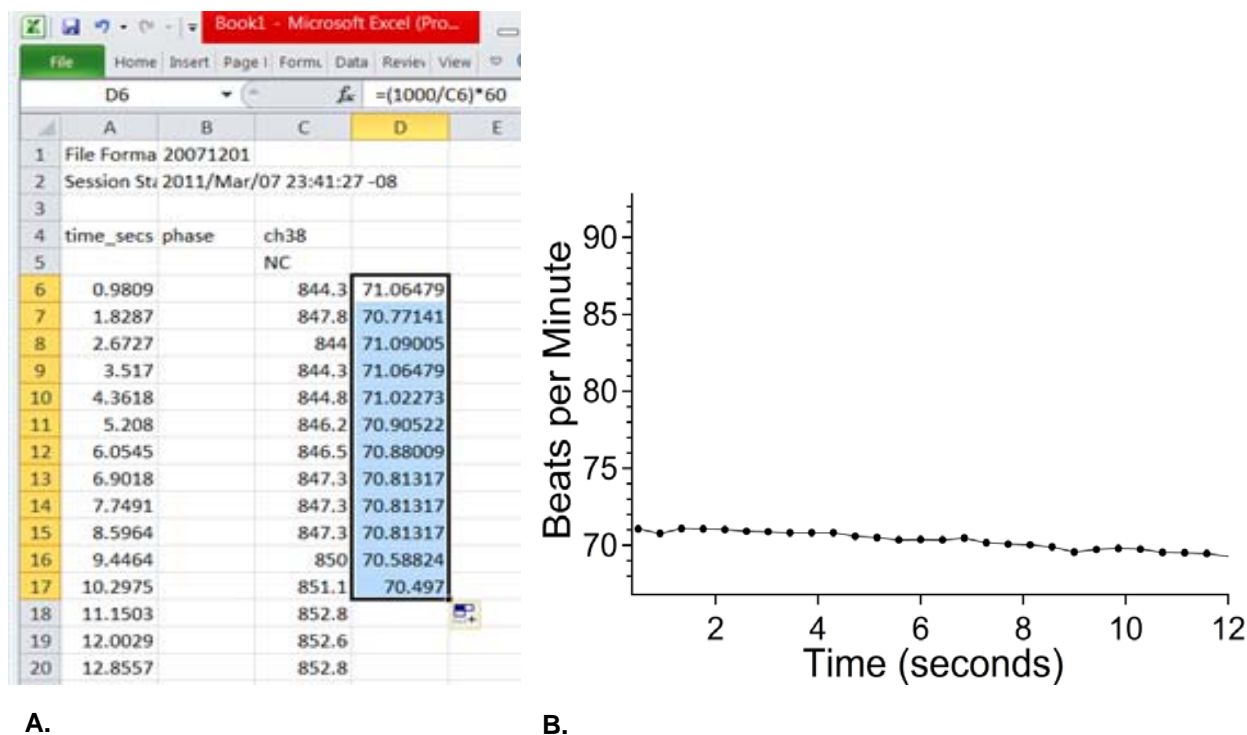


Figure 5. Instantaneous beat frequency calculated and graphed for publication. **A.** The interspike interval (ISI) data exported as an ASCII file contains the timepoints and ISI in milliseconds (Column C). In this case, Microsoft Excel has been used to open the file. The instantaneous beat frequency can be obtained for each timepoint dividing 1000 by the Interspike Interval (in ms) and multiplying the result by 60, as seen in the function dialogue box, $fx = (1000/ISI) * 60$. **B.** Any software package such as Excel, Igorpro, or MATLAB can be used to produce graphs for plotting the instantaneous beat frequency changes over time obtained from the ISI ASCII file. In this example Igorpro has been used.

Graphing chronotropic drug effects

For presentation purposes, it is useful to display the steady state effect of a drug on the hSC-CM preparation's spontaneous beating. This can be done using the "Spike_train" workflow following the procedure below:

1. Download the "Spike_train" workflow from <http://www.med64.com/resources/utilities.html>.
2. In the [Replay Raw Data] control panel, select the file, Trace#, Trace time, and Channel to be exported.
3. In the [Filter Raw Data] control panel, select "Down-sampling" for the Filter Type and select a sampling frequency of 1000 Hz. This can be modified depending on your signals.
4. In the [Export Raw Data] control panel, input the name of your choice for the file to be generated, select the type of data format, and check the "Enable storage" check box.
5. Run the workflow with the Green-Red button.

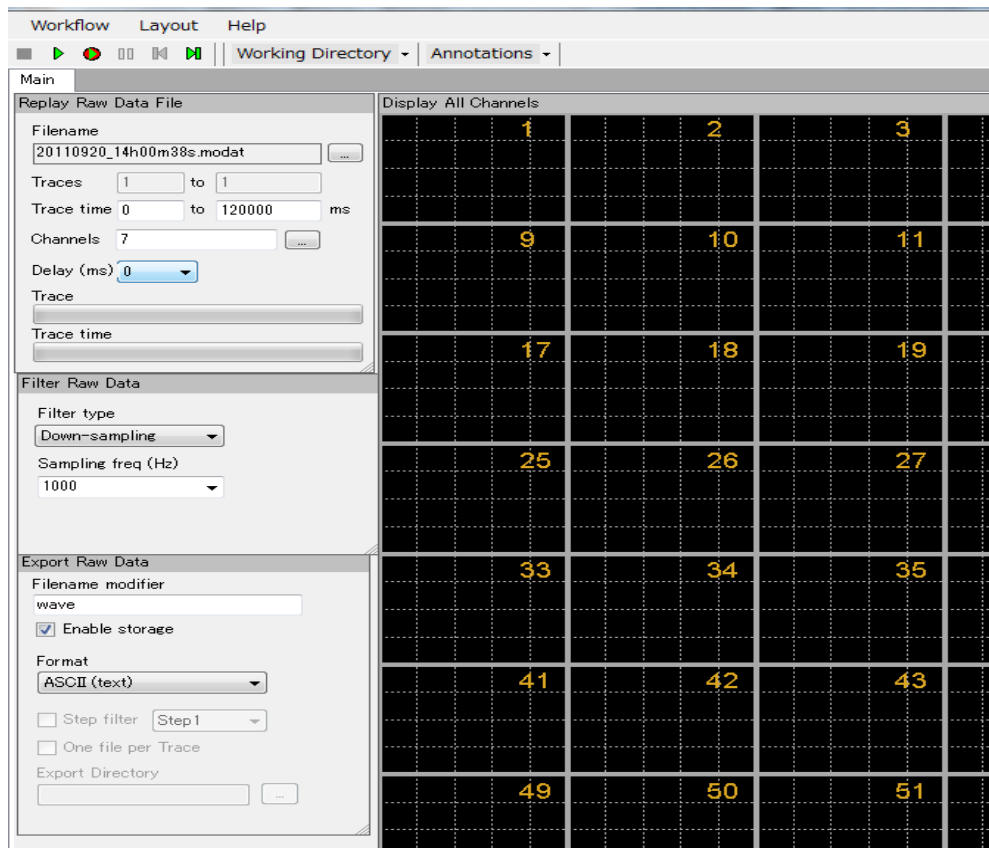


Figure 6. “Spike_train” workflow. The data downsampled to 1 kHz for channel 7 acquired between 0-120 seconds can be exported by clicking the Green-Red button.

- The resulting ASCII or CSV file can then be imported into the software package of choice to produce a graph with the region of interest, as shown in Figure. 7.

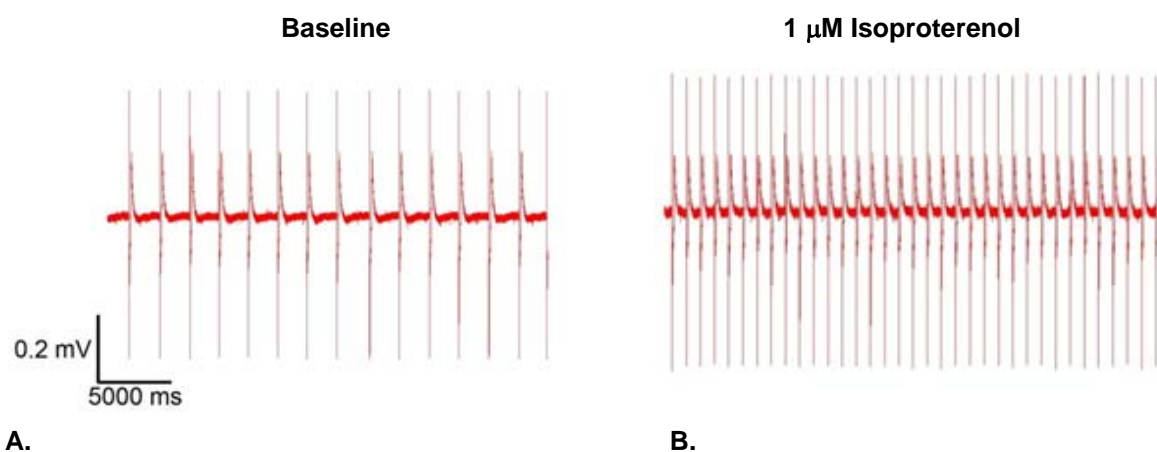


Figure 7. Graphing chronotropic drug effects. **A.** Rhythmic spontaneous field potentials at baseline. **B.** Steady-state effect of isoproterenol ($1 \mu\text{M}$) on beat frequency.

4-2. Field potential duration analysis

The hSC-CM field potential typically starts with a fast depolarizing spike (due mainly to Na^+ influx) followed by a slow repolarizing wave (secondary to K^+ efflux). Field potential duration (FPD) is the electro-physiological correlate of the QT interval obtained via clinical EKG and the cardiomyocyte action potential duration obtained via patch-clamping. QT interval prolongation is a risk factor for Torsades de Pointes, a life threatening arrhythmia. Thus, FPD measurement is a parameter that is used to predict cardiac safety.

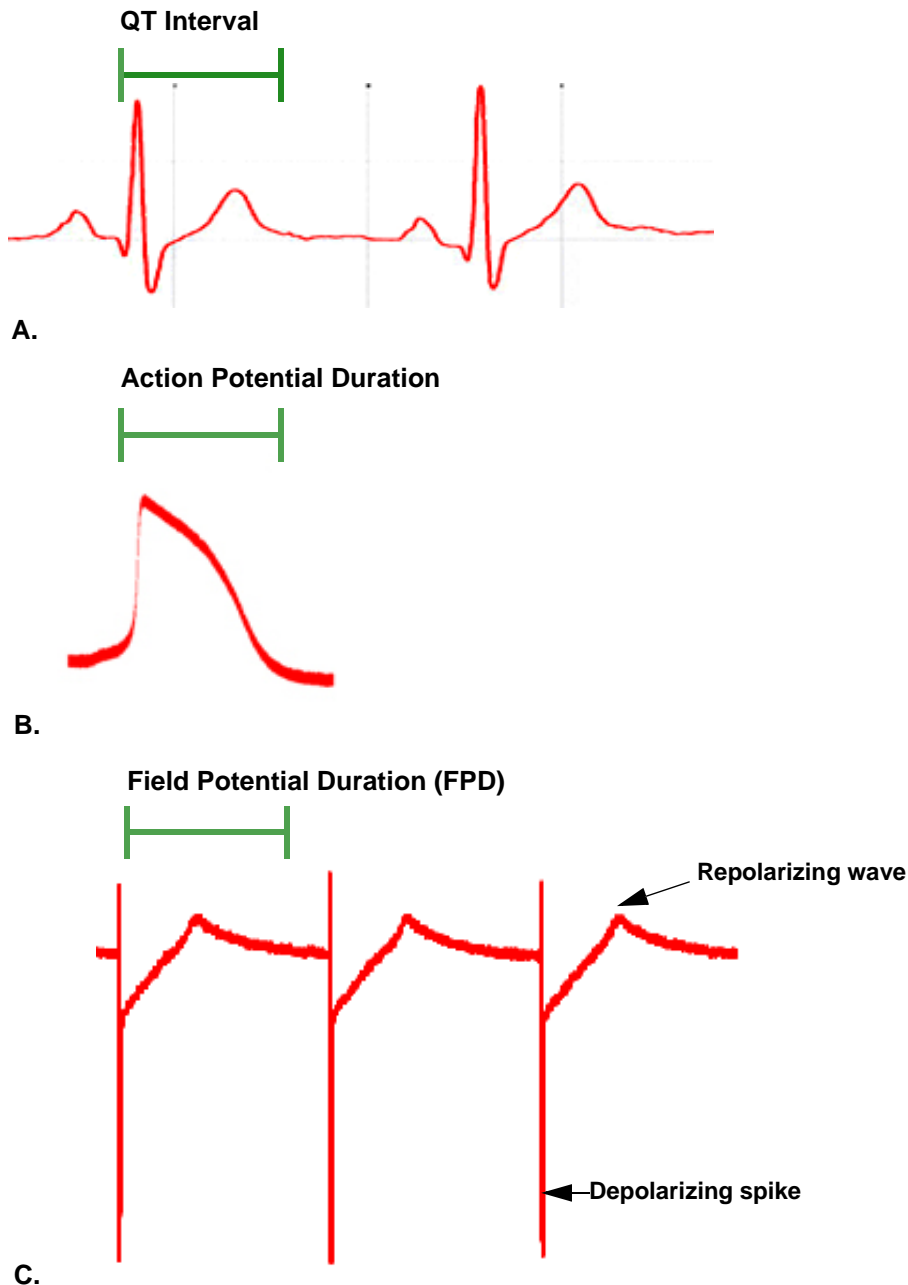


Figure 8. QT interval, action potential duration, and field potential duration. **A.** Classic EKG trace illustrating the QT interval. **B.** Ventricular hSC-CM action potential recorded via whole-cell patch-clamping. **C.** Field potentials recorded from an hSC-CM embryoid body with the MED64. Note the similarity with the EKG in panel A.

Mobius' "QT_recording/QT_analysis" workflow templates are available for FPD analysis (Refer to page 84-85, 96-97 in the Mobius Tutorial in detail). Mobius can automatically extract the FPD using several methods including measurement of the time between the fast depolarizing spike (either positive or negative) and the slow repolarizing wave. Please refer to pages 102 to 105 in the Mobius Tutorial for detailed instructions on performing FPD analyses.

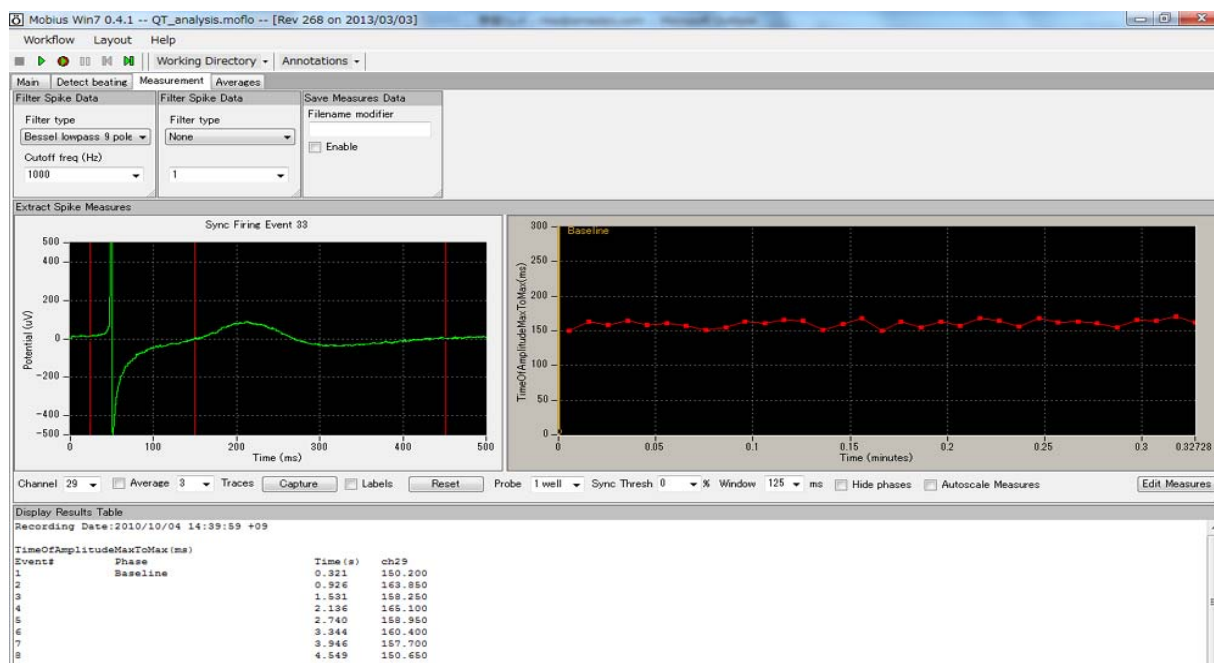


Figure 9. "QT_recording/analysis" workflow template. Signals that surpass user-determined thresholds are extracted and their FPDs are measured using the "Time of Amplitude Max to Max" in this example.

FPD analyses available in Mobius QT software

There are 3 built-in analysis methods for measuring FPD in Mobius QT.

1. Time of Amplitude Max(Min) to Max(Min)

Measures the timepoint at the signal's maximum (minimum) amplitude between the 2 cursors on the left and 2 cursors on the right. (Figure. 10)

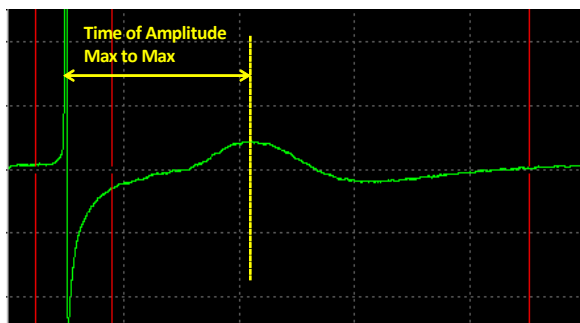


Figure 10. FPD Analysis using the "Time of Amplitude Max to Max" analysis. This method measures the duration between the maximal datapoint in the initial-fast depolarizing spike between the first two cursors and the maximal datapoint in the slow repolarizing wave between the second two cursors.

2. Time of Crossing Horizontal Cursor

Computes the FPD by measuring the duration between the left vertical cursor ("Cursor 1") and the first intersection of the waveform with the horizontal cursor AFTER the right vertical cursor ("Cursor 2", Figure. 11).

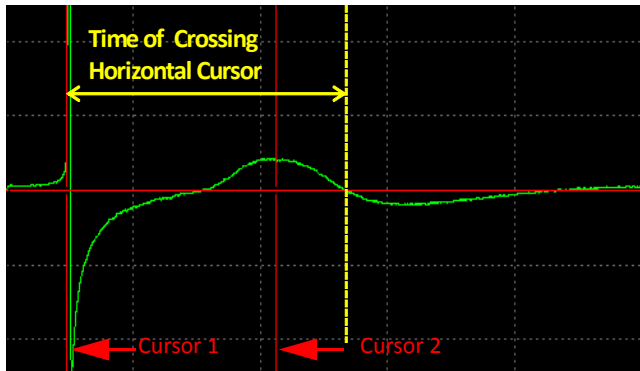


Figure. 11. FPD Analysis using the "Time of Crossing Horizontal Cursor" analysis. The time between the first cursor and the first intersection of the waveform with the horizontal cursor AFTER the right vertical cursor is calculated in this measurement.

3. Time of Slope Crossing Horizontal Cursor

This analysis uses four cursors as shown in Figure. 12, three vertical and one horizontal. It computes the time between the left-most vertical cursor ("Cursor 1") to the intersection of the linear fit slope line and the horizontal cursor. "Cursor 2" and "Cursor 3" are used to select the portion of the waveform to be fitted with the slope line.

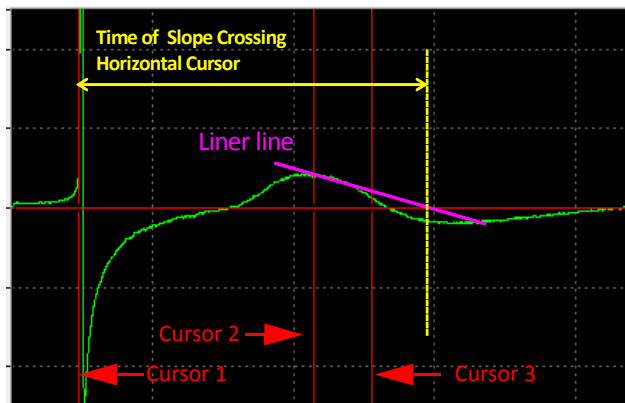


Figure. 12. FPD Analysis using the "Time of Slope Crossing Horizontal Cursor" analysis. This analysis method measures the duration between the first cursor and the timepoint the linear fit slope line between the Cursor 2 and 3 crosses the horizontal cursor.

NOTE: The QT interval is defined as the period between the beginning of the QRS complex and the end of the T wave. The most accurate correlate of this measurement can be obtained by measuring the time between the initial deviation from the isoelectric baseline preceding the beginning of the fast depolarizing spike and the return to baseline after the slow repolarizing wave.

Exporting extracted waveforms

Mobius QT can easily export waveforms as ASCII files. Often pharmacological studies require 10 to 15 minutes to ensure the effect has stabilized, but usually only the last 30 seconds of the recording are analyzed offline. For presentation purposes, comparison of the waveforms averaged for the last 30 seconds of the recording is often desired.

In order to perform this, determine the number of spikes during these last 30 seconds, then average the waveform this number of times in Mobius QT. The averaged waveform can be obtained on the left chart in the "Extract Spike Measures" panel using the following procedure (Refer to Figure. 13):

1. Run the data with the Green button and identify the number of spikes (traces) during the last 30 seconds.
2. Check the [Averages] Check-box.
3. Input the number of spikes during the last 30 seconds in the [Traces] selector. (47 at the Figure. 13.)
4. Select the time for the last 30 seconds in the [Replay Raw Data] module (in Main tab).
5. Run Mobius by clicking the Green button. The waveform for the averaged data is obtained in the left waveform chart.

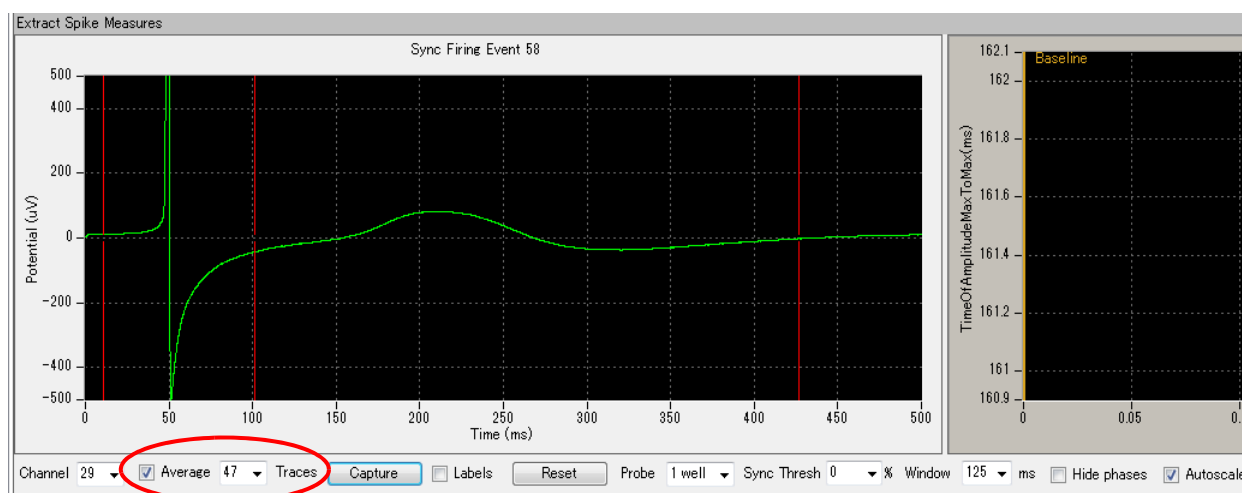


Figure. 13. Averaging waveforms. All traces for the last 30 seconds (47 traces in this example) are averaged and the resulting waveform is displayed in the left chart.

6. Right-click on the left waveform graph, then select [Copy data].
7. Create an Excel spreadsheet file with one column numbered in 0.05 intervals from 0.05 up (to the total amount of milliseconds in the [Extract Spike Measures] module panel so the data is matched to it) (Figure. 14A). Then paste the waveform data in the column next to it (Figure. 14B).

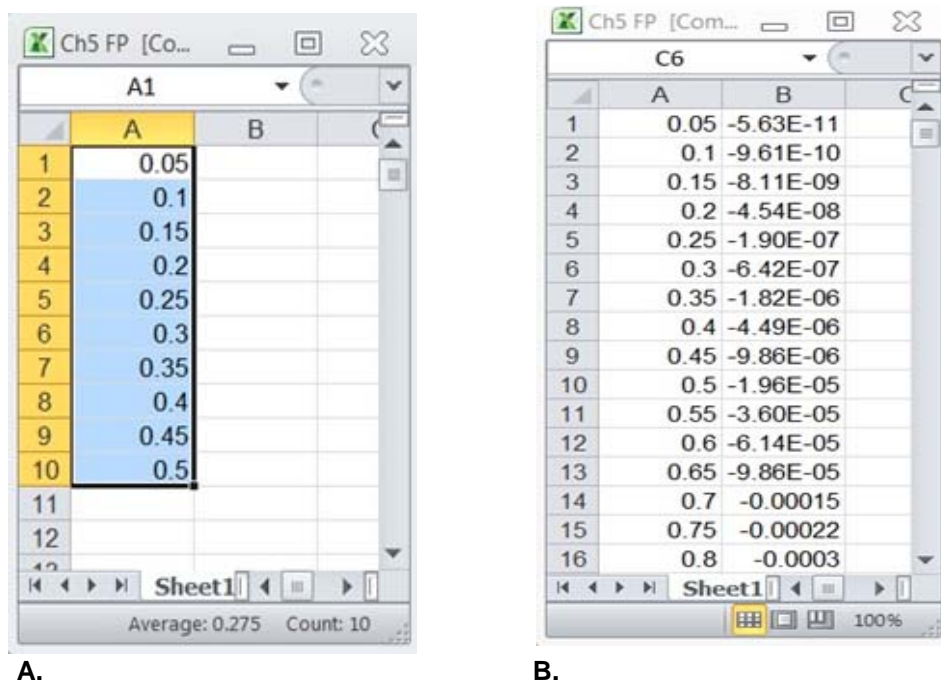


Figure. 14. Creating the Excel spreadsheets to graph a waveform. **A.** Make a new spreadsheet with one column numbered in 0.05 intervals from 0.05 up (to the total amount of milliseconds analyzed in the workflow). **B.** Paste the data copied from the waveform chart in the "Extract Spike Measures" panel.

- Now you can make a graph of the field potential waveform plotting the data vs the time using any software package with graphing capabilities such as Excel, Igorpro, or Matlab. The averaged waveforms for each dose can be superimposed to create a figure demonstrating the dose-dependence of FPD prolongation (Figure. 15).

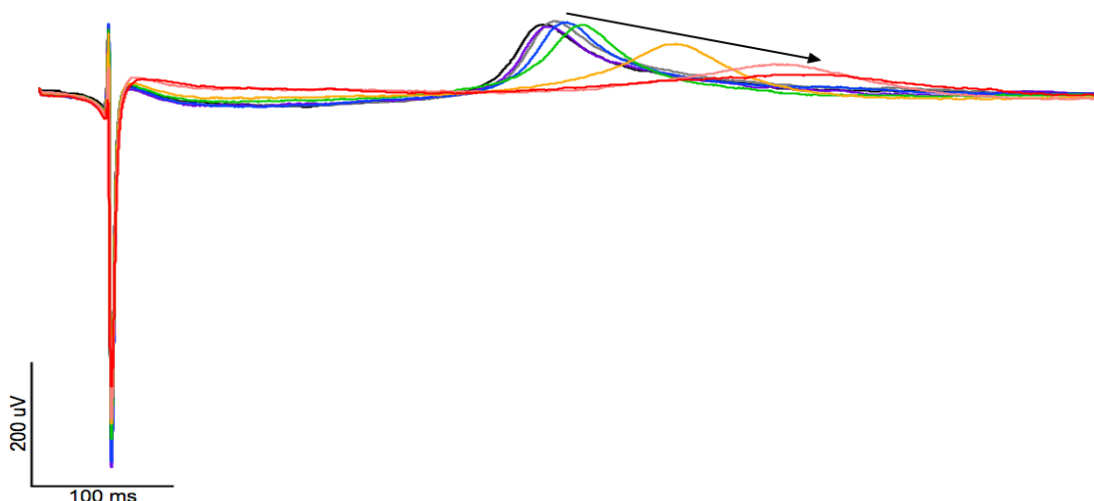


Figure. 15. FPD prolongation with administration of a hERG blocker. FPD prolongation induced by quinidine, a well-known hERG blocker. Any data analysis software can be used to produce graphs obtained from the ASCII file generated by Mobius. In this example, Igorpro (Wavetetrics Lake Osego, OR, USA) has been used to overlay field potential waveforms recorded during administration of incremental concentrations of quinidine.

4-3. Amplitude and slope analysis

Amplitude and slope analysis is useful for studying the ionic components of the hSC-CM field potential. For example, the initial fast depolarizing spike is mainly due to sodium influx, thus block of cardiac sodium channels can be examined by analyzing its amplitude and slope. Below in Figure 16, an expanded view of the fast depolarizing spike in Figure. 15 demonstrates quinidine's well-known blockage of the fast activating sodium current. (Refer to Figure. 16).

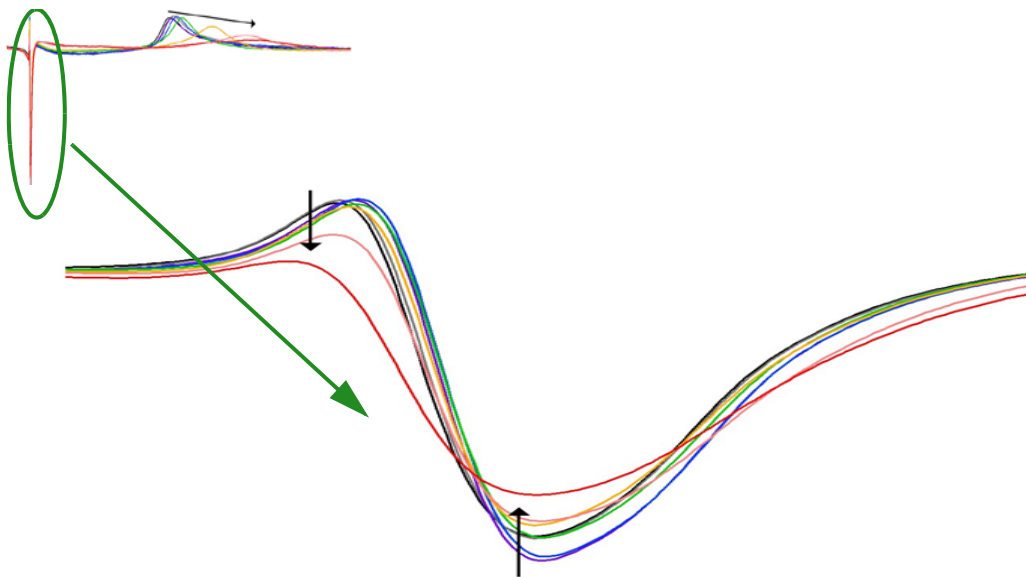


Figure. 16. Dose-dependent changes in field potential amplitude and slope due to a sodium channel blocker. Expanded view of hSC-CM field potential's fast depolarizing spike demonstrating the dose-dependent reduction in amplitude due to blockage of the fast activating sodium current.

These analyses can be performed using Mobius by selecting "Amplitude" or/and "Slope" measurement in the [Extract Spike Measure] module (in the "QT_recording/analysis" workflow template). Please refer to page 108 in the Mobius Tutorial for instructions on performing amplitude and slope analyses.

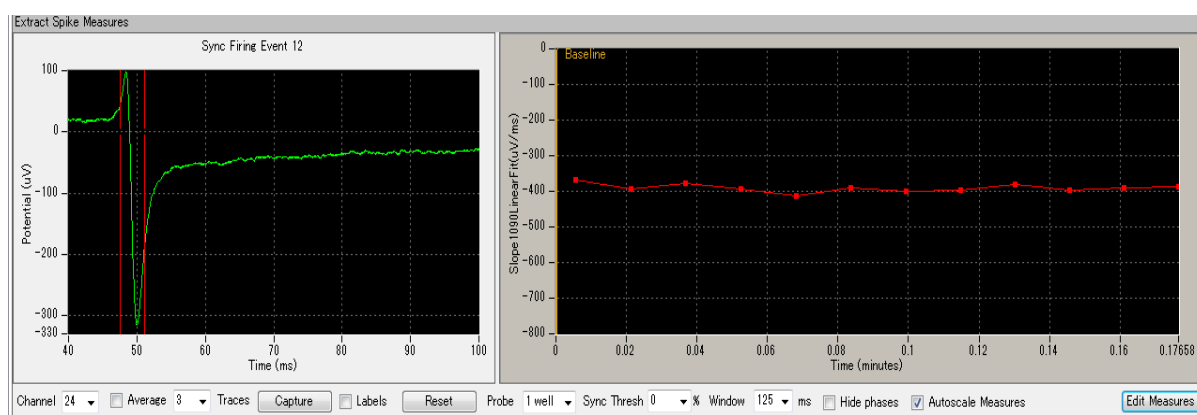


Figure. 17. Analyzing the fast depolarizing spikes with Mobius. In this analysis, "Slope 1090 Linear Fit" has been chosen. On the left graph in the "Extract Spike Measures" panel, the cursors are placed for analyzing the region of interest in the waveform. The right graph shows the slope values over time.

5. Pacing hSC-CMs

Field potential duration (FPD) is correlated with beat frequency. Evaluation of FPD with pacing is useful for normalizing the experiments to the same frequency and also for determining frequency- or use-dependent drug effects.

5-1. Suppressing stimulus artifacts

When pacing, please follow the instructions below to suppress stimulus artifacts:

1) Use of platinum wire reference electrodes

Insert the platinum wire into the MED Probe chamber and ground it using the alligator clip on the top unit of the MED Connector.

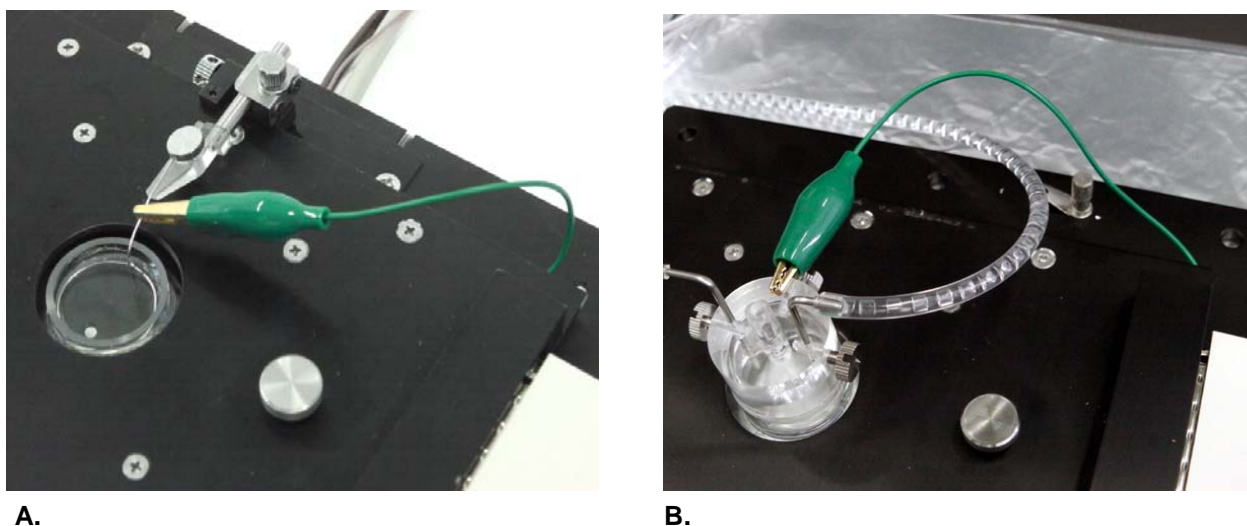


Figure 18. Grounding the platinum wire to the MED Connector. **A.** Grounding the platinum wire with an open chamber. **B.** Grounding the platinum wire in the Perfusion Cap.

2) Applying bi-polar stimulation

Stimulate using 2 adjacent electrodes simultaneously to induce pacing activity more effectively. Make sure **the electrodes' stimuli have opposite polarities** to suppress the stimulus artifact.

For recording paced activities, the "Pacing_recording" workflow template is available. Open the workflow template, and check the "F2" stimulator. Program the stimulus waveform with identical stimulus current amplitude (Amplitude) and duration (Length) but reversed polarity for F1 and F2 (Figure. 19).

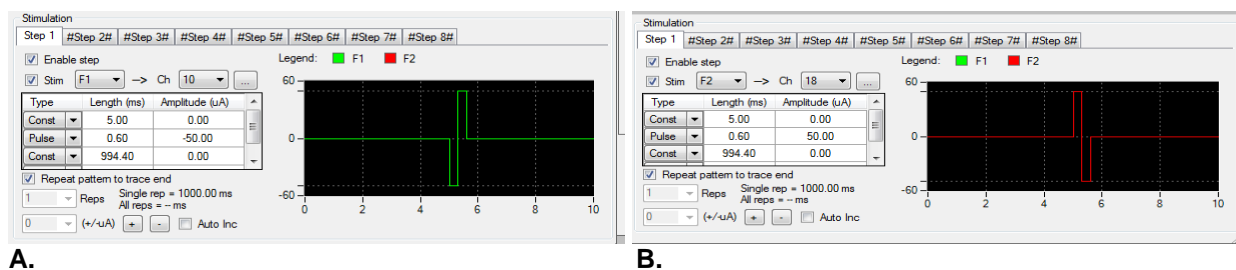


Figure 19. Stimulus parameters for bi-polar stimulation with 2 adjacent electrodes. **A.** Stimulus protocol used for F1 output channel. **B.** F2 stimulus protocol is an identical waveform with reversed polarity. Adjacent electrodes (Ch 10 for F1, Ch 18 for F2) are selected for stimulation.

5-2. Acquiring paced activity

The interval between stimuli should be shorter than the interspike interval during spontaneous activity to drive beating because a pacing stimulus will be ineffective during a spontaneous beat's absolute refractory period. The beat rate at the recommended temperature settings (35.8-36.5°C) is usually less than 50 BPM, so pacing the hSC-CMs at 60-100BPM (the normal adult human heart rate) should not be a problem. The "Pacing_recording" workflow template's default settings will pace the hSC-CMs at 60BPM.

The stimulus parameters below are a good starting point for triggering beating. If this fails to stimulate beating, increase the stimulus amplitude and duration (up to 200 μA x 0.6 msec). Please refer to the MED64 Product manual for optimal stimulus current amplitudes and durations to avoid electrolysis.

Stimulus duration: 0.4-0.6 msec
Stimulus current amplitude: 50 μA

Successful pacing will result in a fast depolarizing spike immediately after the stimulus artifact as shown in the Figure. 20.

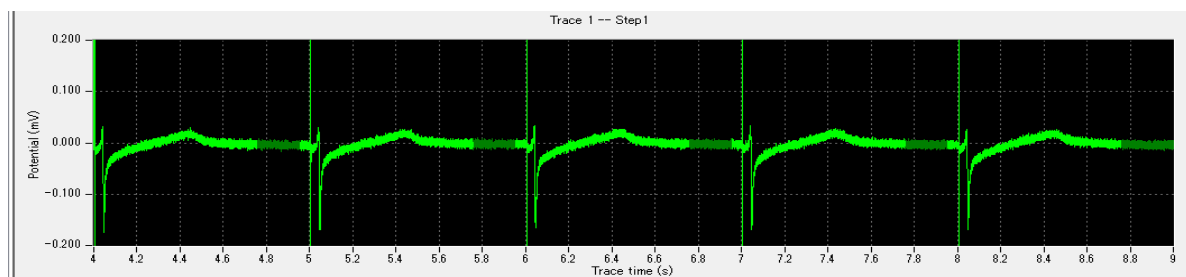


Figure 20. Paced activity in hSC-CMs. Fast depolarizing spikes following the stimulus artifacts demonstrate the pacing stimulus is effective.

6. References

Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells.

Lan F, Lee AS, Liang P, Sanchez-Freire V, Nguyen PK, Wang L, Han L, Yen M, Wang Y, Sun N, Abilez OJ, Hu S, Ebert AD, Navarrete EG, Simmons CS, Wheeler M, Pruitt B, Lewis R, Yamaguchi Y, Ashley EA, Bers DM, Robbins RC, Longaker MT, Wu JC.
Cell Stem Cell. 12(1):101-13 (2013)

Patient-specific induced pluripotent stem cells as a model for familial dilated cardiomyopathy.

Sun N, Yazawa M, Liu J, Han L, Sanchez-Freire V, Abilez OJ, Navarrete EG, Hu S, Wang L, Lee A, Pavlovic A, Lin S, Chen R, Hajjar RJ, Snyder MP, Dolmetsch RE, Butte MJ, Ashley EA, Longaker MT, Robbins RC, Wu JC.
Sci Transl Med. 4:130 (2012)

A novel method of selecting human embryonic stem cell-derived cardiomyocyte clusters for assessment of potential to influence QT interval.

Yamazaki K, Hihara T, Taniguchi T, Kohmura N, Yoshinaga T, Ito M, Sawada K.
J Toxicol In Vitro. 26(2):335-342 (2011)

Usefulness of field potential as a marker of embryonic stem cell-derived cardiomyocytes, and endpoint analysis of embryonic stem cell test.

Koseki N, Deguchi J, Yamada T, Funabashi H, Seki T.
Toxicol Sci. 35(6):899-909 (2010)

Improvement of the embryonic stem cell test endpoint analysis by use of field potential detection.

Koseki N, Deguchi J, Yamada T, Funabashi H, Seki T.
J Toxicol Sci. 35(5):619-29 (2010)

Progressive maturation in contracting cardiomyocytes derived from human embryonic stem cells: Qualitative effects on electrophysiological responses to drugs.

Otsuji TG, Minami I, Kurose Y, Yamauchi K, Tada M, Nakatsuji N.
Stem Cell Res. 4(3):201-13 (2010)

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