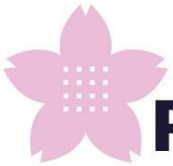


Discover the beauty that only the highest-sensitivity MEA can bring to your research!

MED64 Protocol

iCell® Cardiomyocytes²

ALPHA MED SCIENTIFIC

Version: 1.00; March 27, 2019

Alpha MED Scientific Inc.
Saito Bio-Incubator 209, 7-7-15, Saito-asagi,
Ibaraki, Osaka 567-0085, Japan
E-mail: support@med64.com
Website: http://www.med64.com

Contents

1. Introduction

The MED64 Presto micro-electrode array (MEA) system from Alpha MED scientific is noninvasive low-noise platform that measures local field potentials of electrically active cells. Presto's superior signal-to-noise ratio and carbon nanotube technology affords unblemished extraction of basic electrophysiological properties. Due to the complex yet subtle activity of stem cell-derived cardiomyocytes, a consistent and reliable instrument is needed to measure and examine their activity. The MED64 Presto is a high-fidelity microelectrode array (MEA) platform that is engineered to detect a broad range of action potentials that stem cell-derived cardiomyocytes can exhibit.

iCell® Cardiomyocytes^2 are stem cell-derived cardiomyocytes developed from induced pluripotent stem cells. Due to their human origin, they are the ideal system for investigating human cardiac biology and can be applied to basic research and several stages of the drug discovery and development pipeline. iCell® Cardiomyocytes^2 provide an additional advantage as they can be cultured directly onto the low-impedance carbon nanotube electrodes of the MED64 Presto. The high-sensitivity of the ME64 Presto combined with the human genetic background of the iCell® Cardiomyocytes^2 make them the optimal electrophysiological assay for basic cardiac research and drug discovery.

This Application Protocol describes how to set up experiments using iCell® Cardiomyocytes^2 and the MED64 Presto. This material has been prepared by scientists with expertise in stem cell biology and cardiovascular pharmacology.

1-1. Acknowledgement

Alpha MED Scientific would like to thank the MED64 users that have shared their knowledge:

Kaoru Morimura - FUJIFILM Corporation
Nobuyuki Mochizuki, PhD - FUJIFILM Corporation
Ayako Kamei, PhD – FUJIFILM Corporation
Satoko Yasuoka, PhD - Alpha MED Scientific
Michael Trujillo, PhD - Alpha MED Scientific

1-2. Disclaimer

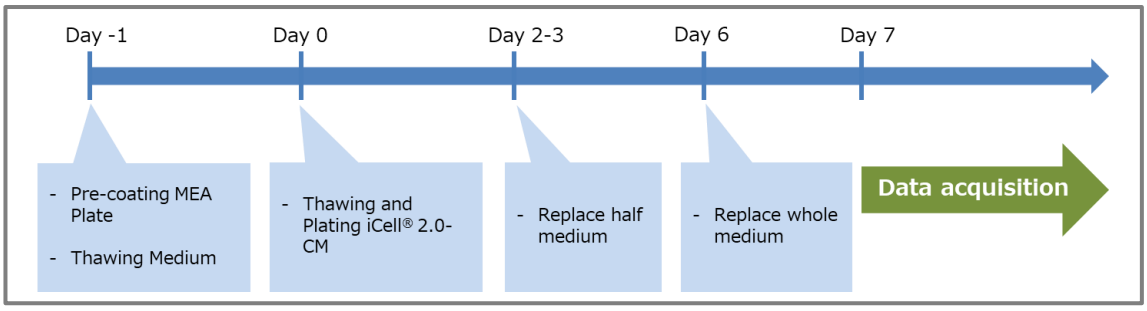
This application note is a summary of information shared by MED64 users and is to be considered marketing material. These methods have been developed, tested, and verified in the course of projects published in peer-reviewed literature. However, Alpha MED Scientific does not guarantee that the information 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 Presto manuals for detailed instructions on use of the MED64 System.

2. Pretreatment of the MEA Plate and plating iCell® Cardiomyocytes²

2-1. Required Materials

Items	Supplier	Cat. No
Cell		
iCell® Cardiomyocytes2 01434 5M Cells	FUJIFILM Cellular Dynamics	C1016
iCell® Cardiomyocytes Plating Medium 30mL	FUJIFILM Cellular Dynamics	M1001
iCell® Cardiomyocytes Maintenance Medium 100mL	FUJIFILM Cellular Dynamics	M1003
Reagents for coating		
Fibronectin	Roche Applied Science	11051407001
Reagents for cell plating and culturing		
70% Ethanol	Various	
Sterlized Distilled Water	Various	
Saline	Otsuka	
TrypLE Express	Thermo Fisher Scientific	12604013
Tergazyme®	ALCONOX	21837-118
Equipment		
MEA Plate24 eco	Alpha MED Scientific	MED-Q2430M
CellDroplet24	Alpha MED Scientific	MED-CRD24
Centrifuge tube (50 mL)	Various	
Microtube (1.5 mL)	Various	
Nunc™ Square BioAssay Dishes 135 mL	Thermo Fisher Scientific	166508
Pipet, Aspirator, Petri dishes, Kim wipes, Waterbath, Safety cabinet, CO2 incubator, Mixed gus, Centrifuges, Microscope		

2-2. Workflow



2-3. Pretreatment of the MEA Plate (Day -1)

CAUTION:

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

Pretreatment of the MEA plate is the most critical step to successfully culture cells onto the electrodes of the Presto MEA Plate. The surface of a new Presto MEA Plate is hydrophobic. Therefore, hydrophilization of its surface is necessary to enhance the adhesion of cells to the electrodes in each well. If cells do not adhere to the electrodes, you will not be able to record from them. It should be noted that some coating agents can affect cell's activity, viability, degree of cell growth, extent of migration, and longevity. Improper coating techniques can cause large-scale clumping and/or the death of cells even if all other cell culture steps are performed properly. The following section contains recommendations for appropriate pretreatment of the MEA Plate.

Sterilizing the MEA Plate

1. Rinse a new MEA Plate with 300 µL of sterilized distilled water (SDW) at least 3 times.
2. Fill the all wells with 70% ethanol (300 µL) and leave it at room temperature for 15 minutes.
    - Higher-grade ethanol is recommended to avoid deposits of organic substances onto the MEA Plate after drying.

    CAUTION:

    - Avoid exposing to ethanol for longer than 15 minutes.
3. Aspirate ethanol from all wells and rinse the wells with SDW at least 3 times.
4. Let the MEA Plate dry under ultraviolet light to irradiate for 15-30 minutes.
    - Or leave the MEA plate in the clean bench for 2 hours.
5. Make sure that MEA Plate is dried. Store and handle the Presto MEA Plate in a sterilized container.

Pre-coating the MEA Plate

This section will guide you to coat only recording electrodes located in the center of each well.

1. Prepare the 50 µg/ml Fibronectin solution (Refer to the instruction in the next page).
2. Install the sterilized CellDroplet24 under the MEA Plate.
3. Dispense 4 µL of Fibronectin solution over the recording electrodes (located in the center) at all wells.
    - Location of electrodes are guided with red mark by CellDroplet24.
    - Pay extra attention so that dropped fibronectin will NOT be evaporated before all wells are coated.

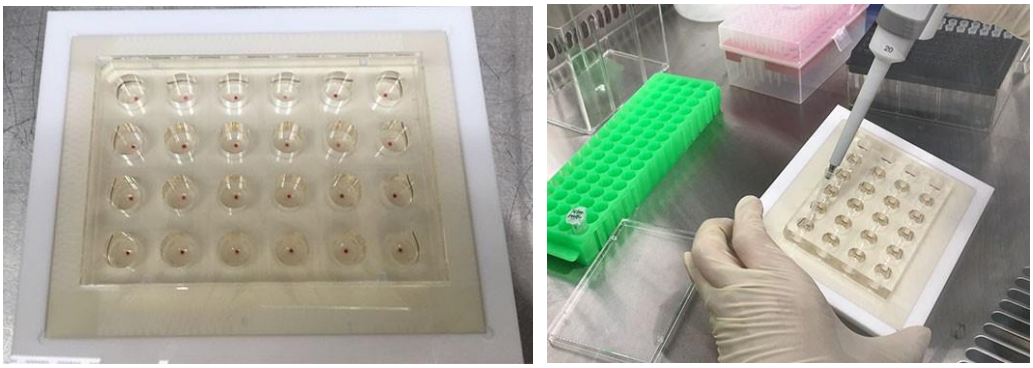
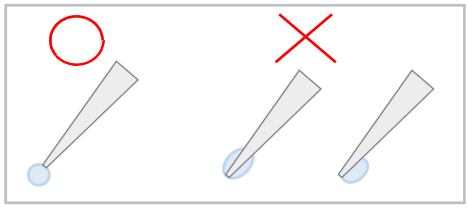


Figure 2-1. MEA Plate24-eco loaded on the CellDlopet24.Location of electrodes are marked with red color (left). Dispensing the fibronectin solution onto the recording electrodes in a well.

Tips for spotting Fibronectin solution

For dropping the Fibronectin solution successfully onto the recording electrodes, have a round droplet on the tip-toe of a pipette first (Left in the figure below), and then drop it onto electrodes. Droplets as seen in the right of the figure below will prevent solution from dropping onto electrodes nicely, or/and could damage the electrodes.



4. Unload the MEA Plate from the CellDroplet24 and store it in a sterilized tray, and leave it overnight at 4ºC.
    - Store the MEA Plate as well as small containers that contains SDW in the tray to prevent coating solution from evaporating.

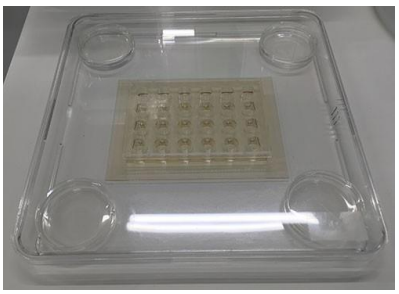


Figure 2-2. Fibronectin solution dropped onto the recording electrodes (left). An example for storing the pre-coated MEA Plate.

Recipe: Fibronectin solution

1) Dilute 1 mg of Fibronectin (Roche, Cat#:11051407001) with 1 mL of saline (Otsuka)

2) Incubate at 37℃ for 30-60 minutes

3) Dispense 50 µL of the Fibronectin stock solution into sterilized 1.5 mL container. Store them at -20ºC.

- Use thawed Fibronectin stock solution within the same day.

Thawing media

Thaw the media overnight at 4ºC according to the iCell® Cardiomyocytes² User's guide.

2-4. Thawing and plating the iCell® Cardiomyocytes² (Day 0)

- Please read and follow the instruction in the iCell® Cardiomyocytes² user's guide.
- Equilibrate the Plating Medium at room temperature before thawing iCell® Cardiomyocytes².

1. Take the MEA Plate that is coated with Fibronectin out of refrigerator and incubate it at 37ºC for 1 hour.

2. Immerse an iCell® Cardiomyocytes² cryovial in a 37ºC water bath for 3 minutes.
   - The time for warming-up is critical and should not be longer or shorter.

3. Remove the cryovial from the water bath, and immediately transfer into the biological safety cabinet. Avoid shaking the cryovial while transferred.

4. Transfer 1 mL of cell suspension gently and slowly from the cryovial to a 50 mL centrifuge tube.

5. Rinse the empty iCell® Cardiomyocytes² cryovial with 1 mL of room temperature Plating Medium, and transfer the 1 mL of Plating Medium rinse from the cryovial drop-wise over 90 seconds (e.g. 1 Drop every 4-5 seconds) to the 50 mL centrifuge tube containing the iCell® Cardiomyocytes² cell suspension. Gently swirl the tube while adding the medium to mix the solution completely and minimize the osmotic shock on the thawed cells.

Figure 2-3. Centrifuge tube

6. Slowly add 8 mL of room-temperature Plating Medium to the 50 mL centrifuge tube. Add the first 1 mL drop-wise over 30-60 seconds, and then add the remaining 7 mL remaining 7 mL over the next 30-60 seconds.

7. Gently swirl the 50 mL centrifuge tube 2-3 times (not over 90 degree).

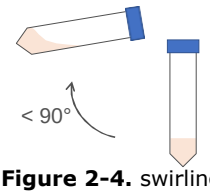


Figure 2-4. swirling the tube.

microtube

8. Transfer the 100 µL of cell suspension to sterilized 1.5 mL microtube, and count live cells.

9. Centrifuge the cell suspension (180 x g) at room temperature for 5 minutes, and then aspirate the supernatant.

10. Calculate the final volume of room temperature Plating Medium needed to obtain **50,000 cells/4 µL (1.25 x 10^7 cells/mL)**.

11. Dispense **4 µL of cell suspension** (containing around **50,000 cells**) over the Fibronectin plated on the recording electrodes.

    - It is NOT necessary to remove the Fibronectin. Drop the cell suspension over the Fibronectin.
    - It is recommended to plate cells by every 4 wells. Pipette the cell suspension after plating every 4 wells.

12. Incubate the MEA Plate at 37ºC, 5%CO_2 for 1 hour.

    - Store the MEA Plate together with containers containing SDW in a tray to prevent plated cells from evaporating.

13. Remove the MEA Plate from incubator and add 300 µL of pre-warmed Maintenance Medium into all wells.

    - Flood the medium gently. Avoid flooding onto plated-cells.
    - It is recommended to add the medium using 6 channel pipettes to save the time.

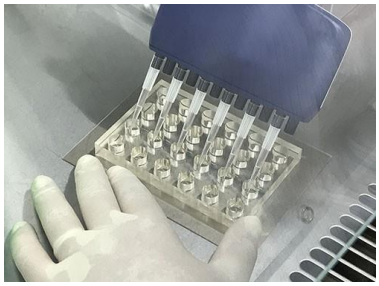


Figure 2-5. Adding culture medium using a 6 triplet-pipettes.

14. Store the MEA Plate together with container containing SDW in the tray and place it back to the incubator (37ºC, 5%CO_2).

15. Replace half the Maintenance Medium at Day 2-3.

16. Replace whole Maintenance Medium at Day 6. It is recommended to record data at Day 7.

    - If data acquisition is not performed at Day 7, change the half-medium every 3 days (e.g. Day 6, 9, Day12). Change the whole medium the day before data recording.

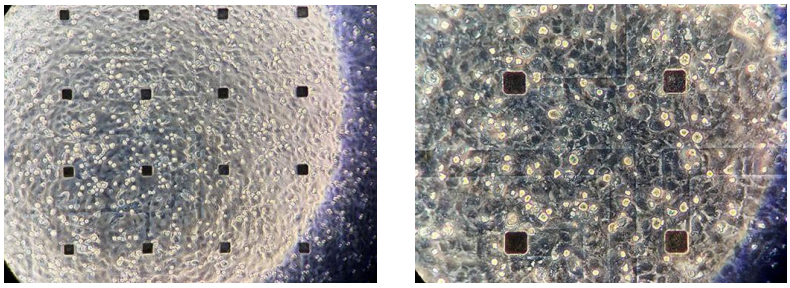


Figure 2-6. iCell® CM2 at the Day 3.

2-5. Cleaning the used MEA Plate

The MED64 Presto MEA Plate's electrical characteristics are best during the first use. High quality signals can be recorded and effective stimulation is possible with the Presto MEA Plate thanks to the electrode's characteristics, the lowest impedance in a commercially available microelectrode array. The electrode's impedance will increase with repeated use of the MEA Plate due to damage in handling and/or residual cellular debris and coating materials. However, the MEA plates can be re-used if they are handled and cleaned very carefully. The following are cleaning procedures recommended by MED64 users:

- **CAUTION:**

  ALWAYS avoid contact with the surface of the MEA Plate to preserve the microelectrodes and insulation layer.

1. Aspirate completely whole medium from all wells. Immediately add 300 µL of TrypLE Express Enzyme (Gibco, Cat# 12604013) into all wells.
2. Incubate the MEA plate at 37°C for 1 hour.
3. Take the MEA Plate out of the incubator. Pipette deliberately and carefully at all wells to detach cells by water pressure.
   - Be extremely careful not to damage the electrodes.
4. Aspirate the TrypLE Express completely from all wells.
5. Rinse the MEA Plate with PBS at least 3 times.
6. Fill the all wells with 300 µL of 1% Tergazyme® solution.
   - Dilute Tergazyme® with sterilized water, and then warm it up (55°C) to dilute completely.
   - Prepare the Teragazyme® solution BEFORE used.
7. Leave the MEA Plate at room temperature for 1 hour.
8. Pipette again deliberately and carefully at all wells.
9. Aspirate the Tergazyme® solution from all wells. Rinse the MEA Plate with SDW at least 3 times.
   - If the MEA Plate is not cleaned well enough, repeat #3-8.
10. Fill the all wells with 70% ethanol. Leave it at room temperature for 15 minutes.
    - Avoid exposing to ethanol for longer than 15 minutes.
11. Rinse the MEA Plate with SDW at least 3 times.
12. Store the MEA plate with electrodes moisten (in SDW) at a refrigerator (4°C).

3. Data acquisition

Cultured iCell® Cardiomyocytes^2 is typically ready for recording and drug testing at Day 7. Change the whole medium the day BEFORE recording.

3-1. Recommended experimental environment

37°C is the typical recording temperature recommended for recording from cardiomyocytes. However, the set temperature can vary depending on samples and nature of your experiments.

The MED64 Presto Amplifier incorporates heater, that heats the MEA Plate from the bottom. Below are the recommended settings for data acquisition.

- Do NOT place the MED64 Presto Amplifier in an environment where temperature changes frequently, for example in the proximity of an air conditioner or heater.
- Keep the acrylic lid closed and secure during acquisition.
- It will take time for the set-temperature to stabilize. **Power on the temperature controller at least 15-30 minutes before starting acquisition**.

If/When the set-temperature has changed, wait for the temperature reported by the temperature controller to stabilize at the new temperature. It can take 5-30 minutes or more but is typically very fast.

Provide gas mixtures (the mix ratio depends on composition of your culture medium, but typically 75% N_2, 20% O_2, 5% CO_2) through the "gas port" on the gas chamber on the top of MED64 Presto Amplifier. Bubble the gas through a beaker containing distilled water to maintain a humidified environment.

Figure 3-1. Experimental environment for the MED64 Presto system (left). Bubble the gas through a beaker containing distilled water to maintain a humidified environment (right).

3-2. Data acquisition and analysis

Each well in the MEA P24well Plate has 16 recording electrodes as well as 16 reference electrodes. The differences between the field potential acquired at each recording electrode is compared to the potential of the average for all reference electrodes. Acquired signals are amplified by x1000, digitized, and sent to an acquisition PC.

MEA Symphony software is available for data acquisition and analysis. Select CardioRecorder in the Start Menu for recording of cardiac signals. Data acquisition starts either with clicking Preview (without saving data) or Record (with saving data).

Figure 3-2.

The Symphony software **always acquires extracellular signals with acquisition bandwidth of 0.1Hz - 5 kHz**. However, acquired raw data can be filtered for analysis. The following parameters are typically recommended for analyzing cardio data.

- Low-pass filter (2 pole): Butterworth 1000Hz
- High-pass filter (2 pole): Butterworth 1Hz (Select 0.1Hz if you want to record slow wave)
- Smoothing: Custom
    - The main purpose for use of this filter is making vague T phase clearer for its Field Potential Duration analysis. The [Custom] filter allows you to smooth beat signals EXCLUDING its fast Q spikes.
    - When [Custom] is selected, the Symphony smooths (moving-average) beat signal in the duration set at the *Smoothing window*. However, the smoothing is deactivated in the time window which signal amplitude's SD goes over the value set at *Activation Threshold*.
    - Start with following parameters and change them depending on your signals:
      Smoothing window (ms): 20 Activation Threshold (μV): 25

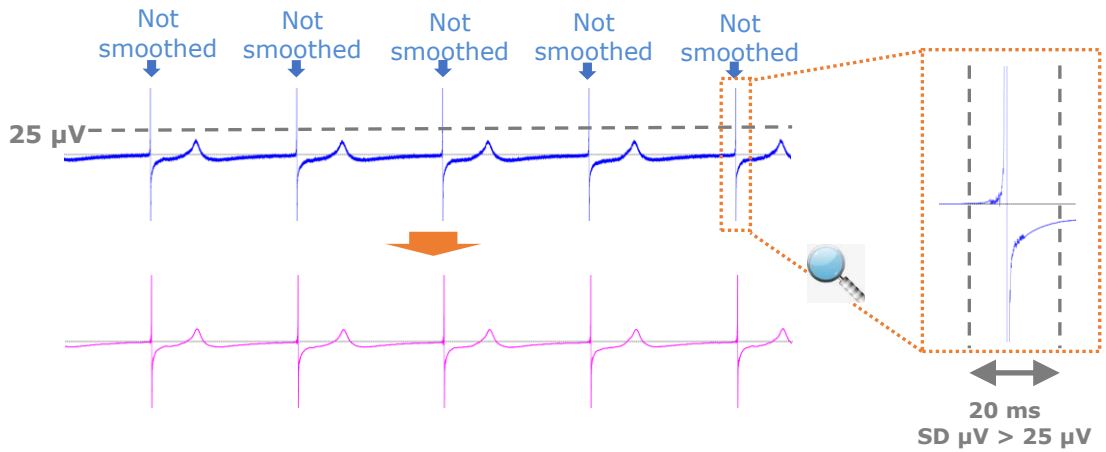


Figure 3-3. Smoothing filter with MEA Symphony software

3-3. Recording paced activities

The MED64 Presto Amplifier incorporates stimulator and will allow you to stimulate physically all wells. However, paced activities from all 24 cardiomyocytes samples in a MEA Plate is not easily achieved because each independent cardiomyocytes has different spontaneous beat rate.

The use of MED64 Stimulator (MED-S09) is recommended to record paced activities with the MED64 Presto. The MED64 Stimulator will allow users to adjust stimulus intensity and intervals WITHOUT stopping pacing until all 24 cardiomyocytes have paced activities.

When using the MED64 Stimulator, select **External** for Stimulator type in the Symphony software, and then click OK. With this option, all parameters for stimulus waveforms becomes inactive, that will be designed in the stimulator.

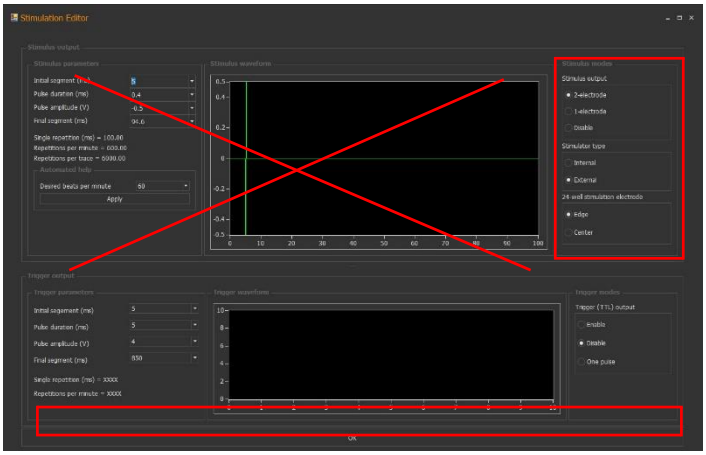
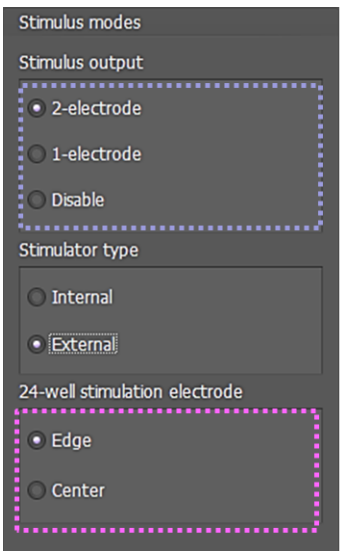


Figure 3-4. Stimulus Editor in the Symphony software. Parameters for stimulus waveforms come to inactive when External is selected in the stimulation type.



Mark	1-electrode	2-electrode
●	Non-Inverted Stimulus pulse	Non-Inverted Stimulus pulse
●	Not Stimulated	Inverted Stimulus pulse

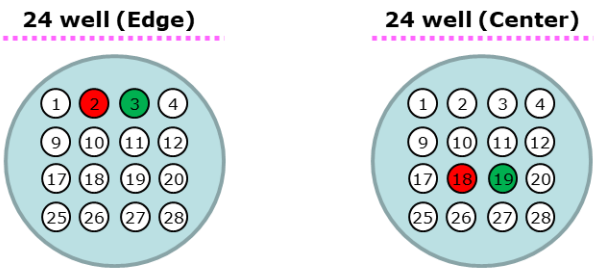


Figure 3-5. Stimulus electrodes for pacing (1 or 2) and the position of pacing electrode(s) (edge or center).

The MED64 Stimulator will stimulate to all wells when the Output button is pushed down (this is indicated with the green lump). It will send bi-phasic stimulus pulses with intervals set in the Interval dial. The duration for each pulse is controlled with the Duration up/down button, and stimulus intensity is with the Output level vernier dial.

The max stimulus intensity that the MED64 Stimulator sends out is 2V, that is equivalent to 10.00 in the Output level vernier dial (1.0 in the vernier dial = 0.2 V). To avoid electrolysis for the electrodes, **do NOT apply stimulation greater than 0.7V (3.5 in the vernier dial).**

Figure 3-5. shows the front panel of the MED64 Stimulator (left) and example of outputted stimulus waveform (right).

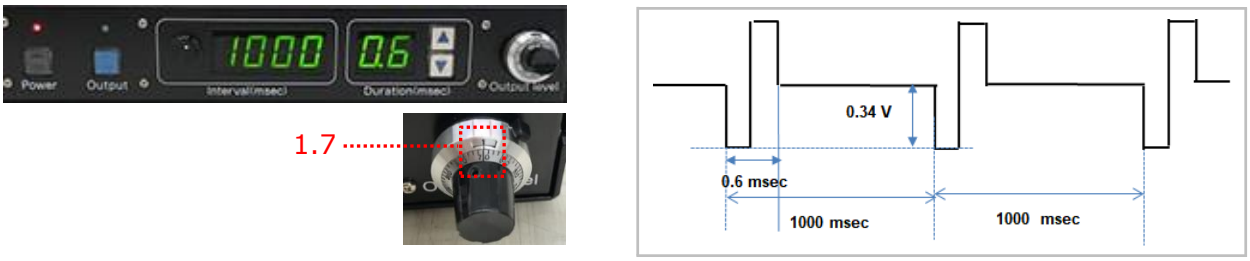


Figure 3-6. Front panel for the MED64 Stimulator (left) and example of outputted stimulus waveform.

Appropriate stimulus intensity and intervals changes depending on cell's maturity and condition. However, following parameters are good to start.

- Stimulus intensity: 0.8
- Intervals: 1000
- Duration: 0.6

3-4. MEA Symphony software

The MEA Symphony software extracts beat signals that go over pre-determined threshold, and perform several analysis for the extracted beat signals. Set the thresholds as well as pre/post time for your extractions at the Beat Setting window.

Figure 3-7. Main screen for the MEA Symphony software, Cardio Recorder.

Analysis performed by Symphony

Following analysis are performed for extracted signals:

- Beat Count
- Beat Frequency
- Interbeat interval (synonymous with "Interspike interval (ISI)")
- Beat rate
- First peak amplitude
- Second peak amplitude
- FPD (Field Potential Duration)
- CFPD Bazett (FPDcB)
- CFPD Fridericia (FPDcF)

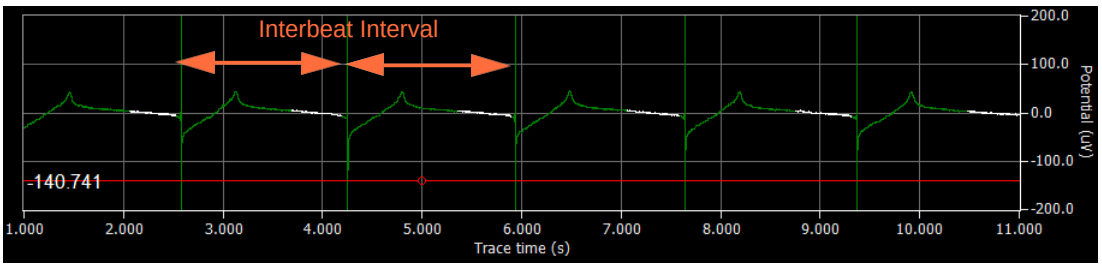


Figure 3-8. Extracted cardio signals. Red cursor shows threshold to extract cardio signals, and extracted signals are color-coded.

Field Potential Duration analysis with the MEA Symphony

The MEA Symphony measures the duration from the "1st peak" to the "2nd peak in the region defined with 2 red cursors".

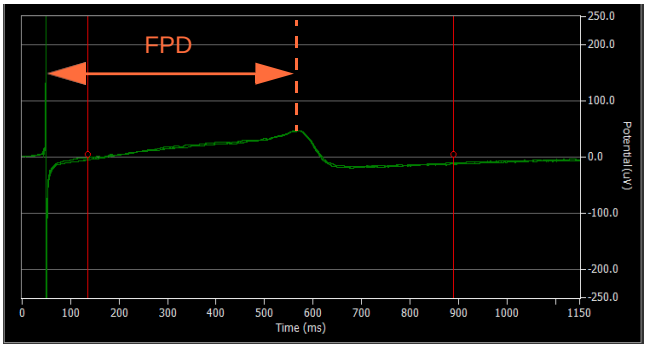


Figure 3-9. FPD analysis with the MEA Symphony software.

Selecting a golden electrode in a well

Electrodes to be used for analysis can be selected in the Beat Settings > Compute Cumulative Statistics. Select your parameter and number of electrodes, and then click [Select Electrode] to activate it. Selected electrodes are color-coded in the Cumulative Beat Statistics, Electrode chart.

In the following example, 1 electrode that "Second Peak Amplitude" is the largest will be analyzed.

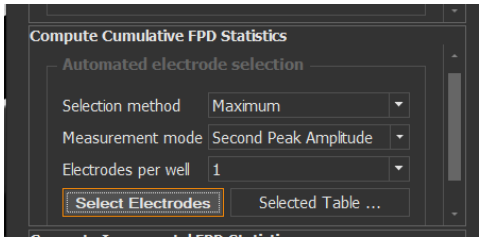


Figure 3-10. Selecting electrodes for cumulative analysis.

Analyzed electrodes can be selected manually in the Cumulative Beat Statistics, Electrode chart. Click the number while holding the Shift button the electrode you wish to select (or the electrode you don't wish to select for off).

Selecting a golden electrode in a well

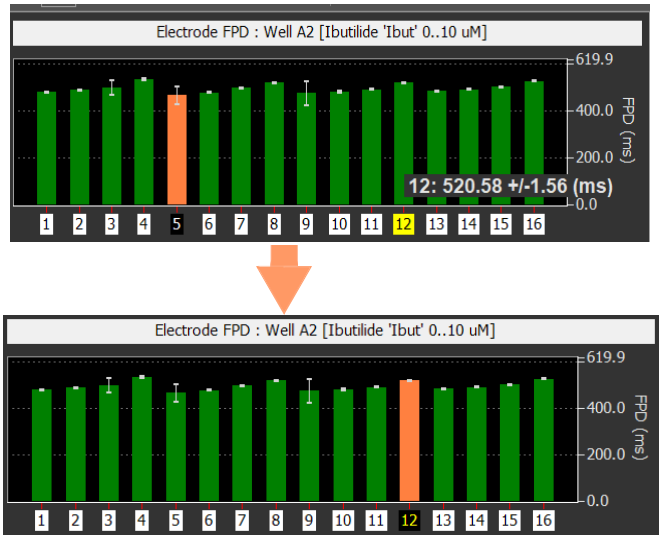


Figure 3-11. Changing an electrode for analysis manually.

3-5. Data Export

In the Export Settings, raw or filtered data and chart data as follows can be exported.

- MED384 Data (raw/filtered data of 384 recording electrodes)
- Beat Data (Extracted beat data)
- Incremental Beat Statistics (Statistical data and its chart displayed on the Main Screen)
- Cumulative Beat Statistics (Statistical data and its chart displayed on the Main Screen)

Data Export of 384 recording electrodes

You can convert raw/filtered data into various formats as below. With this function, specific timing, electrodes' or wells' data can be exported as a new data file.

- ASCII text (.csv file)
- Shorts 16-bit (.bin file)
- Mobius 16-bit (.modat file)
- Floats 32-bit (.bin file)
- Original voltage files (.modax)
- Compressed voltage files (.modax1)
- Nex5 file (.nex5 file)

The MEA Symphony has 384 electrodes' data but Mobius can read only 64 electrodes' data at once. So, 384 electrodes' data of the MEA Symphony will be divided into 6 groups (64 electrodes/group), when you select Mobius format.

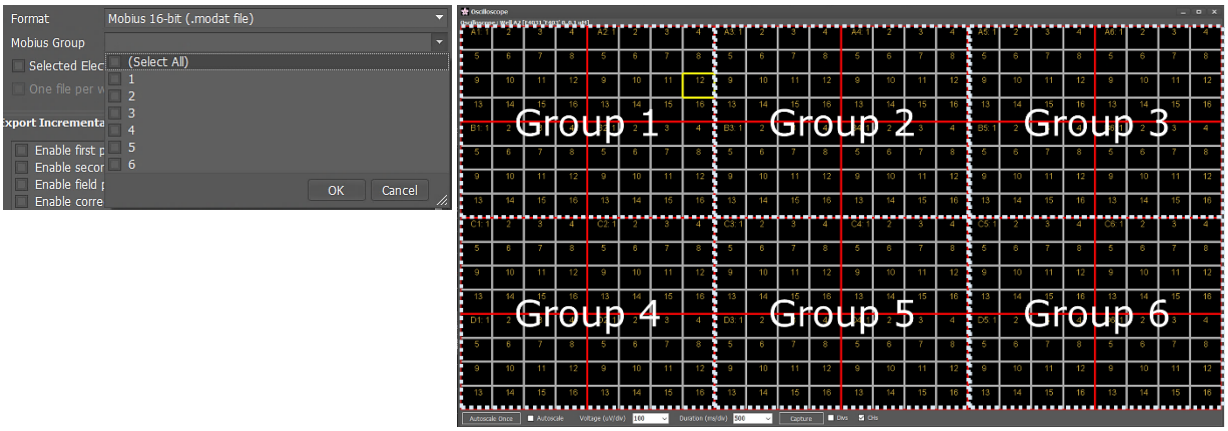


Figure 3-12. Divided 6 groups in Mobius format. When you select Mobius as the format, the group selection box will be available (Left). Select the group that includes your target well. You can select several groups simultaneously.

How to export the data/analysis results

1. Select the well (green well) and time point to export in Protocol Settings.

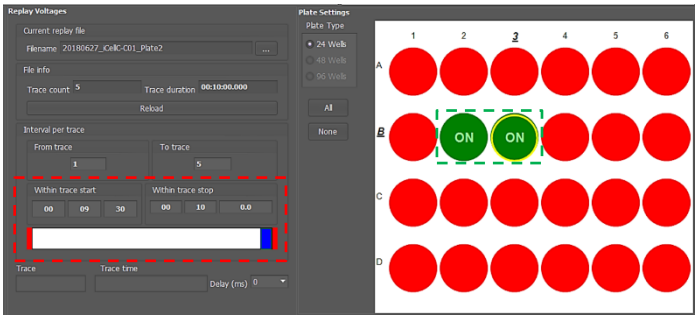


Figure 3-13. Setting of data export. In this figure, only B2 and B3 wells are selected and the data for last 30 seconds (from 9 minutes 30 seconds to 10 minutes) in each trace will be replayed and exported.

2. Select the directory for exporting and enter the filename in Export Settings. Put a check mark on the checkbox depending on respective objectives.

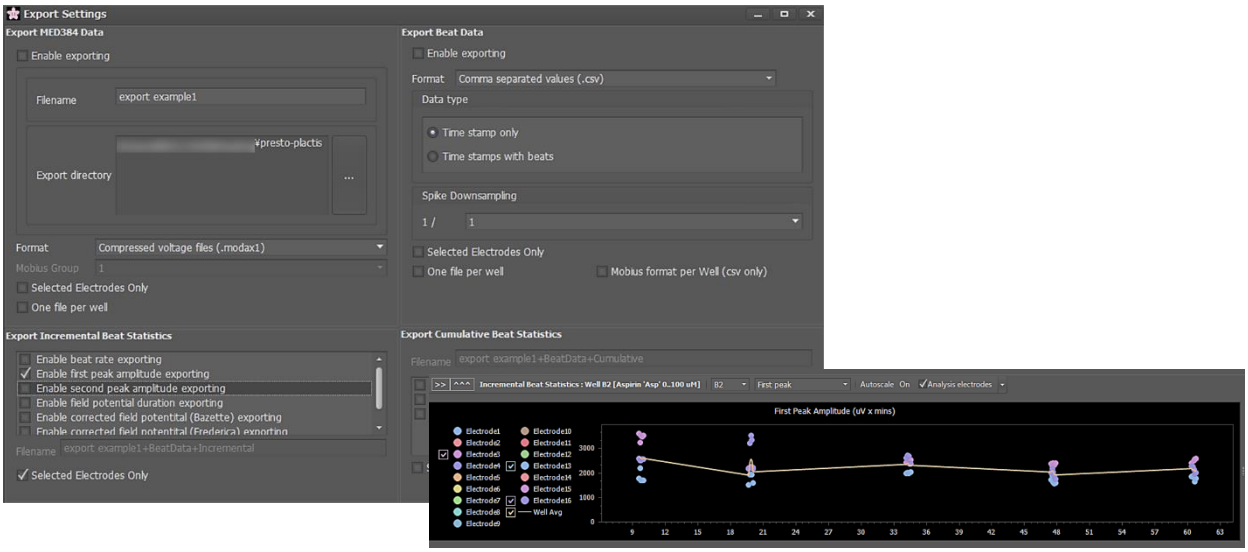


Figure 3-14. Select the data to export. This chart shows the first peak amplitude of selected electrodes (Electrode 3, 13, 16) among Incremental Beat Statistics.

Figure 3-15. Select the Cumulative Beat Statistics data to export. In this figure, the Statistics of [First Peak Amplitude] per electrode on the selected well (B2), per well, and per treatment are selected to export. By checking [Selected Electrodes Only] box, the data of the selected golden electrodes (3 electrodes per well in this chart) are used for analysis.

3. Press the red button on the main screen, data replaying and exporting will start.

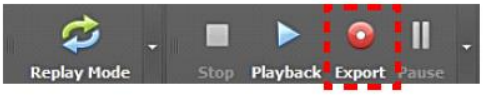


Figure 3-16.

In a simple way, just right-clicking each chart enables you to copy data/image of it.

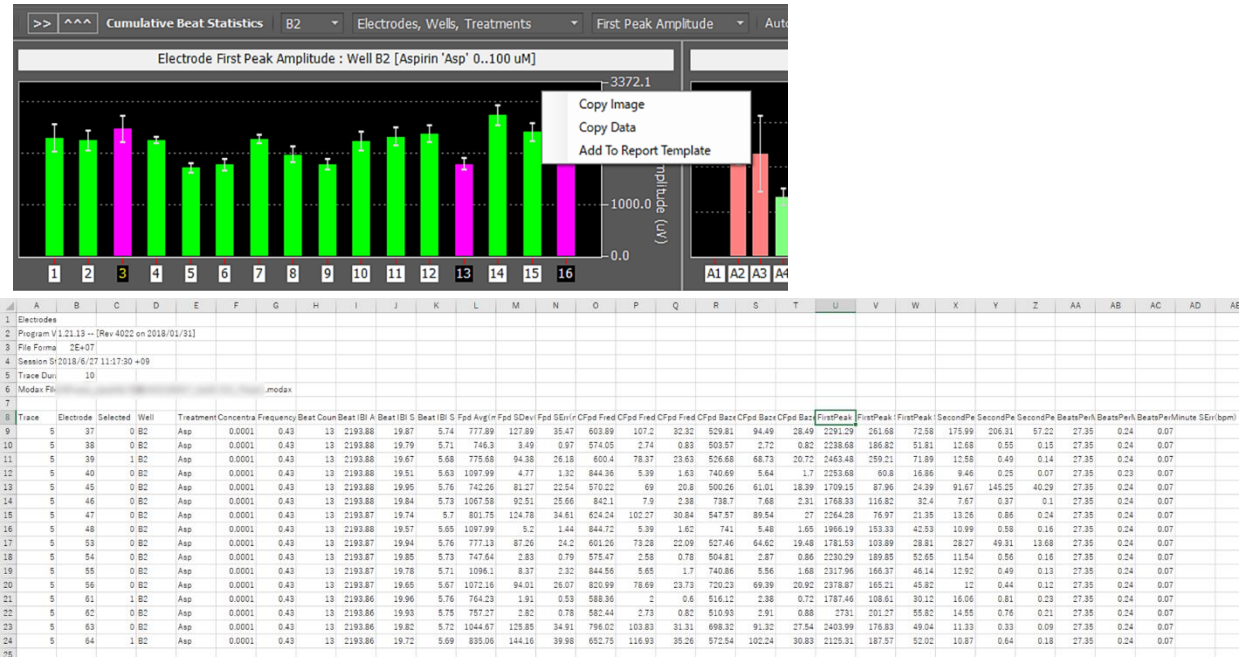


Figure 3-17. Right-clicking the chart enables you to copy the data/image of it (Top). When you select [Copy Data] and past in Excel file, you can get all analyzed data at once (Bottom).

Reporting

The MEA Symphony can create a reporting file that shows the brief results, as well as data exporting. You can select the chart for exporting in Reporting Design. Or you can add the chart on your report by right-clicking them and select [Add to Report Template]

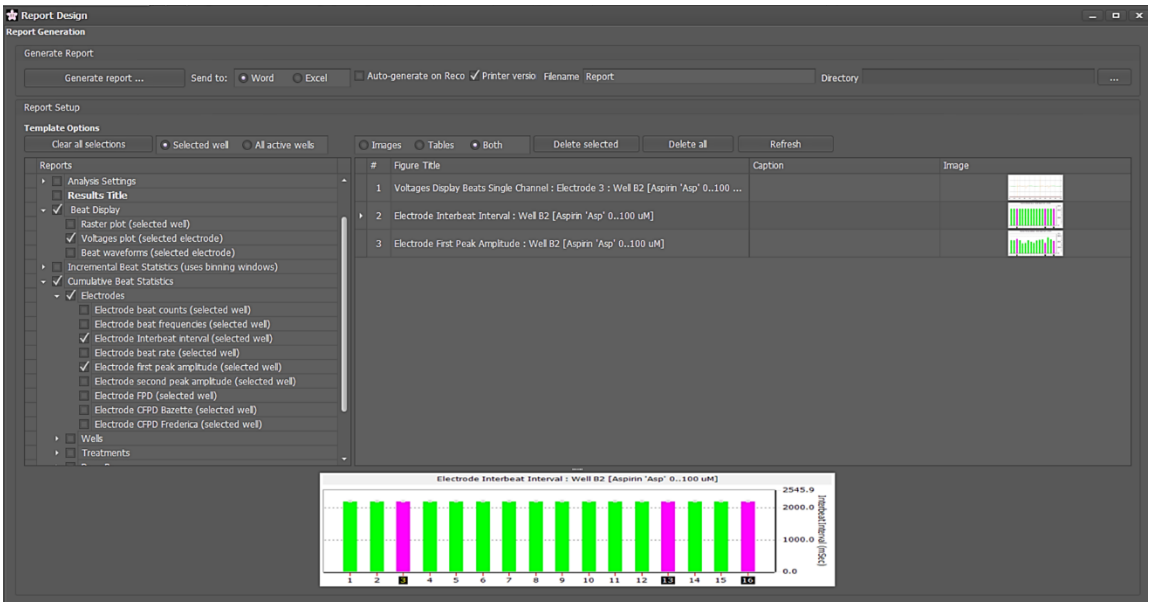


Figure 3-18. Report Design window. You can select analysis results and charts on the left side of "Report Setup". Selected results and charts are displayed in the list on the right side od "Report Setup". Pressing the [Generate report] button will create report files as Word or Excel file (Word style is selected in this figure.)

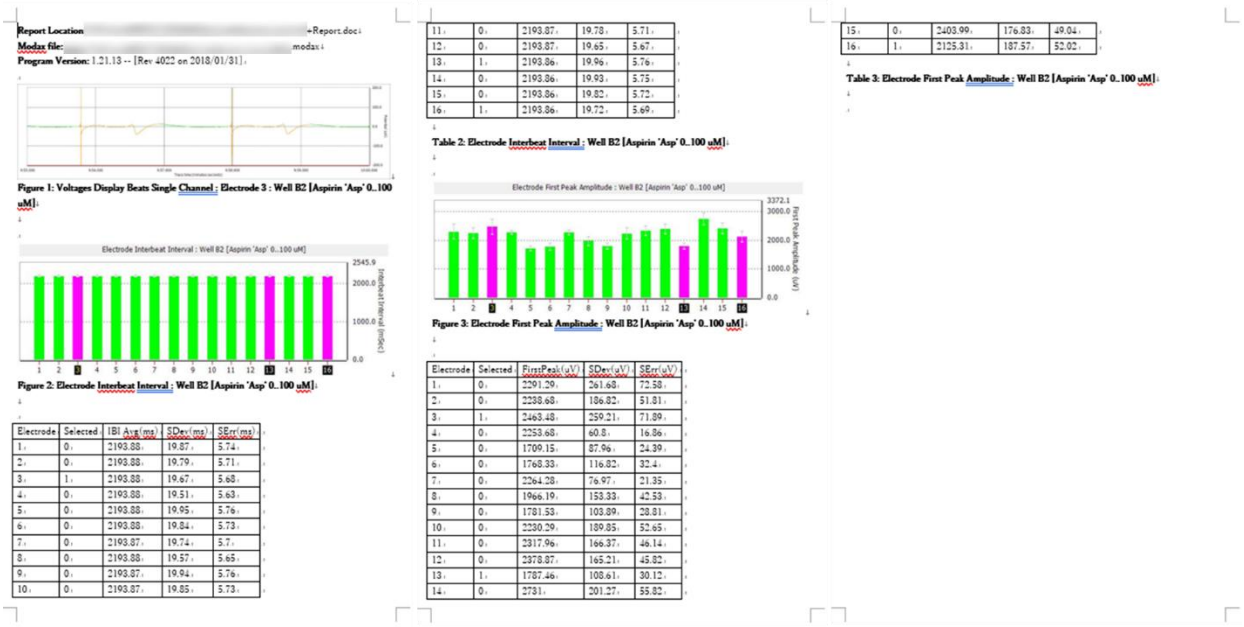


Figure 3-19. Report file created in Figure 3-18.

March 27, 2019

Alpha MED Scientific Inc.
Saito Bio-Incubator 209, 7-7-15, Saito-asagi,
Ibaraki, Osaka 567-0085, Japan
Phone: +81-72-648-7973 FAX:+81-72-648-7974
http://www.med64.com support@med64.com

Manufactured by **Alpha MED Scientific Inc.**