MEA Application Note: Human Embryonic Stem Cell Derived Cardiac Myocytes (hESC-CM)

Imprint

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1 Introduction

1.1 About this Application Note

The intention of the MEA Application Notes is to show users how to set up real experiments with the MEA-Systems on the basis of typical applications that are used worldwide.

The documents have been written by or with the support of experienced MEA users who like to share their experience with new users.

This application note includes a complete protocol for the isolation and cultivation and differentiation of human embryonic stem cells, suggestions for long-term cultures, suggestions for MEA-System configurations or for the Multiwell-MEA-System, example data, and references.

As the various steps in this protocol are performed at different time points, material and methods are indicated for every single step in the culture process.

1.2 Acknowledgement

Multi Channel Systems would like to thank all MEA users and other institutes and persons who shared their experience and knowledge with us, especially the following:

WiCell Wisconsin, USA (contributed the protocols on generation and cultivation of the stem cells)

Prof. Ofer Binah, The Rappaport Institute and the Bruce and Ruth Rappaport Faculty of Medicine, Technion, Haifa, Israel (contributed demo data and images).

1.3 Important Information and Instructions

This application note is only a summary of published information from other sources (see references and acknowledgement) and has the intention of giving an application example for the MEA-Systems. This document is to be understood as a marketing material and not as a handbook. Multi Channel Systems MCS GmbH has not tested or verified the information in this document. Multi Channel Systems MCS GmbH does not guarantee that the information is correct. Multi Channel Systems MCS GmbH recommends to refer to the referenced literature for planning and executing any experiments.

Guarantee and liability claims in the event of injury or material damage are excluded when following the instructions in this document.

Warning: Only qualified personnel should be allowed to perform laboratory work. Always make sure you fulfill the requirements of local regulations and laws. Work according to good laboratory practice to obtain best results and to minimize risks.

2 MEA Recordings from hESCM

Human embryonic stem cell derived cardiac myocytes (hESCM) represent a novel tool in pharmaceutical and biomedical research. Electrophysiological characterization of cardiac myocytes differentiated from human embryonic stem cells (hESC) is crucial for the understanding of specific properties of the tissue generated from these cells.

Beating clusters of hESC can be optically identified and the **spontaneous electrical activity** can be recorded as extracellular field potentials on two-dimensional microelectrode arrays (MEAs). The two-dimensional information allows to analyze the **conduction velocity** and to generate **excitation maps** of the cardiac tissue.

hESCM can be used in the field of **pharmaceutical safety studies**, for testing drugs for their potential cardiac safety risks, namely the **QT interval prolongation** or direct **arrhythmic** effects. Also, hESCM are of interest in the **basic research**, for studying the potential use of hESCM for **implants** and regeneration of damaged cardiac tissue, for example after a heart attack. **Coculture experiments** will reveal new information on the integration of hESCM into native cardiac tissue.

Electrical recording with the **MEA-System** can be combined with other techniques, such as **intracellular calcium imaging**, **optical recordings**, or **patch clamp**.

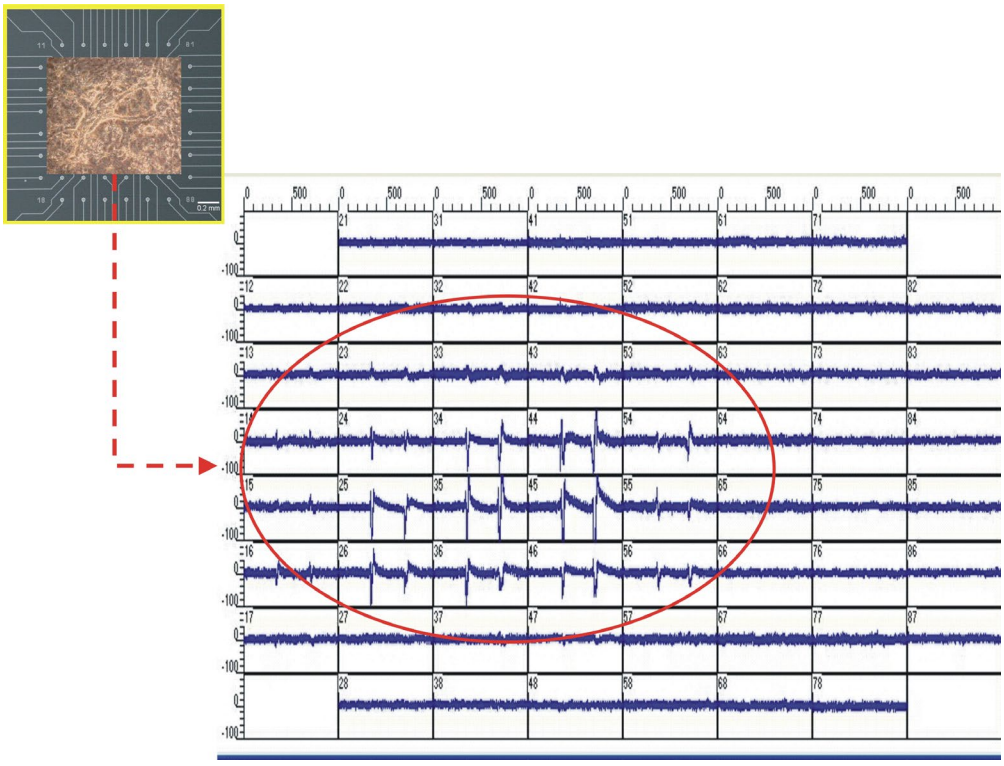


Fig.: hESCM plated on a MEA with 60 electrodes and recording of field potentials

The upper left picture illustrates a spontaneously beating tissue excised from an embroid body and plated onto a MEA on the upper left. The beating correlates with the electrical activity shown in the screen shot from the MC_Rack program.

(Figure kindly provided by Ofer Binah, Rappaport Institute, Haifa, Israel).

2.1 Analysis of Cardiac Waveforms

Simultaneous recordings of action potentials (with intracellular electrodes) and field potentials (with extracellular electrodes) have shown that there is a correlation between the rise time of the cardiac action potential (AP) and field potential (FP) as well as between AP and FP duration. The contribution of **different ionic transmembrane currents** can be identified in the shape of the **FP waveform** as well. The correlation between the waveform components and the ion channel activities was shown by using ion channel blockers or depleting the medium of the respective ions (References: "Determination of Electrical Properties of ES Cell-derived Cardiomyocytes Using MEAs", Jürgen Hescheler et al., Journal of Electrocardiology, Vol. 37 Supplement 2004; "Estimation of Action Potential Changes from Field Potential Recordings in Multicellular Mouse Cardiac Myocyte Cultures", Marcel D. Halbach et al., Cell Physiol Biochem 2003;13:271–284).

The high and short peak of the extracellular field potential can be correlated to the rapid component of the depolarizing sodium current. The following plateau follows the time course of the slow calcium current; and the following positive or negative peak correlates to the slow rectifying K^+ current (IKr). The polarity of this peak depends on several parameters, for example, the proximity of the cell layer to the measuring electrode, and cannot be predicted, but this fact does not matter for this assay.

The field potential duration corresponds to the action potential duration, which can be correlated to a **QT interval** in an electrocardiogram. It is measured from minimum of the Na^+ peak to the maximum or minimum of the IKr current peak.

The effects of tested drugs on QRS duration and amplitude, local activation time, T wave amplitude, time of maximal slope of T wave, QT interval duration, and ARI (activation refractory interval) can be analyzed with the MEA-Systems or with the Multiwell-MEA-System.

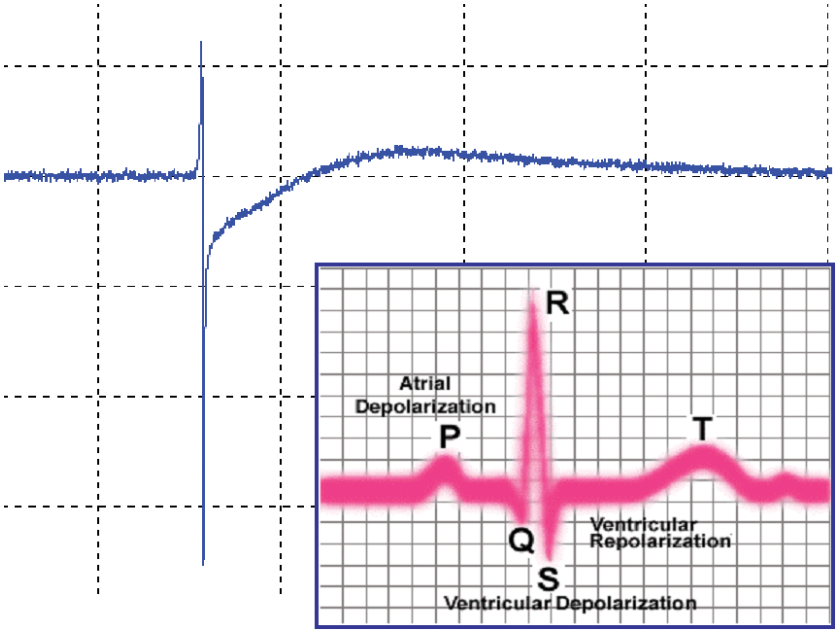


Fig.: Typical cardiac field potential recorded from hESCM

The blue trace shows a typical cardiac field potential recorded with the MEA-System. The exact shape of the waveform depends on the biological material, relative positions of signal source and electrodes, and the amplifier bandwidth. The pink trace shows an electrocardiogram.

(Figure kindly provided by Ofer Binah, Rappaport Institute, Haifa, Israel).

2.2 Pacing of hESCM

As an alternative to studying the spontaneous activity, hESCM can also be paced by **electrical stimulation**. hESCM are usually paced at the lowest rate and voltage as possible, for example, a culture beating with 1 Hz would be paced at an interstimulus interval of 950 ms, with a typical biphasic stimulus pulse of ±2 V and 2 ms for each phase.

As pacing of cardiac tissue requires considerably higher voltages and durations than stimulation of neuronal tissue, the use of **larger stimulating electrodes** is recommended. A special MEA with four pairs of large (250 x 50 µm) stimulating electrodes (60StimMEA) and a special stimulation adapter is provided for such applications by Multi Channel Systems.

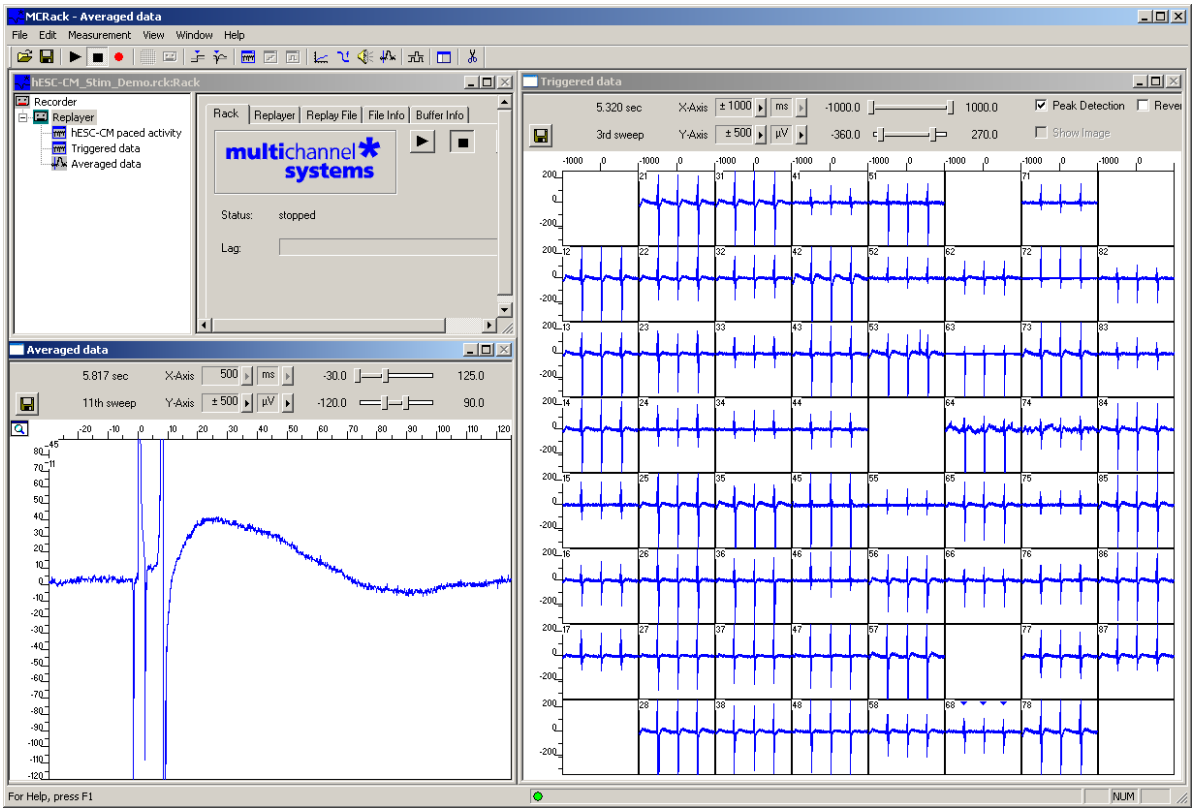


Fig.: MEA Recording from paced hESCM

For this experiment, a 60StimMEA with 4 additional pairs of large stimulating electrodes was used. The right window shows an overview of the activity on 60 MEA electrodes; the left display zooms into a single waveform. Following the stimulus artifact, you can see the fast activation spike and the slow repolarization waveform.

Cell density: 10 - 15.000 cells per square mm, amplifier gain: 1200, voltage input range: -2000 mV to +2000 mV, sampling frequency 10 KHz, electrode diameter: 30 micrometer, electrode spacing: 200 micrometer.

(Data kindly provided by Ofer Binah, Rappaport Institute, Haifa, Israel).

2.3 Arrhythmia Models

Arrhythmias can be induced in rat, mouse, or human cardiomyocytes (hESC-CM) by a variety of experimental procedures, such as catecholamines, ouabain, and by an *in vitro* pressure-overload model newly developed by Ofer Binah that mimics supraventricular arrhythmias. With these models, new leads for the development of antiarrhythmic drugs can be tested.

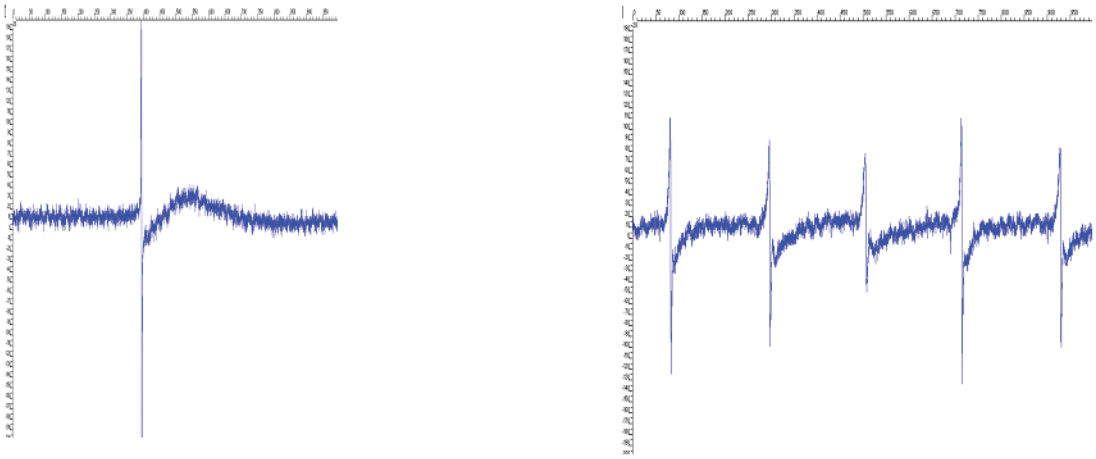


Fig.: This screen shot shows arrhythmia induced in cultured neonatal rat ventricular myocytes.

Use of Commercially Available MEF

The generation of the mouse embryonic fibroblasts (MEF) is possible either from primary cultures as described in the next chapter (Derivation of MEF), or the cells can be obtained commercially (for example from ATCC). The MEF are used as a feeder layer to support the growth of human embryonic stem cells. The growth of these cells must be arrested before they can be used as a feeder layer.

For new users, it may be easier to buy commercially available MEF. In the long run, it may be cheaper to prepare MEF in house. Irradiation is preferable over treatment with Mitomycin C. If you use Mitomycin C, be sure to wash the MEF very well before use, as hESC are very sensitive to chemicals.

2.4 Materials

2.4.1 Biological Materials

- MEF from *Mus muculus* recommended for use as a feeder layer, for example
- MEF CF-1 IRR irradiated with 10 000 rads (order number SCRC-1040.1, ATCC)
- MEF CF-1 MITC treated with Mitomycin C (order number SCRC-1040.2, ATCC)
- MEF CF-1 untreated (has to be arrested before use) (order number SCRC-1040, ATCC)

2.4.2 Technical Equipment

- Sterile workbench
- Incubator set to 37 °C, 100 % humidity, 5 % CO_2

2.4.3 Chemicals

- Phosphate buffered saline (PBS), Ca^{2+}/Mg^{2+}-free (order number SCRR-2201, ATCC)
- EDTA solution
- Dulbecco's Modified Eagle Medium (DMEM), liquid (high glucose) (Gibco/Invitrogen, 11965-092)
- 200 mM L-glutamine solution (Gibco/Invitrogen, 25030-081) (Keep frozen in aliquots. Thaw immediately before use.)
- Fetal bovine serum (FBS) (Gibco/Invitrogen, 16000-044)
- Glucose

2.4.4 Media

Culture medium:

- DMEM with
- 4 mM L-glutamine
- 1.5 g/L sodium bicarbonate
- 4.5 g/L glucose
- 15 % fetal bovine serum

2.5 Plating and Culturing the Cells

The following protocol is based on the recommendations of ATCC for MEF CF-1 IRR cells. Cells should be plated 24 hours before use as a feeder layer for ES cells and kept for no more than 7 days.

1. Thaw the vial by gentle agitation in a 37 °C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water.

2. Remove the vial from the water bath as soon as the contents are half way thawed (approximately 90 seconds), and decontaminate by dipping in or spraying with 70 % ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.

   Transfer the vial's contents plus 5 ml of culture medium to a 15 ml centrifuge tube. Use an additional 1 ml of medium to rinse the vial and transfer the liquid to the 15 ml tube. Add 4 ml of culture medium to bring the total volume to 10 ml.

3. Gently mix and pellet the cells by centrifugation at 270 g for 5 minutes.

4. Discard the supernatant and resuspend the cells with 10 ml fresh growth medium (warm) and transfer to one T75 flask.

5. Add 5 ml more fresh growth medium (warm) to flask.

6. Incubate at 37 °C in a carbogen atmosphere.

7. Medium change twice a week or when pH decreases. It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the culture medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).

3 Derivation of MEF

Skip this part if you use commercially available MEF.

3.1 Materials

3.1.1 Biological Materials

- Mice, CF-1 strain at 13 - 14 days gestation (for example from Charles River Laboratories International, Inc.).

3.1.2 Technical Equipment

- Sterile workbench
- Incubator set to 37 °C, 85 % humidity, 5 % CO_2
- Inverted cell culture microscope
- Centrifuge
- Isopropanol freezing containers (fill every fifth use)
- -80 °C freezer
- Liquid nitrogen racks or canes
- Liquid nitrogen storage container
- Dissecting scissors
- Absorbent paper towels
- Alcohol pads
- Alcohol bath and Bunsen burner, or a hot bead sterilizer (Fine Science Tools Inc.)
- Watchmaker's forceps
- Pipette aid

The following supplies and quantities are for each mouse processed:

- Sterile, disposable petri dishes
- Sterile, disposable 10 ml pipets
- 50 ml conical tubes
- T75 Flasks
- 15 ml conical tubes
- 20 ml Cryopreservation Medium
- 1.5 ml cryovials
- Syringes and needles

3.1.3 Chemicals

- Fetal bovine serum (FBS) (Gibco/Invitrogen, 16000-044)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Inc., D2650)
- Dulbecco's Modified Eagle Medium (DMEM), liquid (high glucose) (Gibco/Invitrogen, 11965-092)
- Non-essential amino acids 100 x solution (Gibco/Invitrogen, 11140-050)
- Penicillin-streptomycin 100 x solution (Pen-Strep) (Gibco/Invitrogen, 15140-122)
- Phosphate buffered saline (PBS) (Gibco/Invitrogen, 14190-144)
- Trypsin (Gibco/Invitrogen, 25300-054)
- Avertin or other form of mouse euthanasia in accordance with local regulations and laws

3.1.4 Media

Note: Filter all media with 0.22 µm pore filter before use. Store at 4 °C.

MEF medium for derivation:

- DMEM 900 ml
- FBS (heat inactivated for 30 minutes at 57 °C) 100 ml
- Non-essential amino acids solution 10 ml
- Pen-Strep 10 ml

Cryopreservation medium:

- DMEM 60 ml
- FBS (heat inactivated for 30 minutes at 57 °C) 20 ml
- DMSO 20 ml

MEF Culture Medium:

- DMEM 450 ml
- FBS (heat inactivated for 30 minutes at 57 °C) 50 ml
- Non-essential amino acids solution 5 ml

3.2 Methods

3.2.1 Dissection

1. Perform an intraperitoneal injection with 0.5 ml Avertin into a mouse at 13 - 14 days gestation.
2. Wait until mouse is anesthetized.
3. Perform cervical dislocation.
4. Place mouse belly up on an absorbent paper towel on a sterile workbench.
5. Saturate mouse abdomen with alcohol pad.
6. Using sterilized instruments, cut back the skin and expose the peritoneum.
7. Re-sterilize instruments with alcohol bath and Bunsen burner or a hot bead sterilizer.
8. Cut peritoneal wall and expose the uterine horns.
9. Remove uterine horns and place them into a sterile, disposable petri dish.
10. Wash uterine horns three times with 10 ml PBS and place horns in a fresh petri dish.
11. Cut open embryonic sacs and release embryos using forceps and scissors.
12. Place embryos in a fresh dish and wash three times with 10 ml PBS.
13. Count and record the number of recovered embryos.
14. Separate visceral tissue from embryos.
15. Place embryos in a new plate; discard visceral tissue.
16. Wash embryos three times with 10 ml PBS.

    Note: Visceral tissue has a darker color.

3.2.2 Enzymatic Digestion

1. Remove excess PBS using a pipette.
2. Mince the tissue with dissecting scissors into grain sized pieces.

   Note: Mince for approximately 5 - 10 minutes. To mince, tip the petri dish with one hand causing the tissue to concentrate on one side of the dish.
3. Add 2 ml Trypsin. Mince for an additional few minutes until pieces are further reduced in size.
4. Add an additional 5 ml Trypsin. Pipette the cells vigorously up and down. Place the dish into an incubator at 37 °C for 20 – 30 minutes.

   Note: During the incubation, another mouse preparation can be started.

3.2.3 Plating und Culturing the Cells

1. Remove the minced tissue from the incubator.
2. Vigorously pipette the mixture up and down with a sterile, disposable 10 ml pipette until the mixture has a sludgy consistency.
3. Add about 20 ml MEF Derivation Culture Media and transfer the contents to a sterile 50 ml plastic conical tube.
4. Rinse the remaining tissue in the plate with a few milliliters MEF Derivation Medium. Transfer the medium to the 50 ml conical tube.
5. Divide the number of recovered embryos (recorded in step 2.2.1 - 13) by 3.
6. This is the number of required T75 flasks. Round-up fractions.
7. Fill each T75 flask with 10 ml MEF Derivation Culture Medium.
8. Transfer 5 ml minced tissue mixture to each flask. Pipette to mix.
9. Distribute the remaining minced tissue mixture 1 ml at a time. Pipette to mix.
10. Incubate the flasks containing the minced tissue mixture in a 37 °C tissue culture incubator overnight.

3.2.4 Observing Fibroblast Cultures and Refreshing Medium

1. Remove flasks from the incubator and view the cultures under a microscope.
2. Inspect the cell layer covering the flask surface.

   Note: If at least 90 % of the flask surface is covered with a cell layer, the cultures are ready for harvesting. Proceed with step 2.2.5.

3. If the flask surface is less than 90 % covered, continue the culture. Aspirate the spent media and replace with fresh MEF Derivation Media.
4. Continue to culture the flask until at least 90 % of the flask surface is covered with cells.

   Note: It is likely that the cell layer will contain raised chunks of tissue.

3.2.5 Harvesting and Freezing Mouse Embryonic Fibroblasts (MEF)

1. Harvest tissue from one mouse at a time, pooling the cells from the flasks.
2. Wash the cells in each flask with PBS.
3. Add Trypsin solution to the flask until the cell layer surface is covered.
4. Incubate for five minutes.
5. Dislodge the cells from the flask surface by slapping the side of the flask with your hand 3 – 5 times.

   Note: Before slapping the flask, ensure the flask cap is screwed on tightly.

   Note: You may need to open flask and use a cell scraper if cells will not come loose.
6. Add 5 ml MEF Culture Media to each T75 Flask. Mix well with Trypsin solution to neutralize.
7. Vigorously pipette up and down with a 10 ml sterile, disposable pipette to break up the cell chunks.

   Note: Do not cause foaming.
8. Pool the suspension from all flasks into a 50 ml conical tube. Allow any large chunks to settle. Remove the supernatant to a new 50 ml conical tube leaving any large chunks behind.
9. Centrifuge the suspension at 200 x g for 5 minutes.
10. Re-suspend pellets in fresh MEF Culture Medium.

    Note: Determine the volume of MEF Culture Medium. Calculate the volume in milliliters needed to produce three 1ml vials per T75 flask, and divide this number by two.
11. Add an equal volume of Cryopreservation Medium and mix.
12. Quickly but carefully add 1 ml suspension to 1.5 ml cryovials. Cap and place in isopropanol freezing container(s).
13. Immediately place freezing container in –80° C freezer. Store overnight.
14. Transfer vials to liquid nitrogen racks or canes.

4 MEF Propagation and Irradiation

Freshly prepared MEF need to be arrested to prevent them from a further cell division, for example by irradiation or by treatment with Mitomycin C. This is not necessary for commercially available MEF that have been irradiated or treated with Mitomycin C.

4.1 Materials

4.1.1 Biological Materials

- Frozen MEF cells in cryovials

4.1.2 Technical Equipment

- Sterile workbench
- Water bath at 37 °C
- 95 % ethanol bath
- Centrifuge
- Incubator set to 37 °C, 85 % humidity, 5 % CO_2
- Aspirator
- Micropipettes and pipette tips
- Cell counter
- Microscope
- Radiation source sufficient to provide 8000 rads of exposure (Mark I 137-Cesium irradiator or Faxitron RX-650 Cabinet X-ray)
- Pipette aid
- 1 ml, 5 ml, and 10 ml glass pipettes
- T75 flasks
- 6-well plate
- 15 ml conical tubes
- Sterile 9" Pasteur pipettes

4.1.3 Chemicals

- 5 ml Phosphate buffered saline (PBS) (Gibco/Invitrogen, 14190-144)
- 2 ml Trypsin - EDTA solution (Gibco/Invitrogen, 25300-054)
- Gelatin
- Endotoxin-free water

4.1.4 Media

- MEF Culture Medium (see Derivation of MEF chapter for the recipe)

Gelatin Coating Solution:

Note: Do not use glass bottles that have been washed with detergents. Glass bottles should be cleaned with NaOH before first use, and then dedicated to sterile gelatin solution only. Do not keep water or gelatin solution in the bottles any longer than a couple of hours before autoclaving.

- Add 0.5 grams gelatin to 500 ml endotoxin-free water (0.1% solution). Gelatin will not be soluble.
- Autoclave for 30 minutes. Gelatin will dissolved and remain liquid. Store at room temperature until use.

4.2 Methods

4.2.1 Gelatin Coating of Culture Plates

1. At least one day prior to plating inactivated MEF, coat the wells of the culture plates with gelatin solution by placing at least 1 ml of gelatin solution into each well of a 6-well MEA.
2. Tilt the plate in several directions so that the liquid covers the entire surface.
3. Incubate plates at 37 °C overnight. Plates can remain for longer than one day, however they may dry out.

4.2.2 Thawing MEF

1. Remove a frozen stock vial of MEF from liquid nitrogen.

Warning: Wear eye protection. Vials can explode when removed from the liquid phase of nitrogen.

2. Roll a vial between hands for about 10 – 15 seconds.
3. Immerse vial in a 37 °C water bath, but do not submerge the lid. Swirl gently.
4. When only an ice crystal remains, remove vial from water bath.
5. Sterilize vial in 95 % ethanol bath. Air dry under sterile culture hood.
6. Pipette cells into a 15 ml conical tube with a 1 ml or 5 ml pipette.
7. Add 4 ml MEF Culture Medium to cells. Do this drop-wisely to avoid an osmotic shock of the cells. Pipette up and down gently to mix.
8. Centrifuge at 200 g for 5 minutes to remove cryoprotectant from the cell culture.
9. Remove and discard supernatant.
10. Reconstitute the small pellet in 10 ml MEF Culture Medium.
11. Transfer suspension into a T75 flask (not coated with gelatin).
12. Place flask into the incubator.
13. Monitor cell density daily.

4.2.3 Splitting MEF Cells

This example illustrates a 1:5 split:

1. Aspirate MEF Culture Medium from the T75 flask.

   Note: The cell monolayer will remain attached to the surface.

2. Wash flask surface with 5 ml PBS. Aspirate PBS.
3. Add about 2 ml Trypsin - EDTA solution for 3 - 5 minutes.
4. Dislodge the cells from the flask surface. Ensure the flask cap is tight. Slap the side of the flask 3 - 5 times with your hand. Continue until the cell layer is dislodged.
5. Add 5 ml MEF Culture Medium to the flask.

   Note: This media contains serum and will completely inhibit the trypsin action.

6. Mix to form cell suspension. Pool all cells into one flask.
7. Add 40 ml MEF Culture Medium to the flask to a total of approximately 50 ml. Mix well.
8. Add 10 ml cell suspension to each of five new T75 flasks.

4.2.4 Enzymatic Digestion and Cell Counting

1. Enzymatically digest the cells as described in the "Derivation of MEF" chapter.
2. Remove 0.5 – 1 ml suspension with a 5 ml pipette and transfer it to a conical tube.

   Note: Be sure the suspension is mixed well before preparing aliquots.

3. Vigorously pipette the cell suspension to break up cell aggregates.
4. Remove 200 µl cell suspension and transfer it directly to the cell counter chamber by capillary forces.

   Note: Carefully touch the edge of the coverslip with the pipette tip and allow the chamber to fill by capillary forces. Do not overfill or underfill the chamber.

5. View two squares of the cell counter chambers with a microscope using a10 x objective lens.
6. Calculate the cell density according to the cell counter instructions.

4.2.5 Irradiating Cells

1. Transfer required cell number to a 15 ml tube.

   Note: Do not exceed 10 ml per tube. Do not spin down cells.

2. Irradiate cells.

   Note: The rads of exposure needed to inactivate MEF can vary from lot to lot and must be tested. 5500 – 8000 rads is typically a good starting point.

3. Centrifuge irradiated cells for 5 minutes at 200 g.

4. Remove supernatant.

5. Re-suspend cell pellet at one million cells per ml in MEF Culture Medium.

   Note: Calculate the cell density based on the preceding cell counts. You do not need to re-count.
   Example: (5.6×10^6 cells) / 1×10^6 cells/ml = 5.6 ml

4.2.6 Plating Cells

1. Further dilute the cell suspension to meet the required cell density.

   Note: The standard cell density of an hES cell feeder layer is 0.75×10^5 cells per ml.
   Example: 2.5 ml/well x 6 wells/plate x 5 plates = 75 ml

2. Remove gelatin coated 6-well MEAs from the incubator and aspirate excess gelatin.

   Add cell suspension drop-wisely to each well of the plate in the following order:

3. Remove 12 ml from the tube with a 10 ml pipette.

4. Aliquot 2 ml into 5 of the 6 wells.

5. Return the remaining 2 ml to the tube and mix the contents by pipetting the suspension.

6. Remove 5 ml with the pipette and add 2.5 ml to the 6th remaining empty well.

7. Add 0.5 ml to the other 5 wells.

8. Place the plates into the incubator and allow cells to attach to the well bottom overnight.

5 Thawing and Plating hESC

The recovery efficiency is about 0.1 to 1 percent from a frozen sample. Therefore, you may not see visible colonies immediately after thawing the cells.

5.1 Materials

5.1.1 Biological Materials

- Cryopreserved hESC: Cell line H1 (WiCell Research Institute, Inc.)

5.1.2 Technical Equipment

- Sterile workbench
- Water bath at 37 °C
- 95 % ethanol bath
- Centrifuge
- Incubator set to 37 °C, 85 % humidity, 5 % CO_2
- Aspirator
- Micropipettes and pipette tips
- Pipette aid
- 1 ml, 5 ml glass pipettes
- Optional: automatic pipette and 1 ml filter tips
- T75 flasks
- 15 ml conical tubes
- 6-well tissue culture plate containing the inactivated MEF feeder layer

5.1.3 Chemicals

- DF12 medium (Gibco/Invitrogen, 11330-032)
- Serum replacer (Gibco/Invitrogen, 10828-028)
- 200 mM L-Glutamine Solution (Gibco/Invitrogen, 25030-081)
- 2-Mercaptoethanol (Sigma-Aldrich, Inc., M-7522)
- Non-essential amino acids 100 x solution (Gibco/Invitrogen, 11140-050)
- 5 ml phosphate buffered saline (PBS) (Gibco/Invitrogen, 14190-144)

5.1.4 Media

B-FGF Solution:

Note: Store frozen in 0.5 mL aliquots.

- B-FGF 10 µg
- 0.1 % BSA in PBS 5 ml

hESC Culture Medium:

Note: Filter the medium with 0.22 µm pore filter before use. Store at 4 °C. The medium can be used for two weeks.

- DF12 Medium 200 ml
- Serum replacer 50 ml
- 200 mM L-glutamine, 1.75 µl
  2-Mercaptoethanol added directly
  to the L-glutamine solution 1.25 ml
- Non-essential amino acids 100 x 2.5 ml
- B-FGF solution 0.5 ml

5.2 Methods

5.2.1 Thawing hESC

1. Remove vial of hESC from liquid nitrogen storage.

Warning: Wear eye protection. Vials can explode when removed from the liquid phase of nitrogen.

2. Roll vial between hands for about 10 - 15 seconds until the outside of the vial is defrosted.
3. Immerse the vial in a 37 °C water bath. Do not submerge the lid. Swirl gently.
4. When only an ice crystal remains, remove vial from water bath.
5. Immerse vial in 95 % ethanol bath to kill microorganisms from the water bath. Air dry under sterile tissue culture hood.

5.2.2 Removal of Cryoprotectant

1. Gently pipette cells into a 15 ml conical tube with a 1 ml or 5 ml pipette.
2. Slowly add 4 ml hESC Culture Medium drop-wise to the cells in the 15 ml conical tube. While adding media, move the tube back and forth to gently mix the hES cells.

   Note: Add the hESC Culture Medium slowly to avoid osmotic shock. Cells are diluted to reduce the DMSO, which is toxic for cells.
3. Gently pipette the cells up and down in the tube a few times.
4. Centrifuge cells at 200 g for 5 minutes.
5. Remove and discard the supernatant.
6. Re-suspend the cell pellet in 2.5 ml hESC Culture Medium.

   Note: Cell will form aggregates. Do not break them up into individual cells.

5.2.3 Plating hESC

1. Aliquot the cell suspension drop-wise into one well of a 6-well tissue culture plate (or 4 wells of a 4-well tissue culture plate) containing the inactivated mouse embryonic fibroblast (MEF) feeder layer with a 1 ml glass pipette or an automatic pipette and a 1 ml filter tip.

   Note: Make sure to wash the well of irradiated MEFs with 1 ml of PBS before adding the HES cells. This removes the FBS, which can interfere with HES cell growth.
2. Place plate into incubator.
3. Refresh hESC Culture Medium daily.

6 Splitting and Expanding hESC

6.1 Materials

6.1.1 Biological Materials

- hESC culture plate
- 6-well tissue culture plate containing the inactivated MEF feeder layer

6.1.2 Technical Equipment

- Sterile workbench
- Water bath at 37 °C
- Centrifuge
- Incubator set to 37 °C, 85 % humidity, 5 % CO_2
- Aspirator
- Micropipettes and pipette tips
- Pipette aid
- Sterile filter
- 5 ml, 10 ml glass pipettes
- Sterile 9" Pasteur pipettes
- 15 ml conical tubes

6.1.3 Chemicals

- DF12 medium (Gibco/Invitrogen, 11330-032)
- Serum replacer (Gibco/Invitrogen, 10828-028)
- 200 mM L-Glutamine Solution (Gibco/Invitrogen, 25030-081)
- 2-Mercaptoethanol (Sigma-Aldrich, Inc., M-7522)
- Non-essential amino acids 100 x solution (Gibco/Invitrogen, 11140-050)
- 5 ml phosphate buffered saline (PBS) (Gibco/Invitrogen, 14190-144)
- Collagenase (Gibco/Invitrogen, 17104-019)

6.1.4 Media

- hESC Culture Medium (see chapter Thawing and Plating hESC for recipe)

Collagenase solution:

- Collagenase 1mg/ml
- Dissolve Collagenase in DF12 Medium

6.2 Methods

6.2.1 When to Split Cells

In general, split cells when the first of the following occurs:

- Mouse Embryonic Fibroblasts (MEF) feeder layer is two weeks old.
- Cell colonies are too dense or too large.
- Differentiation occurs (see Differentiation of human embryonic stem cells into cardiac myocytes).

6.2.2 Collagenase Treatment

1. Remove hESC culture plate from the incubator. Aspirate medium.
2. Add 1 ml Collagenase Solution to each well of the 6-well plate.
3. Incubate for at least 5 minutes.
4. To confirm colony separation from the plate, view surface under a microscope.

   Note: Look for the perimeter of the colony to appear folded back. If necessary, keep collagenase on the cells for another 5 to 10 minutes.

6.2.3 Cell Harvesting

1. Using a glass 5 ml pipette, scrape the cells off the surface of the plate.
2. At the same time, slowly pipette the Collagenase Solution up and down to wash the cells off the surface.
3. Leave the entire contents in the well until the entire plate is finished.
4. After the HES cells are removed from the surface, pool the suspension into a sterile 15 ml conical tube.
5. Gently pipette cells up and down a few times in the 15 ml conical tube to further break-up cell colonies.

   Note: Do not cause foaming.
6. Wash each well with 1ml hESC Culture Medium.
7. Transfer the cells to the 15 ml conical tube.
8. Label the tube and discard used plate.

6.2.4 Spinning and Washing

1. Pellet broken cell colonies by centrifuging at 200 g for 5 minutes.
2. Aspirate supernatant.
3. Wash cell pellet with 2 – 3 ml hESC Culture Medium in the 15 ml conical tube.
4. Gently reconstitute pellet in hESC Culture Medium.
5. Centrifuge at 200 g for 5 minutes.
6. While hESC are centrifuging, aspirate MEF Culture Medium from fresh feeder plates.
7. Add about 1 ml PBS to each well of the 6-well feeder plate to wash away the serum.
8. Aspirate supernatant from hESC pellet. Re-suspend pellet again with 2 – 3 ml hESC Culture Medium.
9. Add a sufficient volume of medium to the 15 ml conical tube to ensure a total of 2.5 ml media per well; 15 ml media per six-well plate.
10. Mix well with pipette.
11. Aspirate the PBS from the wells of the feeder plate.

6.2.5 Plating Cells to Expanded Volume

Add cell suspension drop-wise to each well of the plate in the following order:

1. Remove 12 ml from tube with a 10 ml pipette.
2. Place 2 ml in 5 of the 6 wells.
3. Return final 2 ml to tube and mix contents by pipetting suspension.
4. Remove 5ml with pipette and add 2.5 ml to the sixth well (remaining empty well).
5. Add 0.5 ml to the other 5 wells.

   Note: Do not add drops to the outer perimeter of the well or to the very center.
6. After plating hESC, return the plate to the incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to further disperse the cells across the surface of the wells.
7. Incubate cells overnight to allow colonies to attach to the well bottom.
8. Refresh the cell culture medium daily.

7 Culture Maintenance

The main goal of culture maintenance is to identify and expand undifferentiated cells. The differentiation and isolation of cardiac myocytes is described later.

7.1 Materials

7.1.1 Biological Materials

- hESC culture plates

7.1.2 Technical Equipment

- Sterile workbench
- Water bath at 37 °C
- Centrifuge
- Incubator set to 37 °C, 85 % humidity, 5 % CO_2
- Stereo microscope
- Object marker for the microscope
- Aspirator
- Micropipettes and pipette tips
- Pipette aid
- Sterile filter
- Static enclosure
- 15 ml conical tubes
- 6-well tissue culture plate containing the arrested MEF feeder layer
- Sterile Pasteur pipettes

7.1.3 Chemicals

- DF12 medium (Gibco/Invitrogen, 11330-032)
- Dispase (Gibco/Invitrogen, 17105-041)
- 200 mM L-glutamine solution (Gibco/Invitrogen, 25030-081)
  (Keep frozen in aliquots. Thaw immediately before use.)

7.1.4 Media

- hESC Culture Medium (see chapter "Thawing and Plating hESC" for recipe)
- Collagenase solution (see chapter "Splitting and Expanding hESC" for recipe)

Dispase Solution

- Dispase 5 mg
- DF12 Media 10 ml

7.2 Methods

7.2.1 Discrimination of Differentiated and Undifferentiated Cells

Choose a method to separate differentiated and undifferentiated cells and colonies.

- Remove undifferentiated cells by 'Picking to Keep': If there are a few undifferentiated colonies on the plate with a large number of differentiated single cells and colonies, you will pick the undifferentiated colonies, replate and propagate them. Go to Step 2.
- Remove differentiated cells by 'Picking to Remove': If there are mostly undifferentiated colonies on the plate with only a few differentiated colonies, you will pick the differentiated colonies, remove and discard them. Go to step 3.
- Remove human cells from mouse embryonic fibroblasts (MEF) using Dispase solution: Occasionally, the MEF feeder layer can appear to be uninactivated or abnormal. If you need to passage hES cells without MEFs, you will directly remove the undifferentiated colonies with Dispase solution and plate them on fresh MEF feeder plates. Go to step 5.

Note: Dispase selection is used here for removing hESC colonies from old MEF and then replating hESC colonies to fresh MEF. Dispase solution will remove both undifferentiated and differentiated colonies. This selection procedure should be used when there are too many plates to 'Pick to Remove' severe to moderate single cell differentiation and / or uninactivated MEF. This selection procedure should be not be used to remove hESC colonies from MEF immediately prior to cryopreservation. This may lead to poor recovery.

7.2.2 Picking-to-Keep Method

1. Using an object marker with a 2.5 x or lower objective, mark the undifferentiated colonies you want to keep.
2. Remove the medium from this plate and replace with 1 ml fresh hESC Cell Culture medium.
3. Place plate inside the static enclosure.

Note: Both the interior of the static enclosure and the pulled Pasteur pipettes should be sterilized with UV light for 20 minutes prior to use. Focus and adjust magnification so most of an entire well is visible in the field of view.

4. Remove plate cover. Using a pulled Pasteur pipette, gently scrape off colony or cells inside circular colony mark.
5. Break colony into smaller parts using the pulled Pasteur pipette.

Note: Since enzyme treatment is not used in this procedure, this must be done physically. The aim is to reduce colony size. Undifferentiated colonies reach a threshold size and density beyond which they will differentiate.

6. Replace plate cover and move plate back into the sterile cell culture hood.
7. Remove the medium from each well and transfer to a 15 ml tube.
8. Wash wells with another 1 ml hESC Culture Medium to pick up residual cells.
9. Transfer the medium to the 15 ml tube. Discard the plate.
10. Pipette to disperse cells in the tube.
11. Wash well(s) of a freshly inactivated MEF plate with PBS.
12. Add additional medium to 2.5 ml total per well.

Note: Since only a small amount of cells are transferred, centrifugation is not necessary. Cells may be lost during centrifugation.

13. Plate cells drop-wise. First add 2 ml to each well until 2 ml are left in pipette.
14. Return to 15 ml tube, mix, and add final 0.5 ml to each well.

7.2.3 Picking-to-Remove Method

1. With an object marker, mark colonies or areas of cells to remove.
2. Place plate inside static enclosure.

   Note: Both the interior of the static enclosure and pulled Pasteur pipettes should be sterilized with UV light for 20 minutes prior to use.

3. Focus and adjust magnification so that most of an entire well is visible in the field of view.
4. Remove plate cover. Using a pulled Pasteur pipette, gently scrape off differentiated colonies or cells within the circular colony marking.

   Note: Scrape off cells completely.

5. Proceed swiftly, yet precisely until all marked areas are completely removed.
6. Replace plate cover. Dispose of any used pipettes.
7. Place plate under tissue culture hood and aspirate the medium. Replace with equal amount of fresh hESC Culture Medium.

7.2.4 Removing Undifferentiated Colonies with Dispase Solution

1. Add 1ml 1 mg/ml Collagenase Solution to cells.
2. Incubate the cells with Collagenase Solution for 10 minutes.
3. View colonies under the microscope. If most colonies have nearly peeled off the wells, proceed to next step.

   Note: If not, place back in incubator and check every 5 to 10 minutes. Colonies may take up to 30 minutes to peel away from plate. Proceed to next step after a 30 minutes incubation, even if colonies have not yet peeled off after collagenase treatment and incubation.

4. Add 1ml 0.5 mg/ml Dispase Solution to each well.

   Note: Do not remove Collagenase Solution.

5. Incubate the cells with Dispase Solution for 5 – 10 minutes until colonies are completely peeled off and are floating in the media.

   Note: If necessary, plates can be very carefully tapped against a surface to help release colonies.

6. Under the hood, transfer the cell containing Dispase / Collagenase solution to a 15 ml conical tube.
7. Allow colonies to settle to the bottom of the 15 ml conical tube.
8. Gently remove the supernatant with a pipette.
9. Wash the cells with about 0.5 ml fresh hESC Culture Medium per well.

   Note: For example, if all six wells of a 6-well plate were used, wash with 3 ml. Be careful not to pipette too vigorously.

10. Allow colonies to settle to the bottom of the 15 ml conical tube.
11. Remove the supernatant with a pipette.
12. Reconstitute the pellet in the volume required for re-plating (2.5 ml per well). Resuspend the pellet with a small volume of medium, and then fill up with medium.

    Note: Depending on the final volume, use a 50 ml or larger tube.

13. Remove MEF medium from new feeder plate(s).
14. Wash the wells with PBS.
15. Refer to “Splitting and Expanding hESC “for instructions on plating hESC onto fresh MEF.

8 Differentiation of hESC into Cardiac Myocytes

8.1 Materials

8.1.1 Biological Materials

- hESC culture plates

8.1.2 Technical Equipment

- MEAs (microelectrode arrays) coated with laminin or fibronectin (please see MEA manual for more details)
- Sterile workbench
- Stereo microscope
- Aspirator
- Static enclosure
- Sterile micro scalpel

8.1.3 Chemicals

- Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Invitrogen, 11965)
- Non-essential amino acids 100 x solution (Gibco/Invitrogen, 11140-050)
- 200 mM L-glutamine solution Gibco/Invitrogen, 25030-081) (Keep frozen in aliquots. Thaw immediately before use.)
- Fetal bovine serum (FBS) (Gibco/Invitrogen, 16000-044)

8.1.4 Media

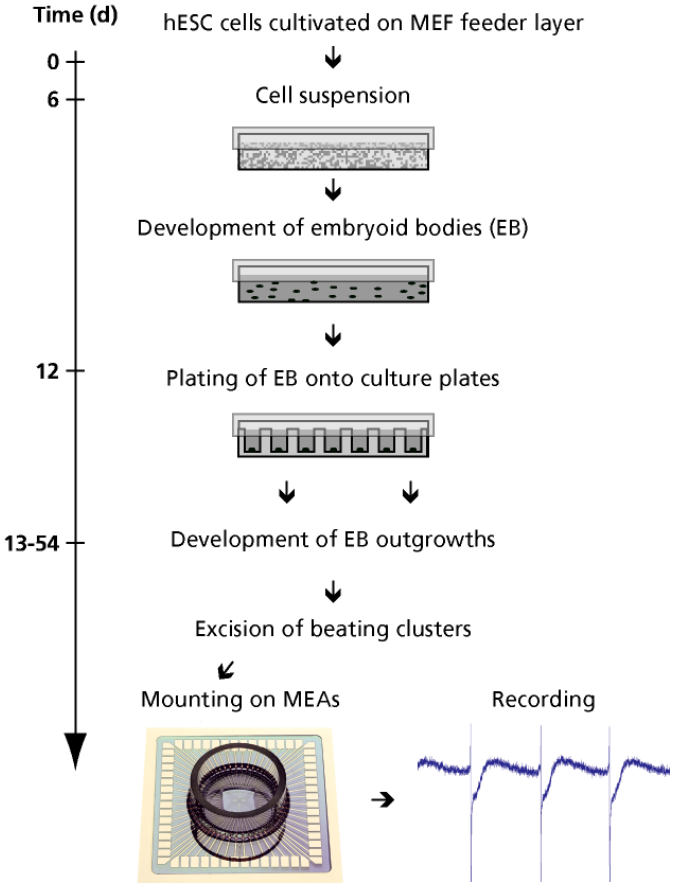
- Collagenase solution (see chapter Splitting and Expanding hESC for recipe)

hESC Differentiation Medium:

- DMEM
- 20 % FBS
- 2 mM L-glutamine
- 1 % non-essential amino acids

Methods

Preparation of hESC derived cardiomyocytes (hESC-CM)



hESC Differentiation

hESC of the line H1 are cultivated on MEF feeder cell layers for approx. 6 days and differentiated into embryoid bodies in suspension culture for 6 days. The embryoid bodies are then plated for different periods of time until spontaneous beating occurs. Clusters of beating areas are isolated with a sterile micro scalpel and plated onto a MEA. The tissue is incubated in medium at 37 °C and can be used for MEA recordings 12 - 72 h after plating. (Reference: Reppel et al. Cell Physiol Biochem 2004; 14:187-196)

Embryoid Body (EB) Formation

1. Disperse hESC colonies into cell aggregates containing approximately 500 to 800 cells using 1 mg/ml Collagenase Solution.
2. Culture cell aggregates in suspension using bacteriological petri dishes for additional 6 days.
3. Embryoid bodies will form in suspension culture.

8.1.5 Cardiac Differentiation and Plating onto MEAs

1. Transfer EB to 6-well plates and culture in hESC Differentiation Medium.
2. During the differentiation process, change medium every second day.
3. Rhythmically contracting clusters will appear 1 to 32 days after plating.
4. Beating areas can be maintained in long-term cultures for up to 61 days after the initiation of contraction.
5. For electrophysiological extracellular field potential (FP) measurements on MEAs, beating areas are cut out of the differentiating EB outgrowths by the use of a micro scalpel and are directly plated onto coated MEAs.

9 Suggested MEA-Systems

9.1 System Configurations

Depending on the throughput and the analysis requirements desired in your laboratory, different system configurations are recommended for the recording from cultured hESC.

- **MEA2100-System**: For the **MEA2100-HS60(2x60)-System**, the same MEAs can be used as for the MEA1060 amplifiers, with the same advantages and disadvantages. The MEA2100-HS60-System allows to operate a maximum of two headstages with up to two 60 channel MEAs each. If all those slots are used with 6-well MEAs (60-6wellMEA) **24 wells** can be tested simultaneously. In addition, a 120-channel MEA layout is available (120MEA200/30iR) for the **MEA2100-HS120-System**, and a 256-channel MEA for a **MEA2100-HS256-System**, which provide larger electrode fields with a resolution identical to the 60MEA200/30iR. This might be interesting for a mapping applications.
- **Multiwell-MEA-System**: The Multiwell-MEA-System is featuring 24- and 96-well plate format, it is the perfect tool for medium and high throughput electrophysiology.
- **USB-MEA60-Inv/Up-System-E**: **60-channel** MEA recording system for inverted or upright microscopes. These systems are not longer available. Please use one of the MEA2100-Systems.
- The **temperature controller TCX** regulates the temperature of the MEA and of the perfusion fluid via the **perfusion cannula PH01**. A **peristaltic perfusion system PPS2** is provided respectively.

9.2 Microelectrode Arrays (MEAs)

Available MEAs differ in electrode material, diameter, and spacing. For an overview on available MEA types please see the MEA manual or the Multi Channel Systems web site (www.multichannelsystems.com) or contact your local retailer.

The microfold structures formed by titanium nitride (TiN) result in a large surface area that allows the design of small electrodes with a low impedance and an excellent signal to noise ratio. For recording from cultured neurons, a medium spatial resolution with an electrode diameter of 30 µm and a spacing of 200 µm is generally sufficient.

As the dimensions of beating areas in EB can be in the range of several hundred µm up to mm we recommend MEAs with rather large interelectrode spacings of 200 – 500 µm.

Recommended MEAs

- **60MEA200/30iR-Ti, 60MEA500/30iR-Ti**: standard 8 x 8 layout, TiN electrodes for recording and stimulation, with substrate-integrated reference electrode.
- **60ThinMEA** for high-resolution imaging and combination with intracellular calcium measurements. 60ThinMEAs are only 180 µm "thick" and mounted on a robust ceramic carrier. Tracks and contact pads are made of transparent ITO.
- **60StimMEA** with 4 pairs of large stimulating electrodes for pacing cardiomyocytes by electrical stimulation.
- **6-well MEAs** (60-6wellMEA) for high throughput when using 24 wells in parallel.
- **120-channel MEAs** (120MEA200/30iR-Ti) for MEA2100-HS120-System only**.**
- **256-channel MEAs** (256MEA200/30iR-Ti) for MEA2100-HS256-System only

Ring Options

The standard glass ring is best for running a perfusion, and will also allow using the ALA MEA-Insert. A plastic ring should only be used if parallel patch clamp experiments are planned.

9.3 Recommended Amplifier Specifications

Though custom MEA1060 amplifiers with gain and bandwidth specified by the user are available, Multi Channel Systems MCS GmbH recommends the following settings for this application.

- Lower cut-off frequency: **1 Hz**
  If you select an even lower value for the lower end of the bandwidth, slow signal drifts can disturb the recordings. A lower cutoff frequency of up to 10 Hz is recommended if you are not interested in Calcium currents, for example, for safety screening or mapping of excitation spreading.
- Upper cut-off frequency: **3 kHz** Sufficient even for recording the rapid depolarization waveforms.
- Gain: **1200**

In **MEA2100-Systems** the sampling rate, signal range and bandwidth can be adjusted via software control and is therefore suitable for a broad range of applications, from single unit spike recordings to field potentials from whole heart preparations.

References

K. Banach, M. D. Halbach, P. Hu, J. Hescheler, U. Egert, Development of electrical activity in cardiac myocyte aggregates derived from mouse embryonic stem cells, Am J Physiol Heart Circ Physiol 284, H2114-23 (Jun, 2003).

J. Itskovitz-Eldor, S. Gerecht-Nir, Human embryonic stem cells: a potential source for cellular therapy, Am J Transplant. 2004;4 Suppl 6:51-7.

I. Kehat et al., Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes, J Clin Invest 108, 407-14 (Aug, 2001).

I. Kehat et al., Electromechanical integration of cardiomyocytes derived from human embryonic stem cells, Nat Biotechnol 22, 1282-9 (Oct, 2004).

J. Hescheler, B. K. Fleischmann, Indispensable tools: embryonic stem cells yield insights into the human heart, J Clin Invest 108, 363-4 (Aug, 2001).

J. Hescheler et al., Determination of electrical properties of ES cell-derived cardiomyocytes using MEAs, J Electrocardiol 37 Suppl, 110-6 (2004).

E. Kolossov , Z. Lu , I. Drobinskaya, N. Gassanov , Y. Duan, H. Sauer, O. Manzke, W. Bloch, H. Bohlen, J. Hescheler, B.K. Fleischmann, Identification and characterization of embryonic stem cell-derived pacemaker and atrial cardiomyocytes, FASEB J. 2005 Jan 19.

M. Reppel et al., Microelectrode arrays: A new tool to measure embryonic heart activity, J Electrocardiol 37 Suppl, 104-9 (2004).

M. Reppel, C. Boettinger, J. Hescheler, Beta-adrenergic and muscarinic modulation of human embryonic stem cell-derived cardiomyocytes, Cell Physiol Biochem 14, 187-96 (2004).

J. Satin et al., Mechanism of spontaneous excitability in human embryonic stem cell derived cardiomyocytes, J Physiol 559, 479-96 (Sep 1, 2004).

T. Xue, H.C. Cho, F.G. Akar, S.Y.Tsang, S.P. Jones, E. Marban, G.F. Tomaselli, R.A. Li, Functional integration of electrically active cardiac derivatives from genetically engineered human embryonic stem cells with quiescent recipient ventricular cardiomyocytes: insights into the development of cell-based pacemakers, Circulation. 2005 Jan 4; 111(1):11-20.

Marcel D. Halbach et al., Estimation of Action Potential Changes from Field Potential Recordings in Multicellular Mouse Cardiac Myocyte Cultures, Cell Physiol Biochem 2003;13:271–284.

The following publications refer to the use of different stem cell lines.

References	H1	H7	H9	H9.1	H9.2	H13	H14	hES2
Kehat et al., JCI 108: 407, 2001					+			
Xu et al., Circ. Res. 91:508, 2002	+	+	+	+	+			
He et al., Circ. Res. 93:32, 2003	+	+	+				+	
Mummery et al., Circ. 107:2733, 2003								+
Kamp lab, unpublished						+		

10 Contact Information

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