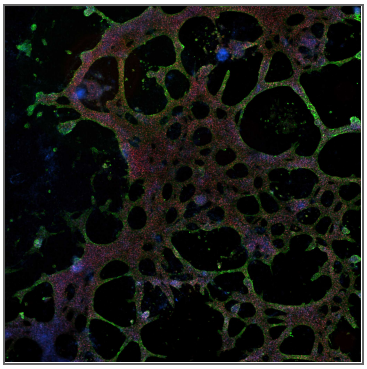

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Human iPSCs culture and cardiomyocyte subtype differentiation in fully chemically defined conditions

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MOSAIC

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ABSTRACT

Cardiomyocyte in vitro differentiation represents a pivotal avenue in stem cell research, offering unprecedented insights into cardiac development and disease understanding. Over the years, protocols for inducing cardiomyogenesis have evolved substantially, particularly with the advent of induced pluripotent stem (iPS) cell technology. The transition from intricate and uncontrolled systems to more refined and robust protocols has been paramount, driven by a cumulative understanding of the molecular and cellular cues governing the process.
This protocol addresses the intricacies of cultivating iPS cells and orchestrating their differentiation into distinct cardiomyocyte subtypes. The protocol encompasses not only the fundamental aspects of human induced pluripotent stem cell (hiPSC) culture but also delves into the nuanced stages of cardiomyocyte differentiation, sub-type specification, and the critical maturation phase. The refined methodologies outlined in this protocol contribute to advancing capabilities in disease modeling, drug discovery, developmental biology, and regenerative medicine.

PROTOCOL MATERIALS

Essential 8™ Flex Medium Kit **Thermo Fisher Catalog #A2858501**

In 2 steps

Penicillin/Streptomycin **Thermo Fisher Scientific Catalog #Invitrogen 15140-12**

Step 2.1

RPMI 1640 Medium, GlutaMAX™ Supplement, HEPES **Thermo Fisher Catalog #72400021**

In 3 steps

Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit **Thermo Fisher Catalog #A24881**

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Protocol status: Working
We use this protocol and it's working

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PROTOCOL integer ID:
91885

Keywords: cardiomyocyte, differentiation, in vitro, cell culture, iPSC

Step 4.1

Penicillin-Streptomycin (5,000 U/mL) **Thermo Fisher Catalog #15070063**

In 2 steps

StemMACS™ CHIR99021 **Miltenyi Biotec Catalog #130-103-926** Step 11.2

Geltrex LDEV Free hESC Quality 5 ml **Thermo Fisher Scientific Catalog #A1413302**

Step 1.1

ROCK inhibitor (Ri) Y-27632 **STEMCELL Technologies Inc. Catalog #72308**

In 3 steps

Lookout® One-Step Mycoplasma PCR Detection Kit **Merck MilliporeSigma (Sigma-Aldrich) Catalog #MP0050-100TST**

Step 5.1

Retinoic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R2625-50MG**

Step 11.2

Fetal Bovine Serum, qualified, One Shot™ format, United States **Thermo Fisher Catalog #A3160501**

Step 18.1

PSC Cardiomyocyte Differentiation Kit **Thermo Fisher Catalog #A2921201**

Step 9

dimethylsulfoxide (DMSO) **Merck MilliporeSigma (Sigma-Aldrich)** In 2 steps

B-27™ Supplement, minus insulin **Gibco - Thermo Fisher Catalog #A1895601**

Step 11.1

B-27™ Supplement (50X), serum free **Gibco - Thermo Fisher Catalog #17504044**

Step 11.1

IWP-2 1 mg **STEMCELL Technologies Inc. Catalog #72122** Step 11.2

Human Cardiomyocyte Immunocytochemistry Kit **Thermo Fisher Catalog #A25973**

Step 16.2

DMEM/F-12 **Thermo Fisher Catalog #11320033** Step 1.2

Trypan Blue Solution 0.4% Sterile-filtered **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8154**

Step 6.6

StemPro™ Accutase™ Cell Dissociation Reagent **Thermo Fisher Scientific Catalog #A1110501**

Step 17.3

SAFETY WARNINGS

Appropriate safety procedures are recommended to be used when handling all cell lines, working with iPSCs and induced cardiomyocytes handle them under at least BSL-1 containment.

BEFORE START INSTRUCTIONS

Keep in mind that while working with cells everything should be kept sterile, every medium or any reagent should be prepared under the sterile hood, autoclaved, or filtered through 0,2um filter.

iPSC culture and maintenance

1 Coating culture plates with ECM proteins for enhanced attachment of iPSCs.

1.1 Thaw Geltrex LDEV Free hESC Quality 5 ml **Thermo Fisher Scientific Catalog #A1413302** 12h on ice 4 °C Overnight. It is prohibited to thaw Geltrex at room temperature or in a water bath. Remember to keep it on ice during the whole aliquoting procedure and use only ice-cold pipette tips and tubes.

Note

Making small aliquot portions of Geltrex is recommended to limit the freeze-thaw cycles. *Storage of aliquots in -20 °C freezer for up to 1 year.

1.2 Resuspend single Geltrex aliquot in cold DMEM/F-12 **Thermo Fisher Catalog #11320033** medium to obtain desired dilution. Store diluted coating medium in the fridge.

Note

1:60-1:100 dilution usually works fine but it should be first tested on a particular cell line.

1.3 Coat 6-well culture plate by adding 1 mL of coating medium to each well and cover the entire 30m surface of each well. Incubate the plate in the incubator for at least 00:30:00 before proceeding further.

> **Note**
>
> Coated plates can be used immediately after removal of the coating medium or stored in the fridge for up to 2 weeks with added PBS and wrapped in parafilm to prevent from drying.

2 iPSC thawing

2.1 Prepare culture medium using the following kit: Essential 8™ Flex Medium Kit **Thermo Fisher Catalog #A2858501** : add 48.5 mL of Essential 8 flex medium to the 50 mL centrifuge tube and 1 mL of 10x supplement. It is not necessary, but it is advised to also add 500 µL of Penicillin/Streptomycin **Thermo Fisher Scientific Catalog #Invitrogen 15140-122** to prevent cell culture from contamination.

2.2 Prepare the previously coated 6-well plate by adding 2 mL of warm culture medium to each well and 2 µL from [M] 10 millimolar (mM) stock solution of ROCK inhibitor (Ri) Y-27632 **STEMCELL Technologies Inc. Catalog #72308** to minimize the cell death.

> **Note**
>
> **Preparing 10mM stock solution of rock inhibitor Y-27632**
>
> 1. Dissolve 5 mg of rock inhibitor in 1.56 mL DMSO.
> 2. Mix well until it dissolves completely.
> 3. Prepare small aliquot portions, for example 10 µL in PCR tubes and keep them in the -20 °C freezer.

2.3 Take out the desired cryovial containing frozen cells from the liquid nitrogen and quickly transfer it to 37 °C water bath. Be careful not to submerge the cap of the cryovial. You can swirl the tube a little to help it thaw quicker but pay attention to save a little ice cube inside the cryovial before taking it out. Disinfect the cryovial with 70% ethanol and put it under the hood.

2.4 Transfer 1 mL of thawed cell suspension to the 15 mL centrifuge tube and add 9 mL of warm culture medium very gently drop by drop, swirling the tube to minimize osmotic shock to the cells.

2.5 Centrifuge the cell suspension 1400 rpm, Room temperature, 00:05:00. Carefully remove and 5m discard the supernatant. Gently add 1 mL of medium to the remaining cell pellet. Gently resuspend cells in medium and seed into 2 wells of a previously prepared 6-well plate slowly drop by drop.

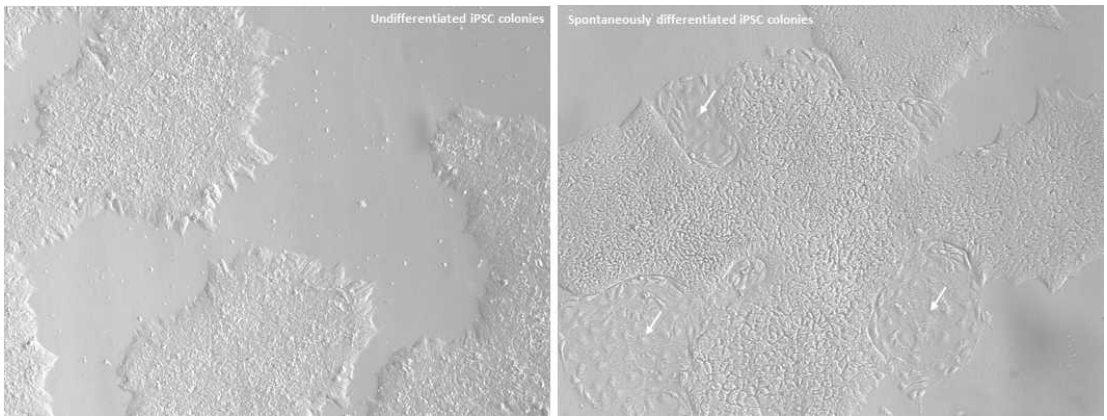
2.6 Gently rock plate side to side, and back and forth to spread the cells evenly across the well. Transfer the plate to the incubator. The next day in the morning change the medium to fresh culture medium without rock inhibitor.

3 iPSC culture

3.1 Incubate the cells at 37 °C/ 5% CO_2 incubator and observe their growth and confluence under the microscope daily. Perfect undifferentiated iPSC colonies should look compact and present distinct borders. Cells present large nuclei and smaller cytoplasm region. It is essential to check iPSCs for the presence of spontaneous differentiation. If any signs of differentiation occur, colonies should be discarded immediately.

Expected result

Morphological differences between correct and incorrect iPSC colonies



white arrows present differentiated regions

3.2 Medium should be changed every day. When using

Essential 8™ Flex Medium Kit **Thermo Fisher Catalog #A2858501**

it is possible to skip weekend medium change by adding 2x more medium on Friday afternoon, but it is only recommended when cells are at low confluency.

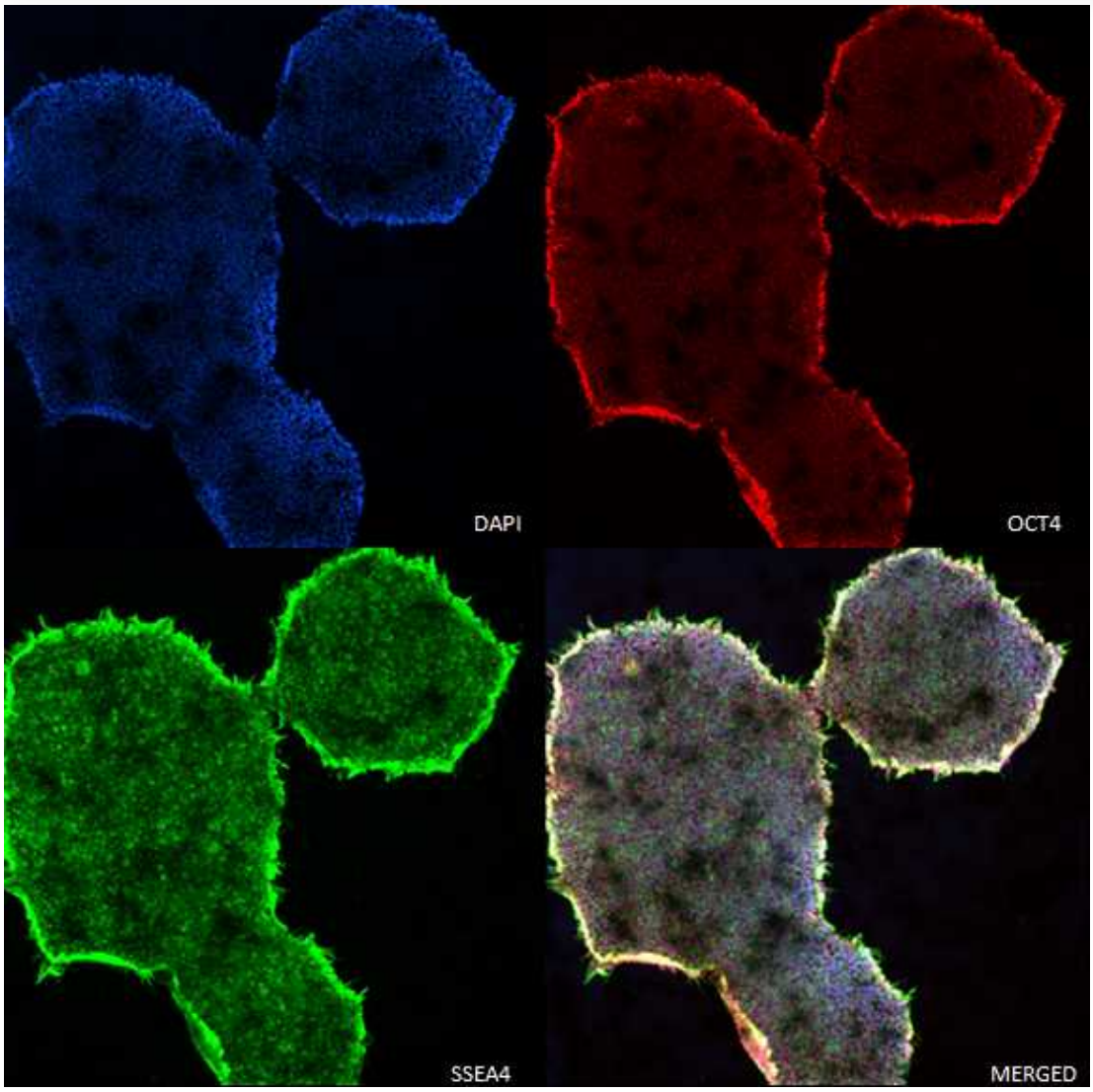
4 Pluripotency characterization

4.1 To guarantee the reliability of the research iPSC lines should be verified for pluripotency markers either by qPCR or immunocytochemistry. The most common pluripotency markers are OCT4, SOX2, SSEA4, TRA-1-60, and alkaline phosphatase.

Expected result

iPSC colonies present positive staining for pluripotency markers OCT4 and SSEA4 using

Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit **Thermo Fisher Catalog #A24881**



5 Mycoplasma testing

5.1 It is very important to maintain contamination-free cultures. A commonly known good laboratory

practice is to regularly check cell cultures for mycoplasma. Mycoplasma contamination is very hard to notice without special tests. This contamination usually does not cause cell death, but it negatively affects the cell metabolism and growth making the research not reliable. The best way to assess mycoplasma contamination in culture is to use available qPCR or immunocytochemistry ready-to-use kits, for example:

Lookout® One-Step Mycoplasma PCR Detection Kit **Merck MilliporeSigma (Sigma-Aldrich) Catalog #MP0050-100TST**

6 iPSC passage

6.1 The optimal time for harvest or passage for iPSCs is typically when cells reach approximately 70-80% confluency.

6.2 To start with prepare mild cell detachment solution, for example PBS with [M] 0.5 millimolar (mM) EDTA. Warm it to Room temperature before adding it to the cells.

6.3 Prepare a new Geltrex-coated plate as in steps go to step #1.3 and #2.2

6.4 Wash cells with warm PBS first, then add 1 mL of cell detachment solution to each well in a 6 3m well plate format. Transfer the plate to the incubator 37 °C. After 00:03:00 take out the plate and observe the cells under the microscope. iPSCs colonies should look loose and be ready to detach, if not please transfer the plate back to the incubator for one minute or two more, but do not exceed the incubation time and do not let the iPSC colonies detach completely and float in the solution.

6.5 Remove the cell detachment solution and rinse the cells with 1 mL of warm culture medium.

Pipette the cells a couple of times, but be careful not to over-pipette the cells. The cells should stay in clumps and easily detach from the well. Transfer the appropriate amount to new wells.

6.6 You can split the cells using ratio for example 1:6 to 1:30 or take a small amount for counting first to have an exact number of cells in a new well.

Note

Cell counting

1. Take 10 µL of cell suspension from the well and transfer it to a small PCR tube.
2. Pipette cell suspension a couple of times to make asingle-cell suspension.
3. Add 10 µL of
Trypan Blue Solution 0.4% Sterile-filtered **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8154**
and mix well by pipetting
4. Take 10 µL for counting using a cell counter or Neubauer chamber.

6.7 Agitate the plate gently from side to side and forwards and backwards to ensure that the cell aggregates are evenly distributed across the surface of the well containing culture medium with theaddition of rock inhibitor.

6.8 The next day in the morning, exchange the medium to fresh culture medium without rock inhibitor. Continue to change the medium daily until iPSCs reach confluency again.

7 iPSC cryopresevation

7.1 Prepare cryopreservation medium: culture medium without antibiotics with 5%-10% dimethylsulfoxide (DMSO) **Merck MilliporeSigma (Sigma-Aldrich)**. The cryopreservation medium should be stored in the fridge and keep cold during the procedure.

7.2 Firstly perform cell detachment as described in steps go to step #6.5 and #6.6 but instead of adding the cell suspension to the new well transfer it to 15 mL centrifuge tube.

7.3 Centrifuge the tube 1400 rpm, Room temperature, 00:05:00 5m

7.4 Remove the supernatant with care not to move the cell debris. Add an appropriate amount of cold cryopreservation medium to the tube, and resuspend the cells. Transfer cryotubes to a freezing container and put them in -80 °C freezer Overnight. The next day, you can transfer cryotubes to the liquid nitrogen for longer storage.

Note

For example, it is recommended to use 1 mL of cell suspension for 1 cryotube and freeze around 1x10^6 live cells per vial.

Equipment

Mr. Frosty Freezing Container	NAME
freezing container	TYPE
ThermoFisher Scientific	BRAND
5100-0001	SKU
https://www.thermofisher.com/order/catalog/product/5100-0001	LINK

Differentiation of hiPSCs into cardiomyocytes

8 **Standard iPSC culture before differentiation**.

8.1 When cells become confluent passage them to prepared geltrex-coated 12-well plate.

8.2 View cells under microscope every day to estimate their confluence. When cells reach desired confluence between 60%-80% they are ready for differentiation.

9 **Differentiation of hiPSCs into cardiomyocytes using commercially available kit**

PSC Cardiomyocyte Differentiation Kit **Thermo Fisher Catalog #A2921201**

9.1 On day 0 of the differentiation remove iPSC culture medium from well and replace it with Cardiomyocyte Differentiation Medium A from the kit.

9.2 On day 2 replace the medium with Cardiomyocyte Differentiation Medium B from the kit.

> **Note**
>
> On this step of protocol cells may start to look different, they become more opaque and many dead cells are floating in the medium.

9.3 On day 4 replace the medium with Cardiomyocyte Maintenance Medium. Change the medium every 2-3 days with Cardiomyocyte Maintenance Medium. Starting from day 8 cells should begin to contract.

Subtype differentiation of hiPSCs into cardiomyocytes

25m

10 **Standard iPSC culture before differentiation.**

10.1 When cells become confluent passage them to prepared geltrex-coated 12-well plate.

11 **Differentiation to cardiomyocyte subtypes**

> **Note**
>
> When iPSCs reach 70-90% confluence they are ready to start differentiation

11.1 In the beginning, prepare two types of medium:
1. Cardiomyocytes induction medium
2. Cardiomyocytes culture medium

Note

1. Cardiomyocytes induction medium:

RPMI 1640 Medium, GlutaMAX™ Supplement, HEPES **Thermo Fisher Catalog #72400021**

with 1x B-27™ Supplement, minus insulin **Gibco - Thermo Fisher Catalog #A1895601** from 50x stock and 1% of

Penicillin-Streptomycin (5,000 U/mL) **Thermo Fisher Catalog #15070063**

2. Cardiomyocytes culture medium:

RPMI 1640 Medium, GlutaMAX™ Supplement, HEPES **Thermo Fisher Catalog #72400021**

with 1x B-27™ Supplement (50X), serum free **Gibco - Thermo Fisher Catalog #17504044** from 50x stock and 1% of

Penicillin-Streptomycin (5,000 U/mL) **Thermo Fisher Catalog #15070063**

11.2 Prepare small molecules working solutions of CHIR99021, IWP2 and retinoic acid (RA)

Note

1. Dissolve StemMACS™ CHIR99021 **Miltenyi Biotec Catalog #130-103-926** [M] 4.66 mg/mL in DMSO 37 °C 00:05:00 to obtain [M] 10 millimolar (mM) working solution, aliquot small portions, and store in the freezer -20 °C
2. Dissolve IWP-2 1 mg **STEMCELL Technologies Inc. Catalog #72122** [M] 2.34 mg/mL in DMSO 37 °C 00:20:00 to obtain [M] 5 millimolar (mM) working solution, aliquot small portions, and store in the freezer -20 °C
3. Dissolve Retinoic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R2625-50MG** [M] 29.94 mg/mL in DMSO to obtain [M] 100 millimolar (mM) master stock, aliquot 10 µL portions, and store in the freezer -80 °C. To obtain [M] 1 millimolar (mM) working solution thaw and dilute RA aliquot in 990 µL EtOH and store in the fridge for 2 weeks, protecting from light.

12 Atrial subtype differentiation

1w 1d

12.1 On day 0 change the iPSC culture medium to cardiomyocytes induction medium with an addition of [M] 5 micromolar (µM) CHIR99021 (0.5 µL /ml from [M] 10 millimolar (mM) working stock solution).

12.2 After 48h on day 2 change the medium to fresh cardiomyocytes induction medium with an addition of [M] 5 micromolar (µM) IWP2 (1 µL /ml from [M] 5 millimolar (mM) working stock solution).

12.3 On day 3, without changing the medium add 1 µL /ml RA [M] 1 micromolar (µM) final from [M] 1 millimolar (mM) working stock solution .

12.4 On day 4 change the medium to fresh cardiomyocytes induction medium with an addition of [M] 1 micromolar (µM) RA.

12.5 On day 6 change the medium to fresh cardiomyocytes induction medium.

12.6 From day 8 change the medium to cardiomyocytes culture medium and continue media change every 2-3 days.

13 Ventricular subtype differentiation

1w 1d

13.1 On day 0 change the iPSC culture medium to cardiomyocytes induction medium with an addition of [M] 5 micromolar (µM) CHIR99021 (0.5 µL /ml from [M] 10 millimolar (mM) working stock solution).

13.2 After 48h on day 2 change the medium to fresh cardiomyocytes induction medium with an addition of [M] 5 micromolar (µM) IWP2 (1 µL /ml from [M] 5 millimolar (mM) working stock solution).

13.3 On day 4 change the medium to fresh cardiomyocytes induction medium.

13.4 On day 6 change the medium to fresh cardiomyocytes induction medium.

13.5 From day 8 change the medium to cardiomyocytes culture medium and continue media change every 2-3 days.

14 Nodal subtype differentiation

1w

14.1 On day 0 change the iPSC culture medium to cardiomyocytes induction medium with an addition of [M] 12 micromolar (µM) CHIR99021 (1.2 µL /ml from [M] 10 millimolar (mM) working stock solution).

14.2 After 24h on day 1 change the medium to fresh cardiomyocytes induction medium.

14.3 On day 3 change the medium to fresh cardiomyocytes induction medium with an addition of of [M] 5 micromolar (µM) final IWP2 (1 µL /ml from [M] 5 millimolar (mM) working stock solution .)

14.4 On day 5 change the medium to fresh cardiomyocytes induction medium with an addition of [M] 3 micromolar (µM) CHIR99021 (0.3 µL /ml from [M] 10 millimolar (mM) working stock solution).

14.5 From day 7 change the medium to cardiomyocytes culture medium and continue media change every 2-3 days.

Cardiomyocytes culture, characterization, passaging and cryo... 25m

15 Longer culture

15.1 Cardiomyocytes and their specific subtypes usually start contracting after 8-10 days from the beginning of the differentiation. After 12-15 days they become defined enough to be safely passaged or cryopreserved. Cells can be kept in a culture for at least 1 month or even longer if such experiments are desired.

Note

With time cardiomyocytes become more mature and gain more cardiomyocyte-like characteristics.

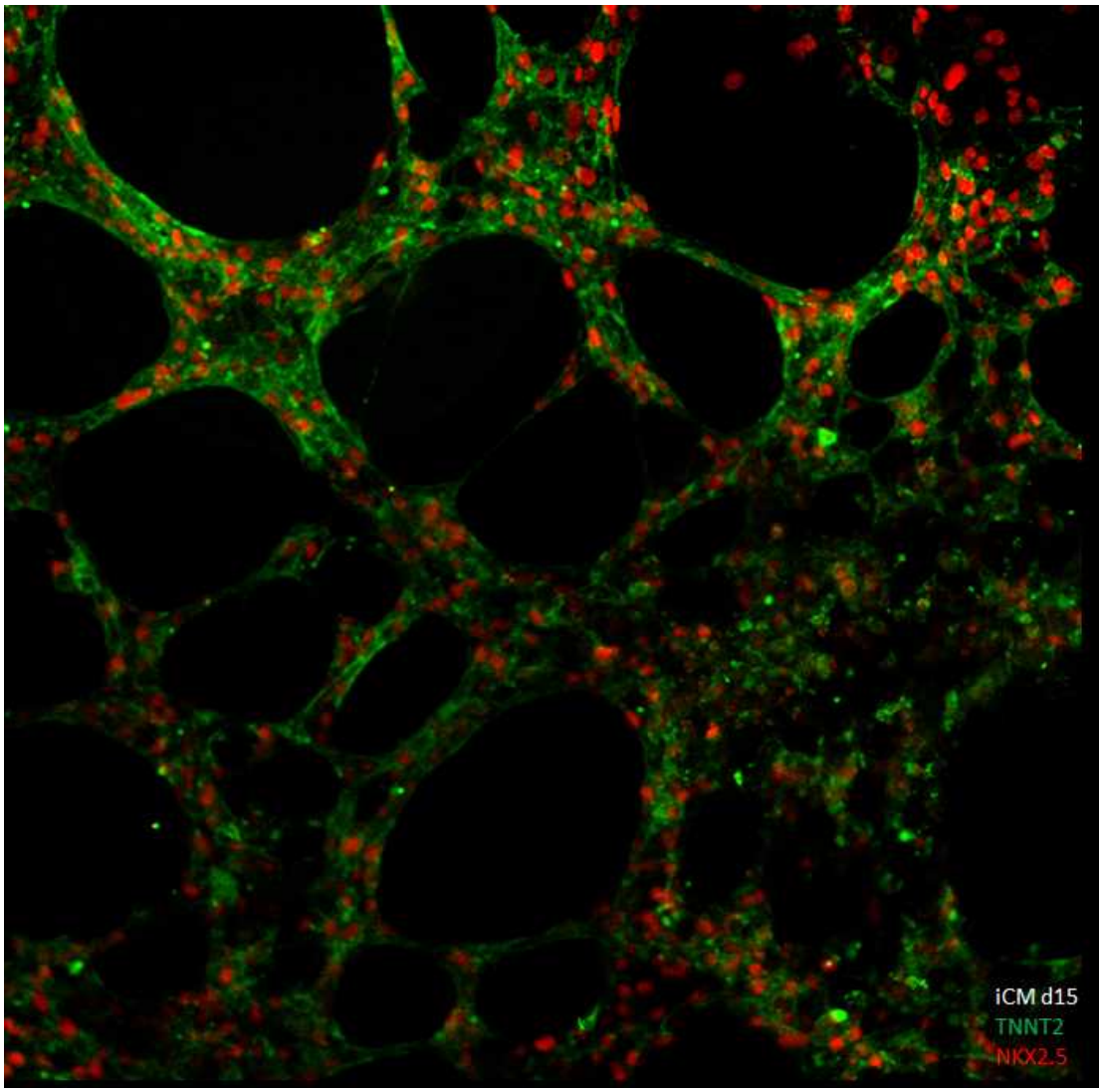
16 Characterization of cardiomyocytes

16.1 Successfully differentiated cells display completely different morphology, with characteristic net-like structure. The most important feature is their ability to contract. It is also recommended to perform PCR analysis or immunocytochemistry to confirm theexpression of key cardiac markers such as TNNT2 and NKX2.5.

16.2 Immunocytochemistry confirmation of key cardiac markers expression in differentiated cardiomyocyte-like cells using commercially available kit

Human Cardiomyocyte Immunocytochemistry Kit **Thermo Fisher Catalog #A25973**

Expected result



17 Passaging cardiomyocytes

17.1 Prepare a new Geltrex-coated plate go to step #1.3

17.2 For better cell survival post-passaging it is recommended to add [M] 10 micromolar (µM) ROCK inhibitor (Ri) Y-27632 **STEMCELL Technologies Inc. Catalog #72308** to the culture medium approximately 1 hour before passage.

17.3 Aspirate the medium and wash cells with warm PBS. Add warm 15m StemPro™ Accutase™ Cell Dissociation Reagent **Thermo Fisher Scientific Catalog #A1110501** to the well (1 mL per well in 6-well plate) and incubate 37 °C 00:05:00 - 00:10:00

17.4 After 5 minutes observe the cells under the microscope to see if they are ready to detach, if not continue to incubate up to 10 minutes maximum. When cells look ready to be detached pipette the solution a couple of times to detach the cells from the well, be careful not to overpipette the cells, because it may disrupt them.

17.5 Transfer them to 15 mL centrifuge tube, add an appropriate amount of culture medium (1:1), 5m centrifuge the tube 1400 rpm, Room temperature, 00:05:00

17.6 After centrifugation aspirate the supernatant and resuspend the cells in cardiomyocytes culture medium. You can take asmall portion for cell counting and place desired amount of cells on a new geltrex-coated plate with addition of [M] 10 micromolar (µM) rock inhibitor for better cell recovery.

17.7 Agitate the plate gently from side to side and forwards and backwards to ensure that the cell aggregates are evenly distributed across the surface of the well. Put the plate in the incubator. The next day in the morning change the medium to fresh culture medium and continue media change every 2-3 days.

18 Cryopreservation of cardiomyocytes

Note

if cardiomyocytes and their subtypes are not required for any ongoing experiment, they can be cryopreserved from day 12-15 onwards and recovered for culture when needed.
It is recommended to freeze around 2x10^6 cells per cryovial.

18.1 After centrifugation from step go to step #17.5 you can aspirate the supernatant and resuspend cells in cold cryopreservation medium.

Note

Cardiomyocytes cryopreservation medium:
[M] 50 % volume
Fetal Bovine Serum, qualified, One Shot™ format, United States **Thermo Fisher Catalog #A3160501**
, [M] 40 % volume
RPMI 1640 Medium, GlutaMAX™ Supplement, HEPES **Thermo Fisher Catalog #72400021**
, [M] 10 % volume
dimethylsulfoxide (DMSO) **Merck MilliporeSigma (Sigma-Aldrich)**

18.2 Add an appropriate amount of cold cryopreservation medium to the tube, and resuspend the cells. Transfer cryotubes to a freezing container and put them in -80 °C freezer Overnight. The next day, you can transfer cryotubes to the liquid nitrogen for longer storage.

18.3 **Post-thaw recovery**

18.4 Prepare the previously coated 6-well plate by adding 2 mL of warm culture medium to each well and 2 µL from [M] 10 millimolar (mM) stock solution
ROCK inhibitor (Ri) Y-27632 **STEMCELL Technologies Inc. Catalog #72308** to minimize the cell death.

18.5 Take out the desired cryovial from the liquid nitrogen and quickly transfer it to 37 °C water bath. Be careful not to submerge the cap of the cryovial and keep a little ice cube in the cryovial before taking it out. Disinfect the cryovial with 70% ethanol.

18.6 Add 9 mL of warm cardiomyocyte culture medium to 15ml centrifuge tube. Centrifuge 5m
1400 rpm, Room temperature, 00:05:00

18.7 After centrifugation aspirate the supernatant and resuspend the cells in culture medium. You can take small portion for cell counting.

Note

It is recommended to seed around 1-3x10^6 cells per well in 6-well plate.

18.8 Transfer the cells to a new plate, agitate the plate gently from side to side and forwards and backwards to ensure that the cell aggregates are evenly distributed across the surface of the well. Put the plate in the incubator. Next day change the medium without rock inhibitor and continue media change every 2-3 days.