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Isolation and Primary Culture of Adult Mouse Cardiac Fibroblasts

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[Abstract] Fibroblasts are often used as a feeder layer for progenitor or stem cells in co-culture systems. In the heart fibroblasts are important for cardiac development, homeostasis, and remodelling. They provide cardiomyocytes and progenitor cells not only with nutrition but also secrete extracellular matrix that forms the microenvironment that ensures cell survival and function. Although different kinds of mouse fibroblasts have been used in co-cultures (embryonic, skin and cardiac fibroblasts) adult mouse cardiac fibroblasts (AMCFs) create the closest microenvironment to the adult murine heart for culturing adult mouse cardiac progenitor cells. This protocol describes the isolation of cardiac fibroblasts from adult mouse hearts as well as their maintenance in culture.

Materials and Reagents

1. Falcon tubes (50 ml) (Sarsdtedt)
2. Sterile pipets (Sarsdtedt)
3. 100 mm cell culture dishes (Sarsdtedt)
4. Aluminum foil
5. Adult mouse hearts from freshly euthanized mice (C57Bl6/J)
6. NaCl (Carl Roth, catalog number: 3957.2)
7. KCl (Carl Roth, catalog number: HNO2.3)
8. Na_2HPO_4 (Carl Roth, catalog number: 4984.1)
9. KH_2PO_4 (AppliChem, catalog number: A2946)
10. $CaCl_2$ (AppliChem, catalog number: A3652)
11. $MgSO_4 \cdot 7H_2O$ (AppliChem, catalog number: A1037)
12. $NaHCO_3$ (AppliChem, catalog number: A1940)
13. Collagenase II (Worthington, catalog number: LS004177)
14. 2.5% Trypsin (Thermo Fisher Scientific, Gibco™, catalog number: 15090-046)
15. DMEM/F12 (Thermo Fisher Scientific, Gibco™, catalog number: 31331-028)
16. PBS (without $CaCl_2$, $MgCl_2$) (Thermo Fisher Scientific, Gibco™, catalog number: 14190-94)
17. FBS (Thermo Fisher Scientific, Gibco™, catalog number: 10270-106)
18. PenStrep (Thermo Fisher Scientific, Gibco™, catalog number: 15140-122)
19. L-glutamine (Thermo Fisher Scientific, Gibco™, catalog number: 25030-024)

20. Ascorbic acid (AppliChem, catalog number: A1052)
21. PBS (see Recipes)
22. Hanks balanced salt solution (HBSS) (see Recipes)
23. Collagenase II stock (see Recipes)
24. Digestion buffer (see Recipes)
25. Fibroblast medium (see Recipes)

Equipment

1. Scalpel, forceps and scissors (Fine Science Tools)
2. Autoclaved glass beaker (100 ml) with magnetic stirrer
3. Sterile cell culture hood
4. Cell culture incubator (Labotect Labor-Technik Göttingen)
5. Magnetic stirrer with thermostat (IKA®-Werke GmbH & CO. KG)
6. Cell culture centrifuge (Eppendorf, model: 5417R)
7. Autoclave

Procedure

1. Remove 3 adult mouse hearts from freshly euthanized mice and place them on a Petri dish containing ice cold PBS.
2. Place the hearts under a sterile cell culture hood and perform the following steps under sterile conditions.
3. Pump the blood out of the hearts by the use of forceps.
4. Mince the 3 hearts on a Petri dish on ice. By the use of scissors cut the heart into 10 pieces and by the use of a scalpel reduce them into a size of 1 mm.
5. Transfer the minced tissue (using the scalpel as a spatula) in an autoclaved glass beaker with a magnetic stirrer and cover the beaker with aluminum foil.
6. Add 25 ml digestion buffer and digest tissue under constant stirring at 37 °C for 5 min.
   Note: The stirring speed should be adjusted so that all the pieces of tissue just flow and do not sit at the bottom but should not be too strong to destroy the cells.
7. Leave the mixture for 1 min for the tissue to sit down and discard the first supernatant that contains debris and blood cells.
8. Add 25 ml digestion buffer and digest tissue under constant stirring at 37 °C for 10 min.
9. Leave the mixture 1 min for the tissue to sit down and collect the supernatant with a 25 ml pipette (around 22 ml). Do not collect the tissue pieces that tend to float.
10. Place the supernatants in plastic 50 ml falcon tubes on ice containing 2 ml fibroblast medium. If possible supernatants can be combined in one falcon but for each supernatant 2 ml of fibroblast medium is required.

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11. Repeat steps 6-10 until all the tissue is dissolved (typically 7-10 times).
12. Spin the cells down for 5 min at 300 *x g* at 4 °C.
13. Resuspend cells in 20 ml fibroblast medium.
14. Plate the cell suspension into two 100 mm cell culture dishes (10 ml cell suspension/dish) and incubate them at 37 °C in a cell culture incubator with 5% CO_2 for 2 h (“Pre-plating”).
15. 2 h upon plating alive and healthy fibroblasts should have adhered to the dish. Check the cell confluence under the microscope (it should be around 50%). At this point fibroblasts resemble little round dots.
16. Collect and discard supernatant. Wash 3x with 2.5 ml warm PBS and replenish with 10 ml fresh fibroblast medium. Cultivate at 37 °C and 5% CO_2 in a cell culture incubator.
17. Primary adult mouse fibroblasts divide less than embryonic ones and under our culture conditions have a doubling time of 36-48 h. Typically, 2-3 days upon plating the fibroblasts reach 90% confluence.
    Note: Caution! Do not split the fibroblasts until they reached a minimum confluence of 80% because the fibroblasts growth rate will decrease and they will differentiate.
18. Once confluence is reached fibroblasts are trypsinized as follows.
19. Wash two times with 2 ml pre-warmed PBS and then add 2 ml warm trypsin. Incubate for 5 min at 37 °C, in a cell culture incubator. Control if fibroblasts detached from plate. If not prolong incubation until the cells detach.
20. Neutralize trypsin by adding 7 ml pre-warmed fibroblast medium.
21. To achieve a re-plating confluence of 30%, split 1:3 into 3 new 100 mm plates. Add 3 ml of fibroblast suspension in a plate containing 7 ml fresh and warm fibroblast medium (final volume/plate 10 ml)

Representative data

For representative photos and results please refer to Zafiriou *et al.* (2014).

Notes

Note that primary isolated cells as fibroblasts are differentiated when cultured for a long time period (more than 20 days) cease to divide (Bayreuther *et al.,*1988). Moreover, using cells from the same passage leads to better reproducibility. Therefore, we recommend using passage 4 fibroblasts for all co-culture experiments.

Recipes

1. PBS, 1 L
   Dissolve the following in 800 ml distilled H_2O (dH_2O)

8 g of NaCl
0.2 g of KCl
1.44 g of Na_2HPO_4
0.24 g of KH_2PO_4
Adjust pH to 7.4 with HCl, add dH_2O to 1 L, sterilized by autoclaving

2. Hanks balanced salt solution (HBSS) (100 ml)
   a. Stock solution 1: 8.0 g NaCl, 0.4 g KCl in 100 ml dH_2O
   b. Stock solution 2: 0.358 g Na_2HPO_4, 0.6 g KH_2PO_4 in 100 ml dH_2O
   c. Stock solution 3: 0.72 g $CaCl_2$ in 50 ml dH_2O
   d. Stock solution 4: 1.23 g $MgSO_4 \cdot 7H_2O$ in 50 ml dH_2O
   e. Stock solution 5: 0.35 g $NaHCO_3$ in 10 ml dH_2O
   f. HBSS buffer: 10 ml Solution 1, 1 ml Solution 2, 1 ml Solution 3, 86 ml dH_2O, 1 ml Solution 4, 1 ml Solution 5 (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 4.2 mM $NaHCO_3$)
3. Collagenase II stock (1 ml)
   Weight the appropriate amount of collagenase (varies from lot to lot) to obtain 10,000 U and dissolve in 1 ml sterile water
4. Digestion buffer (100 ml)
   95 ml HBSS buffer
   1 ml collagenase II (final conc. 100 U/ml)
   4 ml 2.5% Trypsin (final conc. 0.1%)
   Filter sterilize
   For optimal enzymatic activity always prepare fresh.
5. Fibroblast medium
   DMEM/F12
   10% FBS
   100 U/ml PenStrep
   1x L-glutamine
   100 µM ascorbic acid
   Filter sterilize the whole medium and keep at 4 °C

Acknowledgments

This protocol was adapted by methods described by Lafontant *et al.* (2006) and Ohkubo *et al.* (1997). The HBSS recipe was retrieved from the internet site labrat.com (link: http://www.thelabrat.com/protocols/Hanks.shtml).

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