SOP Co-Culture Hepatocytes with Macrophages (BMDM)

Step 1

Isolation of bone-marrow cells and differentiation to BMDM

1. Dissect the upper and lower leg bones of the hind legs free of tissue
2. Put the bones in PBS (w/o Ca/Mg, Biochrom) before incubation in 70 % [v/v] ethanol for 3–5 minutes.
3. After short washing with PBS and transfer the bones into rinse medium (DMEM 1 g/ml Glucose; Biochrom, Berlin, Germany, supplemented with 1% [v/v] Penicillin/Streptomycin; PAN Biotech, Aidenbach, Germany), cut both ends of the bones and flush out the bone marrow by irrigation with rinse medium by a syringe (needle: 23G x 1“).
4. Resuspension the bone marrow by pipetting cells vigorously to get a single cell solution.
5. Centrifuge (300 x g, 4°C, 10 minutes) the cell suspension, discard supernatant.
6. Resuspended the cells in 20 ml culture medium (DMEM 1 g/ml Glucose, 1% [v/v] Penicillin/Streptomycin, 10% [v/v] FCS).
7. Culture the cell in a 175 cm^2 cell culture flask overnight.
8. Day1: Transfer non-adherent cells in a 50 ml Falcon and harvested by centrifugation (300 x g, 4°C, 10 minutes).
9. Resuspend cells in culture medium, supplemented with 10 ng/ml murine M-CSF (mM-CSF) (Peprotech) and cultivated in five cell culture dishes with 15 cm diameter and 20 ml medium (including mM-CSF).
10. Day 3: **add** 20 ml of medium (do not remove the old medium!) supplemented with mM-CSF of medium (do not remove the old medium!) supplemented with mM-CSF
11. Day 7: wash cells twice with PBS, add 3 ml Trypsin/EDTA (PAN Biotech) for approximately 10 minutes.
12. Stop Trypsin/EDTA by adding 20 ml medium, rinse cells from the surface, if necessary detach them from the surface with a spatula, transfer into a 50 ml falcon.
13. Centrifuge the cells (300 x g, 4°C, 10 minutes), discard supernatant, resuspend cells in medium with mM-CSF.
14. Determination of the vital cell count.

15. Subcultivate BMDM´s in transwells [BD Falcon #353090] (0,1 mio per transwell in 2 ml Medium with mM-CSF and 2 ml Medium below the transwell).
16. Incubate cells for 24 h for attachment (37°C, 5% CO2, humidified atmosphere).

Step 2

Isolation of primary mouse hepatocytes

HANKS buffer I:
137 mM NaCl, 5 mM KCl, 15 mM Hepes, 0.34 mM $Na_2HPO_4x2H_2O$, 0.44 nM KH_2PO_4, 2 mM EGTA and 0.1 % [w/v] Glucose, pH 7.4

HANKS buffer II:
37 mM NaCl, 5 mM KCl, 15 mM Hepes, 0.34 mM $Na_2HPO_4x2H_2O$, 0.44 nM KH_2PO_4, 0.1 % [w/v] Glucose, 5 mM $CaCl_2$ and 240 U/ml collagenase, pH 7.4

1. Perfuse the liver with HANKS buffer I through the portal vein by using a peristaltic pump at a flow rate of 8 ml/min for 2 min.
2. To reduce the pressure incise the vena cava and the right heart ventricle.
3. Perfuse the liver with HANKS buffer II until the liver tissue swells up (~ 5 min).
4. Remove the liver from the animal and transferred into a tube with William´s medium E (Biochrom) supplemented with 10 % fetal calf serum (FCS, Biochrom), 2 mM glutamine and 1 % penicillin/streptomycin (Gibco).
5. In a sterile hood open the capsule of the liver carefully by a tweezers and flush out liver cells by gentle shaking.
6. Filter cell suspension through a70 µm cell strainer to remove tissue residues.
7. Wash cells with William´s medium by centrifugation (50 x g, 3 minutes, acceleration/deceleration 2, 20°C), discard supernatant carefully.

Using MACS separation columns to remove liver macrophages from the primary hepatocytes

1. Wash the cells twice with 20 ml MACS-buffer [autoMACS rinsing solution, Miltenyi Biotec #130-091-222 + 0,5 % BSA]

2. Centrifuge the cells (50 g, 3 min, acceleration/deceleration 2, RT), discard supernatant carefully

3. Resuspend cells in remaining volume and add 100 µl CD11b Beats [Miltenyi Biotec, #130-049-601]. Incubate the cells 15 min at 4°C (in the fridge, not on ice!)

4. Place the MACS Separation Columns [Miltenyi Biotec, #130-042-202] in the OctoMACS Separation Unit [Miltenyi Biotec, #130-042-109] and attach the flow resistor (23G needle). Equilibrate the Columns with 1 ml MACS-buffer.

5. Resuspend the cells in 10 – 20 ml MACS-buffer. Pipette the cell suspension gradually onto the columns (max. 1 ml per column per step) and let it run through. Collect the effluent.

6. Wash the columns by adding 1 ml MACS-buffer per column.

7. Centrifuge the flow-through (50 rcf, 3 min, acceleration/deceleration 2, RT) and resuspend the cells in 10 ml Attachment-Medium (William's E [Biochrom #F1115] supplemented with 10 % FCS, 1 % Penicillin/Streptomycin, 2 mM L-Glutamine and 100 nM Dexamethasone.)

8. Determine the vital cell number.

Step 3

Hepatocyte cultivation in Collagen-Sandwich

Adapted from SOP: "Preparation of collagen sandwich cultures of primary hepatocytes" by Jan Hengstler and Patricio Godoy

1. Dissolve 10 mg rat-tail tendon collagenI lyophilisate (# 11179179001, Roche Mannheim) in 9 ml sterile-filtered (0.2 µM) 0.2 % (v/v) acetic acid (complete solution will take ≥ 3 h, better over night in fridge).

2. Add 1 ml 10x concentrated DMEM medium (Sigma, # D2429) and adjust pH 7,4 with 1M NaOH ⇒ 1 mg/ml collagenI solution. (Prepare neutralized collagenI solution always fresh (immediately before use), i. e. for the bottom gel layer and the top gel layer each and keep ice-cooled)

3. Dispense neutralized collagenI solution in cell culture vessels using a cell scraper [BD Falcon #REF353085].

Cell culture vessel	Neutralized collagenI solution (for bottom and top gel layer each)
6 well companion-plate [BD Falcon, #353502]	350 µl per well

(Do not process more than 1 - 2 plates at a time to prevent premature gelation before having dispensed collagen completely in all wells)

4. Put collagen-coated cell culture vessels in incubator (37°C, 5% CO2, humidified atmosphere) for ≥1 h to allow gelation.

5. Plate cells onto collagen gel

Cell culture vessel	Number of cells per well / dish	Media volume per well / dish
6 well plate	0.8×10^6	2 ml per well

6. Incubate cells for ~3 h for attachment (37°C, 5% CO2, humidified atmosphere).

7. Remove medium and wash cells twice with cold PBS to remove dead and unattached cells (shake well but not vigorously), aspirate PBS completely.

8. Overlay cells with 350 µl neutralized collagenI solution. Dispense cells well by softly shaking the plate in the shape of the figure 8.

9. Put collagen-coated cell culture vessels in incubator (37°C, 5% CO2, humidified atmosphere) for ~1 h to allow gelation and the formation of the sandwich.

10. Dispense 2 ml Pre-Starvation-Medium (William's E [Biochrom #F1115] supplemented with 1 % Penicillin/Streptomycin, 2 mM L-Glutamine and 100 nM Dexamethasone) onto the sandwich cultures (be careful not to disrupt the collagen sandwich).

11. Incubate sandwich cultures over night (37°C, 5% CO2, humidified atmosphere).

Step 4

Co-Cultivation

1. Remove medium from sandwich culture and add 2 ml Starvation-Medium (William's E [Biochrom #F1115] supplemented with 1 % Penicillin/Streptomycin and 2 mM L-Glutamine). Incubate for 3 hours (37°C, 5% CO2, humidified atmosphere) before starting the experiment.

2. Remove medium from the BMDM´s, transfer the transwells to the hepatocytes and add 2 ml Starvation-Medium per transwell.

Step 5

RNA-Isolation

Hepatocyte RNA-Isolation from Sandwich-Culture with RNeasy Mini Kit

1. Remove Medium and wash the cells with PBS
2. Transfer the scraped cells within the collagen-matrix into a 1.5 ml reaction tube
3. Spin the collagen-cell-mix at 5400 g and 4°C for 2 min and remove the supernatant – repeat this step three times
4. Add 600 µl RLT-buffer with 6 µl β-ME, vortex
5. Continue by following the RNeasy Mini Kit (Qiagen, #74106) manual