Murine Bone Marrow-Derived Macrophage (BMM) culture

Last updated: 9/2/2022

L929 media **(keep a bottle for ≤3 months in the fridge):**

- DMEM high glucose, sodium pyruvate
- 10% FBS
- L-Glut
- Pen/strep
- 10 mM HEPES

BMM base media **(keep a bottle for ≤3 months in the fridge):**

- DMEM high glucose, sodium pyruvate
- 10% FBS
- L-Glut
- Pen/strep
- β-mercaptoethanol

BMM complete media **(make fresh):**

- BMM base media
- 20% L929-conditioned media

L929 conditioned media generation

Materials needed:

- L929 cells (frozen in LN2)
- T75 flasks
- L929 media

1. Pre-warm a 15 mL tube containing 10 mL L929 media at 37 °C
2. Pull vial of frozen L929 cells from LN2 and gently swirl in 37 °C water bath to thaw, then transfer to tube containing pre-warmed media
3. Pellet cells (250 x g, 21 C, 8 minutes), decant, and resuspend in 10 mL L929 media
4. Count cells on hemocytometer or Moxi
5. Seed T75s with 4.7e5 L929 cells in 55 mL L929 media
    a. Try to do as many flasks as possible, freezer space permitting (each flask will produce 50 mL conditioned media, 5 x 15 mL tubes containing 10 mL) – the less frequently you make conditioned media, the better!
6. Culture cells for 7 days in 37 °C, 5% CO_2 incubator
7. Collect supernatant from flasks and filter through 0.2 µm PES vacuum filter
8. Store 10 mL aliquots of conditioned media at -20 °C

Bone collection

Materials needed:

- 50 mL tube containing ~40 mL 70% EtOH
- 50 mL tube containing ~40 mL sterile PBS
- 96 x 22 mm dishes, x 2, labeled with “EtOH” and “PBS”
    - Tape together to prevent lids from coming off during transport
- Sterile surgical instruments in autoclave pouches (store pouches in plastic bag to keep from getting wet in ice bucket)

  - Large and small scissors
  - Forceps
- 15 mL tube containing 10 mL BMDC base media per mouse
- 4-5 Kimwipes per mouse (store in plastic bag to keep from getting wet in ice bucket)
- Tube rack
- Ice bucket

Do all steps in a tissue culture hood to keep tissues as clean as possible

1. Euthanize mouse by CO_2 inhalation and cervical dislocation
2. Douse skin with 70% ethanol, then use the large scissors to make a transverse incision all the way around the midsection of the mouse (separating the skin into "pants" and "shirt")
3. Use the large scissors to clip off the feet below the ankle, then expose the legs by pulling the lower skin down and off the mouse
4. Using the small scissors, carefully trim away as much of the muscle and fascia on the legs as possible
    a. In particular, try to expose as much of the femoral head as possible—the goal here is to be able to visualize where the hip socket is, in order to avoid cutting the femoral head in the next step
5. Using the large scissors held in a vertical position (i.e., blades pointed straight down), cut through the hip about 1 cm superior (towards the mouse's head) of the hip socket
    a. You will feel a good amount of resistance for this cut
6. Pull the leg up and away from the mouse, and use the scissors to cut through the remaining fascia/bone and separate the leg from the body
    a. You should feel less resistance for these cuts
7. Place the excised bone in the lid of one of the petri dishes, then process the other leg
8. Clean both legs of remaining muscle/fascia by "polishing" them between your gloved fingers using Kimwipes. You should be able to see the remaining tissue coming off onto the Kimwipe. Switch to a clean one whenever they get too dirty.
9. Immerse cleaned bones in petri dish containing 70% EtOH; gently agitate for 30 sec with sterile forceps
10. Immediately transfer bones to second petri dish containing sterile PBS; gently agitate for 30 sec, then transfer to 15 mL tube containing media

Marrow extraction

Materials needed:

- 96 x 22 dish
- Sterile surgical instruments
  - Large scissors
  - Forceps
- 5 mL syringe
- 1 mL syringe (rubber plunger)
- 18 G and 27 G needles
- 50 mL tube containing BMDC base media
- 70 µm cell strainer
- Labeled 50 mL collection tube
- ACK lysis buffer

- **Do all steps in a tissue culture hood to keep cells sterile**

- **Keep cells on ice until plating**

1. **Invert the 15 mL bone tube several times to dislodge the bones, then quickly dump the contents into a 96 x 22 mm dish**
2. **Using the scissors and forceps, carefully separate the femur and tibia, then clip off the ends of each bone**
    a. **The marrow cavity of the tibia does not extend all the way down to the distal head (where it meets the ankle). Look for where the read streak of the marrow ends, and clip just proximal (towards the knee) of this point.**
    b. **Ideally for each end of the bone where you have cut, you should be able to visualize a small red dot (representing an opening to the marrow cavity)**

3. Using the 18 G needle, fill the 5 mL syringe with base media, then switch to the 27 G needle
4. Hold each bone with forceps over the labeled 50 mL catch tube with a 70 µm strainer, and carefully insert the needle into the marrow cavity
    a. This step can be tricky, but you'll know you've hit the marrow cavity when you can move the needle without resistance up and down, parallel to the bone

5. Expel media while gently moving the needle up and down until the bone is completely blanched; Repeat until marrow has been flushed from all bones
6. Use the rubber plunger of a 1 mL syringe to dissociate the marrow clumps on the filter, then rinse the filter 2-3 times with ~5 mL of the collected cell suspension
7. Pellet cells (350 x *g*, 8 minutes, 4 °C), decant into waste, rake to resuspend, and add 2 mL ACK lysis buffer
8. Vortex and incubate for 1 minute at room temp, then dilute to 12 mL with 10 mL base media
9. Pellet cells (350 x *g*, 8 minutes, 4 °C), rake to resuspend, decant into waste, and add 4.85 mL base media (5 mL with residual volume)
10. Pass cells through a 70 µm cell strainer into a new 50 mL tube
11. Dilute an aliquot of cells 1:20 in PBS and count on the Moxi cell counter (4.6 µm left gate, max right gate)

Expected yield: 15-45e6 cells per mouse

Cell plating and culture

Cells can be plated and grown in a variety of different formats, but in general:

- Always use NON-TC-TREATED plastic, otherwise cells will adhere very firmly and be difficult to remove
- Growing in a large format and then harvesting/replating for assays significantly reduces noise associated with well-to-well variability

Seed cells at 4e4 cells/cm^2 in BMM <u>complete</u> media (BMM base + 20% L929 conditioned media)

Format	Well area (cm^2)	Seeding cells	Seeding cells/mL (final)	mL media
96 well plate	0.37	1.48E+04	1.18E+05	0.125
24 well plate	1.91	7.64E+04	7.64E+04	1
6 well plate	9.5	3.80E+05	1.90E+05	2
150 x 25 mm dish	147.41	5.90E+06	1.97E+05	30

Example: 150 x 25 mm dish

- 30e6 ACK-lysed bone marrow cells in a total volume of 5 mL (6e6 cells/mL)
- For 150 x 25 mm dish and a final well volume of 30 mL, we want to plate 5.9e6 cells in 30 mL complete media
- Add 1.02 mL 6e6 cells/mL bone marrow cells, 6 mL L929 conditioned media, and 23.98 mL base media into a labeled 150 x 25 mm dish, gently rock to mix, and place in incubator

Feeding schedule:

Day 0 – Plate cells
Day 3 – Add 2/3rd well volume of complete media to each well (e.g., 20 mL to 150 x 25 mm dish)
Day 7 – Harvest cells

BMM harvest and replating

Materials needed:

- PBS
- PBS + 2.5 mM EDTA

1. When ready to harvest cells, aspirate the supernatant, then rinse dish/wells with 1 well volume PBS, aspirate, and repeat once
2. Dispense 1 well volume of PBS + 2.5 mM EDTA into dish/wells and incubate in the fridge for 30 min
    a. EDTA disrupts the action of integrins which hold the remaining adherent cells to the plastic
    b. At the end of this incubation, the cells should be visibly rounded under the microscope

3. Vigorously pipette cells to detach from the dish/well bottom
    a. For large culture formats like the 150 x 25 mm dishes-- tilt the plate towards you and use the pipette gun to eject the solution near the top of the dish so that it flows over the whole plate and collects at the bottom. Rotate the dish every few cycles to change where the ejected stream of solution is hitting the plate. The cell layer should be visible to the naked eye (especially from certain angles with the hood light off), so you can visually track cell removal

4. Count on the Moxi cell counter (6.6 µm left gate, max right gate)

Expected yield – ~4e4 cells/cm²

Format	Well area (cm²)	Expected recovered cells
96 well plate	0.37	1.48E+04
24 well plate	1.91	7.64E+04
6 well plate	9.5	3.80E+05
150 x 25 mm dish	147.41	5.90E+06

5. Pellet cells (350 x *g*, 8 minutes, 4 °C) and resuspend in BMM complete media to desired cell concentration
    a. “Desired cell concentration” will strongly depend on the downstream assay

6. Replate cells in desired format for downstream assays
    a. In most cases, it is wise to plate cells in a volume that allows for treatments to be added on top of the existing solution in the wells

A "general purpose" D7 seeding density (good for ELISA, flow) is **1.56e5 cells/cm^2**

Format	Well area (cm^2)	Cell count	Well volume (mL)	Cells/mL
96 well plate	0.37	5.00E+04	0.12	4.17E+05
24 well plate	1.91	3.00E+05	0.75	4.00E+05
6 well plate	9.5	1.50E+06	2	7.50E+05

- Allow cells to rest and re-adhere for at least 8 hours before treating or manipulating them
- In general, the plating volumes above allow for 1 well volume of treatments to be added on top of the existing solution
- If collecting cells at the end of the experiment is necessary (e.g., for flow), follow the harvest and replating procedure again