ISOLATING BONE MARROW-DERIVED MØ'S

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BACKGROUND

- The bone marrow of the leg bones of mice is a great source of monocyte/macrophage (Mø) precursors which can be easily cultured.
- This protocol explains how to isolate macrophage progenitors and culture them to the point where they can be used for *in vitro* assays.

NOTES

- It is very important that the bones remain completely intact from the point when they are harvested from the mice [see protocol entitled 'Harvesting Femurs and Tibias From Mice'] to when the bone marrow is to be flushed from the bone.
- All steps in this protocol should be performed in a BioSafetly Level 2 (BSL2) laminar flow hood to maintain sterility. Gloves and a lab coat should be worn at all times.
- This protocol is for bone marrow flushed from 4 femurs and 4 tibia (ie. 2 mice). Adjust amount accordingly.

EQUIPMENT

- Mouse femurs and tibia in R10 media [Fresh or kept in 4˚C fridge for less than two days]
    - See protocol entitled 'Harvesting of Femurs and Tibia from Mice'
- Tweezers and scissors in 70% EtOH
- 4 Petri dishes (10cm)
- 19- and 26-gauge needles
- 20mL syringe filled with sterile PBS [sufficient for 4 femurs and 4 tibia]
- Empty 20mL syringe
- 50mL Falcon tube
- Centrifuge for 50mL tubes cooled to 4˚C
- 2 large bacterial culture dishes [NOT tissue culture plastic]
    - Mø's will adhere to TC plastic in such a way that they cannot be removed without liberal use of Trypsin
- Reagents
    - R10 medium containing 15% L929-cell conditioned medium (LCM) [recipe below]
    - Sterile PBS
    - EtOH

PROTOCOL

1. Pour out the medium containing the bones into the first small petri dish. Transfer the bones with sterile tweezers to the second petri dish containing 70% EtOH for one minute and then to the third petri dish containing sterile PBS.
2. Remove a bone from the PBS with tweezers and cut the ends as follows:
    - For the **femur**, holding the bone with the tweezers, rest the hip side on the tissue. Place the scissors just above the joint and hold the bone with the tweezers just above the scissors to prevent the bone from shattering. Cut off the hip joint. Turn the bone over and cut off the knee joint, similarly keeping the scissors and tweezers very close together.
    - For the **tibia**, holding the bone with the tweezers, rest the knee side on the tissue. Place the scissors just above the joint and hold the bone with the tweezers just above the scissors to prevent the bone from shattering. Cut off the joint. Turn the bone over and identify where the red bone marrow ends in the bone, about halfway down the tibia. Cut bone just below this point, again keeping scissors and tweezers close together.
    - Note: While cutting each bone, and until the bone marrow is flushed (see next step), DO NOT let the cut ends of the bones touch anything as this will compromise the sterility of the cells within. The marrow is very delicate.
3. Flush out the bone marrow into the 50mL Falcon tube by inserting a 26-gauge needle attached to the 20mL syringe filled with PBS at the knee side of both types of bone. Pass the PBS through the bone until the colour of the bone turns from red to white, indicating that all the marrow has been expelled. Discard the bone into an empty petri dish.
    - Perform this step for each bone IMMEDIATELY following the cutting of the bone. DO NOT put a bone down between cutting and flushing.
    - You should be able to see red bone marrow flecks in the 50mL tube.
4. When all bones have been washed of marrow, add any remaining PBS from the syringe into the Falcon tube.
5. Disperse marrow by sucking it into an empty 20mL syringe through an 19-gauge needle TWICE.
    - Doing this more than twice will shear cells.
6. Centrifuge cells at 1500rpm for 5 minutes at 4˚C.
    - * The directions from here on are for cells that will be used ~7 days later. If you wish to freeze down the cells, resuspend in 3mL of FCS and aliquot 1mL each into 2mL cryotubes and add 100µL DMSO dropwise. Freeze at -80˚C then transfer to liquid nitrogen for longterm storage.
7. Discard supernatant into container with bleach or virkon and resuspend cells in 15mL R10 medium containing 15% LCM
    - 15mL volume is enough to seed 3 large plates or 3 T175 flasks (5mL/plate)
8. Each plate or flask should be prepared with 20mL R10 media + 15% LCM. Add 5mL of cell suspension and swirl to distribute cells in medium.
9. Incubate at 37˚C and 5% CO_2. Do not seal plate with parafilm as this will not allow Mø's to 'breahe' appropriate amounts of CO_2

10. Culture the cells for a week, allowing the Mø progenitors to attach to the plastic and divide until there is a confluent monolayer of Mø's. The media is specific for Mø's and all other cells present will die within two days of culture.
11. Feed Mø's on day 3 by adding 15mL R10 medium + 15% LCM.
12. Replace all media on day 6 with 25mL fresh R10 + 15% LCM. If there are non-adherent cells then decant used medium into a 50mL Falcon tube, centrifuge, resuspend in 5mL R10 + LCM and place them in a new plate or flask containing 20mL R10 + LCM.
13. When ready to use, review 'Harvesting Bone Marrow-Derived Macrophages' for protocol

DISPOSAL

- Once the bones have been flushed they, and any other animal tissue, should be disposed of according to your institution's animal facility's suggested disposal protocol for animal tissues.
- Excess media and reagents should be diluted in 10% bleach for 30 minutes and then flushed down the sink with plenty of running water.
- Tools should be cleaned immediately after use with 70% EtOH and rinsed with water.
- Excess tubes should be disposed of via Biosafety Level 2 protocols for your facility.

RECIPES FOR MEDIA

R10 medium

RPMI-1640	500 ml
Foetal Calf Serum	55 ml
1 M Hepes	5 ml
Penicillin/Streptomycin	1 vial (5 ml), defrosted at 37°C, containing 10 000 U Penicillin/ml; 10 mg/ml Streptomycin; 200 mM L-Glutamine

Using 5 ml syringes, add the Hepes and Penicillin/Streptomycin to the RPMI through a 0.2μm filter.
Using a 50 ml syringe, add the Foetal Calf Serum through a syringe filter with 0.2μm membrane.
Store at 4°C.

L-cell conditioned medium (LCM)

Thaw a vial of L929 fibroblasts at 37°C.
With a 5 ml pipette, gently decant into a 50 ml falcon and add 10 ml DMEM-10.
Centrifuge at 900 rpm, 5 min RT.
Resuspend pellet carefully in 5 ml DMEM-10.
Pipette into at T25 tissue culture flask, incubate at 37°C, 5% CO_2 overnight.
Lift cells with 5 ml 1x trypsin/EDTA/PBS, centrifuge as before, resuspend in 15 ml DMEM-10 and transfer to a T75 tissue culture flask. Gas the culture and incubate in warm room overnight.
Lift cells with 10 ml 1x trypsin/EDTA/PBS, centrifuge as before, resuspend in 30 ml DMEM-10 and transfer to a T175 tissue culture flask.

Grow them to about 80-90% confluency and split them 1:5 each time into a new T175.
When there are enough cultures going to make about 40-50 flasks, split them 1:5 into new T175 containing 50 rather than 30ml DMEM-10 and then leave them for 10 days.
Harvest the SPN, spin hard at about 3000 rpm and then filter the SPN through a 0.45 µm filter before freezing in 50ml aliquots.
When adding to R10, filter through 0.22µm filter