Osteoclast Differentiation (*in vitro*)

Day 1:
Sacrifice & dissect:
1. Sacrifice mouse
2. Dissect out both femurs and tibias
3. Dip bone briefly in 70% EtOH then place in sterile PBS in 6 well plate

Isolate cells:
1. Prefill 10 mL syringe with 25G needle with cold sterile PBS.
2. In sterile 6-well plate, cut off both ends of bone to be flushed. For tibia cut off right where you can see the red marrow.
3. Hold bone with forceps and insert 25G needle into bone end, crunch up and down while flushing PBS through bone into an empty well of 6-well plate. Flip bone over and repeat on other end. Repeat procedure until bone is white. If cut off ends look like they have marrow, flush them also.
4. Pipet PBS + marrow repeatedly and transfer to a 50 mL conical.
5. Spin cells at 1000rpm for 5 minute
6. Add 5 mL RBC lysis buffer, incubate RT for ~5 minutes, then spin at 1000rpm for 5 minutes.
9. Resuspend cells in α-MEM media complete + MCSF (10ng/ml) or 5% CMG; Plate cells at 2 legs/ 6well plates with 6-8 mL media per well

Day 2: Harvest Non-Adherent cells (bone marrow macrophages):
1. Stromal cells and some macrophages will adhere to the plastic within 4 hours. You can collect non-adherent cells for differentiation into macrophages or OCL anytime between 4hrs to day 3. The longer you wait, the lower your yield of non-adherent cells in my experience. I usually plate bone marrow in the evening and collect non-adherent cells after overnight culture first thing in the morning (12-15 hrs).
2. Collect non-adherent cells and count
3. Spin cells and resuspend in α-MEM media + MCSF (10 ng/ml or 5% CMG).
    a. Plate **125K cells**/well of 48 well plates
    b. Volume of media should be 200ul/96well; 0.5ml/48 well; 2ml/24 well; 4-5ml/6well
    c. pH of media should be 7.2-7.4 for good osteoclast development
    d. Plate in multiples so that 2-3 wells are for TRAP staining alone, 1-3 wells for dentine, 1-2 wells for BD biocoat, 3 wells for RNA.

Day 5: Change to fresh media
1. Change media to fresh α-MEM + MCSF/CMG.

Day 8: Change to fresh media
1. Change media to fresh α-MEM + MCSF/CMG.
2. Note: you may change to add RANKL depending on the density of the expanding macrophages; if dense enough you can skip to day 11.

Day 11: Change to fresh media

1. Change media to fresh α-MEM + MCSF/CMG + 50-100 ng/mL RANKL.
2. Begin to monitor daily for formation of osteoclasts; once large osteoclasts have formed, they will apoptose in 24-48 hours, so fix once large osteoclasts are observed

Day 14: Change to fresh media

1. Change media to fresh α-MEM + MCSF/CMG + 50-100 ng/mL RANKL.
2. Continue monitoring for osteoclasts.

Fixation for TRAP staining:

1. Remove media and rinse 1x in PBS
2. Fix in 3.7% formaldehyde in PBS for 10 minutes at RT.
3. Rinse 2x in PBS. Flick to empty plates, **do not** aspirate as this will suck up osteoclasts
4. Plates can be stored in PBS/1% sodium azide before staining (if so, remember to rinse with PBS 2x before starting staining as sodium azide inhibits TRAP staining)

TRAP staining in plates:

1. Make up TRAP staining solution as per instructions
2. Prewarm plates with PBS at 37˚C
3. Add substrate to fixed cells and incubate 15 min – 1 hr (usually an hour).
4. Rinse with water or PBS several times; can be stored in PBS/1% sodium azide at 4˚C.
5.

However, If good osteoclasts are visible, then consider stopping assays.

a) TRAP staining – see below
b) resorption assays – either change the media to acidified alpha MEM complete +RANKL and MCSF or allow assays to continue without changing media which will acidify naturally. Acidify alpha MEM complete by: aliquot out 50cc to conical tube. Add 1 drop 12N HCL. Check that pH is 6.8-7.0 with pH paper. Sterile filter.