Replication of *Salmonella typhimurium* in Mouse Macrophages

I. Setting up infection:

1. Harvest and count macrophages to be seeded. Seed 2 x 10^5 cells per well in a 24-well dish, 1 x 10^6 cells in a 6-well dish. Seed enough wells to infect 3 wells per strain per timepoint.

   Note: If activating macrophages, seed cells early in the morning and activate 3 hrs. later (once the cells have adhered to the plate); or seed the cells late in the afternoon and activate early the next morning. Allow cells to be activated for 18-24 hr. before infection
   Activated RAW 264.7 cells will stop dividing and become granular/vacuolated
   I activate the cells with 100 U IFN-γ + 50 ng LPS. A Griess assay for NO production can be performed to monitor the activation state.

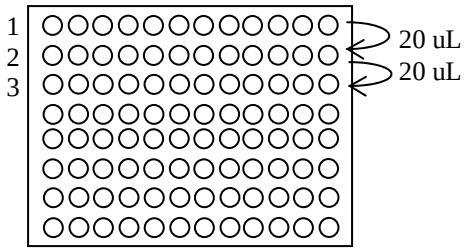
2. The same day inoculate overnight cultures of *S. typhimurium* strains to be used in infection into LB + appropriate antibiotics, grow on the wheel at 37 °C O/N (at least 12 hrs, preferably 16)

   Note: growing the bacteria in this way minimizes bacterial cytotoxicity to the macrophages. Growing the bacteria standing O/N in high-salt LB increases bacterial invasiveness, but also bacterial cytotoxicity.

3. The next morning dilute bacterial cultures 1:10 in PBS and measure the OD_{600}
4. Opsonize bacteria in 50% normal mouse serum for 20-20 minutes at 37 °C
5. Wash bacteria 1X in excess PBS, and resuspend in PBS using equivalent volume to volume in step #4.
6. From OD_{600}, calculate CFU/mL concentration using OD_{600} 1 = 1.2x10^9 CFU/mL
7. Dilute bacteria in DMEM to appropriate concentration for infection
8. Wash macrophages 1X with PBS
9. Add 100 µL DMEM.
10. Infect cells with 100 µL bacteria in DMEM
11. Spin cells 500 RPM in clinical table-top centrifuge
12. Place infected cells in incubator for 30-45 minutes
13. Aspirate medium, wash cells 1X with PBS, add back 1 mL DMEM containing 100 µg/mL gentamicin. Place cells back in incubator for 90-120'
14. No more than 2 hrs after adding DMEM+gent, aspirate medium, wash 1X with PBS, add back 1 mL DMEM containing 10 µg/mL gentamicin. At this time, bacterial CFU may be harvested for a 2 Hr timepoint. I harvest bacterial CFU 2 hr, 8 hr, and 24 hr after infection (see below)

II. Harvesting bacteria for CFU counts:

1. Aspirate medium from infected wells that you are planning to harvest. Add 200 µL 1% Triton X-100 in PBS. Pipette up-and-down 8-15 times. Don't worry too much about the frothing but try not to make it too frothy. Add 800 µL LB. Pipette up-and-down several times, harvest into 1.5 mL Eppendorf tubes.
2. Prepare dilutions: I use 96-well plates to do the dilutions, because it saves a lot of time and pain. Some people prefer to use 1 mL tubes or 5 mL glass tubes because it reduces the variation within the triplicate wells; however, I feel that the 96-well plates are consistent enough to make the cost-benefit analysis worth it. Using a multi-channel pipette, add 180 µL PBS to each well. Make sure the pipette tips are all sitting evenly on the pipetter, since this is often the source of systematic error along the
wells.



3. Pipette 20 µL from Eppendorf tubes containing bacteria and lysed cells into appropriate wells of row 1 in 96-well dish
4. Using a multichannel pipette, pipette 20 µL from row 1 into row 2 and then 20 µL from row 2 into row 3.
5. Depending on your MOI and the activation state of the macrophages, plating 100 µL from row 3 or 100 µL from row 2 will give countable CFU. I call row 3 a $1:10^4$ dilution and row 2 a $1:10^3$ dilution, although technically they are 1:2000 and 1:200 respectively since I plate 100 µL.

> ***Note: with RAW 264.7 cells and MOI of 10, if the cells are activated, plating a $1:10^4$ dilution gives countable counts at all timepoints. If the cells are not activated, the $1:10^3$ dilution gives countable counts at 2 and 8 hr, but at 24 hr you need to plate the $1:10^4$ dilution.***