Mt extraction protocol:

**Prepare –**

* Put pestles and tubes on ice
* Place 1 aliquot 5X Mito extraction buffer on ice to melt
  + Once melted, add 8 ml nanopure H2O
* Place 1 aliquot of BSA on ice to melt
* Place aliquots of JC-1 and CCCP at room temp ***(protected from light)***
* Place one aliquot of 5X mito storage buffer on ice, add 400 ul nanopure H­2O to tube once melted
* Place 1 aliquots of JC-1 assay buffer at room temp, when melted, add 14.4 ml H2O
* Label flow cytometry tubes for each assay; Do three tubes for bacterial control (UNstained, + dye, +dye+CCCP). Remove caps and ready tubes in rack. **This assay is set up at room temp and run at 37˚C.**

**Wash –**

1. Collect snail tissue into pre-chilled 1.5ml tube
2. Add 200 ul Mito Extraction Buffer A to each tube
3. Spin tubes at 3000 rpm for 30 seconds
4. Aspirate liquid gently with filtered pipet or vacuum

**Grind –**

1. Add 340 ul BSA to remaining Mito Extraction Buffer
2. Add 500 ul Mito Extraction Buffer A + BSA to each tube
3. Homogenize with pre-chilled pestle
4. Centrifuge at 600 x g @ 4ºC for 5 minutes
5. Collect supernatant into waiting 1.5ml tubes on ice
6. Repeat homogenization steps 6 – 9

**Enrich –**

1. Spin supernatant tube at 10,000 – 11,000 rpm @ 4ºC for 10 minutes
2. Set up JC-1 assay **(see below)**
3. Pipet/pour away supernatant very *carefully*
4. Add 60 ul Mito Storage Buffer

**Set up JC-1 Assay – KEEP EVERYTHING DARK AS LONG AS POSSIBLE**

1. Beginning with stock JC-1 at 5 mg/mL (7.65 mM), create a new JC-1 aliquot at 306 uM –add 2 uL of JC-1 to 50 uL DMSO. Vortex to mix and return to darkness. This makes 200 ug/ml (306 um) JC-1 stock in DMSO. It should be bright pink.
2. Add 49.6 ul of the new JC-1 stock **(JC-1 Working Solution)** to 7.6 ml of JC-1 assay buffer; this is the JC-1 Dye you will use on your samples. The final concentration is 2 uM. It should be very pale pink; **return it to darkness**
3. Add 500 ul JC-1 Assay Buffer (without JC-1 Dye) to each 1.5ml **Unstained** tube
4. Add 470 ul JC-1 Dye to all 1.5ml JC-1+ tubes and all JC-1+/CCCP+ tubes
5. Add 3 ul CCCP stock solution to all JC-1+/CCCP+ tubes
6. Return to dark until needed

**Perform JC-1 Assay –**

1. Add 30 ul sample to all JC-1+ and JC-1+/CCCP+ tubes
2. Cover with foil and incubate at 37ºC for 20 minutes
3. Centrifuge at 11,000 rpm at room temperature for 10 minutes
4. Pour off supernatant
5. Resuspend in 300 ul of JC-1 assay buffer, transfer to flow cytometry tubes
6. Collect data on all indicated parameters. Use the unlabeled bacterial control to confirm that the dye-labeled samples are significantly higher in both FL1 (green) and FL2 (red fluorescence). I collect FL3 (farther red) as well, just in case we may need it in the future. CCCP should diminish both the red signal, but the real change is in the FL2/FL1 ratio. This ratio is independent of particle size, which will affect the median FL1 and FL2 fluorescence individually.

**flow cytometer**, collecting in channels that are comparable to a Beckton Dickinson Facscalibur channels FL1, FL2, FL3. Settings are: FSC= E00 (lin), SSC= 350 (lin), FL1 = 400 (log), FL2 = 320 (log), FL3 = 300 (log). **analysis software**: comparable to FlowJo; for plotting FL2 vs. FL1, calculating FL2/FL1, medians for all measured and calculated parameters, and for plotting overlay graphics of medians.