ISAKSON LAB

ATP Release from RBCs

Collect blood from mice via cardiac puncture.

- 3mL syringe, 25G needle
- Lubricate syringe/needle by drawing up and expelling 250mM EDTA; leave last drop or two in needle prior to blood draw
- Anaesthetize mouse, cut open chest and through diaphragm, clamp and fold back upper chest to expose heart, draw gently from right ventricle, not collapsing the chamber & upstream.
- Should be able to draw 0.5 - 1mL per mouse
- Collect in EDTA tube or heparinized tube.

Spin whole blood down in microcentrifuge tube at 800g for 10 minutes. Pipet off supernatant and buffy coat, keeping only red blood cell pellet. Err on the side of removing top of RBC pellet so as to ensure near-complete removal of other blood cells from the buffy coat.

Wash with DPBS, resuspending pellet, and repeat spin and supernatant removal 3 times until pellet is clean with no buffy coat or color to supernatant visible.

Add ARL to 300uM final concentration in stimulation buffer (Krebs-HEPES + 2mM Ca^{2+}, pH 7.4), as well as any inhibitors desired (e.g., 100uM CBX). Re-suspend RBCs at 0.5% hematocrit, e.g., 50uL RBC pellet into 10mL stim buffer.

Add 500uL diluted RBCs into each well of a 24-well or 96-well plate, or into individual 1.5 mL tubes. Due to the variability of ATP measurement (below), use enough wells or tubes to run each condition in triplicate. Let sit for two hours on the bench top for extracellular ATP to be eliminated and reduce background level for experiments.

If RBC lysis with any stimulation reagents is a potential issue, dilute RBCs to half the final dilution in stim buffer while saving the other half of the buffer, add ARL/inhibitors to the remaining buffer, and add stimulation reagents to this buffer and stimulate via doubling the RBC buffer volume. E.g., RBCs can be pre-incubated with an inhibitor half the final volume, then an equal volume of buffer + inhibitor with 2x desired concentration of stimulation reagent can be added to reach 1x inhibitor + 1x stimulation condition in the final mixture. This prevents the need to add a stimulation reagent in a small volume at high concentration, which would risk shocking the RBCs and causing lysis.

During incubation of RBCs, make an ATP standard curve from 1nM to 1uM.

Stimulate RBCs with stimulation reagents, incubate desired time on benchtop at RT (e.g. five minutes), and pipet 200uL from each sample into a 96 well plate. (Setting aside 150uL of that in case of repeat testing) pipet 50uL from each sample into a white 96 well plate for ATP measurement. Spin the remaining samples at 500g for 5 min to pellet cells, and pipet 50uL pf supernatant from each sample into a clear 96 well plate for cell-free hemoglobin measurement.

Use ATP analysis kit to perform the luciferin-luciferase reaction and measure ATP content from white 96-wp. Must run alongside ATP standard curve to have a point of reference, as the assay may not be internally consistent from use to use or batch to batch – but if it can be standardized to known ATP concentrations run alongside, that error can be negated via back-calculation from luminescence. Using ATP Bioluminscence Assay Kit HS II (Roche), set plate reader to inject 50uL of reagent into 50uL sample per well. Check that the sample results are all on the calibration curve – if not, use the remaining 100uL of supernatant collected to dilute out an appropriately lower concentration that will fall on the calibration curve and re-run.

With 50uL per well in a clear 96-wp, collect an absorbance spectrum at every integer nanometer wavelength in the range from 400 – 700 nm. An isosbestic point for oxy- and deoxy-hemoglobin at 570nm can be used to determine hemoglobin content, with a subtraction of 700nm absorbance to eliminate background.

Compare ATP release data to hemoglobin content of samples to determine whether ATP levels are due to controlled ATP release or to increased cell-free hemoglobin (i.e., RBC lysis and release of all ATP in the cell).