**CEC Assay**

**Updated 9-15-22**

**Cell Plating (Day before experiment ~ 4 pm)**

1. Plate J774A1 cells at **50,000 cells per well** in **96-well black-clear bottom plate** in complete media
   1. **Complete Media** = RPMI, 10% Fetal bovine serum, 1% streptomycin + penicillin
2. Incubate for **18 Hours**
   1. **REASONG BEHIND THIS**
      1. 18 hours is the doubling time for J7741A cells so final concentration is ~100,000 cells per well next day.
      2. Cells are more adherent with overnight plating, more robust, CVs very nice.

**Prepare cholesterol loading (ABCAM Kit) (Day of experiment ~9 am)**

1. Prepare cholesterol loading mix (100 ul/well) with the following:
   1. **BODIPY-cholesterol** (Kit): 1:4
   2. **Equilibrium Media** (Kit): 1:4
   3. **SERUM FREE RPMI MEDIA** (No FBS/antibiotics): 2:4
   4. **Reagent A** (ACAT Inhibitor or cAMP): 0.5 ul per 1mL of BODIPY-cholesterol used
   5. **Reagent B** (ACAT Inhibitor or cAMP): 0.5 ul per 1mL of BODIPY-cholesterol used

**Cholesterol loading**

1. Wash cells with SERUM FREE RPMI MEDIA x 1, flick it in the sink
2. Load cells with **100 uL of cholesterol loading mix**
3. Incubate for **4 Hours**

**Prepare Acceptor (HDL in your case) at least 1.5-2HRhr before the end of loading**

1. **12.5 ug of HDL** or **100 ug/mL** HDL per replicate
   1. It was 10ug HDL before when the total acceptor volume = 100 uL
      1. **REASON BEHIND THIS**
         1. CVs are more consistent when you have more volume inside well, evaporation has minimal effect.
2. 125 uL of total volume/replicate with **FLUOROBRITE DMEM (PHENOL RED FREE) SERUM FREE MEDIA**
   1. **E.g.** Subject 1 has 3000ug/mL of HDL. You have one 25 uL aliquot to use for this assay. The formula goes like this for duplicates (and a little extra)
      1. 2.25 replicates x 125 uL total volume = 281.25 uL
         1. 3000ug/mL \* x = 281.25 uL \* 100 ug/mL
            1. x = **9.38 uL of isolated HDL + 271.87 uL FluoroBrite media = 281.25 uL**

**Acceptor loading**

1. Wash cells with **FLUOROBRITE DMEM (PHENOL RED FREE) SERUM FREE MEDIA** x1**.**
   1. **REASON BEHIND THIS**
      1. This special media has minimal background fluorescence.
      2. FBS has lipoproteins, so you don’t want your efflux to be confounded by the FBS
2. Load cells (125 uL of HDL/FluoroBrite media mixture) per well.
   1. Cover with Breath-Easy Film
      1. **REASON BEHIND THIS**
         1. Minimizes evaporation
   2. Incubate **4 Hours**

**Remove Supernatant and Lyse Plate**

1. Remove supernatant into **96-well black-opaque bottom plate** (**note** black-opaque bottom and not clear bottom
2. Lyse remaining cells with 125 uL of M-PER lysis reagent
   1. Put lyse fraction in shaker for 30min no temperature
      1. No need to transfer this lyse fraction, keep in original plate

**Reading**

1. Read supernatant in Microplate reader Ex/Em = 485/523 nm
   1. Settings
      1. Set Plate to Black-opaque bottom
      2. **3 Reads** fluorescence **Endpoint**
         1. **Normal Range**
2. Read Lyse Fraction in Microplate reader Ex/Em = 485/523 nm
   1. Settings
      1. Set Plate to Black-clear bottom
      2. **1 Read** on fluorescence **AREA SCAN**
         1. **Extended Dynamic Range**
      3. OPTIONAL:
         1. 3 additional reads on fluorescence Endpoint