ESC derivation – Paul updated 1-5-17

- *I combined different protocols (see supplemental protocols). The major changes are:*
    a. *Using 2i/LIF medium (M15 medium, 1μM PD0325901 [Sigma #PZ0162], 3μM CHIR99021 [Millipore #361559], 100U/ml LIF [1/10 LIF used for regular ES culture]) and MEF feeders during the first few (P1-P3) passages*
    b. *Using accutase (Thermo Cat. # A1110501) to dissociate blastocysts*

1. Isolate blastocysts from uteri following "Manipulating the Mouse Embryo" textbook (check the supplemental protocols for more detail)
    a. Before opening the mouse, put 2i/L on top of MEF feeders on 12-well plate (I usually prepare 8 wells). Pre-warm in the CO2 incubator for at least 30 min.
    b. Put 5ml Knockout DMEM (Thermo #10829018) + 20 mM HEPES on each of the three 6 cm plates (Thermo #15630106). Pre-warm in the CO2 incubator for at least 30 min before opening the mouse.
    c. Isolate uteri from the mouse. Cut of oviducts and ovaries from both ends. Then cut the "Y"-shaped uterus from the middle to break it into two uterine horns.
    d. Flush the uterine horns using syringe from both ends with the pre-warmed Knockout DMEM+HEPES (use 4-8 ml in total).
    e. Transfer individual blastocyst to each well of the 12-well plate (with MEF feeders and 2i/L). If there are >8 embryos, put 2 blastocysts in one well.

2. Culture blastocysts in 2i/L without ANY disturbance for 4 days. After the 4^{th} day, check the outgrowth under the microscope. The blastocysts with outgrowth can be trypsinized and transferred at 5-6th days following **Step 3.**
    a. Blastocysts hatched faster need to be transferred earlier.
    b. To find the blastocyst, look through the bottom of the plate towards the light. The blastocysts appear as white dots and can be circled with the pen.
    c. 80-90% blascocysts should give rise to outgrowth.

3. Handpick blastocysts with outgrowth (P1)

a. Pre-warm ESC medium (M15+LIF) on top of feeders growing on the 24-well plate. The number of wells = the number of blastocysts to be passaged.
b. Don't change the medium (2i/L) before handpicking. The blastocysts are loosely attached to the feeder. Also avoid shaking while moving the plate.
c. Put 30 μl accutase (don't warm up, directly take from refrigerator and bring it to the room temperature. Warming up accutase decreases its activity) into eppendorfs (= the number of blastocysts going to be picked)
d. Handpick the colonies with 10 μl tip. Directly transfer the blastocyst to the accutase in the eppendorf and pipet several times to ensure the transfer. It's better to pick all the colonies asap (within 5-10 min).
e. Incubator all the eppendorfs in the CO2 incubator for 15-20 min.
f. Use 100 μl tip to put 100 μl M15 medium to the eppendorf, pipet up and down 5-6 times until blastocysts are dissociated. Don't pipette more than 10 times. Cells still aggregate together can be dissociated at P2.
g. Centrifuge at 800g for 5 min
h. Carefully remove the top 100 μl medium from the eppendorf. Use the pre-warmed ESC medium on 24-well plate (**Step 3a**) to resuspend the pellet and put back into the same well.
i. Rock the plate to evenly distribute cells. Grow cells in the CO2 incubator.

4. Change the ESC medium daily until the colony are visible with appropriate size
    a. Blastocysts that not dissociated well (**Step 3f**) can be trypsinized with 0.25% TE (red TE) and passaged to the new 24-well plate the 1-2 days after P1

5. Trypsinize and transfer (using 0.25% TE) the blastocysts on 24-well plate until it reaches 50-60% confluency. Save ESC stocks at early passages. Then ESC lines can be expanded for experiments.