**HEK Cell Aggregation Assay (Antony Boucard, Rewritten)**

**Incubation Media:**

HEK media with 10 mM CaCl2 and 10 mM MgCl2

**Day 1: Split HEK cells for tranfection**

1. From a confluent T75 flask, trypsinize and resuspend cells in 8 ml total volume.
2. Aliquot 250 ul of cells to 1 well of a 6 well plate (1.5 ml total).
3. Add HEK media to a total volume of 2ml per well (1.75 ml per well).
4. Make sure cells are evenly seeded to ensure uniform transfection.

**Day 2: Transfect HEK cells**

FOR ONE WELL:

1. In tube A, add 62.5 ul 2x HBS.
2. In tube B, add 4 ug total DNA (3 ug cell-adhesion molecule and 1ug fluorescent protein), water to 56.25 ul and 6.25 2.5 M CaCl2.
3. Slowly add contents of tube B to tube A while vortexing.
4. Incubate for 10-15 or until you start to see precipitates on a glass slide.
5. Add transfection mixture to well and incubate O/N

**Day 3: Harvest cells and perform aggregation assay**

1. 24 hours after transfection, wash transfected HEK cells with PBS 2x.
2. Add 1 ml PBS + EDTA (same one used for AAV prep) and incubate at 37 for 5’.
3. Gently collect cells and add to tube with 5 ml pre-warmed HEK media.
4. Centrifuge at 500 xg for 5’.
5. While cells are centrifuging, prepare incubation tubes. For each condition, add 360 ul **Incubation media**.
6. Gently resuspend cells with 500 ul **Incubation media**.
7. Pipette up and down to break up any clumps of cells (you can put the 1 ml pipette tip near the bottom of the tube and expel the cells against the botton of the tube to increase success of breaking up clumps).
8. Take 10ul and put on hemocytometer - count number of cells.
9. Add 40,000 - 75,000 cells of each condition to the incubation tubes (total V = 500ul).
10. Invert the tube several times and take 30 ul of cells and add to one well of a 24-well plate.
11. Take image of T=0 sample (no aggregation).
12. Begin rotating tubes at RT.
13. Image 30 ul of cells every 15 minutes to find the optimal timepoint.

\*\*Note: We was seeing aggregation after 30’\*\*

1. *Example of aggregation assay - left panel: T=0, right panel: T=5*
2. 