**A549 NTC/RIG-I KO FFU Assay**

Day 1: seeding cells:

1. Seed A549 cells in 6 well plate at density of 6x105 cells/well (2ml/well)

Day 2: infecting cells:

1. Serially dilute virus stocks in DMEM containing 2% FBS. Have each dilution in triplicate.
2. Remove media from each well and add 300ul of virus dilution to the appropriate wells. Rock 2-4 times.
3. Incubate infection for 2 hours at 37C on rocker
4. Add 2ml of 1% methylcellulose overlay (2% FBS, 1% methylcellulose, Pen/Strep, 10mM HEPES) to each well
5. Incubate plate at 37C until appropriate timepoints

Day 3: fixing, permeabilizing, and imaging cells:

1. Remove overlay from each well and add 500ul 4% PFA in 1X PBS to each well to fix cells for 20min. at room temperature inside TC hood
2. Wash cells/each well 3x with 1ml 1X PBS
   1. Plate can be removed from TC after wash step following fixation
   2. Plates can be stored at 4C until ready for permeabilization/staining and imaging
3. Dilute primary antibody in perm/wash/block buffer
   1. To make perm/wash/block buffer:
      1. 2.5% normal goat serum
      2. 2.5% normal donkey serum
      3. 0.2% BSA
      4. 0.1% Triton X-100 (in 1X PBS)
      5. 1X PBS
4. Remove PBS from wells and add 300ul/well of diluted primary antibody to each well
5. Incubate plate on rocker for 2 hours at room temperature
   1. This can also be done on the bench
   2. Cells can be labelled for longer, but background staining will increase, so additional/longer washes may be needed
6. Wash plate 3x with 1ml 1X PBS
   1. If cells were stained for longer periods of time (like overnight), leave final wash on plates and incubate at room temperature for 2+ hours (or overnight at 4C)
7. Dilute secondary antibody in perm/wash/block buffer
8. Remove PBS from wells and add 300ul/well of diluted secondary antibody to each well
9. Incubate for 1 hour at room temperature on rocker covered in foil (HRP is somewhat light sensitive)
   1. Cells can be labelled for longer, but background staining will increase, so additional/longer washes may be needed
10. Wash cells 3x with 1ml 1X PBS
11. To develop assay, add 300ul/well of Trublue peroxidase substrate (at AL’s bench)
12. Incubate for 2-10min at room temperature or until spots are fully defined and minimal background is seen
13. Wash with 1ml dH2O
14. Flick plates over sink to remove excess water
15. Image plates on Chemidoc white light setting