**Immunofluorescence for Tissue Culture**

**Reagents**

* 6-well TC plate
* TC Media
* TC PBS
* Cover slips
* Slides
* Tweezers
* PBS pH 7.4
* PBST pH 7.4
* 70% EtOH
* 90% EtOH
* Image-iT FX (Thermo Fisher)
* Petri dish
* Whatman paper
* Parafilm
* Kimwipes
* Aluminum foil
* di-H2O
* ProLong Gold (Thermo Fisher) mounting media
* Nail polish
* Permeabilization solution: 0.2% Triton X100 in PBS
* \*Optional\* poly-l-lysine solution: 0.01% poly-l-lysine w/v in PBS
* Glyoxal Fixing Solution: 3.15% glyoxal, 19.87% EtOH, 0.76% Acetic Acid in PBS. pH 4.3
* Blocking solution: 5% goat serum in PBST
* Primary solution: 1/250 - 1/1000 primary antibody in 1% goat serum PBST
* Secondary solution : 1/250 - 1/1000 secondary antibody in 1% goat serum PBST
* Hoechst solution: 3ug/ml of Hoechst stain in PBST
* Pyronin solution: 0.5uM Pyronin Y in PBST

Prior to using cover slips - wash with 70% EtOH, let dry and then autoclave

**Day 0:**

1. Using tweezers take an autoclaved cover slip, wash in 90% EtOH, flame dry and place into a well of a 6-well plate. Let cover slips air dry for ~30 min before adding media

 *\*Optional - for cells that do not adhere well to slides\* Treat slides with poly-l-lysine before adding media*

1. Add 250ul of poly-l-lysine solution to each cover slip and gently rock of 10 min
2. Rinse once with TC PBS, aspirate and then place the 6-well plate into 37C incubator for ~90 min to dry
3. Then begin to add media and cells. Fill to 2ml
4. Seed cells to be a final density of ~1.8x105 cells/ well at time of harvesting

 Place the plate into the 37C incubator until the next day (or whenever you are ready to harvest)

**Day 1** (or whenever you are ready to harvest)**:**

The following steps are all done at RT.

1. Aspirate wells and rinse once with PBS.
2. Fix cells: add 1ml of glyoxal fixing solution to each well and incubate for 15 min
3. Wash 3x with ~1.5ml of PBS.
4. Permeabilize cells: add 1ml of 0.2% Triton X100 PBS to each well and incubate for 5 min.
5. Wash 3x with ~1.5ml of PBS.
6. Add 1-2 drops of Image-iT FX to each cover slip and incubate for 30 min.

 During this incubation, prepare a petri dish with a piece of damp whatman paper on the bottom of the dish and covered with a piece of parafilm (this prevents the cover slips from drying out during the blocking and antibody incubations).

1. Wash 3x with ~1.5ml of PBS.
2. Block cover slips: remove the cover slip from the 6-well plate with tweezers. Gently dab the back side of the cover slip with a Kimwipe to remove excess buffer (**Do not completely dry the cover slip) (Do not apply the Kimwipe to the cell side of the cover slip)**. Place the cover slip in the petri dish, cell side facing up. Add 150ul of blocking solution to the cover slip. Rock slowly for 1 hour
3. Applying primary: remove the cover slip from the petri dish, gently dab a corner of the cover slip with a Kimwipe. Place the cover slip back into the petri dish, add 125ul of primary antibody and rock gently for 1 hour.
4. After the primary antibody, remove the cover slip from the petri dish, gently dab with a Kimwipe and place the cover slip back into the 6-well plate

 Add ~1.5ml of PBST to each well of the 6-well plate and rock for 10 min. (rock slightly faster than was done for the antibody incubation). Repeat this wash for a total of 3 times

1. Applying secondary: remove the cover slip from the 6-well plate, gently dab a corner of the cover slip with a Kimwipe. Place the cover slip into the petri dish, add 125ul of secondary antibody and rock gently for 1 hour. Cover the petri dish with aluminum foil.
2. After the secondary antibody, remove the cover slip from the petri dish, gently dab with a Kimwipe and place the cover slip back into the 6-well plate.

 Add ~1.5ml of PBST to the 6-well plate and rock for 10 min. (rock slightly faster than was done for the antibody incubation) repeat this wash for a total of 3 times. **Each time removing and re-covering the plate with foil.**

1. Add 1ml of Hoechst solution to each well and rock for 15 min. Cover the plate with foil.
2. Remove the Hoechst solution, add ~1.5ml of PBST and rock for 5 min. Cover the plate with foil.
3. Add 1ml of Pyronin solution to each well and incubate for 30 sec. Remove the Pyronin then gently and quickly rinse the wells with di-H2O.
4. Add a 15ul drop of ProLong Gold to a clean slide and place the cover slip cell face down onto the drop of ProLong Gold.

 Anchor the cover slip to the slide by adding a drop of nail polish to each of the four corners of the cover slip. Cover the slides with foil and store at +4C overnight.

1. The following day seal all of the sides of the cover slip with nail polish, cover with foil and allow to dry for ~1 hour.
2. Gently wash the slides and cover slips with di-H2O and a Kimwipe

Analyze slides that day, or at longest the following day. After ~48 hours the Pyronin staining starts to diffuse.