**Immunofluorescent Staining of 10μm Human Cardiac Cryosections**

* Pre-Stain Notes
  + This protocol takes 2 days. Make sure you have the time to dedicate consecutive days for these experiments.
  + Make sure you have at least 1L of 1X PBS, 1L of PBT, each, and ~10mL of 10% normal donkey or goat serum (NDS or NGS) prepared prior to starting this protocol. 1X PBS is diluted from a 10X stock with DI water. PBT is 0.25% Triton X-100 / PBS (2mL of Tritin X-100 for every 800mL of 1X PBS). 10% NDS or NGS is diluted from a 100% stock with PBT.
  + Hoechst 33342 counterstain is prepared with 6μL of Hoechst 33342 in 60mL of 1X PBS.
  + Prepare dark, humidified box prior to starting by adding tap water and closing the lid. The same water can be used throughout this protocol but needs to be exchanged outside of this time frame.
  + Each wash step indicates that slides are placed in a Coplin jar on the rocker. In Coplin jars, slides are to be placed back-to-back (with sections facing outward) for the inner slots, but only 1 slide can be placed on the corner slots (with sections facing inward).
  + Unless otherwise stated, all steps of this protocol are performed at room temperature in a fume hood.
  + When handling the dark, humidified box, be careful not to disturb the solutions on top of the sections.
* Day 1
  1. Place slides in a fume hood and allow them to equilibrate to room temperature (~30 minutes).
  2. Wash in PBS for 5 minutes.
  3. Wash in 4% paraformaldehyde for 10-15 minutes.
  4. Wash in PBS for 5 minutes
  5. Repeat the previous step once, totaling 2 PBS washes.
  6. Wash in PBT for 5 minutes.
  7. Draw a hydrophobic barrier around each section with an ImmEdge Hydrophobic Barrier PAP Pen.
  8. Place slides in dark, humidified box and add 50-80μL of 10% NDS or NGS to each section. Put on the rocker for 1 hour.
     + The blocking buffer used must match the species of the secondary antibody.
  9. During incubation with the blocking buffer, prepare the primary antibody (see next page) and store it on regular ice.
  10. Suction the blocking buffer off the sections using a hose connected to a vacuum line. Do not remove the slides from the dark, humidified box.
  11. Add 50-80μL of the primary antibody solution to each section. Place the dark, humidified box on the rocker in the 4oC fridge overnight.
* Day 2
  1. Remove the dark, humidified box from the 4oC fridge and allow slides to equilibrate to room temperature (~30 minutes).
  2. Wash in PBT for 5-10 minutes.
  3. Repeat the previous step twice, totaling 3 PBT washes.
  4. During PBT washes, prepare the secondary antibody (see next page) and store it on regular ice.
  5. Redraw a hydrophobic barrier around each section with an ImmEdge Hydrophobic Barrier PAP Pen.
  6. Place slides in dark, humidified box, and add 50-80μL of secondary antibody solution to each section. Place dark, humidified box on the rocker for 1-2 hours.
  7. Wash in PBT for 5-10 minutes.
  8. Repeat the previous step twice, totaling 3 PBT washes.
  9. Wash with Hoechst 33342 for 10 minutes.
  10. Wash in PBS for 5 minutes. Allow slides to completely dry prior to proceeding to the next step.
      + The drying process can be expedited by using a hose connected to an air line.
  11. Add 2-3 drops of VECTASHIELD Vibrance Antifade Mounting Medium (H-1700) to each slide and apply a coverslip. Gently push down on the coverslip to disperse trapped air bubbles.
  12. Allow slides to dry for one hour and store in the -80oC freezer.
* Preparing primary antibodies (all diluted in 10% NDS or NGS)
  1. Phospho-Histone H2A.X (Ser139) Rabbit mAb (from CST; 20E3)
     + 1:200 (2.5μL in 500μL)
  2. Anti-α-Actinin Mouse mAb (from Sigma)
     + 1:200 (2.5μL in 500μL)
  3. p16 INK4A (E6N8P) Rabbit mAb (from CST; 18769)
     + 1:500 (1μL in 500μL)
* Prepare secondary Abs:
  1. Anti-Mouse IgG F(ab’)2 Fragment (AlexaFluor 488 Conjugate) Goat Host (from CST; 4408)
     + 1:500 (1μL in 500μL)
  2. Anti-Rabbit IgG F(ab’)2 Fragment (AlexaFluor 647 Conjugate) Goat Host (from CST; 4414)
     + 1:500 (1μL in 500μL)