**Multiplex Labeling with Tyramide Fluorophores (Free-Floating Tissues)-Killinger Lab 2024**

**Day 1:**

1. Wash free-floating tissue (3 x 10 minutes) in dilution media (DM)
2. Heat water bath 1.5 hrs before the antigen retrieval step.
   1. Human samples: 90-95°C
   2. Mouse samples: 80-85°C
3. Place the dish containing sodium citrate buffer in the water bath and heat it for 10 minutes.
   1. Sodium Citrate Buffer, pH 6.0 (1L)
      1. 2.94g Sodium citrate-Trisodium salt(Dihydrate)in 1000 mL DI water.
      2. Adjust pH to 6.0
      3. 0.5mL Tween-20. Mix well.
4. Wash the tissues in sodium citrate buffer (1 x 5 minutes).
5. Incubate the tissues in the heated sodium citrate buffer for 30 minutes.
6. Cool down the tissues by placing container in an ice bucket for 15 minutes.
7. Wash in DM for 10 min x 2 times.
8. Endogenous peroxidase inhibition and serum blocking step (1-hour incubation): 0.3% H2O2+0.1% Sodium Azide in blocking buffer.
   1. Blocking buffer:
      1. 100mL dilution media
      2. 3mL normal serum
      3. 2g bovine serum albumin
      4. 0.4mL of Triton x100.
      5. Mix well so the Triton is completely dissolved.
   2. In 50 mL blocking buffer, add 0.5mL 30% H2O2 + 0.5mL 10% Sodium Azide
9. Dilute primary antibody in blocking buffer. Incubate overnight at 4°C.

**Day 2:**

1. Wash (3 x 10 minutes) in dilution media
2. HRP-Secondary antibody incubation 1:1000 dilution (1 hour).
   1. Solvent is 100 mL DM/1 mL normal serum/1g BSA
3. Wash (2 x 10 minutes) in dilution media
4. Wash in borate buffer (1 x 10 minutes)
   1. 0.05M Borate buffer pH 8.5
      1. 300mL DI H2O + 5.72 g of sodium tetraborate decahydrate (P17, big bottle)
         1. Mix well to dissolve completely.
         2. Adjust to pH 8.5
5. Incubate with tyramide fluorophore (TF) for 30 minutes while blocking light.
   1. 10 mL Borate buffer + 1 uL H2O2 + 5 ul TF
6. View under the microscope to confirm successful staining.
7. Store in PBS and leave at 4°C. It can be stored for up to 2 weeks. Otherwise, proceed with the antigen retrieval step.

**Day 3:**

1. Heat water bath 1.5 hrs before the antigen retrieval step.
   1. Human samples: 90-95°C
   2. Mouse samples: 80-85°C
2. Place the dish containing sodium citrate buffer in the water bath and heat it for 10 minutes.
   1. Sodium Citrate Buffer, pH 6.0 (1L)
      1. 2.94g Sodium citrate-Trisodium salt(Dihydrate)in 1000 mL DI water.
      2. Adjust pH to 6.0
      3. 0.5mL Tween-20. Mix well.
3. Wash the tissues in sodium citrate buffer (1 x 5 minutes).
4. Incubate the tissues in the heated sodium citrate buffer for 30 minutes.
5. Cool down the tissues by placing container in an ice bucket for 15 minutes.
6. Wash in DM for 10 min x 2 times.
7. Endogenous peroxidase inhibition and serum blocking step (1-hour incubation): 0.3% H2O2+0.1% Sodium Azide in blocking buffer.
   1. Blocking buffer:
      1. 100mL dilution media
      2. 3mL normal serum
      3. 2g bovine serum albumin
      4. 0.4mL of Triton x100.
      5. Mix well so the Triton is completely dissolved.
   2. In 50 mL blocking buffer, add 0.5mL 30% H2O2 + 0.5mL 10% Sodium Azide
8. Dilute primary antibody in blocking buffer. Incubate overnight at 4°C.

**Day 4:**

1. Wash (3 x 10 minutes) in dilution media
2. HRP-Secondary antibody incubation 1:1000 dilution (1 hour).
   1. Solvent is 100 mL DM/1 mL normal serum/1g BSA
3. Wash (2 x 10 minutes) in dilution media
4. Wash in borate buffer (1 x 10 minutes)
   1. 0.05M Borate buffer pH 8.5
      1. 300mL DI H2O + 5.72 g of sodium tetraborate decahydrate (P17, big bottle)
         1. It takes a while to dissolve completely.
         2. Adjust to pH 8.5.
5. Incubate with tyramine fluorophore (TF) on a shaker for 30 minutes while blocking light.
   1. 10 mL Borate buffer + 1 uL H2O2 + 5 ul TF
6. View under the microscope to confirm successful staining.
7. DAPI staining (20 minutes)
   1. 1:2000 dilution PBS. Block the light.
8. Mount the tissues on a slide, cover the slide with Fluoroshield, and coverslip. Seal with nail polish on all sides of the coverslip.
9. When the nail polish is completely dried, view under the microscope. Always protect the slides from light. Slides can be stored at 4°C