Protocol for whole-mount RNA FISH of *Drosophila* adult CNS on coverslips

FISH Probes:

FISH probe libraries are designed based on transcript sequences. Probe libraries typically have 48 oligos. Each probe is typically 18-22nt long with a 3' end amine-modified nucleotide for an NHS-ester dye coupling reaction. Probes should be pooled if necessary, then labeled with desired dyes via dye manufacturer NHS-ester dye coupling reaction instructions.

Solutions (RNase-free):

Water, 1x PBS, 0.5% PBT (1x PBS, 0.5% Triton)
2% paraformaldehyde (PFA) in PBS
5% (v/v) CH_3COOH (acetic acid) in water; store 5% stock at 4 ℃.
1x PBS with 1% (wt/v) $NaBH_4$ (sodium borohydride); make fresh with 4 ℃ PBS.
Pre-hybridization solution (15% formamide, 2x SSC, 0.1% triton)
    Use Hi-Di formamide. Store stock at -20 ℃.
Hybridization solution (10% Formamide, 2x SSC, 5x Denhardt's solution, 1mg/ml Yeast tRNA, 100 ug/ml Salmon sperm DNA, 0.1% SDS)
    Make 400 µL aliquots and store at -20 ℃.
Formamide wash solution (30% formamide, 2x SSC, 0.06% triton)
    Can use cheaper formamide, but still needs to be deionized.
    Aliquot deionized formamide and store at -20 ℃.
    Store wash solution at -20 ℃.
SSC wash solution (2X SSC, 0.06% triton); store stock at -20 ℃.
70%, 50%, and 30% EtOH

Reagents and equipment:

RNase-free 1x PBS, Fisher BP2438-4
Acetic Acid, Glacial, Fisher A38S-500
Sodium borohydride, 99%, VenPure™ SF powder, Acros Organics AC448481000
SSC (20X), Fisher AM9763
Hi-Di formamide, Applied Biosystems 4311320
Denhardt's solution (50X), Alfa Aesar™ AAJ63135AD
tRNA from baker's yeast, Roche 10109495001
UltraPure™ Salmon Sperm DNA Solution, Fisher 15632011
SDS, 10%, Corning 46-040-CI
Deionized formamide, Ambion AM9342
RNaseZap, Fisher AM9780
Polypropylene Humid Chamber, Ted Pella 2249-6
poly-L-lysine, Sigma Aldrich P1524-25MG
Cover glass 22x22mm, Corning 2845-22
Cover glass staining jar, Electron Microscopy Sciences 72242-24
Programmable incubator
Plexiglass Drosophila Hybridization Dish (https://www.janelia.org/open-science/plexiglass-drosophila-hybridization-dish)
Plexiglass Drosophila mounting T-dish (https://www.janelia.org/open-science/drosophila-mounting-t-dish)

Stepwise protocol

Day 1

Dissection:

- Dissect fly CNS or other tissue at desired age, no special RNase control.
- Fix dissected tissue in 2% PFA at 25 ℃ for 55 min.
- Wash with 0.5% PBT, 3x, 10 min each.
- Chemical tagging before end of day, as necessary.
- Dehydrate within 1-2 days.

Day 2

Dehydration:

- Dehydrate tissue with graded EtOH series: 30%, 50%, 75%, 100%, 100%, 100%, 10 min each. Move to 4 ℃, preferably for at least multiple hours, until mounting. Protect from light.
- Dehydrated tissue can be stored at 4 ℃ in 100% EtOH for at least one week with minimal impact on FISH or chemical tag signal.

Mounting:

- Note that each coverslip will get single hybridization condition.
- Mount samples in 75% EtOH. Samples don't stick to poly-L-lysine in 100% EtOH.
- Repeat 100% EtOH dehydration and store in 100% EtOH at 4 ℃.

Day 3

Environment: RNase-free zone. Wipe tweezers & pipettor with RNase away/RNase Zap on a kimwipe. Clean 10 mL coverslip jars.

Permeation:

- Rehydrate tissue with EtOH series: 100% rinse, then 70%, 50%, 30%, 5-10 min each.
- Move samples to cold room.
- Rinse in 4 ℃ 5% CH_3COOH then incubate for 5 (up to 8) min in cold room.
- Rinse in 4 ℃ 1x PBS then wash for 5 min in cold room.
- Move to room temperature and wash 2x with RT PBS for 5 min.
- Fix with 2% PFA at room temperature for 55 min.
  Meanwhile, prepare pre-hybridization solution and warm to 50 ℃.
- Rinse with 1x PBS, then 3 washes, 10 min each.

Autofluorescence quenching:

- Prepare fresh 1% $NaBH_4$ solution in 4 ℃ 1xPBS.
  Put 10 mL cold PBS into four tubes, one for rinse and each round of incubation.
  Add 100 mg $NaBH_4$ to 10 mL solution. Start with first two tubes in parallel.
  $NaBH_4$ reacts with water. Close tube to mix, but quickly open to release pressure.
- Rinse in 4 ℃ 1% $NaBH_4$ then incubate 3x for 10 min in cold room.
  While each incubation proceeds, prepare next tube of 1% $NaBH_4$. Do not seal tightly.
- Rinse with 4 ℃ PBS, then wash for 5 min in cold room.
- Move out to room temperature and wash 2x with RT PBT for 5 min.

Pre-Hybridization:

- Warm pre-hybridization solution to 50 ℃.
- Incubate tissue with pre-hybridization solution at 50 ℃ for 2 hours. Agitate if possible.

Hybridization:

- Preparation requires about 30 minutes. Start prep during pre-hybridization.
- Thaw hybridization buffer aliquots. Prepare probes.
  If probe has been frozen, heat at 90 ℃ for 3 min, then quickly put on ice to snap chill.
- Start with 170 µL hybridization solution per coverslip. (Total reaction volume is 180 µL.)
- Add 3-6 µL of 100ng/µL FISH probes to the hybridization solution. The extra probe is useful for densely-populated coverslips.
- Add hybridization solution to hybridization dish, then gently place coverslip with samples facing downward, avoiding introducing bubbles. Place dishes in humidified chamber.
- Incubate at 50 ℃ for 10hr, then at 37 ℃ for 10hr.

Day 4

Washing:

- Thaw wash solutions and warm the pre-hybridization solution to 37 ℃.
- Transfer coverslips to jar of 37 ℃ pre-hybridization solution for 10 min.
- Wash with pre-hybridization solution, 1x, 37 ℃, 10 min.
- Wash with pre-hybridization solution, 1x, at room temperature, 10 min.
- Wash with formamide washing solution, 2x, at room temperature, 30 min each.
- Wash with 2xSSC washing solution, 3x, at room temperature, 10 min each.
- Wash with 1xPBS, 1x, at room temperature, 10 min.
- Fix tissue at 2% PFA at room temperature for 55 min.
- Wash with 0.5% PBT, 3x, at room temperature, 10 min each.
- Hold up to two days in cold PBT.

Day 5

Clearing and mounting:

- Dehydrate with graded EtOH series: 30%, 50%, 75%, 100%, 100%, 100%, 10 min each.
- Clear in xylene, 3X, 5 min each.
- Mount in DPX (Electron Microscopy Sciences). A detailed protocol of DPX mounting is described in “DPX Mounting” at https://www.janelia.org/project-team/flylight/protocols