Whole Brain Free-Floating TSA Fluorescent ISH protocol

**DAY 1 – PERFUSION / FIXATION**
1) Perfuse animal with 4% PFA / 1x PBS. Dissect out brain and post-fix overnight (o/n) at 4C.

**DAY 2 – PROBE HYBRIDIZATION**
1) Wash brains in 3x5 min. in 1x PBS at 4C (on ice).
2) Section tissue (25 - 50um sections for adult brain) with a vibratome.
3) Post-fix sections 15 min. in 4% PFA / 1x PBS at RT.
4) Wash sections 3x3 min. in 1x PBS
5) Incubate sections in 3% hydrogen peroxide / 1x PBS (for 10mL total, use 1mL 30% hydrogen peroxide in 500ul 20x PBS and 8.5mL H2O) for 15 min., to quench endogenous peroxidases
6) Wash sections 3x3 min. in 1x PBS.
7) Acetylate sections for 10 min. (for ~20mL total acetylation solution: add 234uL Triethanolamine (viscous) to 20mL H2O, mix, and then add 50uL acetic anhydride, shake vigorously, and immediately add to sections), to allow better probe binding.
8) Turn on oven if necessary to prepare next steps (and reserve for o/n with post-it note) and dry heat bath
9) Wash sections 3x5 min. in 1x PBS
10) Prehybridize sections at 65C in 700uL hyb buffer per well (buffer is prewarmed to 65C, about 10 mins.) in enclosed, humid chamber (use 50% formamide / 50% 5x SSC in petri dish as humidifier) for 1 hr.
11) Incubate sections in hyb buffer+probe o/n (18-24 hr.) at 65C (optimal probe concentration and temperature is determined empirically). A good starting concentration is 3uL probe per 750uL hyb buffer (so add 3uL probe to 50uL buffer on ice (to prevent sticking to itself), then denature in 80C dry bath for 5 min., then back to ice shock for 1 min. and finally add to prehyb, mixing gently before putting in oven).

**DAY 3 – TYRAMIDE AMPLIFICATION AND MOUNTING**
1) Remove sections & wash 3x20 min. in 0.2x SSC (controls stringency) at 65C (prewarm first). Also, thaw TNB if necessary
2) Equilibrate sections in TN buffer for 5 min. at RT
3) Block sections (of nonspecific residues) 30 min. in TNB at RT
4) Dilute Anti-DIG-POD 1:500 - 1:2500 in TNB (needs to be determined empirically to minimize background), and incubate at RT for 30 min.
5) Wash sections 3x5 min. in TNT
6) Dilute Cy3-Tyramide (powder already dissolved in DMSO) 1:50 in amplification reagent, add 300-400ul to wells and incubate at RT for 10 min.
7) Wash sections 3x5 min. in TNT and then mount (for single label, see last step, or proceed to next step for double label ISH)

8) Quench peroxidase activity by incubating 1 hr. in 3% hydrogen peroxide / 1x PBS.
9) Wash sections 3x5 min. in TNT
10) Dilute Anti-FITC-POD 1:500 - 1:2500 in TNB (needs to be determined empirically to minimize background), and incubate at RT for 30 min.
11) Wash sections 3x5 min. in TNT
12) Dilute FITC-Tyramide (powder already dissolved in DMSO) 1:50 in amplification reagent, add 300-400ul to wells and incubate at RT for 10 min.
13) Wash sections 3x5 min. in TNT
14) Incubate sections in DAPI and/or TO-PRO (thaw first, use 1:10k dilution if fresh, down to 1:1k in 1x PBS) nuclei counterstain for 10 min., then mount on slides using paintbrush and anti-fade reagent
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Reagents:

Hybridization solution (pre-prepare and store at -20C):
50% formamide (prevents secondary structure)
5X SSC (saline-sodium-citrate, controls stringency)
5X Denhardts (mix of HMW polymers to saturate non-specific binding sites and thus artificially increase probe concentration)
250 μg/ml yeast tRNA
500 μg/ml herring sperm DNA
50 μg/ml Heparin (anti-coagulant, can occupy DNA binding sites on RNA poly.)
2.5 mM EDTA (chelating agent to sequester metal ions)
0.1% Tween-20 (surfactant/detergent to remove nonpolar residues)
0.25% CHAPS (zwitterionic detergent to solubize proteins, etc.)

Antibodies & tyramide amplification:
anti-FLUORESCINE-POD, Fab fragments (Roche 1 426 346)
anti-DIGOXIGENIN-POD, Fab fragments (Roche 11 207 733 910)
TSA™ Plus Fluorescein Fluorescence System (Perkin Elmer NEL741)
TSA™ Plus Cy3 Fluorescence System (Perkin Elmer NEL745)
Roche Anti-digoxigenin-POD (peroxidase), Fab fragments Catalog 11 207 733 910
Roche Anti-Fluorescein-POD (peroxidase), Fab fragments 1 426 346

Buffers for tyramide amplification:
TN
100 mM Tris-HCl (pH 7.5)
150 mM NaCl
TNB (for nonspecific blocking)
0.5% Blocking Reagent (Perkin Elmer) in TN buffer (1g in 200mL, add slowly & heat gradually while stirring)
TNT (for all washing steps)
0.05% Tween20® (100uL in 200mL) in TN buffer
(NOTE: all washes are in dishes and incubations can be performed on parafilm or with hybrislips).
Making RNA probes (one s/b conjugated to DIG, other to FITC):
Keep everything on ice:

8.5uL dH2O (add first)
2uL 10X transcription buffer
4uL linearized cDNA template (1ug)
2uL DIG (or FITC) RNA labeling mix
1uL 0.1M DTT (to relax cDNA)
0.5uL RNase OUT
2uL T7 RNA Polymerase (enzymes last, added quickly)
[= 20uL total]

…Mix up and down with pipette ~10 times and incubate at 37C for 2 hr. for transcription (cover w/ foil for FITC); can make overlapping probes for entire ROI to increase SNR

from Roche’s instructions: 1ug of template generates 10ug of labeled probe; dissolve this 10ug in 50ul (add 30uL dH2O after transcription), and pass through a G50 column to separate by size, resulting in ~200ng probe/uL concentration

Misc. Notes:
• Smaller brain structures (i.e. VNO) will require OCT embedding, cryostat sectioning, and slideglass ISH
• For young brains or small sections, incubate in 0.1% hydrogen peroxide / 1x PBS for 30 min.
• Use heat-closed Pasteur pipette (in hook shape) to handle tissue, or Corning Netwells (15mm diameter, 74um mesh)
• Use foil to protect fluorophores if necessary
• 4% PFA: 85mL DEPC or dH2O, 5mL 20xPBS, 10mL PFA (Electron Microscopy Sciences)