**Fractin immunostaining protocol**

updated 9/15/21, Caleb Wood and Gaby Perez

\*All steps done at room temperature except where noted.

\* All incubations done on rotating platform to keep sections separated and moving.

**Day 1**

1. Rinse free-floating sections with 1X TBS 3x10’.
2. Quench endogenous peroxide by incubating free-floating sections for 30 min with 0.9% H2O2 in 1X TBS + 0.3% Triton X-100 and 0.05% Tween 20 (TBS-TT).
3. Wash free-floating sections with 1X TBS 3x10’.
4. Block free-floating sections with 5% NGS in TBS-TT for 1 hour.
5. Incubate free-floating sections in primary antibody at 4°C for 72 hours.

Primary antibody: 1:3,000 chicken anti-GFP, Abcam, ab13970; 1:5,000 rabbit anti-Fractin, PhosphoSolutions, 592-FRAC

(Note: we typically co-stain for GFP with fractin, but other antibodies could of course be used instead.)

**Day 4**

1. Wash sections 3x10’ with TBS-TT.
2. Incubate sections O/N at 4°C in secondary antibody diluted in blocking solution (5% NGS in TBS-TT).

(1:1000 goat anti-chicken Alexa 488, Life Technologies, A11039 (for GFP primary); 1:5000 biotinylated goat anti-rabbit, Vectastain Elite ABC HRP kit, Peroxidase, PK-6101, Vector Laboratories, Burlingame, CA).

Prepare Vectastain A + B solution:

1 drop A = 50 uL, 1 drop B = 50 uL

1 drop of A and 1 drop of B in 30 mL 1X TBS total. (Note that this is used at a significantly thinner dilution here than the manufacturer's recommendation.)

For smaller volumes, use a dilution factor of 1 part A + 1 part B in 600 parts buffer (ie, 50 ul + 50 ul in 30 mL)

**Day 5**

1. Wash sections 3x10’ with 1X TBS.
2. Incubate sections with diluted Vectastain A + B in 1X TBS for 30 min.
3. Wash sections 2x10’ in TBS-TT.
4. Wash sections O/N at 4°C in 1X TBS.

**Day 6**

1. Incubate sections with Cy3 Plus Amplification Reagent diluted 1:300 in 1X Plus Amplification Diluent for 10 min.

(TSA Plus Cyanine 3 System 50 – 150 Slides, Perkin Elmer, Waltham, MA, NEL744001KT; Akoya Biosciences ($373.15 + $60 shipping as of 1/2021) .

(1X Plus Amplification Diluent, 50 mL (Akoya Biosciences, Catalog # FP1498, # NC1345235 at Fisher; $582 as of 7/2021)

1. Wash sections 3x10’ with 1X TBS.
2. Incubate sections with 0.2 ug/mL DAPI diluted in 1X TBS for 10 min.
3. Wash sections 3x10’ with 1X TBS.