Conventional immunostaining of smFP-V5 reporter in thin MORF3 brain sections

- Wash 4% PFA fixed sections 3 times in PBS for 15 minutes each, shaking at room temperature (RT).
- Block in 3% bovine serum albumin (BSA) and 3% normal goat serum (NGS) with 0.1% Triton X-100 for 1 hour, shaking at RT.
- Incubate in rabbit polyclonal anti-V5 (Bethyl, A190-120A; 1:500) or chicken polyclonal anti-V5 (Bethyl, A190-118A; 1:500) added to blocking solution for 24 to 72 hours, shaking at 4°C.
- Following incubation in primary antibody solution, wash 3 times for 15 minutes each, shaking at RT.
- Incubate in secondary antibody of choice (1:500) added to blocking solution for 24 hours, shaking at 4°C.
- Following incubation in secondary antibody solution, wash 3 times for 15 minutes each, shaking at RT.
- Counterstain with DAPI or NeuroTrace following manufacturer's protocol.
- Wash 3 times for 15 minutes each, shaking at RT.
- Mount sections on slides with mounting medium or antifade reagent or choice.

iDISCO+ clearing and immunostaining of smFP-V5 reporter in thick MORF3 brain sections (adapted from Renier et al., 2016)

- Transcardially perfuse with 50 mL of 0.1 M phosphate buffered solution (PBS) followed by 50 mL of ice cold 4% PFA.
- Post-fix dissected brain in 4% PFA overnight, gently shaking at at 4°C.
- Tissue can be stored at 4°C in 0.01M PBS with 0.02% sodium azide.
- Vibratome section tissue at 300-600 µm.
- Wash sections in PBS for 15 minutes, shaking at room temperature (RT).
- Dehydrate sections with a series of methanol (MeOH) washes (20%, 40%, 60%, 80%, 100%) for 1 hour at each step, nutating at RT.
- Remove membrane lipids with an overnight incubation in 66% dichloromethane (DCM) and 33% MeOH, nutating at RT.
- Bleach sections to reduce autofluorescence with incubation in 5% hydrogen peroxide in MeOH overnight, at 4°C.
- Rehydrate sections with decreasing concentrations of MeOH (80%, 60%, 40%, 20%, then 0.01M PBS) for 1 hour at each step, nutating at RT
- Incubate in permeabilization solution (0.01M PBS with 0.2% Triton X-100 and 20% DMSO) overnight, nutating at 37°C.
- Block in 0.01M PBS, 0.2% Triton X-100, 6% NGS, and 10% DMSO overnight, nutating at 37°C.
- Incubate in rabbit polyclonal anti-V5 (Bethyl, A190-120A; 1:500) or chicken polyclonal anti-V5 (Bethyl, A190-118A; 1:500) in 0.01M PBS, 0.2% Triton X-100, 3% NGS, 5% DMSO, and 10 µg/ml heparin for 72 hours, nutating at 37°C. The primary antibody solution should be replaced every 24 hours at the concentration.
- Wash in 0.01M PBS with 0.2% Triton X-100 overnight, nutating at RT. Replace wash solution several times during the first several hours.
- Incubate in fluorescent-conjugated secondary antibody of choice (1:500) and a fluorescent counterstain of choice (such as NeuroTrace, 1:300) in 0.01M PBS, 0.2%

Triton X-100, 3% NGS, and 10 µg/ml heparin for 72 hours, nutating at 37°C. The secondary antibody and counterstain solution should be replaced every 24 hours at the concentration. Protect sections from light, from this step on.
- Wash in in 0.01M PBS with 0.2% Triton X-100 and 10 µg/ml heparin overnight, nutating at RT. Replace wash solution several times during the first several hours.
- Dehydrate sections with a series of methanol (MeOH) washes (20%, 40%, 60%, 80%, 100%) for 1 hour at each step, nutating at RT.
- Incubate in 66% dichloromethane (DCM) and 33% MeOH overnight, nutating at RT.
- Wash in 100% DCM 2 times for 15 minutes each, nutating at RT.
- Clear sections by incubating in dichloromethane (DBE) for at least 2 hours, at RT.
- Mount cleared and immunostained tissue in DBE on glass microscope slides with silicone spacers, glass coverslips, and edges sealed with silicone. Allos silicone seal to cure overnight, at RT.