Double in situ hybridization (ISH)

Mouse embryos were fixed overnight in PBS/4% PFA at 4°C and processed as for immunolabeling experiments. 14 µm cryostat sections were cut and double ISH protocol was performed essentially as previously described with slight modifications (Pelosi et al., 2014). Sections were simultaneously hybridized overnight at 65°C with a DIG-conjugated *Vsx1* (NM_054068.2, nucleotides 121-2728, gently provided by C. Cepko) and a fluorescein-labeled *Foxn4* (NM_148935.2, nucleotides 78-1643) riboprobe. After hybridization, sections were washed four times in 50% Formamide, 1× SSC, 0.1% Tween-20 for 1 h at 65°C, twice in MABT buffer (100 mM maleic acid, 150 mM NaCl, 0.1% Tween20, pH 7.5) for 30 minutes before blocking in blocking buffer (MABT, 2% blocking reagent from Roche, 20% inactivated horse serum) for 2 h at room temperature. Sections were then incubated overnight with anti-DIG-alkaline phosphatase (AP)-conjugate antibody (Roche) at 4°C. After washing for 30 minutes in MABT, *Vsx1* probe was visualized by AP-catalyzed chromogenic reaction using NBT-BCIP substrates (Roche) according to manufacturer's instructions. The color reaction was stopped in 1X PBS and AP was inactivated by incubating the slides for 30 minutes with 0.1M glycine/HCl, pH 2.2. The sections were then washed twice in MABT buffer for 30 min, blocked again in blocking buffer for 2 h, and incubated to a 1:2000 dilution of anti-Fluo-AP-conjugate antibody (Roche) in blocking buffer overnight at 4°C. Slides were washed and incubated with HNPP/Fast Red kit (Roche) according to the manufacturer's instructions to visualize *Foxn4* expression. The reaction was stopped by washing in 1x PBS. Sections were then counterstained with DAPI and slides were mounted with Fluorescent mounting medium (DAKO).