Double Fluorescence Immunohistochemistry Protocol for Brain Cryosections (floating sections)

Updated 6-22-15

Perfusion Preparation

On the day of the perfusion, make up the clearing solution (0.01M PBS) and the 4% PFA. The following recipes are for one rat:

Clearing solution- (0.01M PBS
Fixative- 250ml 4% PFA. Dissolve 10g PFA powder in water (+2 NaOH pellets) by heating, then add the mixture to (88ml 0.2M Sodium Phosphate dibasic +37ml 0.2M sodium phosphate monobasic), this will bring the pH down to ~8.3.

Once the clearing solution and fixative are made up, put them on ice so they are ice-cold when you start to perfuse.

Perfusion

When the animal is anesthetized (by Nembutal or isofluorane), bring it over to the perfusion tub under the hood and lay it on its back. Check for reflexes.
When the animal has no reflexes, pin it down on the black rubber mat. Pin the animal through the fleshy part of the paws.
Cut the diaphragm and be sure to cut the ribs to expose the heart. Cut far enough up so that the heart is readily visible. Clamp the flap of skin over the animal's head so you will have a clear view of the animal's heart.
Cut open the right atrium,

With a 5cc syringe and a 27 gauge needle filled with cold clearing PBS, insert in the left ventricle, push the PBS slowly to clear the circulating blood.

Now use 5cc syringe filled with 4% fresh PFA, repeat the perfusion.

Collect the brain,

Post-fixation: overnight in 4% PFA, kept at 4 degree

Cryoprotection and tissue embedding

Embedding the perfused brains in the gradient of 10%, 20% and 30% sucrose solution, each condition for overnight, make sure the brain descends to the bottom of the container. After the cryopretection, embed the brains with embedding medium in a cutting mold. Immediately freeze the sample on dry ice, then keep it at -80 degree until sectioning on a vibratome.

Vibratome Sectioning

Switch brain samples into the Leica vibratome chamber, let the temperature be in balance, this will take 45min.

Before sectioning make sure you have plenty of ice-cold 0.01M PBS, and clean, labeled beakers with cold 0.01M PBS. Bring all supplies to the core room where the vibratome is

located. Bring single use vibratome cutting blade, single side blades, brushes, PBS container to collect the floating sections.

Cut the brain into 40um sections,

Transfer the section to 0.01PBS, and wash for 15 min.

Permeabilization

Carefully transfer the sections to a 6-well plate with a nylon mesh insert using a plastic transfer pipette. Add between 2.5 of 0.05% Triton-PBS. Leave on RT for 2hrs. This will poke holes in the tissue and allow better penetration of the antibody.

Blocking

This step will help to block non-specific binding, increase the signal specificity. In 0.05% Triton- 0.01M PBS add 10% normal donkey serum (NDS), 1% bovine serum albumin (BSA). Leave the blocking on either overnight at 4°C or for 1hr at RT.

Primary Antibody

Make primary antibody dilution solution in the blocking solution.
Primary antibody dilution is typically 1:200-1:2000; mix two antibodies of interest that are produced in different species.
To minimize the use to primary antibody, use a 2ml tube, and use a fire polished glass needle to transfer the brain sections to this tube.
Incubate in cold room for overnight.

Water with ample amount of PBS in the 6-well plates for 3X10min.

Secondary Antibody

Dilute fluorescent secondary antibodies in 0.01M PBS
dilution is typically 1:100-1:200
using Alexa Fluor secondary antibodies (as compared with FITC or Rhodamine) to increase the signals.
Make sure the species are correct
Incubate at room temperature in the dark for 4 hours.
Water with ample amount of PBS in the 6-well plates for 3X10min.

Mounting on the slides

Seal with Prolong Gold or Vectashield antifading reagent.