Immunohistochemistry Protocol for frozen sections from mice spinal cord.
Staining noradrenergic fibers by an Indirect Method (ABC-DAB reaction)

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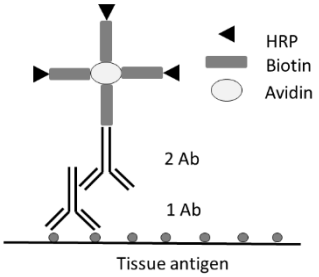
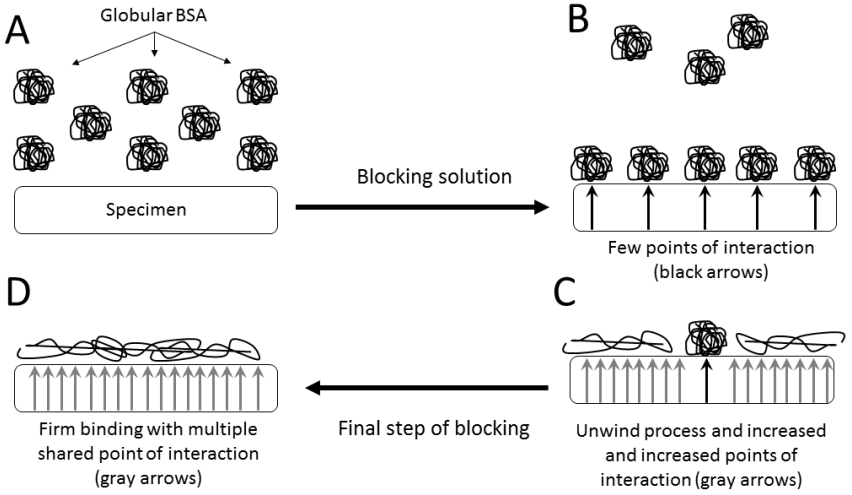
Introduction



Immunohistochemistry (IHC): [*Immuno (antibodies and histo (tissue)]* is a technique commonly used in scientific basic research and clinical diagnosis laboratories to identify specific targets (Antigens) located in cells of a tissue by using antibody (Ab) combinations.

Appropriate blocking of specimens is the first step while performing IHC. This can be done for instance with solutions containing bovine serum albumin (BSA) and pH close to the iso-electric point of the blocking agent. These will guarantee that the blocking agent has little net charge, thus favoring hydrophobic interaction. Although two of the most commonly used protein blockers are BSA and dry milk, most of the commercially available choices are contaminated with bovine IgG, which might cause problems when a goat or sheep primary antibody is used because the labeled secondary anti-goat will significantly cross-react with bovine IgG since goat and sheep are very closely related to cow.



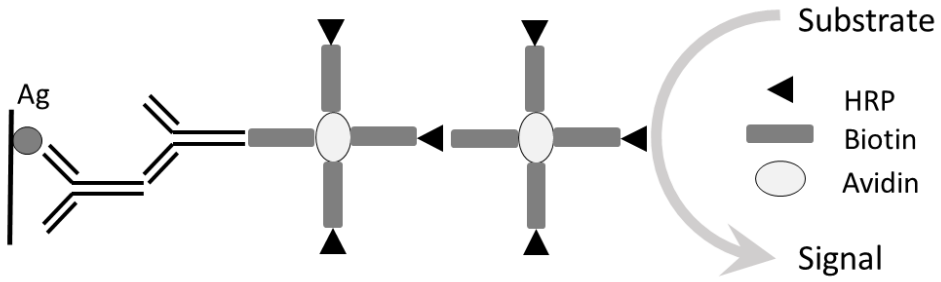
Step A: Applying blocking solution (BSA)
Step B: BSA adsorbs on the sample surface. Initially there are not many points of interaction (represented by arrows).
Step C and D: As incubation time increases, BSA interaction with the tissue surface will increase as well and become irreversible.

An alternative to BSA is to incubate the tissue with normal serum from a different host species as the primary antibody and from the same the same host species as the labeled secondary. For example, in this lab we will block with donkey serum because we will use a primary Ab produced in mouse followed by a secondary Ab made in donkey. In addition, if a biotinylated antibody will be used in tissues, it may contain

cross-reacting endogenous immunoglobulins. To avoid unspecific signal from endogenous immunoglobulins is recommended to dilute this biotinylated antibody in a solution containing ~2% generic blocker from horse or donkey.

There are several methods to visualize the antibody-antigen reaction. The method that we will learn in this laboratory involves the use of:

- A primary Ab that detects an enzyme responsible for the production of the neurotransmitter Norepinephrine (or Noradrenaline) in neuronal fibers.
- A secondary Ab linked to Biotin
- Signal amplification step by producing Avidin Biotin Complex (ABC).
- The enzyme Peroxidase (HRP) and its substrate (DAB). The result of HRP catalysis is a Color-producing reaction.



Avidin is a glycoprotein with a very high affinity for the vitamin Biotin (more than one million times higher affinity than an antibody for its antigens). Avidin have four biotin-binding sites to Biotin that allows optimal macromolecular complexes to be formed and to remain stable for many hours after formation (ABC complex).

When preparing ABC solution, Reagent A contains Avidin and Reagent B the biotinylated enzyme horseradish peroxidase (HRP). HRP is commonly used to amplify weak signals and we will be able to visualize it using a chromogenic substrate 3,3'-Diaminobenzidine (DAB). When DAB is oxidized, it catalyzes the conversion of the substrate (DAB) into colored products. Colored products will be located in the noradrenergic neuronal fibers on the cross sections of mice spinal cord.

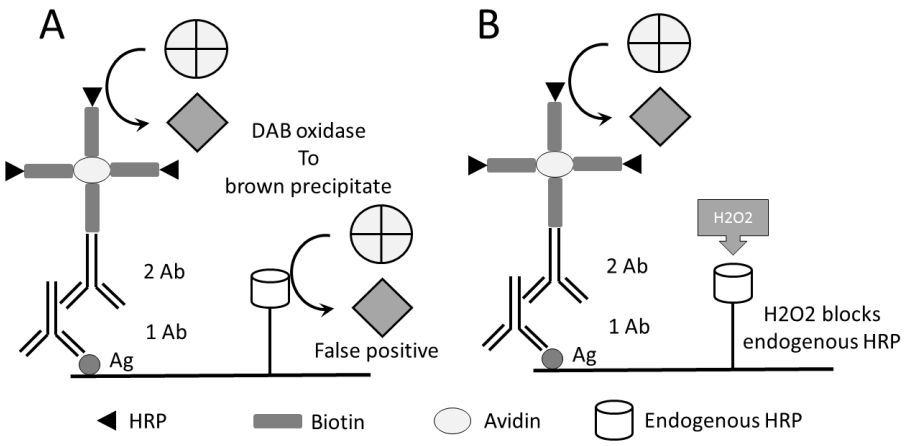
While working with tissue there are several aspects that you will need to address to be able to obtain appropriate staining of sections and simultaneously minimizing the costs of the reactions:

- The use of pens with hydrophobic properties will allow you to draw barriers on the slides, limiting the amounts of valuable reagents (such as antibodies) because it confines the volume of reagents to the selected area.

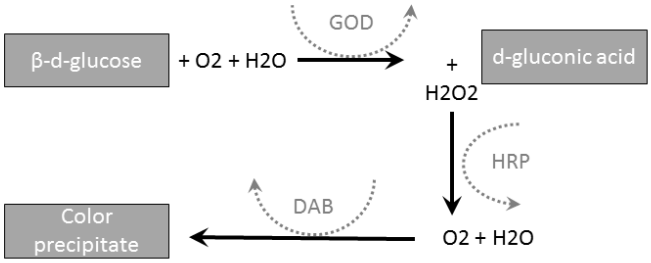
- Tissue permeabilization is necessary when the target protein is located in the cell cytoplasm or its organelles**.** Triton or NP-40 will partially dissolve the membranes and are therefore very appropriate for nuclear antigen staining. However as they are harsh detergents, they will disrupt proteins when used at higher concentrations or for longer amounts of time.

- An appropriate Signal/Background is necessary so the level of a desired signal is higher to the level of background noise. A higher Signal/Background ratio guarantees a clear signal with low interference caused by unwanted noise making a signal stand out

Endogenous peroxidases are an important source of nonspecific signal because they are physiologically present in many cells and tissues with high blood content. If the endogenous peroxidases activity is not depleted it will react with the substrate increasing unwanted non-specific background and leading to false positives. Hydrogen peroxide can act irreversibly inhibiting the peroxidase enzyme activity if used at the appropriate concentration. Therefore, sections can be pretreated with hydrogen peroxide before incubation with HRP-conjugated antibody to quench endogenous peroxidases activity.



The following diagram represents the staining reaction used in the second part of this lab using glucose oxidase (GOD), horseradish peroxidase (HRP), and 3'3'-diaminobenzidine (DAB).



After the staining is completed (when the colored precipitate is formed on the tissue), next required steps are dehydration, clearing and mounting.

- Dehydration: This step will allow you to remove fixatives and water and replace with a dehydrating compound (ethanol, methanol, and acetone). In this lab this step will be done in gradients to minimize the distortion of delicate nervous system samples.
- Clearing: This step will replace the dehydrating compound with an intermediate compound that is both miscible in it and in the embedding medium. Zylene and citrisolv are examples of clearing agents.
- Mounting: This step uses a mounting medium that holds the specimens in place between the cover slip and the slide. Generally, mounting media for permanent slides can be categorized into

water-based and organic solvent based mounting media. In this lab we will use an organic based mounting media that inhibits photobleaching of dyes and have a very good refractive index and that can be stored without sealing for long term analysis.

Materials

Tris Buffered Saline-Stock: 0.5 M (pH=7.4)
PBS
Triton X-100
Hydrophobic pen (*Ex: Advanced Cell Diagnostics, 50-489-330FP*)
Hydrogen peroxide
Ethanol
Normal Donkey Serum (JACKSON IMMUNORESEARCH LAB, S017-000-121)
Primary antibody Anti-Dopamine β Hydroxylase Antibody (Millipore, MAB 308)
Biotinylated secondary antibody Biotin Donkey anti-mouse (JACKSON IMMUNORESEARCH LAB 715-065-150
ABC HRP Kit (Vector Labs PK-6100)
DAB Peroxidase (HRP) Substrate Kit (Vector Labs, SK-4100)
NH4NiSO4
Glucose
Glucose oxidase (G0050, TCI).
Nuclear fast red (Vector labs, H-3403)
Vectashield Mounting Media (Vector labs, H-1000)
Citrisolv Clearing Agent (Ex: Fisher brand 22-143-975)

Equipment:
Vortex
Slides racks and Cuvettes for staining (Ex: Simport Slide Staining System M90012B
Microscope (Transmitted light with 40X or 60X)
Slides
Tissue

Experimental Protocol

Day 1

1. Draw one oval line around all 3 sections of the spinal cord using the hydrophobic pen. The oval will hold the liquid so be sure there are no gaps or the solutions will leak out.
   IMPORTANT: Guarantee that the sections are covered with the solutions all the time by adding as many drops of each solution as needed.
2. Wash tissue in PBST twice (5 minutes each time) at room temperature
3. Cover tissue in 0.3% H2O2 for 15 min at room temperature (This step deplete the endogenous peroxidase activity. Look for bubbles on the tissue)
4. Wash tissue in PBST twice (5 minutes each time) at room temperature
5. Incubate in 50% Ethanol for 45 minutes at room temperature
6. Wash tissue in PBST 3 times (5 minutes each time) at room temperature
7. Incubate in 1.5% Normal Donkey Serum in PBST for 1 hour at room temperature

8. Dilute the primary antibody (DbH) at 1:1000 in 1.5% Normal Donkey Serum/PBST solution
9. Incubate the side with the antibody prepared on Step 7 overnight at 4°C

Day 2

10. Wash tissue in TBST twice (5 minutes each time) at room temperature on a shaker
11. Dilute the Biotinylated secondary antibody Biotin Donkey anti-**mouse** at 1:500 in 1.5% Normal Donkey Serum
12. Cover sections with the Biotinylated antibody prepared in step 11, 1 hour at room temperature
13. Prepare ABC solution: Add 5 ml of 1.5% Normal Donkey Serum. Stir (use a magnet) then add 1 Drop of Agent A, stir few seconds then add 1 Drop of Agent B. Mix for 1 minute. Afterwards, do not stir or move the solution again until use.
14. Wash tissue twice in TBS (5 minutes each time) on a shaker
15. Cover tissue with solution prepared in step 13 for 1 hour at room temperature
16. Wash tissue twice in TBS (5 minutes each time) on a shaker
17. In 25 ml, dissolve in order in a vortex:
    a. 0.625g NH_4NiSO_4
    b. 1 % aliquot DAB
    c. 0.05 g glucose
    d. ~1mg glucose oxidase
18. Shake vigorously in a vortex until dissolved
19. Cover sections with the solution prepared in step 17 watching reaction until sufficient staining occurs (2-12 minutes)
20. Wash tissue in TBS 3 times (5 minutes each time)
21. Counterstain by immersing the slide in 0.05% Neutral Red for 45 seconds
22. Transfer the slide to a Slide holder and rinse the slides in running tap water for 5 min. Allow sections to air dry 1-2 hours or overnight.
23. Dehydrate the tissue slides using the following solutions of ethanol (70%, 95%, 95%, 100% and 100%), 3 min in each solution. Important: Do not allow sections to dry between solutions
24. Clear the tissue slides in 2 changes of Citrisolv 3 min each and coverslip using mounting solution. The mounted slides can be stored at room temperature permanently.
25. Observe the antibody staining in the tissue sections under a microscope and draw your cross section with the stained noradrenergic fibers.

26. Answer the following questions:

a. Describe the configuration of antibodies. How are its chains joined?

b. What is an epitope?

c. How would omitting the blocking step (step 7) affect the signal?

d. Why is the Avidin-Biotin Complex formation considered to be an amplification step?

e. What is the role of the noradrenergic fibers in the central nervous system?

f. Explain how Parkinson's disease, ADHD or stress are related with the noradrenaline system.