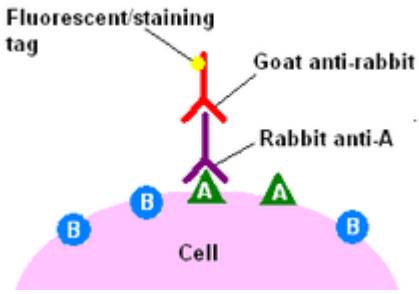
LAB 5: IMMUNOHISTOCHEMISTRY I
Protein detection in tissue – PRINCIPLES AND PREPARATION

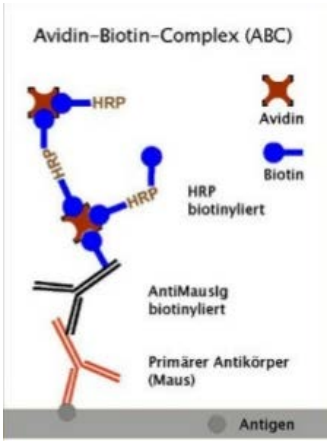
Prelab: watch the following videos - https://www.youtube.com/watch?v=M69B7j_usB8
& https://www.youtube.com/watch?v=HdBgTAAi3rU (DAB staining)

Neurochemistry is the branch of neuroscience interested in the molecules, like neurotransmitters and other chemical molecules, which affect neuron function. It studies how dopamine, serotonin and other molecules regulate neural pathways and influence neural processes, like movement control, reward, mood states and so on.

In order to define what a neurotransmitter was and where it acted, for many years scientists relied on electrophysiological studies. The limitations of those studies were related to exact concentration levels in the solutions and in the pipettes and comparison of pharmacological compounds became tedious and unreliable. The introduction of radiolabeled compounds improved the studies on receptor locations, densities and binding affinities. However, artifacts due to low binding capacity or specificity of the radiolabeled compounds used were reported. Today, most of these studies have been replaced by a much safer and in most cases more reliable technique called immunohistochemistry (IHC).

A technique borrowed from Immunology, IHC is a method for detecting the presence of specific proteins in cells or tissues based on the binding of a primary antibody to a specific protein and the formation of an antibody-protein complex which can be then recognized and bound by a secondary antibody (indirect method). This secondary antibody can be conjugated to an enzyme or a fluorophore to be detected. For more information, refer to http://www.ihcworld.com/_intro/intro.htm





General protocol:
We will be using an **indirect method** to detect our proteins of interest. From www.ihcworld.com: The indirect method involves an unlabeled primary antibody (first layer) which react with a tissue antigen, and a labeled secondary antibody (second layer) which reacts with the primary antibody (Note: The secondary antibody must be against the IgG of the animal species in which the primary antibody has been raised). This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. The second layer antibody can be labeled with a fluorescent dye or an enzyme such as Horse Radish Peroxidase (HRP), and this is

called indirect immunofluorescence method. The enzyme label can be visualized by means of enzyme histochemical methods via chromogenic producing color reactions, essentially a soluble colorless substrate is converted to a water-insoluble colored compound. HRP is a 44kDa glycoprotein enzyme label that can be visualized by chromogenic reactions such as diaminobenzidine (DAB)

Control and experimental conditions:

Controls:

Special controls must be run in order to test the protocol and for the specificity of the antibody being used.

- Positive control is to test a protocol or procedure and make sure it works. It will be ideal to use the tissue of known positive as a control. If the positive control tissue showed negative staining, the protocol or procedure needs to be checked until a good positive staining is obtained.

- Negative control is to test for the specificity of an antibody involved. First, no staining must be shown when omitting primary antibody or replacing a specific primary antibody with normal serum (must be the same species as primary antibody). This control is easy to achieve and can be used routinely in immunohistochemical staining.

Goal of the Immunohistochemistry lab series:

1) to become familiar with a very useful technique to localize specific molecules or proteins in neurons;
2) to identify specific neurons based on their protein content;
3) to learn how to set-up a useful IHC experiment;
4) to run your own experiment, collect data and write a lab report in the format of a scientific manuscript

METHOD

PART I: BECOMING FAMILIAR WITH THE ADULT MOUSE BRAIN

Different preparations are used today in neuroscience to study brain anatomy and function, such as (but not limited to) thin sections or thicker slices, embryonic tissue, adult brain tissue, neural tissue from simple or more complex organisms, explants and cultures of individual cells.

You will have access today to neural tissue preparations, in order to become familiar with neuronal morphology and staining. Our starting material for the next labs will be 25µM sections of perfused adult mouse brain of a control or a transgenic Alzheimer's model mouse strain, fixed in 4% paraformaldehyde and cut using a vibratome.

Material used: Mice were anesthetized using Isofluorene (30% working solution), perfused with a 0.9% saline solution, followed by a 4% Paraformaldehyde (PFA) solution. Whole brains were post-fixed at +4°C overnight in 4% PFA. 48 hours later, the brains were washed extensively in PBS at RT, 1x brief rinse and 3x 25min washes. They were then sectioned at 25µM thickness, collected in plates and preserved at -20°C. The following video highlights the method used to collect samples:
https://www.youtube.com/watch?v=M69B7j_usB8

Now, obtain your samples and get ready to observe a neuron in its original setting! To orient yourself and to start getting familiar with the mouse brain anatomy, help yourself with online mouse atlases available at: http://www.mbl.org/atlas170/atlas170_frame.html or http://mouse.brain-map.org as well as the atlas provided in class.

PART II: IHC
Please watch the following videos:
- https://www.youtube.com/watch?v=HdBgTAAi3rU (DAB staining)

- You can check the following web site for an idea about different immune-techniques/methods: http://www.immunohistochemistry.us/

Our tissue will be exposed to a set of solutions in a specific sequential manner. The main steps are: blocking the tissue, exposure to the primary antibodies, wash of unbound antibodies, exposure to secondary antibodies and wash of unbound antibodies.

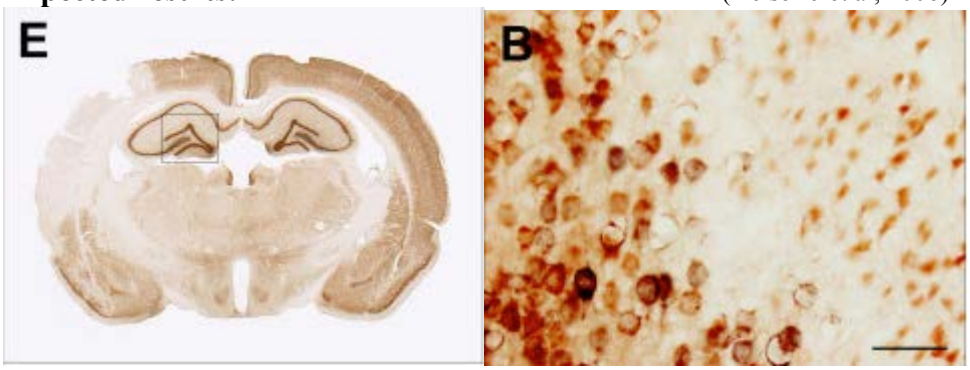
Solutions used in this experiment:

1. Phosphate Buffered Saline – PBS
2. Block Solution: PBS + Triton X-100 (final conc 0.5%)+ Serum (final conc 5%)
3. I° antibody solution: Block solution + I° ab
4. II° antibody solution: Block solution + II° ab
5. DAB staining solutions
6. Semi-permanent mounting medium

Why do we need a Blocking step?

Blocking is used to prevent background staining, which results often in false positive results. The cause might be due to the fixation step or the I° antibodies. The main cause of non-specific background staining is non-immunological binding of the specific immune sera to certain sites within tissue sections. This form of background staining is usually uniform and can be reduced by blocking those sites with normal serum. Autofluorescence or natural fluorescence exists in some tissues and can cause background problems when fluorescent dyes are used in the experiments.

Expected results: (Kelsen J et al, 2006)



PART III: PRIMARY ANTIBODY INCUBATION

You will be assigned a primary antibody from a list of possible antibodies – e.g. NeuN, GFAP, Iba, or Amyloid-B, caspase-3. Each antibody recognizes a specific protein (possibly) present in your tissue.

Procedure for staining free floating brain sections:

1. Use 24 well plates for antibody incubation to decrease the amount of reagent required
2. Wash sections several times in PBS to remove antifreeze.
3. If using Vectastain ABC (HRP-DAB) for development, quench endogenous peroxidase activity by incubating sections in 0.6% peroxide in PBS for 30 min at RT
4. Wash 3 x 10 min with PBS
5. Block for 1h at RT in 250uL prediluted normal blocking horse serum.
6. Incubate primary antibody diluted in 250uL prediluted normal blocking horse serum at 4° C.
7. Leave overnight at +4C

NOTES:

<u>Lab 5 ASSIGNMENT:</u>

1. By the end of lab, your group should turn in a sketch of the experiment that you are carrying out over the next 2 labs. Draw tissue, protein of interest (antigen), primary and secondary antibodies, enzyme used for labelling,…

LAB 6: IMMUNOHISTOCHEMISTRY II – STAINING SECTIONS

Here is a video about the procedure - https://www.youtube.com/watch?v=HdBgTAAi3rU (DAB staining)

By the end of lab **a. You should have a draft of the Methods section (group draft)**

1. Find your sections!

Preparing to detect signal from antibody application:

2. wash 3 x 10 min with PBS.
3. Incubate in secondary antibody diluted in block 30 min at RT. Use with 3-4 drops diluted biotinylated secondary antibody solution.
4. Wash 3 x 10 min in PBS.
5. Incubate sections in 3-4 drops VECTASTAIN® ABC Reagent [a peroxidase (HRP) detection system] for 30 min at RT.
6. Wash 3 x 10 min in PBS.
7. Apply 400 µl DAB substrate working solution to the sections on the slides to reveal the color of the antibody staining. Allow the color development for 5-10 minutes until the desired color intensity is reached. Optimal development times should be determined by the investigator.
8. Rinse sections in tap water.
9. Mount sections onto Superfrost Plus slides. Let dry overnight.

LAB 6 ASSIGNMENT:

1. **By the end of lab, your group should sketch a draft of the Methods section based on procedures from Labs 5&6. You should then revisit the assignment individually and turn in the FINAL version before LAB 8.**

LAB 7: IMMUNOHISTOCHEMISTRY III – OBSERVING STAINED SECTIONS

Prelab: **a. Read attentively Results, Figure and Figure legends Handouts**
By the end of lab **a. You should have figures and sketches of Figure legends (group draft)**

You will have a chance this week to observe the results of your experiments. You will collect pictures of your data. The instructors will be around to help you use the microscope, answer any question about your results and so on. However try to work as much as possible with your partners.

Today you will also be working on your data representation. Those are the images, tables and graphs that will go on your final manuscript. Following are the tasks to accomplish before leaving the lab:

1. Coverslip dry brain slices with mounting media.

2. Once you have decided which sections are the best, **you will take pictures**. Remember, those pictures will be the figures of your report. They are important! Try to take multiple pictures so you will have more freedom when writing your report.

3. You will count the number of positive cells for staining. You will make a graph representing each stain results. Is there another way to convey your results?

4. Think about alternative ways to represent your data and create one or more graphs that best support your results.

5. Design a figure or multiple figures (sometimes multipaned figures are the way to go)

6. Write a figure legend for each figure created.

7. Store your slides at +4C in the dark

LAB 7 ASSIGNMENT:

1. **By the end of lab, your group should sketch a draft of the figures and figure legends based on what you observed today and turn those in at the end of lab.**
2. **You will then individually write the results section. Each of you should turn in a Result section, Figures and Figure legends before the start of Lab 9.**