Lab 2. Introduction to Immunocytochemistry

The goal of this lab is to learn how to process juvenile crayfish brains for both serotonin and bromodeoxyuridine (BrdU) using immunocytochemistry.

BrdU is a synthetic thymidine analog that is incorporated into DNA during cell replication. It can then be located using immunocytochemistry using an anti-BrdU antibody. Cells that label with this antibody can be used as evidence that the cell has divided.

> *Juvenile crayfish have already been incubated in BrdU labeling reagent at 5mg/mL for 24 hours dissected, fixed and rinsed six times in PB.*

In this lab you will learn about the principles of Immunocytochemistry. Then you will set up for immunocytochemical staining for serotonin and BrdU. This will involve coming into the lab several times during the week to follow the procedure. Make sure that you understand the protocol before you leave the lab.

Immunocytochemical Localization of Neuronal Transmitters: In Situ

The explosion of the field of molecular biology in the last 30 years has led to a wealth of information, including an understanding of the exquisite specificity of antibodies. This knowledge has spawned a variety of research tools, including ELISA (enzyme-linked immunosorbent assay) and RIA (radioimmunoassay) that allow precise quantitative measurements of cellular molecules. Another of these tools, immunocytochemistry, combines the understanding of antibody specificity with microscopy techniques to locate a tissue antigen (cellular component or specific molecule) in its original position in the cell. Antibodies can be raised against these antigens, labeled with a visual marker, and then added to the tissue containing the antigens to determine precisely where these antigens are located in the cell. This useful method allows the neuroscientist to map neurotransmitters, or other neurologically relevant molecules, in neurons of any species. These exercises are intended as an introduction to these immunocytochemical methods and their importance in the study of the nervous system.

In this experiment, a neurotransmitter and a cytoplasmic protein are the antigens and their location will be identified in the crayfish brain. The two-step fluorescent method will be used to identify the monoamine neurotransmitter, **serotonin** and the **bromodeoxyuridine (BrdD),** an indicator for cell division.

A. Definitions:

Antigens are the molecules whose location can be identified using the technique of immunocytochemistry. They can be one of a variety of cellular components: cell membrane molecules, enzymes, neurotransmitters, receptors or almost any cell constituent. In order to locate the antigen using these methods, the antigen must first be "fixed" in the tissue. This is accomplished using a fixative which crosslinks the antigen molecules, rendering them insoluble and holding them in their "natural" place. The tissues (whole mounts or sections) are then incubated in antibody solutions where antigen/antibody binding occurs.

Antibodies (see Fig. 1) are serum glycoproteins, known as immunoglobulins (Igs), which are secreted by terminally differentiated B cells (leukocytes). In the vertebrate immune system, antibodies are produced as a defense against foreign substances. They

are also used a biochemical tools to identify and locate specific cell components. Of the five classes of antibodies, IgGs are the predominant class produced in the immune response.

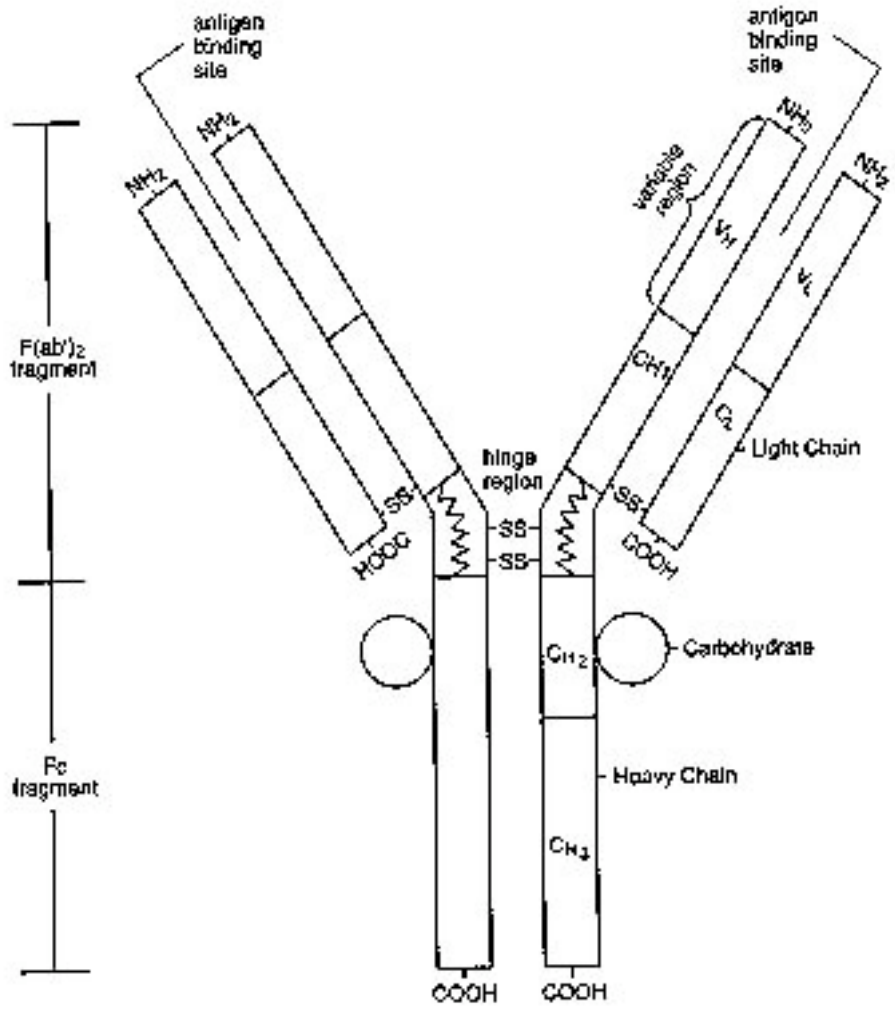


Fig. 1 Basic structure of an IgG molecule
This molecule consists of four polypeptide chains – two identical heavy chains (~440 a.a.s) and two identical light chains (~220 a.a.s). These chains are held together with covalent disulfide linkages and non- covalent interactions. The characteristic Y shape molecule has antigen-binding sites on the arms of the Y, which are free to bend with respect to each other, increasing binding strength of the antibody for antigens. The molecule can be enzymatically cleaved into two Fab fragments, the antigen binding fragments, and one Fc (constant) fragment. When antibodies are generated against the serum from another species, most of the antibodies are directed against the Fc fragment.

Modified from Beltz and Burd 1989

Polyclonal antibodies can be made against any antigen by injecting the antigen (isolated cell constituent or molecule) into a host animal (e.g. goat, rabbit or mouse), harvesting the plasma, and removing clotting factors to produce serum containing the antibodies.

Antibodies can be tagged for later identification using fluorescent markers, enzymes, colloidal gold, or other markers. A wide variety of antibodies are now commercially available with a choice of markers. Two or more antigens in the same tissue sample can be labeled simultaneously using different antibodies, for double and triple labeling studies, where the position of more than one antigen can be located.

B. Anatomy of the Crustacean Brain.

Certain aspects of the crustacean anatomy make these animals good models for studying neurogenesis. Because of their relatively large cells and accessible brain and nerve cord, and the compartmental organization of neuronal cell bodies into neatly packed ganglia, neurogenesis can be examined with relative ease. In addition the olfactory system has been highly conserved throughout evolution so that there is a high degree of similarity among the elements in the olfactory pathway of crustaceans and vertebrate organisms.

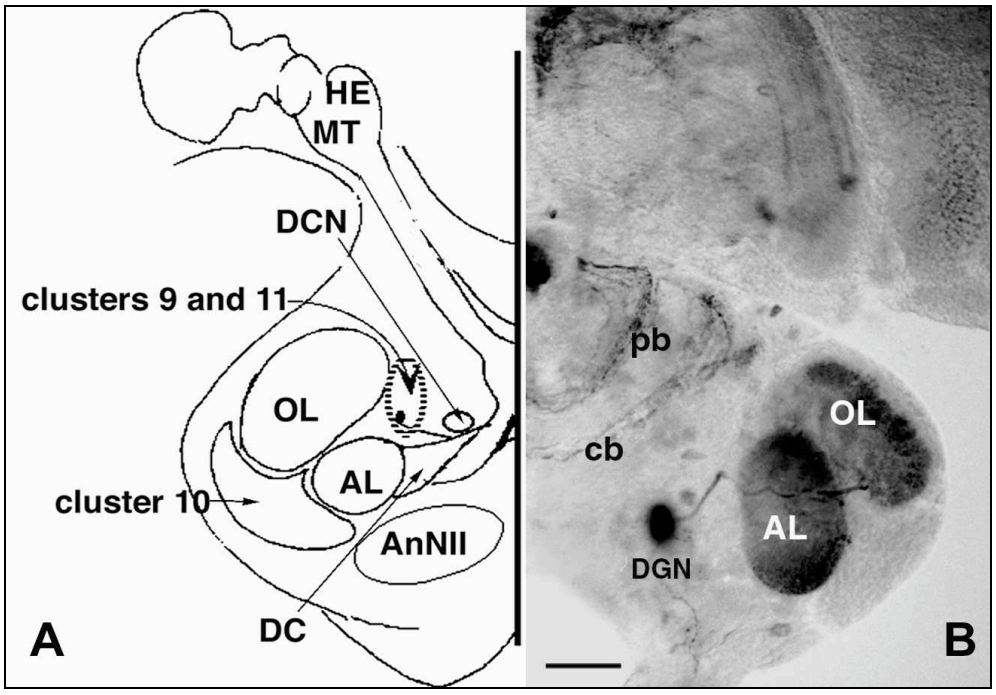


Fig. 2 Drawing of a crustacean brain A, beside a photograph of a serotonin stained section of the brain, B.

The crustacean brain is separated into three main divisions: protocerebrum – concerned with vision whose primary input is the *eyes*, the deutocerebrum concerned with *olfaction* having input from the 1st antennae and the tritocerebrum which is primarily *mechanosensory* and receives input from the 2nd antennae (see lab 1, Fig. 1). The

deutocerebrum, the "mid brain", includes the olfactory lobes (OL), accessory lobes (AL), deutocerebral commissure neuropil (DCN), and other small neuropills (synaptic regions). Figure 2 illustrates the layout of the crayfish brain.

C. Labeling Methods:

Many types of methods are available: one-step, two-step, three-step, etc., where each step involves another layer of antibody and produces a more intense visual label. To localize both antigens, serotonin and BrdU (known as double labeling, see Fig. 3B), in the crayfish brain, the two primary antibodies must be raised in different hosts (e.g., mouse anti-BrdU and rabbit anti-serotonin). Two kinds of secondary antibodies would be necessary to differentially label the primary antibodies (e.g., goat anti-rabbit and goat anti-mouse). Thus two antigens are identified with two distinct sets of antibodies.

TIP: *The following methods refer to numerous antibodies that have been raised in various animals: rabbits, mice, goats, etc. For the neophyte this can be confusing, but remember these animals are the hosts used to raise antibodies. They should be kept track of because the specificity of each layer of antibody used will depend upon the host in which the previous antibody was raised.*

i) One Step fluorescent method – used for labeling BrdU

A one step method is used when the antibody raised against the antigen is, itself conjugated to a fluorescent label. The antibody to BrdU is raised in a mouse and is conjugated to Alexa 488 (a fluorescent green label).

ii) Two-step fluorescent method - used for labeling Serotonin

In the two-step method, two layers of antibodies are used: the primary antibody, which binds to the antigen, and the secondary antibody, labeled with a fluorescent tag, which attaches to the primary antibody. Several secondary antibody molecules can bind to one primary antibody, providing intensification of the visual image. The visual label on the secondary antibodies reveals the location of the primary antibodies, which in turn bind to the original antigen. In this case the primary antibody to serotonin is raised in rabbit, the antigen is injected into a rabbit and the antibodies are harvested in the serum. To generate the secondary antibody, IgGs from a rabbit are injected into a goat and the serum containing the secondary antibodies (formed against the rabbit antibodies) are harvested and conjugated to a visual marker. These labeled secondary antibodies (goat anti-rabbit IgG-Texas Red,)

will bind only to the primary antibody (rabbit anti-antigen IgG) and thus indicate the presence of the original antigen to which the primary antibody is attached.

The bright colors and high contrast that result from fluorescent labeling produce exciting and dramatic data.

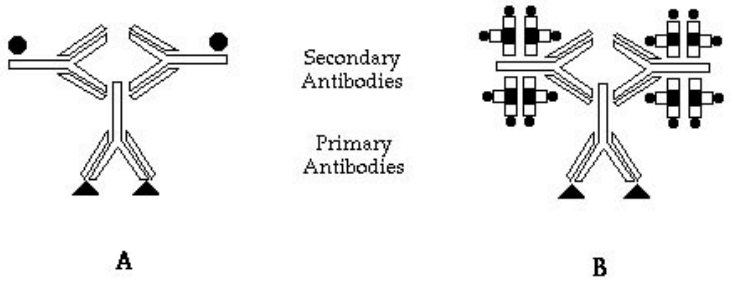


Fig. 3A. Simplified diagram illustrating two immunocytochemical methods:
A) Two-step method - note the intensification of the visual label with the secondary antibody
B) Three-step ABC method - note further intensification with the avidin/binding complex
modified from Beltz and Burd 1989

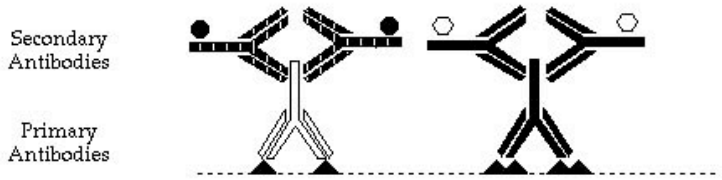
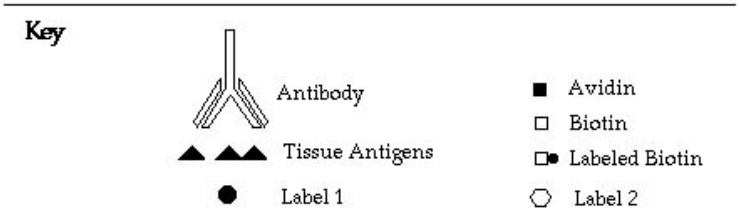


Fig. 3B. Diagram to illustrate double labeling in the same tissue.
Note that the different antigens bind to different primary antibodies, and their secondary antibodies carry different labels, thus making the antigens visually distinguishable in the same tissue.



iii. Triton. PBTx is phosphate buffer that contains Triton X-100, a detergent that permeabilizes the membrane. Antibodies are large compounds that do not easily penetrate through tissues; therefore an agent such at Triton X-100 that opens up "holes" in the membrane will insure better penetration of the immunoreagents. The fixative used will "hold" molecules of interest in place in spite of Triton X-100 treatment.

iii. Presoaking to Reduce Background **-** A presoak in normal serum should also be used to reduce non-specific labeling. Secondary antibodies often stick non-selectively to tissue components via ionic or low affinity interactions producing a level of background labeling. In order to "pre-bind" any sticky sites in the tissue, the tissues are incubated for approx. 1 hour in 5% normal goat serum (in PBTx). Goat serum is often utilized, since many secondary antibodies are generated in goats.

iv. Control Experiments: Special controls must be done to establish the specificity of the immunocytochemical method and the antibodies to be used:

'No primary' control: For one or more tissue samples, the primary antibody is not added, but the tissue is processed through all the subsequent steps, including the secondary antibody. If any staining results, then the secondary antibodies are binding non-specifically to different sites on the tissue and are not binding specifically to the primary antibodies. This is a test for method specificity.

Preadsorption: An antibody is pre-incubated at its working dilution with its antigen. If the antibody recognizes the antigen, they will bind and form precipitating complexes. These antigen/antibody complexes can be spun down with a centrifuge, and the resulting supernatant (called the preadsorbed serum) is used in place of the primary antibody. Staining should not result from incubation with the preadsorbed serum, since this serum should not contain any free antibody. This is a test for antibody specificity.

iv. Dilutions - Before doing this lab it is important to be able to understand and 'do' dilutions. A supplement at the end of this lab explains antibody dilutions and how to do them. Review this material before coming to lab.

D. Flowchart Preparation (see Fig. 4)

The following procedures require many different steps, so it is essential to be alert and aware of what step is next and the reason for each one. A flow sheet is a method of organization that can be very helpful in protocols like this. Prior to starting, prepare a flow sheet for each experimental sequence. Use the drawings of the 9-well plate (Fig. 5) to label which wells are to be used for each of the primary antibodies and to plan your rinses and secondary antibody incubations.

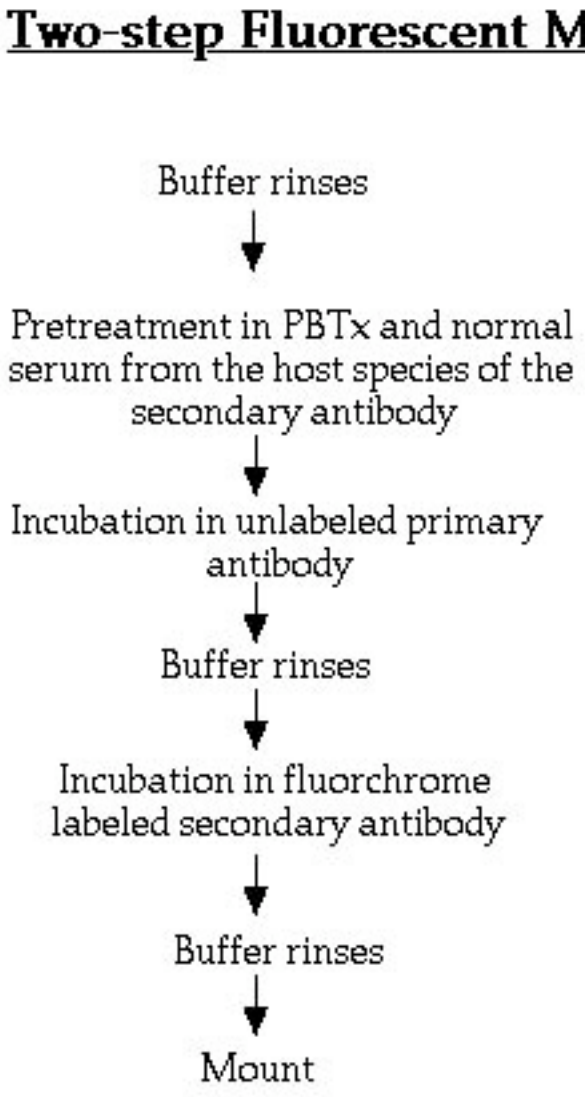


Fig. 4 Condensed flow chart for the Two-step Fluorescent Method.
Modified from Beltz and Burd 1989

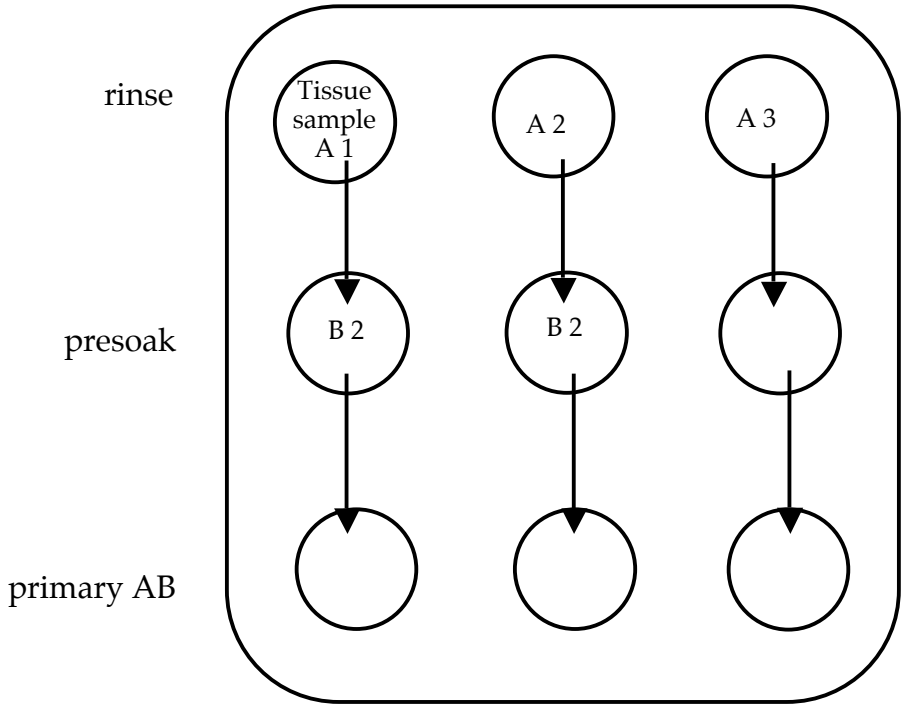


Fig. 5 – Diagram of a 9 well plate for planning incubations.

E. Fluorescent Protocol.

In this experiment one antibody will be used in a one step process (i.e. the fluorescent tag is attached to the primary antibody) and the other in a two-step process. Double labeling is accomplished by incubating tissue in the two primary antibodies, which were raised in different host animals.

The primary antibodies that will be used are:

anti-serotonin--raised in rabbit and anti-BrdU/Alexa 488 (a green fluorophore) --raised in mouse.

The tissue will be incubated in both antibodies sequentially, each at its appropriate dilution. The tissue is then rinsed and incubated in a secondary antibody:

anti-rabbit labeled with Texas Red (a red fluorophore) --raised in goat

Note that these antibodies are generated against IgG of the host animals in which the primary antibodies were raised, and that they have different labels. This feature allows specific labeling of the two different antigens in the same tissues simultaneously.

Use the data sheet on p.11 to record when you did each step.

1. Distribution of tissue samples into a well plate

Divide the crayfish brains into three wells in PBTx in the top row of a 9-well plate:

a. Crayfish brains to be processed for serotonin and BrdU immunoreactivity.
b. "No primary antibody" control for fluorescent secondary antibodies
c. Pre-adsorbed control. Pre-adsorbed serum is prepared using serotonin antibody at working dilution (1/1000) that has been incubated with serotonin antigen (serotonin conjugated to BSA at a final concentration of 10μg/ml.) overnight, and then spun in a high-speed centrifuge. The preadsorbed serum is the supernatant that results after this centrifugation.

2. Incubation in 2N HCL

In order for the anti-BrdU antibody to reach the label, it must be able to penetrate the nuclear membrane, which can be made more permeable with HCl. Fill the second row of wells with the HCl solution, and transfer the pieces of tissue into these wells with forceps. Be careful to handle tissue by the connectives to minimize tissue damage. Soak the brains in 2N HCl for 20 mins.

3. Presoak with Triton

One ml aliquots of non-immune goat serum will be provided. Dilute this with PBTx to make a 5% solution of non-immune goat serum (remember to vortex). Add PBTx to the third row of wells. Rinse three times for 20 mins in 0.1M PBTx + 5% NGS..

4. Primary antibody

TIP: *Antibodies are very expensive, so if very small quantities of tissue are being processed, it is best to use a very small container. 150 µL of Ab should be sufficient to cover brains in the 9 well depression plates.*

a. Place the test brains (not the controls) in the primary anti-BrdU antibody at a dilution of 1:20 in PBTx at room temperature for 2 1/2 hr. ***NOTE:*** Cover the well plate with foil since Alexa 488 (attached to the BrdU) is a light-sensitive fluorophores.

b. Rinse the tissues six times (minimum 15 min/rinse).

c. Place theses brains in anti-serotonin at a dilution of 1:1000 in PBTx.

d. Place the control tissue sample in a "no primary" (PBTx) well.

e. Place the brains for the preadsorbed control the preadsorbed serum.

f. Incubate the tissues in the well plate (covered in foil) overnight, in a refrigerator at 4ºC.

5. Secondary antibody *(during week)*

a. Rinse the tissues

b. Incubate the tissues in the secondary antibody (anti-rabbit-Texas Red [goat], at a dilution of 1:50 in PB. Tissue is incubated in this secondary antibody for 24 hours at 4ºC. Secondary antibodies are supplied in 20 μl aliquots of whole serum and need to be diluted.

c. Rinse tissues at least six times over three or more hours with PB (not PBTx) and leave in PB until mounting.

Preparation for Week 3

For next week's lab you should:

1. ***Complete the rinses described in section 5 above.***
2. ***Make sure you understand the solutions to the sample problems – there will be a dilution problem in the hourly exam.***
3. ***Read about fluorescence microscopy and the confocal microscope.***

BrdU Processing

NOTES

	DATE	TIME	
1. **Incubate specimen in BrdU**	_____	_____	_____
2. **Dissect and fix**	_____	_____	_____
3. **Rinse 6x/ 20min in 0.1M PB**	_____	_____	_____
4. **Soak in 2N HCl -- 20 min**	_____	_____	_____
5. **Rinse 3x/ 20 min 0.1M PBTx + 5%NGS**	_____	_____	_____
6. **mouse α BrdU for 2 1/2 hr at room temp.** *cover with foil*	_____	_____	_____
7. **Rinse 3x/ 20 min 0.1M PBTx**	_____	_____	_____
8. **Rabbit α5HT (1:1000 in PBTx); over night**	_____	_____	_____
9. **Rinse 3x/ 20 min 0.1M PBTx**	_____	_____	_____
10. **2°AB -α rabbit TR (1:50),**	_____	_____	_____
11. **Rinse 3x/ 20 min in 0.1M PB**	_____	_____	_____
12. **Mount in mounting medium**	_____	_____	_____

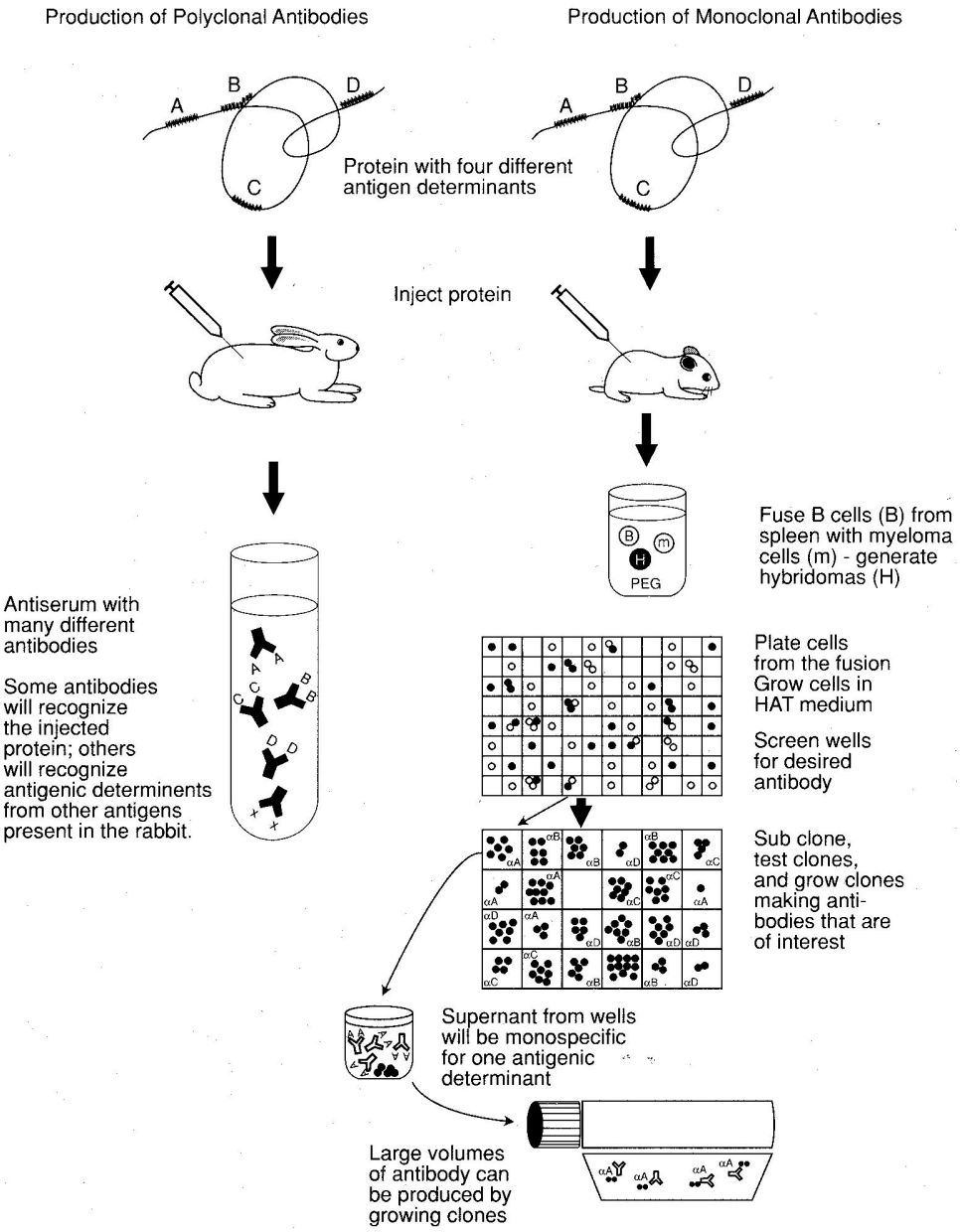


Fig 6 Production of Polyclonal and Monoclonal Antibodies.
Polyclonal and monoclonal antibodies can be generated against proteins with several different antigenic determinants. In the illustration, a protein with four different antigenic determinants (A, B, C and D) is used to inject a rabbit or a mouse. Polyclonal antiserum contains many different antibodies generated against the 4 antigenic determinants. To generate monoclonal antibodies, spleen cells (B) from an immunized mouse are fused with the aid of polyethylene glycol (PEG), to myeloma cells (m). Fused cells (hybridomas. H) that survive will go on to produce clones of cells that secrete antibodies and have the ability to live forever. Using low plating densities, antibody-producing hybridomas are grown in 96 well plates. Wells that test positive during screening with immunocytochemistry on tissue sections or with ELISA are subcloned in 96 well plates at average densities of one cell per well. This and subsequent observations of the clones assures that the cells will be monoclonal. Later clones from positive wells are grown in mass cultures to generate large volumes of monclonal antibodies.

Modified from Beltz and Burd 1989

Solutions

1. Crayfish saline
   Add the following to: 1L(1000ml) ddwater

NaCl	12.0g
KCl	0.4g
$CaCl_2$-$2H_2O$	1.5g
$MgCl_2$-$6H_2O$	0.5g
$NaHCO_3$	0.17g

2. 4% Paraformaldehye solution
   To make 200 ml:
   50 ml dd H_20 at 60°C
   8 g Paraformaldehyde
   100 ml 0.2M Phosphate Buffer (pH 7.4)
   50 ml ddH_20
   Add paraformaldehyde to the 50 ml of 60°C ddH_20 and stir solution for 10 mins. Clear solution with 1 M NaOH. Add remaining water and PB. Filter solution and adjust the pH to 7.4. Store the solution in an airtight container and in the fridge. Use within 1 week of making it. It works much better that way.

3. O.1M PB
   Stock A: (pH 4.5) 26.7 $Na_2HPO_4.H_2O$/1000ml ddH_2O
   Stock B: (pH 9) 28.4 Na_2HPO_4 /1000ml ddH_2O (53 g for heptahydrate)
   Mix one part stock A, 4 parts stock B, for a) 0.2M solution; adjust pH to 7.3-7.4 with A or B. Mix 0.2M buffer with an equal volume of ddH_2O for a O.1M solution.

4. PBTx
   Add 400μl Triton x-100 for every 100 ml 0.1 M PB

5. PBTx NGS
   Add 5% non-immune goat serum (NGS) to PBTx.

6. Mounting Medium
   80% glycerol and 20% 0.1MPB; also commercially available from Sigma

HOW TO MAKE ANTIBODY DILUTIONS

In immunocytochemistry, it is critical that dilutions be accurate and repeatable. It is suggested that adjustable pipettes (for instance, Gilson Pipetmen) be used. Since the greatest accuracy of such pipettes is in their midrange, ideally one should have access to three ranges of pipettes: 5-50 μl, 50-200 μl, and 200-1000 μl. A word of caution about pipettes: be very careful to keep them clean and do not use pipettes for antibody work that are used routinely for other chemicals. Fixatives and other substances can get inside the shaft of the pipette and could contaminate the reagents. Even with pipettes reserved for immunocytochemistry, it is important to prevent fluids from being sucked into the shaft: fluids will corrode the inside of the pipette and destroy the calibration. It is also important to use clean pipette tips to avoid cross-contamination of the antibodies and buffers. If it is not possible to be sure that a tip is clean, it should be discarded. The rule of thumb here is "when in doubt, throw it out!"

By convention, 1:100 means 1 part in a total of 100 parts, or 1 part primary antibody plus 99 parts of the diluent.

There are two ways to make dilutions:

Direct dilution is generally used when diluting an antibody by a small amount (1:10 - 1:50). For instance, when initially aliquoting whole serum, one often dilutes the serum 1:10 in phosphate buffer for long-term storage.

Serial dilutions are used for larger dilutions and are defined as making further dilutions from a previously diluted stock. For instance, to make a 1:10,000 dilution, one might begin by taking 10 μl of a 1:10 stock and adding to that 990 μl (10+990=10/1000) of buffer for 1:1000

$$[1/10]*[10/1000] = 1/1000 = 1:1000$$

If one then took 100 μl of 1:1000, and added to that 900 μl of buffer, this would make 1 ml of 1:10,000 dilution

$$[1/1000]*[100/1000]=1/10,000=1:10,000$$

If you had diluted to 1:10,000 directly from whole serum, you would have used 1 μl of serum, plus 9,999 μl of buffer - and ended up with 10 ml of diluted serum - a very large amount of antibody to use up! Furthermore, it is difficult to accurately measure 1 μl of serum. Therefore, by making a series of dilutions, one can make more accurate dilutions, conserve antibody, and avoid ending up with huge volumes of the final dilution. Additionally, if it is necessary to test a variety of dilutions of a single antibody, it is possible to generate those dilutions in one rapid set of serial dilutions (i.e., 1:100 - 1:500 - 1:1000 - 1:10,000), rather than making up each dilution separately from a 1:10 stock. This saves both antibody and time!

DILUTION EXAMPLES

Example 1. 1 ml of a 1:100 dilution is needed

Add 10 μl of undiluted antibody plus 990 μl of diluent to give a total of 1000 μl or 1 ml.

Example 2A. Many manufacturers sell small samples of serum lyophilized from an original volume of 100 μl. Since the reconstituted volume of 100 μl is difficult to aliquot, the antibody is usually reconstituted to 1:10 with buffer, aliquoted in small volumes into microcentrifuge tubes, capped, and frozen for later use.

If you need 500 μl of a 1:200 dilution of your antibody and the antibody is aliquoted at 10 μl of a 1:10 dilution, how many aliquots will you need for 500 μl at 1:200?

Two ways to solve this are:

1:200	1 μl 'whole' antibody in 200 μl total
or, if antibody is 1:10	10 μl antibody in 200 μl total 5 μl antibody per 100 μl total 25 μl antibody in 500 μl total

Therefore, 3 aliquots of primary antibody at 1:10 gives you 30 μl, more than enough to give you the 500 μl you need.

OR

(X μl) (at 1/10) = (500 μl) (at 1/200 dilution)

$$(X\ \mu l) = (500\ \mu l)\ \frac{(\text{at } 1/200)}{(1/10)}$$

X μl = (500 μl) (0.005/0.1)
X μl = 25 μl

Therefore, 3 aliquots gives 30 μl, enough to yield the 500 μl you need.

Example 2B If you use all 3 aliquots (30 μl at 1:10), how much diluted antibody at 1:200 will you have?

(30 μl antibody) (at 1/10) = (X μl final) (at 1/200)
(30 μl) (0.1/0.005) = X μl
600 μl = X μl final

Therefore, 3 aliquots (or 30 μl) at 1:10 added to 570 μl diluent gives 600 μl of 1:200 diluted antibody.

SAMPLE PROBLEMS: DILUTIONS

1. You are given two antibodies (anti-A and anti-B), and are asked to localize the two compounds in the same tissue section by applying both antibodies simultaneously (double-labeling). You are given 100 μl of whole A serum and told to use it at 1:10. You are given 50 μl of B that has already been diluted to 1:10, and told to use it at 1:100. How would you work out the proper dilution for each? (Note: you may use part of the aliquot if you wish).

2. You are given 10 μl of anti-substance P antibody at a dilution of 1:10 in phosphate buffer and 10 μl of anti-neurotensin antibody at a dilution of 1:5. You want to incubate your tissue in both primary antibodies simultaneously. The working dilutions you need are:

anti-substance P	use at 1:1000
anti-neurotensin	use at 1:200

How would you dilute these antibodies so that each is used at its appropriate dilution when they are placed on the tissue simultaneously? (You do not necessarily need to use up all of each aliquot that is given to you--i.e., you may pipette smaller quantities from the 10 μl aliquot if you wish.)