Laboratory methods

Immunohistochemistry and in situ hybridization

Immunohistochemistry

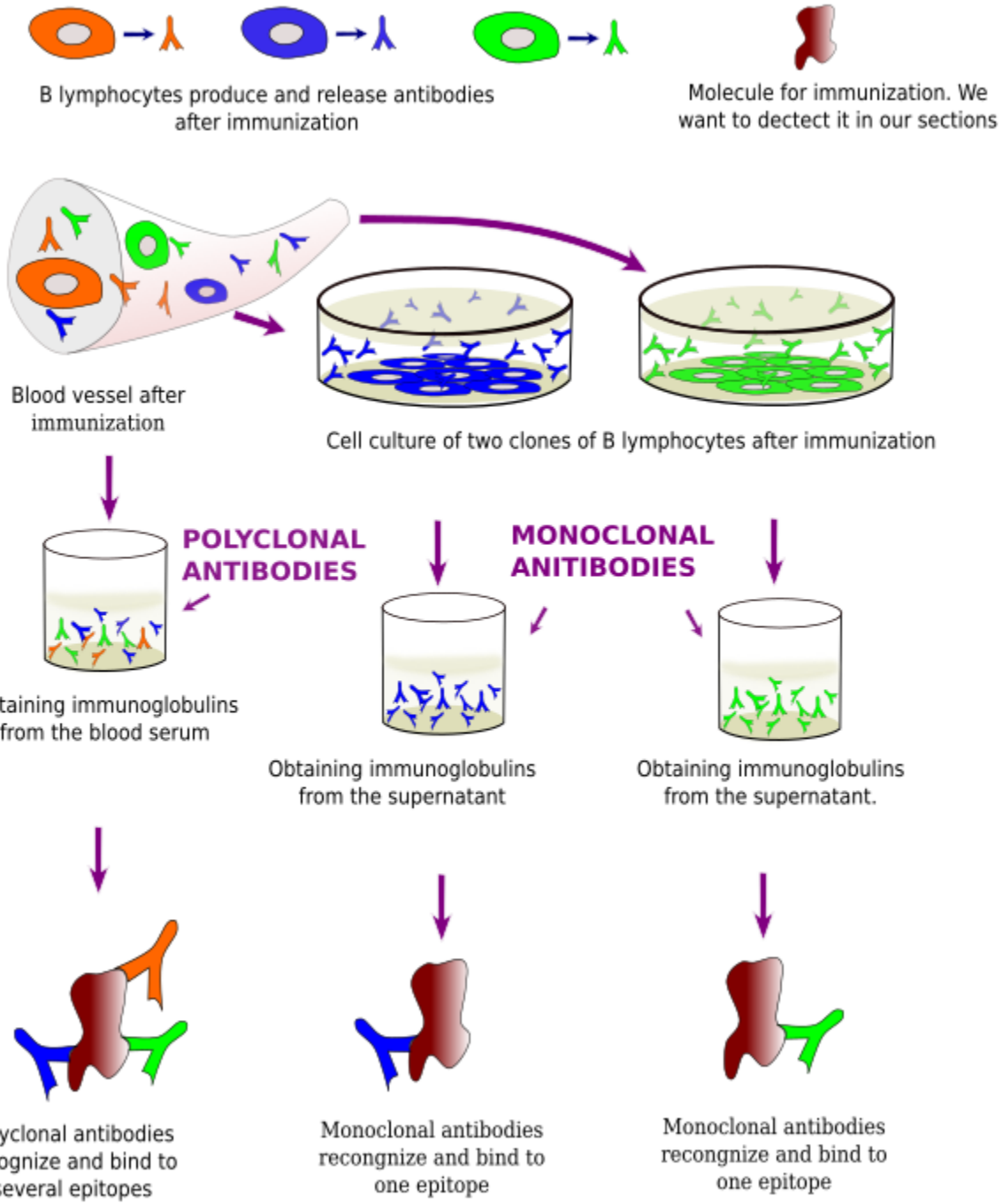
- is a technique for studying the tissular location of **particular molecules** by using **antibodies** as tools.
- a straightforward and powerful lab technique.
- relies on the **high affinity** and **specificity** of the **antibody-antigen** recognition.
- to visualize the antibody-antigen binding, antibodies are conjugated with **enzymes or fluorescent molecules**

Antibodies

- Antibodies used in immunohistochemistry are **G type immunoglobulins**, produced by the **B lymphocytes**
- They are purified from blood serum and used for immunohistochemistry.

B lymphocyte

- Complex molecules like proteins may have several antigenic determinant sites.
- An antigenic determinant activates a B lymphocyte clone, that produces the same type of G immunoglobulin against that molecular domain.
- Immunoglobulins from all activated B lymphocyte lineages after the injection of a complex molecule.
- A polyclonal antibody is the purified serum containing the immunoglobulins from all the activated B lymphocyte clones


B lymphocytes produce and release antibodies after immunization
Molecule for immunization. We want to dectect it in our sections
Blood vessel after immunization
Cell culture of two clones of B lymphocytes after immunization
POLYCLONAL ANTIBODIES
MONOCLONAL ANITIBODIES
Obtaining immunoglobulins from the blood serum
Obtaining immunoglobulins from the supernatant
Obtaining immunoglobulins from the supernatant.
Polyclonal antibodies recognize and bind to several epitopes
Monoclonal antibodies recongnize and bind to one epitope
Monoclonal antibodies recongnize and bind to one epitope

- G immunoglobulins have two molecular domains or regions: variable and crystallizable.
- The variable region (Fab) is responsible for recognizing the antigenic determinant, or epitope, in the foreign molecule.
- Each G immunoglobulin molecule has two equal variable domains, i.e., they recognize the same antigenic determinant, so that each single G immunoglobulin may bind two foreign molecules at the same time.

- If the epitope is modified, the antigen-antibody reaction will be weak or not happening at all because the antibody is not able to recognize the epitope.
- Thus, the fixative and fixation method have to be chosen carefully to preserve the features of the epitope in the tissue.

Immunoglobulins

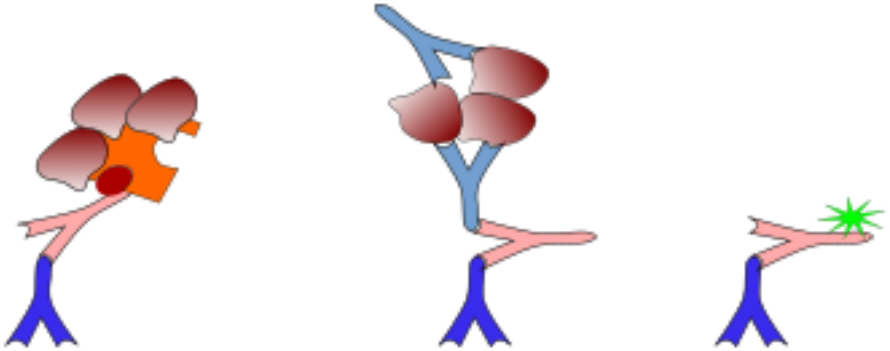
- Immunoglobulins are molecules that cannot be visualized with a light microscope.
- They must be conjugated (bound) to other molecules that make possible visualizing the antigen-antibody reaction.
- There are two types of molecules that are commonly conjugated with antibodies: fluorescent molecules and enzymes.

- Fluorescent molecules can be directly visualized with fluorescent microscopes, while the enzyme activity produces colored and insoluble products that can be observed at light microscopy.
- In both cases, the signal (what we can observe) are located where the antigen-antibody reaction occurred.

- Fluorescent-conjugated immunoglobulins have advantages.
- However, one disadvantage is that the fluorescence of the fluorescent-molecule is exhausted with time.
- On the other hand, sections processed for immunohistochemistry, i.e., using enzymes, can be dehydrated, mounted, and maintained nearly forever.
- **Peroxidase** and **alkaline phosphatase** are the typical enzymes that are conjugated with immunoglobulins.

- Direct immunohistochemistry means that the primary antibody is conjugated with a molecule, either an enzyme or a fluorescent molecule.
- Nowadays, indirect immunohistochemistry is more common. It means that the primary antibody is not conjugated with any molecule and there are other molecules between the primary antibody and the conjugated molecule.

Direct immunohistochemistry. Primary antibody is conjugated.



Indirect immunohistochemistry.
Primary antibody is not conjugated

Primary antibody specific for the molecule we are interested in

Secondary antibody agains the primary antibody

Enzimes: peroxidase alkaline phosphatase

Fluorescent molecule

Biotin

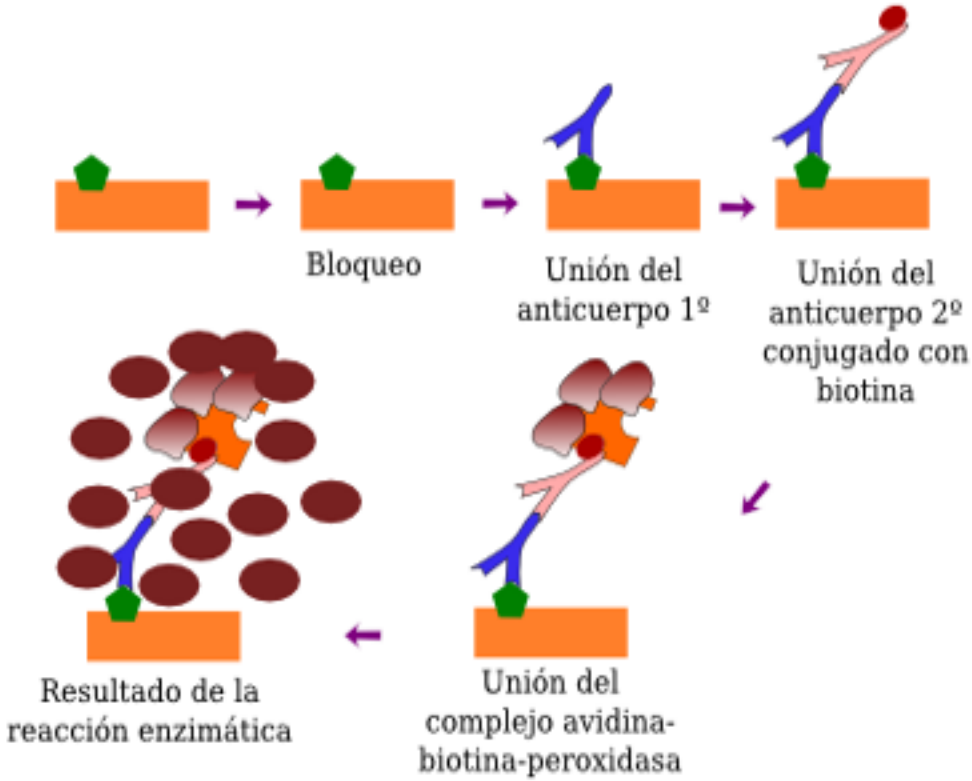
Avidin biotin complex

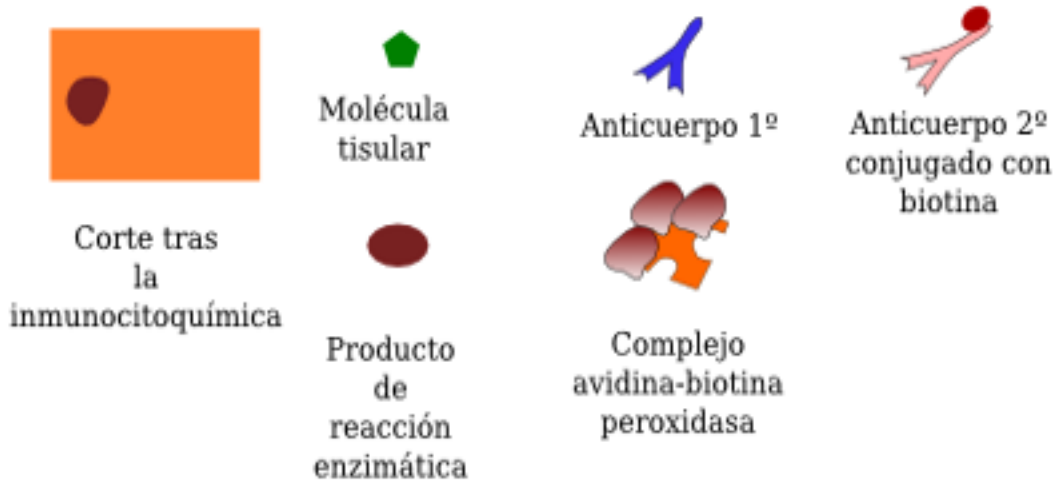
Peroxidase antiperoxidase complex

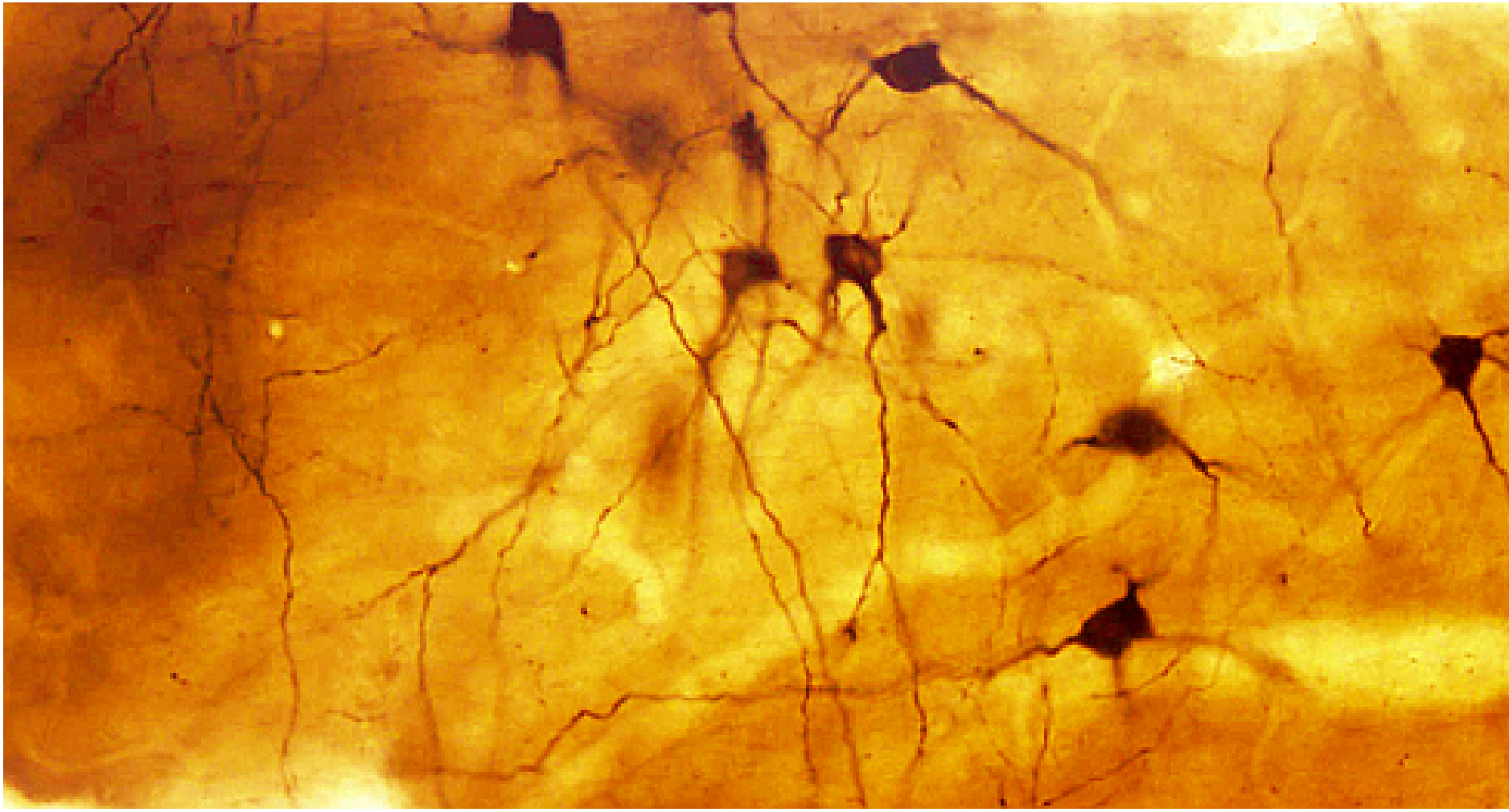
- Nowadays, biotin-conjugated secondary antibody combined with avidin-biotin-peroxidase or streptavidin-peroxidase is the most common method
- Indirect methods provide higher versatility and more sensitivity (more signal for an amount of epitopes).

The general procedure

- The general procedure begins with sections from fixed material.
- Then, sections are incubated in the primary antibody, and subsequently in biotin-conjugated antibody, and in the enzyme complex.
- Finally, the enzyme activity is developed with its substrate and a substance, such as diaminobencidine for peroxidase or nitroblue tetrazolium for alkaline phosphatase, that is reduced and precipitates as a colored complex.

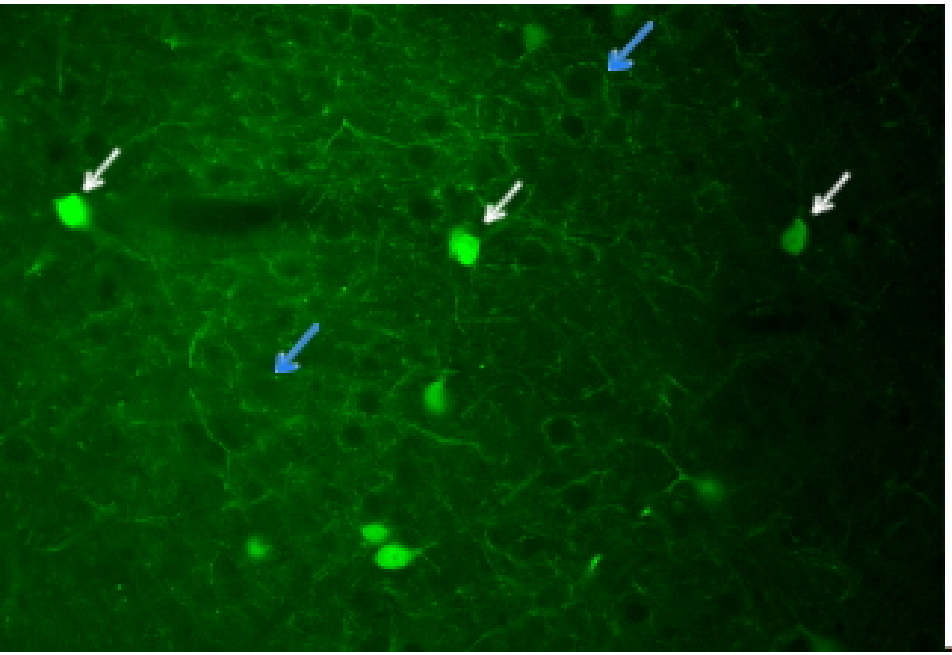

Bloqueo
Unión del anticuerpo 1º
Unión del anticuerpo 2º conjugado con biotina
Unión del complejo avidina-biotina-peroxidasa
Resultado de la reacción enzimática

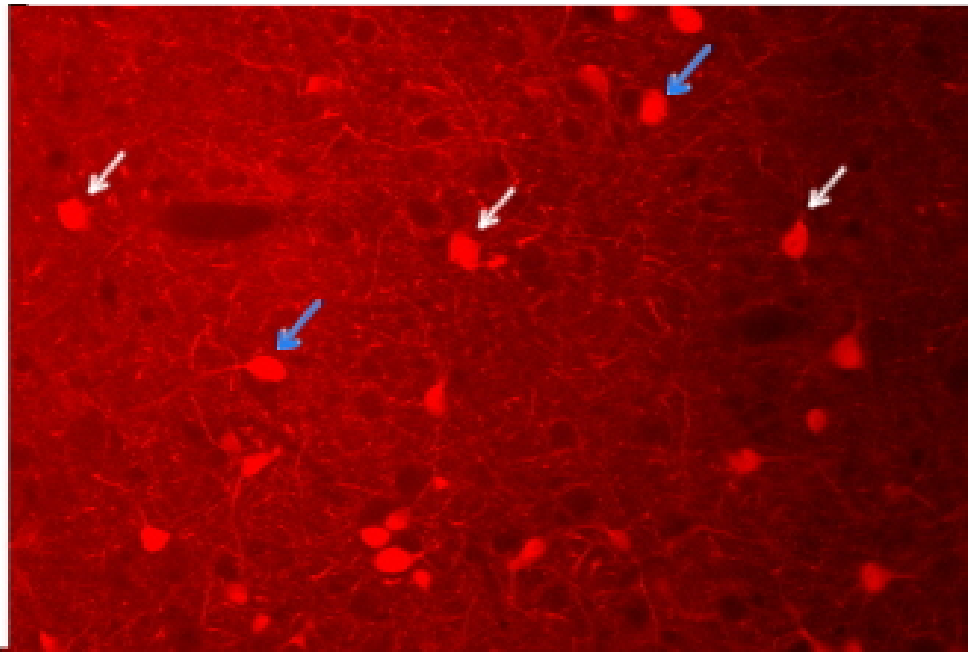

Corte tras la inmunocitoquímica
Molécula tisular
Producto de reacción enzimática
Anticuerpo 1º
Complejo avidina-biotina peroxidasa
Anticuerpo 2º conjugado con biotina

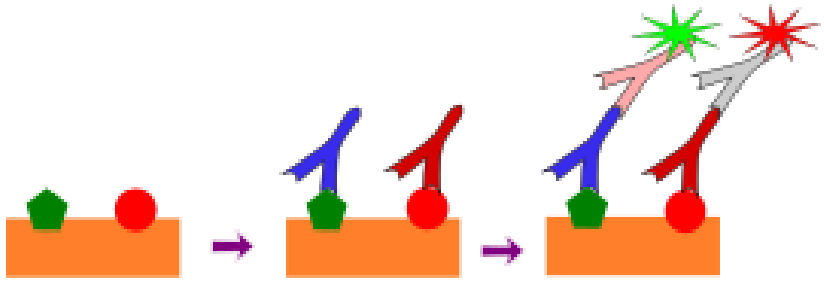


Immunofluorescence

- Immunofluorescence uses fluorochrome-conjugated immunoglobulins.
- is not immunohistochemistry because there is no chemical reaction. It takes advantage of fluorochromes, molecules that emit visible light when stimulated by a specific light wavelength.



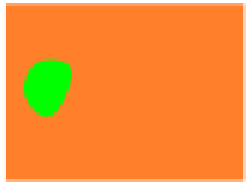




Tissue and molecules

Primary antibodies

Flurochrome conjugated secondary antibodies



Excitation with a specific wavelength for fluorochrome A

Tissular molecule A

Primary antibody obtained in mouse

Goat fluorochrome conjugated secondary antibody against mouse Ig-G

Excitation with a specific wavelength for fluorochrome B

Tissular molecule B

Primary antibody obtained in rabbit

Goat fluorochrome conjugated secondary antibody against rabbit Ig-G

IN SITU HYBRIDIZATION

- is intended to detect a nucleotide sequence of interest by using its complementary nucleotide sequence, called **probe.**
- hybridization between the **nucleotide sequence** and the probe, is the base for the specificity of this technique.
- it can be studied when and where the expression of a **particular gen** is happening in a tissue, by detecting the messenger RNA, which tell us about the cell physiology.
- can also be used for detecting the **physical localization** of a gen in a chromosome.
- is not as widely used in histology labs as immunohistochemistry. However, it provides information about the **physiology behavior of the cell** that cannot be studied with other techniques.

Tissue

- As usual, samples need to be **fixed**.
- It is better to perform hybridization on **frozen sections**, instead of paraffin sections, because the messenger RNA is better preserved.
- after fixation and cryoprotection, samples can be stored **at -80 ºC for a long time**, or samples can be fixed and immersed in methanol and stored **at -20 ºC for months**.
- Single strands of RNA are easily degraded by **RNAases**
- It is mandatory to wear gloves because there is a huge amount of **RNAase in our fingerprints**, and lab-ware needs to be sterilized in an autoclave (not to remove bacteria but to inactivate RNAase).

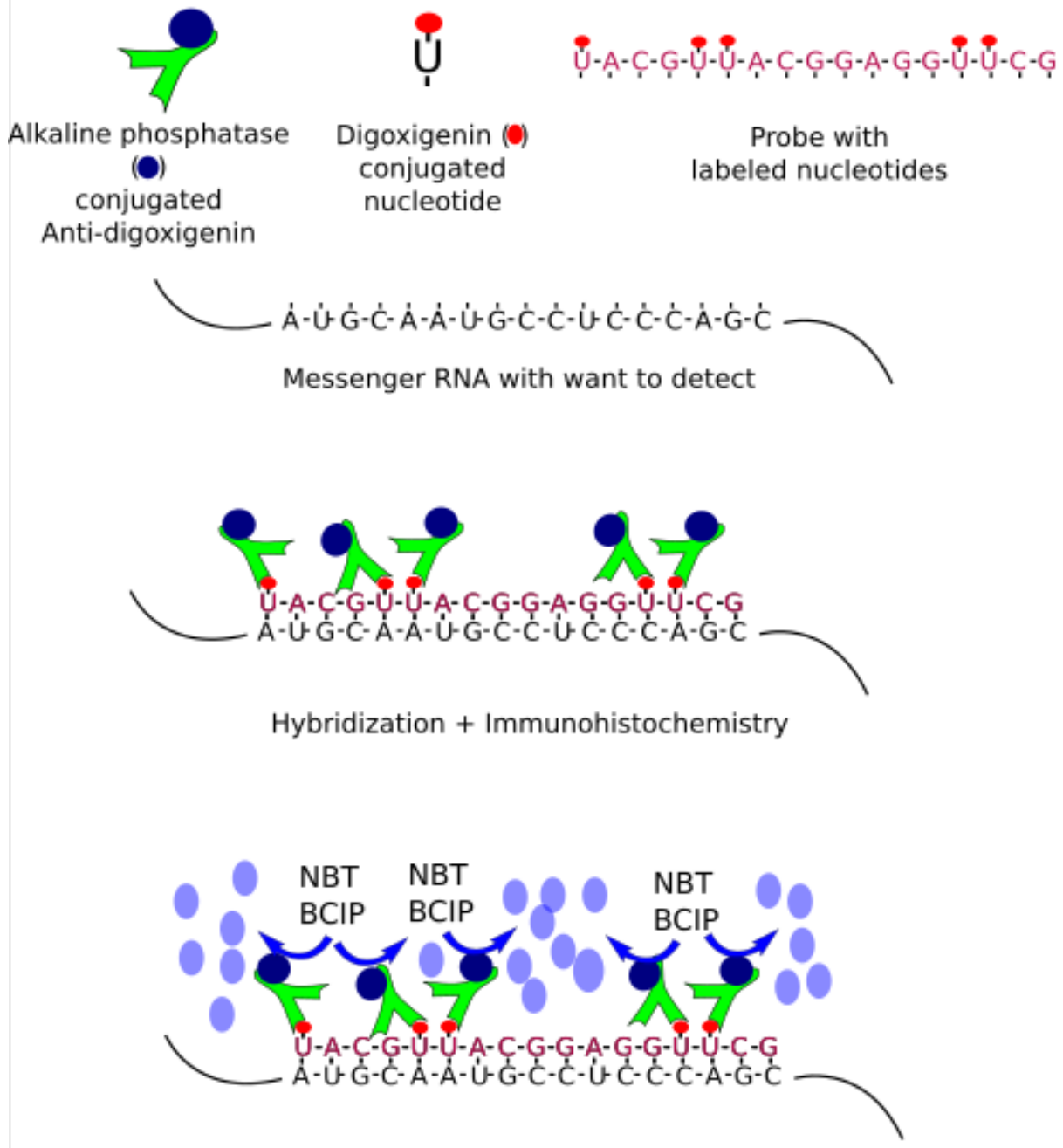
Probe

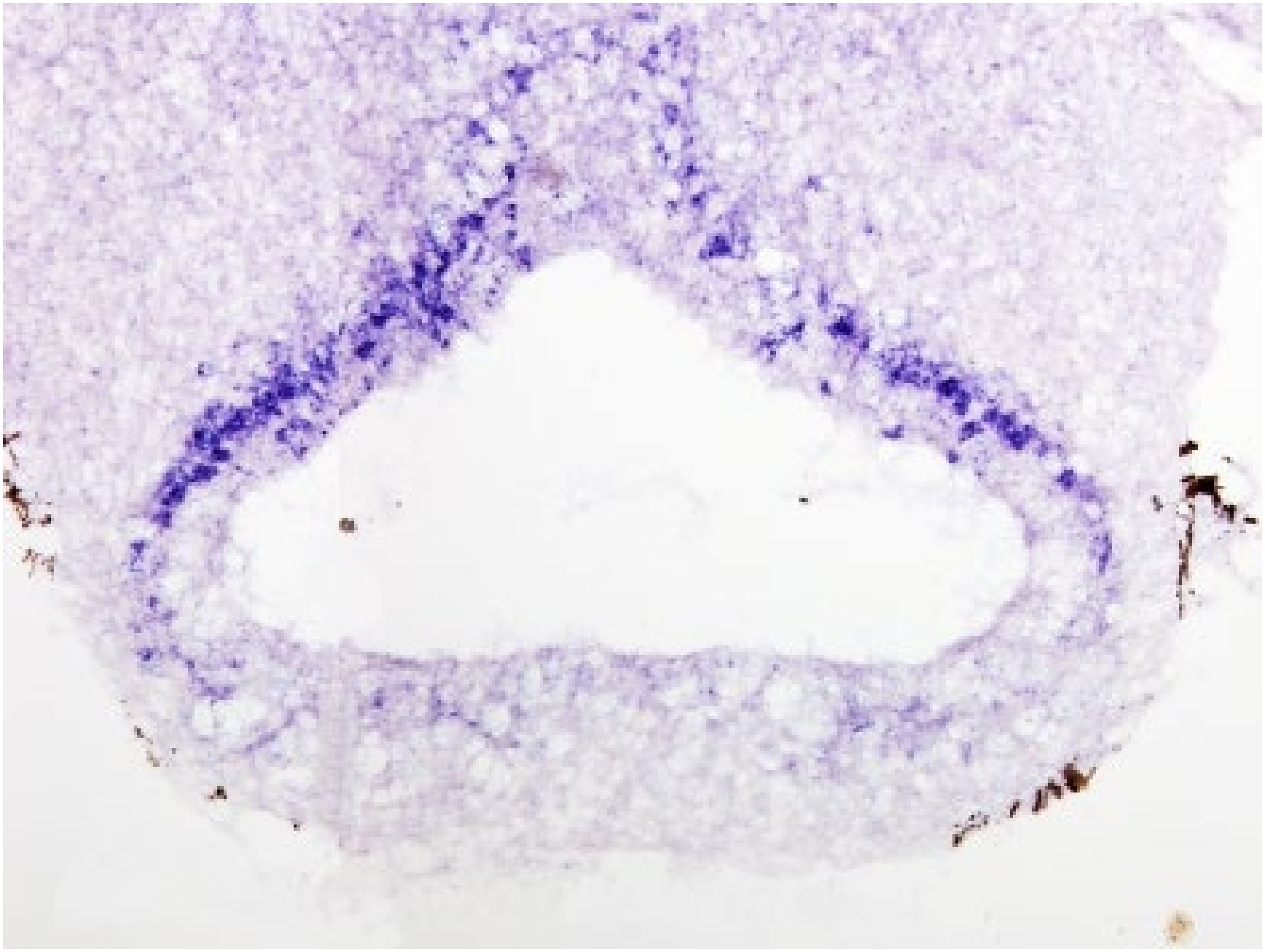
- is based on **complementary** nucleotides (A-T or A-U, and G-C).
- In the same way that immunohistochemistry uses antibodies, in situ hybridization uses a nucleotide sequence, **or probe**, which is complementary to the RNA sequence we want to detect.
- The **size of the probe** (the number of nucleotides that forms the sequence) usually ranges between 50 and 300 nucleotides.
- Probes can be labeled with **radioactive isotopes** (isotopic hybridization) or conjugated with non-radioactive molecules (non-isotopic hybridization).

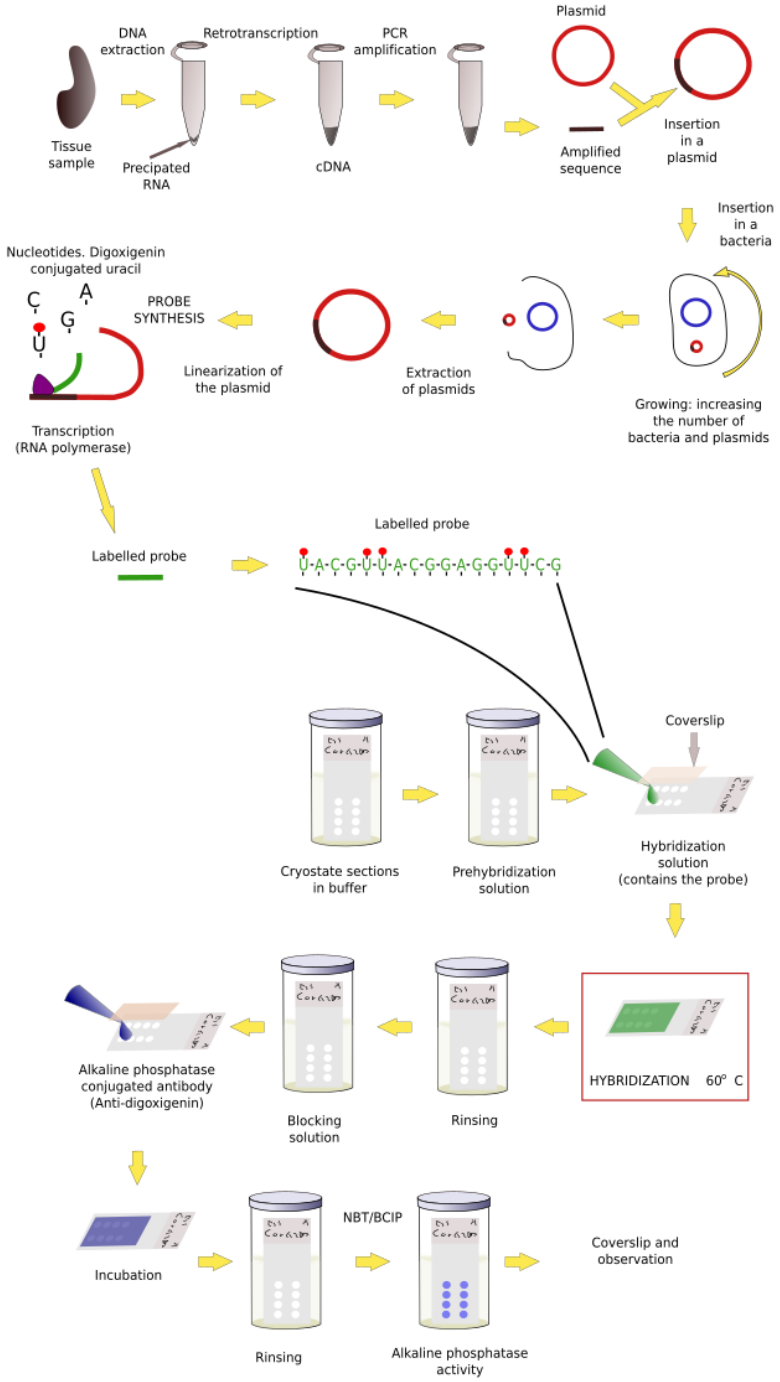
- The first thing to get a working probe is to know the nucleotide sequence of the mRNA with want to detect.
- DNA strands up to 1000 nucleotides are easy to get.
- Probes can be obtained by transcribing these sequences.

Cloning

- 1) Purification of the total RNA of the tissue and retrotranscription of the RNA into **cDNA**.
- 2) By using short sequences (primers), which are specific for our sequence, and the PCR technique, multiple copies of our sequence are obtained. This process is known as **amplification**.
- 3) After purifying the amplified fragments, they are inserted in **plasmids in bacteria**.
- 4) Bacteria are grown and yield many **copies of the plasmid**. Plasmids divide together with the bacteria DNA in each cell division.
- 5) Plasmids are then **purified** from bacteria colonies.
- 6) The sequence is now transcribed, as in during **S phase in normal cells**, so that many RNAs are obtained. During the synthesis, some modified nucleotides are inserted in the probe. These nucleotides are conjugated with molecules, like biotin or digoxigenin that can be detected later by immunohistochemistry
- These RNA labeled **transcribed sequences** are our probes.


Alkaline phosphatase
conjugated
Anti-digoxigenin
Digoxigenin
conjugated
nucleotide
Probe with
labeled nucleotides
Messenger RNA with want to detect
Hybridization + Immunohistochemistry
NBT
BCIP
NBT
BCIP
NBT
BCIP
Alkaline phosphatase reaction




DNA extraction
Retrotranscription
PCR amplification
Plasmid
Tissue sample
Precipated RNA
cDNA
Amplified sequence
Insertion in a plasmid
Insertion in a bacteria
Nucleotides. Digoxigenin conjugated uracil
PROBE SYNTHESIS
Linearization of the plasmid
Extraction of plasmids
Growing: increasing the number of bacteria and plasmids
Transcription (RNA polymerase)
Labelled probe
Labelled probe
Coverslip
Cryostate sections in buffer
Prehybridization solution
Hybridization solution (contains the probe)
HYBRIDIZATION 60° C
Rinsing
Blocking solution
Alkaline phosphatase conjugated antibody (Anti-digoxigenin)
Incubation
Rinsing
NBT/BCIP
Alkaline phosphatase activity
Coverslip and observation