IMPRS NeuroCom

Neurohistology II

Methods of “staining” different Cell types

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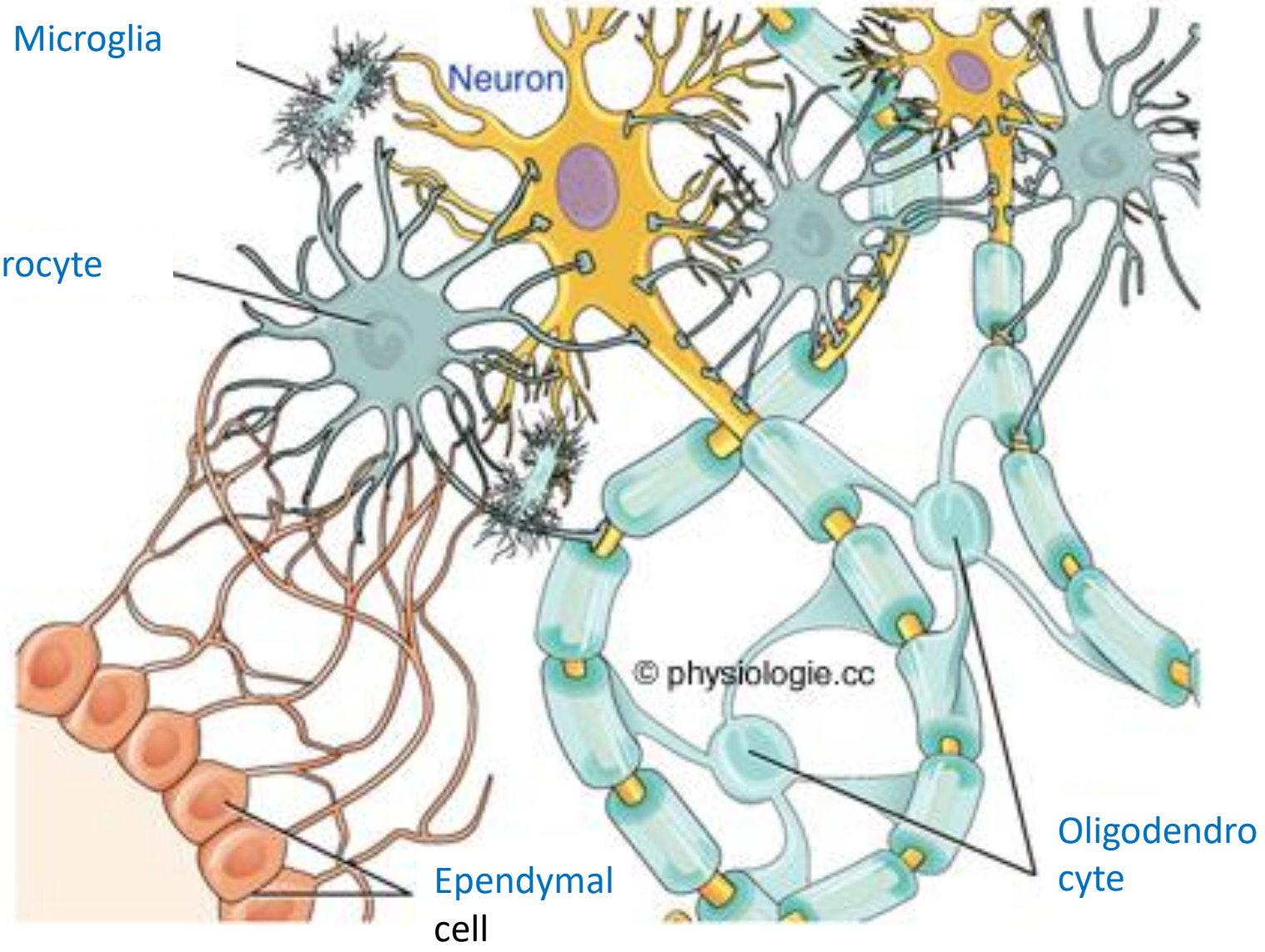
Histology

- Histology (Greek ἱστός histos "tissue" and logic, Gr. Λόγος logos "teaching") is the science of biological tissues. Tissue science is a branch of medicine and biology. Histopathology is the science of abnormal tissue changes.

History of Neurohistology

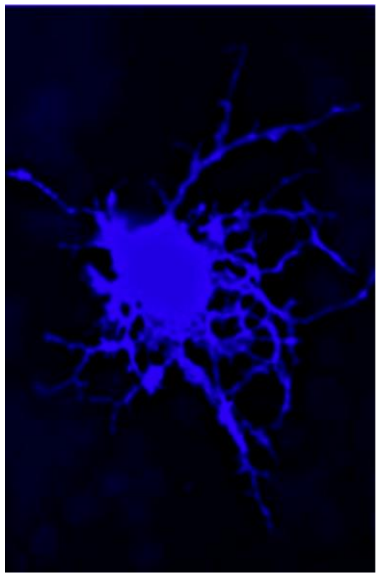
- **Important historical names and techniques in CNS histology are:**
- **First dyeing of tissue by Malphigi around 1650**
- **In 1863 Waldeyer used an extract from the bloodwood tree (Haematoxylum campechianum) for the hematoxylin staining of nerve cells.**
- **Nissl is known for his histopathological brain studies based on the Nissl clods he discovered in 1894 and for the Nissl staining.**
- **The Golgi silver stain was developed in 1873 by the later Nobel Prize winner Camillo Golgi for contrasting nerve tissues; it was later referred to as “la reazione nera” (“the black reaction”).**
- **Nobel laureate Santiago Ramón y Cajal used and modified Golgi stain to further determine neural anatomy. For their knowledge of the structure of nerves, both were jointly awarded the Nobel Prize in Physiology or Medicine in 1906.**

Celltypes of the CNS

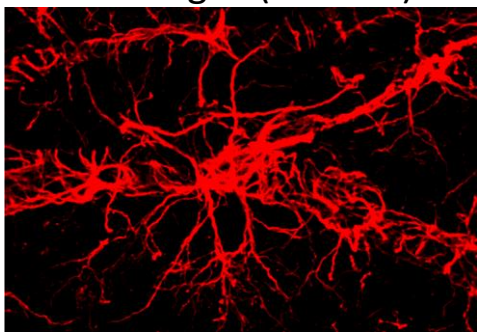


Celltypes of the Brain

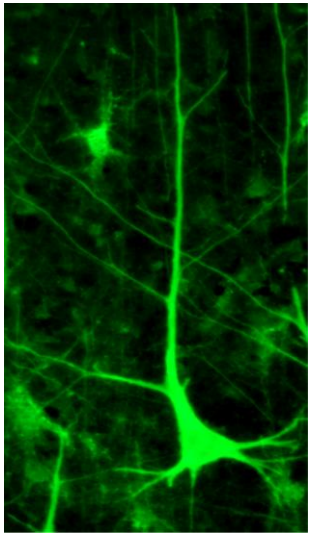
Oligodendroglia (25-35%)

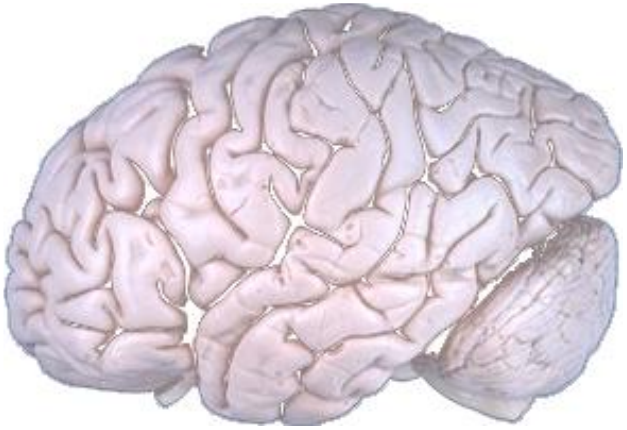


Astroglia (10-20%)

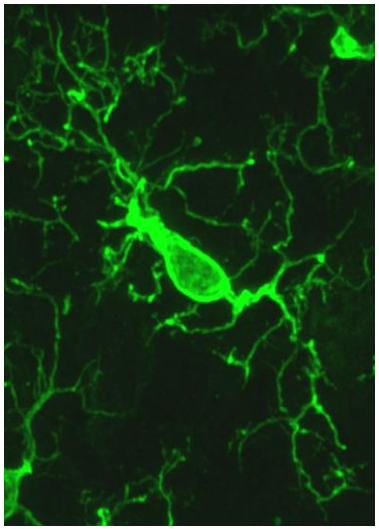


Neuron (40-60%)

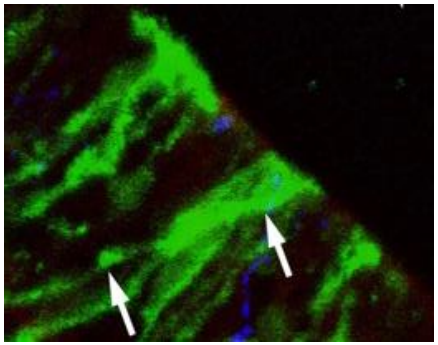




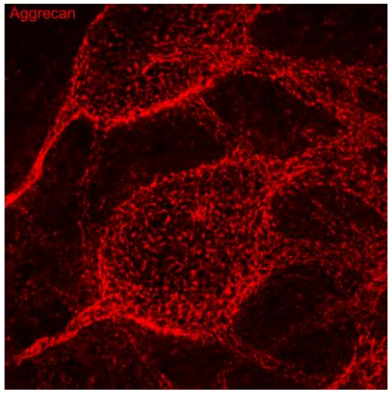
Microglia (2-5%)



Ependymal cells (?%)



Extracellular Matrix (~20%)



Dyes

- The theory of biological staining is mostly based on the ability of certain tissue structures to react to certain dyes. The cell structures and tissues are classified into basophilic, acidophilic and neutrophilic structures based on the coloring behavior of the dyes.

- **Basophilic structures** are, for example, the cell nucleus, the ribosomes and the rough endoplasmic reticulum; they contain acid groups and are therefore stained with **basic dyes** (hematoxylin, iron hematoxylin, azo carmine, methylene blue, toluidine blue, cresyl violet).

- **Acidophilic structures** are the cytoplasm, collagen fibers. These are basic and are therefore colored with **acidic dyes** such as eosin, picric acid and acidic fuchsin.

- **Neutrophil structures** of the cell are not stained by either basic or acidic dyes. They are mainly lipophilic components.

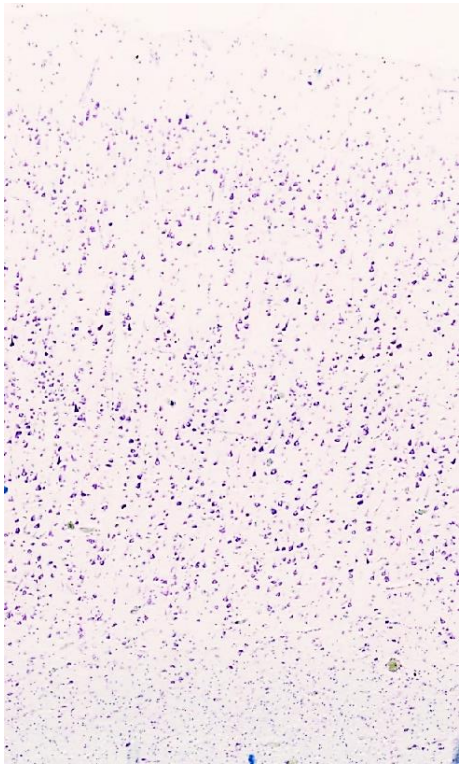
- **Argyrophilic structures** bind silver ions, argent-affine structures bind and reduce silver ions to elemental silver.

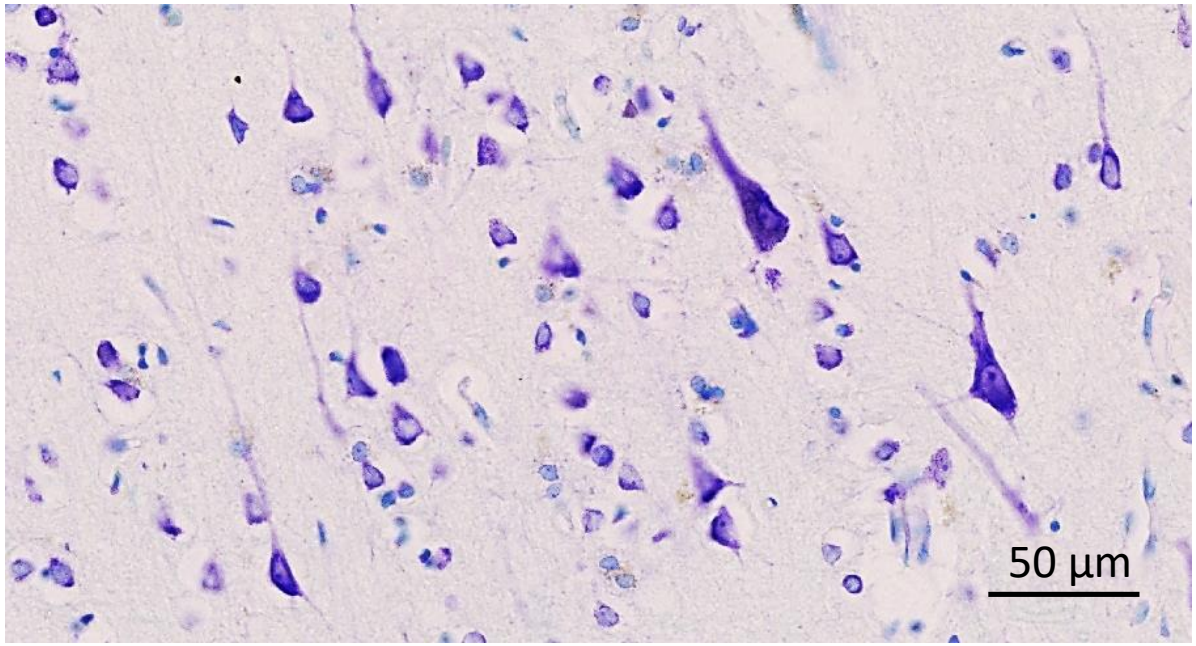
- The main **binding force** is the **ionic bond** (acidic dyes bind basic proteins and basic dyes bind acidic proteins).

Classical Dyes

Most common Neurohistological stains, you should have heared off:

Nissl-Stain:
Staining of the cell body, and in particular endoplasmic reticulum.
This is done by using basic dyes (**cresyl violet**) to stain the **negatively charged RNA blue**, and is used to highlight important structural features of neurons. Individual granules of extranuclear RNA are named Nissl granules (ribosomes). DNA in the nucleus stains a similar color.

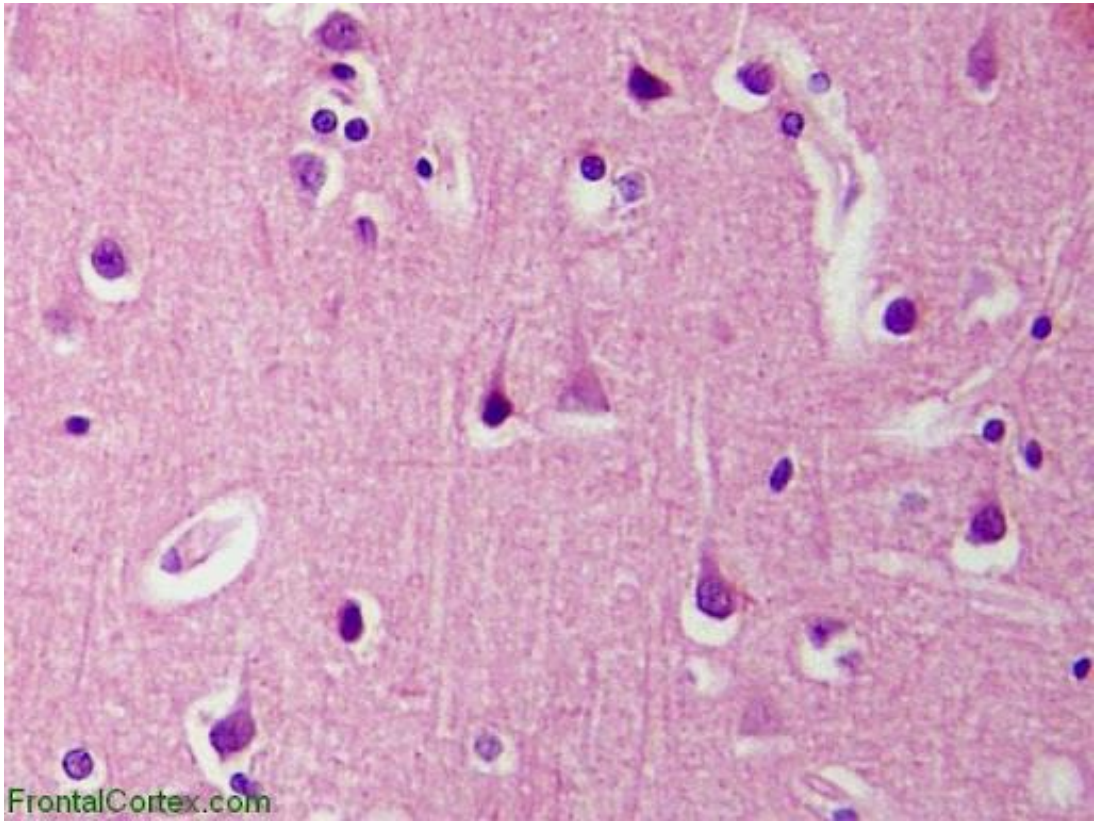




Classical Dyes

- **HE- Stain**
- **Hematoxylin** is a natural dye from the bluewood tree. In order to develop its coloring properties, it must be oxidized to hematein. Alum hematoxylin, a basic hematin varnish, often used as "hemalum" in histological technology, stains all acidic or basophilic structures blue, especially cell nuclei with the deoxyribonucleic acid (DNA) they contain and the rough endoplasmic reticulum enriched with ribosomes.

- **Eosin Y** is a synthetic acidic dye and colors all acidophilic or basic (eosinophilic) structures red, especially cell plasma proteins, mitochondria, the smooth endoplasmic reticulum, collagen and keratin.



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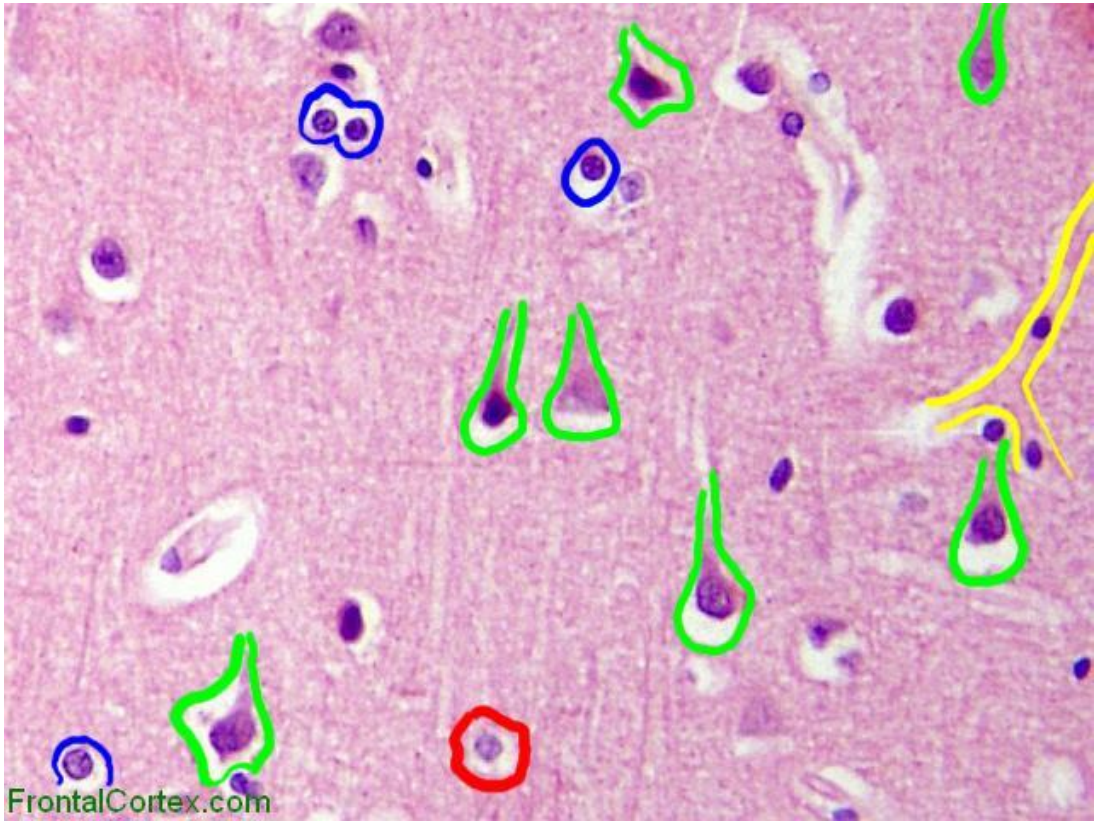
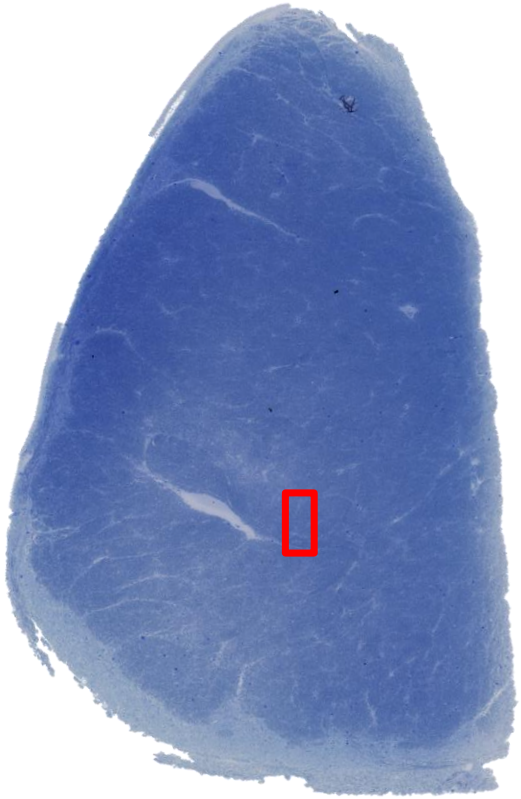
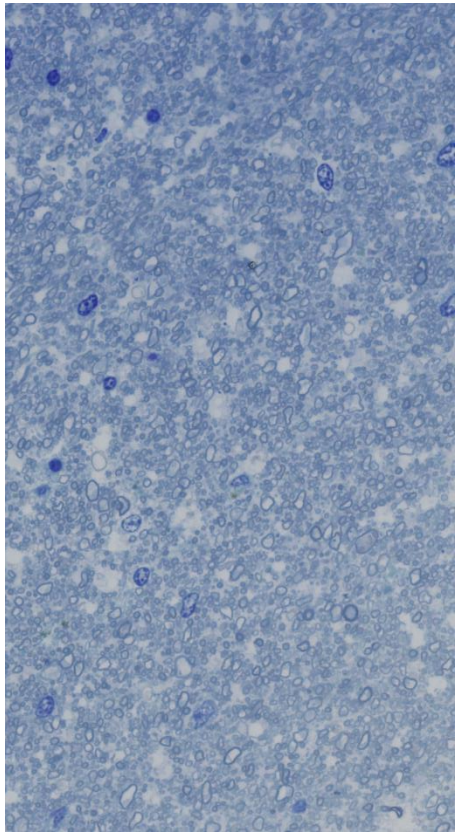


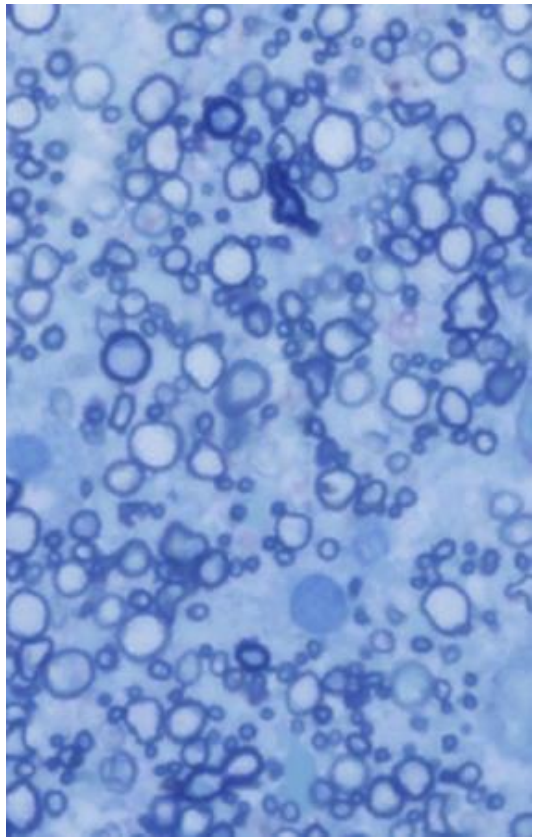
Neurons (green)
Oligodendrocytes (blue)
Capillary (yellow)
Astrocyte (red)

Classical Dyes

- **Toloidine blue:** is a blue cationic (basic) dye used in histology commonly used for staining semi-thin (0.5 to 1 µm) sections of resin-embedded tissue. At high pH (about 10) the dye binds to nucleic acids and all proteins. Although everything in the tissue is stained, structural details are clearly visible because of the thinness of the sections. Semi-thin sections are used in conjunction with ultra-thin sections examined by electron microscopy.

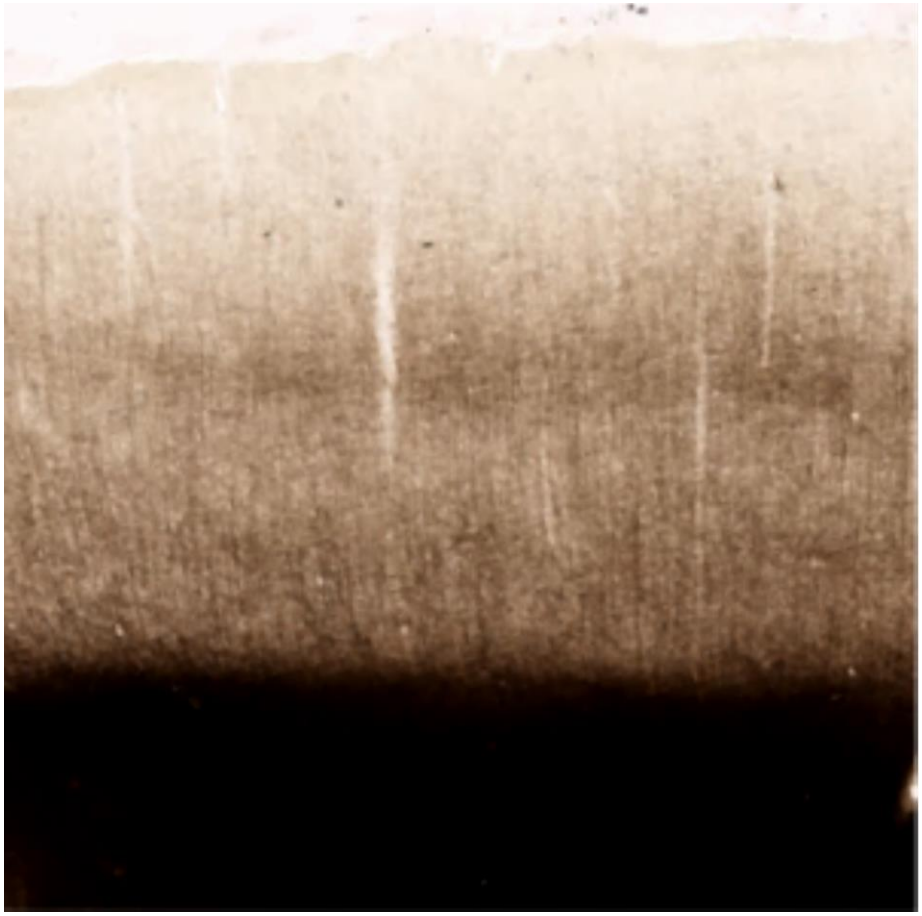






Silver impregnation

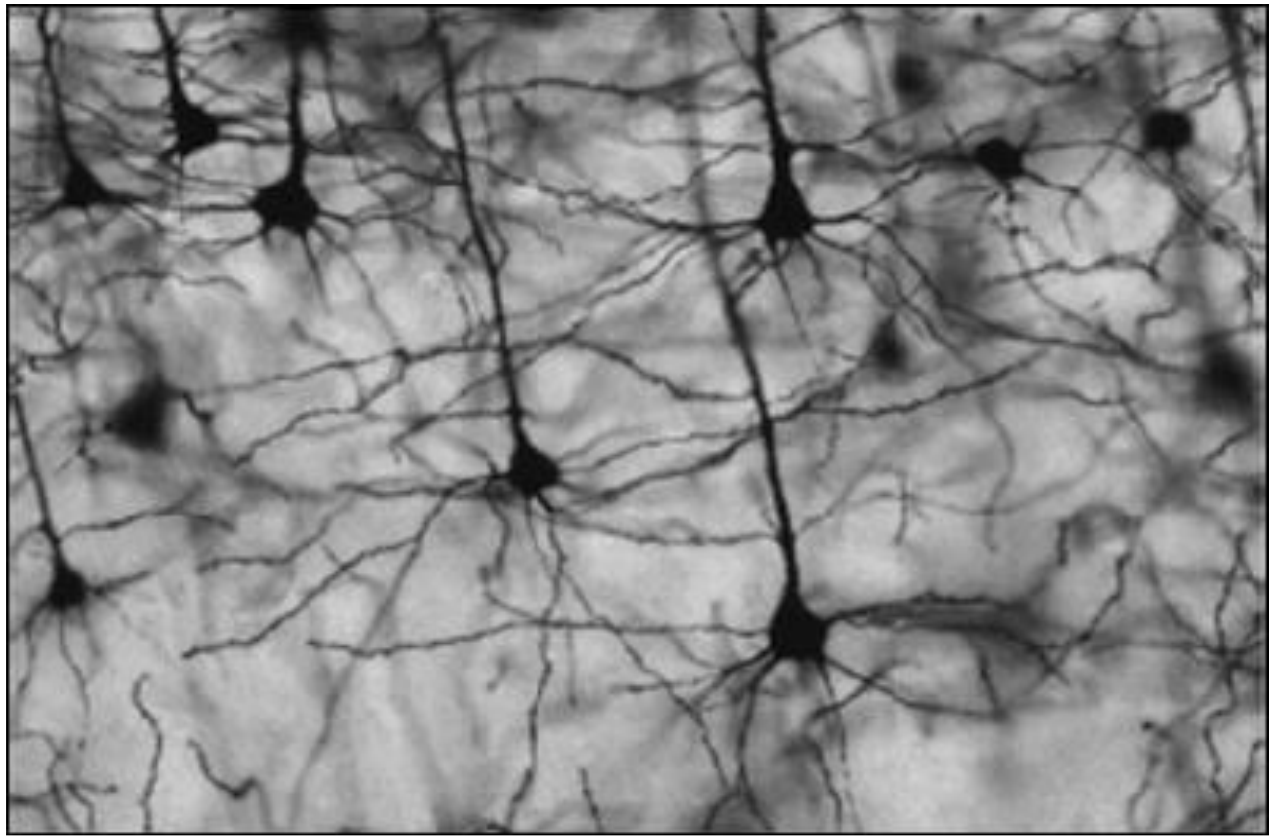
- Gallyas Myelin Silver impregnation

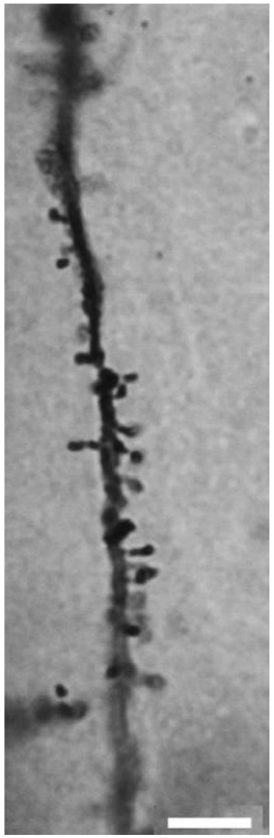


Joseph, S., Werner, H. B. and Stegmüller, J. (2019). Gallyas Silver Impregnation of Myelinated Nerve Fibers. Bio-protocol 9(22): e3436. DOI: 10.21769/BioProtoc.3436.

Silver impregnation

- The **Golgi silver impregnation** technique is a histological procedure that reveals complete three-dimensional neuron morphology. This method is based in the formation of opaque intracellular deposits of silver chromate obtained by the reaction between potassium dichromate and silver nitrate (black reaction)





Comprehensive Review of Golgi Staining Methods for Nervous Tissue
Kang et al., 2017 https://doi.org/10.9729/AM.2017.47.2.63

Enzyme Histochemistry

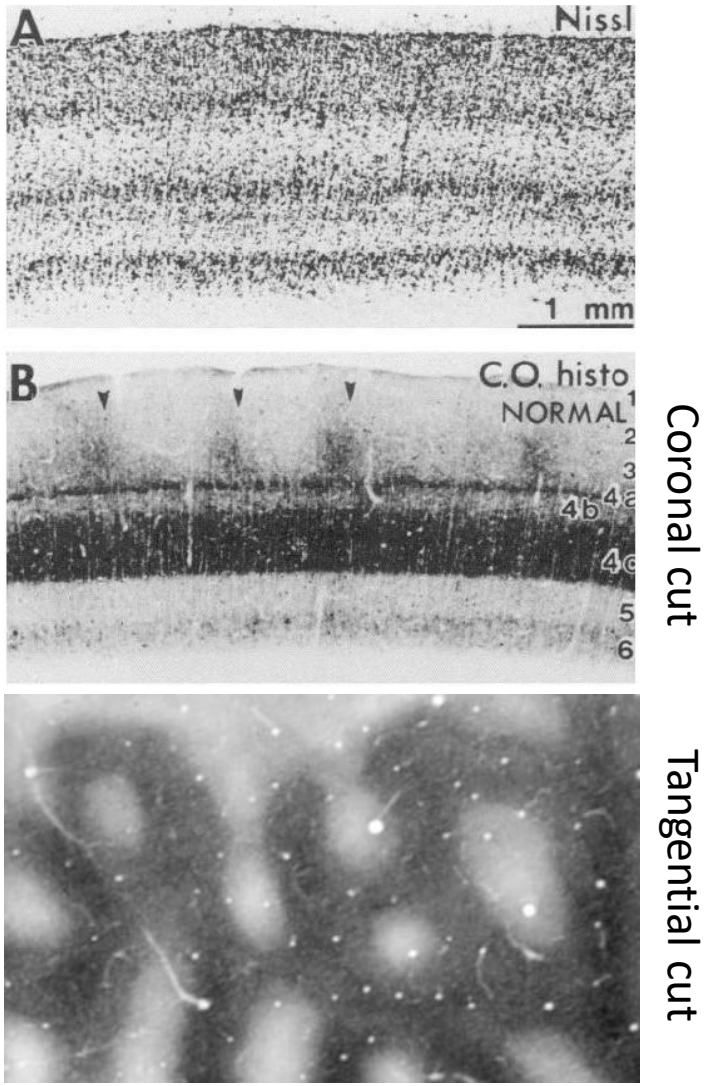
Is based on metabolisation of a substrate provided to a tissue enzyme in its orthotopic localization. Visualization is accomplished with an insoluble dye product.

Cytochrome oxidase : Metabolic enzyme in the mitochondria, useful to reveal the cytoarchitecture of the brain and potentially activity of neurons

NADH diaphorase: Demonstrates mitochondria and the fine detail of the sarcoplasmic reticulum.

Acid phosphatase: Demonstrates macrophages and abnormal lysosomal activity.

Phosphorylase, Adenosine triphosphatase, Acetylcholinesterase and so on



Cytochrome oxidase

Sargaiyan V, Bansal A. Enzyme Histochemistry: A Review. J Adv Med Dent Scie 2014;2(2):191-195.

Immunochemistry

- This classic histology has been supplemented by immunochemistry since the 1980s. Here, the detection of “cell properties” is based on an antigen-antibody reaction. In a one to multi-step technique, the reaction becomes visible through a color reaction at the location of the antigen (protein).

Immunochemistry

- **Principle:**

Visualization and localization of proteins and thus specific structures in cells and tissue sections with the help of labeled antibodies. These should be characterized by high specificity and affinity and should not show any cross-reactions with similar epitopes.

Immuno**cyto**chemistry vs. Immuno**histo**chemistry

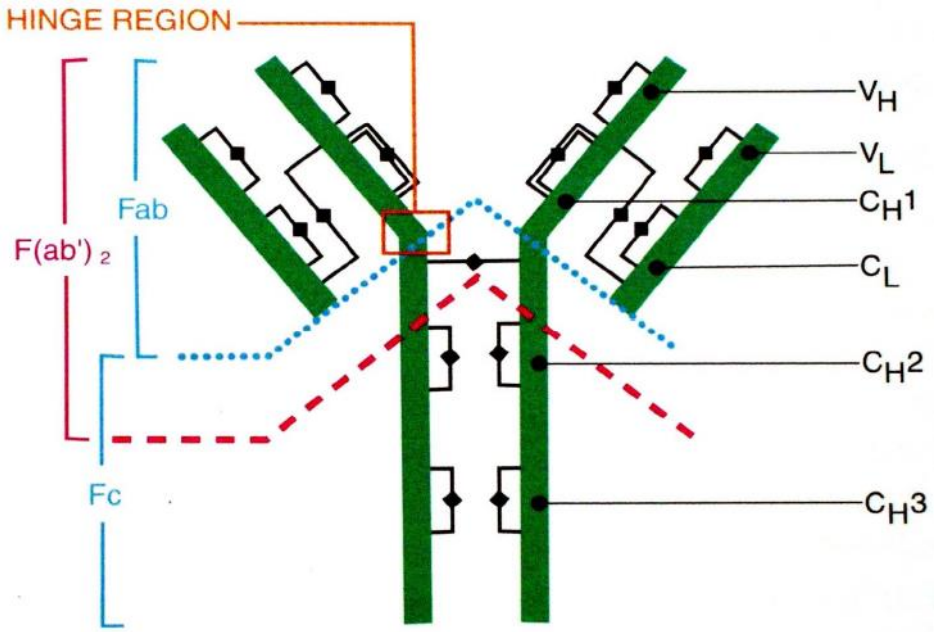
Samples include blood smears, aspirates, swabs, cultured cells, and cell suspensions. surrounding tissue and extracellular matrix Is removed

sections of biological tissue, where each cell is surrounded by tissue and ECM architecture and other cells normally found in the intact tissue

Don‘t mess this up ist often used incorrectly/interchangeably!

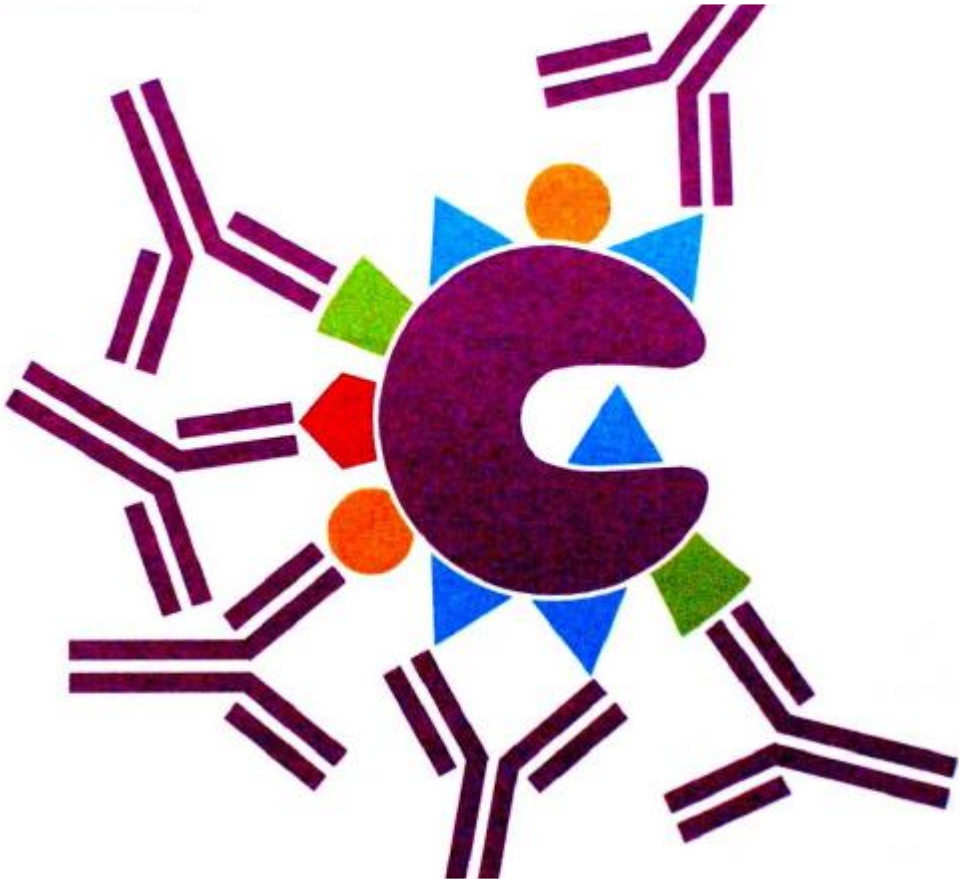
Immunohistochemistry

- Antibody:



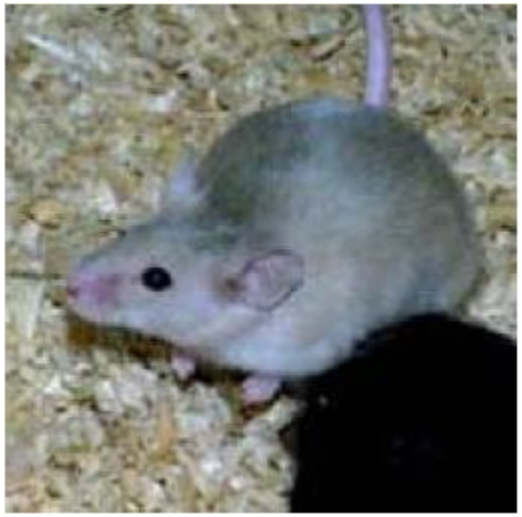
Immunohistochemistry

Polyclonal antibodies (often extracted from rabbit serum)
- Polyclonal antibodies (mix of antibodies, produced by different B-cells in the body against one target) find an antigen via the various epitopes
If one epitope remains masked (formalin), it can still recognize the target over the other epitopes



Immunohistochemistry

Monoclonal antibodies (often mouse)
-You only recognize an epitope of your antigen
-You seem more specific
-Masking mostly necessary



Primary Antibodies

- More than 2,5 million research antibodies on the market at least at the “The Antibody Registry” (https://antibodyregistry.org/search)
- Problem is specificity, comparability and reliability
- Cross validation is strongly needed and positive and negative controls for your experiments

Primary Antibodies

Most common Antibodies for Neurohistology:

Neurons:
Anti-NeuN (neuronal nuclei)
Anti-Hu C/D (Elav family HuC, HuD neuronal proteins)
Anti-Parv/Calb/Calr/Somato (Calcium binding proteins)
Anti-SMIs (pan neuronal filament marker)

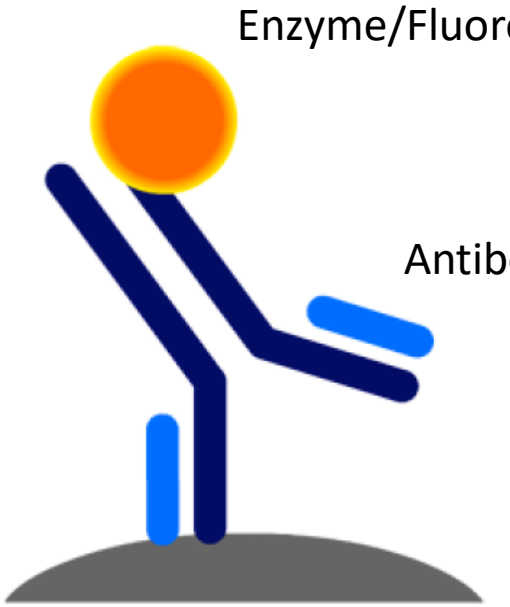
Astroglia:
Anti-GFAP (glia fibrilary acidic protein)
Anti-EAATs (astrocyte-specific glutamate transporter)
Anti-GS (Glutamine synthetase)

Microglia:
Anti- IBA-1 (Ionized calcium binding protein)
Anti HLA (major histocompatibility class (MHC) class II)

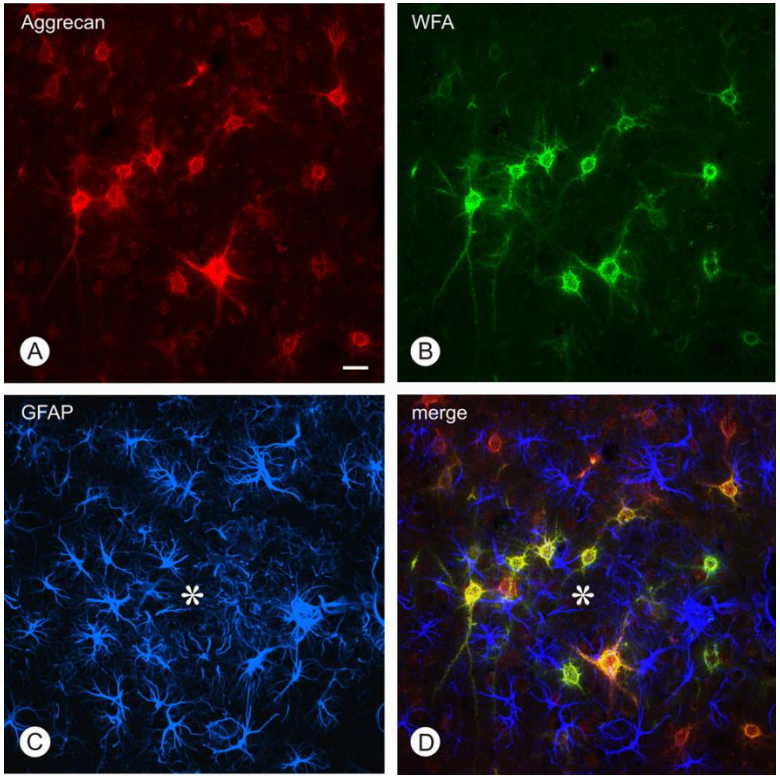
Oligodendroglia:
Anti-Olig (Transcription factors necessary for oligodendrocyte development)
Anti-CNPase (myelin-associated enzyme)
Myelin:
Anti-MBP/PLP (Myelin basic protein/Proteolipid protein)

Immunohistochemistry

- Direct Method:



In the direct method,
the specific antibody for
the protein to be examined is
directly coupled with the enzyme
/ fluorochrome

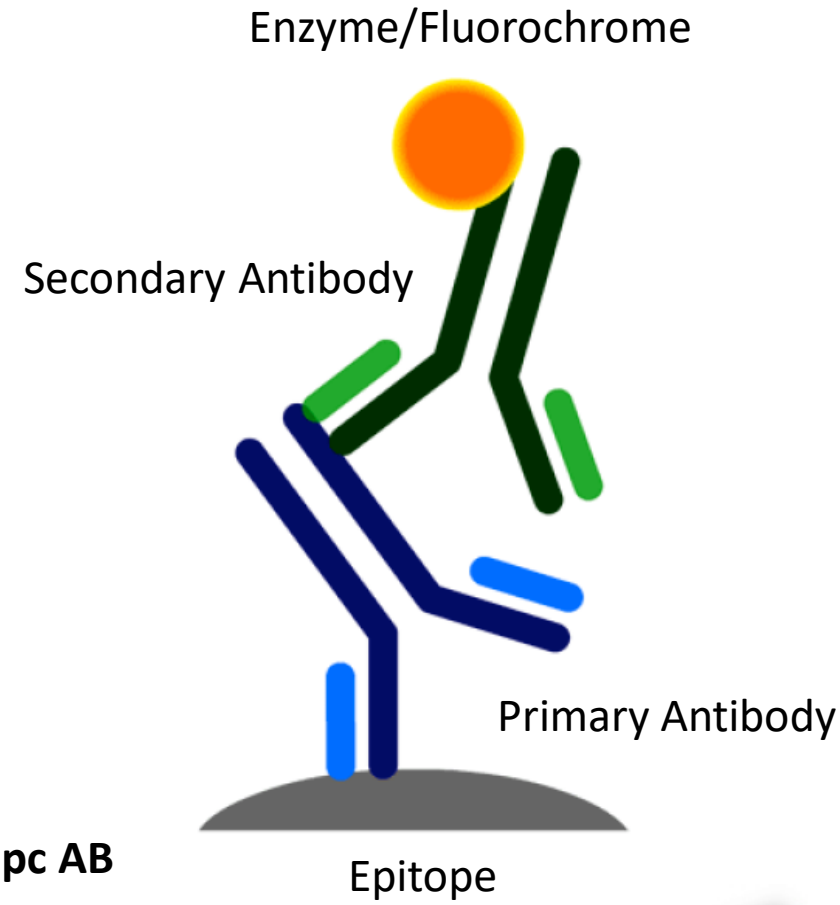


Potentially „quantitative“ in monoclonal AB

Immunohistochemistry

- Indirect Method:

The epitope-specific antibody is unmarked in the indirect method. The staining takes place in a second step in which a second enzyme / fluorochrome labeled secondary antibody is applied to the sample, which specifically binds to the first antibody.
This method is many times more sensitive than the direct method because
several secondary antibodies (and thus several enzymes / fluorochromes) can bind to one primary antibody (signal amplification)



Not really „quantitative“, especially when using pc AB

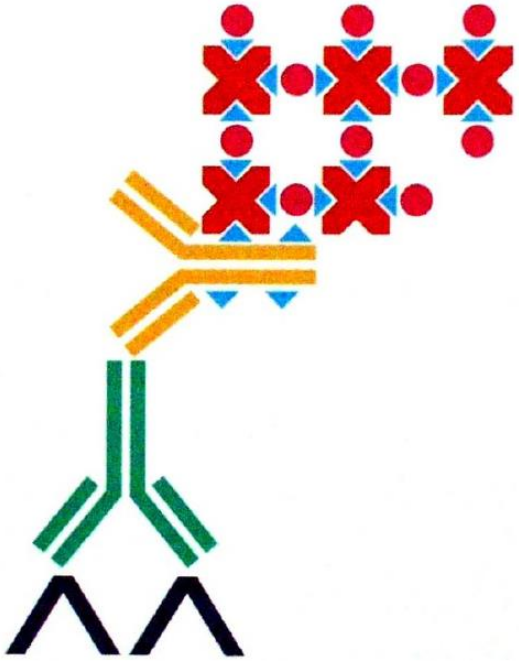
Immunohistochemistry

The indirect method has many different "varieties“

- **ABC Methode:** (Strept)**A**vidin-**B**iotin-**C**omplex**-** Methode
- **PAP Methode: P**eroxidase-**A**nti-**P**eroxidase
- **APAAP Methode: A**lkalische **P**hosphatase **A**nti **A**lkalische **P**hosphatase
- **LSAB Methode: L**abeled (**S**trept) **A**vidin-**B**iotin-**Methode**
- **Envision: Polymerkonjugat-Methode**
- **EPOS: E**nhanced **P**olymer **O**ne **S**tep Staining
- **CAS-Methode: C**atalyzed **S**ignal **A**mplifikation

Immunohistochemistry

- **ABC method: (Strept) Avidin-Biotin-Complex**
- 1. Unconjugated primary AK binds to epitope
- 2. Biotin-labeled (biotinylated) secondary antibodies
- binds to primary AK
- 3. Enzyme-labeled (strept) avidin-biotin
- reacts with biotinylated secondary AK



- Common enzymes:
- Horseradish Peroxidase (HRP)
- Alkaline Phosphatase (AP)

Immunohistochemistry

- Indirect / ABC method:
- The last step in the above methods is visualization by adding a chromogen (substrate). The enzyme (e.g. peroxidase) converts the colorless chromogen into a color **complex.**

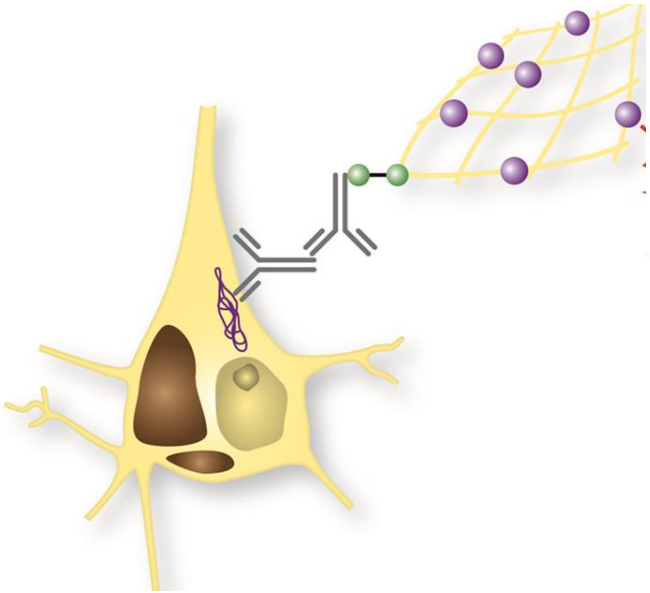
Immunohistochemistry

- DAB-Reaktion:

$$\underbrace{HRP + H_2O_2 + \text{Electron donor}}_{(\text{Substrat} + \text{Chromgen})} \xrightarrow{\text{Oxidation}}$$

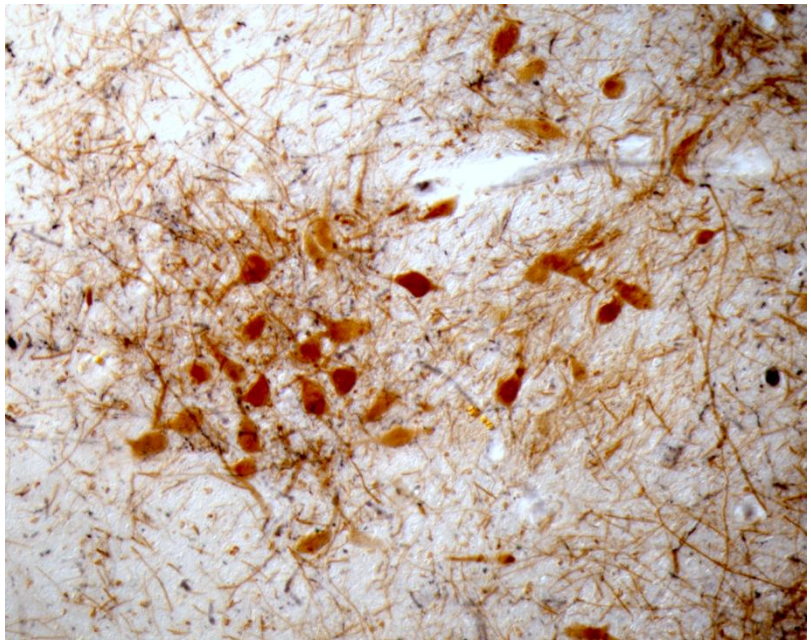
$$\underbrace{HRP - H_2O_2 + \text{Electron donor}}_{(\text{Chromogen})} \longrightarrow$$

Colored Endproduct $+ HRP + H_2O$

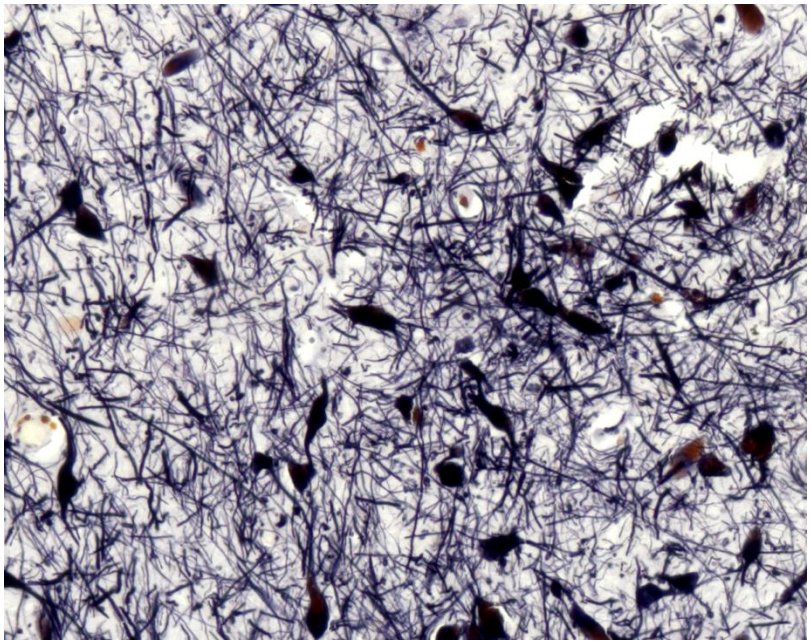


DAB-Reaction:

Anti-Tyrosine hydroxylase / DAB



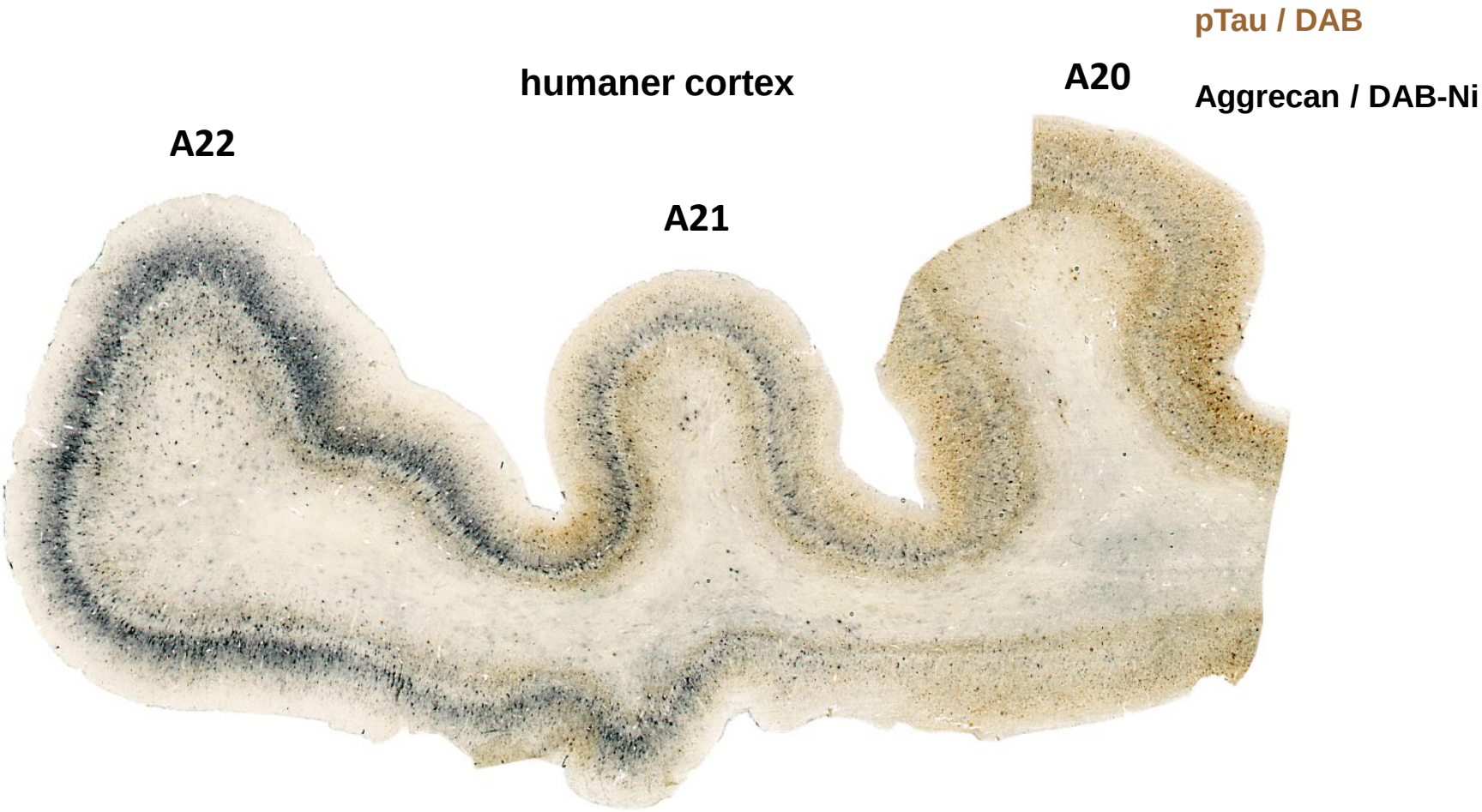
Anti-Tyrosine hydroxylase / DAB-Ni(ckel)



Dopaminergic neurons human substantia nigra

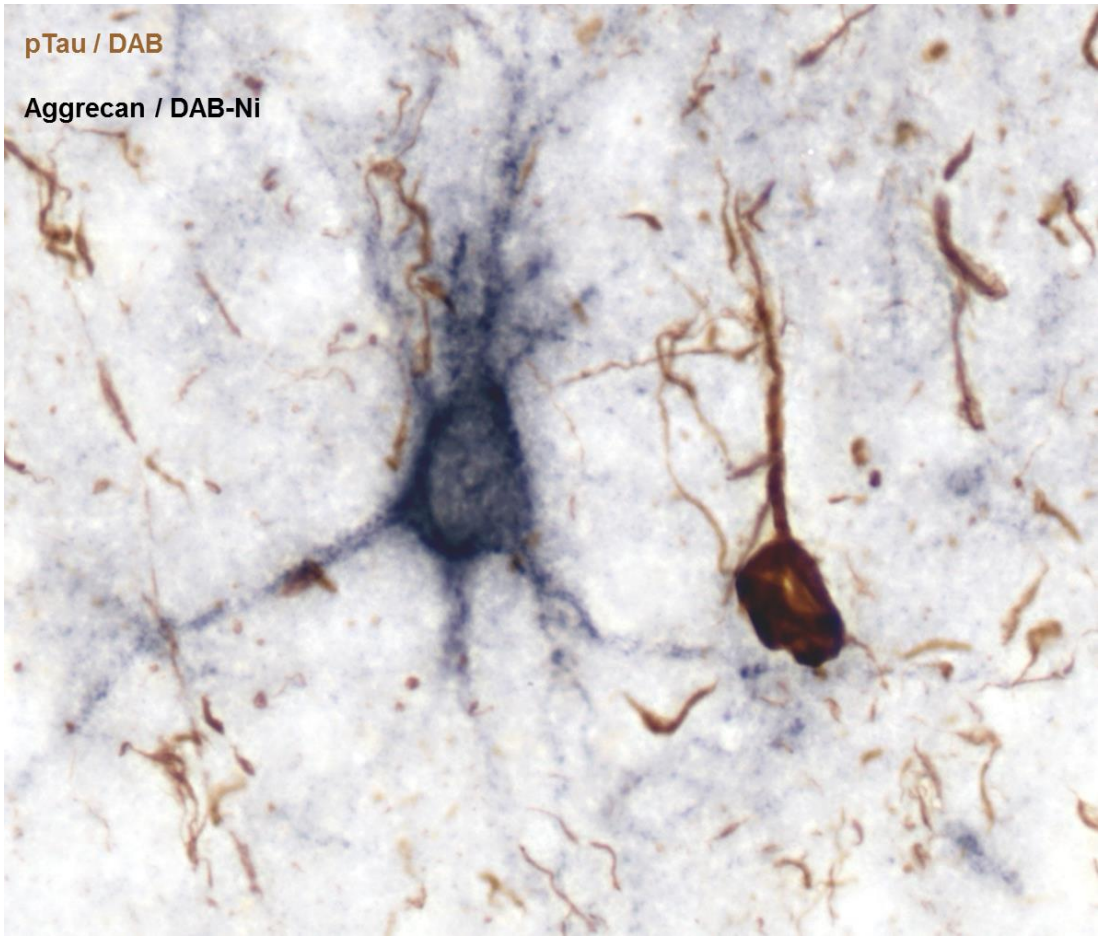
DAB-Reaktion:

Combination DAB + DAB-Ni(ckel)



DAB-Reaction:

Combination DAB + DAB-Ni(ckel)



DAB vs. Fluoreszenz