IMMUNOLÓGIAI ÉS BIOTECHNOLÓGIAI INTÉZET

Immunohistochemistry, fluorescent microscopy

Basic Immunology

University of Pécs, Clinical Center
Department of Immunology and Biotechnology
Pécs

Direct immunohistochemistry practice

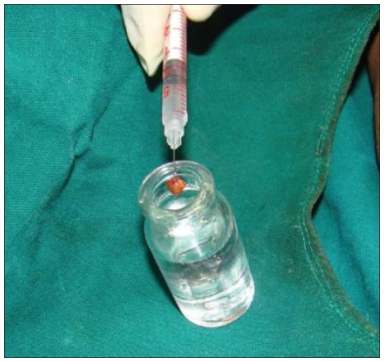
Steps of the practice:

1. Isolation of murine spleen, tissue section and fixation. (already done)
2. Inhibition of **endogenous peroxidase** enzyme activity with phenylhydrazine (**POISONOUS**) dissolved in PBS for 10 minutes.
3. Wash with PBS for 2x2 minutes. (PBS: phosphate buffered saline)
4. **Block non-specific protein binding sites** with 5% BSA-PBS solution of 10 minutes. (BSA: bovine serum albumin)
5. Label the **antigen** (murine Thy-1, T cell marker) with HRP-conjugated anti- mouse Thy-1 **monoclonal antibody** for 30 minutes.
6. Wash with PBS for 3x2 minutes.
7. **Add the chromogen** (AEC: amino ethylcarbazole, **POISONOUS**) in the presence of hydrogen peroxide (substrate) dissolved in 0,1 M Na-acetate buffer. (pH 5.2)
8. View the tissue slides with a microscope.

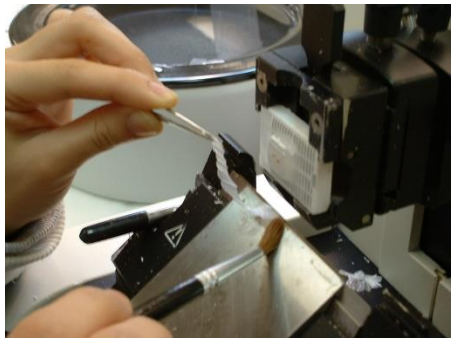
WEAR GLOVES!

Immunohistochemistry 1.

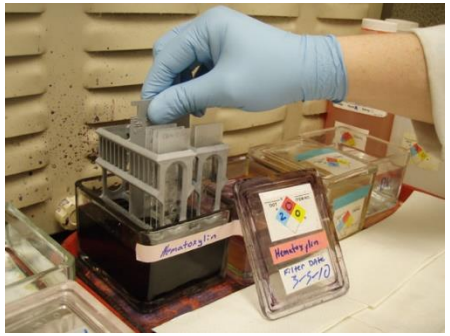
1. Why is it called „histo”? → **histology** (you have already seen such tissue slides)



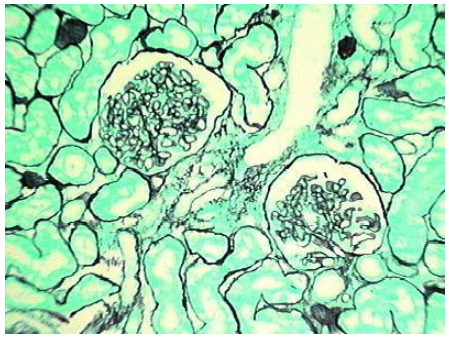
1. Obtaining a tissue sample



2. Cutting sections of the tissue

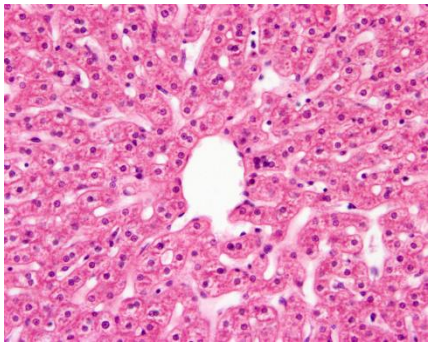


3. Staining of the slides



Glomeruli (Gömöri’s silver impregnation)

4. Examining the slide under a microscope

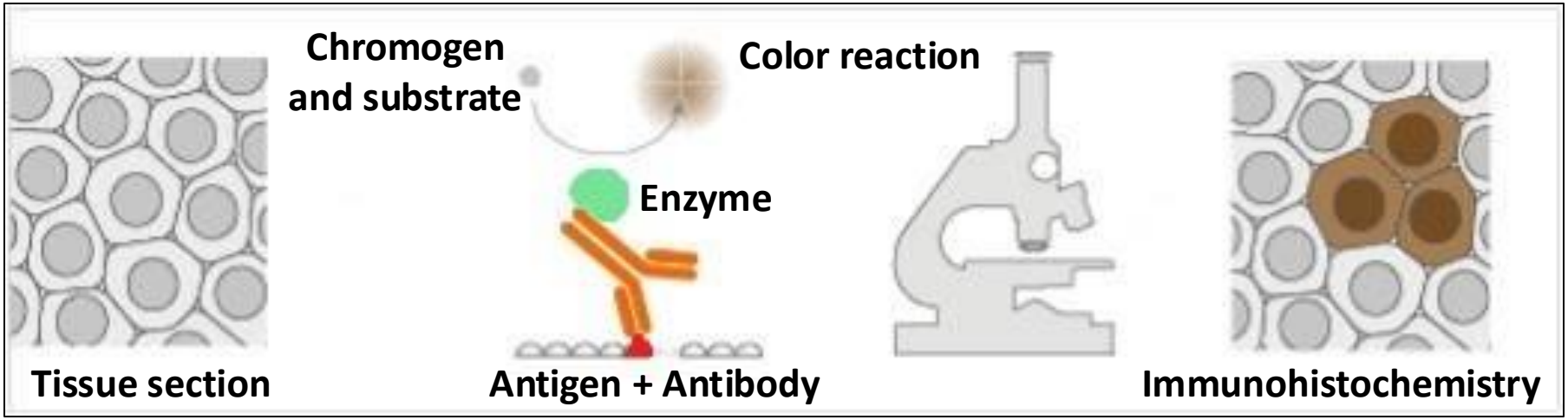


Liver (H&E staining)

Immunohistochemistry 2.

2. Why is it called „histochemistry”?
   - Amalyzes the chemical composition of tissues.[1.]
   - E.g. in the case of H&E staining:
     - hematoxylin → binds to acidic molecules („basophilic” e.g. DNA in the cell nucleus)
     - eosin → binds to basic molecules („eosinophilic” e.g. proteins in the cytoplasm, collagen in the extracellular matrix, etc.)
3. Why is it called „**immuno**histochemistry” (IHC)?
   - It is based on the **antibody-antigen reaction.**
   - Goal: Detection of an **antigen** in the tissue with the use of an **antigen-specific antibody**. (on the cell surface, inside the cells or in the extracellular space)
   - The antibody-antigen reaction is **undetectable on its own**, but can be visualized by conjugating **reporter molecules** to the antibodies. (see in the previous practice)
   - In the case of enzyme immunohistochemistry the reporting molecule is an **enzyme** which turns the chromogen to an **insoluble and colored end-product** that can be visualized under the microscope once the **chromogen** and the **substrate** of the enzyme were added.
   - In the case of fluorescent IHC the reporting molecule is a fluorochrome. (see later)

Enzyme immunohistochemistry

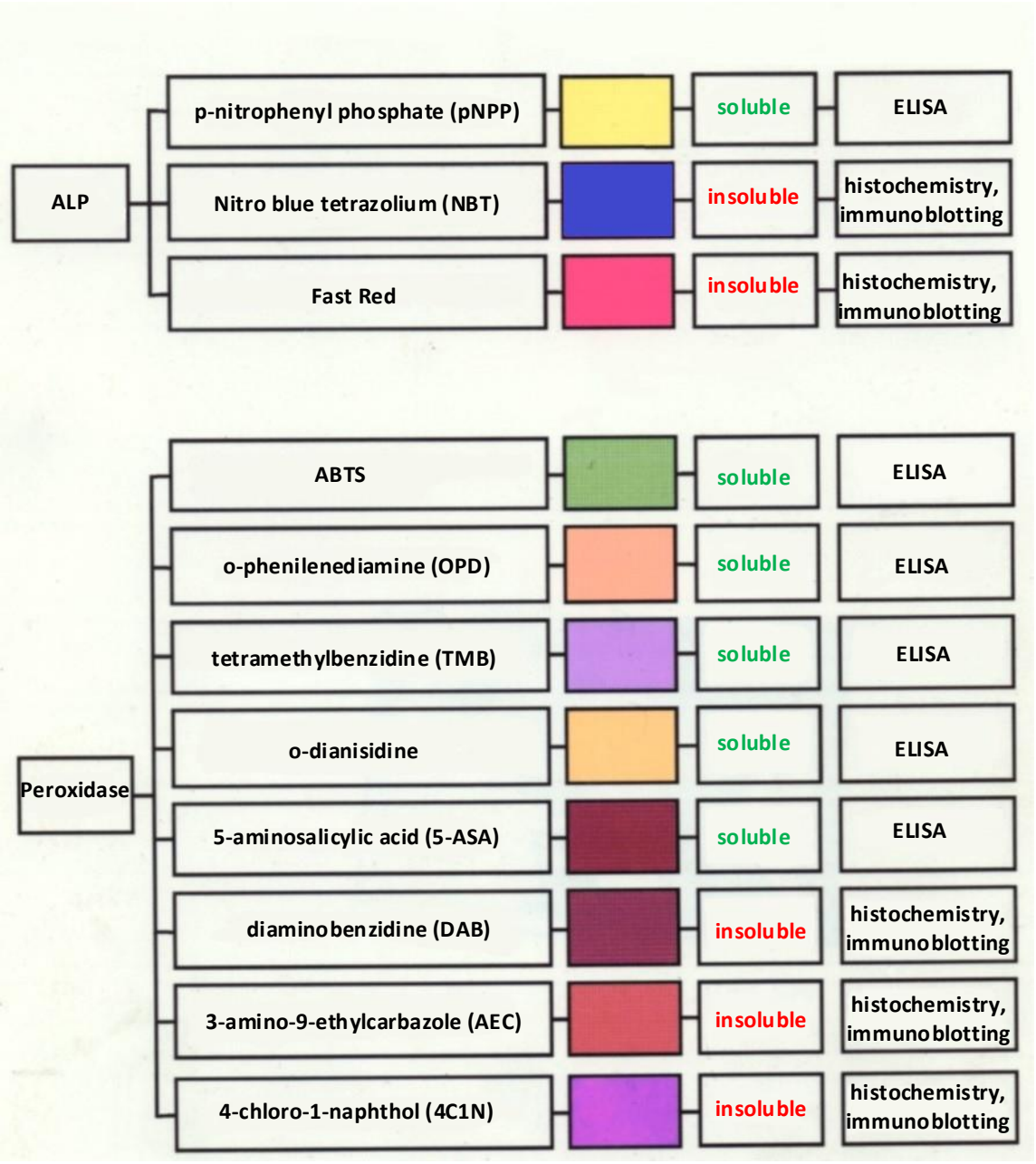


Frequently used enzymes:

1. **HRP** = Horseradish peroxidase
2. **ALP** = alkaline phosphatase

Their most widely-used chromogens:

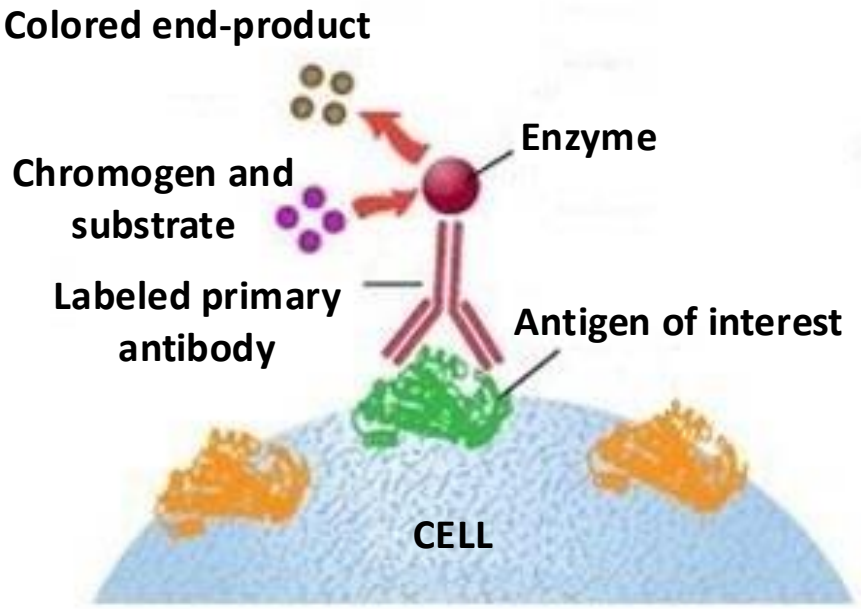
- **DAB (diaminobenzidine)**
- **AEC (amino ethylcarbazole)**
- **NBT (nitro blue tetrazolium)**



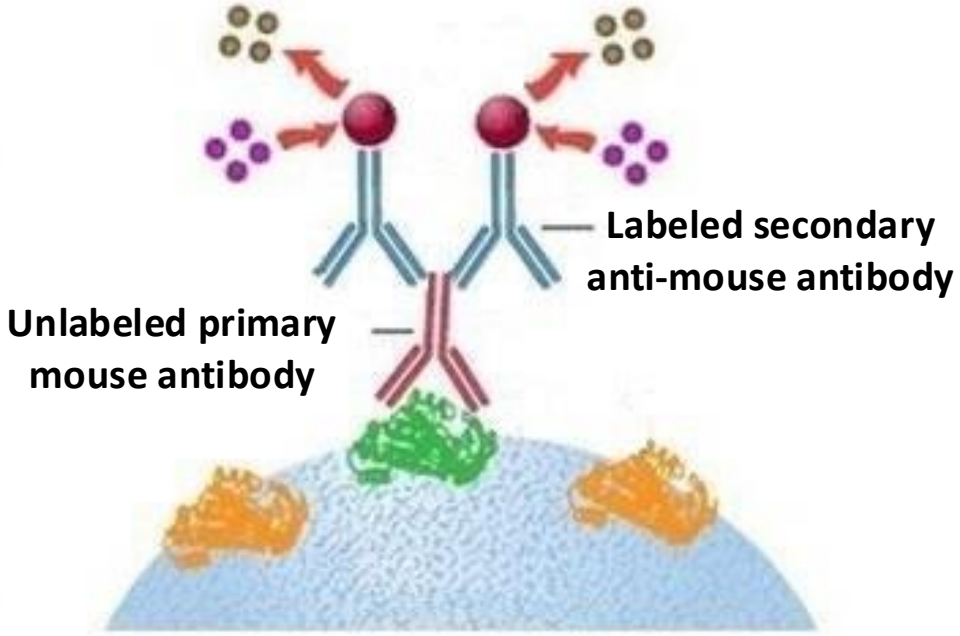
It is important to consider whether the **end-product** of the chromogen is **soluble or insoluble**:

- In case of enzyme IHC the end-product **must be insoluble**, otherwise it will diffuse away. An insoluble end-product will stay at the site of the reaction allowing the visualization of antigen-antibody reaction under the microscope.
- In case of **ELISA** a chromogen with a **soluble** end-product must be chosen. (see later)

Direct or indirect?



Direct IHC



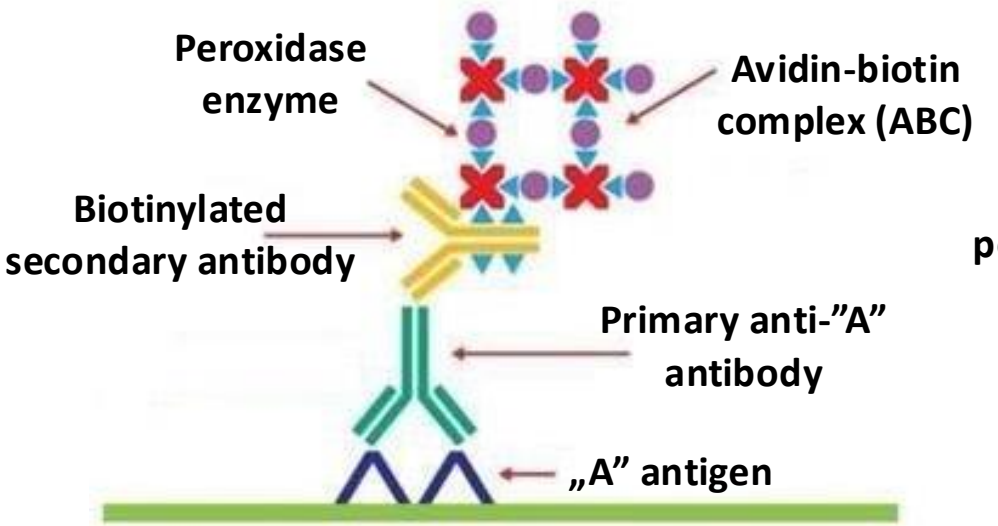
Indirect IHC

The **indirect method** is usually preferred for enzyme IHC because it has several advantages (although is more time-consuming):[2.]

- **The signal is stronger.** (This is especially useful when the antigen can be found in low amounts in the tissues.)
- It is **cheaper** in the long run. (The very same labeled secondary antibody can be used for the detection of various primary antibodies. Labelled antibodies are usually more expensive)

Complex detection systems

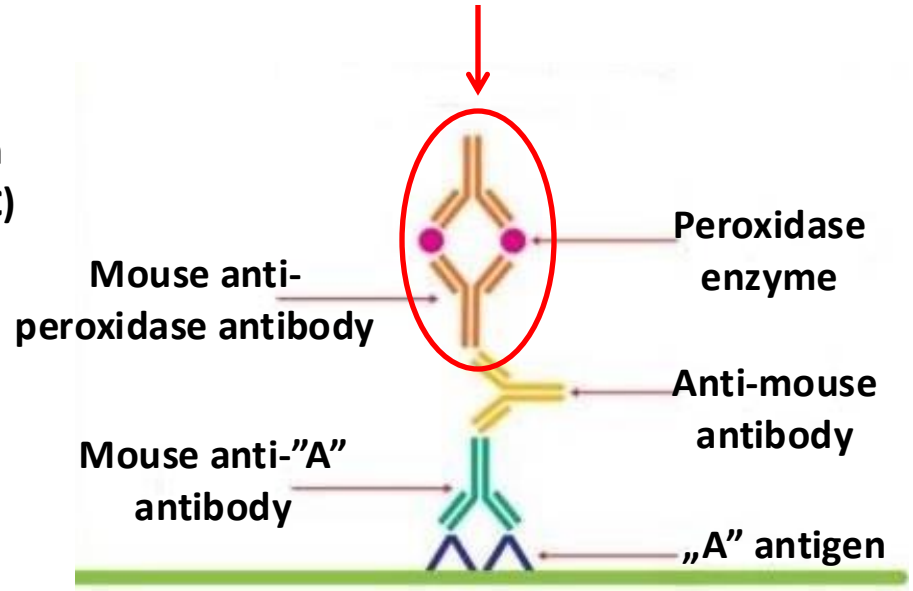
Avidin-biotin complex (ABC)



The biotin-avidin bond is the strongest known non-covalent interaction between a protein and its ligand. The enzyme is bound to biotin and the avidin will crosslink the biotinylated antibody and the biotinylated enzymes forming large enzyme complexes.[3.]

Benefit: **Signal amplification**

PAP (peroxidase anti-peroxidase complex)

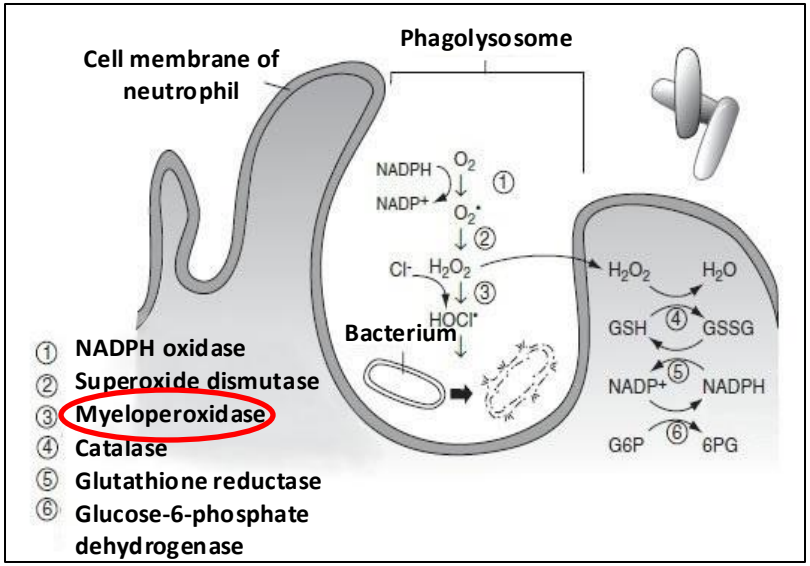


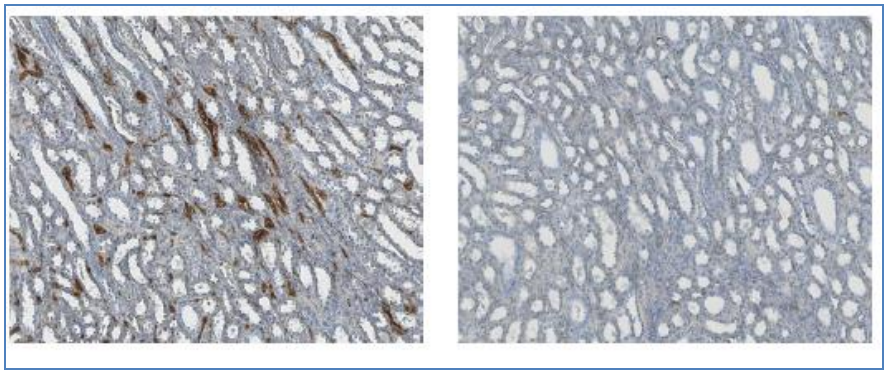
Animals are immunized with peroxidase enzyme, then anti-peroxidase antibodies are purified from the serum of the animal. They add both the peroxidase and anti-peroxidase antibodies which will form complexes. (PAP)[4.]

Benefit: **Signal amplification**

Inhibition of endogenous peroxidase

- Why is it necessary?
  - Several cell types contain peroxidase. Do you remember? → e.g. **respiratory burst** in the myeloid WBCs (e.g. neutrophils, monocytes/macrophages → 2nd practice)
  - Their enzymes will also transform the substrate. → **non-specific background**
- The endogenous peroxidase must be inhibited **before** adding the labeled antibody.[5.]





Activity of endogenous peroxidase in the kidney

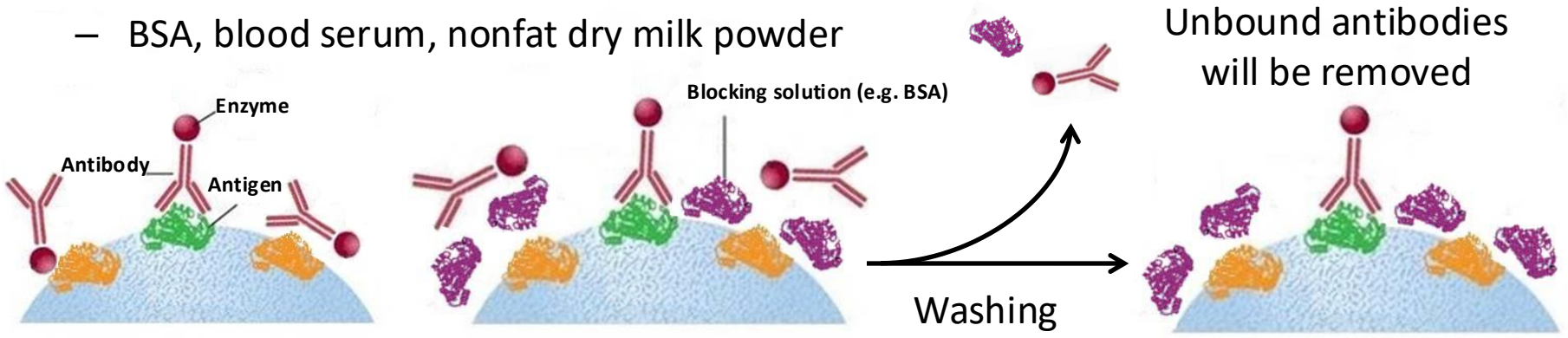
No non-specific signal

Main inhibitors:[6.]

- Phenylhydrazine
- Hydrogen peroxide
- Azide

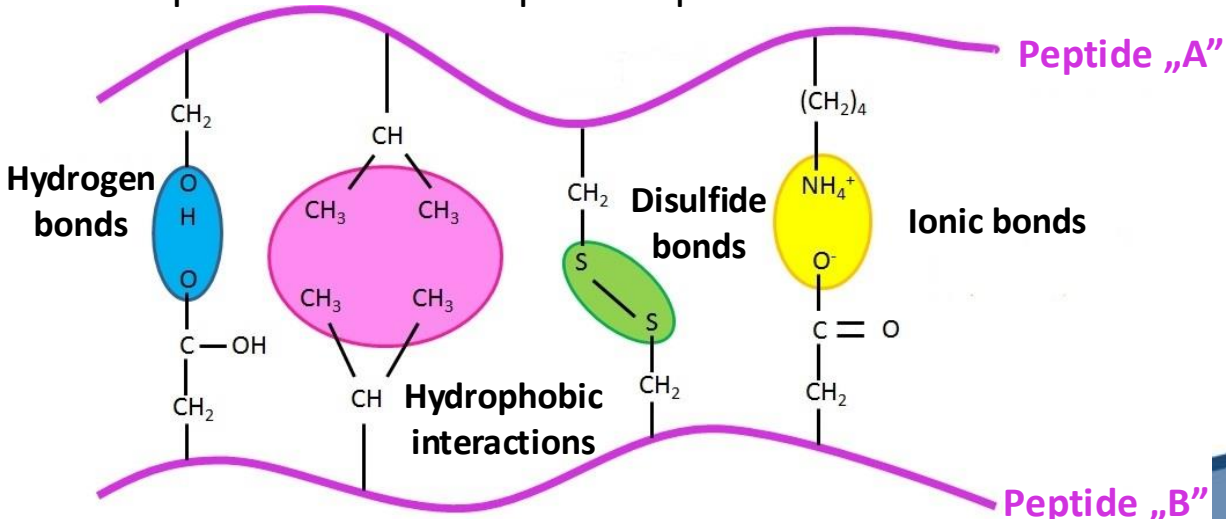
Blocking

- Why is it necessary?
  - Although the antigen-antibody reaction is specific, **non-specific protein-protein interactions** can also occur between the antibodies and proteins in the tissues. (see on next slide) → **non-specific background**
- Non-specific binding sites should be blocked **before** adding the antibody. Several protein solutions can be used for blocking depending on the tissue and the used antibody, such as:[7.]
  - BSA, blood serum, nonfat dry milk powder

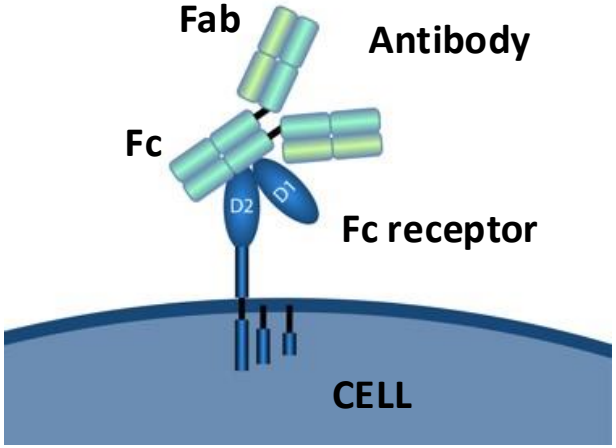


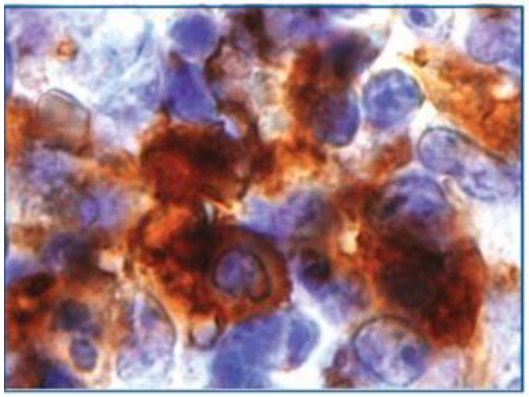
Non-antigen-specific protein-protein interactions

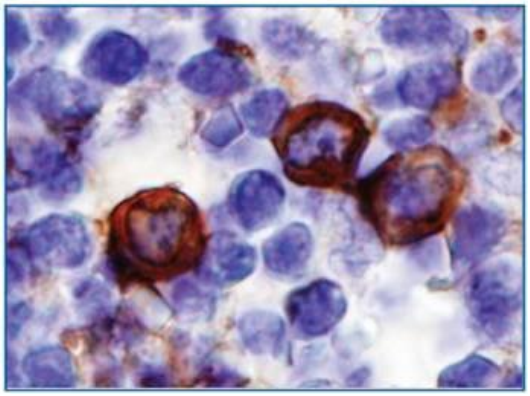
Some possible forms of protein-protein interactions:



Problem only related to antibodies:





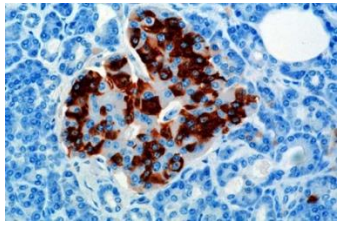


Fc receptors can be blocked with unlabeled antibodies.

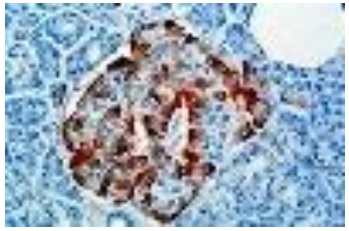
Detection of CD14 (LPS receptor) in a human tonsil without blocking (left) or with blocking. (right)

Double staining

- Only one antigen can be detected in the tissue with the use of classical enzyme IHC. If more than one antigen needs to be detected, they must create additional tissue sections and stain them with different antibodies. E.g.:

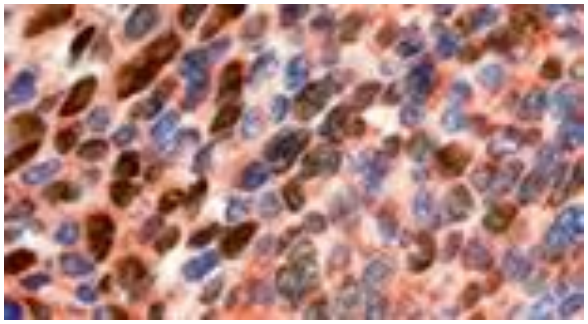


1. section : anti-insulin



2. section: anti-glucagon

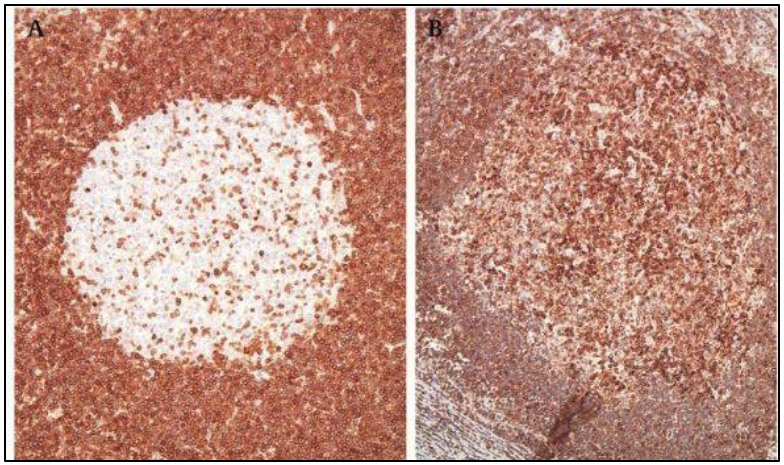
- One possibility is the use of double staining. → They add 2 types of antibodies each **conjugated with a different enzyme** which will create **different color reactions** that can be distinguished.



An example of double staining in a human prostate cancer tissue. p53 was detected by a **brownish color** (DAB) while AIF (apoptosis-inducing factor) is marked by the **reddish color** reaction (Fast Red) in the same tissue section.[9.]

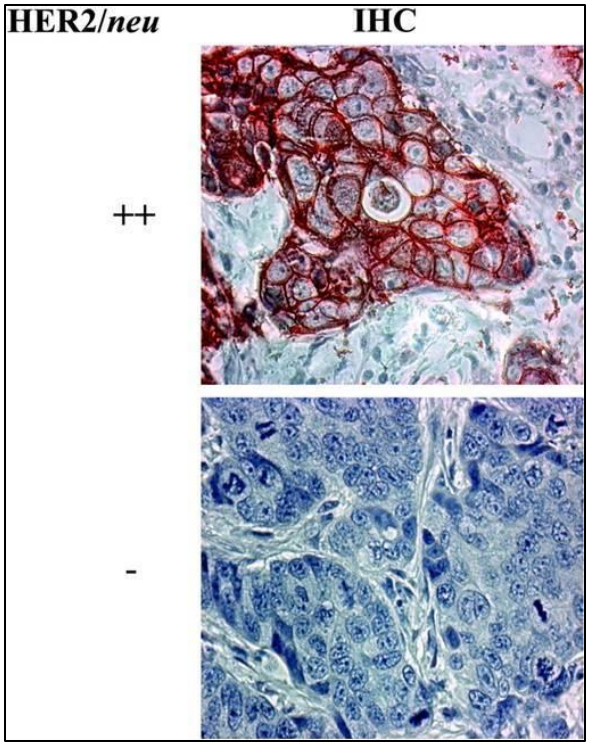
The clinical significance of IHC

1. Some pathological conditions cannot be distinguished solely by their morphological appearances. Detecting markers that are specific to certain diseases are of great importance for **diagnosing** them. (see in pathology next year)
2. The presence or absence of certain markers have **prognostic significance** and can influence the therapy of the patient.



Detection of the anti-apoptotic Bcl-2 in a normal follicle (left) and in follicular lymphoma. (right)

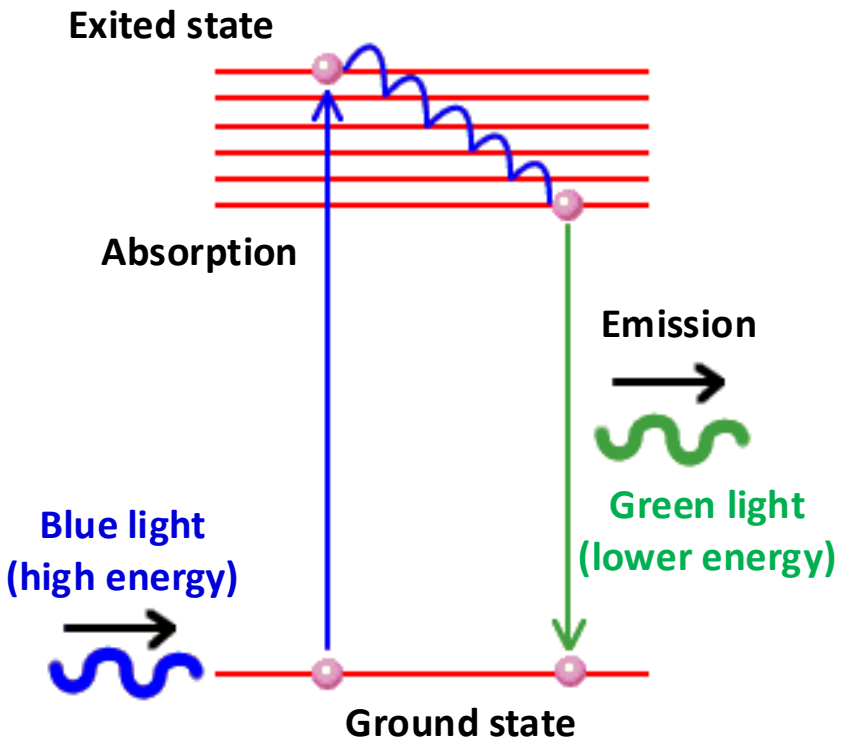
Detection of HER2 in breast cancer:



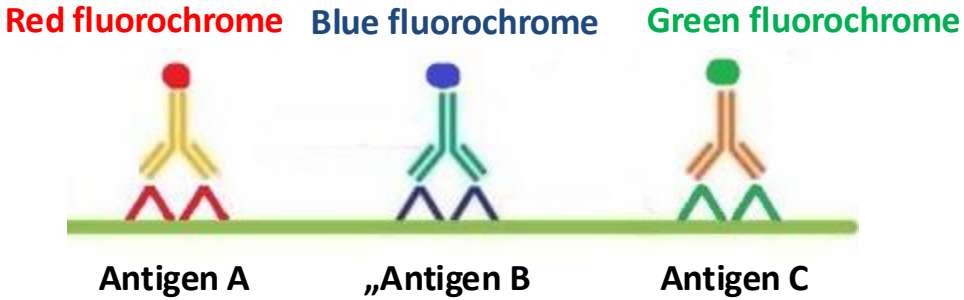
The cancer cells of the upper patient show high levels of HER2 expression making the patient a possible candidate for Herceptin® treatment.[8.] The cells of patient below do not express HER2.

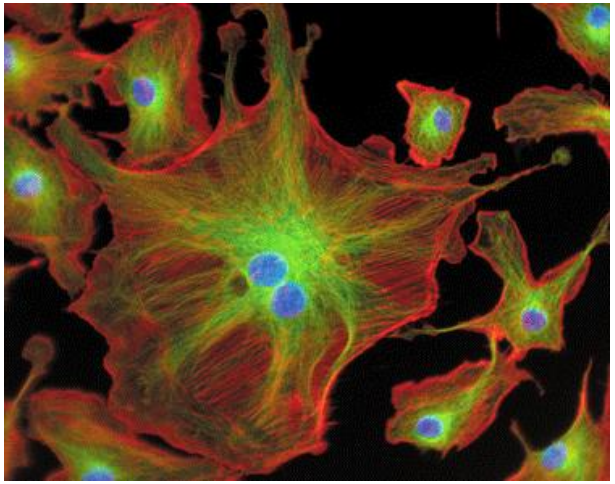
Immunofluorescent staining

- **Several different antigens** can be detected in the **same sample** simultaneously with the use of antibodies labeled with different **fluorochromes**.[10.] (e.g. fluorescent microscopy, flow cytometry, see later)



The basic principle of fluorescence

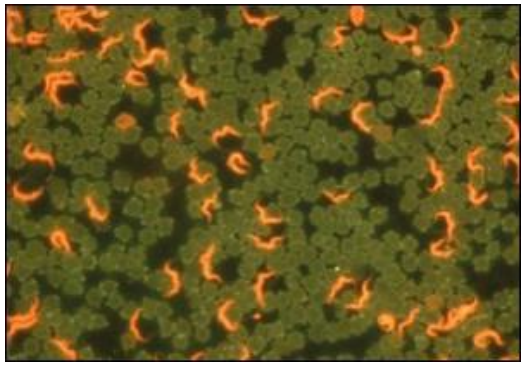




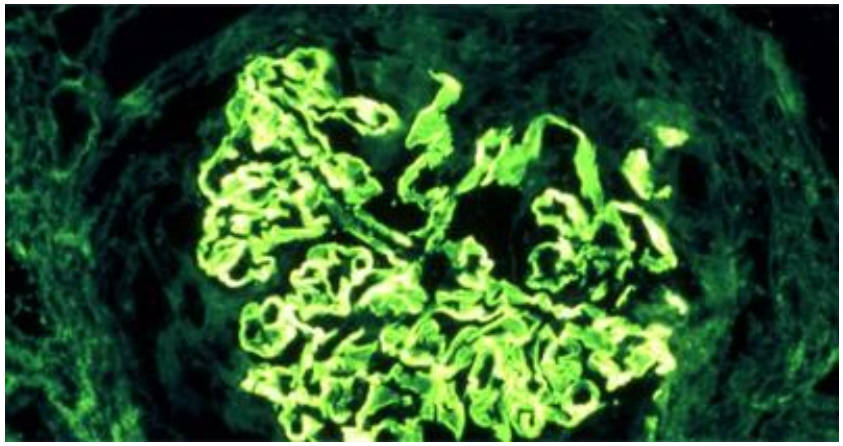
Green: tubulin
Red: actin
Blue: cell nucleus

Fluorescent microscopic image of endothelial cells

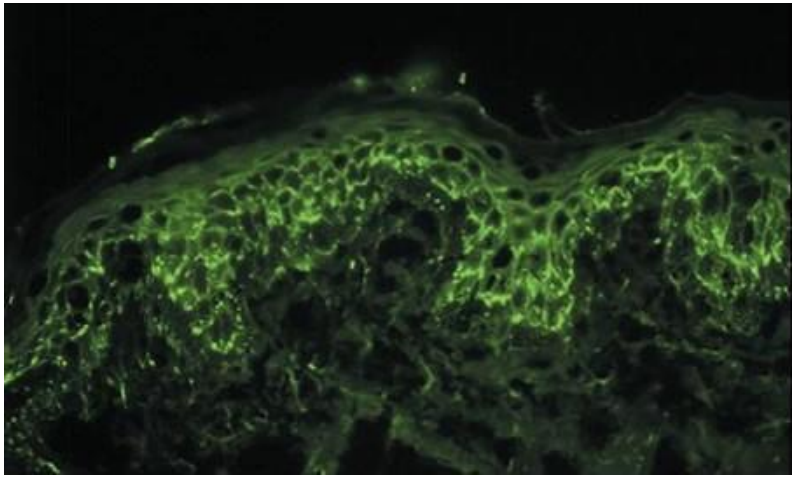
The clinical significance of fluorescent microscopy



Parasites (*Trypanosoma*) in the peripheral blood of a patient stained with an antibody that had an orange fluorochrome attached to it. (Acridine Orange)



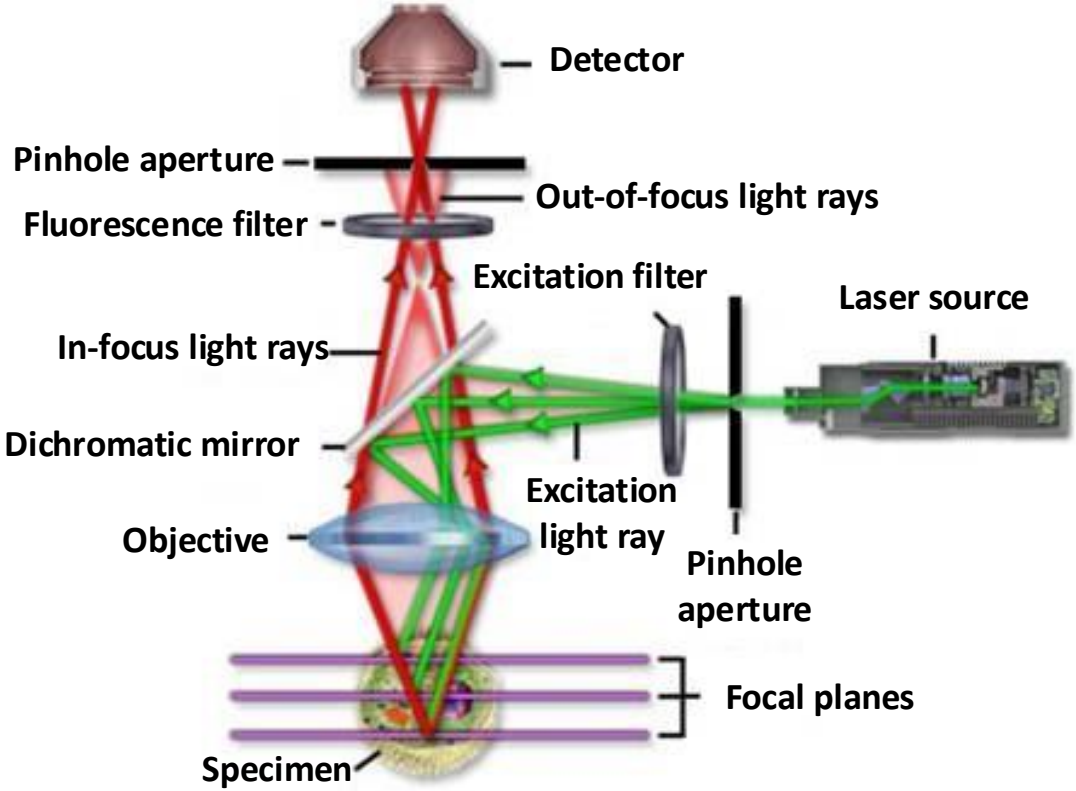
Linear deposition of IgG along the glomerular basal membrane of a patient with Goodpasture syndrome. (see later)



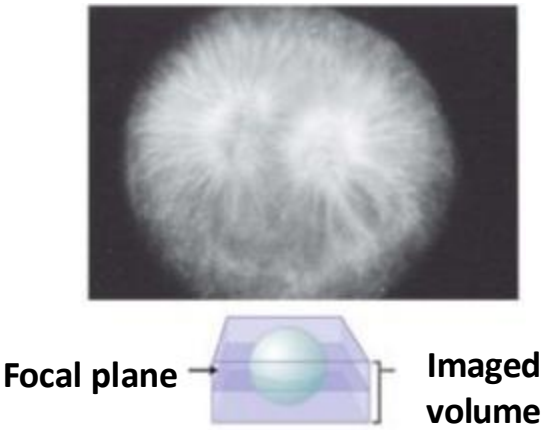
Intercellular (=between cells) deposition of IgG in the epidermis of the skin in a patient with pemphigus vulgaris. (see later)

Confocal microscopy

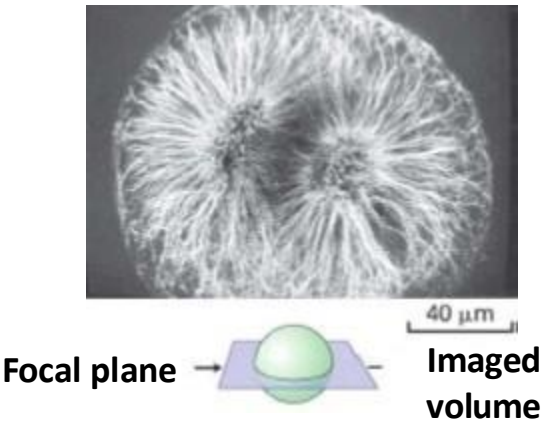
- Ordinary microscopes: They provide of a summed image of the entire volume of the section (similar to classic X-rays)
- Confocal microscopes: They create an image of a very thin optical section only.[11.] (similar to CTs)



Ordinary:

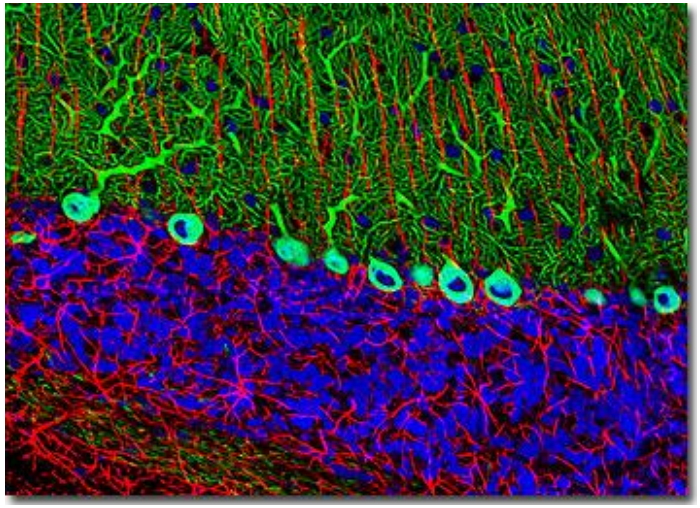


Confocal:

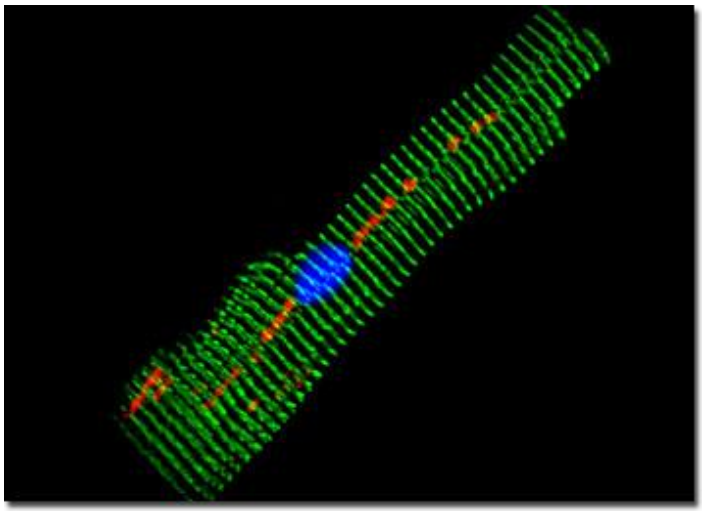


Labelling of a tubulin in a mitotic cell with an anti-tubulin antibody.

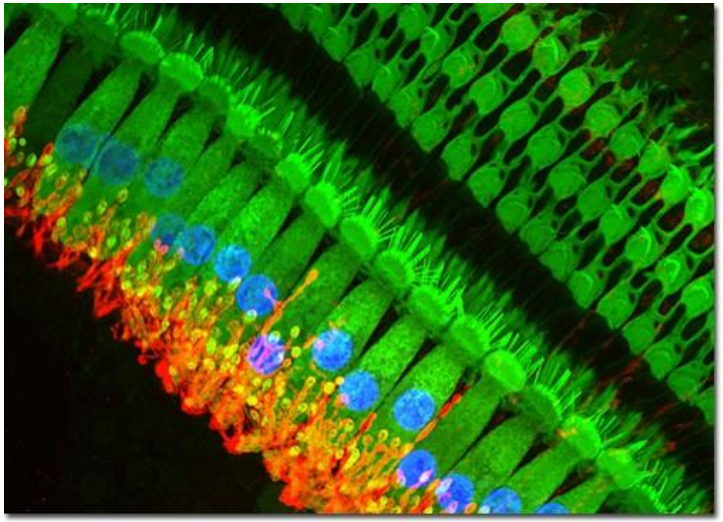
Confocal microscopy



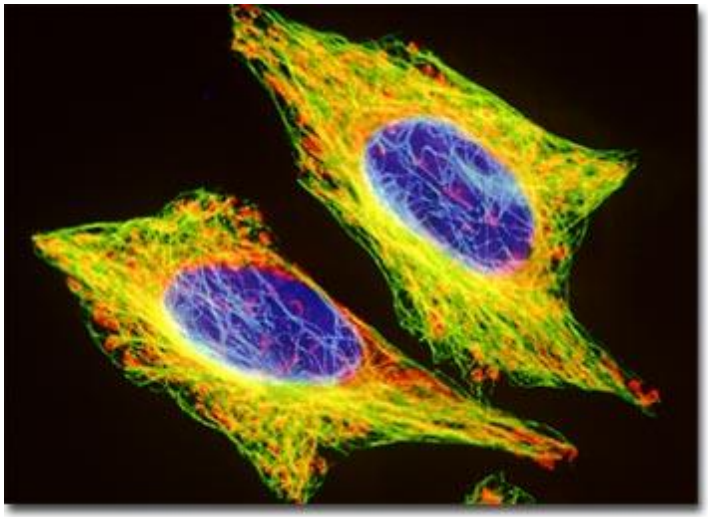
Rat cerebellum



Heart muscle



Organ of Corti

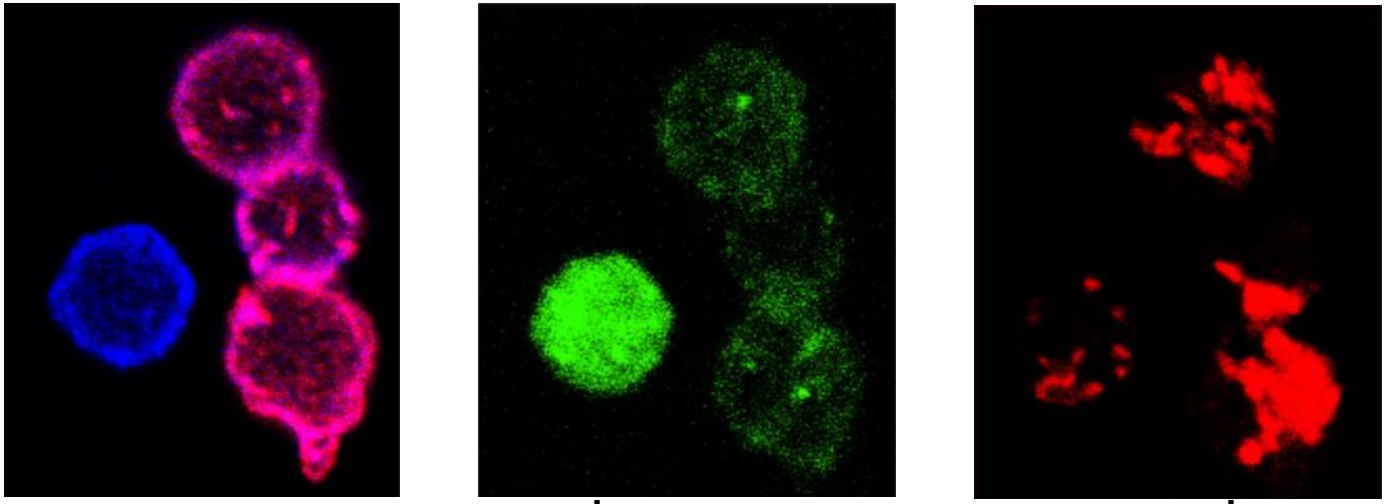


HeLa cells

Detecting colocalization

Green: glucocorticoid receptor

Red: mitochondrion



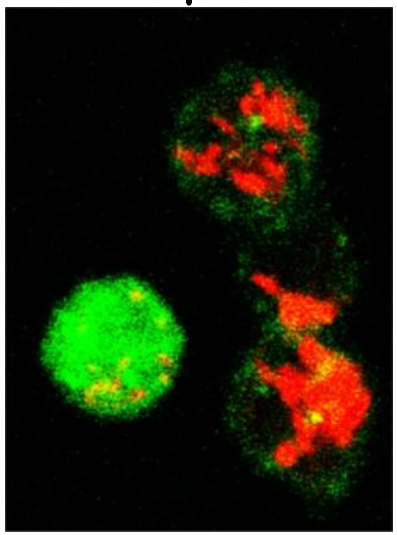
Blue: CD4

Reddish: CD8

Purple: CD4/CD8 double positive

↓

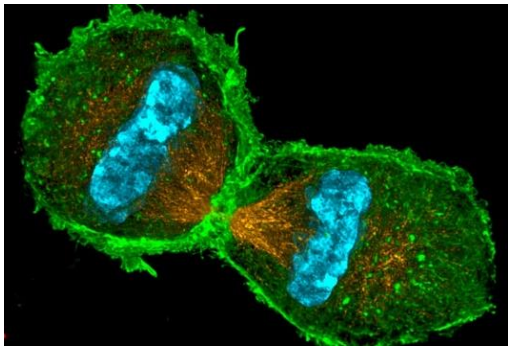
DP (double positive): Immature T cell precursor expressing both CD4 and CD8, see later



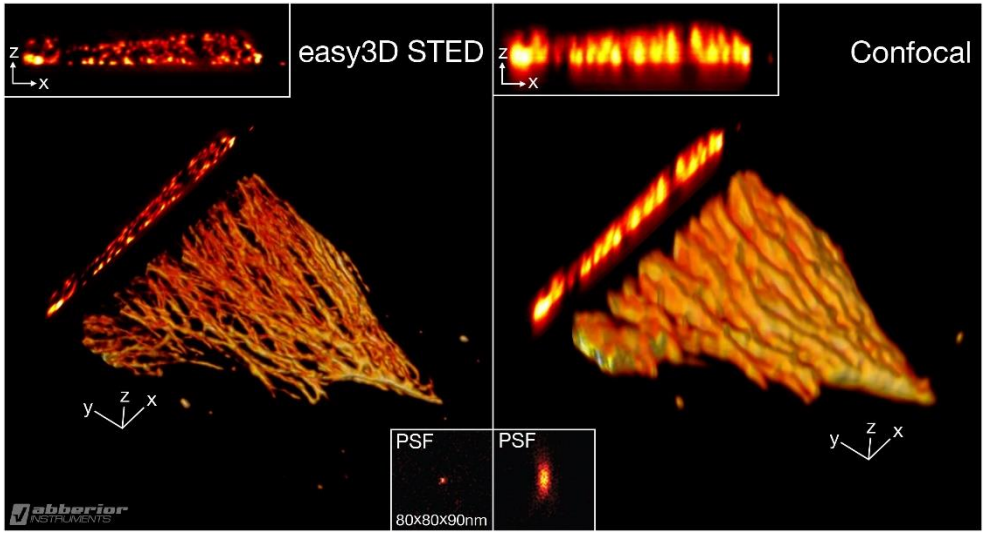
Yellow: GR+mitochondria

Super-resolved fluorescent microscopy

In the range of 20-50 nanometers→ protein complexes[12,13.]



Two mouse cells in telophase
Orange: tubulin
Green: actin
Blue: chromatin



Comparison of the resolution of confocal (right) and super-resolved STED microscopy (left) on microtubules.

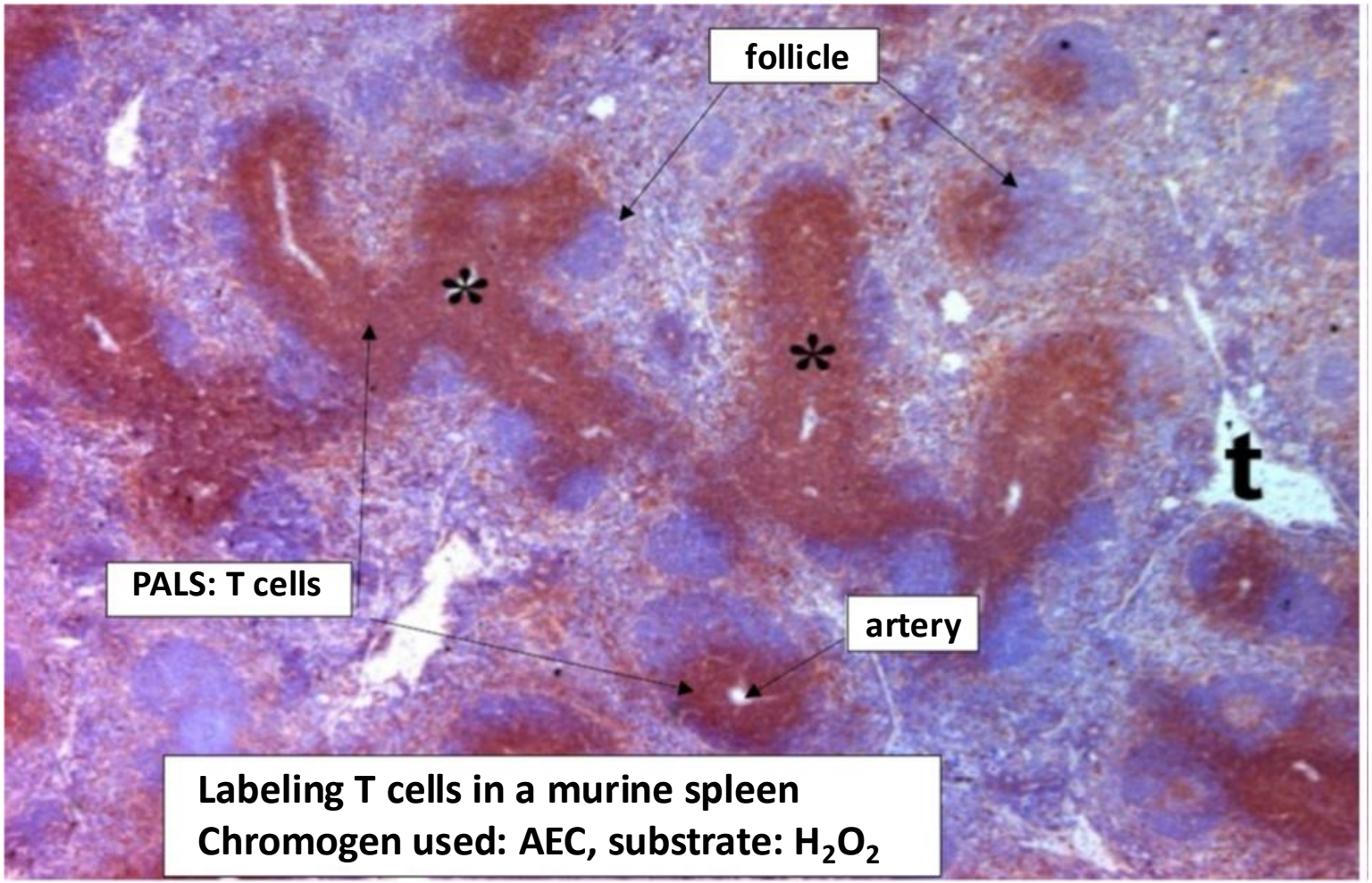
Eric Betzig

Stefan W. Hell

William E. Moerner

Were awarded the 2014 Nobel Prize in Chemistry:
„for the development of super-resolved fluorescence microscopy.”[14.]

What you should see in the section:



Tissue distribution of lymphocytes

	Peripheral blood	Lymph nodes	Spleen
Th cells	50-60%	50-60%	35-40%
Tc cells	20-25%	15-20%	10-15%
B cells	10-15%	20-25%	40-45%
NK cells	≈10%	Very few	≈10%

References 1.

1. Pellicciari C[1]: **Histochemistry as an irreplaceable approach for investigating functional cytology and histology.** *Eur J Histochem.* 2013 Dec 19;57(4):e41. doi: 10.4081/ejh.2013.e41.
2. Chen X[1], Cho DB, Yang PC: **Double staining immunohistochemistry.** *N Am J Med Sci.* 2010 May;2(5):241-5. doi: 10.4297/najms.2010.2241.
3. Bratthauer GL[1]: **The avidin-biotin complex (ABC) method and other avidin-biotin binding methods.** *Methods Mol Biol.* 2010;588:257-70. doi: 10.1007/978-1-59745-324-0_26.
4. Bratthauer GL[1]: **The peroxidase-antiperoxidase (PAP) method and other all-immunologic detection methods.** *Methods Mol Biol.* 2010;588:243-55. doi: 10.1007/978-1-59745-324-0_25.
5. Liu G[1], Amin S, Okuhama NN, Liao G, Mingle LA: **A quantitative evaluation of peroxidase inhibitors for tyramide signal amplification mediated cytochemistry and histochemistry.** *Histochem Cell Biol.* 2006 Aug;126(2):283-91. Epub 2006 Mar 1.
6. Li CY[1], Ziesmer SC, Lazcano-Villareal O: **Use of azide and hydrogen peroxide as an inhibitor for endogenous peroxidase in the immunoperoxidase method.** *J Histochem Cytochem.* 1987 Dec;35(12):1457-60.
7. Buchwalow I[1], Samoilova V, Boecker W, Tiemann M: **Non-specific binding of antibodies in immunohistochemistry: fallacies and facts.** *Sci Rep.* 2011;1:28. doi: 10.1038/srep00028. Epub 2011 Jul 1.
8. Figueroa-Magalhães MC[1], Jelovac D[1], Connolly RM[1], Wolff AC[2]: **Treatment of HER2-positive breast cancer.** *Breast.* 2014 Apr;23(2):128-36. doi: 10.1016/j.breast.2013.11.011. Epub 2013 Dec 19.
9. Abcam.com: **Perfect for immunohistochemistry (IHC): the third RabMAb® advantage** (http://www.abcam.com/primary-antibodies/perfect-for-immunohistochemistry-ihc-the-third-rabmab-advantage)
10. Sanderson MJ[1], Smith I[2], Parker I[2], Bootman MD[3]: **Fluorescence microscopy.** *Cold Spring Harb Protoc.* 2014 Oct 1;2014(10):pdb.top071795. doi: 10.1101/pdb.top071795.

References 2.

11. Nwaneshiudu A[1], Kuschal C, Sakamoto FH, Anderson RR, Schwarzenberger K, Young RC: **Introduction to confocal microscopy.** *J Invest Dermatol.* 2012 Dec;132(12):e3. doi: 10.1038/jid.2012.429.
12. Betzig E[1], et al.: **Imaging intracellular fluorescent proteins at nanometer resolution.** *Science.* 2006 Sep 15;313(5793):1642-5. Epub 2006 Aug 10.
13. MacDonald L[1], Baldini G, Storrie B: **Does super-resolution fluorescence microscopy obsolete previous microscopic approaches to protein co-localization?** *Methods Mol Biol.* 2015;1270:255-75. doi: 10.1007/978-1-4939-2309-0_19.
14. NoblePrize.org: **The Nobel Prize in Chemistry 2014** (http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/)