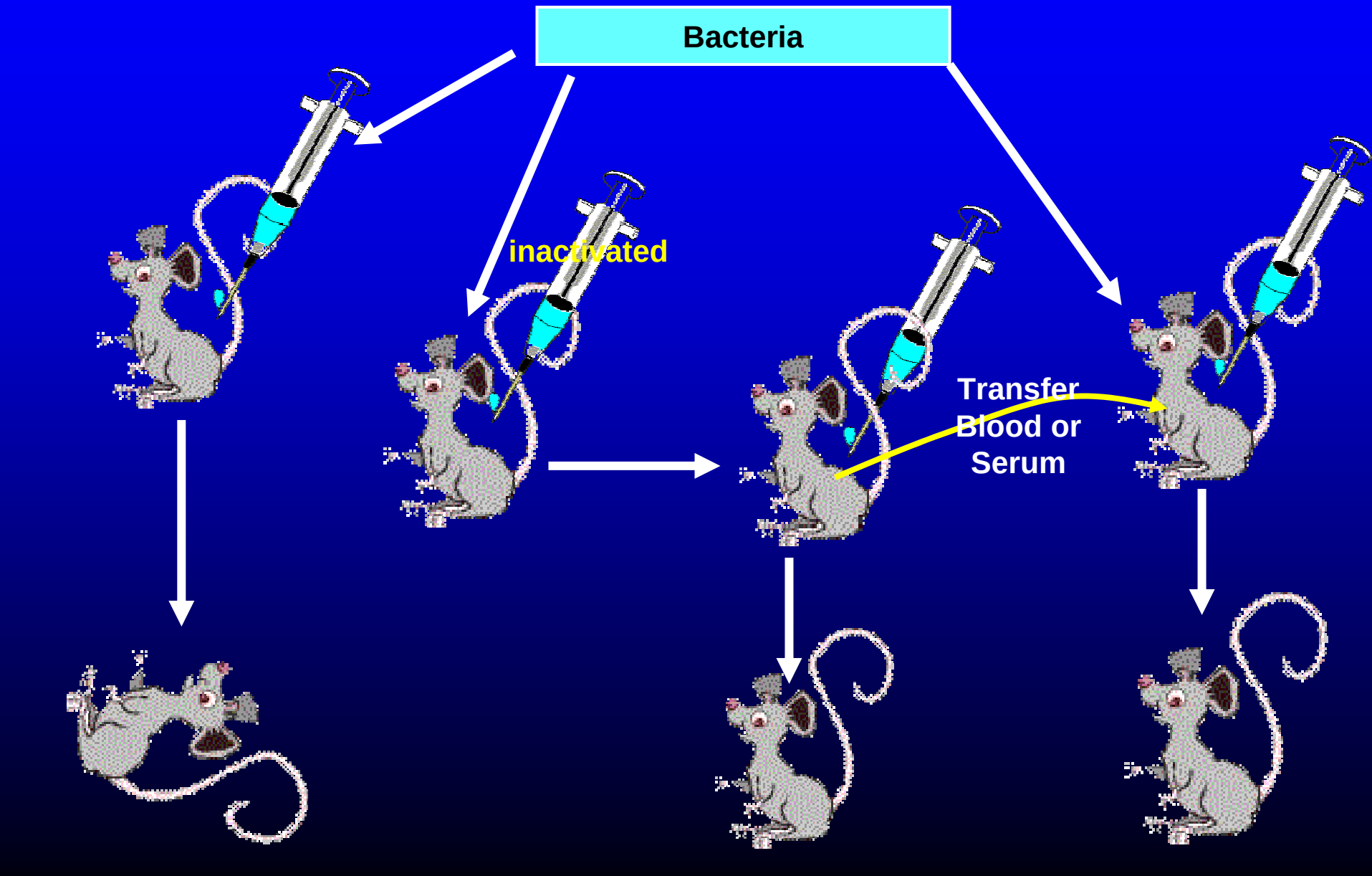
Methods of Biomaterials Testing
Lesson 3-5

Biochemical Methods

Antibodies

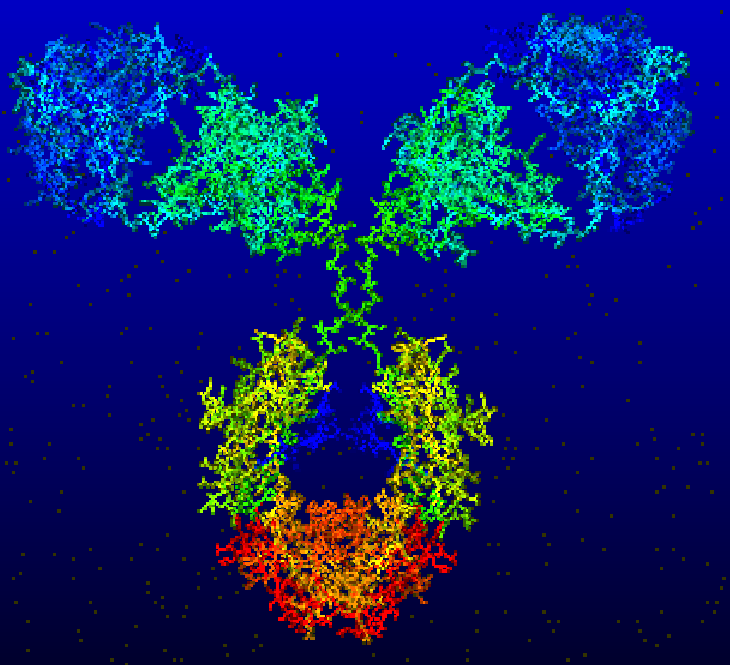
Vaccination



Conclusion

Second Mouse produced neutralizing “antibodies” against the bacteria

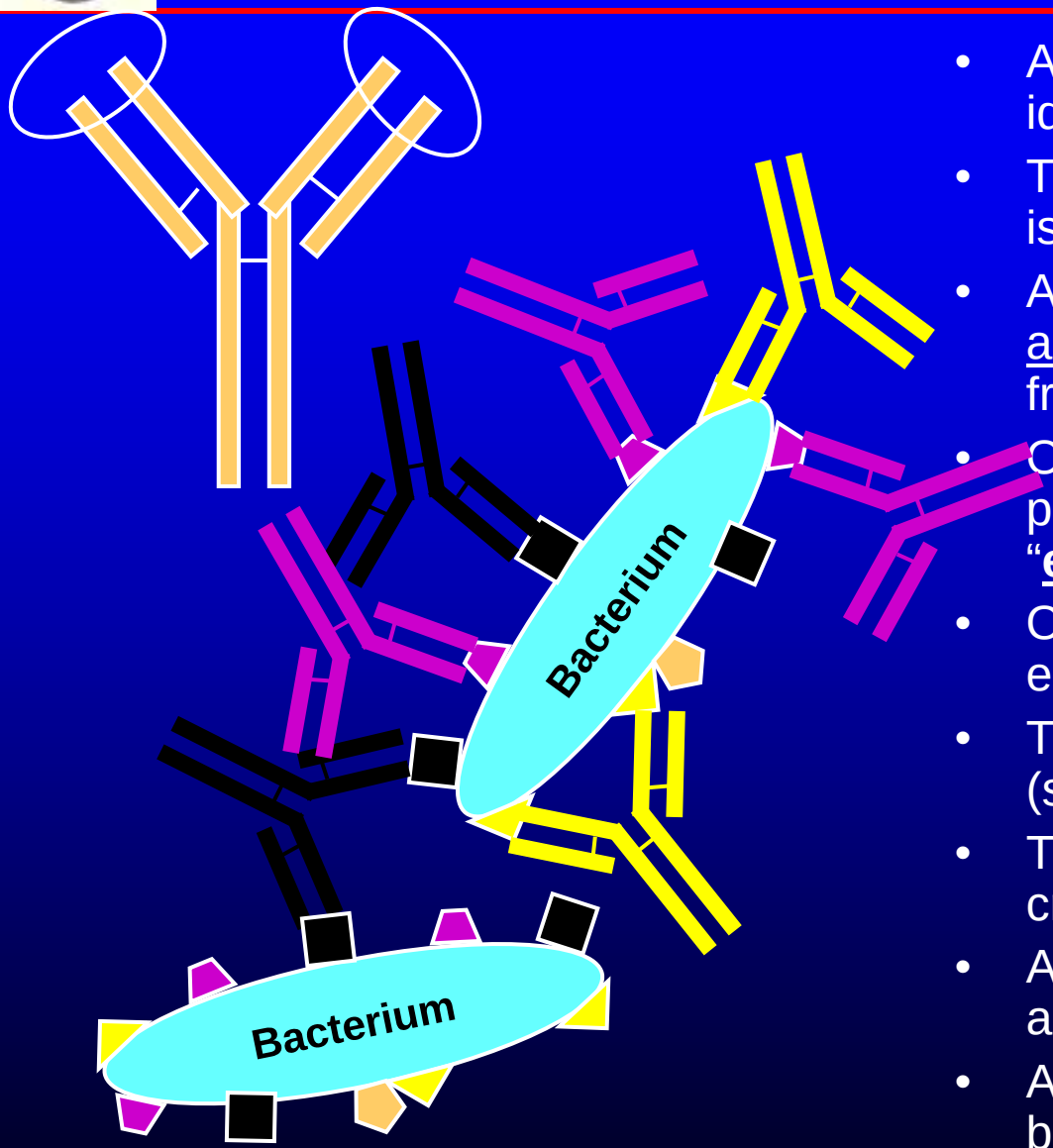
Much later the Antibody (“Ab”) was identified as a protein:



Ball-like, globular Protein
→ “Immunoglobine” (Ig)

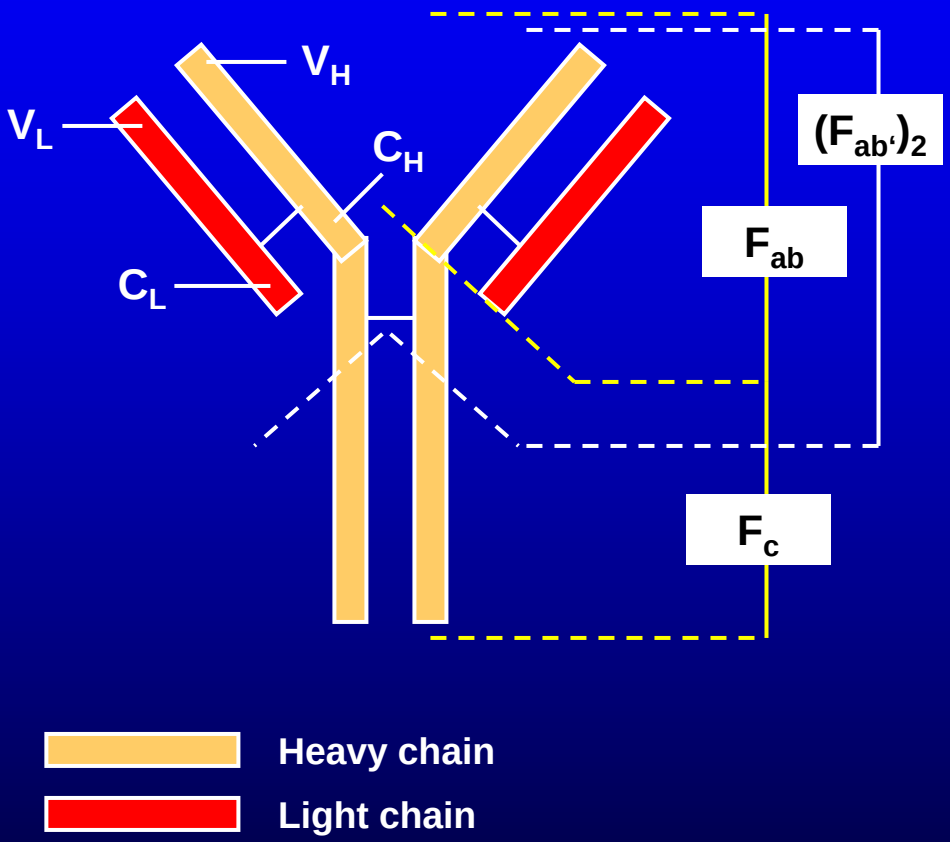
Different classes: IgG, IgM, IgA, IgD, IgE

Antibody



- Antibodies have Y shape with two identical binding sites at the ends
- The counterpart/ target of an antibody is called “antigen”.
- Antigens can be bacteria, viruses or any type of protein, especially proteins from an other species of animal.
- One antibody recognizes only a small part of an antigen (~ 7 aminoacids): “**epitope**”
- One antigen usually has several epitopes (antibody binding sites)
- The recognition is highly specific (sequence and 3D structure)
- The antibody binding can lead to crosslinking of the antigens
- Antibody binding is a reversible association, but with very high affinity
- Antibody binding *per se* does not kill the bacterium

Antibody Structure



- An antibody consists of two identical parts
- **Molecular description:**
  Each part consists of a heavy and a light chain
  The heavy chain determines the class (IgG, IgM, IgA, IgD, IgE)
  The light chain can be either kappa or lambda
- **Functional Description:**
  crystalline (constant) fragment (F_c)
  antigen binding fragment (F_{ab})
  - contains whole light chain
  - contains part of the heavy chain
  - both chains in the F_{ab} fragment have a constant (C) and a variable (V) domain

Terminology

Antiserum: Serum of a vaccinated animal with many antibodies in it

Polyclonal antibody: Many different antibodies against different epitopes of one antigen.

Monoclonal antibody: One type of antibody, which is directed only against one epitope of the antigen.

Affinity purified antibody/ antiserum: Polyclonal antibody which only has antibodies against the target antigen.

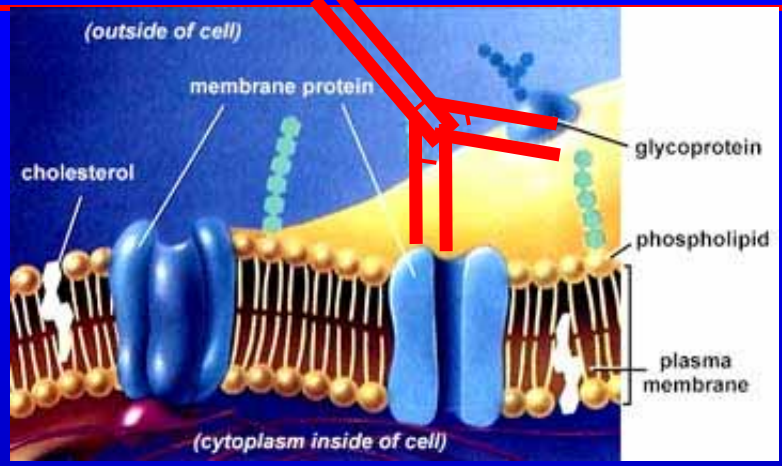
Cross reactivity: (1) The antibody reacts with the equivalent protein of an other species

(2) The antibody reacts with other/ similar proteins of the same/ other species

Preadsorbed antibody: Polyclonal antibody, where those antibodies with crossreactivity to other antigens are removed.

What for?

- Antibodies can block or activate receptors and channels



- It is possible to produce antibodies against antibodies (constant fragments). i.g. “Rabbit anti Mouse-IgG kappa chain”. Or “Goat anti Rabbit IgG F_c Fragment”
- It is possible to label the antibody at the F_c fragment with
  - An enzyme (Alkaline Phosphatase (ALP) or Horseradish Peroxidase (HRP). Both can induce a color reaction
  - A fluorescent molecule/ dye (Fluoresceinisothiocyanate (FITC), Rhodamin, Phycoerythrin (PE), APC…..
  - Gold particles (TEM contrast)
  - Biotin: Has very high and specific binding to avidin
  - A radioactive isotope

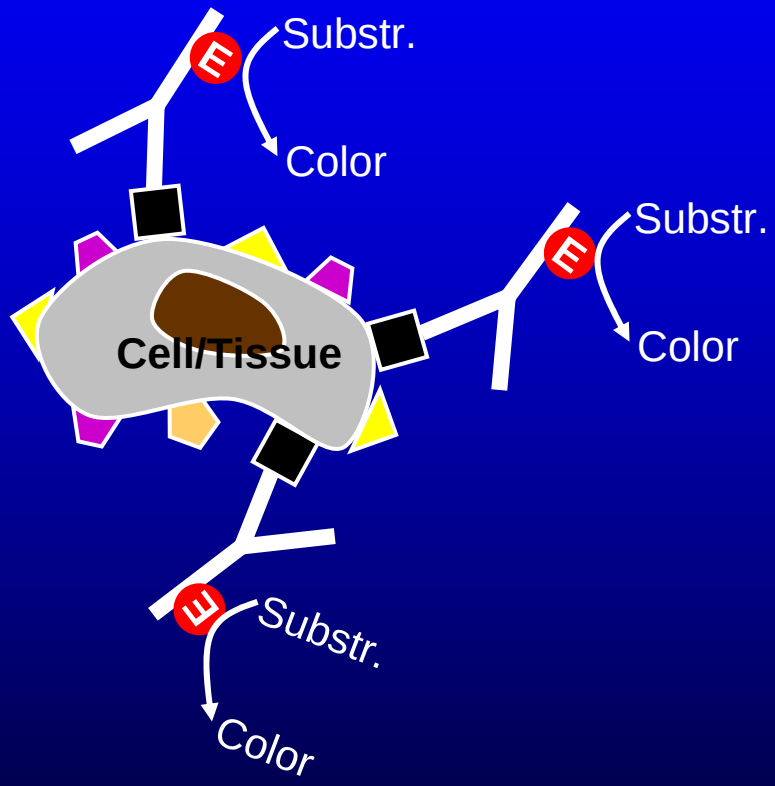
What for? II

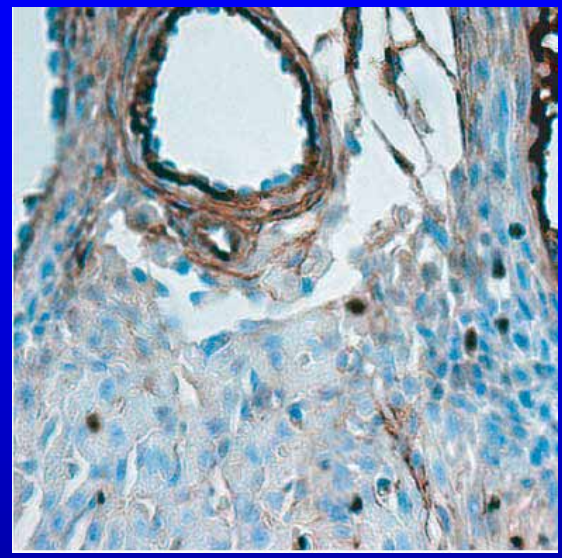
Labeled antibodies provide a tool for selective labelling of a desired protein

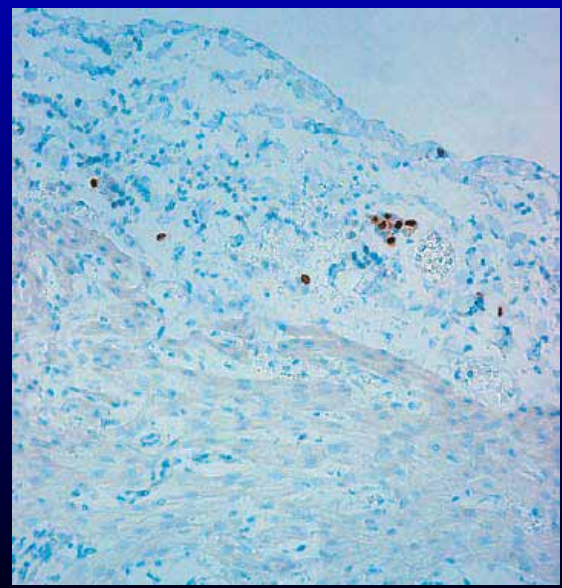
- Very high sensitivity
- Very high specifity

Immunohistochemistry I

Principle





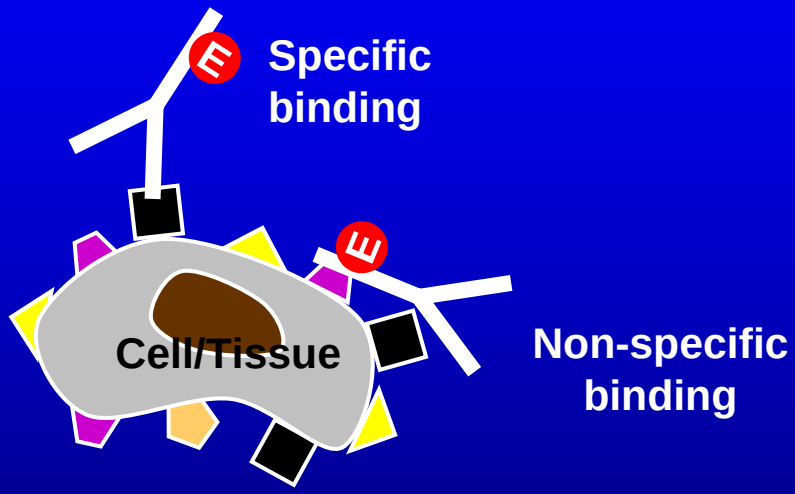


Isl-1 positive cells

Laugwitz, K; nature 433:647 (2005)

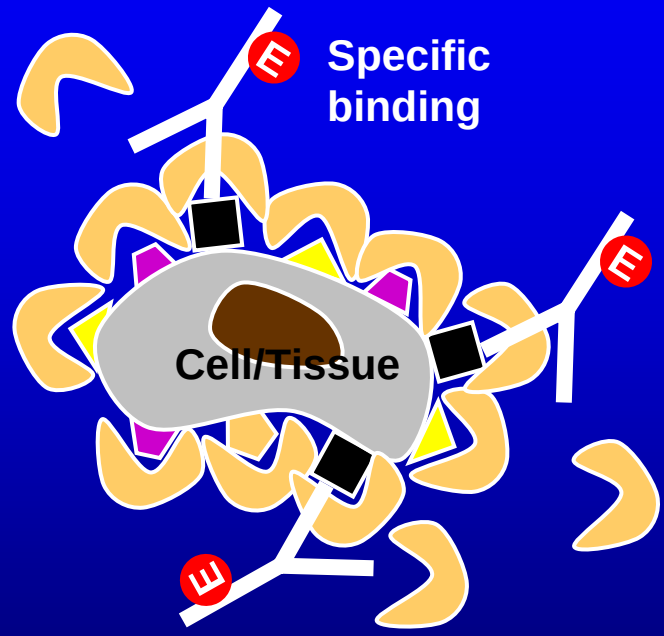
Immunohistochemistry II

Problem: Non-specific binding



- Binding of antibodies occurs to all types of proteins
- Relevant in all antibody applications
- Lower affinity
- Increased after fixation
- Increased at crushed cells, necrotic or apoptotic cells

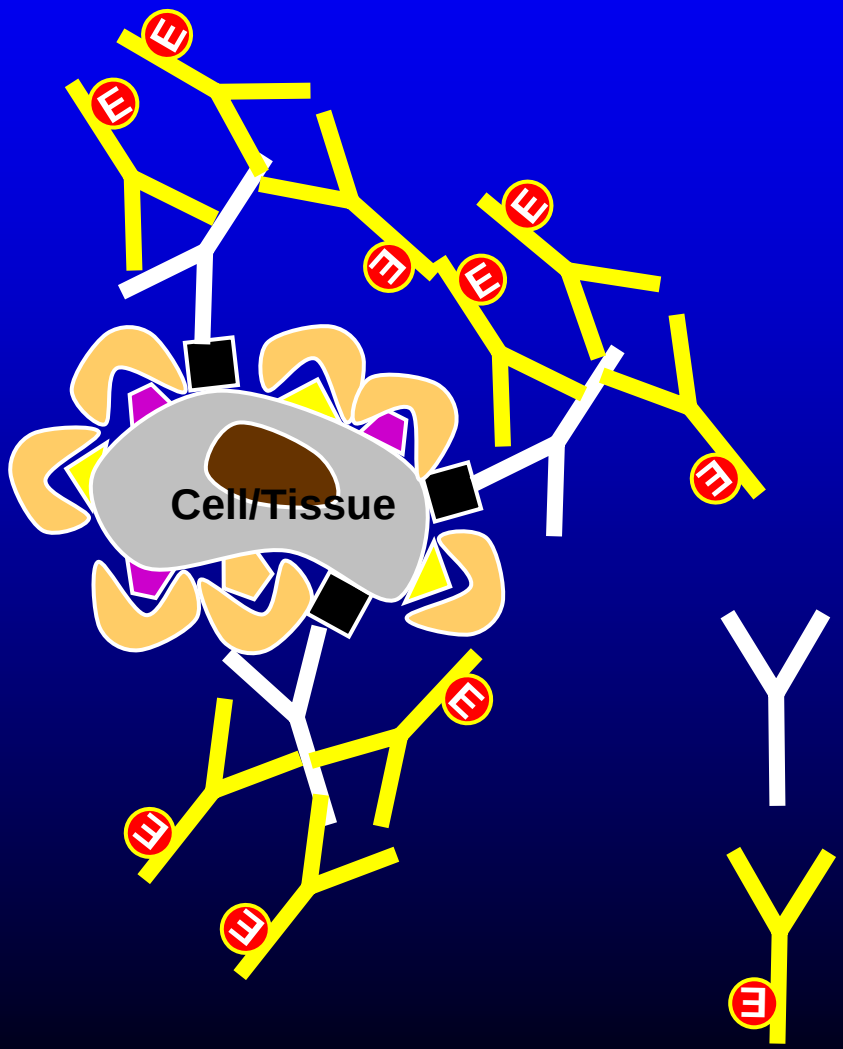
Solution: Blocking



Blocking Reagents

- Binding by same non-specific, low-affinity mechanism as Antibodies
- Ideal: (non-vaccinated) Serum of donor-species as the antibody
- Typically: Fat-free dry milk powder, casein, gelatin, albumin, (PEG, Tween), commercial mixes

Indirect Immunochemistry



Purpose

- Antibody is not available in the labeled form
- Signal amplification when polyclonal secondary Ab.

Disadvantage

- Loss of antigen – signal linearity

Primary antibody

Secondary antibody: labeled; against the species of the primary Ab.

Substrates for Immunochemistry

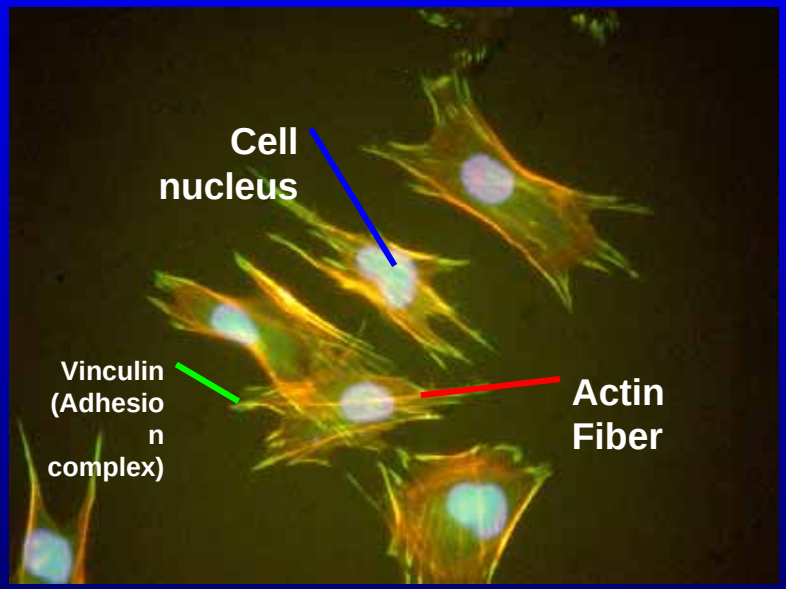
Peroxidase

- 3,3’ Diaminobenzidine (DAB) brown product, product insoluble in water and ethanol; highly toxic
- 3-amino-9-methylcarbazole (AEC): rose-red product, product soluble in ethanol. Fading in light
- 4-Chloro-1-naphthol (CN): blue product, product soluble in alcohol, product tends to diffuse
- TMB: blue product, with H_2SO_4 yellow. Product soluble in water

Alkaline Phosphatase

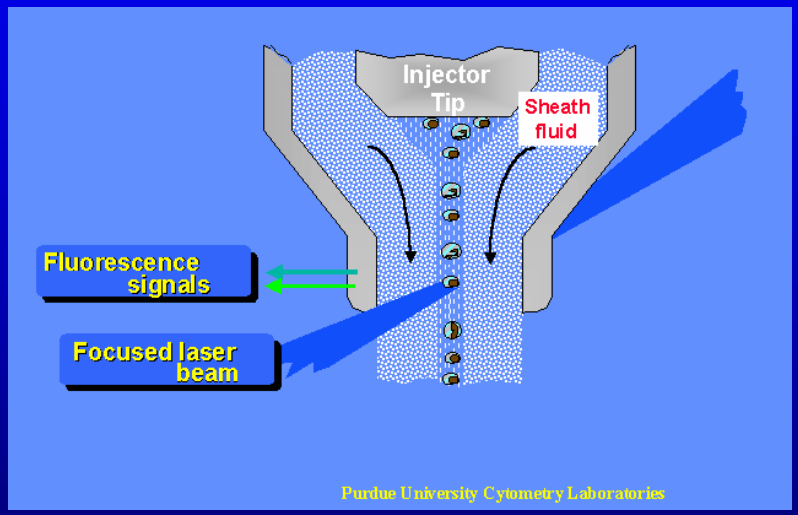
- Fast Red TR, Fast Blue BB: bright red or blue product; product soluble in alcohol
- New Fuchsin: red product; product non soluble in alcohol
- 4-Nitrophenyl Phosphate (4-NPP): Yellow product, soluble in water

Immunofluorescence



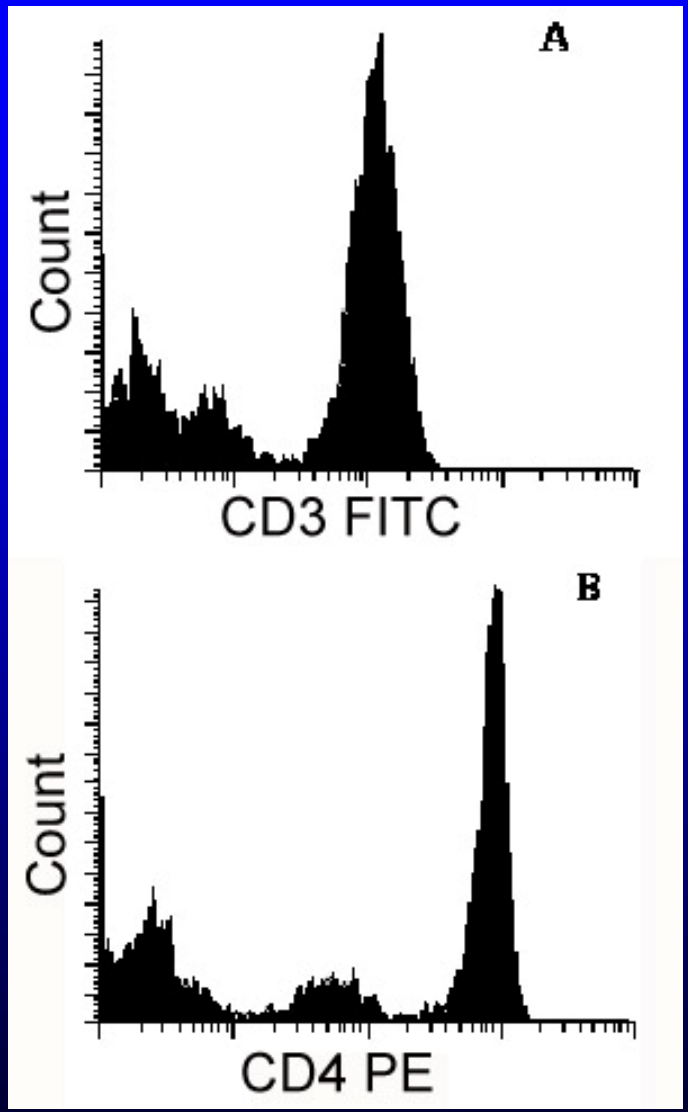
- Same methods as for immunochemistry
- Antibody is labeled with a fluorescent molecule (FITC, PE)
  - No chemical reaction necessary
  - More colors possible
  - Higher sensitivity
  - Inspection requires a specially equipped microscope (Hg high pressure lamp, filter blocks)
  - Not a transmission microscopy but reflection microscopy → suitable for non-transparent materials

Flow Cytometry (FACS)

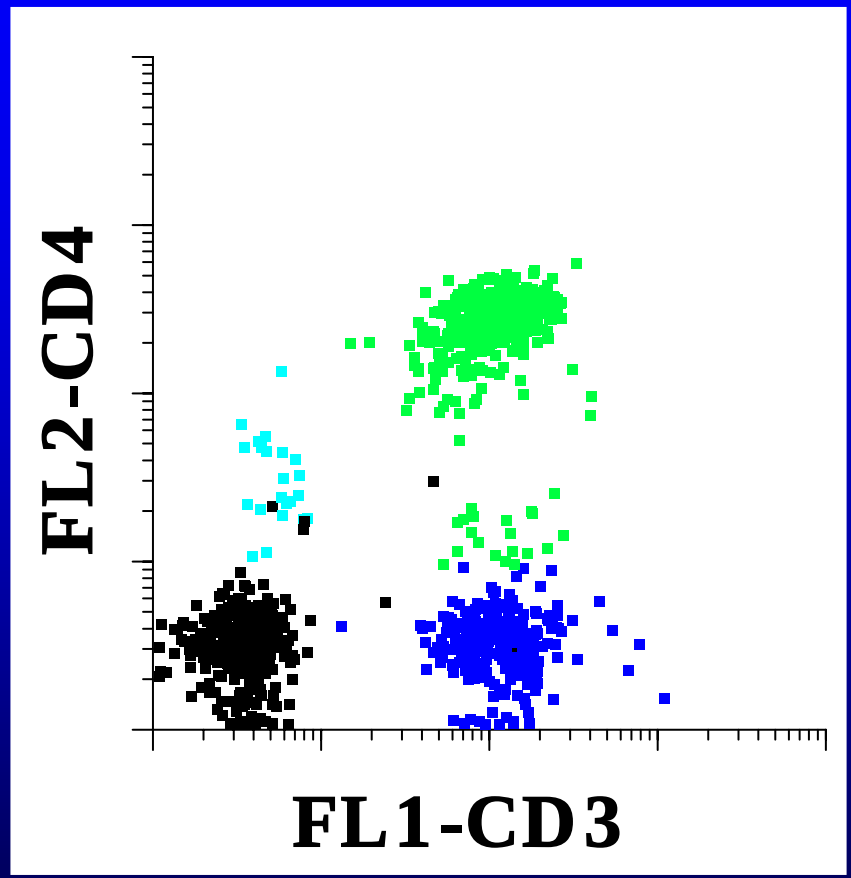


- Single-cell suspension
- Cells are stained with fluorescent antibodies
- Cells pass one-by one through a laser beam, which induces the fluorescence
  - Scattering of the laser light as such gives information about the size and structure of the cell
- Fluorescence (several colors) is detected (quantatively) for each individual cell
- Information, which antigens are expressed together...
- Sorting according to antibody staining possible

Flow Cytometry



1D Plot



2D Plot

Adapted from C.C. Steward, Purdue Cytometry Site

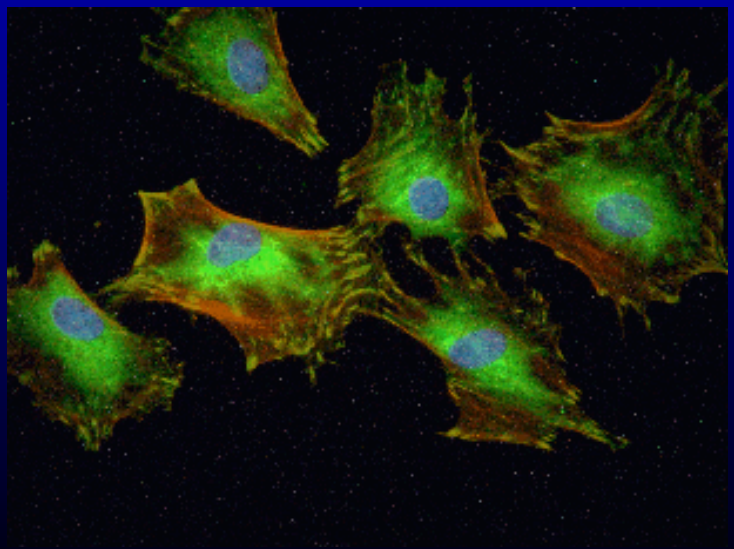
Pitfalls

- No cross-reactivity of the antibody with the species of your sample
  - This almost certainly happens if the antibody is produced in the same species as your sample (Ab from mouse and tissue or cells also from mouse)
- Cross-reactivity of the antibody with other proteins
- Antibody recognizes only native epitopes, but not after formalin or ethanol fixation
- Epitopes may be not stable at high temperature
- (antibodies cannot pass a cell membrane)

Immunofluorescence Protocol

Staining of Cells on Stainless Steel Samples

20min	Fixation in PBS-Formalin
3x10min	wash in PBS
5min	0.2% Triton X/PBS-FBS pH 7.3, 0°C
3x10min	wash in PBS-FBS
min 1h	primary antibody in PBS-FBS at RT
3x10min	wash in PBS-BSA
min 1h	sec. antibody 1:50 in PBS-FBS
2x10min	wash in PBS-FBS

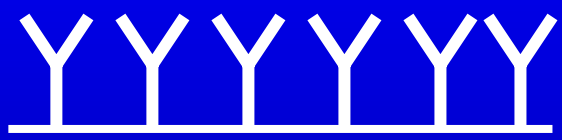


Rat Bone Marrow Cells on stainless steel. 8h adhesion time.

Green: Vinculin
Red: f-Actin
Blue: Nucleus

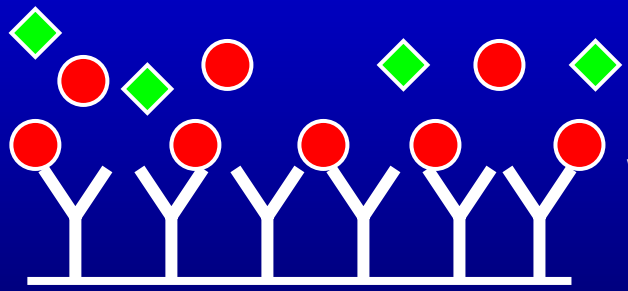
Enzyme Linked Immunosorbent Assay
ELISA

Principle of the classical „Sandwich ELISA“

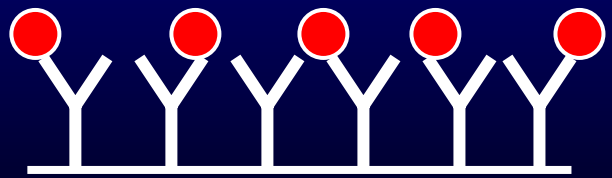


Catching Ab

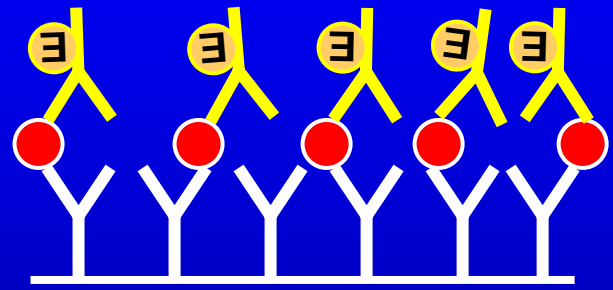
Carrier, ELISA-Plate



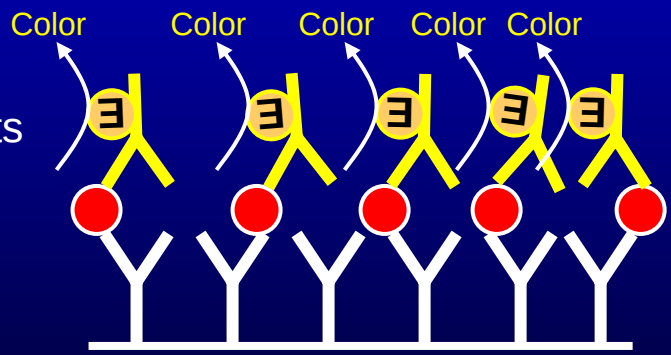
Incubate with protein solution: Protein will bind to the antibody proportional to its concentration in the solution



Wash: remove free and other proteins

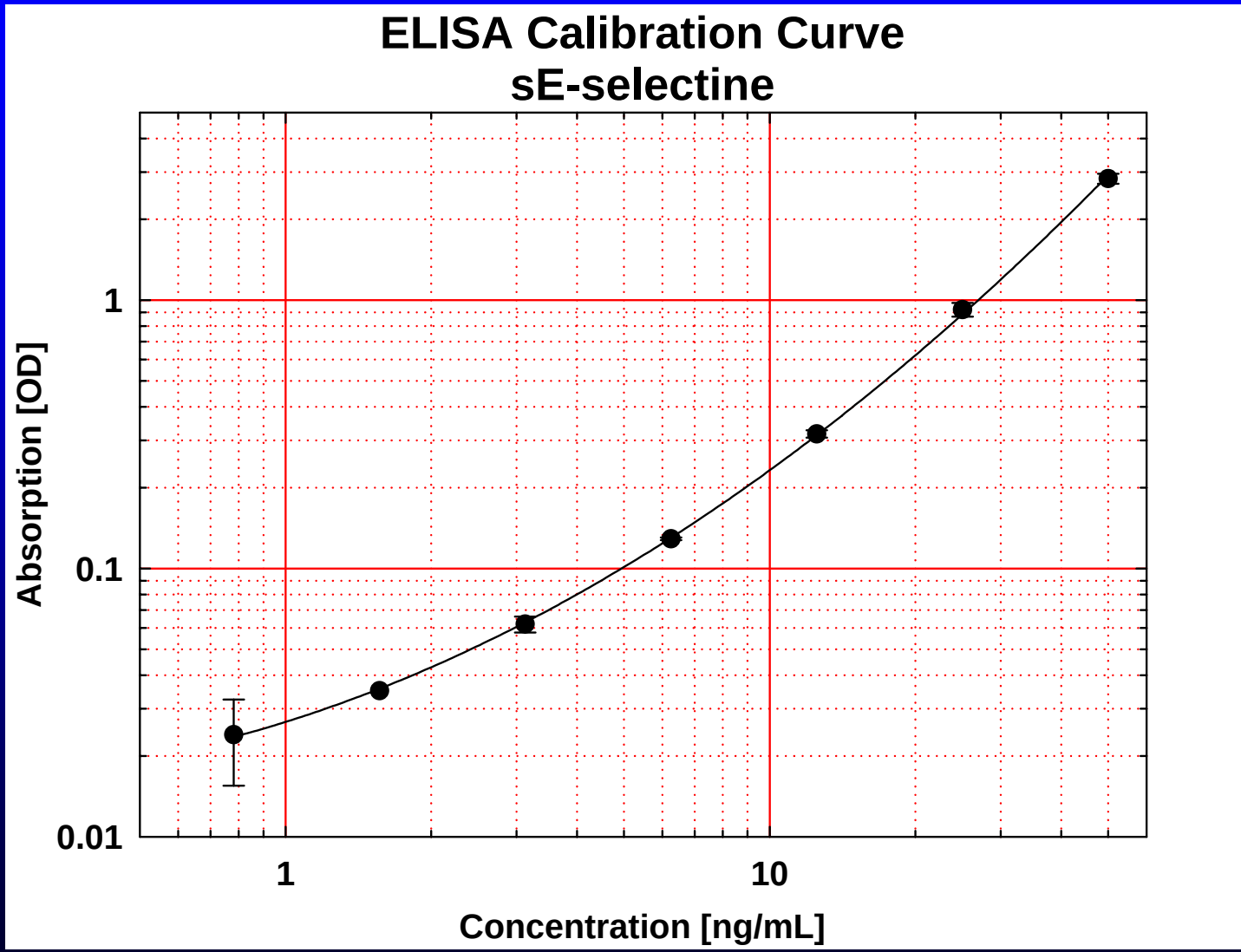


Incubate with detection Ab and wash



Color Reaction and photometrical analysis against standards

Calibration Curve



ELISA (II)

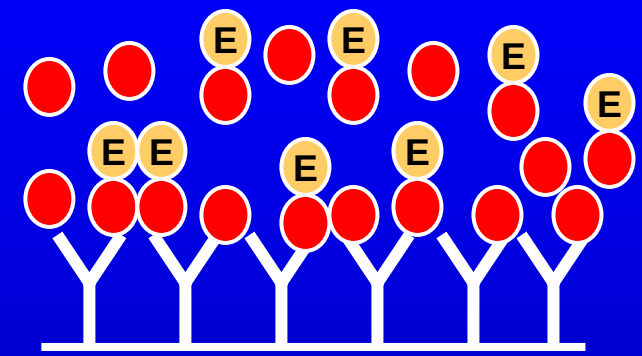
Purpose:

- Measuring the concentration of a solvable protein: mediator, enzyme, dissolved cell component...; frequently in cell culture supernatant
- Concentration often in µg/ml or pg/ml range

Specifics

- Requires a pair of antibodies: capture Ab and detection Ab against two epitopes of the same protein
- Calibration curve with known concentrations must be determined for every test
- Double measurement of the samples is recommended

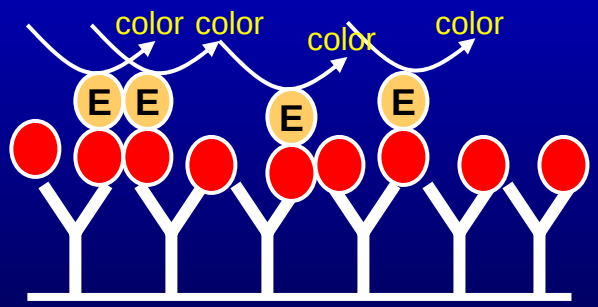
Competitive ELISA



Incubate with labeled substrate and unknown concentration of non-labeled substrate

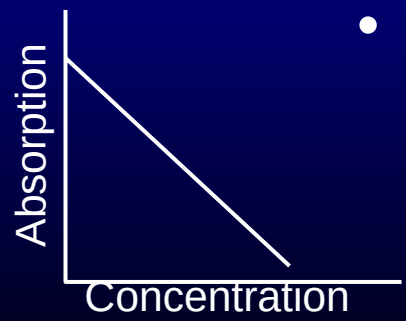
E Labeled substrate

Sample substrate (unknown concentration)



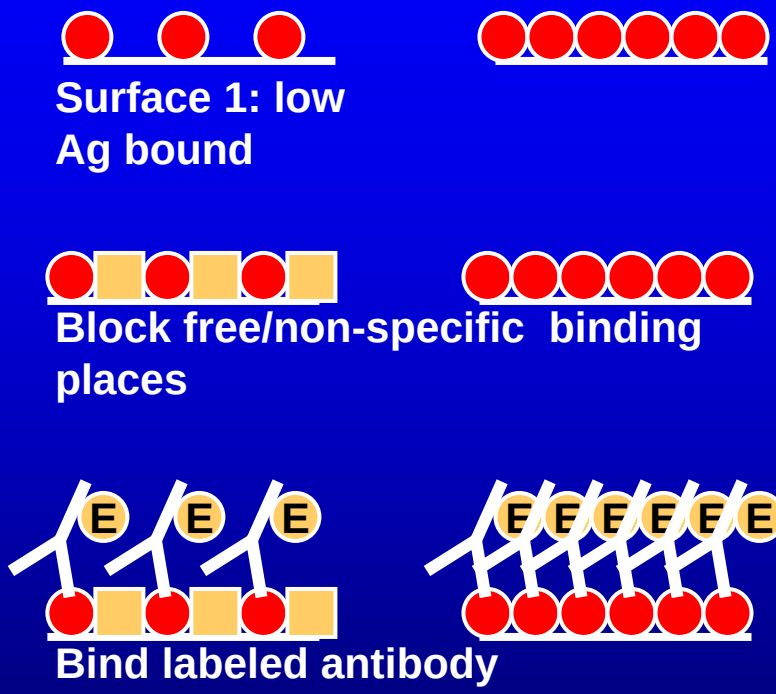
(1) Wash

(2) Color reaction



- Use if only one antibody is available
- Requires a labeled substrate at a constant concentration
- The non-labeled molecules of the sample and the labeled test compete with the binding places of the antibody
- Signal decreases with increasing concentration of the target molecule

Quantification of Surface-Bound Molecules



- Antibody binding and detection in the standard method
- Mainly it allows only a relative quantification and no absolute quantification (ng/cm^2)
- False low result, if the antigen binds with the target-epitope of the antibody to the surface (steric hindrance)

Limitations of ELISA

- False negative/ low results
  - No cross-reactivity between ELISA-AB's and the species of your sample
  - Epitopes are hidden/ covered
- False positive results: ELISA says nothing about the biological activity of the molecule
  - ELISA detects also degradation products/ fragments
  - ELISA detects also products with inhibitor
  - ELISA detects also inactive precursor molecules

SDS Polyacrylamide Gel Electrophoresis SDS-PAGE

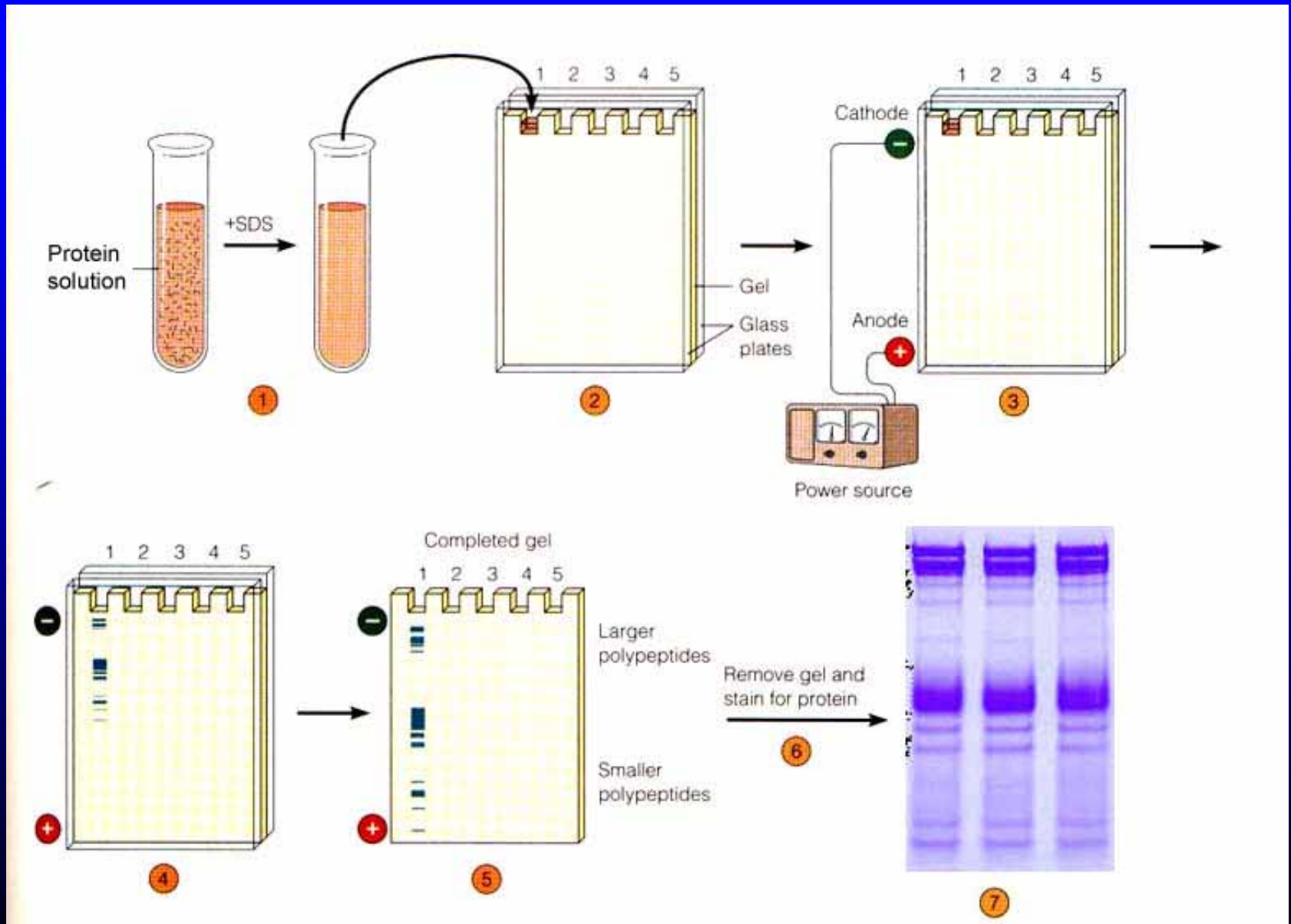
Purpose

- Use to split up a mixture of proteins by their size (molecular mass)

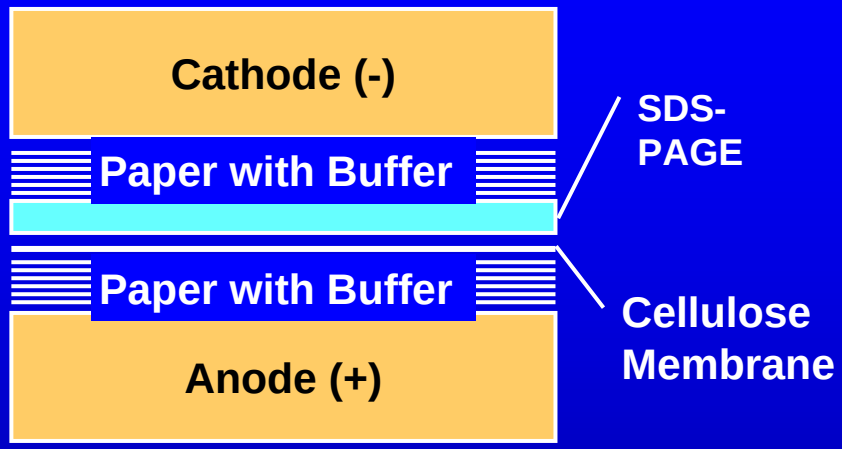
Background

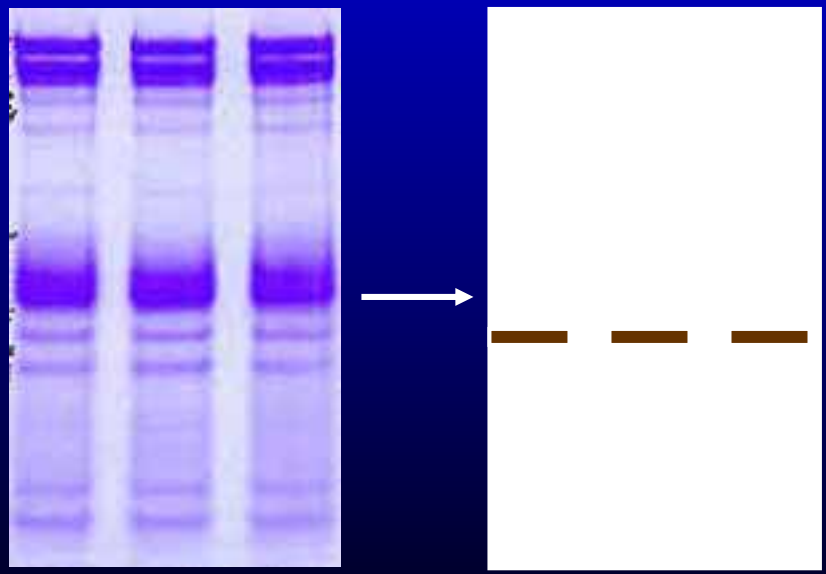
- Sodium dodecylsulphate (SDS) is a negatively charged detergent, which binds to proteins and gives them a constant charge-to-mass ratio
- Polyacrylamide is a gel. It forms a meshwork with adjustable pore size in the range of the size of a protein
- In an electric field the lighter proteins with SDS migrate faster than the heavy ones.

SDS Gel Electrophoresis



Western-Blot (Immuno-Blot)





SDS-PAGE Westernblot

Method

- Transfer the proteins from the SDS-PAGE to a (cellulose) membrane
- Incubate in blocking solution
- Stain with antibodies for your desired protein

Purpose

- Higher sensitivity than PAGE
- Direct identification of the protein
- Correlation with size/ molecular weight