SPECIAL HISTOLOGICAL TECHNIQUES

SPECIAL TECHNIQUES OF LIGHT MICROSCOPY

CONVENTIONAL HISTOCHEMISTRY

Histochemical reaction:
searched structures reveal
coloured reaction product
in situ
(reagent originally colourless)

Detection of
- elements (ions)
- nucleic acids
- lipids
- saccharides
- pigments
- proteins (aminoacids) – currently unusual, immunohistochemistry used instead

EXAMPLES OF HISTOCHEMICAL REACTIONS I

elements (ions)

- Kossa reaction (substitution of $AgNO_3$) = Ca^{2+} (calcification)

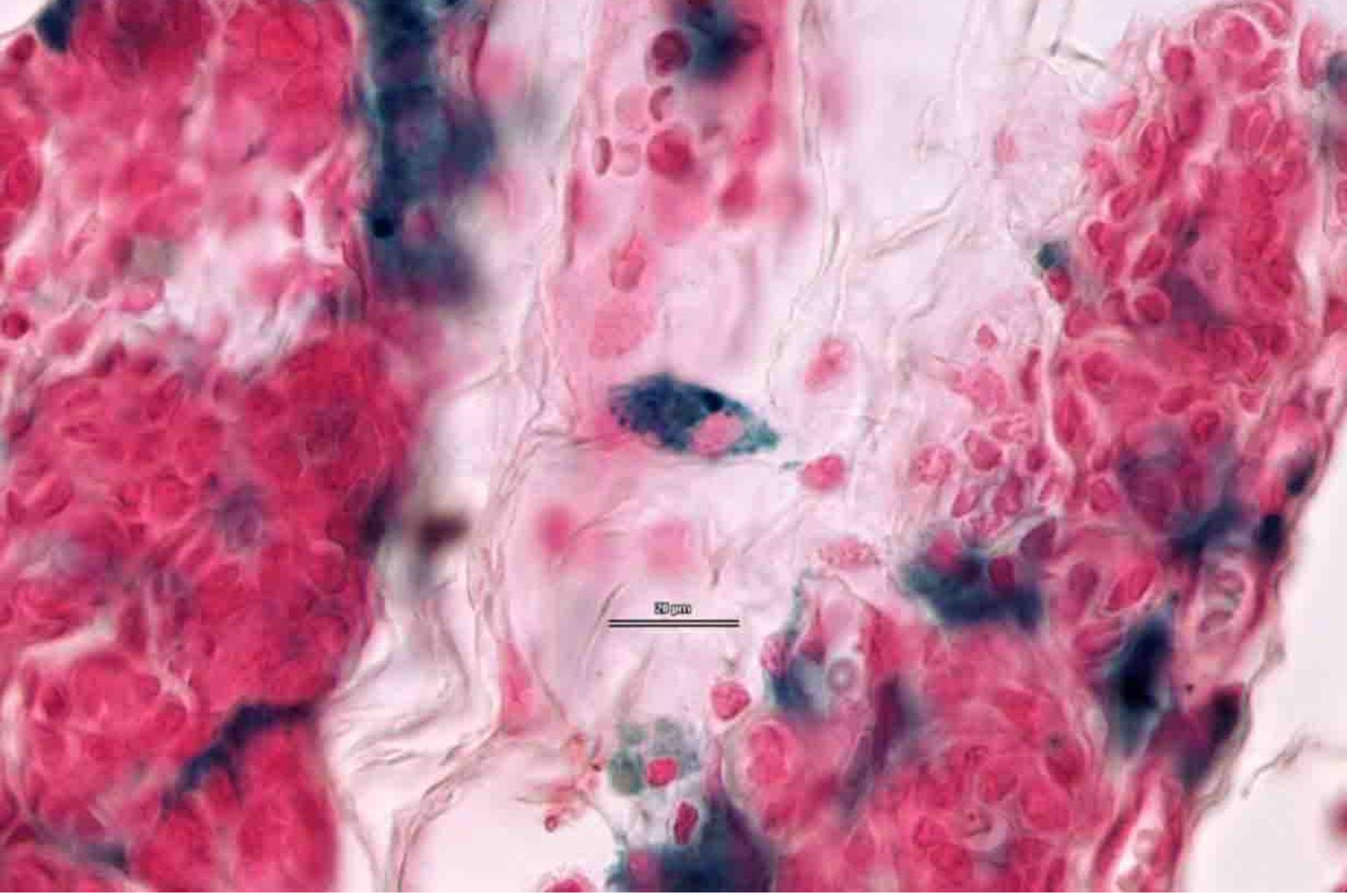
- Perls reaction (production of Prussian blue) = Fe^{3+} (hemosiderin)

nucleic acids

- Feulgen's reaction (HCl + Schiff reagent) = DNA

lipids

- PFAS reaction (PerFormic Acid + Schiff reagent) = double bonds (phospholipids, lipofuscin, also keratin)



Perls' reaction

hemosiderin - siderophages

EXAMPLES OF HISTOCHEMICAL REACTIONS II

saccharides (carbohydrates)

- PAS reaction	= polysaccharides with vicinal glycols (neutral polysaccharides)
- diastase or α-amylyse + PAS	= glycogen
- HID (high iron diamine)	= sulphated glycoconjugates
- Hale-Müller (colloidal Fe)	= acid mucopolysaccharides

pigments

- Gmelin reaction (oxidation)	= bilirubin, hematoidin

proteins (aminoacids)

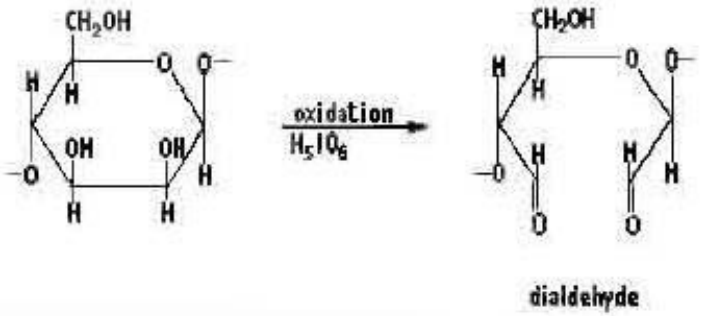
- Million reaction	= tyrosine
- Sakaguchi reaction	= arginine
- tetrazote benzidine reaction	= tryptophan

special	= e.g. detection of superoxide or peroxide radical (Babbs' method)

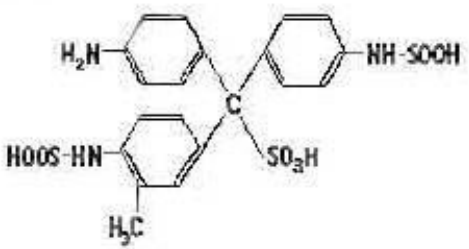
PAS reaction (periodic acid – Schiff)

incompletely specific oxidative method detecting complex carbohydrate in cells and tissues

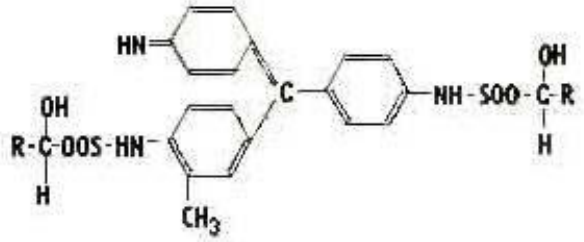
1 oxidation of free *vic* glycol groups by periodic acid (Malaprade reaction)

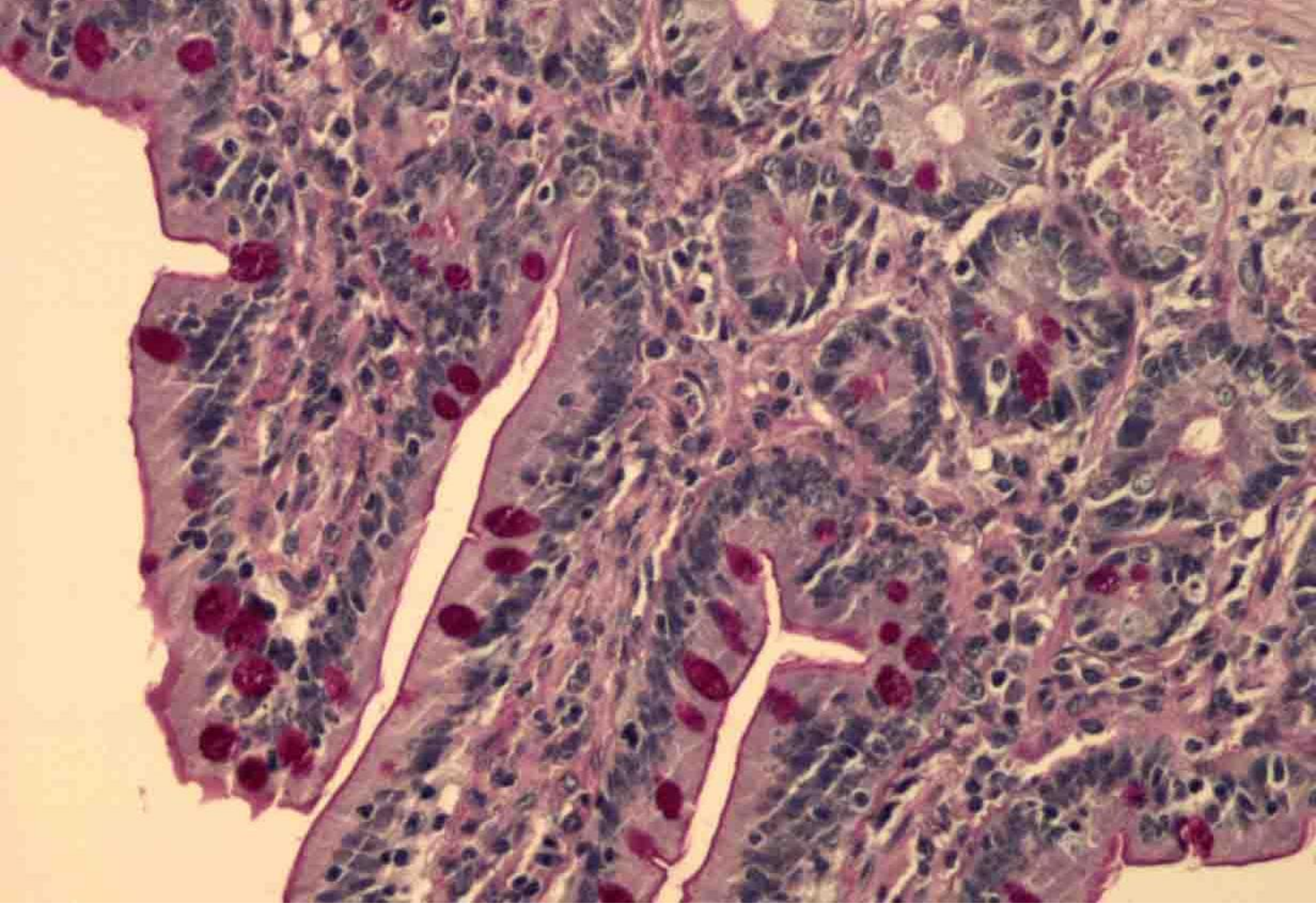


2 detection of just formed aldehyde groups by Schiff reagent
Schiff reagent – leukoform of basic fuchsin (colourless)



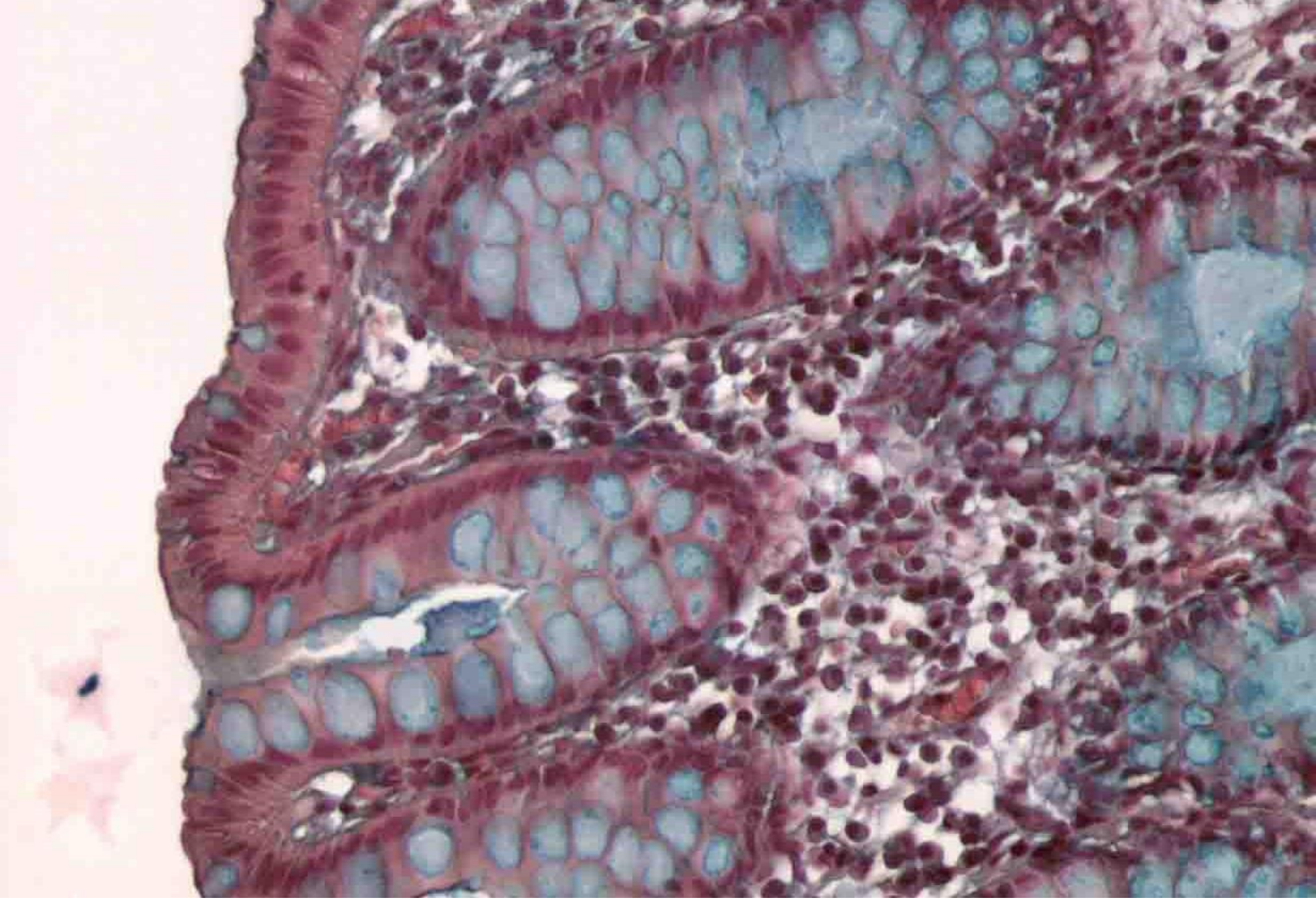
having reacted with aldehydes, a new magenta compound arises





PAS reaction

neutral mucins



Hale-Müller

acidic glycoconjugates

ENZYME HISTOCHEMISTRY

(catalytic)

looking for localization and/or activity of certain enzyme

- first; a reaction product is formed after processing a **substrate** by the enzyme looked for,
- then, the reaction product is visualized by formation of a **coloured compound *in situ***

What should be preserved:

- activity of the enzyme („native“ specimen, proper pH, and temperature)
- stability of the reaction product as well as of the final coloured compound
- tissue structure

thus, an enzyme is offered with a suitable **substrate** at first

- phosphatases — various phosphates - glycerolphosphate
  - NP (α-naphtylphosphate)
  - BCIP (5-bromo-4-chloro-3-indolylphosphate)
- dehydrogenases — oxidizable substrate releasing H^+
- oxidases — oxidizable substrate
- peroxidases — hydrogen peroxide
- esterases (specific and non-specific) — hydrolyzable ester
- glycosidases — glycosidic bond (sugars, glycoproteins, glycolipids)
- sulphatases — sulphoesters
- nucleotidases — nucleotide chain
- reductases — reducible substrate
- proteases — protein or peptide

then, the reaction product is visualized by a **chromogene** reaction

by

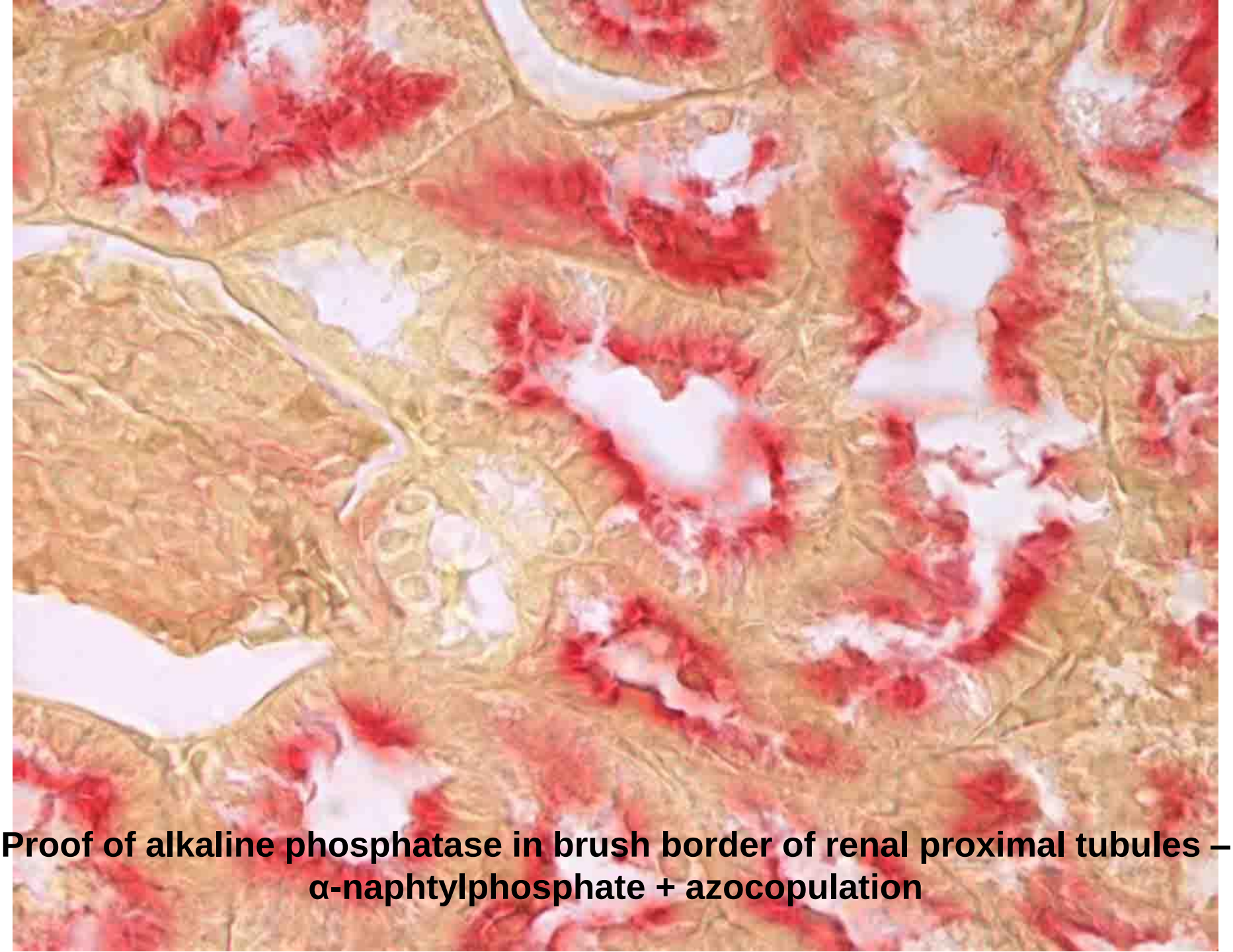
- precipitation reaction (coloured unsoluble metallic salts Pb, Co, Sr, Ce – e.g. sulphates)

- simultaneous azocopulation (naphtol split from a substrate is transformed by a diazonium salt to an unsoluble azocolour)

- indigogenous reaction (indoxyl split from a substrate is oxidized with air O_2 to indigo)

- tetrazolium method (colourless tetrazolium salt is reduced to formazan)

- diaminobenzidine reaction (oxidation of DAB to brown precipitate)



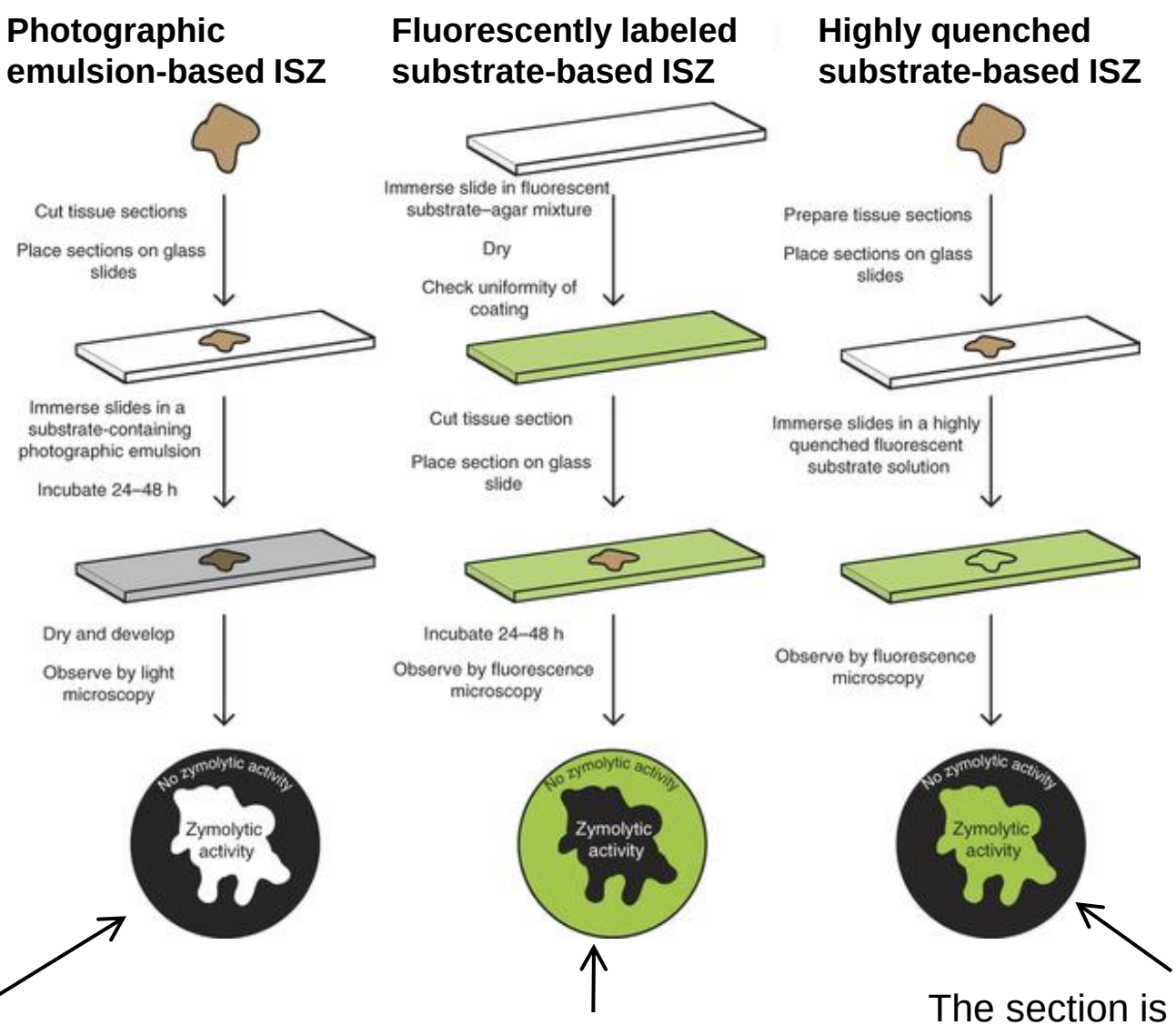
Proof of alkaline phosphatase in brush border of renal proximal tubules – α-naphtylphosphate + azocopulation

ZYMOGRAPHY *in situ*
(ISZ)

identification of localization or activity of a **protease**

metalloproteinases	(MMP-Zn)
serine proteases	(chymotrypsin, trypsin, carboxypeptidase, thrombin, chymase, tryptase, callicrein)
cystein proteases	(cathepsins, calpains, caspases, papains, bromelain)
aspartate proteases	(pepsin, chymosin, renin, retropepsin)
undefined proteases	(kedarcidin, chromoprotein, apoprotein)

enzyme activity reveals as
consumption (cleavage) of a substrate *in situ*
and is visualized either via photography or via fluorescence

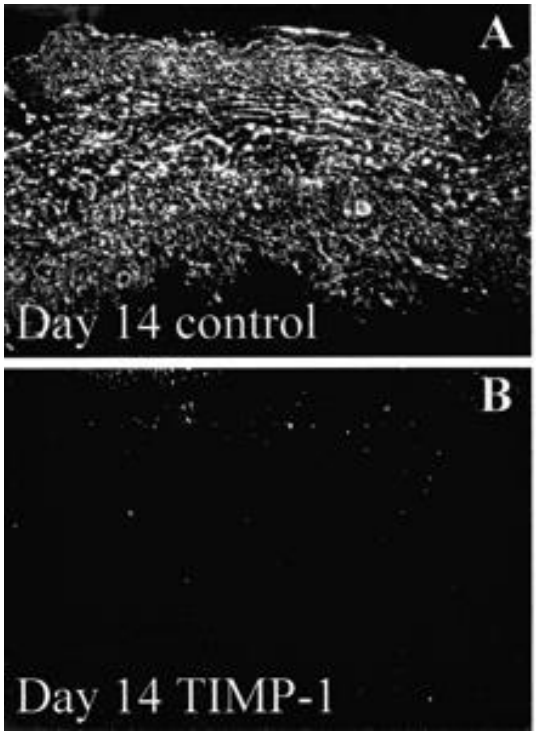


Photographic emulsion contains a substrate, which is digested. After development, empty areas are seen.

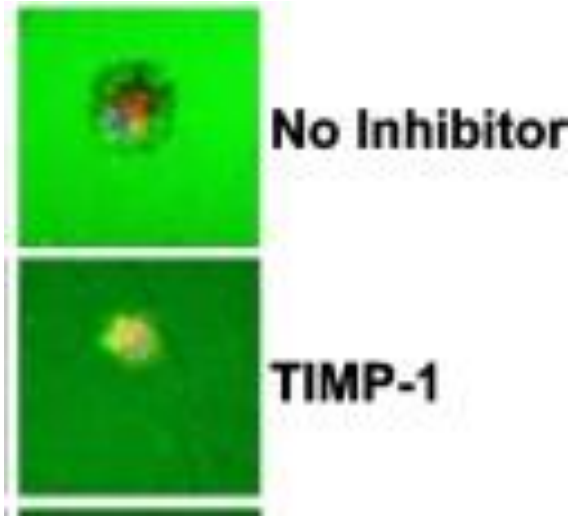
The slide is coated by a substrate conjugated with a fluorochrome. After digestion, empty (non-fluorescing) areas are seen.

The section is incubated with a substrate conjugated with a fluorochrome and its quencher. After digestion, quenching is lost and fluorescent areas are seen.

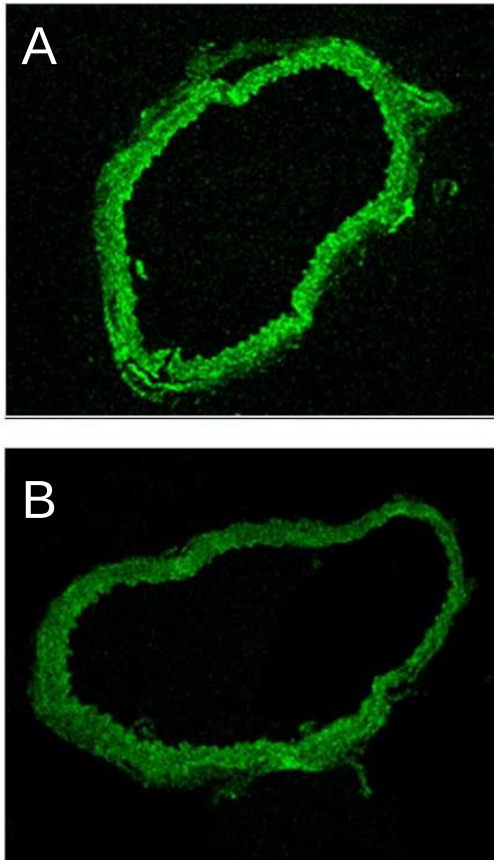
Vandooren et al.: Nature Methods 10, 2013, 211-220



ISZ based on photographic emulsion. White areas indicate metalloproteinase activity in the wall of vein (A). Metalloproteinase inhibitor TIMP-1 was used in B.



ISZ based on fluorescently labeled substrate. Dark areas indicate metalloproteinase activity of human breast carcinoma cells, which is inhibited by a TIMP-1.



ISZ based on highly quenched substrate. Green areas indicate metalloproteinase activity in the wall of mouse aorta, which was partly inhibited in figure B.

Aguilera et al.: Cardiovasc. Res. 58, 2003, 679-688
Remacle et al.: J. Biol. Chem. 286, 2011, 21002-21012
Basu et al.: Arch. Physiol. Biochem. 117, 2011, 270-282

AFFINITY HISTOCHEMISTRY

IMMUNOHISTOCHEMISTRY

looking for **location of certain substance** in a cell or in extracellular matrix

the molecule of detected substance must contain at least one antigenic determinant (**epitope**), the detected substance is then called the **antigen**

the epitope is a specific chain of aminoacids or monosaccharides, thus the detected substance can be **protein, glycoprotein,** polysaccharide, lipoprotein or glycolipid

an artificially prepared **antibody** (immunoglobulin) binds specifically to the epitope

The antibody is **labeled** by

- fluorochrome
- enzyme
- hapten – small non-immunogenic molecule, which becomes immunogenic after a bond with protein (biotin, digoxigenine)
- heavy metal in EM

Then, the label is visualized.

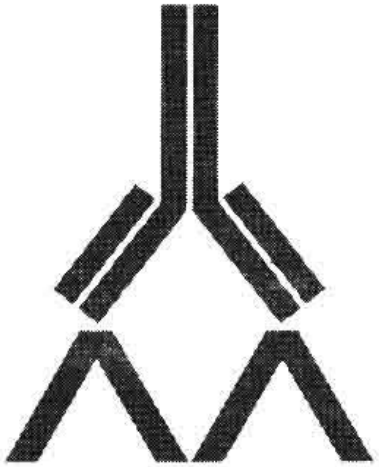
BOND OF AN ANTIBODY TO AN ANTIGEN INFLUENCED with

- incubation temperature
- incubation pH
- incubation time
- environment humidity
- antibody concentration
- lighting
- fixation method and course

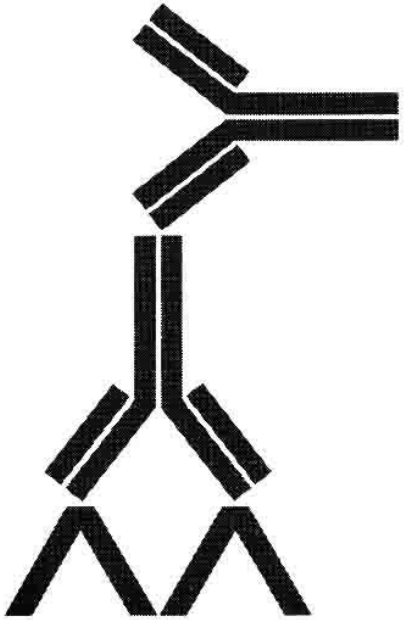
ANTIBODIES USED IN IMMUNOHISTOCHEMISTRY

1/ division by application

a/ primary antibody – an antibody binding directly the detected antigen

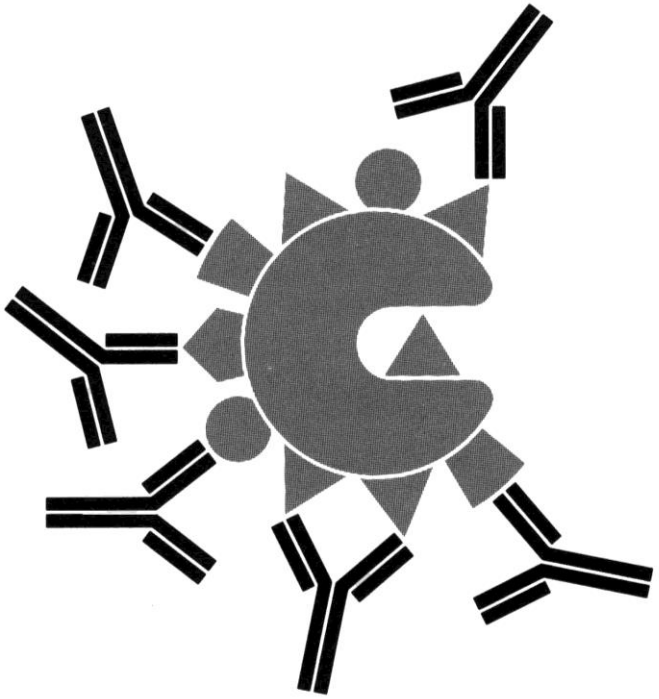


b/ secondary antibody – an antibody against the Ig of the animal species producing the primary antibody

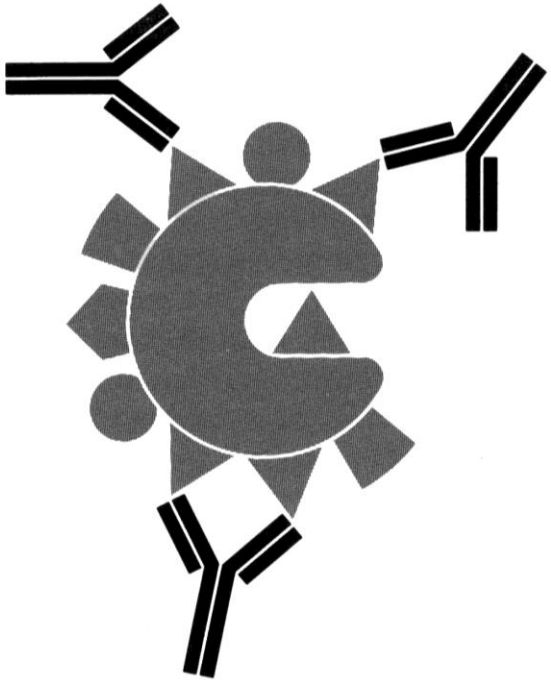


2/ division by specificity

a/ polyclonal antibody – an antibody prepared by immunization of an animal by the selected antigen, it can bind various epitopes of the given antigen



b/ **monoclonal antibody** – an antibody prepared by the hybridoma technique, it can bind only one epitope



POLYCLONAL ANTIBODIES

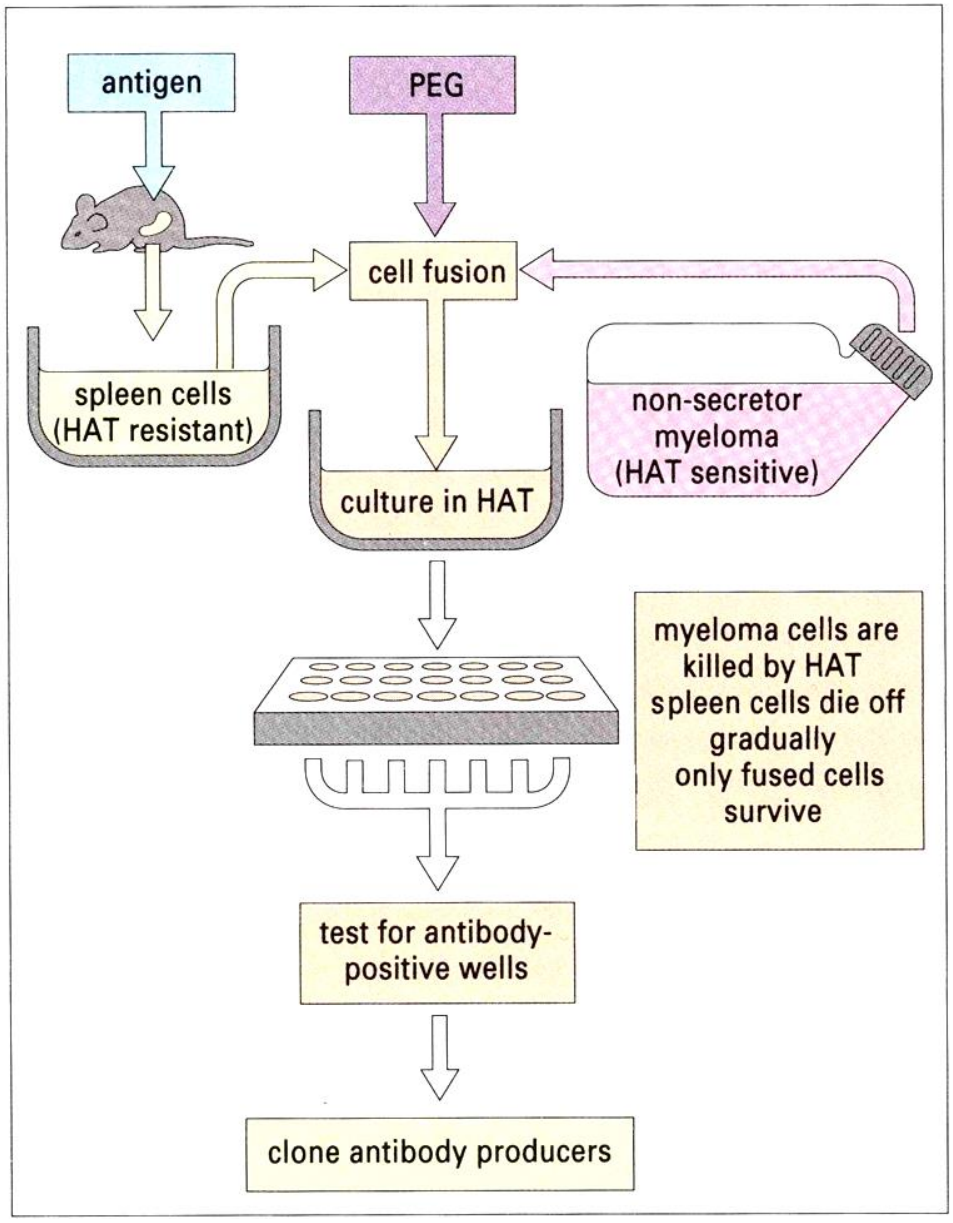
immunization of a laboratory animal
(mouse, rabbit, goat, rat, swine, donkey, dog, horse)
with an **antigen**

blood serum fraction (antiserum)

advantage
speed and simple

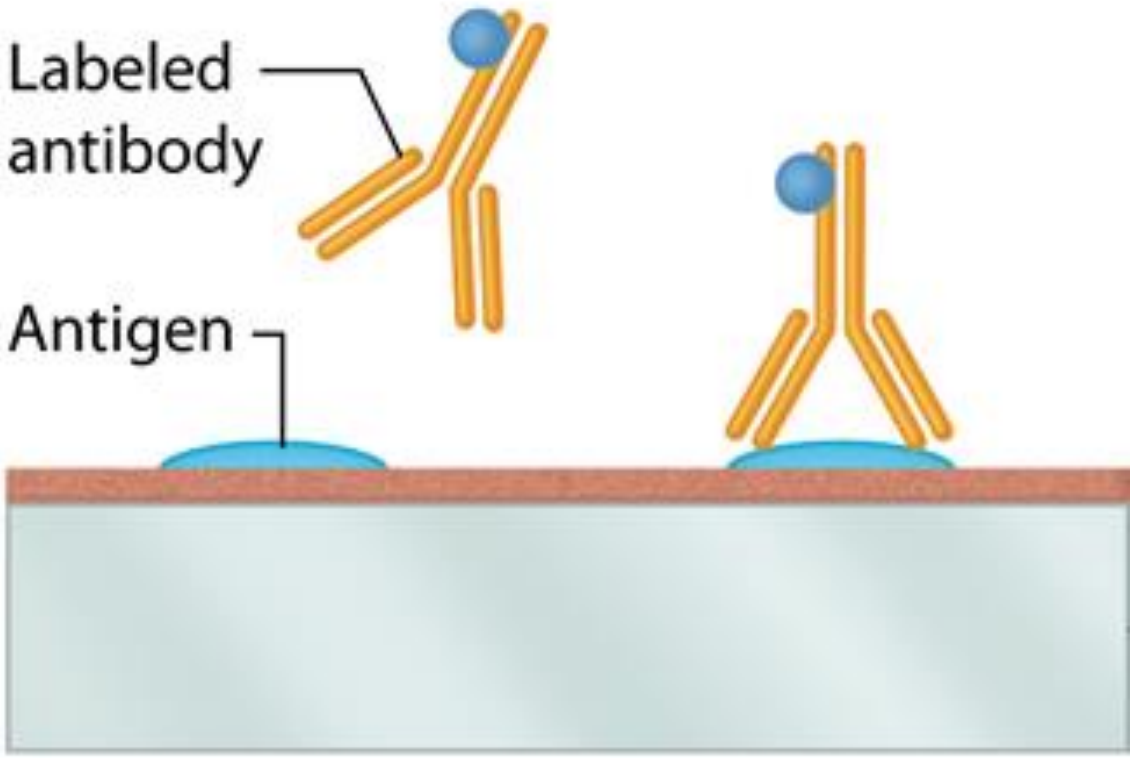
problem
non-specific cross reactions to identical or similar epitopes of different antigens

Monoclonal antibody production

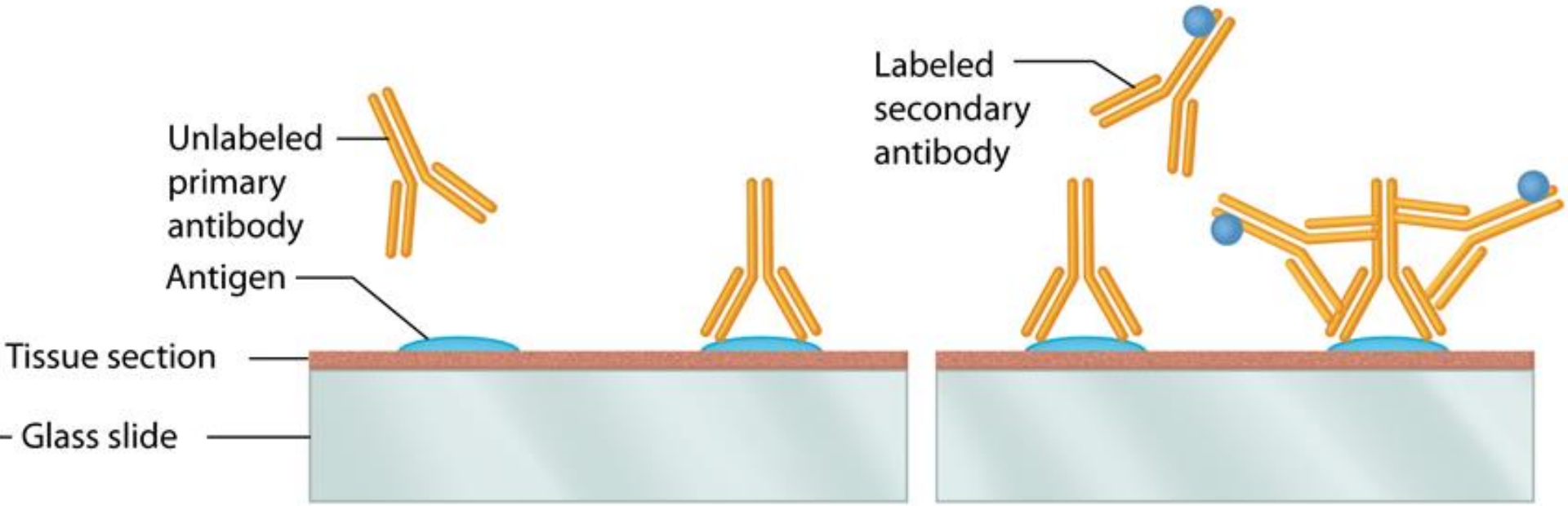


Animals (usually mice or rats) are immunized with antigen. Once the animals are making a good antibody response the spleens are removed and a cell suspension prepared (lymph nodes cells may also be used). These cells are fused with a myeloma cell line by the addition of polyethylene glycol (PEG) which promotes membrane fusion. Only a small proportion of the cells fuse successfully. The fusion mixture is then set up in culture with medium containing 'HAT'. HAT is a mixture of Hypoxanthine, Aminopterin and Thymidine. Aminopterin is a powerful toxin which blocks a metabolic pathway. This pathway can be bypassed if the cell is provided with the intermediate metabolites hypoxanthine and thymidine. Thus spleen cells can grow in HAT medium, but the myeloma cells die in HAT medium because they have a metabolic defect and cannot use the bypass pathway. When the culture is set up in HAT medium it contains spleen cells, myeloma cells and fused cells. The spleen cells die in culture naturally after 1–2 weeks and the myeloma cells are killed by the HAT. Fused cells survive however as they have the immortality of the myeloma and the metabolic bypass of the spleen cells. Some of them will also have the antibody producing capacity of the spleen cells. Any wells containing growing cells are tested for the production of the desired antibody (often by RIA or ELISA) and if positive the cultures are cloned, that is, plated out so that only one cell is in each well. This produces a clone of cells derived from a single progenitor, which is both immortal and produces monoclonal antibody.

Hybridoma technology (G.J.F. Köhler and C. Milstein, 1975 – Nobel Prize 1984)

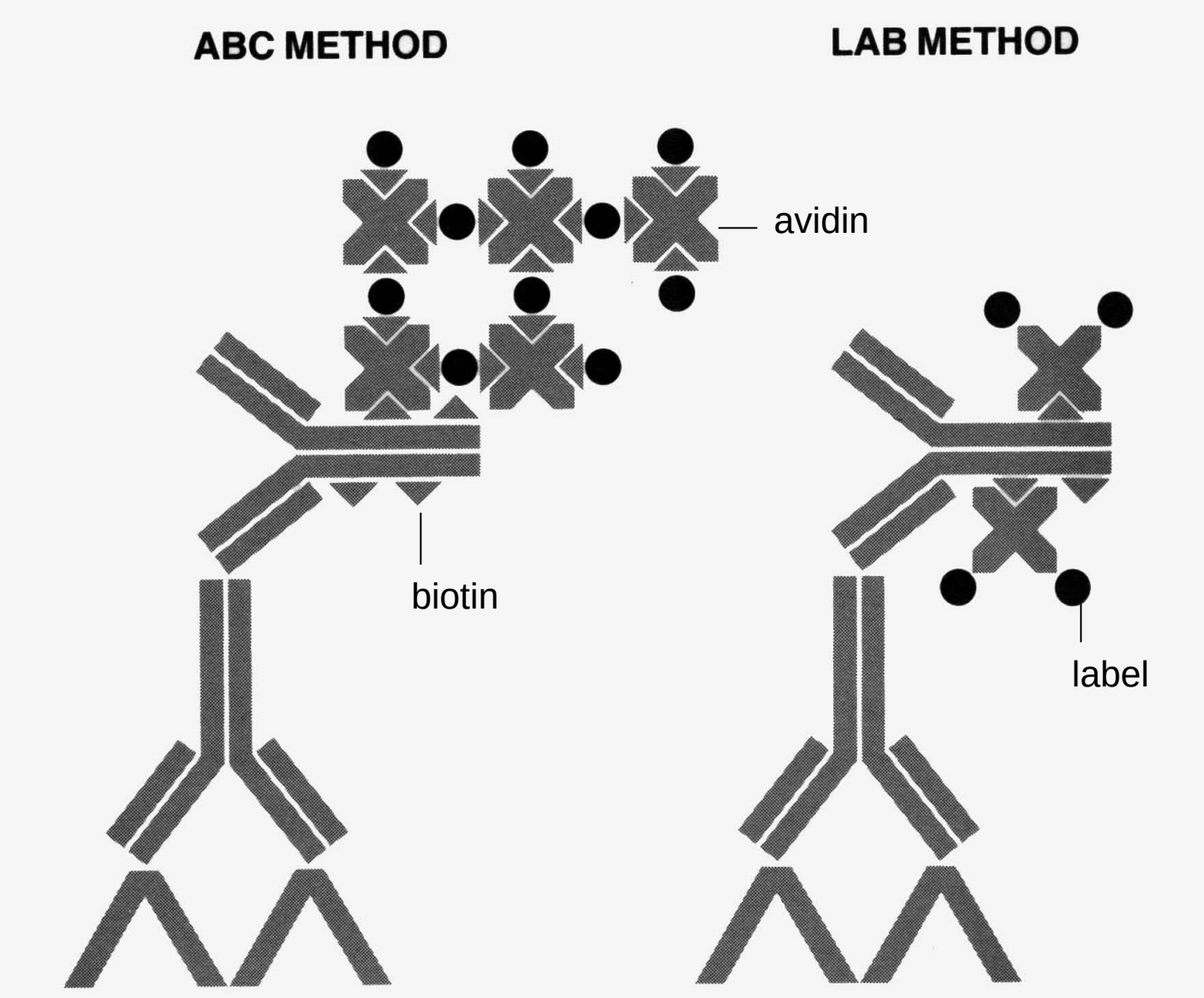


Direct method
labelled **primary** antibody reacts with a tissue (cellular) antigen directly



Two-step indirect method

labelled **secondary** antibody reacts to **primary** antibody bound to a tissue (cellular) antigen



Three-step methods using avidin-biotin
pre-prepared complex of avidin and biotin (ABC) or enzyme-labelled avidin (LAB) react with the biotinylated **secondary** antibody

Performance of the two-step indirect method

1. cryostat or paraffin (deparaffinated and rehydrated) section on the slide
2. revitalization of antigens (antigen retrieval)
(3. blocking endogenous enzymes – if the enzyme visualization is used)
4. blocking background staining
5. incubation with the primary antibody
6. incubation with the labeled secondary antibody
7. visualization
8. counterstaining
9. mounting

ANTIGEN RETRIEVAL

During tissue processing, some epitopes can be damaged by fixation, clearing, drying, overheating or repeated thawing and freezing

The revitalization of antigen properties of a biological material:

- **using proteases digestion (**trypsin, pepsin, pronase, ficin, bromelain)

or

- **using high temperature and special buffer** (citrate, TRIS, EDTA)
  - -water bath heating (95-98°C)
  - -microwave radiation
  - -pressure cooker boiling

BACKGROUND BLOCKING

unwanted non-specific background reaction multifactorial

- section or reagents contamined
- non-specific bond – non-covalent hydrophobic interaction
- electrostatic interactions
- non-specific bond to Fc of tissue Ig

- solution
  - different fixation
  - different buffer
  - **saturation with a non-specific serum (BSA, FCS)**
  - or milk

BLOCKING OF ENDOGENOUS ENZYME ACTIVITY

important in visualization using enzyme histochemistry with horseradish peroxidase or alkaline phosphatase as a label

integral peroxidases and phosphatases contained in tissues and cells

false positivity

solution: the enzyme is offered with its substrate (hydrogen peroxide)
or blocked (sodium azide, levamisole)

VISUALIZATION

Specific bond of an antibody with an antigen on kryostat, paraffin or resin sectons is visualized by formation of

a **coloured compound *in situ*** using enzyme histochemistry
or by
a **fluorochrome**

Examples of fluorescence visualization

excitation light (UV, green or blue) excites electrons in the stain molecule onto a higher energy level,
by return, energy is released in the form of fluorescence light of longer wavelength, which is observed

labels mostly used:

fluorescein isothiocyanate (FITC)	**green**
Alexa Fluor® 488	**green**
rhodamine (TRITC)	**orange-red**
Alexa Fluor® 405	**blue**
cyanine dyes	
Cy2	**green**
Cy3	**orange-red**
Cy5	**deep red**

Examples of visualization using enzyme histochemistry

labels mostly used

alkaline phosphatase – AP
substrates/chromogenes:

bromo-chloro-indolylphosphate (**BCIP**)/nitroblue tetrazolium (**NBT**) blue-black reaction (insoluble)

α-naphtylphosphate (**NP**)/**Fast Red**
red reaction (soluble in alcohol and xylene)

horseradish peroxidase – HRP
substrates/chromogenes:

hydrogen peroxide/diaminobenzidine (**DAB**)
brown-black reaction (insoluble, osmiophilic)

hydrogen peroxide/aminoethylcarbazole (**AEC**)
red reaction (soluble in alcohol and xylene)

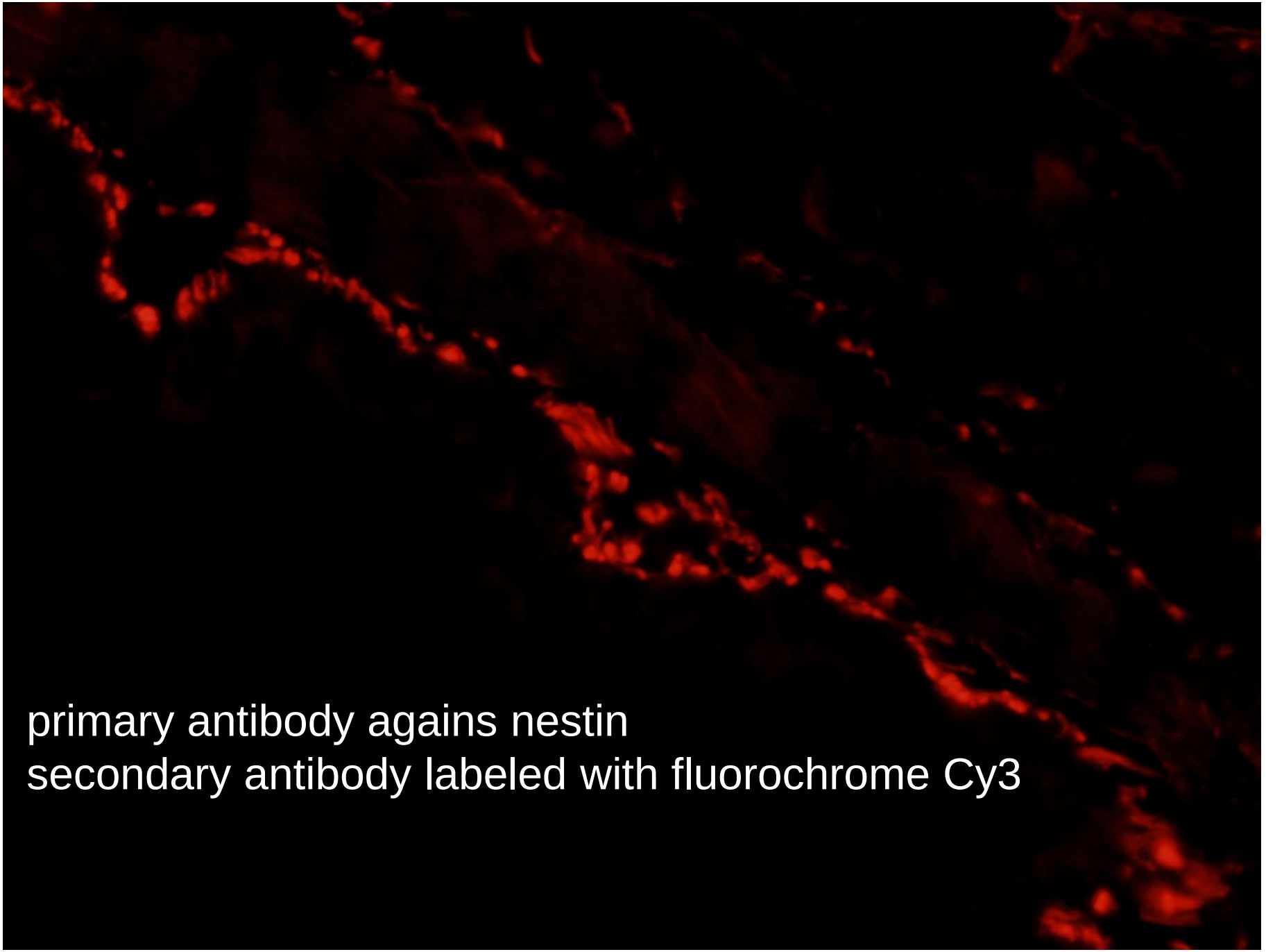
COUNTERSTAINING

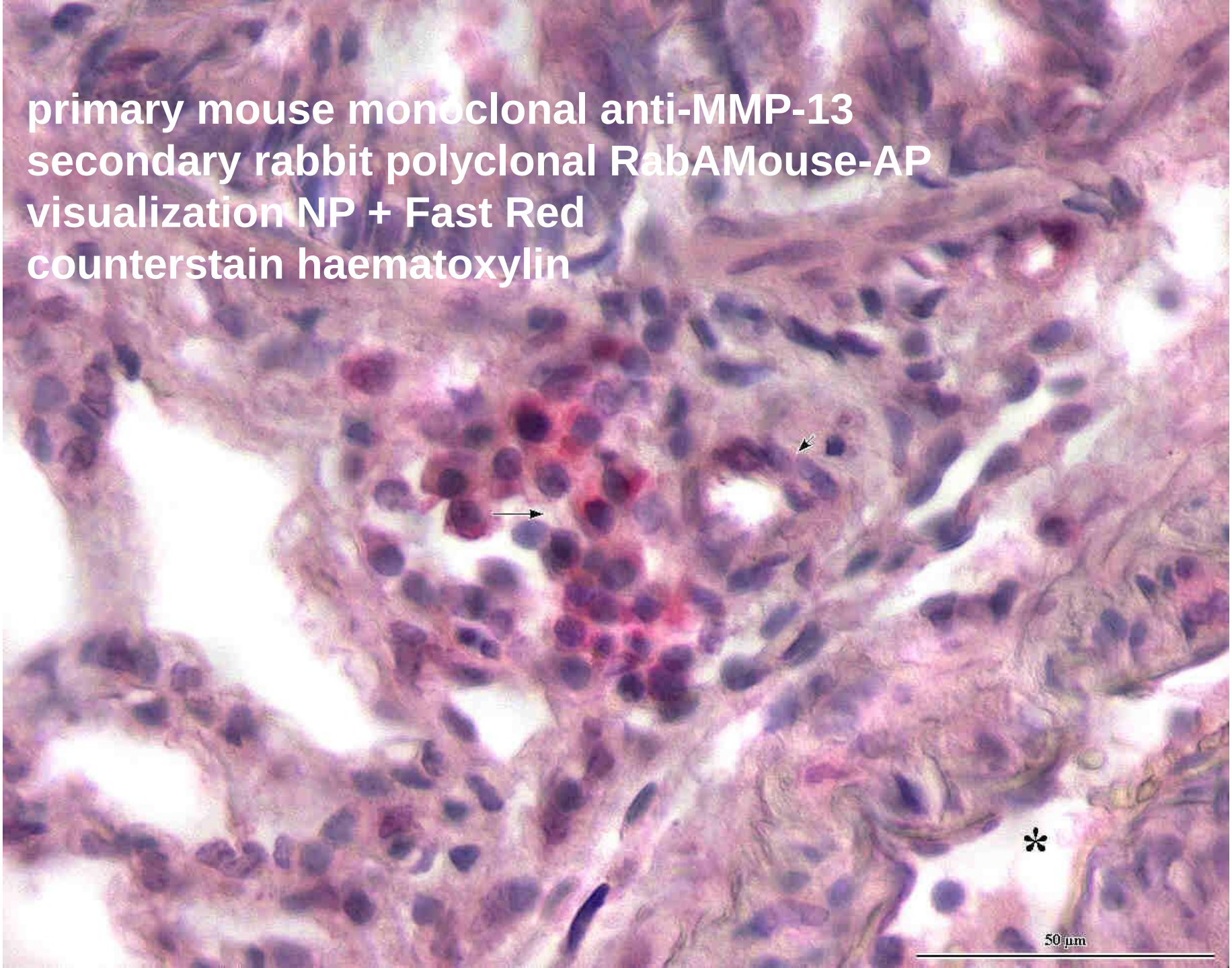
Counterstaining of nuclei: haematoxylin, nuclear red, methylene green, toluidine blue

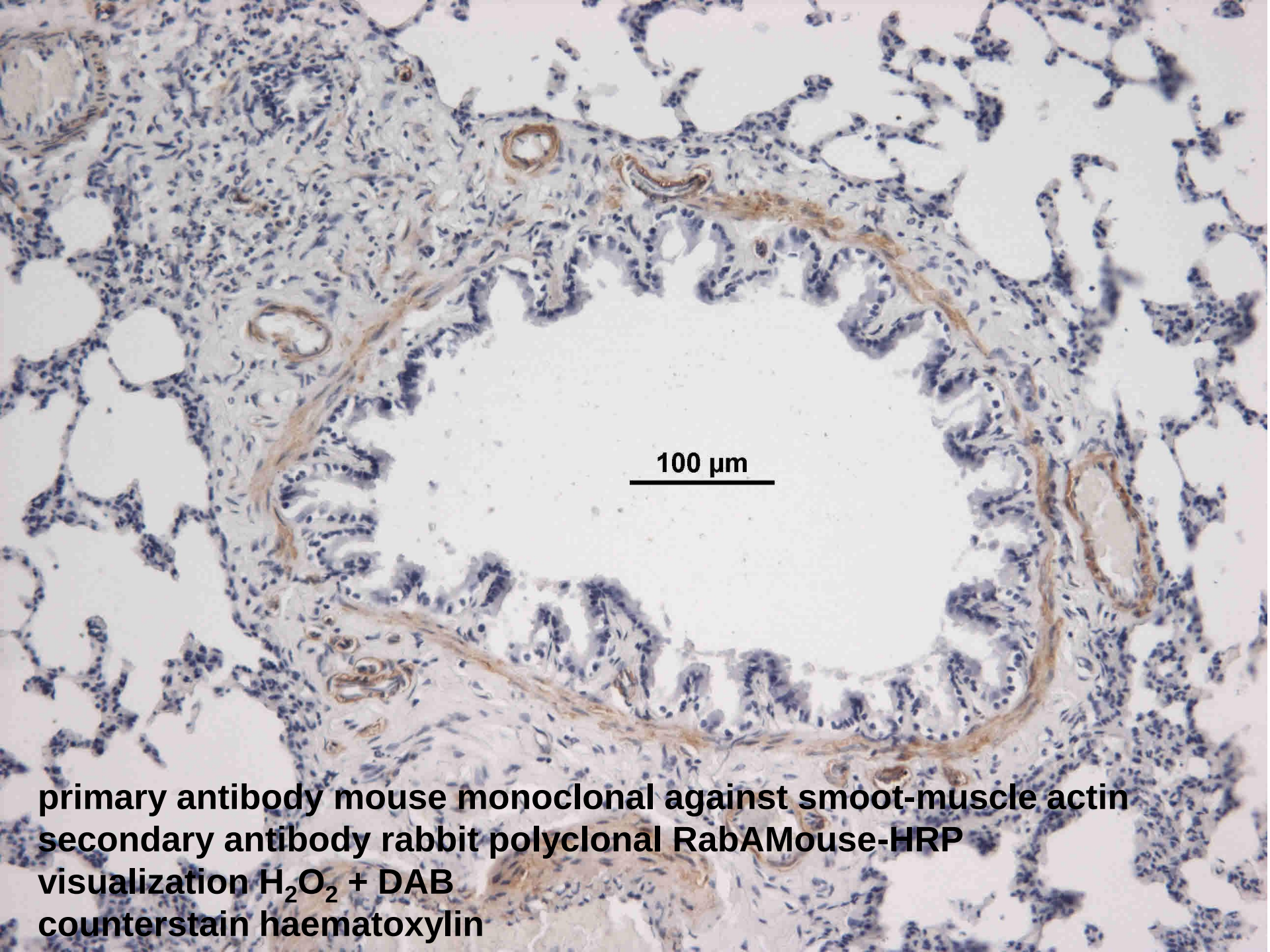
diamidinophenylindol (DAPI) blue fluorescence

propidiumjodide (PI) red fluorescence

Counterstaining of cytoplasm: e.g. light green


primary antibody agains nestin
secondary antibody labeled with fluorochrome Cy3


primary mouse monoclonal anti-MMP-13
secondary rabbit polyclonal RabAMouse-AP
visualization NP + Fast Red
counterstain haematoxylin
50 µm



primary antibody mouse monoclonal against smoot-muscle actin
secondary antibody rabbit polyclonal RabAMouse-HRP
visualization H_2O_2 + DAB
counterstain haematoxylin

Simultaneous detection of several antigens

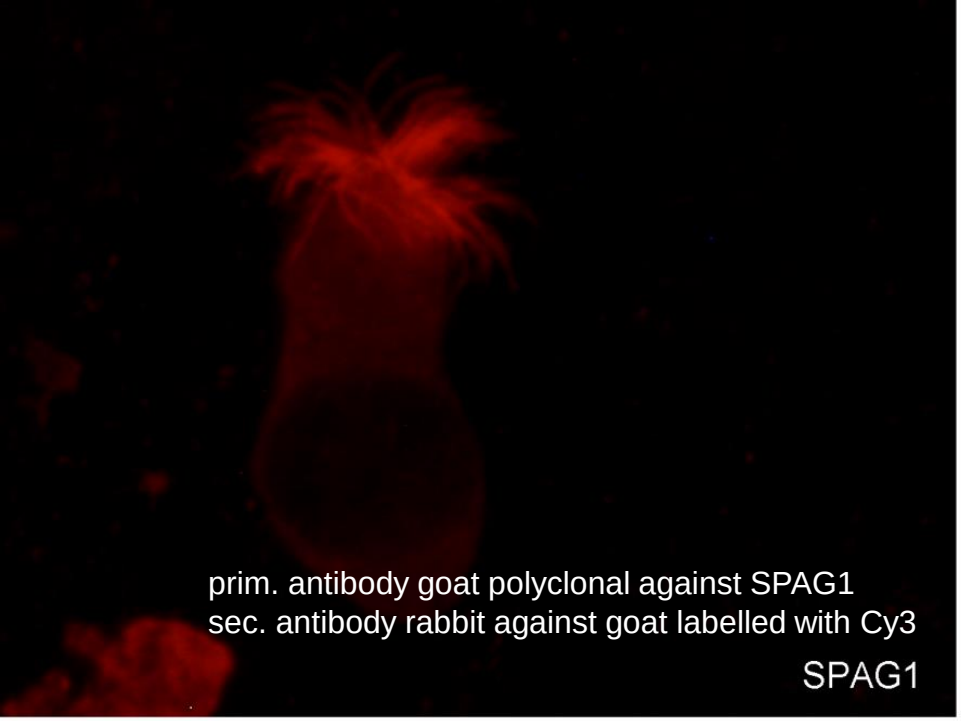
- in an ideal case, we could compare series sections with the evidence of individual antigens – in real, it is difficult

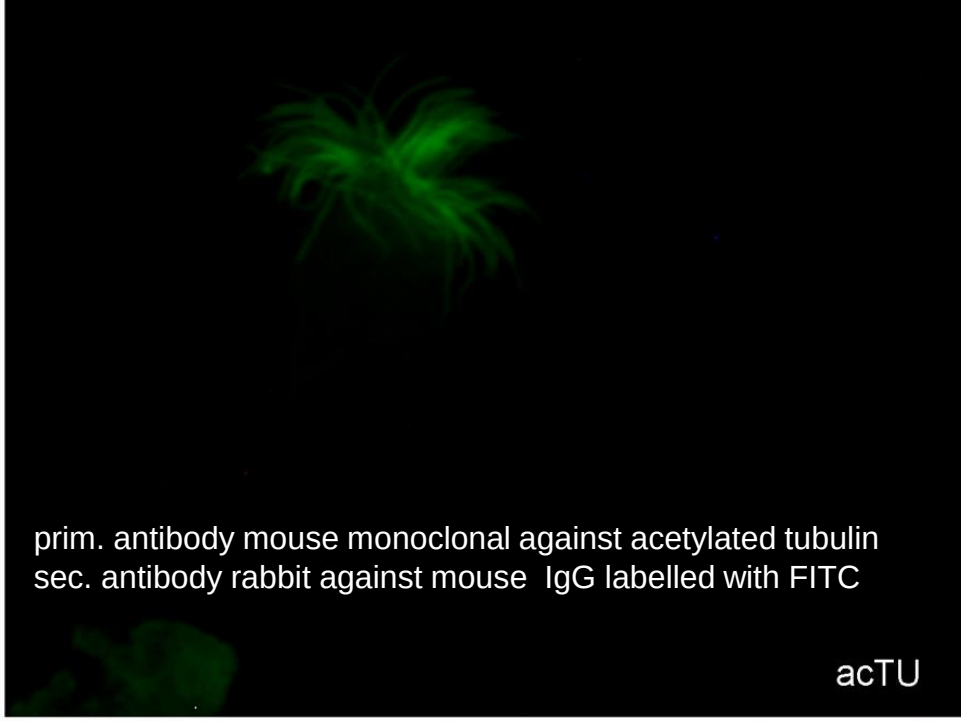
that is why we use on one section

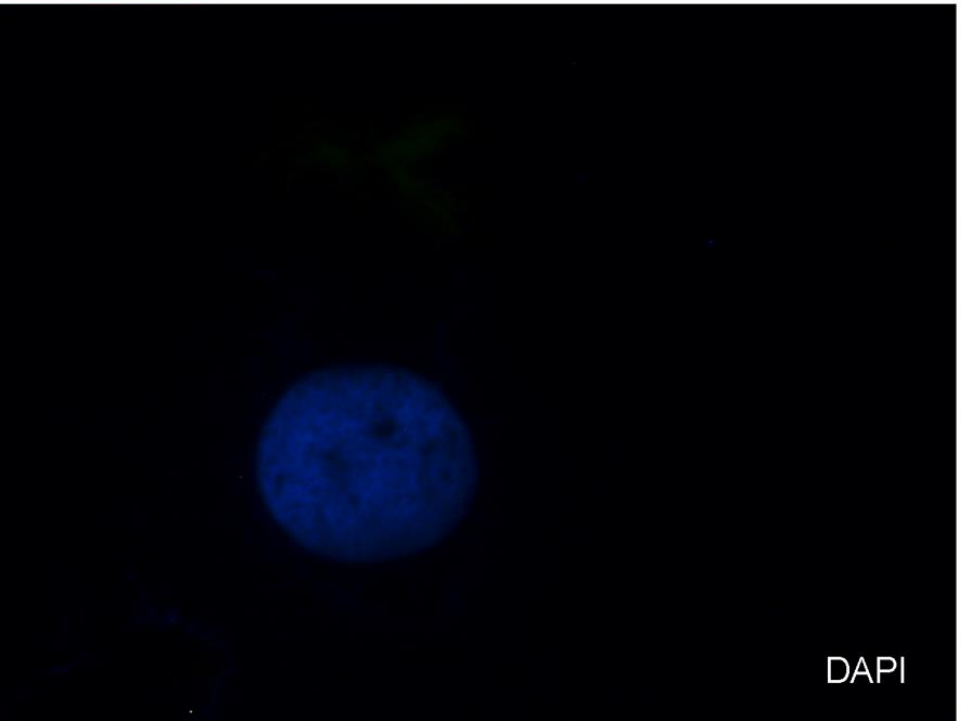
- primary antibodies of different species (e.g. Rb, Go, Sw)
- corresponding secondary antibodies (e.g. SwARb, RbAGo, GoASw)

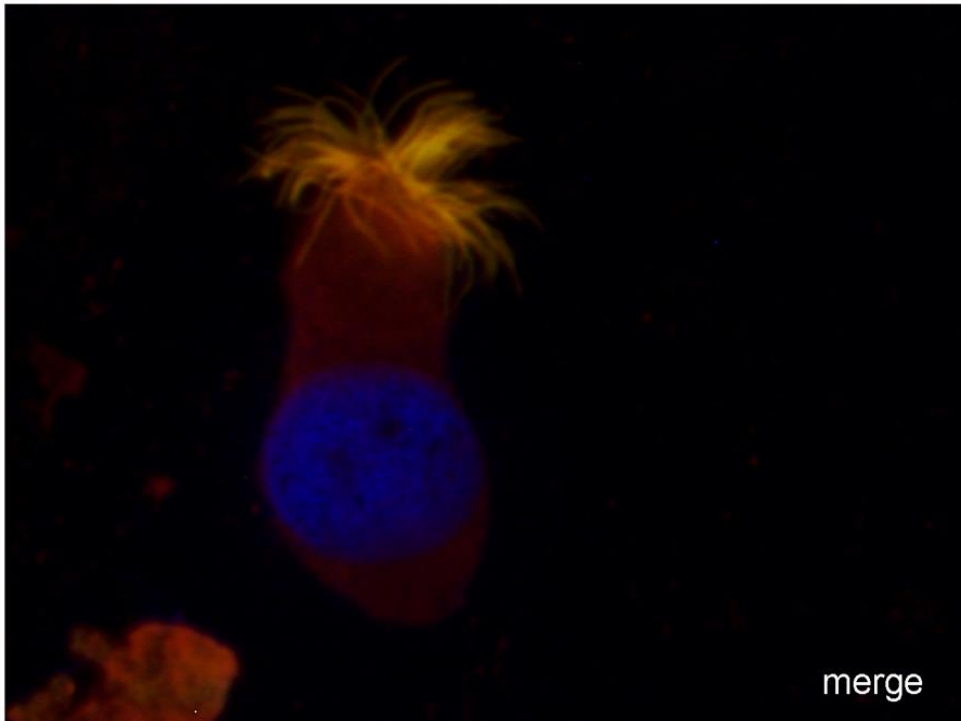
then, we need the latter labelled with

- different fluorochroms
- colloid-Au particles of different sizes
- different enzymes
- or the same enzymes, but we use different substrates and chromogenes


prim. antibody goat polyclonal against SPAG1
sec. antibody rabbit against goat labelled with Cy3
SPAG1


prim. antibody mouse monoclonal against acetylated tubulin
sec. antibody rabbit against mouse IgG labelled with FITC
acTU


DAPI

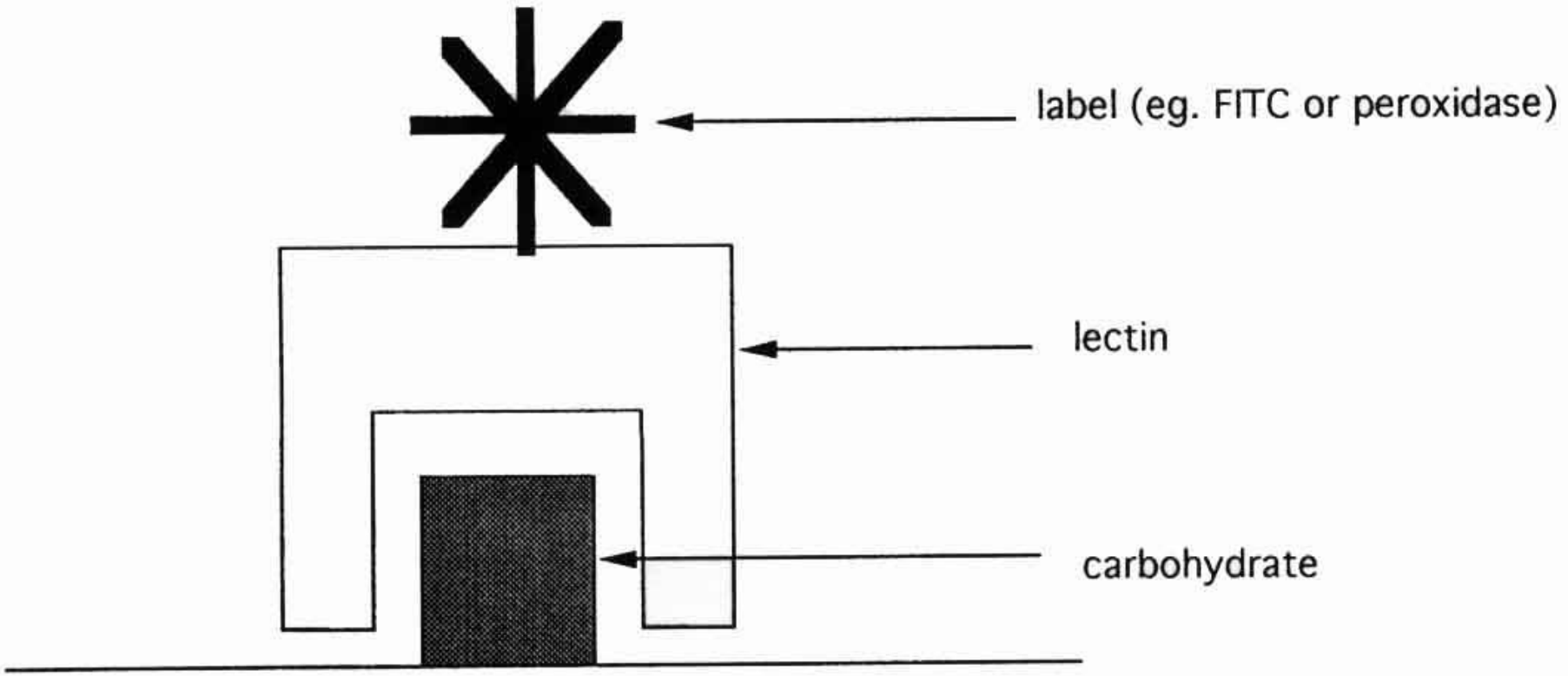

merge

LECTIN HISTOCHEMISTRY

Lectins - proteins or glycoproteins capable of specific recognition and binding to certain mono- or oligosaccharides. They are natural components of some plants, animals or microorganisms – called by the latin name of the species.

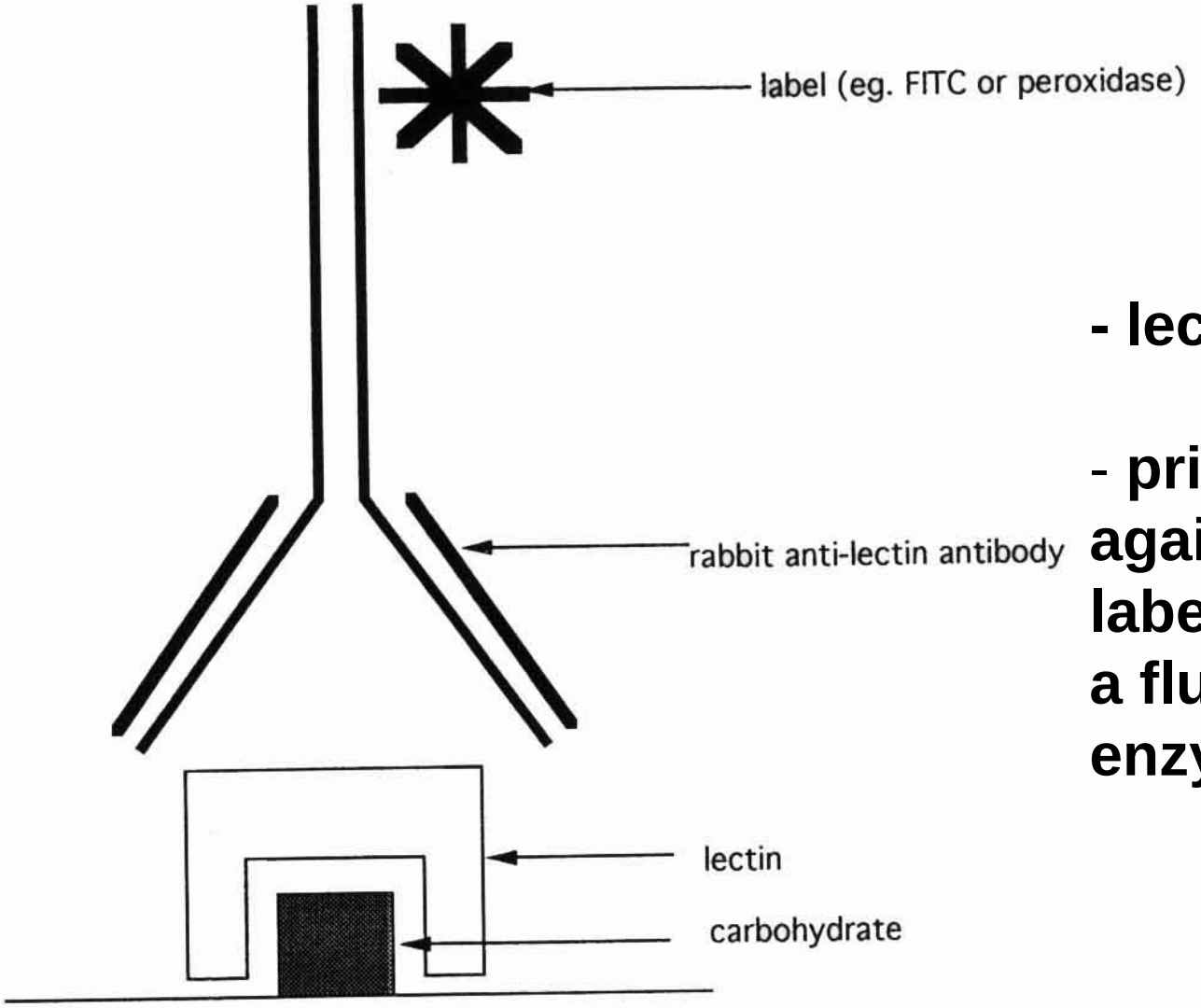
Practical use: 1/ looking for localization of a specific sugar (usually a component of a complex molecule)
2/ analysis of a carbohydrate chain *in situ*

specific bond of a sugar and a lectin is then visualized by formation of a **coloured compound *in situ*** using enzyme histochemistry or a **fluorochrome**



1 - Direct method

lectin is labelled with a fluorochrome or enzyme directly



- lectin is not labelled

- primary antibody against the lectin is labelled with a fluorochrome or enzyme

2 – Indirect antibody method

Two ways of using

1. looking for a specific sugar in the oligosaccharide chain

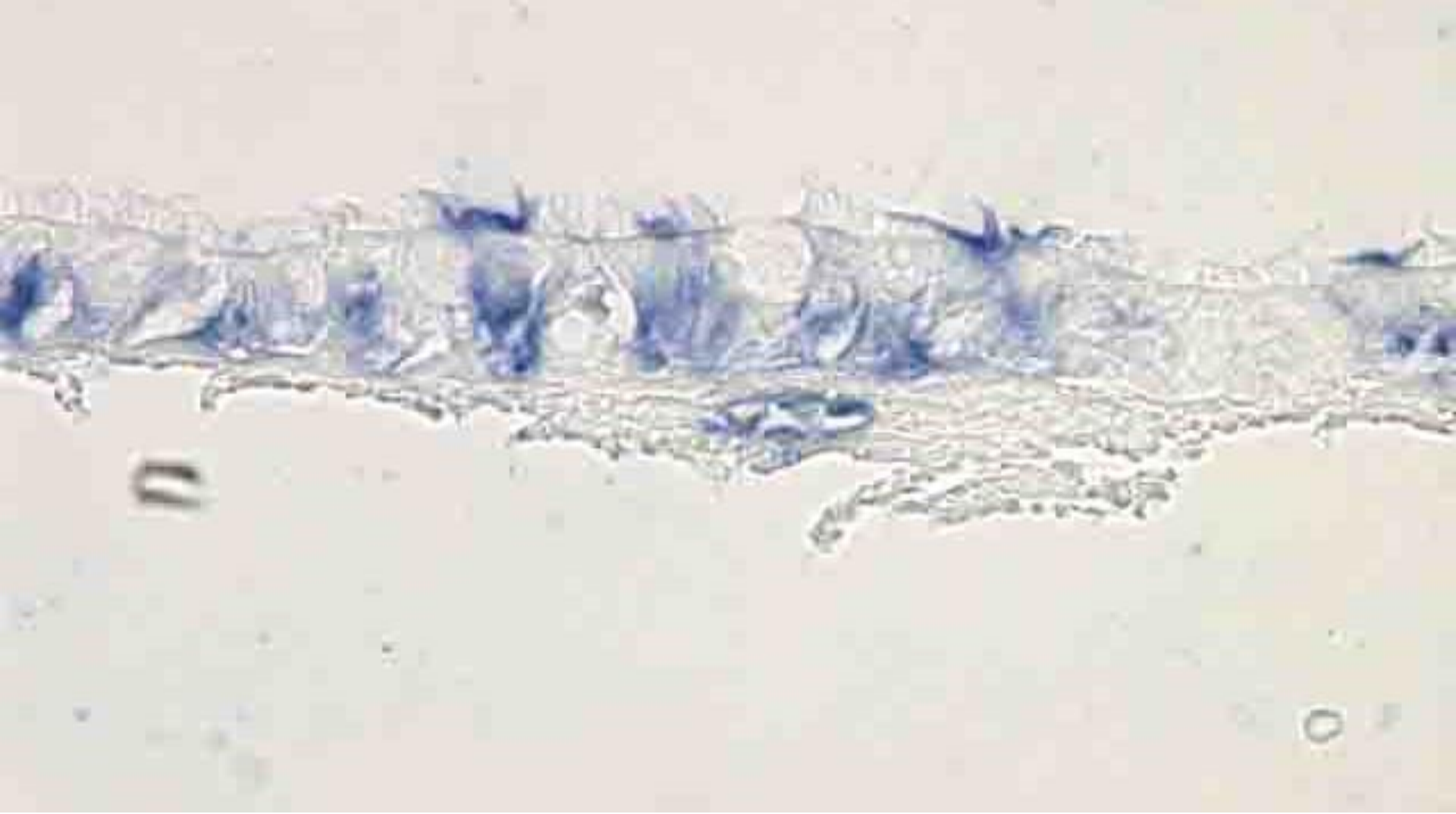
   => specific lectin

2. not knowing exactly, which sugars are components of an oligosaccharide chain

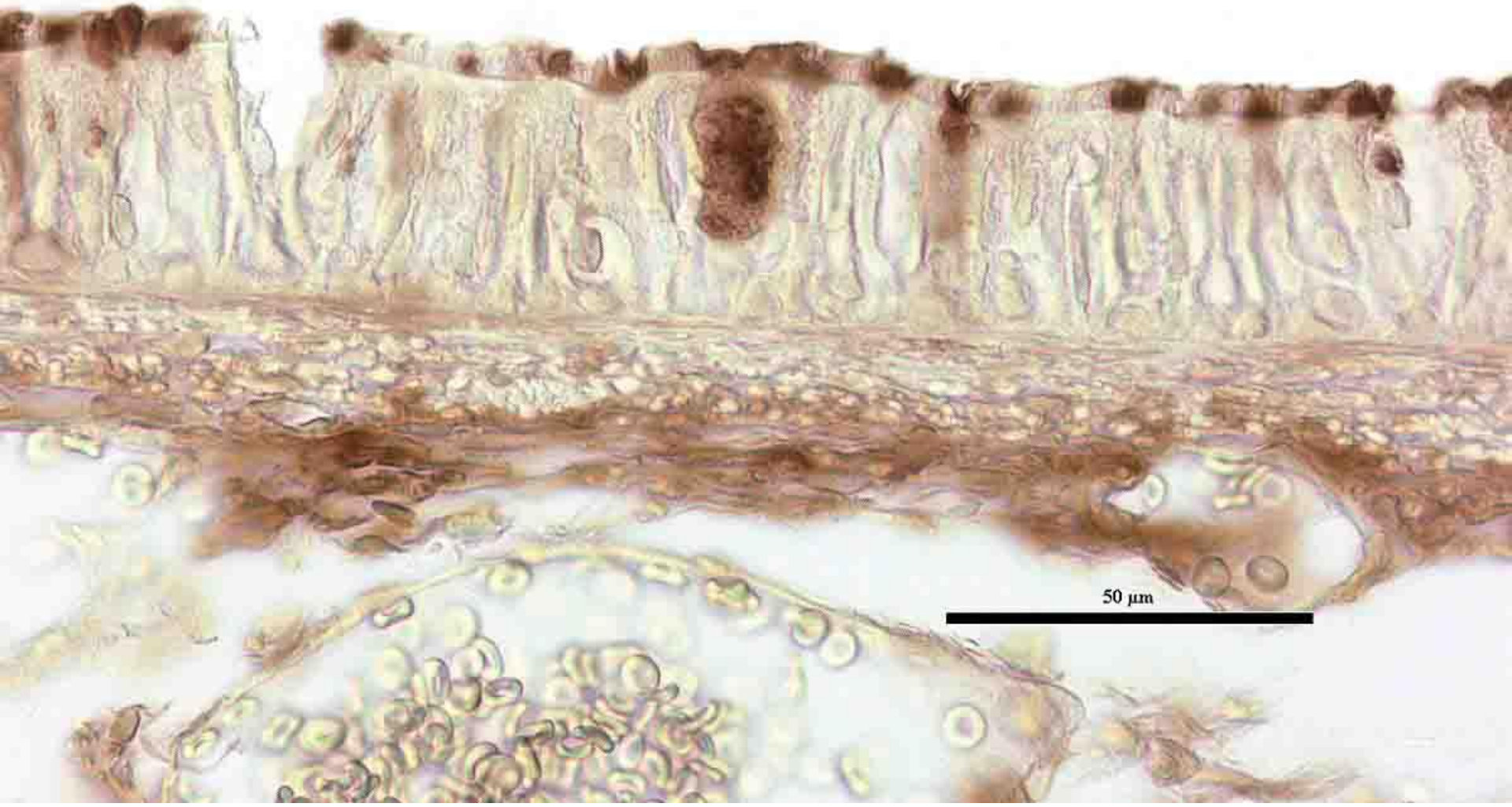
   => panel of lectins

Example of panel of lectins (shortened)

abbreviation	latin name	english name	saccharide specificity
AAA	Allium ascalonicum	Shallot	terminal trisaccharide α–D-Man-(1-6)[α-D-Man(1-3)]-Man
AAL	Aleuria aurantia	Orange Peel (Fungus)	L-Fuc
ABA	Agaricus bisporus	Common mushroom	Gal and β-D-Gal-(1-3)-GalNAc, Gal and β-D-Gal-(1-3)-GlcNAc, GlcNAc on degalactosylated N-glycans
ACA	Amaranthus caudatus	Love-lies-bleeding	β-D-Gal-(1-3)-GalNAcα-Ser/Thr
AMA	Arum maculatum	Lords and Ladies	β-D-Gal-(1,4)-GlcNAc, desialylated fetuin
Achatinin H, (ATNH),	Achatina fulica	Giant African land snail	9-O-acetylated sialic acids
AOL	Aspergillus oryzae	Rice mold	α-L-Fuc-(1-6) bound on the core of N-bound oligosaccharide, α-L-Fuc-(1-2,3,4)
ASA I, ASA III	Allium sativum	Garlic	N-glycans rich in mannose
BanLec	Musa sapientum	Banana tree	internal α-D-Glc-(1,3)-Man
BDA	Bryonia dioica	Red Bryony	GlcNAc
BPL	Bauhinia purpurea, alba	Orchid Tree	β-D-Gal-(1-3)-GalNAc
CCL	Ciborinia camelliae	Petal blight	GalNAc
concanavalin A ConA	Canavalia ensiformis	Jackbean	α-mannosyl branches (without GlcNAc), glucosylated structures, hybrid types and biantennary complex types of N-glycans
DBA	Dolichos biflorus	Horse grain	α-D-GalNAc-Ser/Thr a α-D-GalNAc(1-3)-GalNAc (Forssman disaccharide) > α-D-GalNAc-(1-3)[α-L-Fuc-(1-2)-Gal (A-trisaccharide)
DSA	Datura stramonium	Jimson weed	poly-LacNAc and branched LacNAc



sialic acid Neu5Ac α(2-6) terminal on Gal, GalNAc
lectin of Black Elderberry (Sambucus nigra, SNA) labelled with digoxigenin
antibody against digoxigenin labelled with alkaline phosphatase (AP)
visualization: 5-bromo-4-chloro-3-indolylfphosphate +nitroblue tetrazolium (BCIP/NBT)



fucose α(1-2) terminal or branched
lectin of Common gorse (Ulex europaeus, ULE I) labelled with biotin
streptavidin labelled with horseradish peroxidase (HRP)
visualization: hydrogen peroxide + diaminobenzidine (DAB)

ISH, FISH

in situ hybridization, fluorescence *in situ* hybridization

looking for a nucleotide sequence
(DNA – genes, viral DNA; RNA – transcripts of mRNA, viral RNA)

specific bonds of complementary nucleotides
(A-T, A-U, C-G)

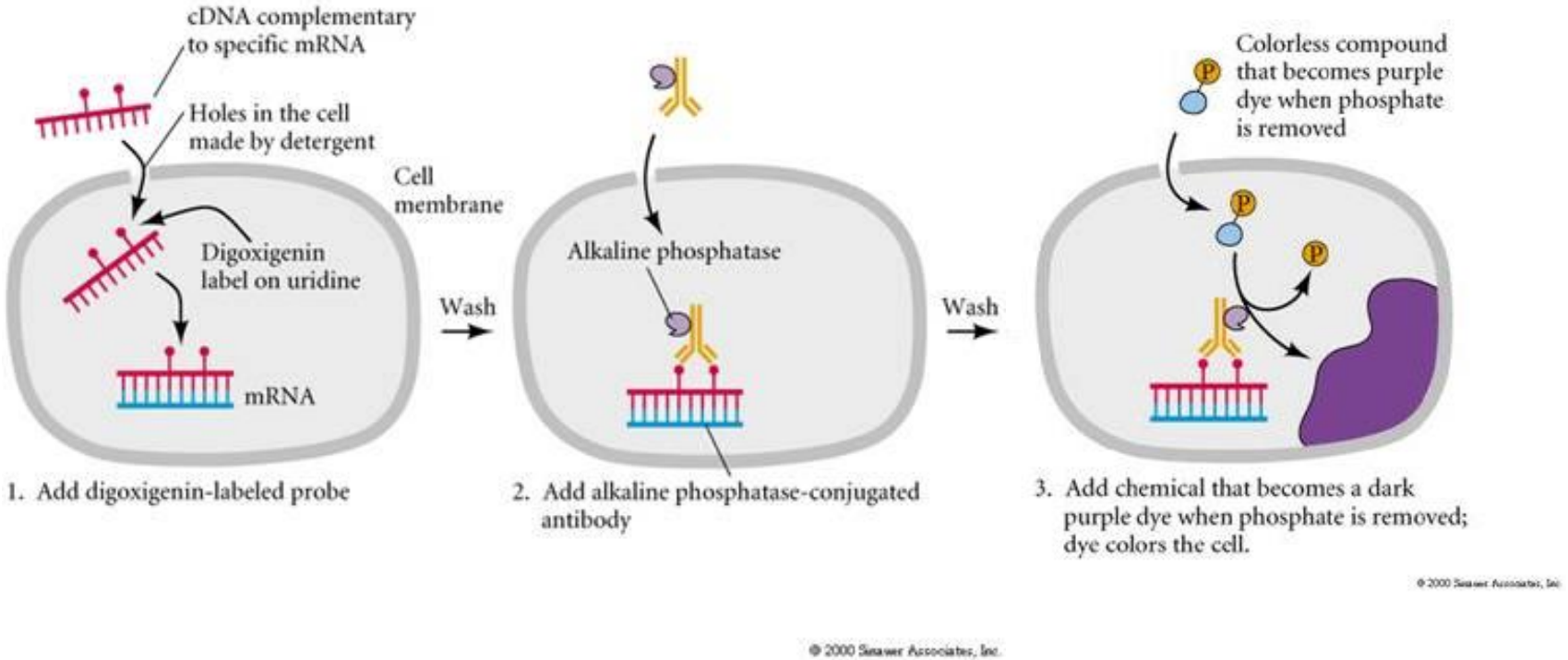
PROBE

synthetic test sequence, complementary to a sequence looked for

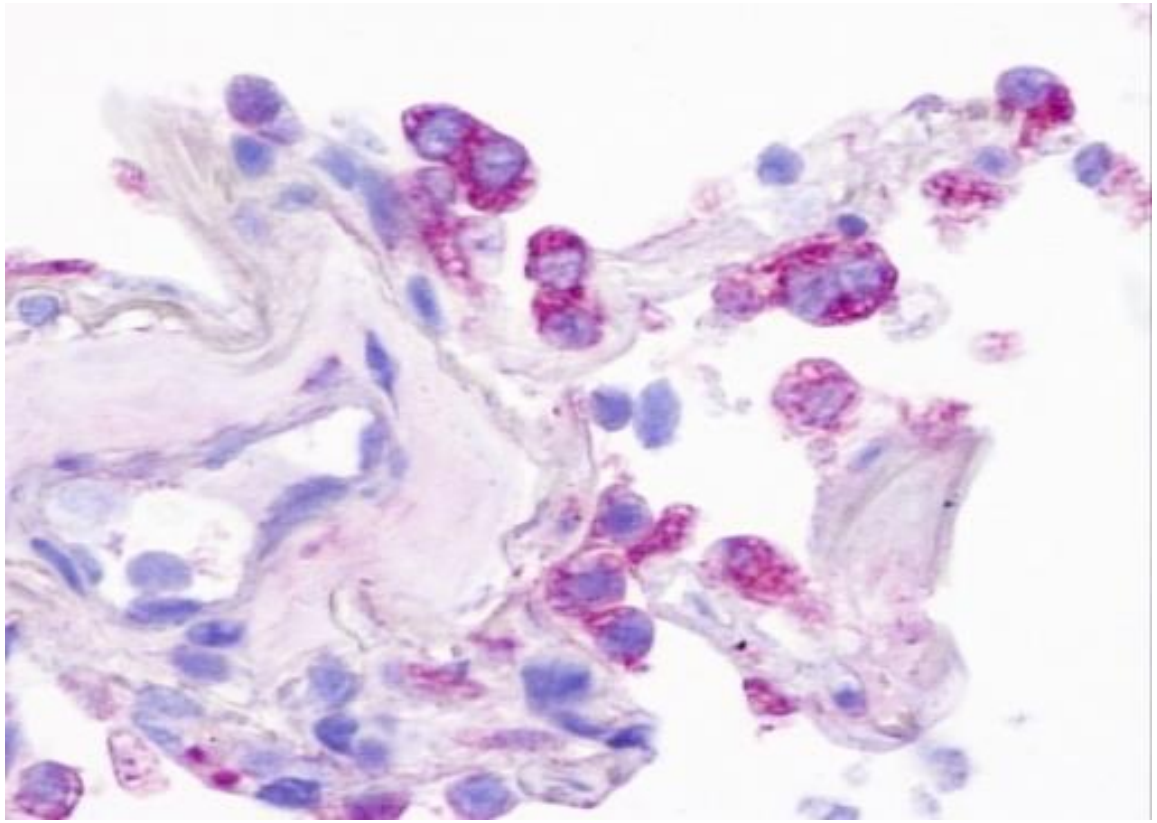
for visualization, a probe is labeled with e.g. digoxigenin, biotin, fluorochromes or radioisotopes (e.g. ^{32}P)

visualization is then provided by formation of a **color compound *in situ*** by means of enzyme histochemistry or **fluorescence** and **radioautography,** respectively

Example of ISH detection of specific mRNA using cDNA probe labelled by digoxigenin

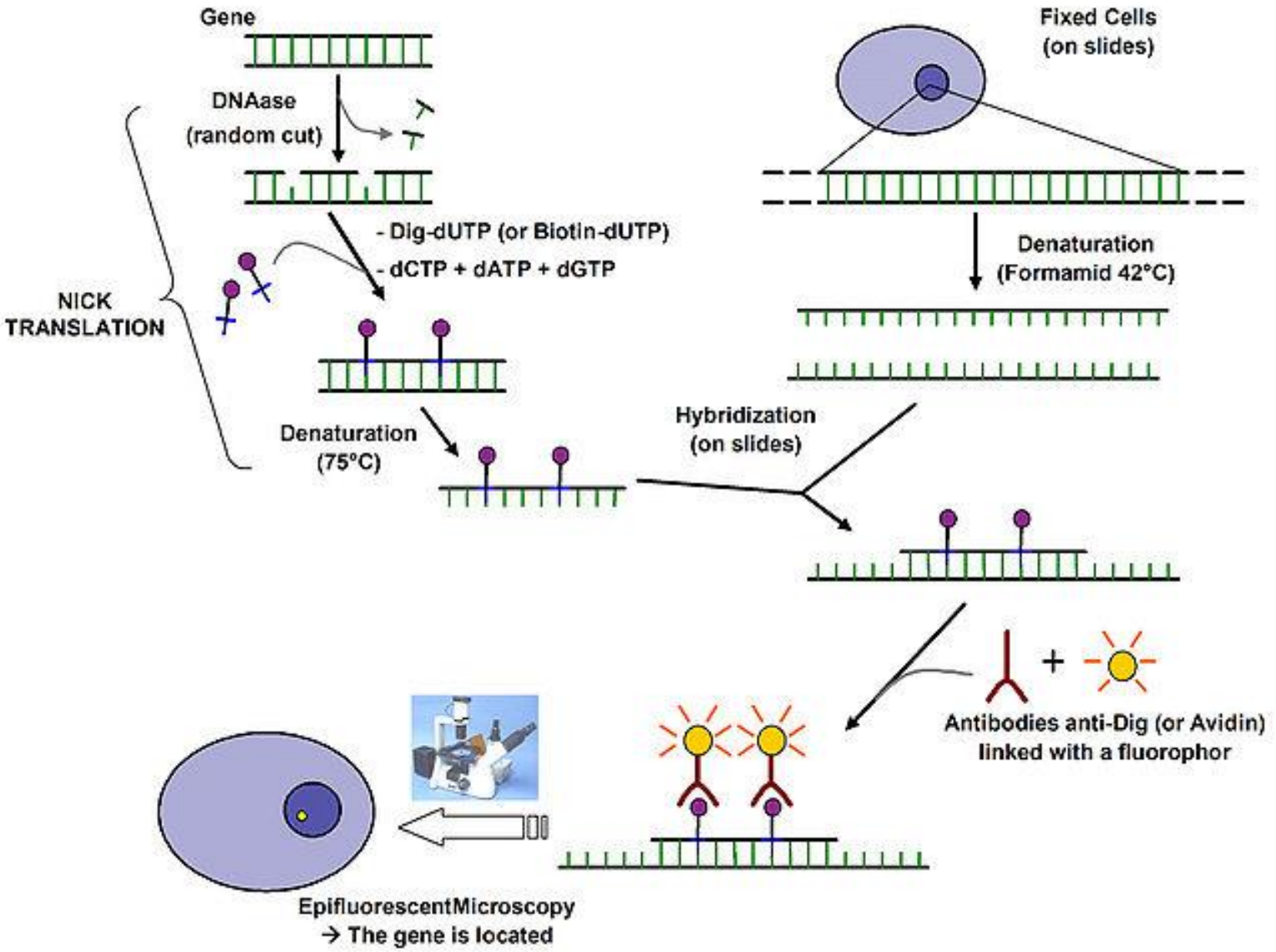


ISH with the 663 bp DNA-probe complementary to mRNA for surfactant-protein A labelled by digoxigenin. Detection in human lung by an anti-digoxigenin antibody conjugated with AP.

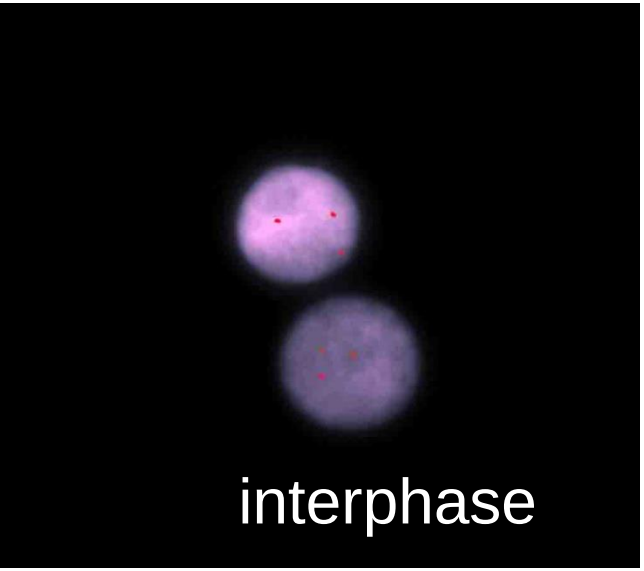


Goldmann et al., Diagn Pathol. 2009; 4: 8.

FISH (Fluorescent In Situ Hybridization)



FISH



Down syndrome
(trisomy 21)

Down-specific region

Spectrum Orange / DAPI

<

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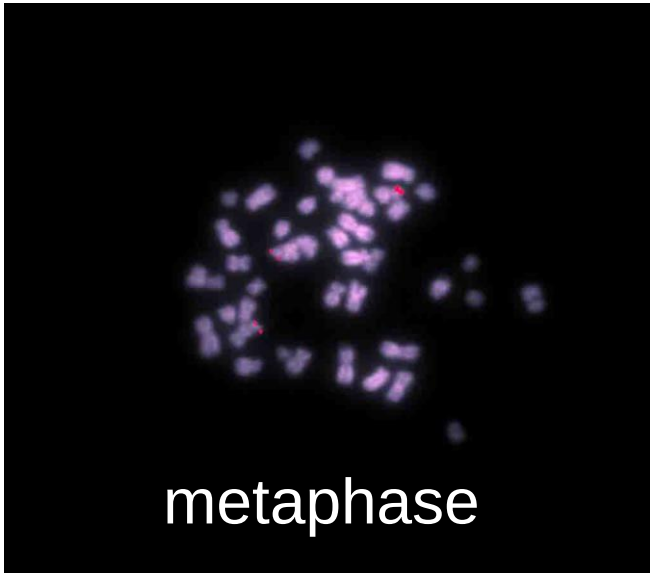
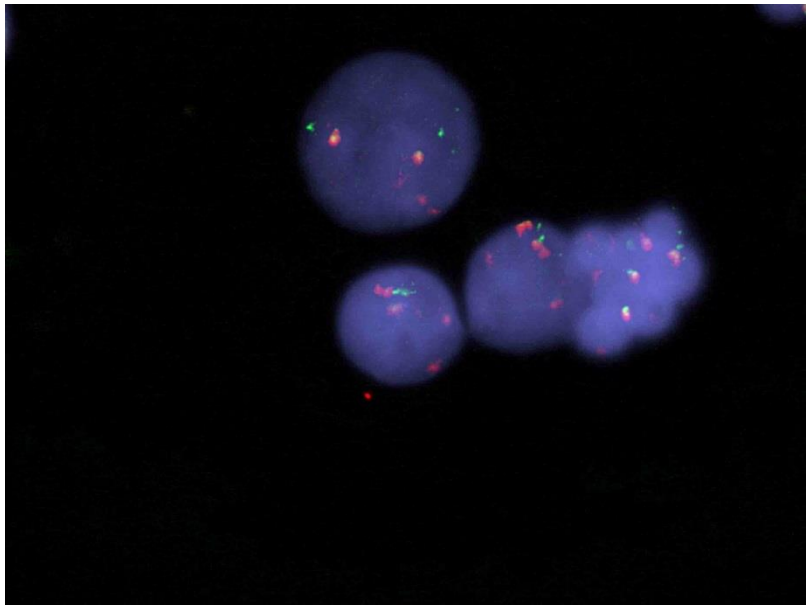


suspection of X-linked ichthyosis
(a deletion mutation of the StS gene
on the X-chromosome),
negative result (normal finding)

gene StS (steroid sulphatase) /
centromere X

Spectrum Orange /
Spectrum Green / DAPI >



SPECIAL TECHNIQUES OF ELECTRON MICROSCOPY

Fixation methods in TEM

- PHYSICAL
  - **quick and deep FREEZING (cryofixation)**
  - microwaves
- CHEMICAL
  - SOLUTIONS of chemical substances

Cryofixation

- specimens can be observed by a microscope in their natural extra- and intracellular environment
- denaturation of enzymes and antigens does not occur
- cells are instantly immobilised, which makes possible to observe dynamic actions
- cells can be damaged with crystal formation and volume increase by freezing
- this can be prevented by quick freezing, when water solidifies earlier than crystals are formed

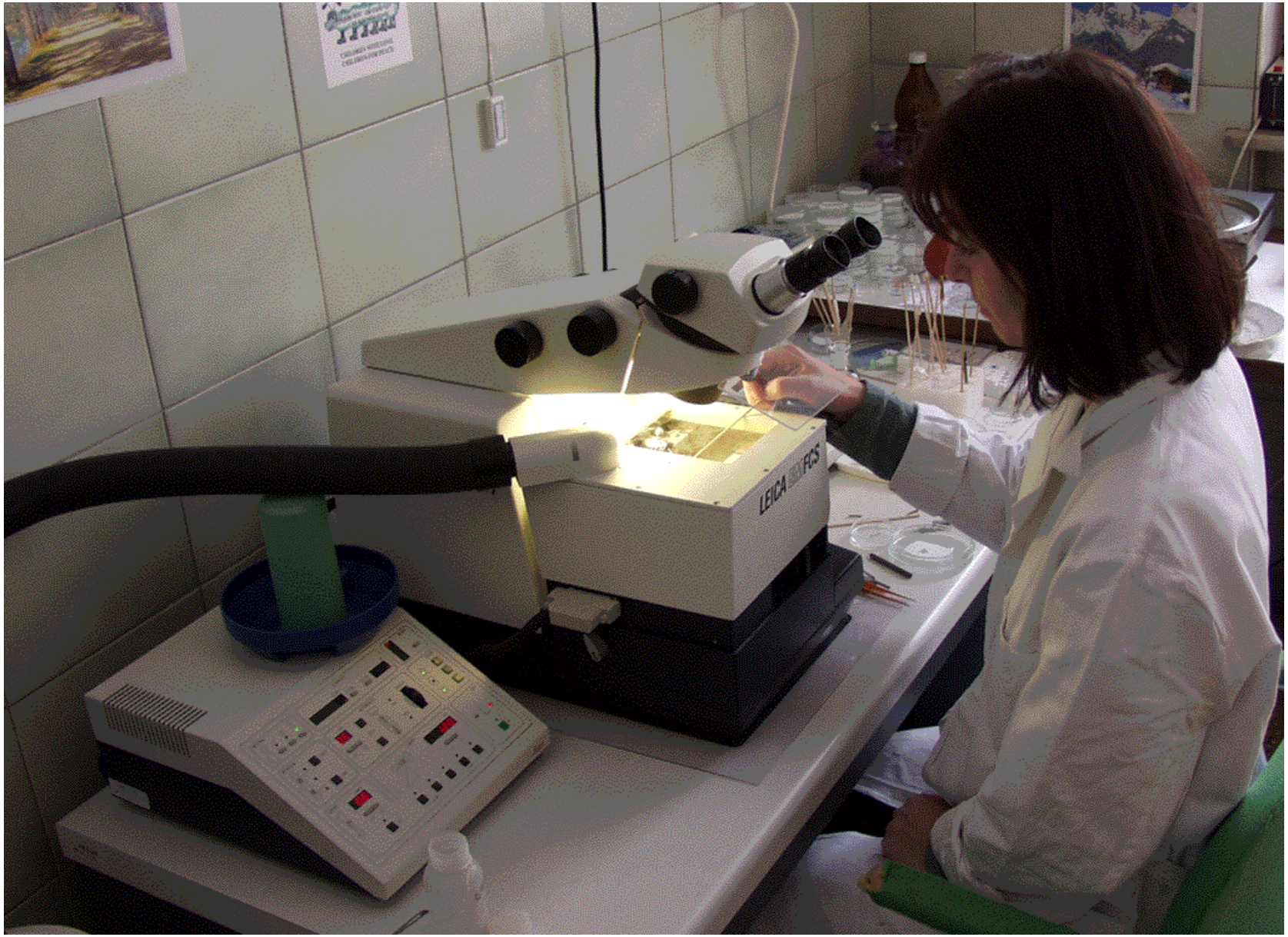
High-pressure cryofixation device

Processing of frozen specimen

- cryosectioning – sectioning by an ultramicrotome equipped with the cryochamber (cca -100°C)
  - sections can be observed on grids by a TEM equipped with the cryoadaptor (expensive and technically complicated)
  - or dried-up by controlled sublimation of ice (more suitable for SEM)
  - or thawed (danger of fast autolysis)
    - autolysis is prevented if the specimen is fixed chemically before freezing (Tokuyasu)

- freeze substitution – ice in the specimen is substituted by an organic dissolvent and subsequently by the acrylic resin (-50°C), next processing in room temperature

Ultramicrotome with the cryochamber



Conventional histochemistry in EM

-all methods are based on the formation ot electron-dense reactive products containing metal elements e.g.

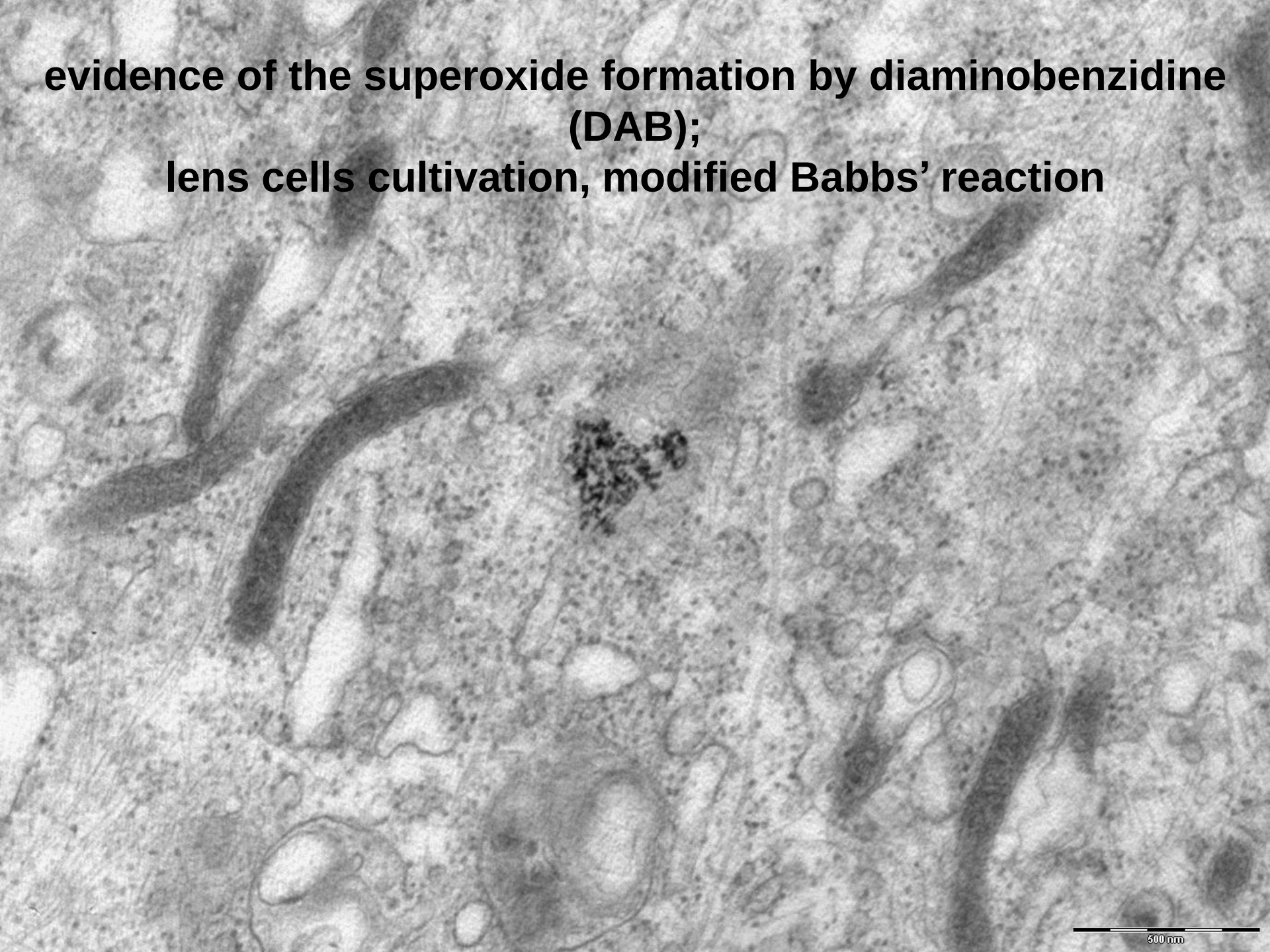
- polysaccharide-evidence (Thiery's reaction) – analogous to PAS; instead of the Schiff's reagent, a silver proteinate is used

-evidence of sulphated polysaccharides by ruthenium red

- evidence of acidic polysaccharides by alcian blue (Cu) or colloid iron (Hale-Müller's method modified)

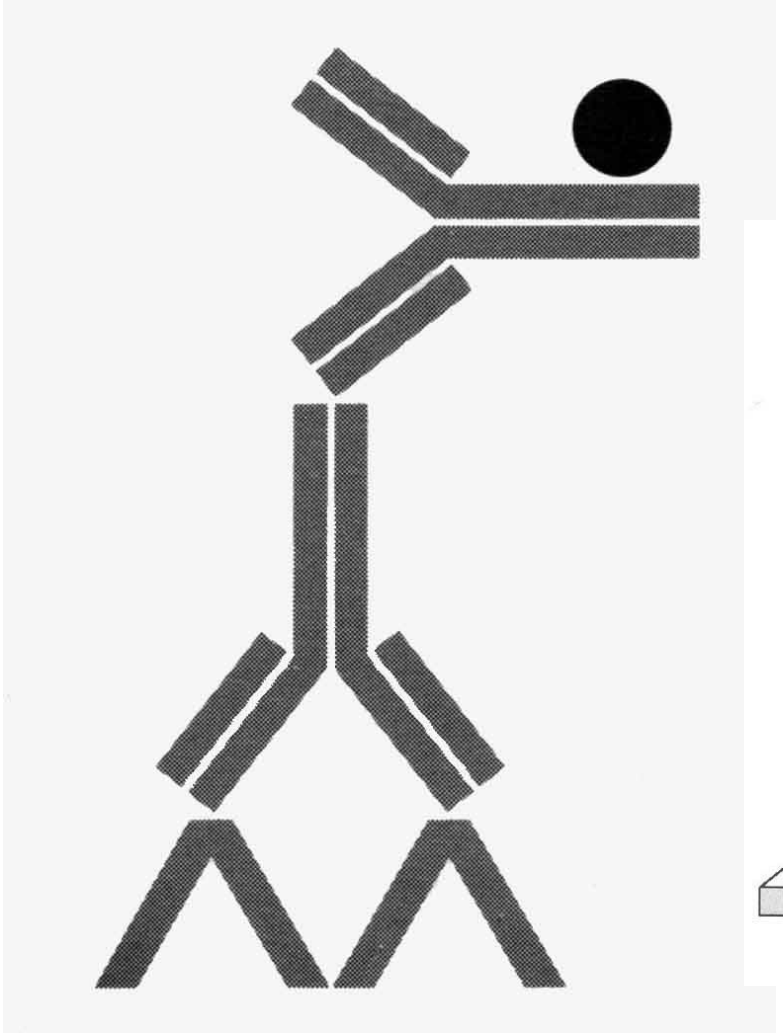
- evidence of the superoxide formation by diaminobenzidine (DAB); (modified Babbs' reaction) – oxidized DAB binds osmium (is osmiophilic)

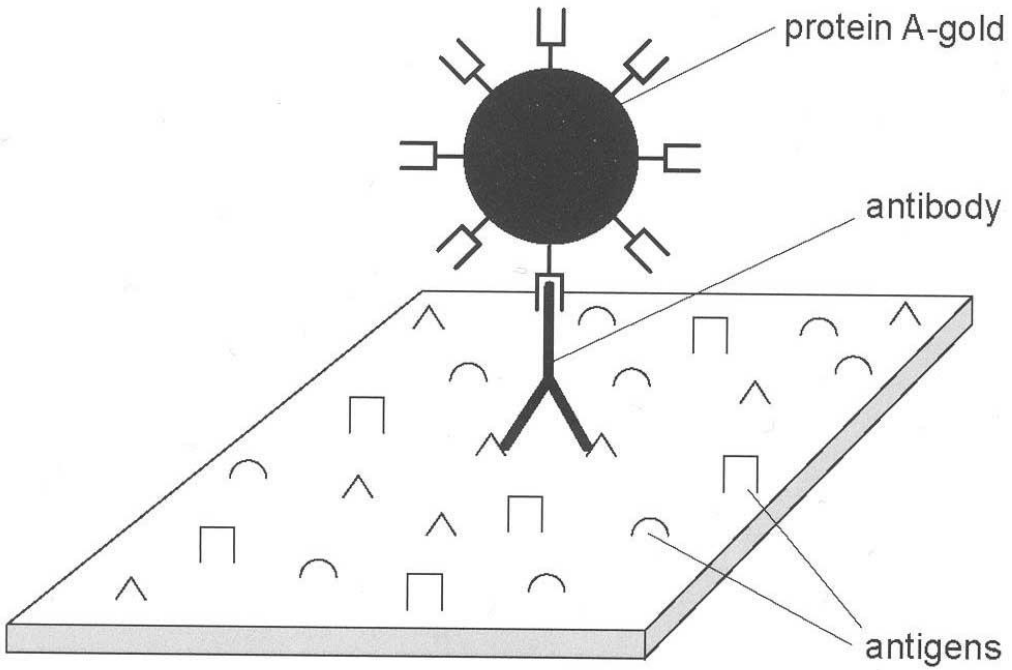
evidence of the superoxide formation by diaminobenzidine (DAB);
lens cells cultivation, modified Babbs' reaction



EM IMMUNOHISTOCHEMISTRY

1/ antigen revealed before embedding
(pre-embedding)
2/ antigen revealed in resin sections
(post-embedding)
3/ antigen revealed in frozen sections
(method after Tokuyasu)





Two-step indirect method – the most frequently used in EM
labelled **secondary** antibody or **protein A** (protein of Staphylococcus aureus binding most of Ig) reacts to **primary** antibody bound to a tissue (cellular) antigen

Antigen proof in resin sections (post-embedding) in EM

- Fixation – paraformaldehyde is the best
  (glutaraldehyde denatures antigens)
- Embedding – acrylic resin miscible with water
  (LR-White, Lowicryl)
- Ultrathin sections: background saturation with non-specific serum (FCS)
- Primary antibody
- Rinse in buffer
- Secondary labelled antibody (or Protein A, G, L™)
- Rinse in buffer and water
- Contrasting – e.g. 2% uranylacetate

Antigen proof on frozen sections – method after Tokuyasu

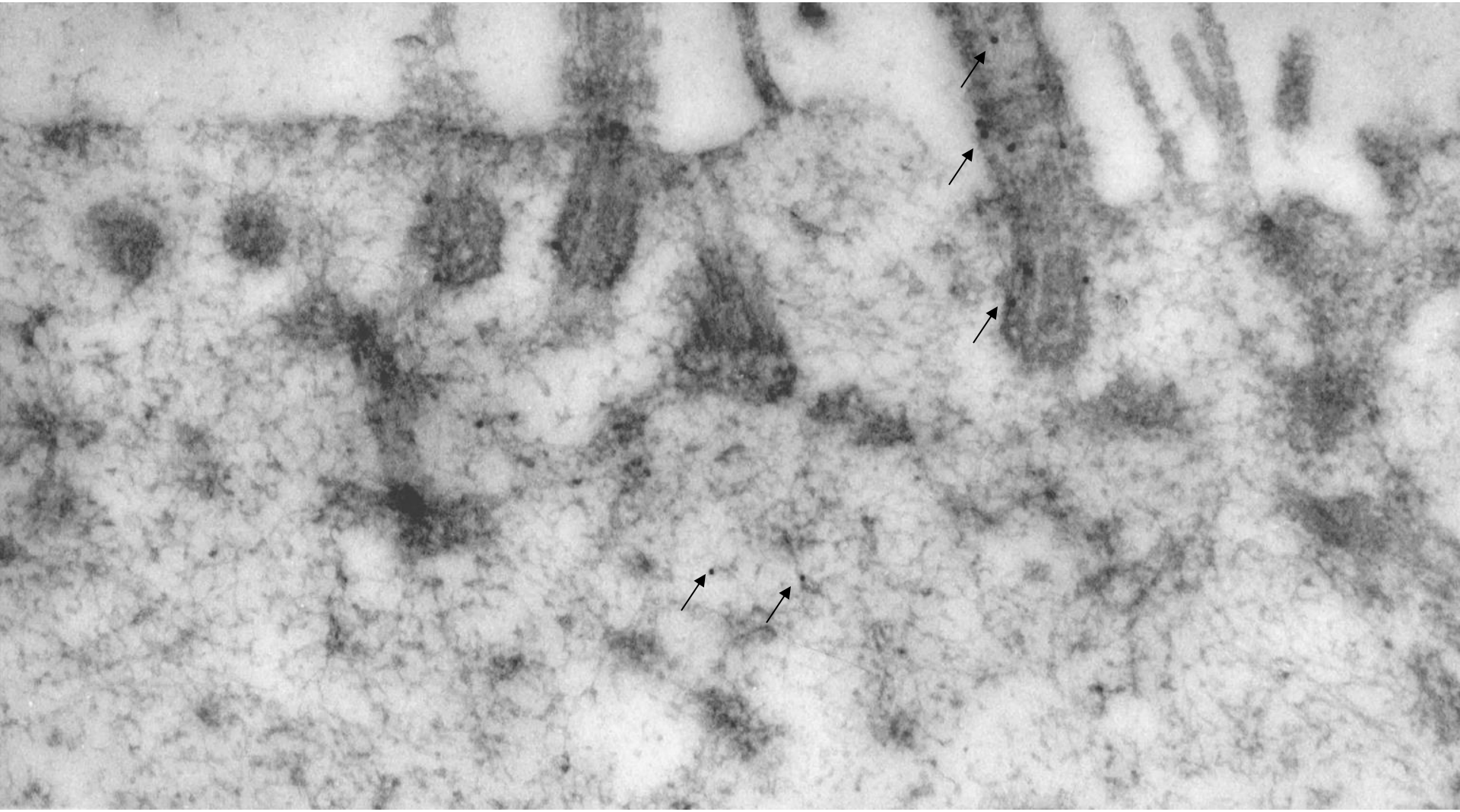
- Fixation – paraformaldehyde is the best
- Cryoprotection – specimen infiltration with concentrated sucrose
- Freezing a specimen on a metallic holder with liquid nitrogen
- Cutting the frozen specimen by the ultramicrotome with cryoadaptor
- Ultrathin frozen sections posted on the grid using a wire loop submerged in a mixture of methyl cellulose and sucrose
- Next procedure same as in resin sections
- Advantage – antigens are not damaged by specimen dehydration and embedding

Visualization

The bond of an antibody to an antigen epitope is visualized using a **metal** (high atomic mass → electron density)

Standard label – colloid gold particle
Particle size 5 – 30 nm → multiple labelling for more antigens on one section possible

Alternatively horseradish peroxidase labeling and DAB visualization (osmiophilia) can be used



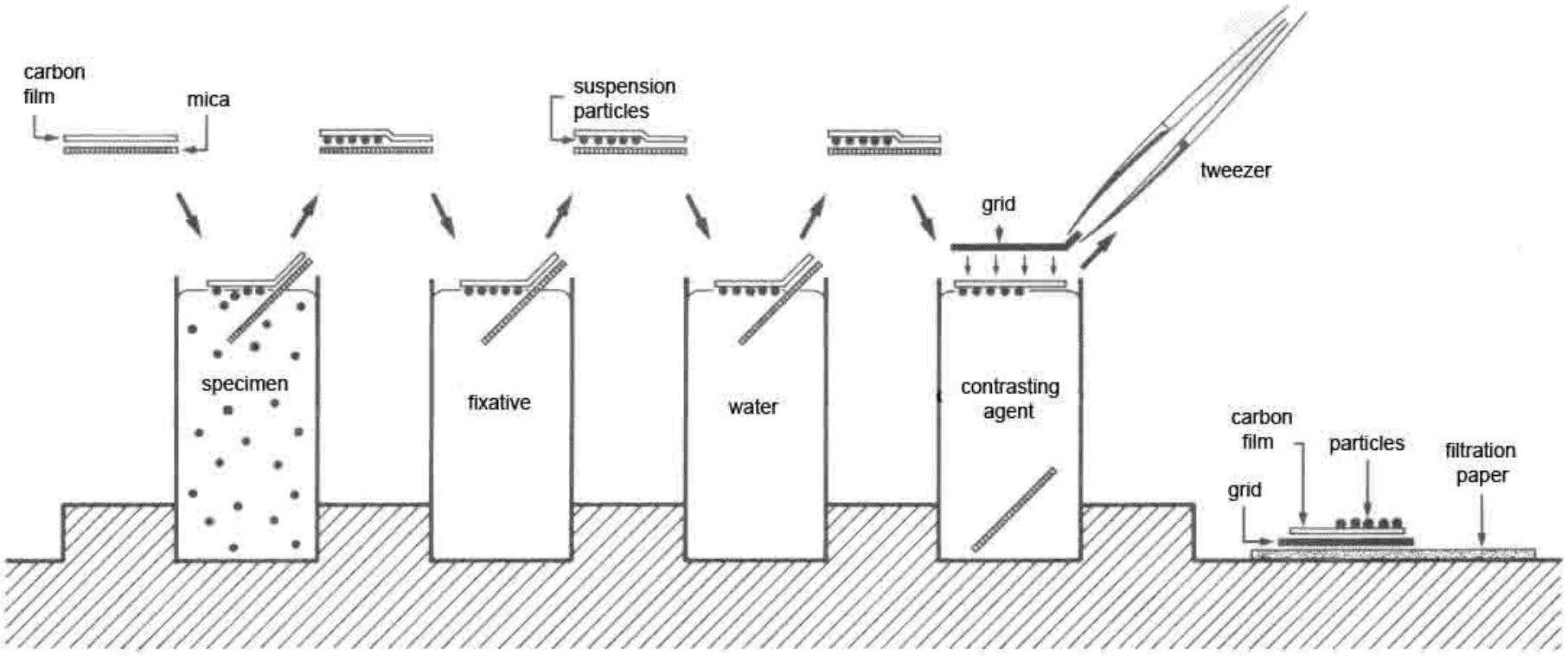
immunohistochemical evidence of tubulin α, colloid-gold labelled (10 nm)
apical portion of a ciliated cell, trachea, rabbit

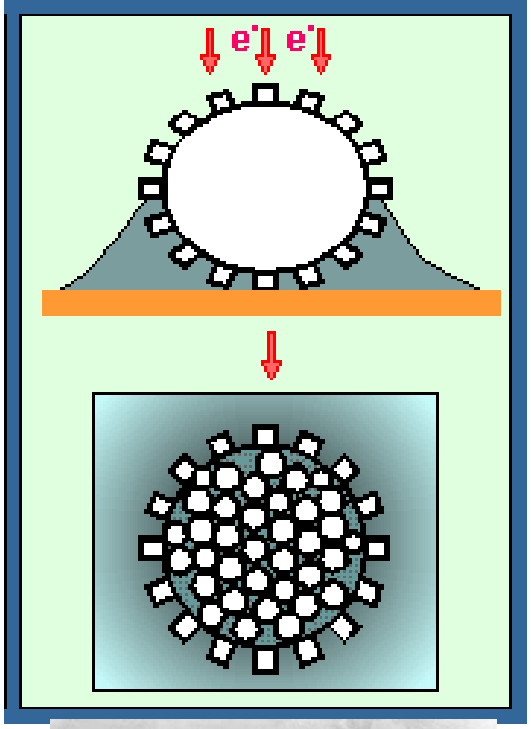
NEGATIVE CONTRAST

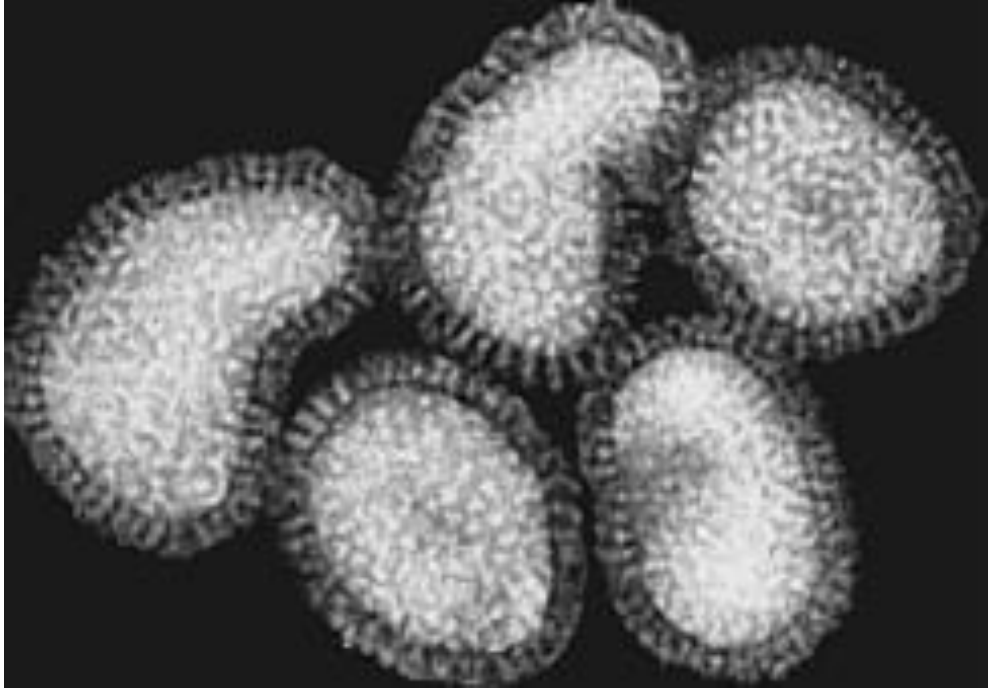
- observation of specimens, size of which is far below the ultrathin section thickness
  - macromolecules (proteins, lipoproteins, polysaccharides, nucleoprotein complexes)
  - isolated cell organelles (mitochondria, membrane systems)
  - bacteria, **viruses**
- specimen entrapped on a supporting film on the grid
- contrasting with an agent containing a heavy metal
- agent surrounds the specimen and forms the dark background, in which individual objects reveal as light ones; the agent also penetrates surface irregularities of the object enabling thus their observation

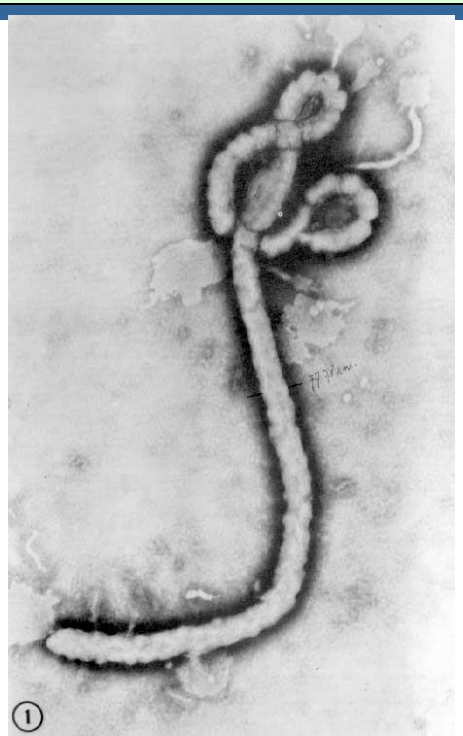
The most simple way: a suspension drop is mixed with the agent and posted on the grid prepared with the formvar film. Having displaced abundant fluid and dried, a thin layer with agent-coated particles is formed on the formvar film.

More sophisticated way:




e⁻ e⁻

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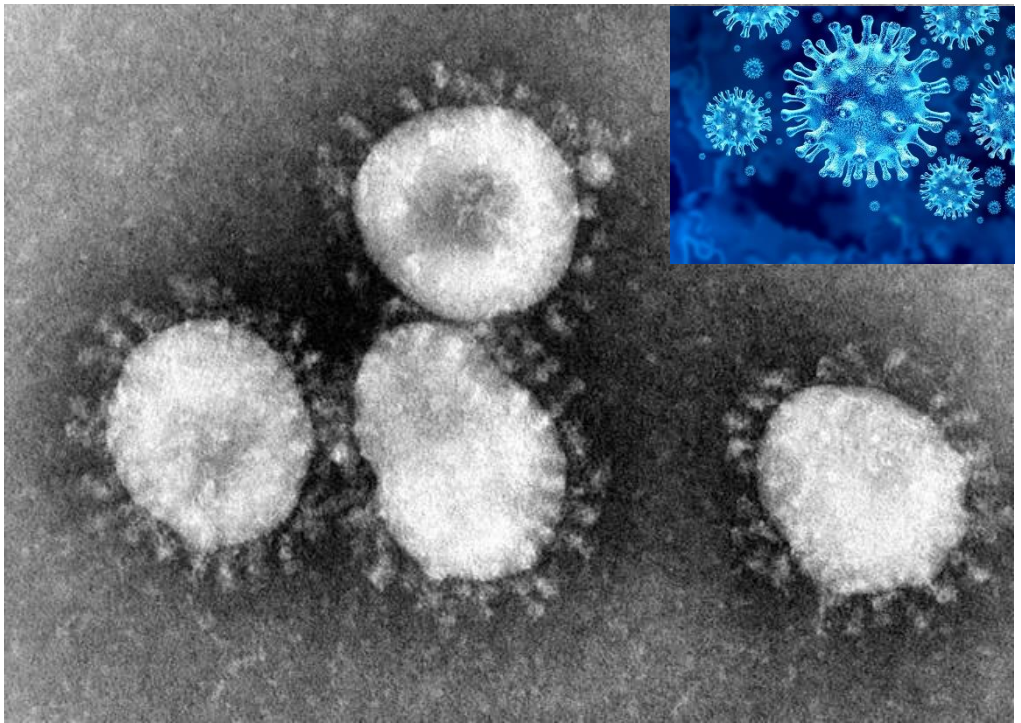


Special methods of the SEM-processing

- cryofixation (same as in TEM)
- cryofracture - permits assessing the inner surfaces revealed by fracturing a frozen specimen
- freeze etching – revealing surface details by ice sublimation under low pressure

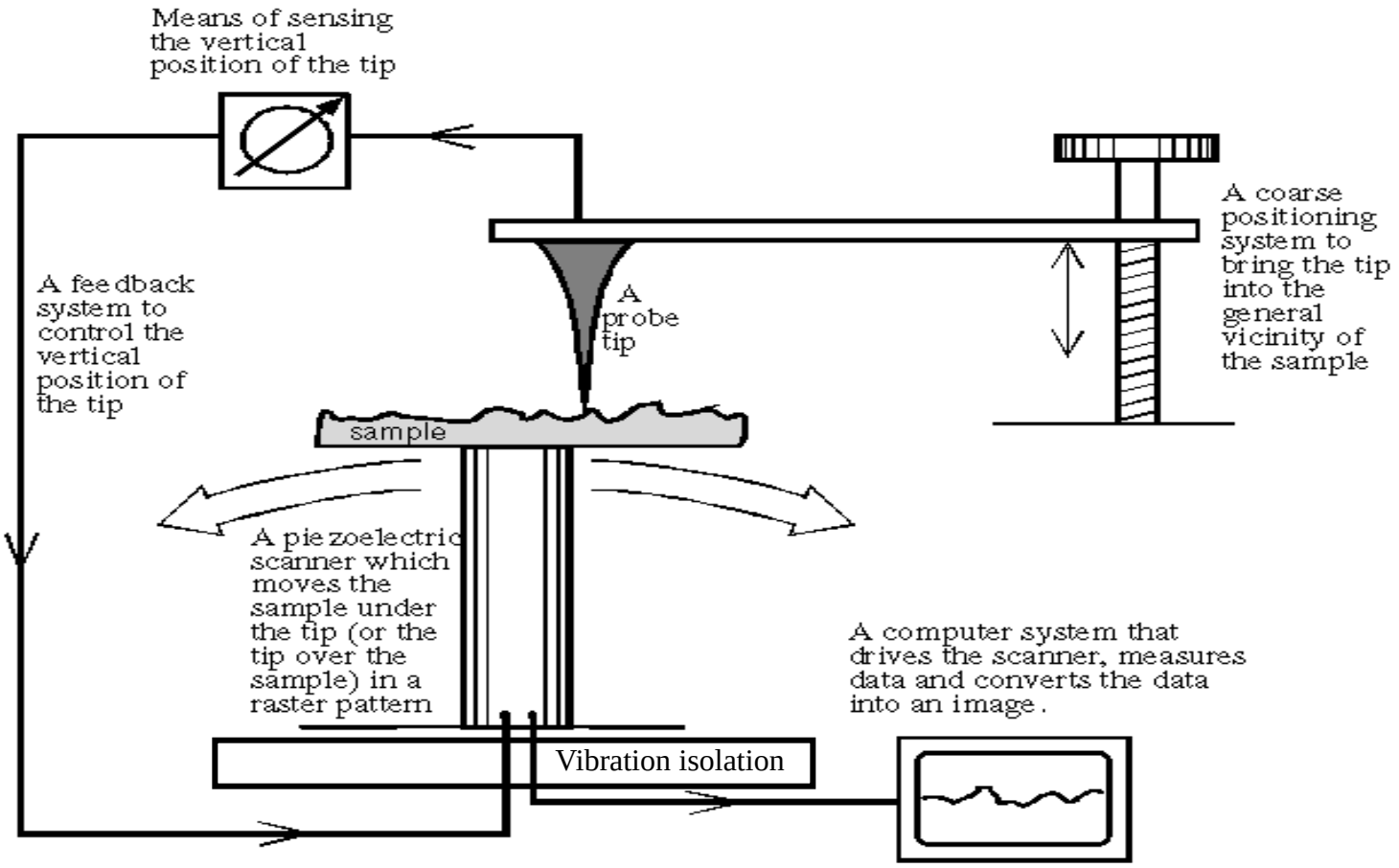
in all cases, the specimen surface should be shaded (sprayed with a metal)

Tight junction – cryofracture

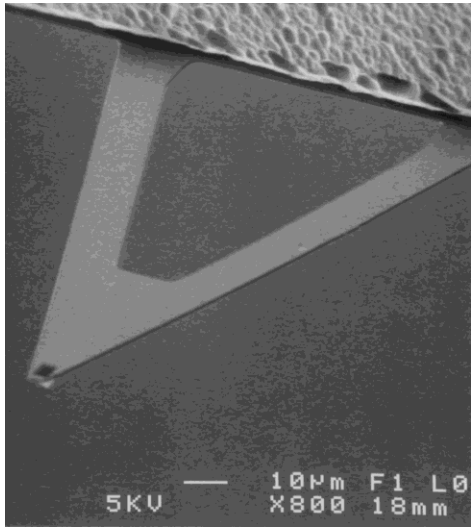
Scanning Probe Microscopy – SPM

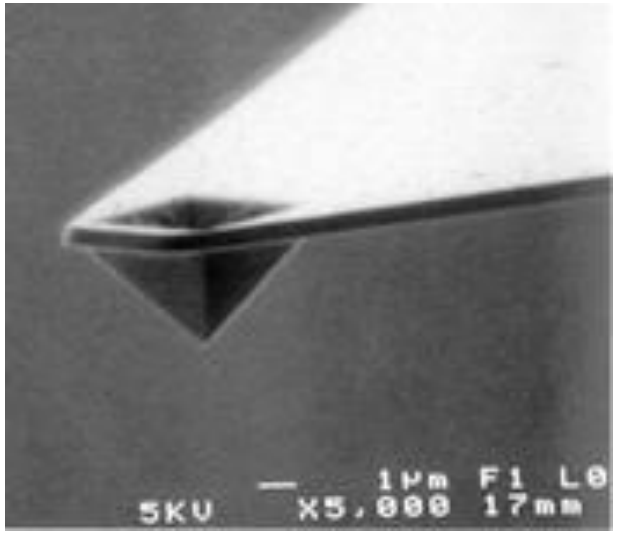
- methods designed to study of surface structures
- not based on any type of light or radiation, but on succesive scanning of the surface by a probe
- many options differing by physical principle
  - **atomic force microscopy (AFM) – today mostly used, developed 1986, based on attractive and repulsive forces among atoms**
  - scanning tunneling microscopy (STM) – oldest method (1981), based on the monitoring of electric currents between specimen and probe, it needs a conducting specimen and vacuum
  - many other options – lateral force microscopy (LFM), force modulation microscopy (FMM), magnetic force microscopy (MFM), electrostatic force microscopy (EFM), ballistic electron emission microscopy (BEEM) ……..

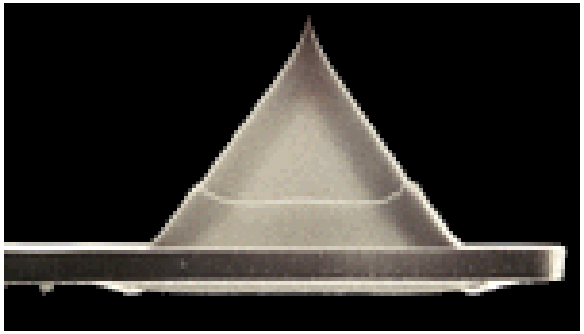
Basic SPM-Components



Atomic force microscopy (AFM)





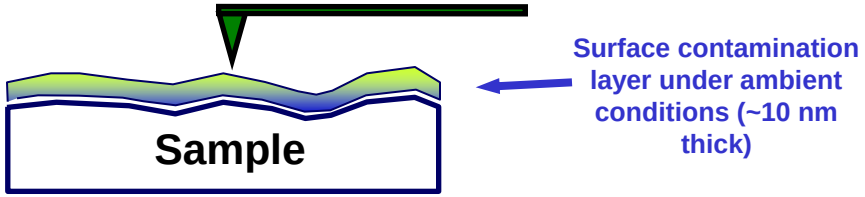


- **elastic cantilever** and **probe** made of Si_3N_4
- pyramidal shape with apex radius around 10-50 nm
- cantilever length : 50-500µm
- spring constant ~0.1 - 0.7 N/m
- used both in air and liquid for contact
- laser and photodiode are used for detection of cantilever position

Topographical AFM-Modes

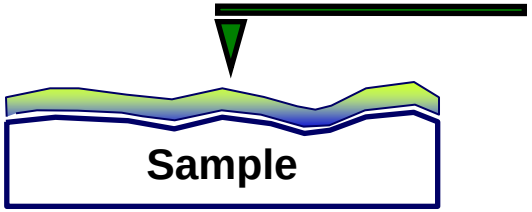
Contact Operation

Feedback results from “repulsive” electric forces



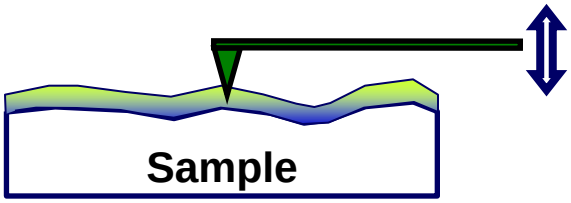
Non-Contact Operation

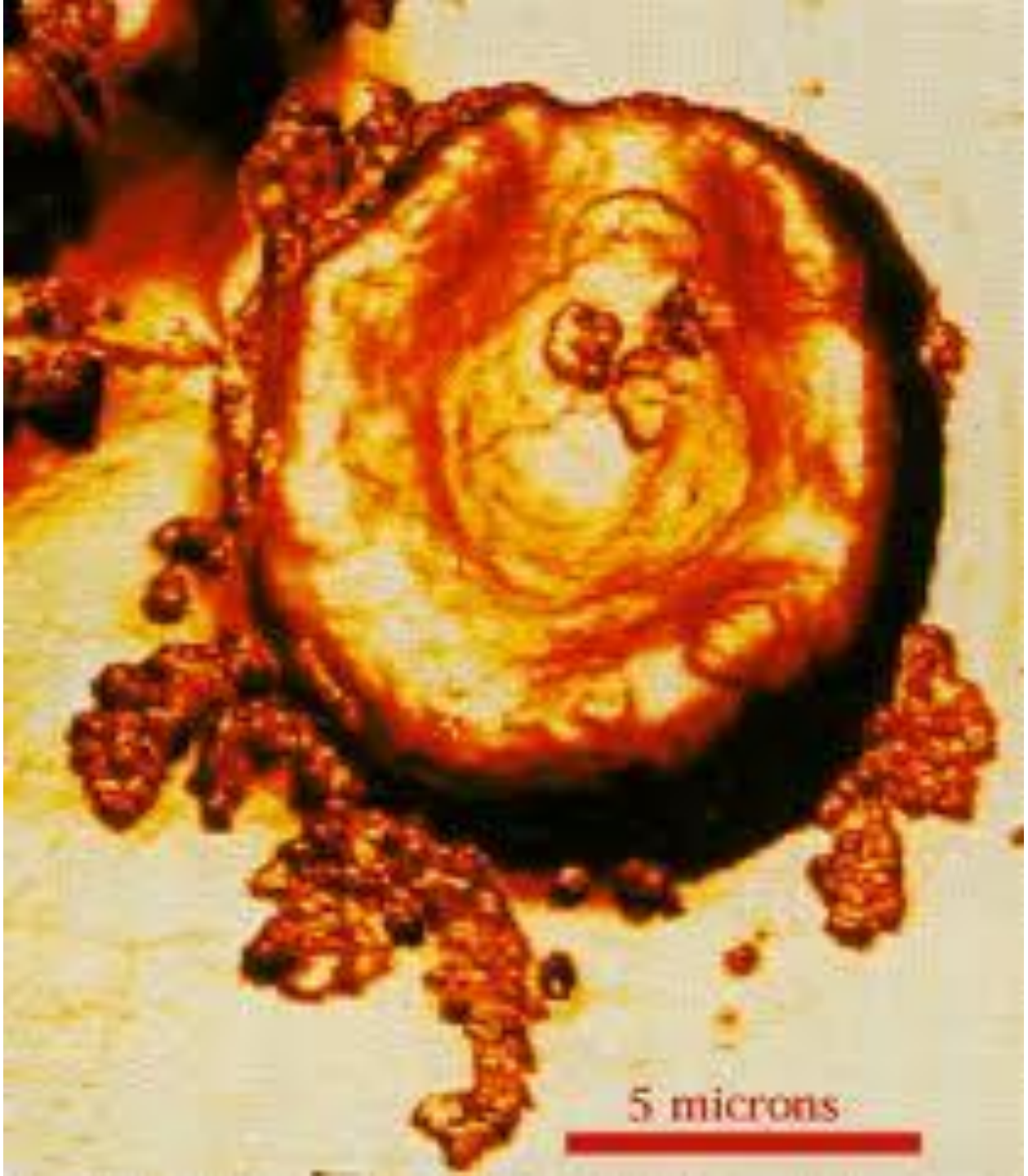
Feedback results from “attractive” Van der Waals forces



Tapping Mode Operation

Probe oscillates between Non-Contact and Contact Regions
The most suitable for biological specimens





red blood cell


NT-MDT
SOLVER
NEXT
SOLVER
NEXT