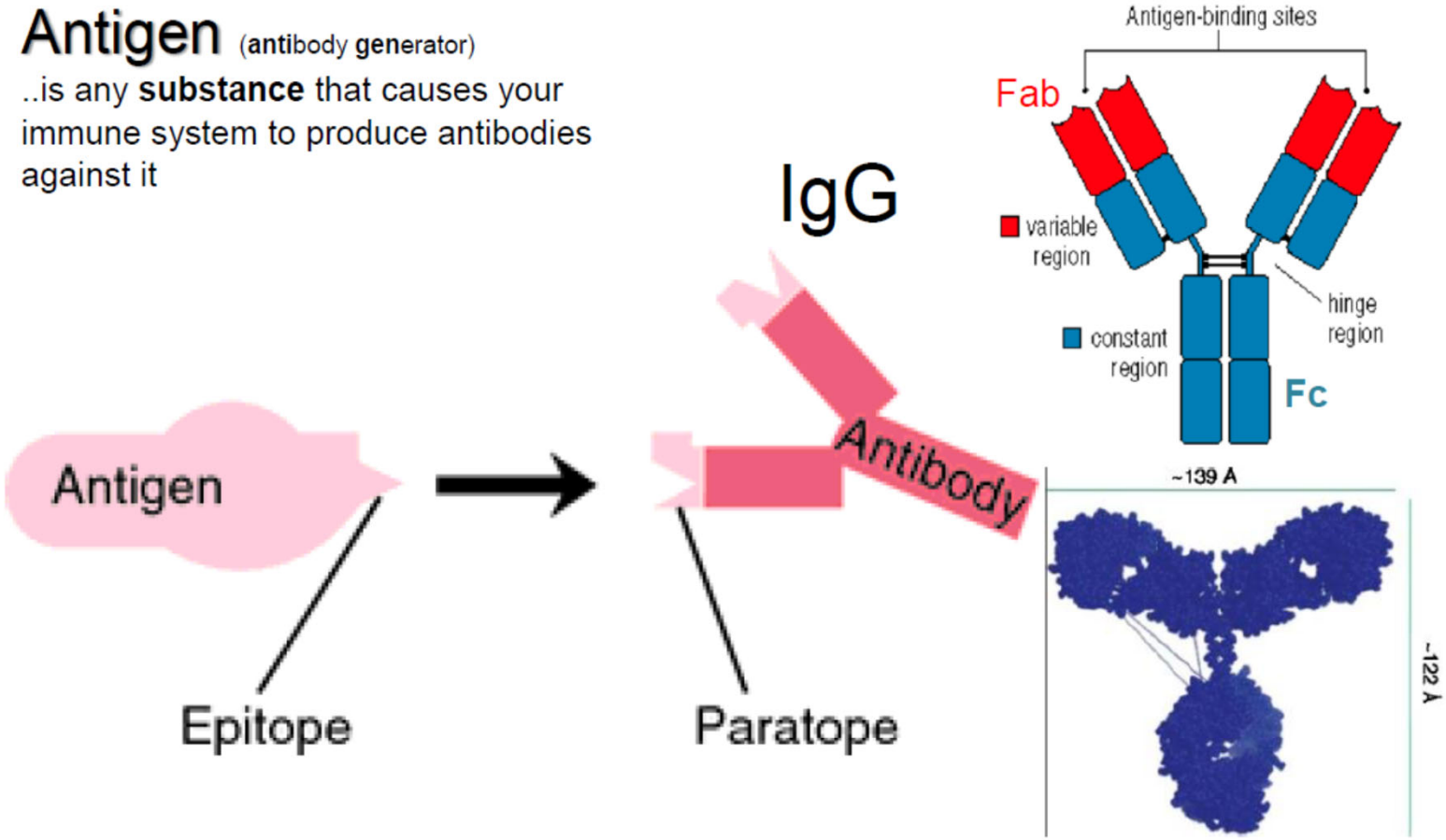
Methods of immunolocalization in SEM

Jana Nebesářová
Biology Centre CAS, České Budějovice
Faculty of Science, Charles University in Prague
nebesaro@natur.cuni.cz

The goal of the immunolabeling is to localize biochemically defined antigens using antibodies or high-affinity interactions (e.g. biotin-avidin; proteinA-IgG)

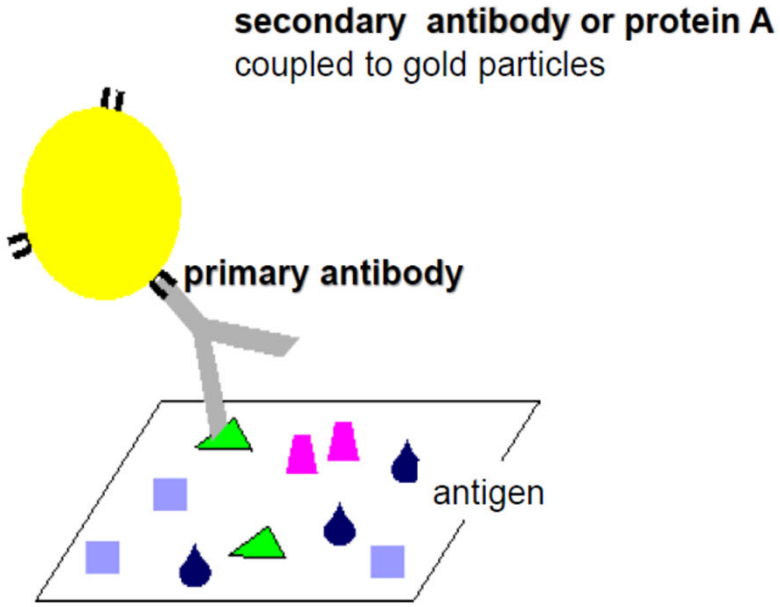
Antigen (antibody generator)

..is any **substance** that causes your immune system to produce antibodies against it



An epitope, antigen-binding site, also antigenic determinant, is the part of an antigen that is recognized by the immune system, specifically by antibodies, B cells, or T cells. For example, the epitope is the specific piece of the antigen that an antibody binds to. The part of an antibody that binds to the epitope is called a paratope.

Immunolocalization principle



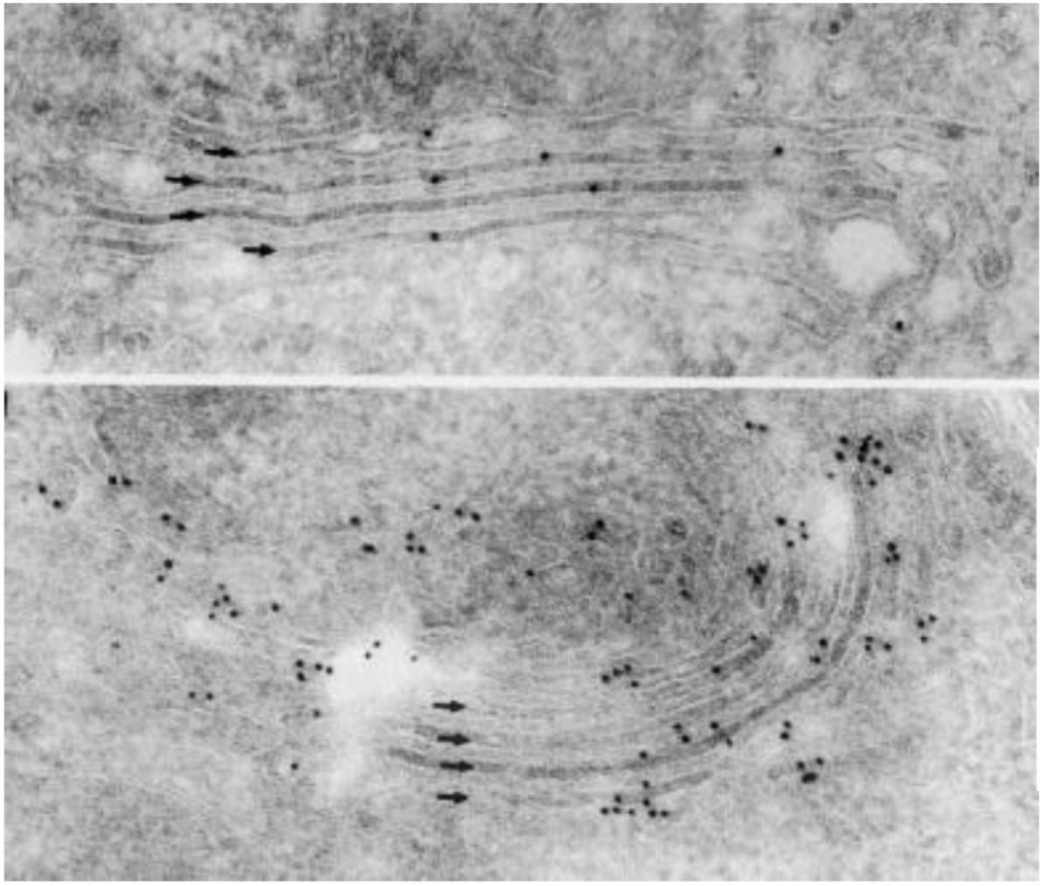
The methods of immunolocalisation in EM are based on the specific bound between epitops/antigens and tagged antibodies.
Each antibody contains a paratope which recognizes a specific epitope on an antigen, acting like a lock and key binding mechanism.
The indirect method represents the most common strategy:

- Antigen is first exposed to a primary antibody
- Then a secondary antibody containing the tag and directed against IGs of the primary antibody is applied

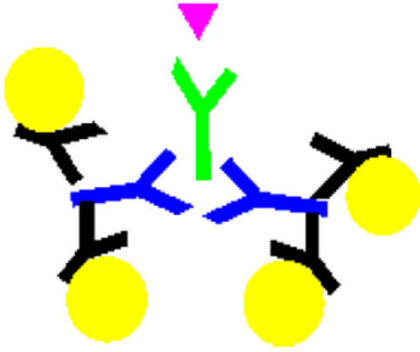
Advantages:

- Only small amounts of primary antibodies is necessary
- Conjugation of secondary antibodies is not so risky
- Amplification of localisation by binding more the tagged secondary antibodies due to the polyclonal nature of most antisera to a single antigen (the primary antibody)

Signal amplification



Indirect –two steps



Indirect –three steps

Gareth Griffiths

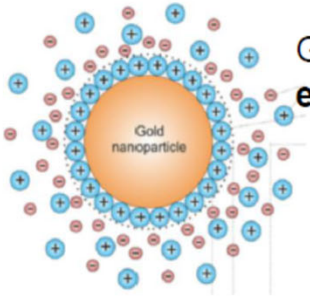
Visualization probes

Visible markers coupled to antibodies or other molecules which bind to primary antibodies to make their binding sites visible

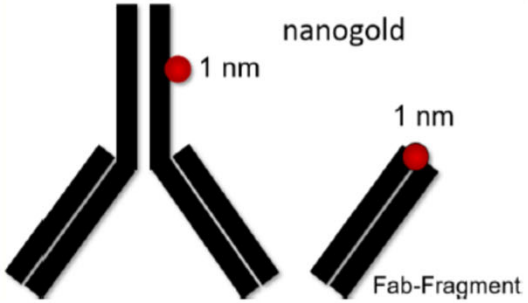
Visible markers

- Light microscopy
  - *Colorimetric dyes*
  - *Enzyme reactions*
    - HRP, AP
  - *Fluorescent dyes*
  - *Quantum dots*
- Electron microscopy
  - *Colloidal gold*
  - *Nano-gold (1-4nm)*
  - *Silver*
    - Silver enhanced gold
  - *Enzyme reactions*
    - HRP with DAB

The most common tag currently used is colloidal gold.
Gold NPs are easily detected on tissue sections.
Colloidal gold is suspension
of Au nanoparticles in water.
Particles of different sizes (1-20 nm) are easily distinguishable in electron micrographs, allowing simultaneous multiple-labelling experiments



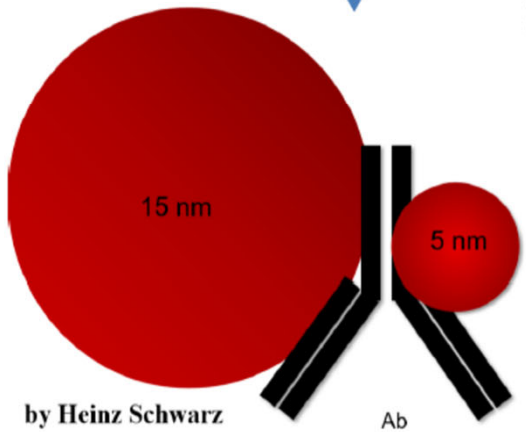
Gold colloids is the term for suspension of finely divided gold particles **or elemental gold.**



difficult to see on sections

10-15% size variation

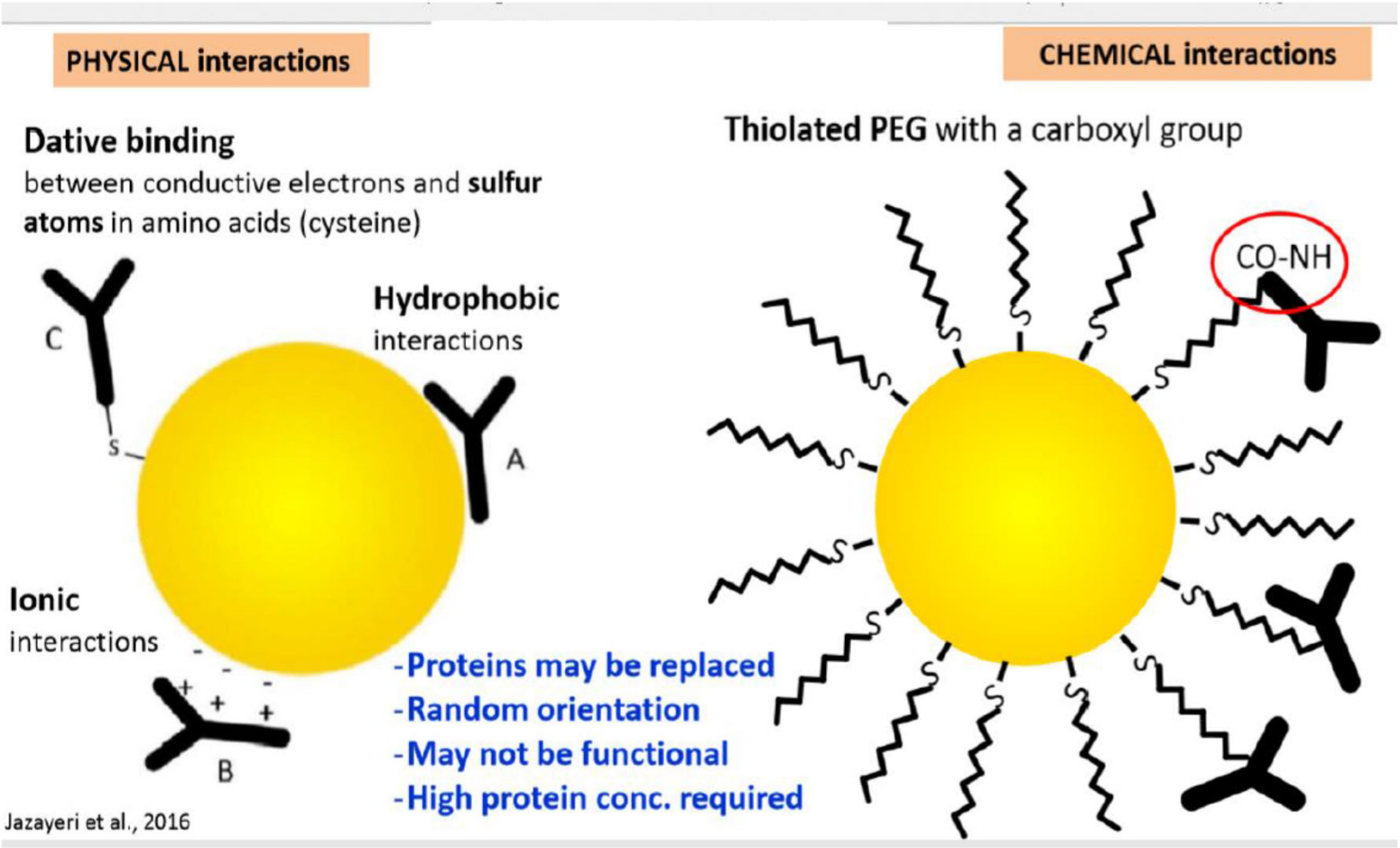
Considerably lower efficiency due to steric hindrance - electron repulsion between Au NPs



Colloidal gold particles can be attached to many traditional biological probes such as antibodies, lectins, glycans, nucleic acids and other receptors.

Slide from: Urska Repnik Max Planck Institute for Molecular Cellular Biology and Genetics , EMBO 2019 CB

Gold-protein conjugates



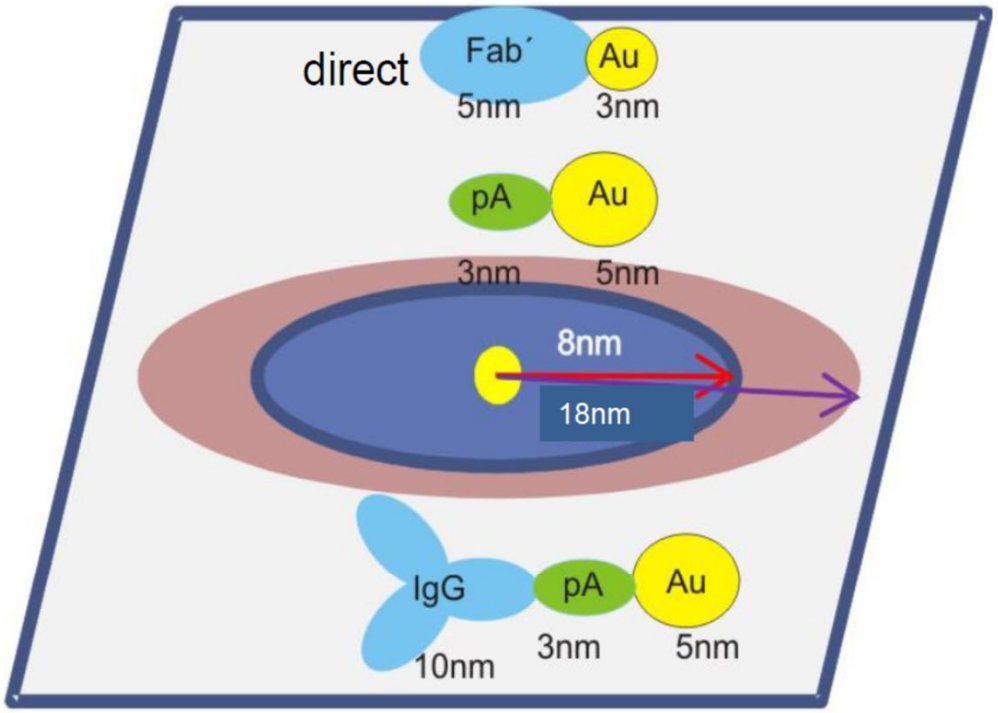
Affinity Labelling

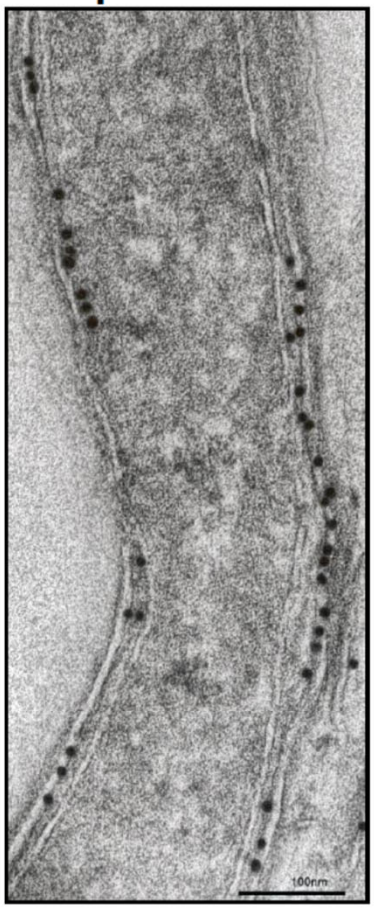
Using a molecule to bind specifically to a target in the cell

- Most common:
  - *Antibodies*
    - Polyclonal, monoclonal
    - IgG's
    - Fab fragments
    - Affinity purified
    - Well characterized
- Specificity varies
  - *Proteins with similar characteristics to target*
- Other affinity markers
  - *Lectins*
  - *Nucleic acids (in situ)*

Resolution power

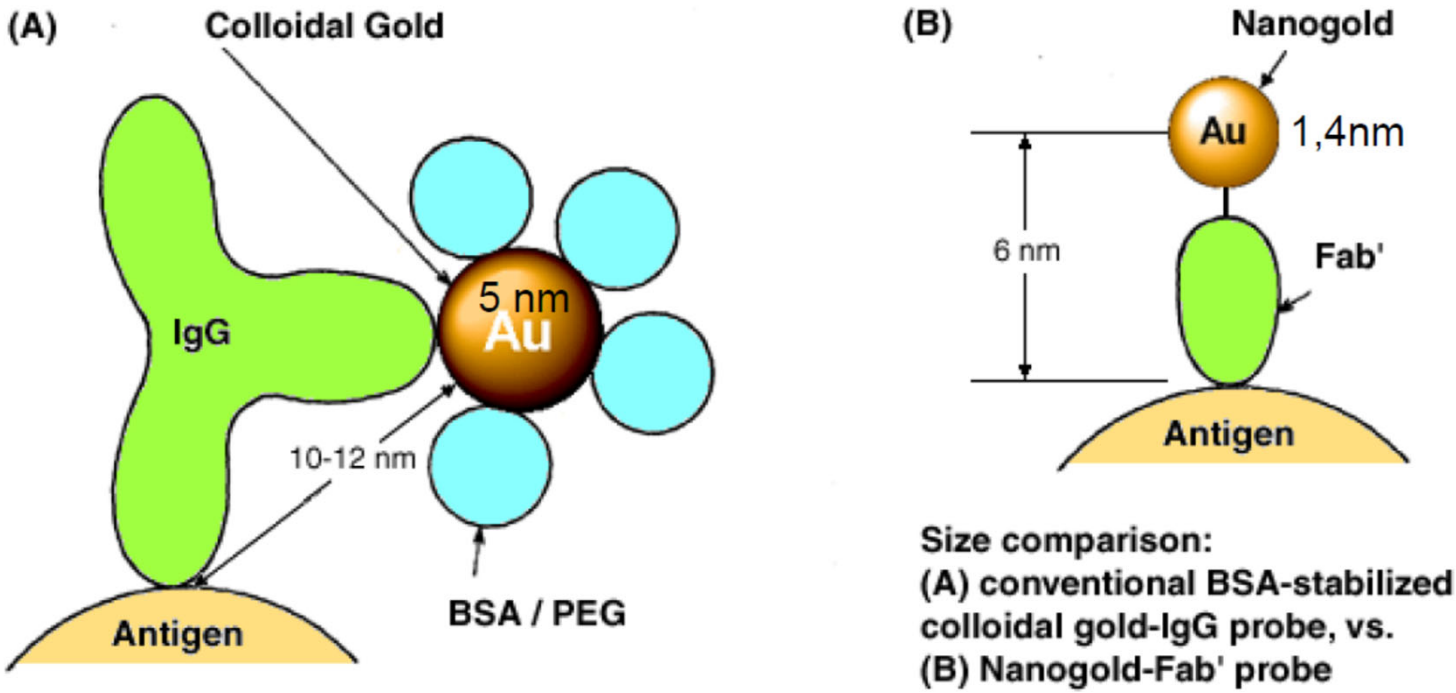
To reduce the size of the labelling complex : antibody + gold





The best theoretical resolution: Fab fragments conjugated directly to very small Au particles giving „worst case"- resolution of 8 nm radius
Specific IgG+ 5 nm Au/protein A = 18 nm

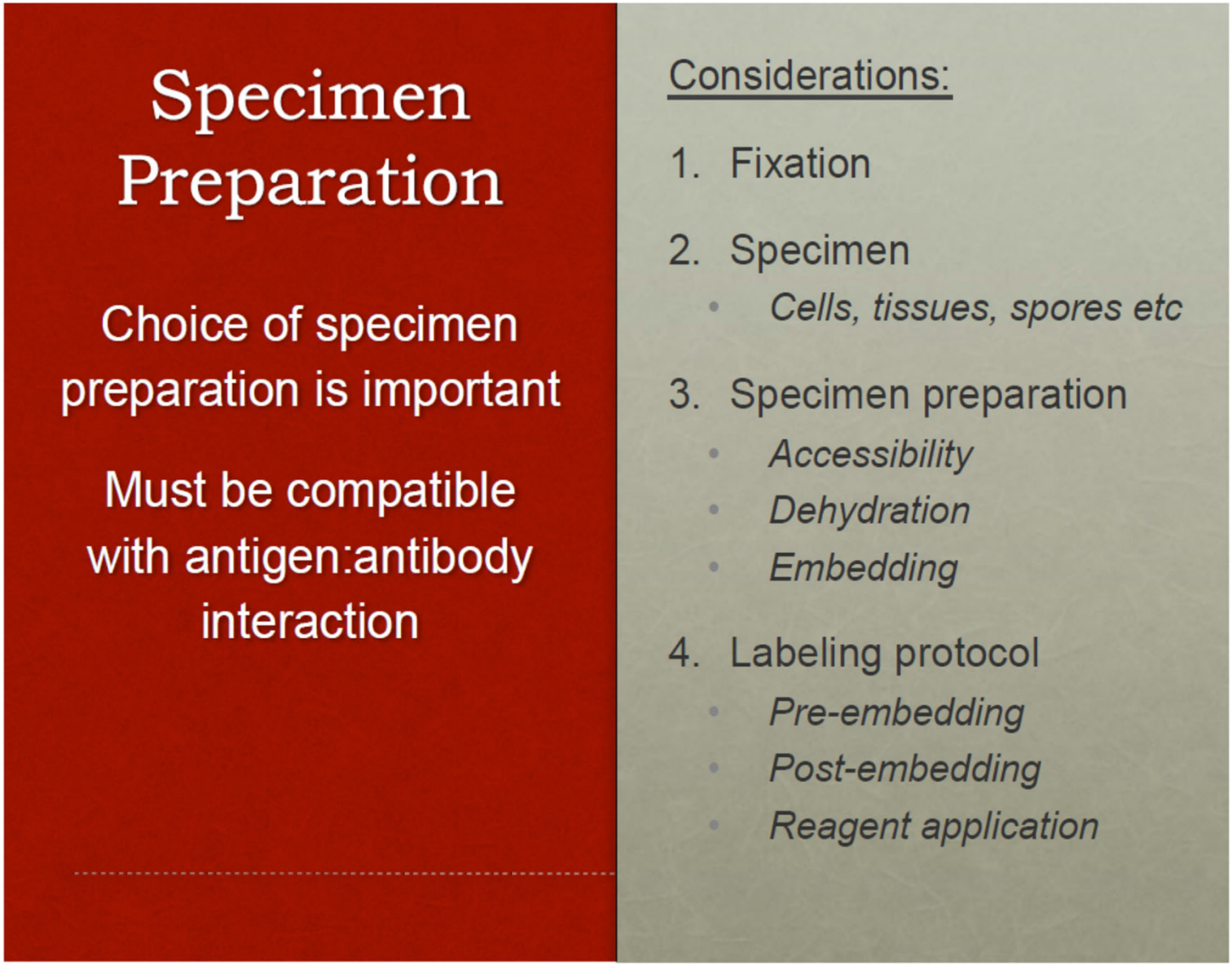
Nanogold®-Fab' is the smallest commercially available immunogold reagent capable of visualization in a regular transmission electron microscope; a comparison of the size of Nanogold-Fab' with a conventional 5 nm colloidal gold-IgG conjugate is shown below.



Size comparison:
(A) conventional BSA-stabilized colloidal gold-IgG probe, vs.
(B) Nanogold-Fab' probe

Pre-embedding: Immunolabeling is performed before dehydration and embedding the sample. (permeabilization, smallest gold conjugates x without antigen denaturation)

Post-embedding: Immunolabeling is performed on ultrathin sections



Methods-overview

HPF, plunge freezing

Freeze substitution (UA)

Methacrylate resins, UV

formaldehyde / glutaraldehyde

dehydration and infiltration/

Sucrose infiltration

plunging into IN2 no resin and dehydration!!!!

Tokuyasu cryosections

Labeling

Embedding UA/MC

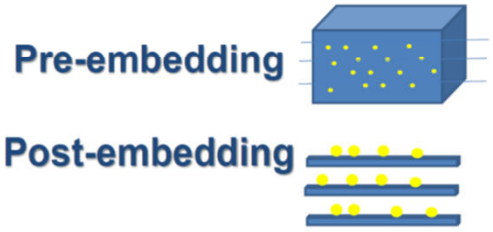
Ultrathin sections

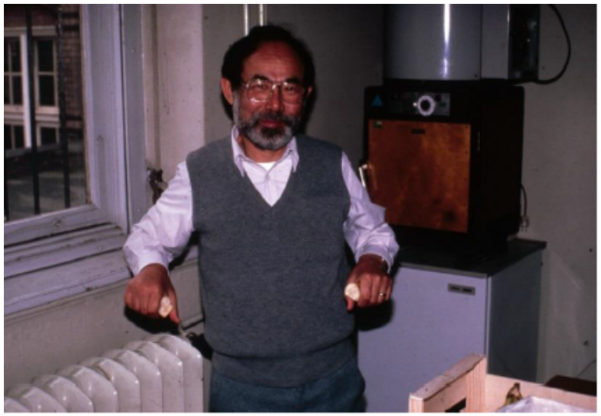
Labeling

Contrasting

Pre-embedding

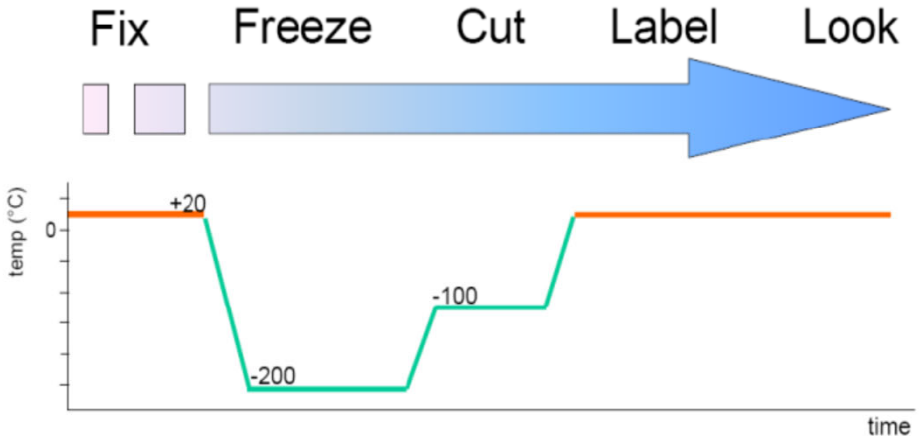
Post-embedding

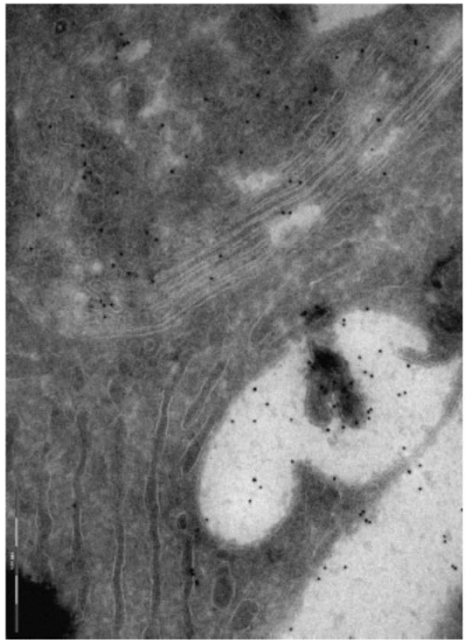




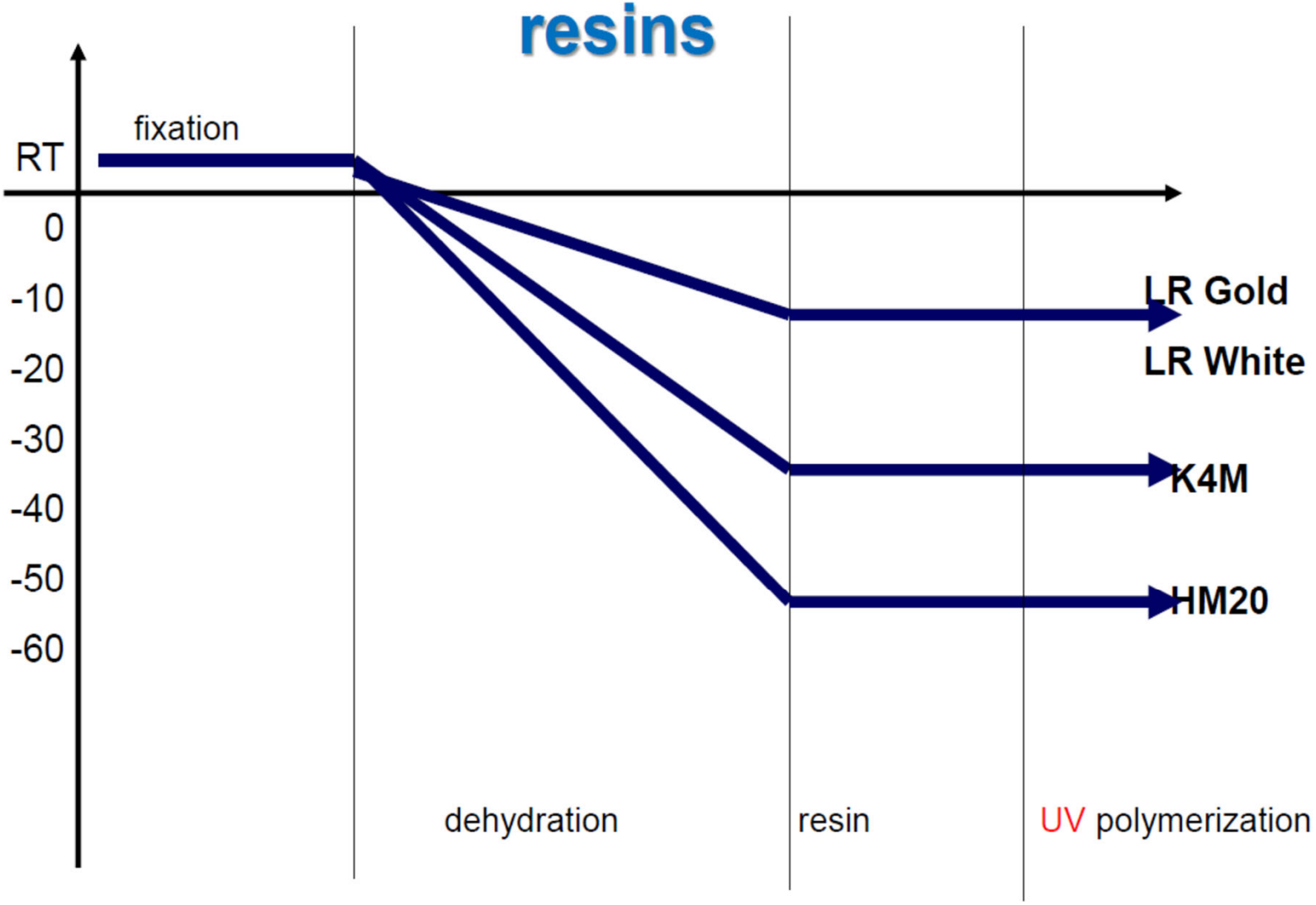
Tokuaysu technique

Tokuyasy, KT. 1973: A technique for ultracryotomy of cell suspensions and tissues. J Cell Biol. May;57(2):551-65,

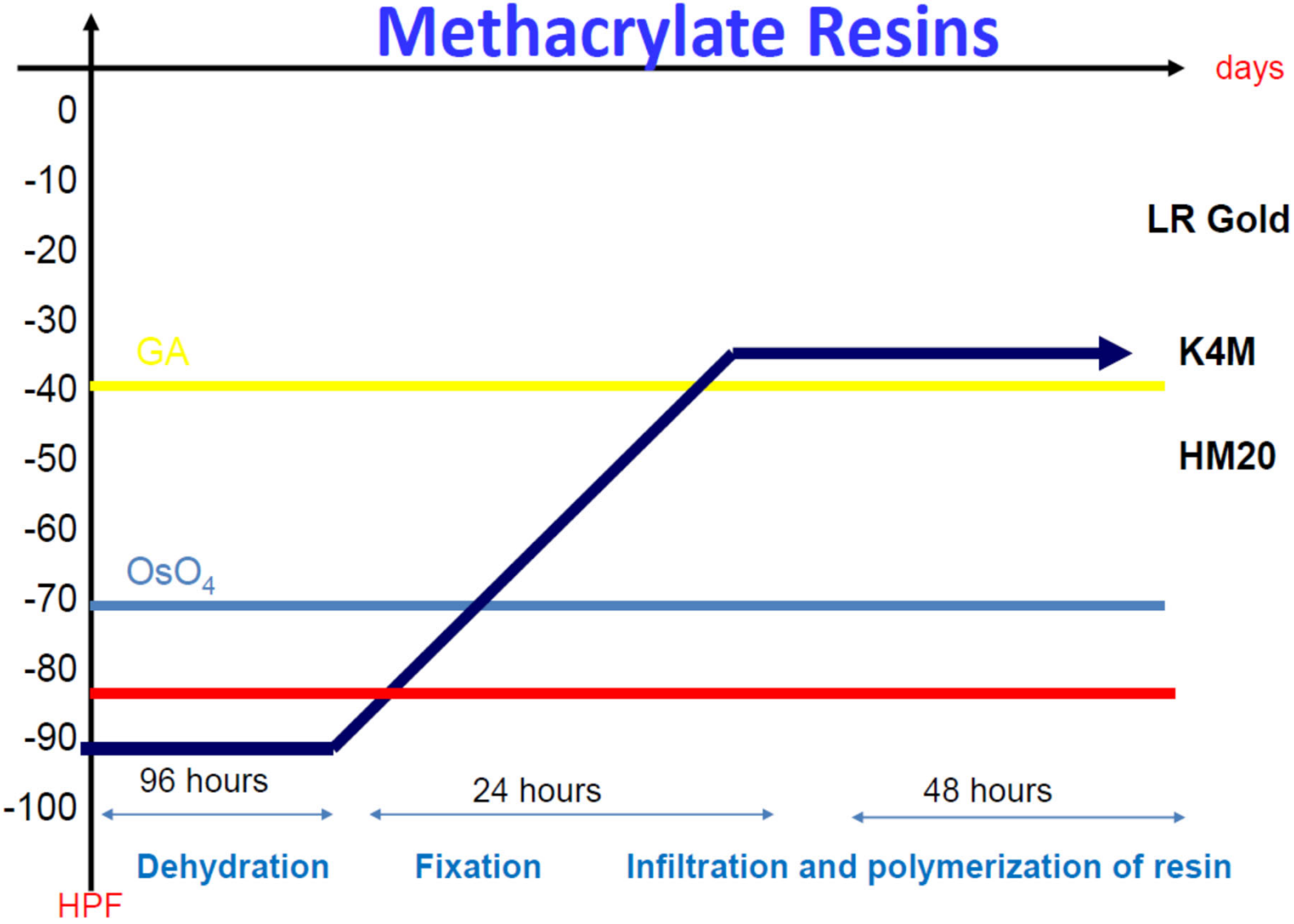




Chem. fix- dehydration-methacrylate resins



HPF- FS-reeze Substitution- Methacrylate Resins



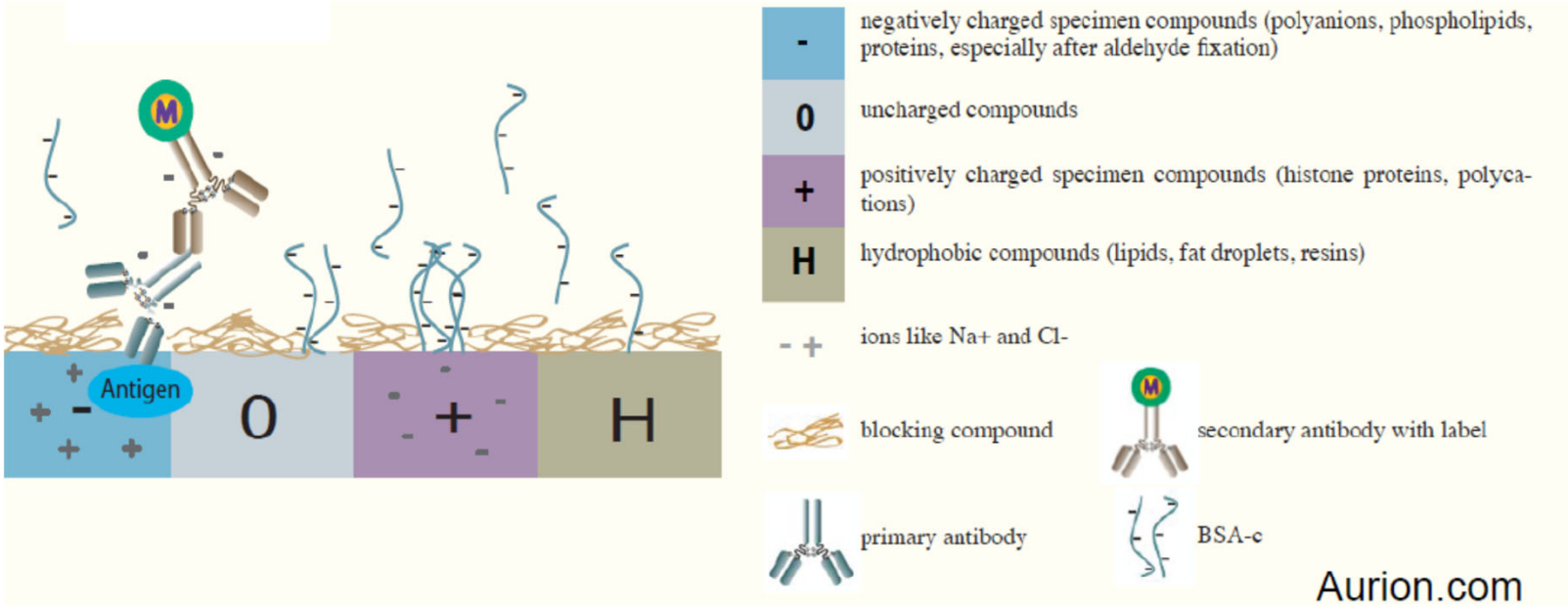
Blocking

Aim:

To eliminate non-specific binding of antibody and visualization probe

- Immunoreagents may bind to components in the specimen
  - *Aldehydes*
  - *Hydrophobic areas*
  - *Highly charged regions*
- Blocking agents:
  - *Low mol. wt. block*
  - *Protein block*
  - *Wash buffers*
    - (pH, high salt)
- Exposure time may be more important than concentration

Blocking agents



Used to prevent non-specific binding

A dilute solution for specific Ab and secondary/gold Ab

Applied to section prior 1st Ab

Blocking agents

Coldwater fish skin gelatine (0.1%)
Bovine serum albumin (0.5%)
Non-fat dry milk
Non-immune sera (same species as 2nd Ab/gold conjugate)
Goat serum (useful for secondary goat Ab/gold)
Tween 20, glycin, ammonium chloride, high salt solution (NaCl)

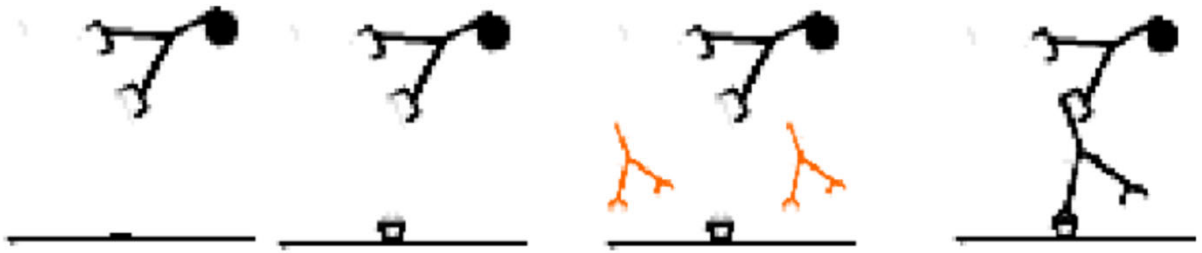
BUT Can be a source of labelling artifacts

rabbit serum binds to protein A; serum bind to streptavidin; serum will react with Ab to serum proteins, serum may bind to lectins, BSA and dry milk can contain bovine IgG and cross-react with anti-goat or anti-sheep IgG!!

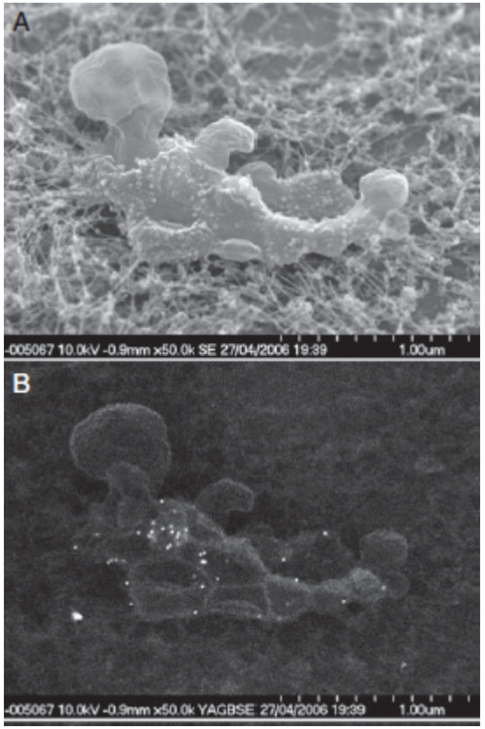
The protein blocking step is unnecessary ?? Buchwalow et al. 2011: Non-specific binding of antibodies in immunohistochemistry: fallacies and facts. Sci. Rep. 1, 28; DOI:10.1038/srep00028 (2011).

Controls

- Determining specific labelling using knockdown/knockout – two samples
- Negative control (binding of 2nd Ab to specimen)
- Preimmune serum: cross reactivity, proof of specificity
- Positive control (function of 1st Ab)
- Pre-adsorption



Immunolabeling evaluation in HRSEM

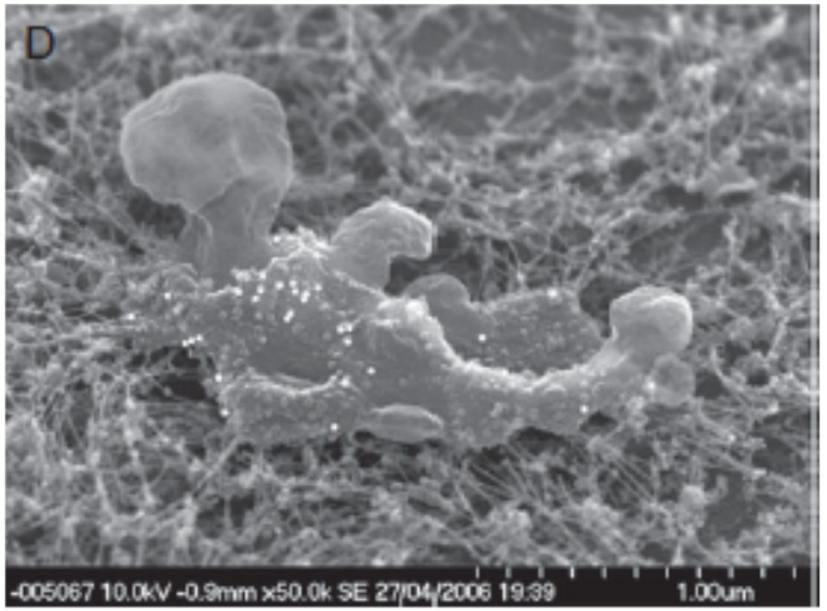


Superimposition of the backscattered image of the gold particles onto the secondary image

5 nm – the magnification 50 000 x
10 nm - the magnification 20-30 000 x
1 nm – the enhancement to 5-10nm

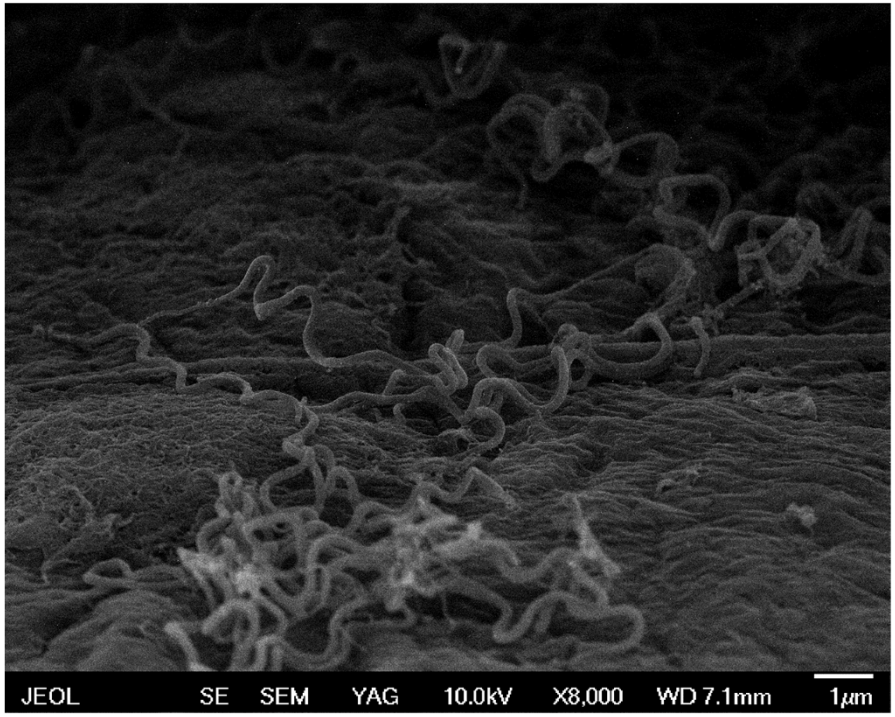
SE Imaging

BSE Imaging

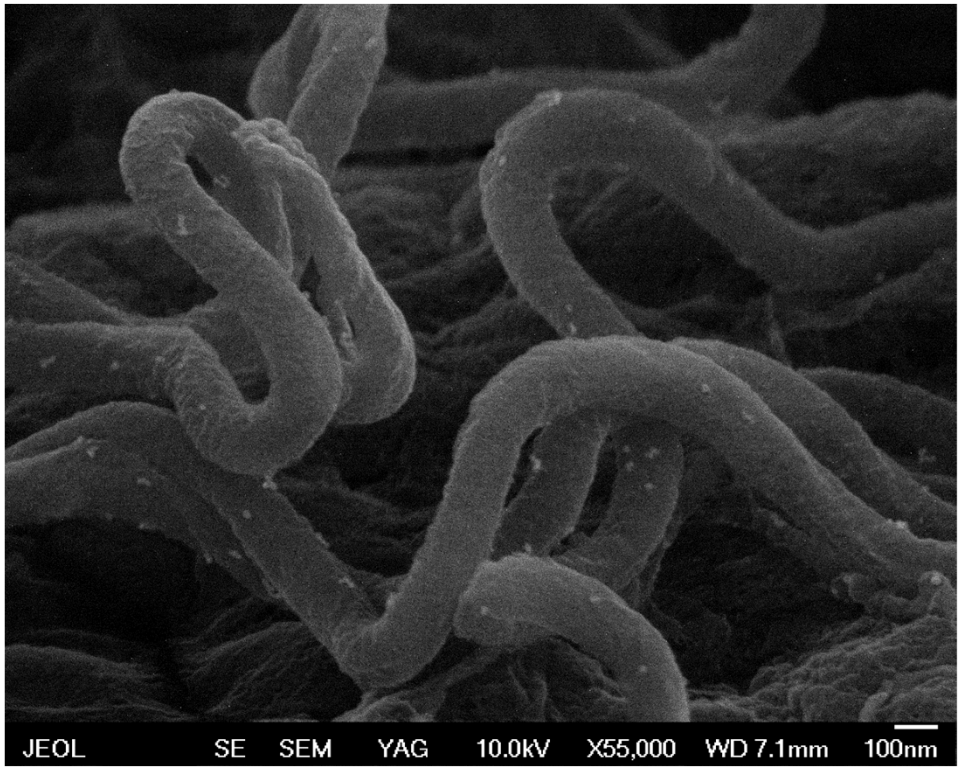


Goldberg M.W., Meths in Cell Biol. 88, 2008

Immunolocalisation on the specimen surface

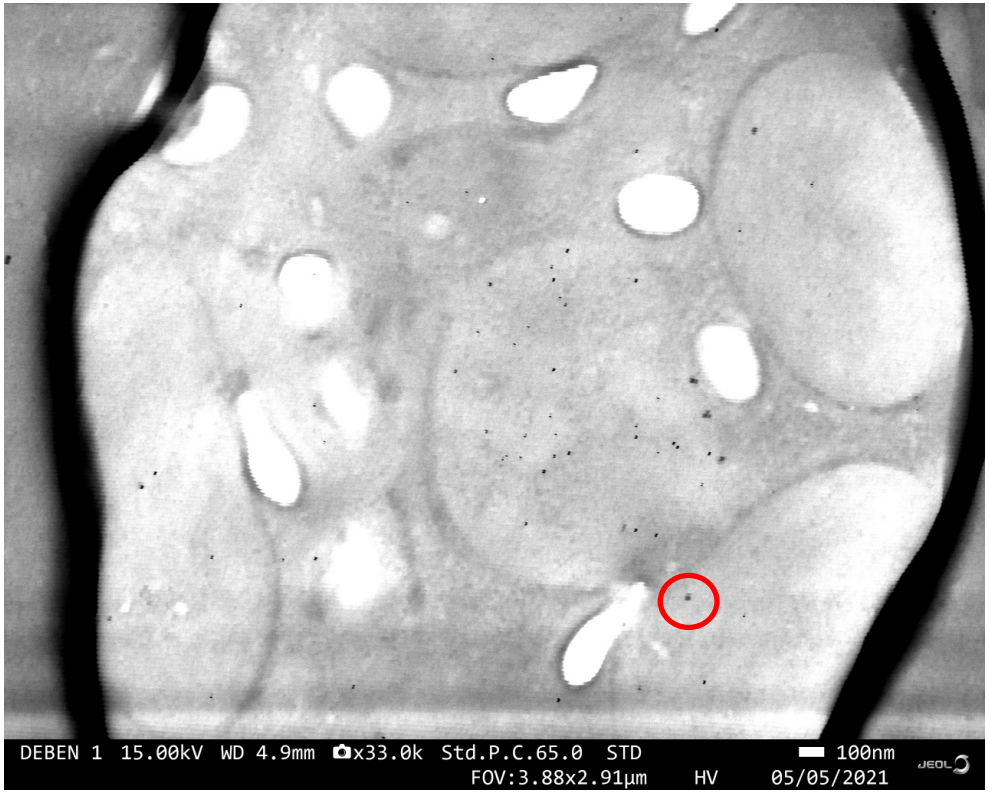


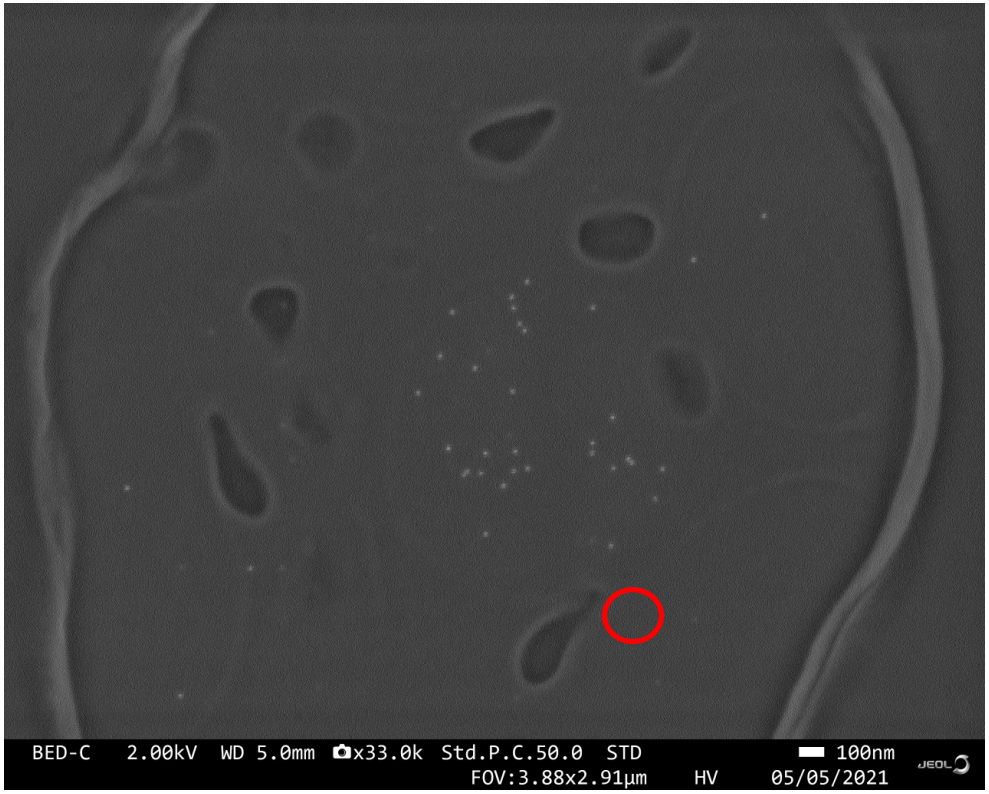
Salivary glands of ticks infected with Borrelia



Immunolocalisation on ultrathin sections

Low Voltage STEM





Thanks for your attention!