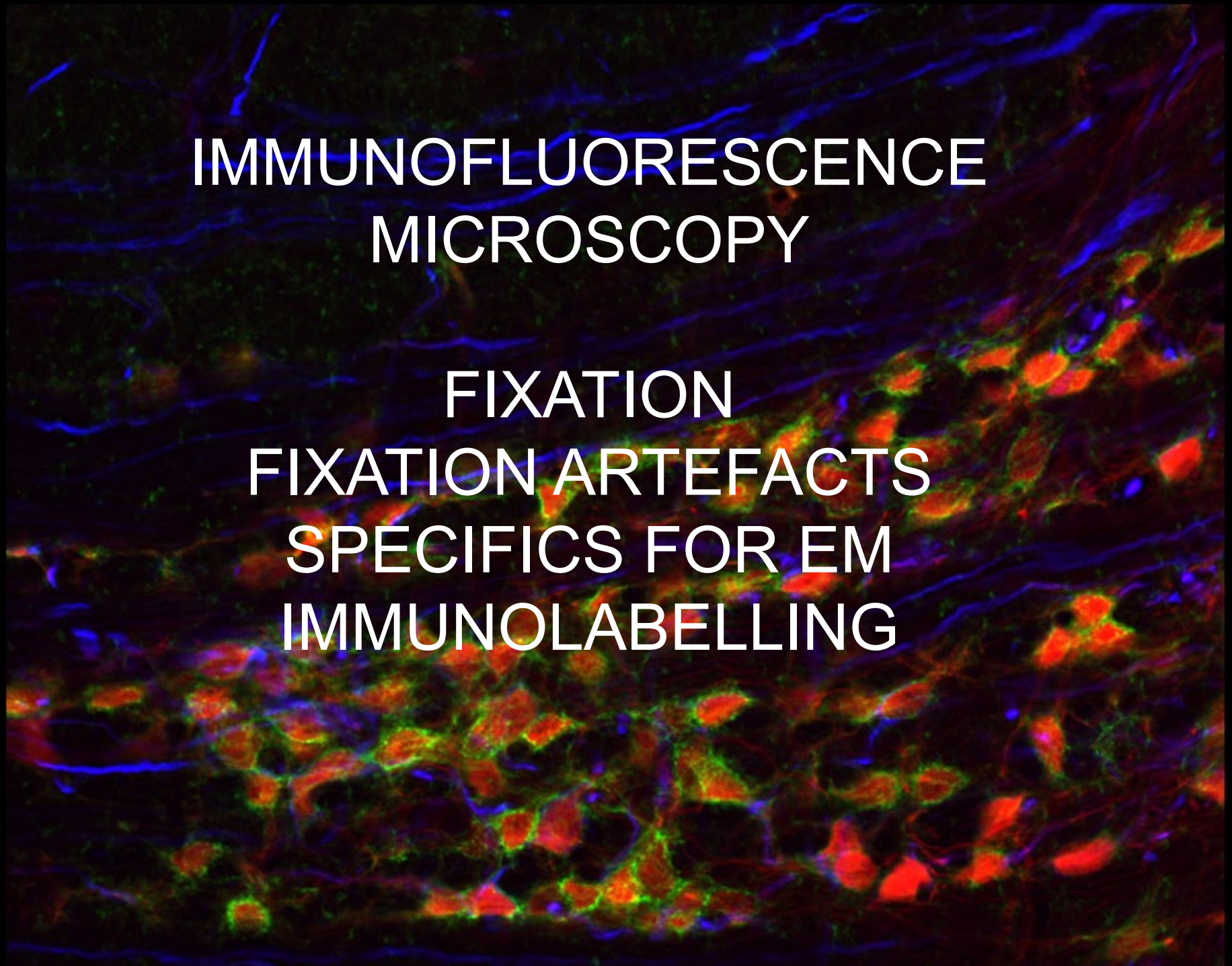
From a living cell to microscopical sample

Pavel Hozák


IMMUNOFLUORESCENCE MICROSCOPY
FIXATION
FIXATION ARTEFACTS
SPECIFICS FOR EM IMMUNOLABELLING

Fixation

- structure preservation
- abolishing biochemical reactions
- preserving antigens

Fixation schemes

- 4% formaldehyde/PBS + permeabilization 1% Triton X
- 4% formaldehyde/1% Triton X
- -20°C acetone + formaldehyde
- methanol

0022-1554/91/$3.30
The Journal of Histochemistry and Cytochemistry

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Brief Report

Effects of Formaldehyde Fixation on Protein Secondary Structure: A Calorimetric and Infrared Spectroscopic Investigation[1]

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We investigated the effects of formaldehyde fixation on the secondary structure of isolated proteins (bovine serum albumin, ribonuclease A, and hemoglobin) using high-sensitivity differential scanning calorimetry and Fourier transform infrared spectroscopy. Whereas thermograms obtained by scanning calorimetry on unfixed purified proteins demonstrated denaturation transitions in the 70–90°C temperature range, the thermograms showed no denaturation transitions in this temperature range when the proteins had been placed in formaldehyde solutions. Thus, fixation destroyed the denaturation transition of bovine serum albumin, ribonuclease A, and hemoglobin. Infrared spectra obtained on the unfixed and fixed proteins were essentially identical. This demonstrates that the "fixed" proteins retain the secondary structure present before fixation. We therefore conclude that the cross-linking of proteins that occurs in the process of formaldehyde fixation "locks in" the secondary structure of these protein molecules. (*J Histochem Cytochem 39:225–229, 1991*)

KEY WORDS: Formaldehyde; Fixation; Calorimetry; Infrared; Spectroscopy; Proteins.

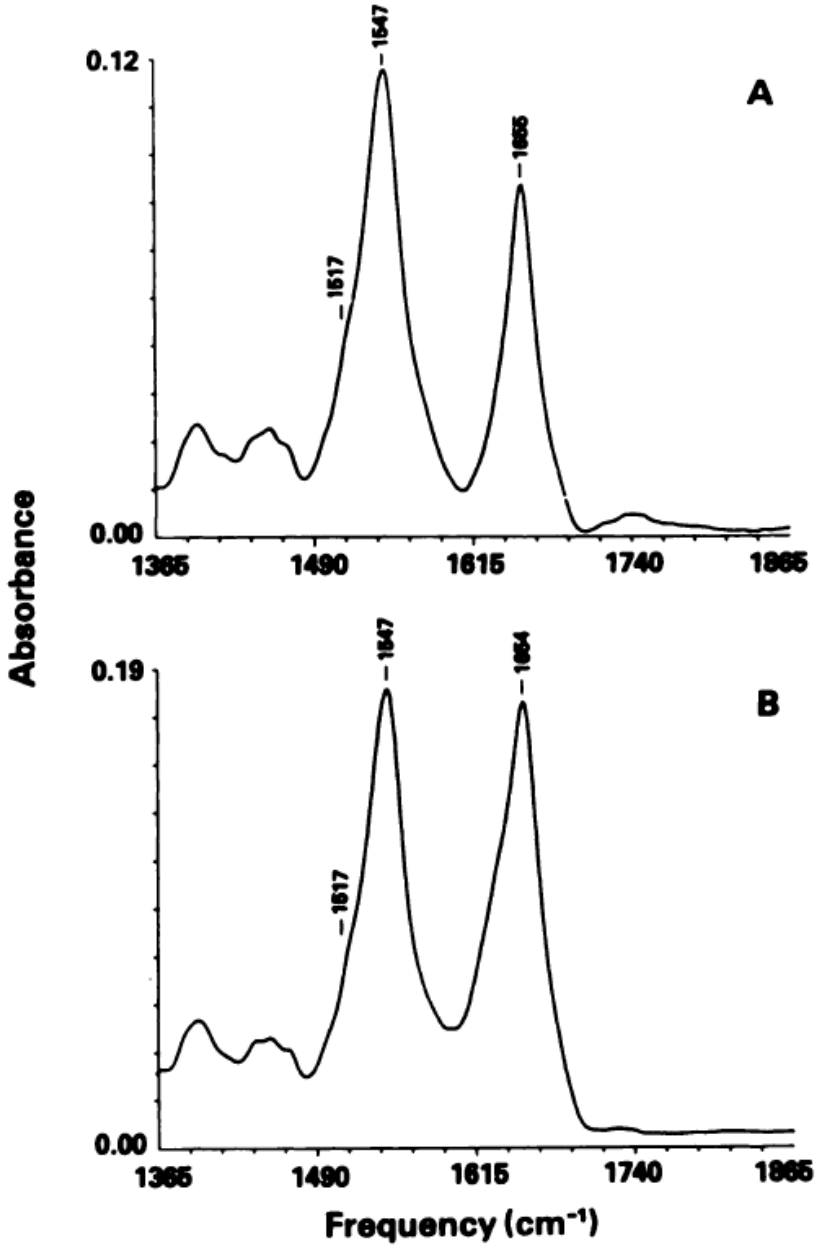


Figure 5. Amide I and II region infrared spectra of human hemoglobin in the absence (**A**) and presence (**B**) of formaldehyde. Although baseline differences are seen, the spectra are otherwise nearly identical, indicating that similar secondary structure is present in the two specimens.

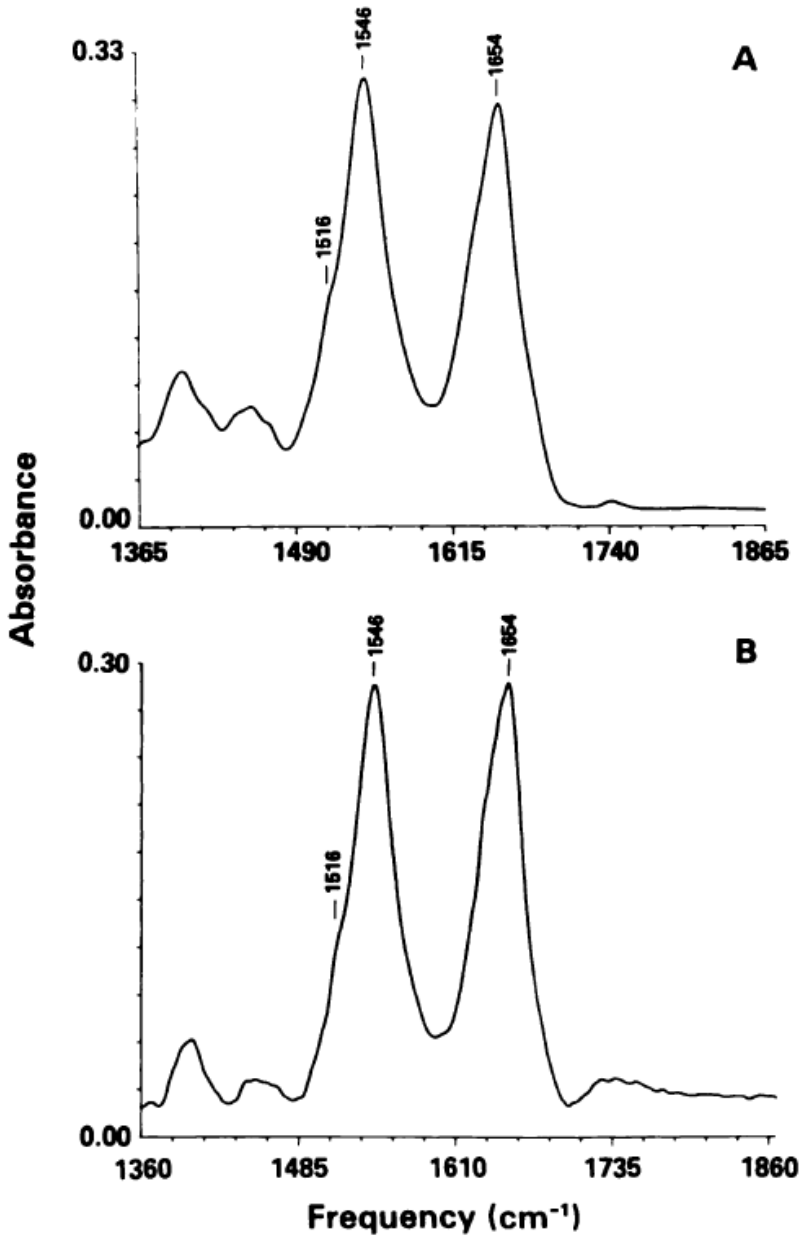


Figure 6. Amide I and II region infrared spectra of ribonuclease A in the absence (**A**) and presence (**B**) of formaldehyde. Although baseline differences are seen, the spectra are otherwise nearly identical, indicating that similar secondary structure is present in the two specimens.

Fixation artifacts

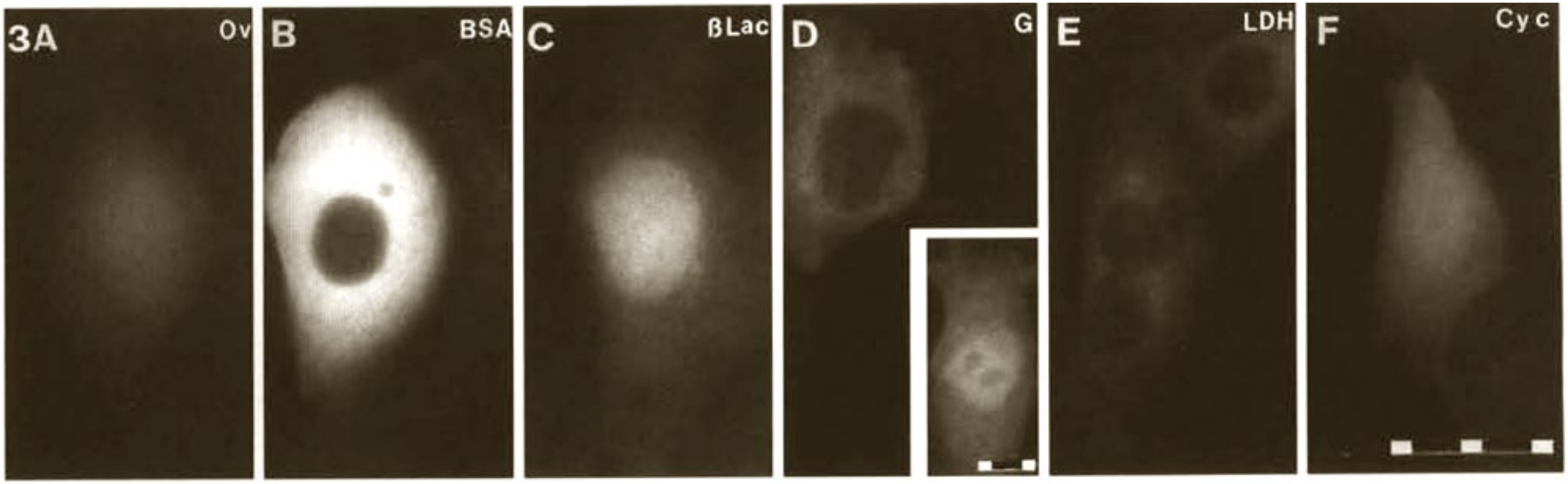
Journal of Cell Science 101, 731-743 (1992)
Printed in Great Britain

Redistribution and differential extraction of soluble proteins in permeabilized cultured cells

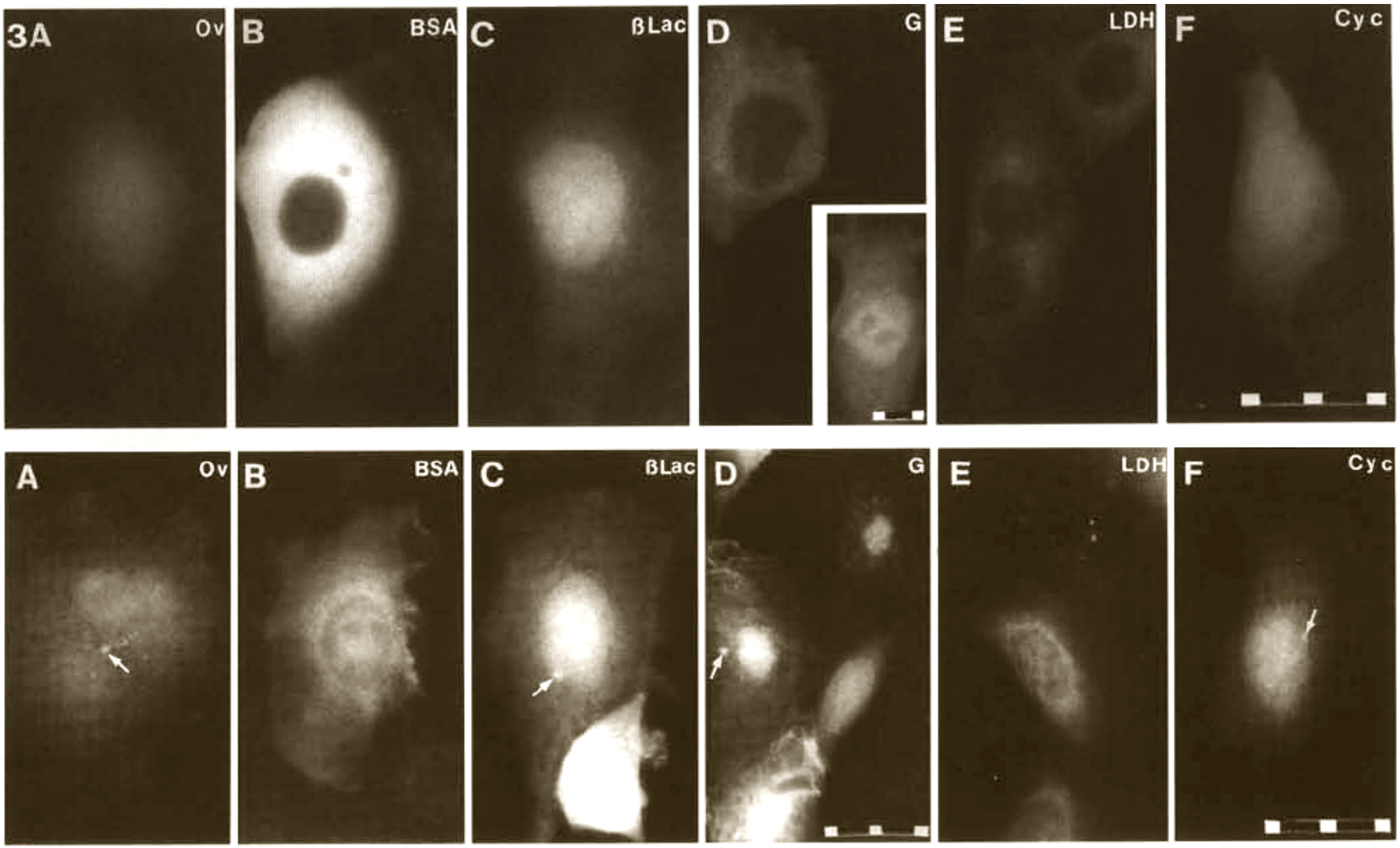
Implications for immunofluorescence microscopy

MELISSA A. MELAN* and GREENFIELD SLUDER

FITC-protein distribution in living cells



FITC-protein distribution in living cells



FITC-protein distribution: 30 min. F + 0,1% Triton 10 min.

FITC-BSA in various cel llines

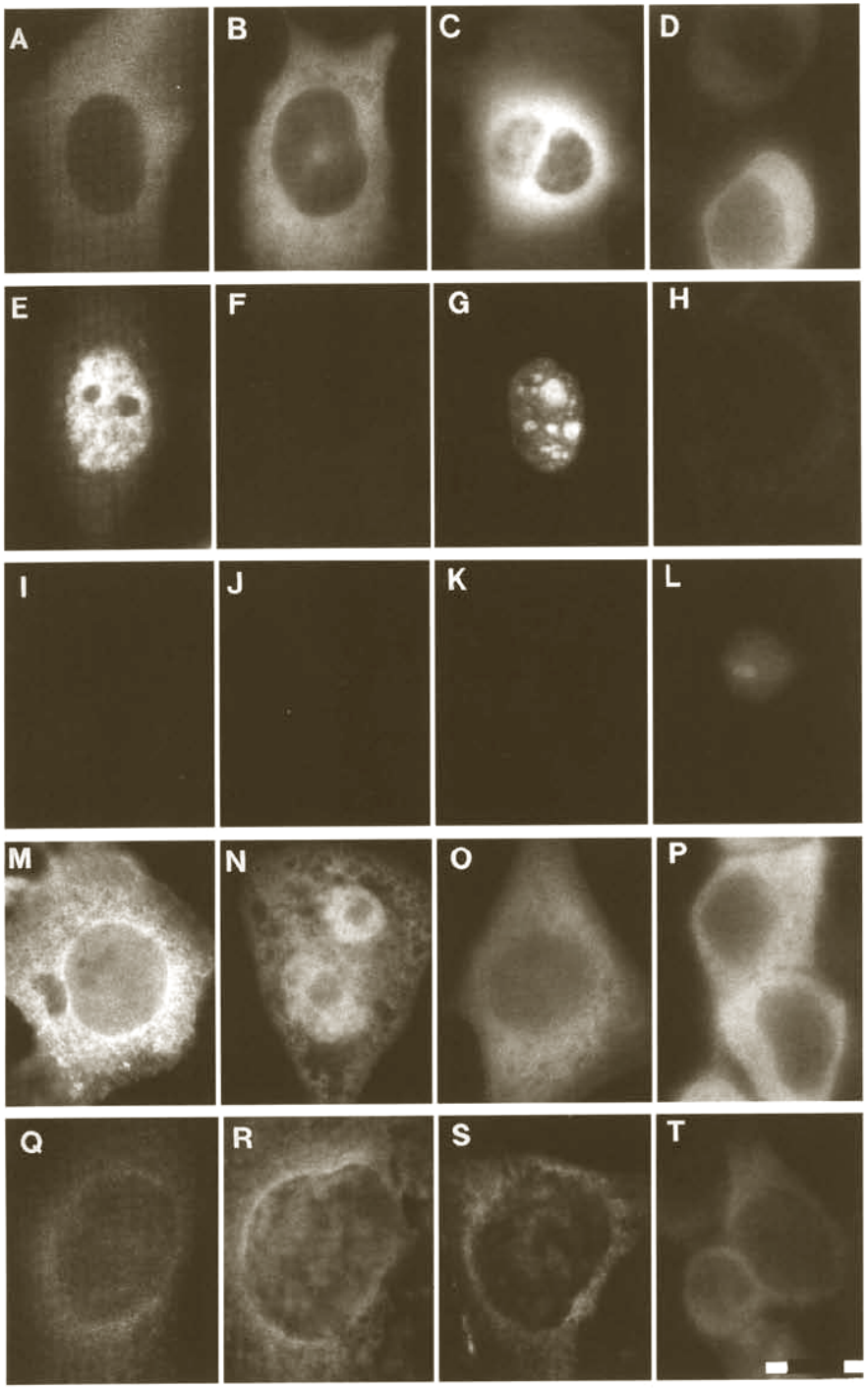


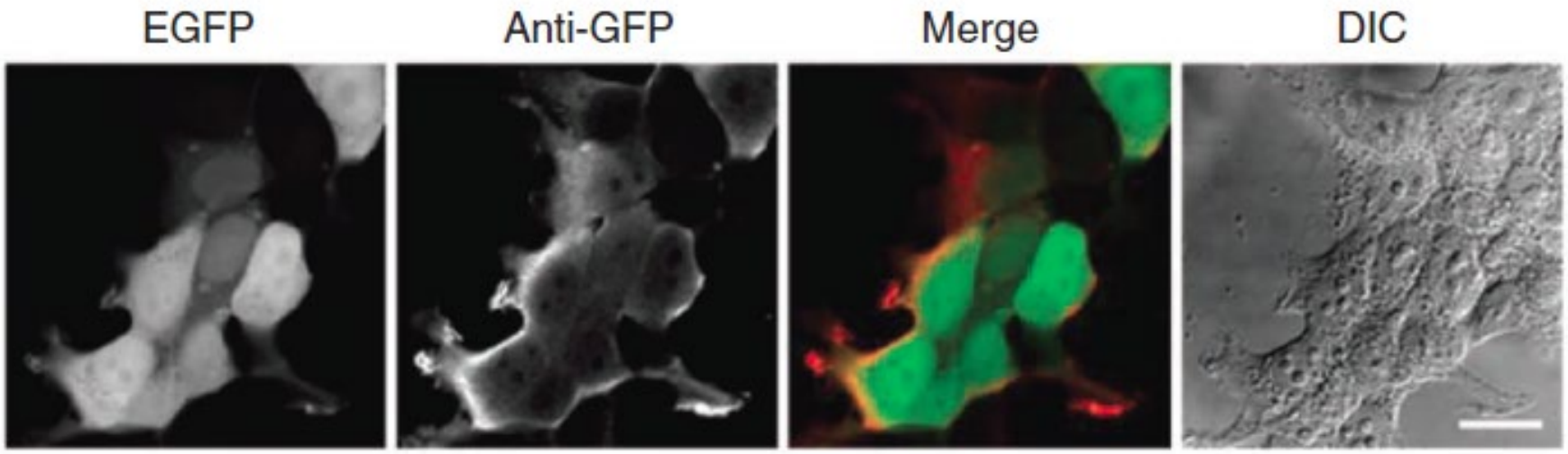
Fig. 10. Distribution of FITC-BSA in various cell lines. (A- D) Protein distribution in living cells, PtK_1, CHO, 3T3 and HeLa cells, respectively. The protein is excluded from the nuclei of all cells. (E-H) Protein distribution in cells extracted for 10 min with 0.1% Triton X-100 before fixation for 30 min with 3.7% formaldehyde, PtK_1, CHO, 3T3 and HeLa cells, respectively. Nuclear fluorescence is seen in PtK_1 (E) and 3T3 (G) cells. (I-L) Protein distribution in cells extracted for 10 min with 1% Triton X-100 before fixation for 30 min with 3.7% formaldehyde, PtK_1, CHO, 3T3 and HeLa cells, respectively. No fluorescence is detected in the cells with the exception of some nuclear fluorescence seen in HeLa cells (L). (M-P) Protein distribution in cells fixed for 30 min with 3.7% paraformaldehyde before permeabilization for 10 min with 0.1% Triton X-100. Fluorescence is seen primarily in the cytoplasm with the exception that nuclear fluorescence is seen in PtK_1 (M) and CHO (N) cells. (Q-T) Protein distributions in cells fixed for 5 min with 90% methanol, 50 mM EGTA at −20°C, PtK_1, CHO, 3T3 and HeLa cells, respectively. All cells show an overall low fluorescence, fibrous textured cytoplasmic fluorescence and bright staining at the periphery of the nucleus. 10 μm per scale division (black bar).

Immunolabeling artifacts and the need for live-cell imaging

Ulrike Schnell, Freark Dijk, Klaas A Sjollema & Ben N G Giepmans

Fluorescent fusion proteins have revolutionized examination of proteins in living cells. Still, studies using these proteins are met with criticism because proteins are modified and ectopically expressed, in contrast to immunofluorescence studies. However, introducing immunoreagents inside cells can cause protein extraction or relocalization, not reflecting the *in vivo* situation. Here we discuss pitfalls of immunofluorescence labeling that often receive little attention and argue that immunostaining experiments in dead, permeabilized cells should be complemented with live-cell imaging when scrutinizing protein localization.

Fixation and permeabilization can affect epitope accessibility



Ultrastructural changes after fixation and permeabilization

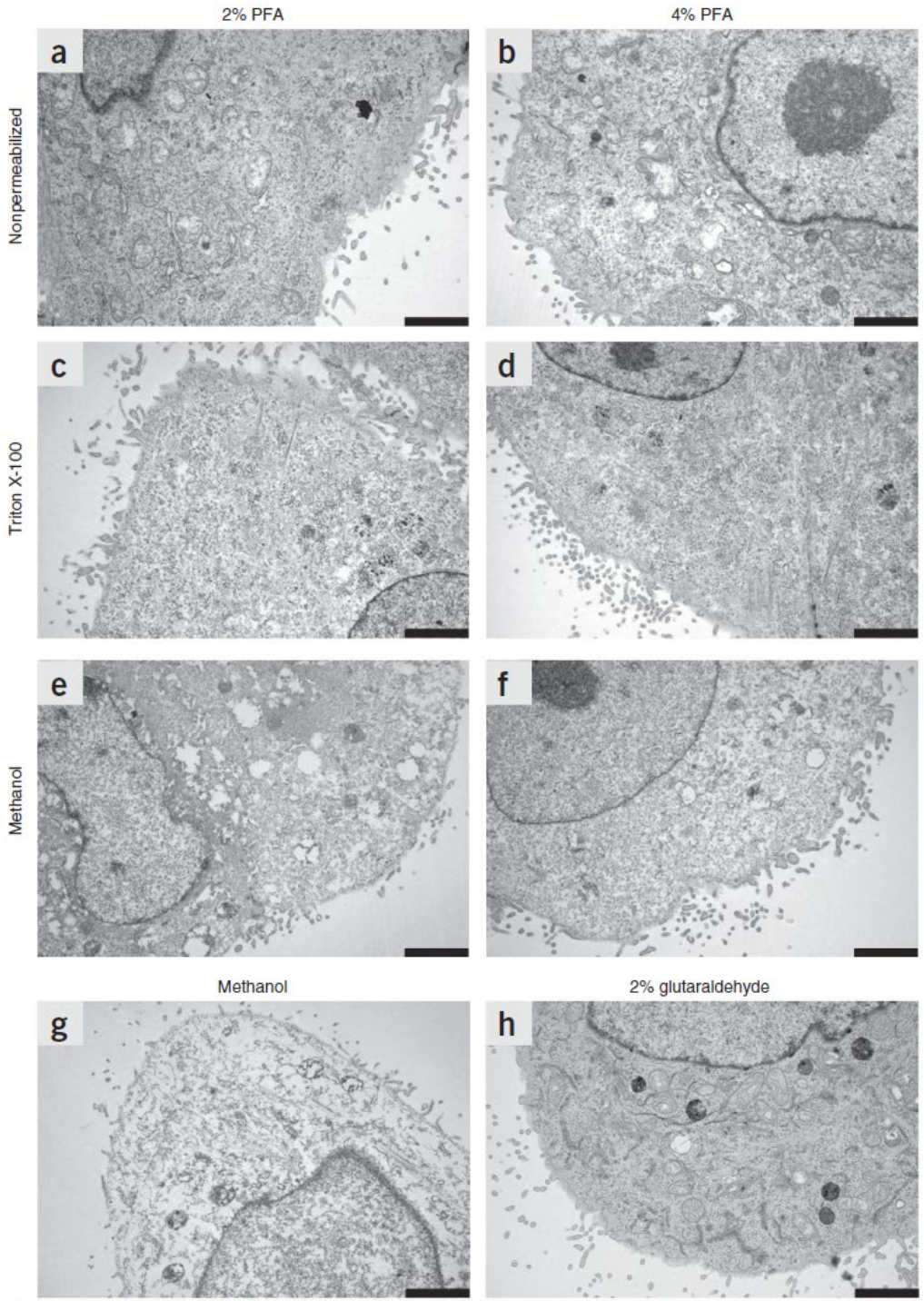
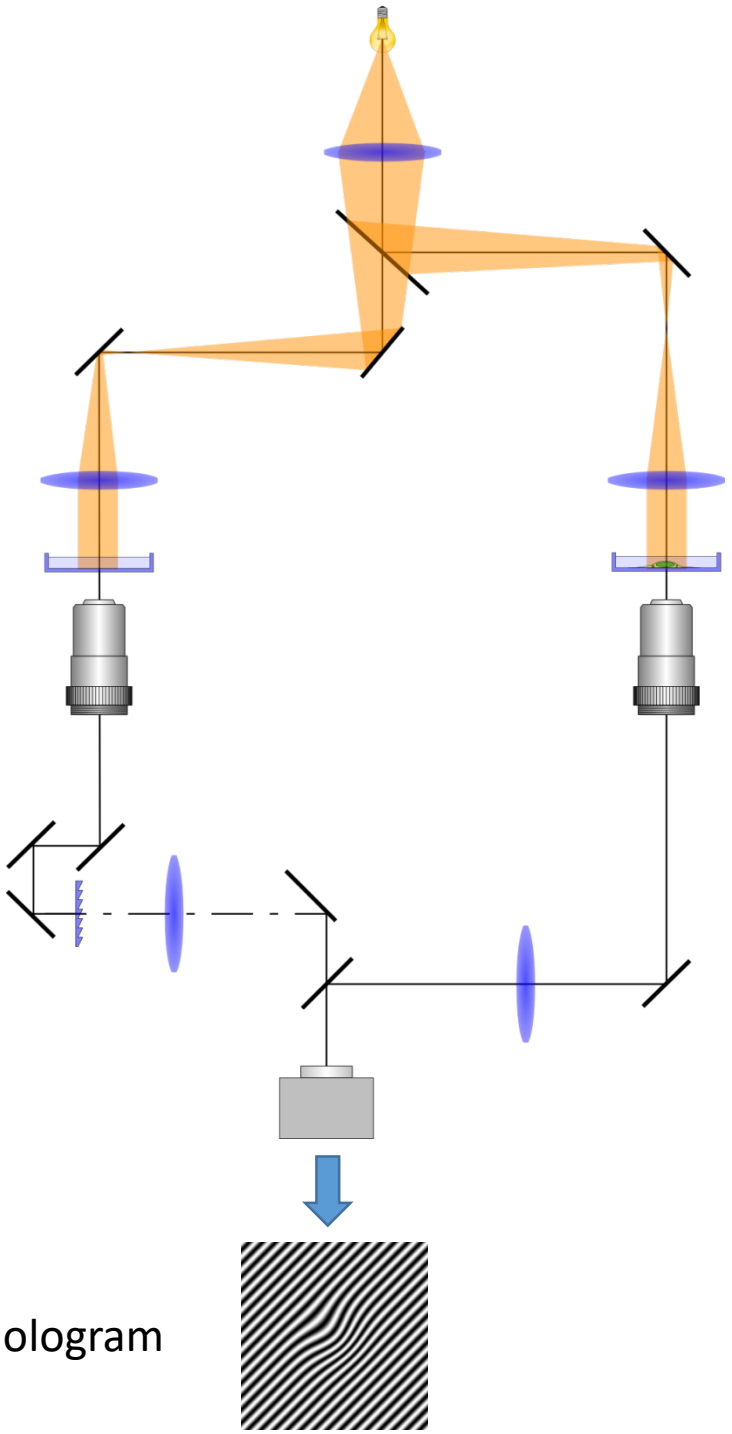


Figure 4 | Ultrastructural changes after fixation and permeabilization. (**a**–**g**) Electron micrographs of MDCK cells fixed with 2% or 4% PFA (**a**–**f**), methanol (**g**) or glutaraldehyde (**h**). To mimic the immunostaining procedure, PFA-fixed cells were permeabilized with 0.05% Triton X-100 (15 min; **c**,**d**) or MeOH (1 min at –20 °C; **e**,**f**). All samples were washed (6× PBS), then fixed with 2% glutaraldehyde for 10 min and processed for electron microscopy. Scale bars, 2 µm.

Reduction of fixation artifacts ?

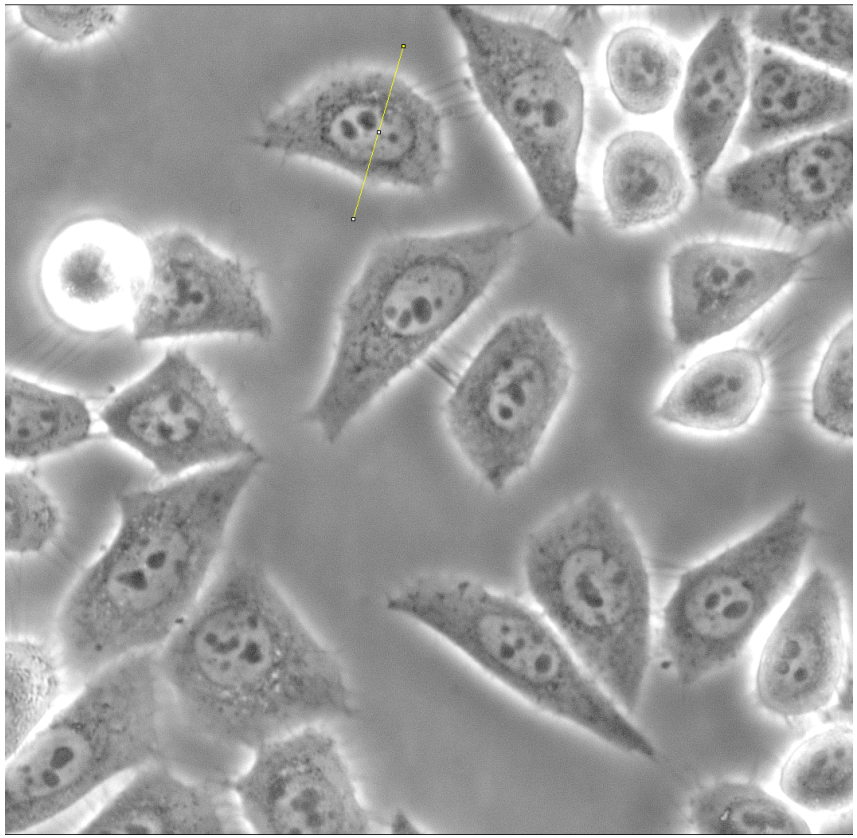
Principles of HM



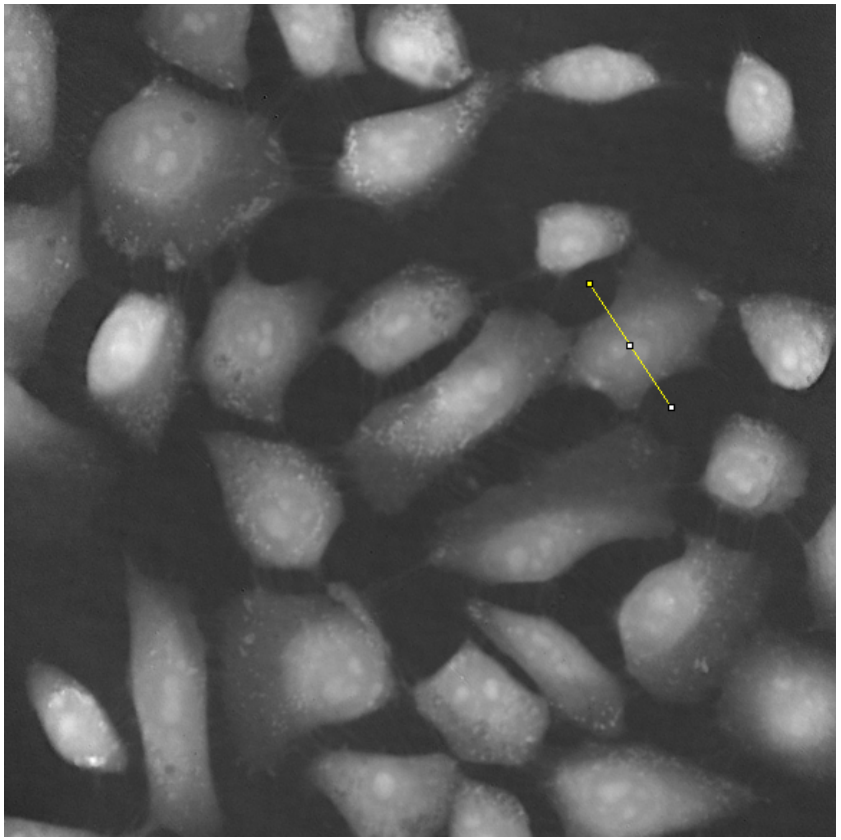
interference and diffraction induced pattern = hologram

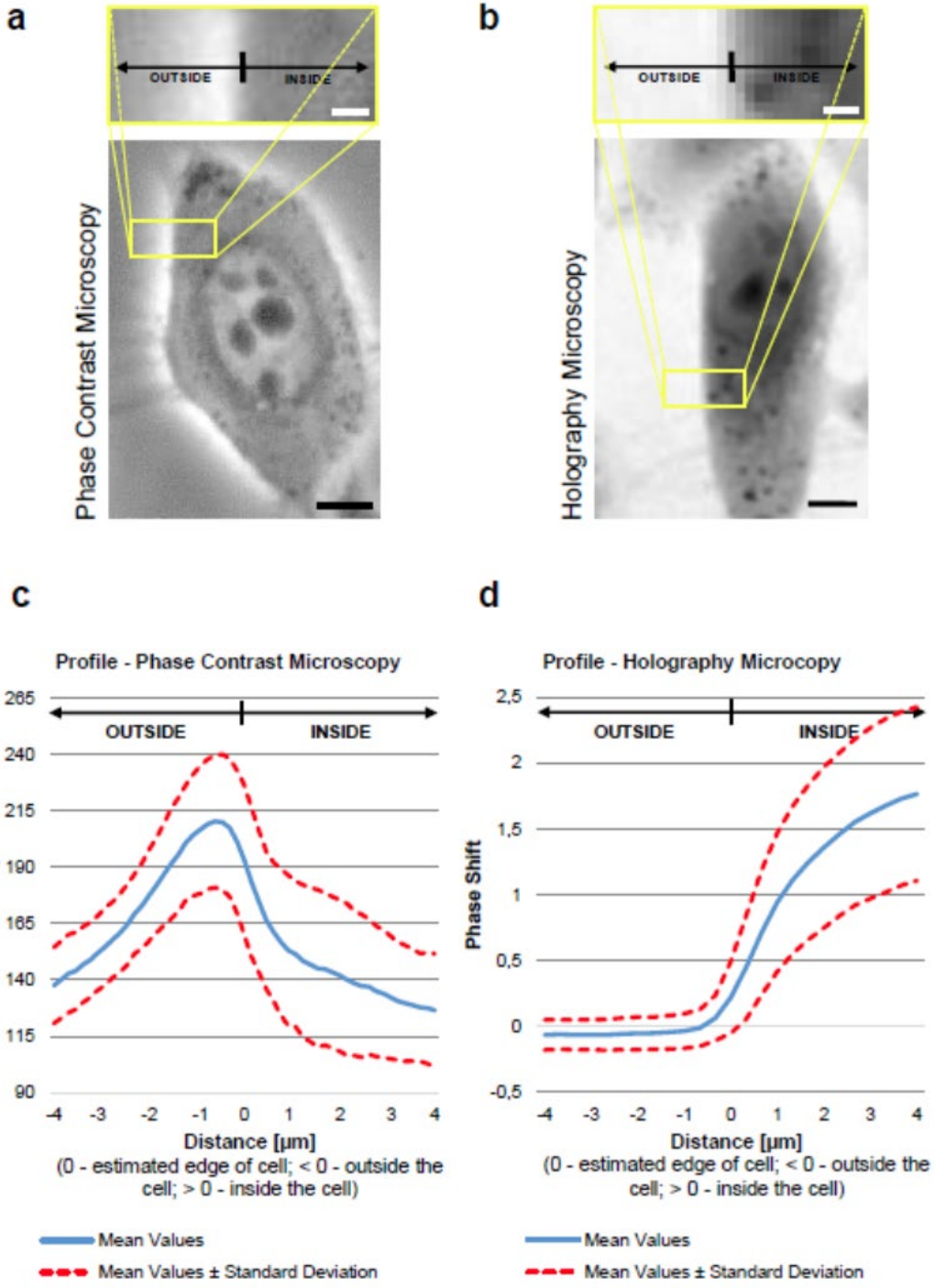
Holographic microscopy of HeLa cells

PC

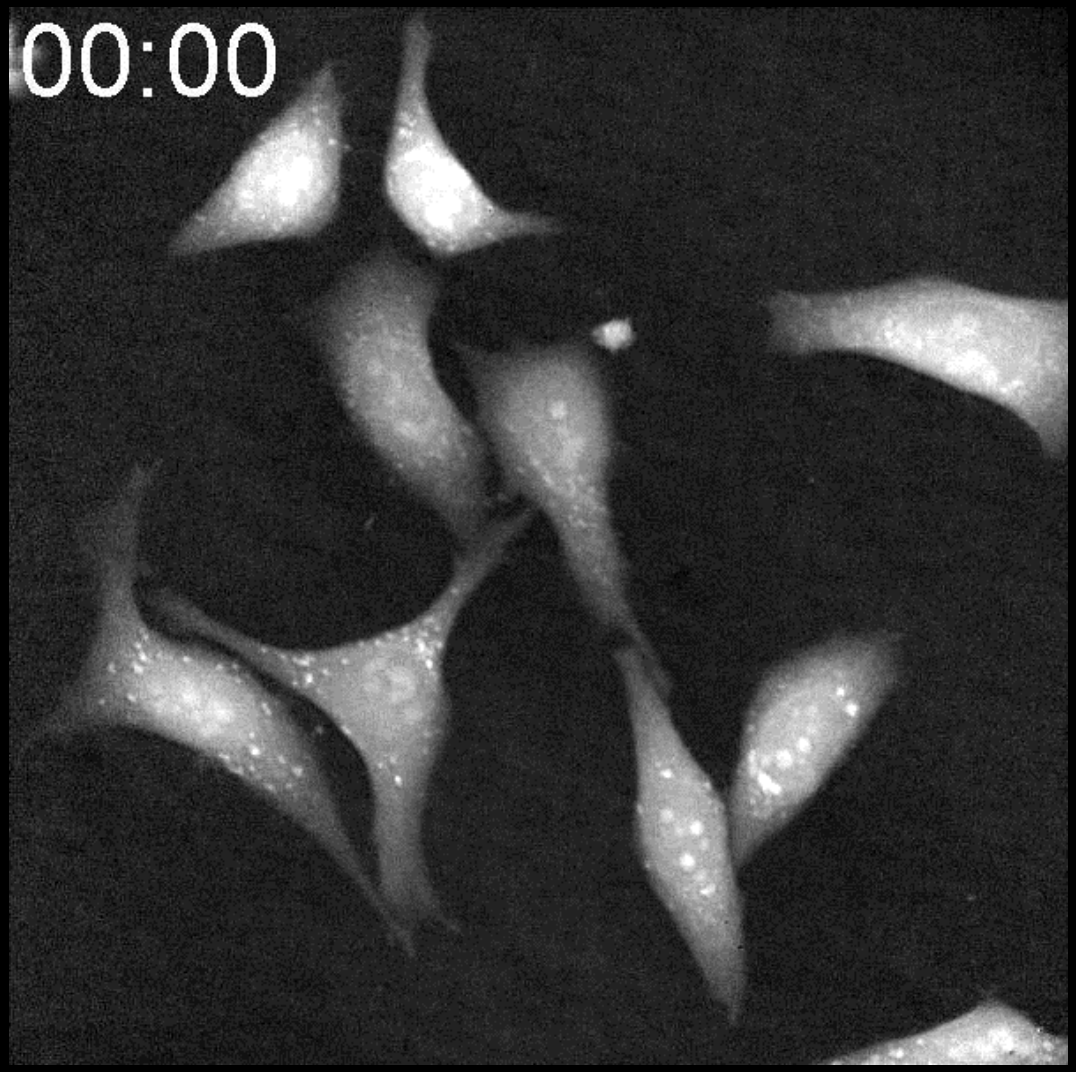


HM



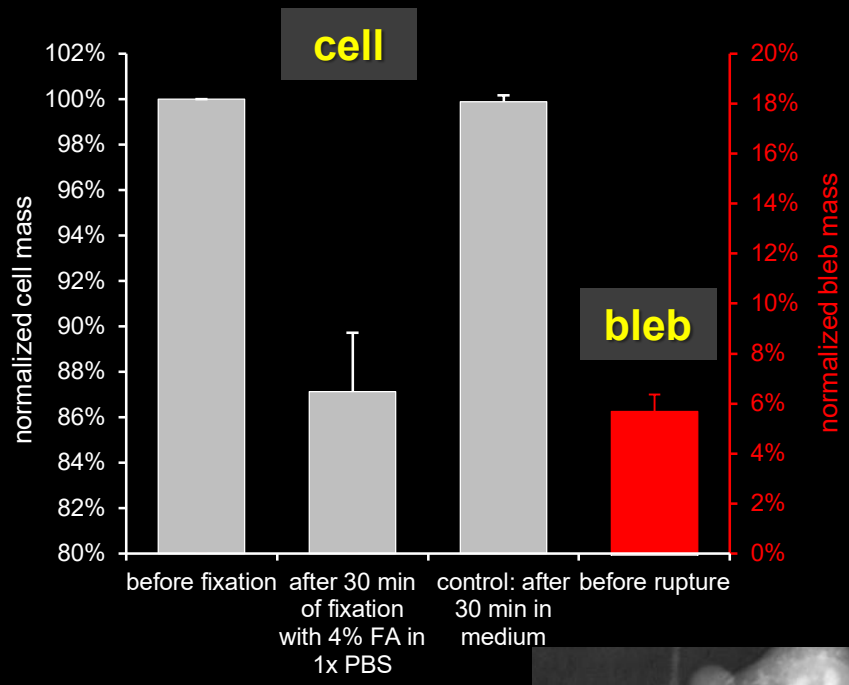

a
OUTSIDE
INSIDE
Phase Contrast Microscopy
b
OUTSIDE
INSIDE
Holography Microscopy
c
Profile - Phase Contrast Microscopy
265
240
215
190
165
140
115
90
Intensity
OUTSIDE
INSIDE
-4 -3 -2 -1 0 1 2 3 4
Distance [µm]
(0 - estimated edge of cell; < 0 - outside the cell; > 0 - inside the cell)
Mean Values
Mean Values ± Standard Deviation
d
Profile - Holography Microcopy
2,5
2
1,5
1
0,5
0
-0,5
Phase Shift
OUTSIDE
INSIDE
-4 -3 -2 -1 0 1 2 3 4
Distance [µm]
(0 - estimated edge of cell; < 0 - outside the cell; > 0 - inside the cell)
Mean Values
Mean Values ± Standard Deviation

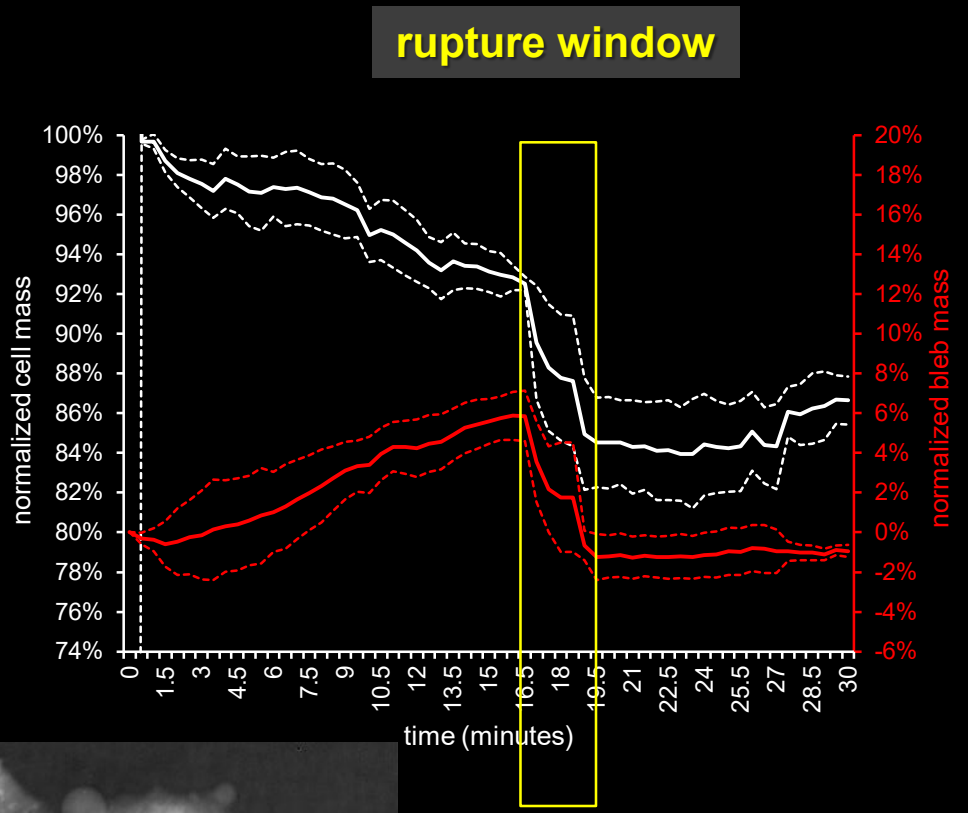
HeLa cells - 30 min – 4% FA/PBS

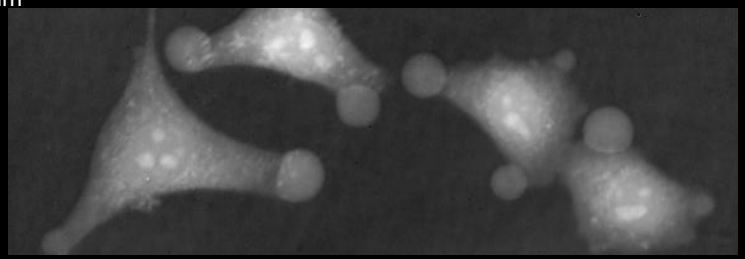


Holographic microscopy documents fixation-induced blebbing

Fixation-induced blebbing results in the loss of cellular material



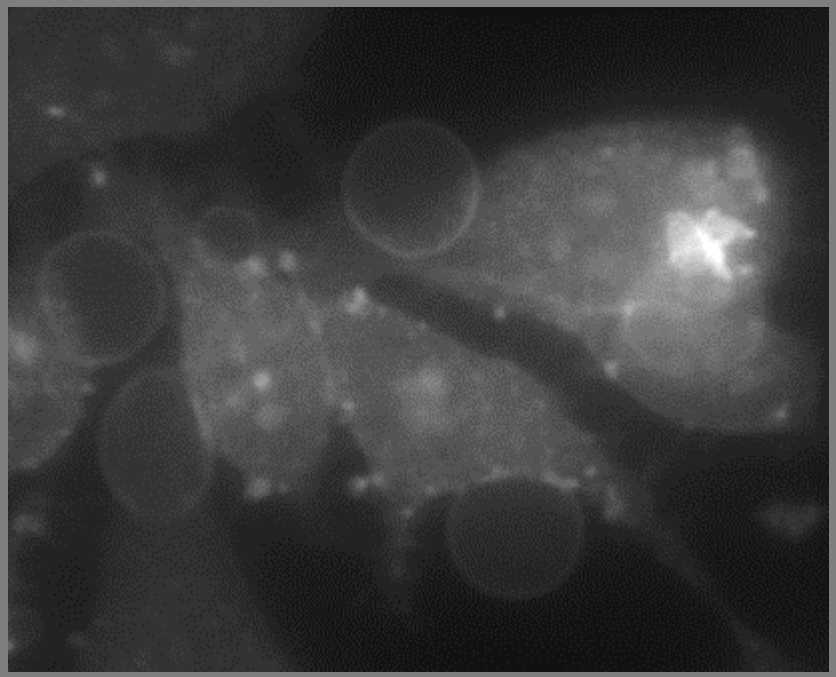




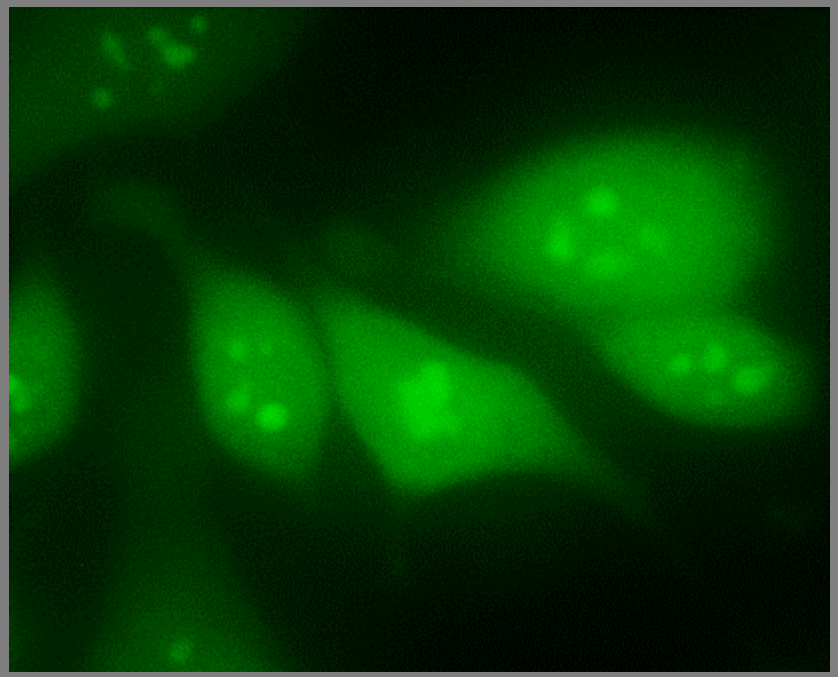
holographic microscopy

What material is lost due to the rapture of the fixation-induced blebs?

Membrane (Cell Mask)



RNA (SYTO 14)

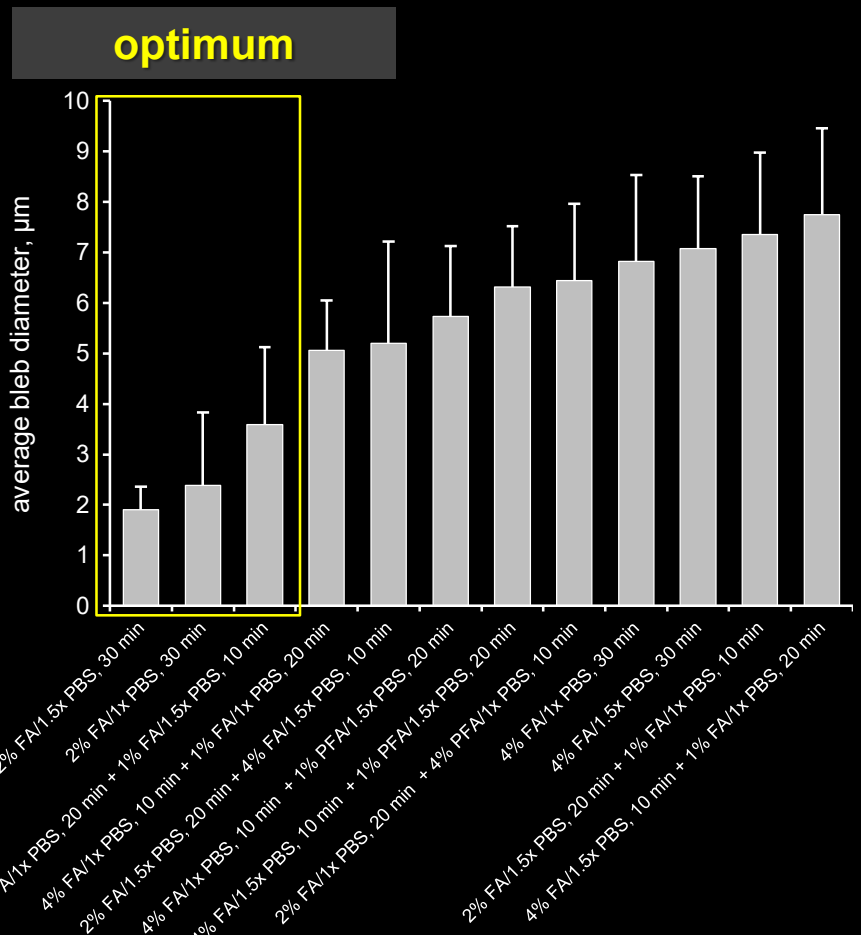


1x CellMask™Plasma Membrane stain/PBS + 5µM SYTO 14 Green RNA stain/PBS

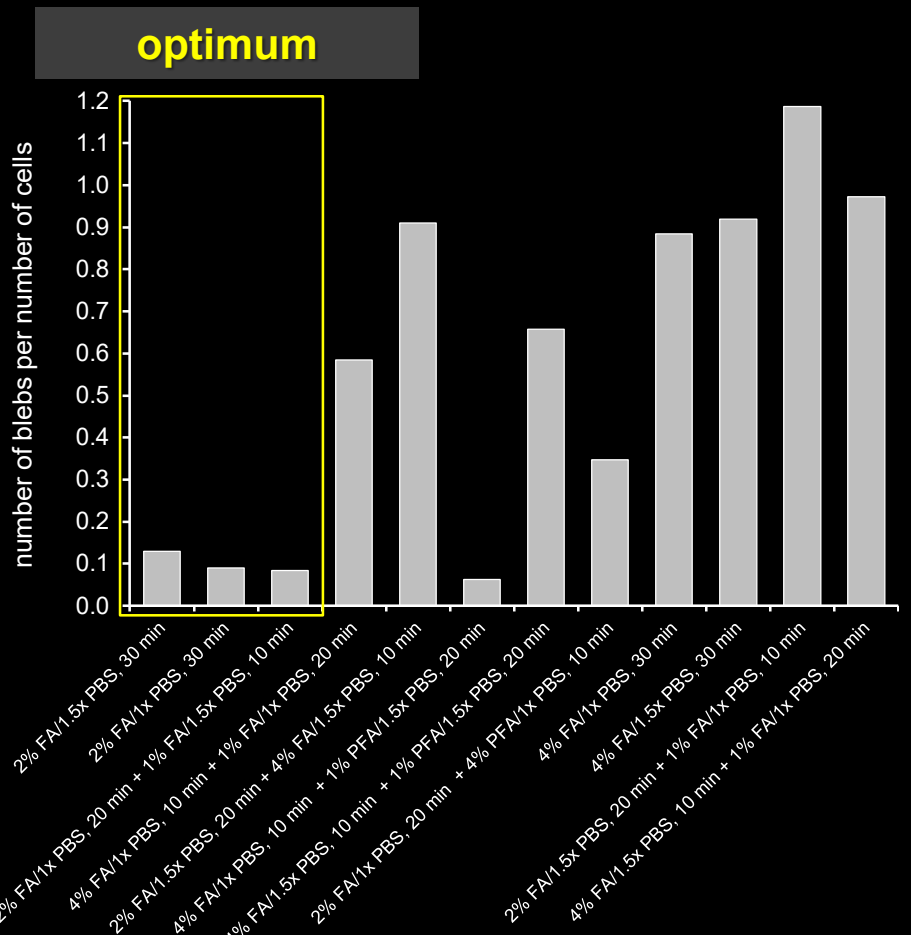
RNA is not significantly present in the induced blebs.

Minimal loss of cellular material is achieved by optimal combining the fixative concentration + diluent + timing

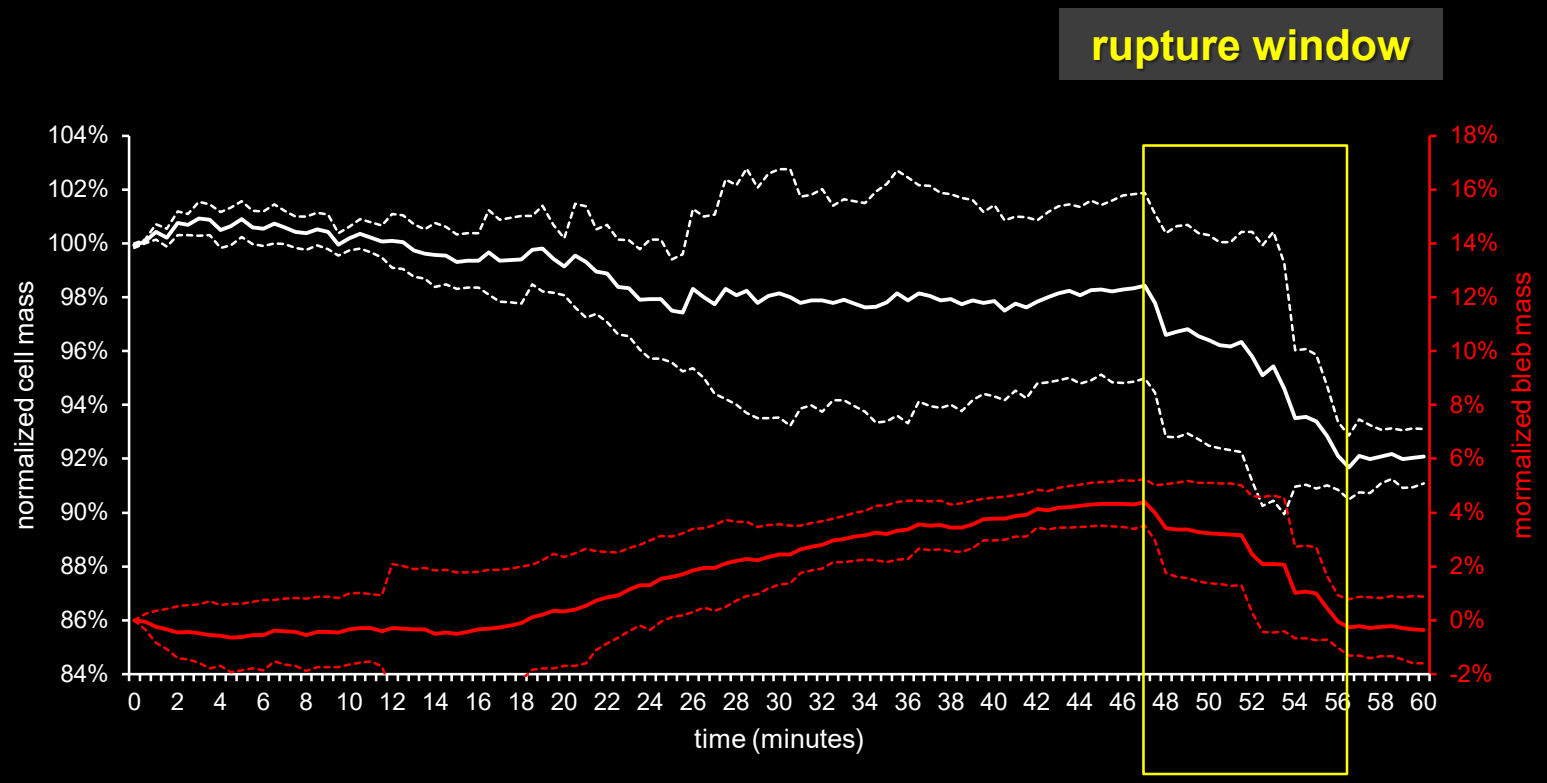
Size of blebs



Number of blebs



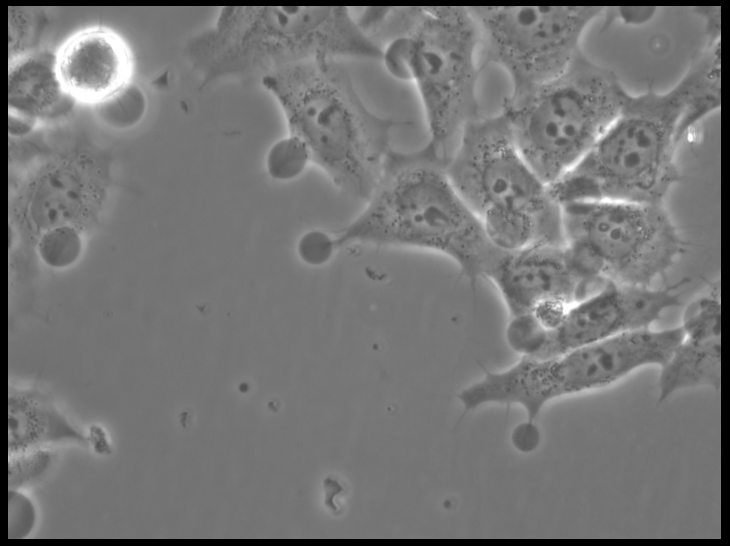
The negative effect of blebbing is reduced by a decrease in fixative concentration from 4% FA to 2% FA



holographic microscopy

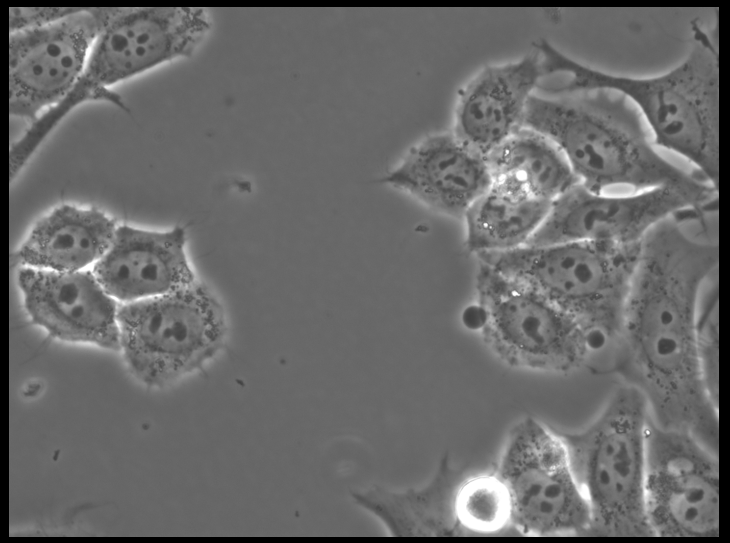
Negative effect of blebbing is reduced by a decrease in the fixative concentration from 4% FA to 2% FA

4% FA/1x PBS



20 min of fixation

2% FA/1x PBS

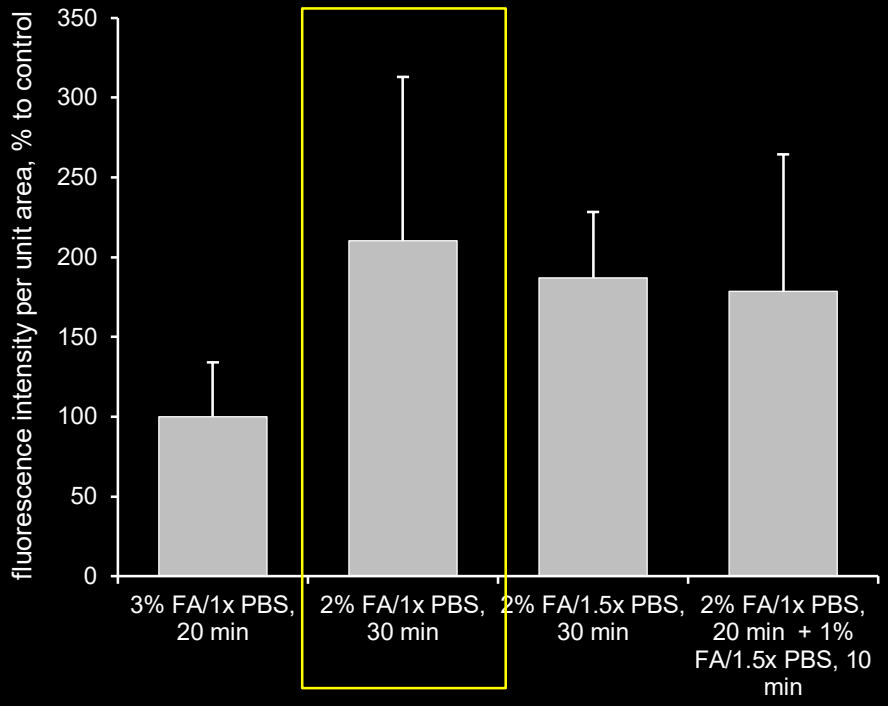


20 min of fixation

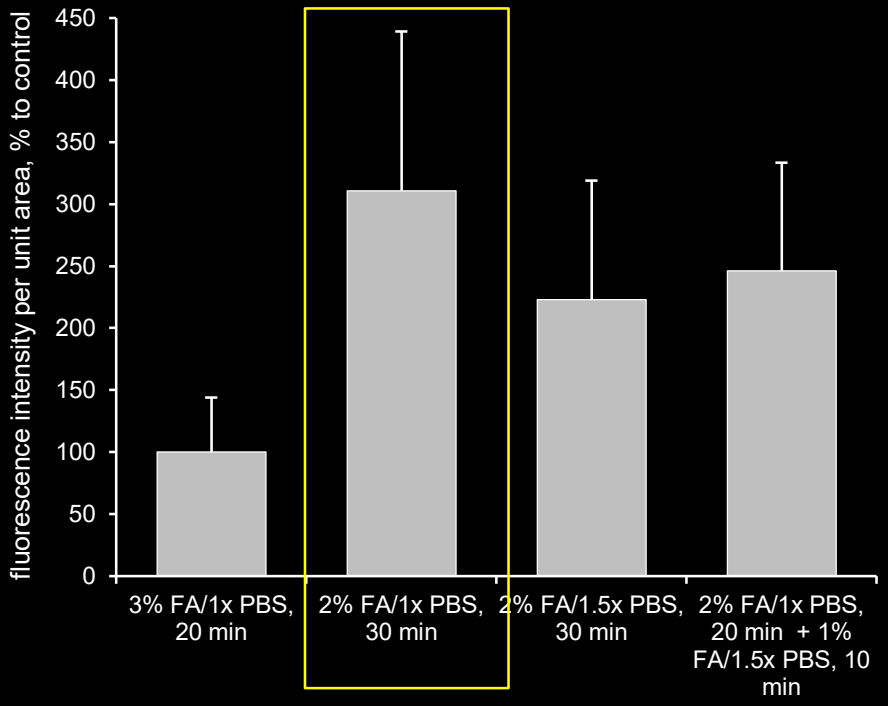
phase-contrast microscopy

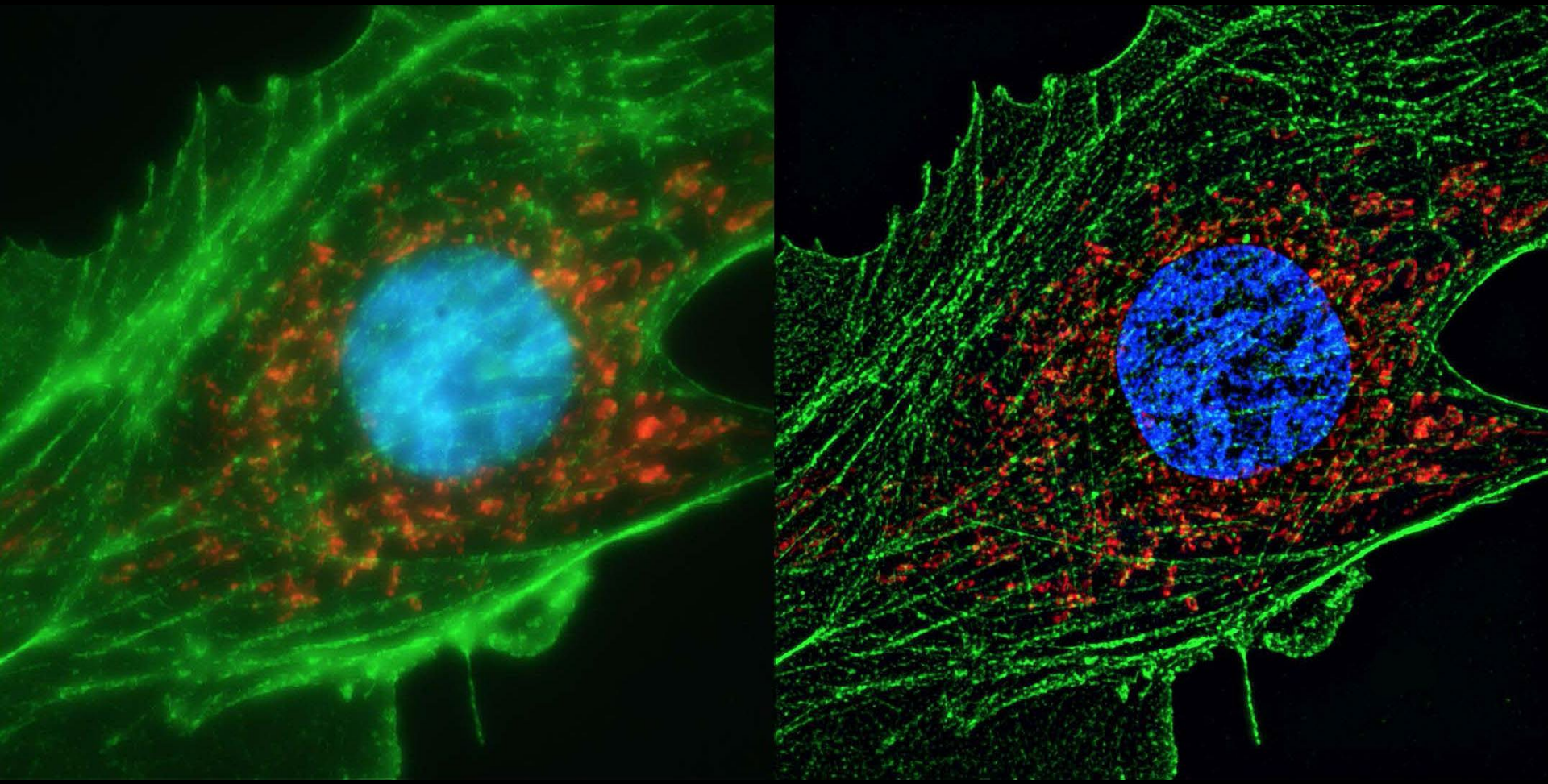
Maximal fluorescence (minimal loss) achieved by optimalization the fixative and timing

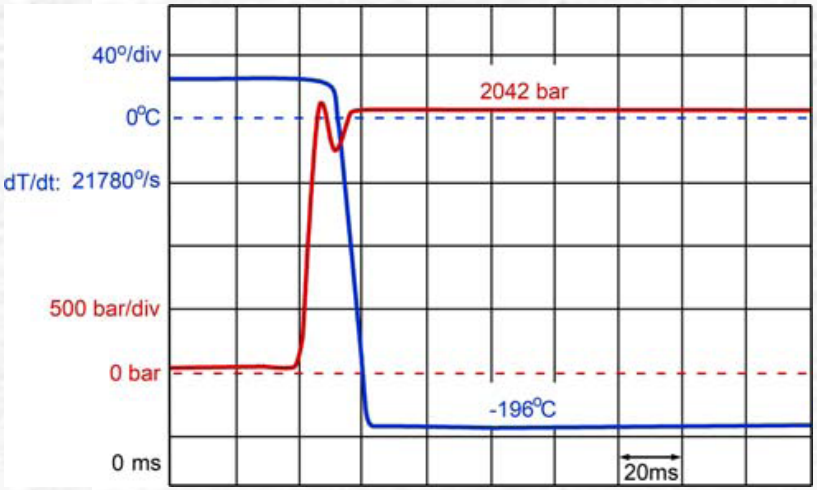
p190RhoGAP



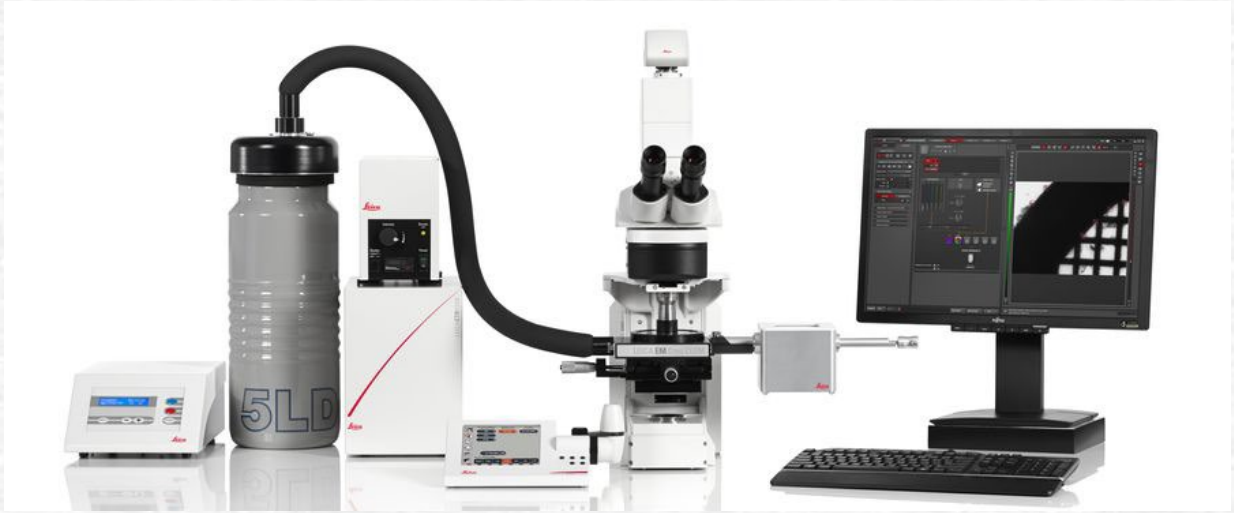
Akt1







cryofixation (HPF)
+ cold-stage microscopy?

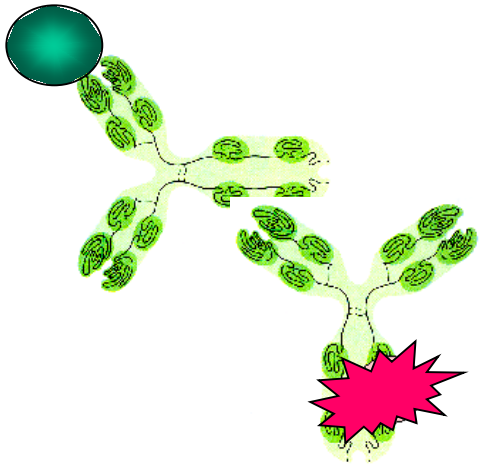


IMMUNOLABELLING

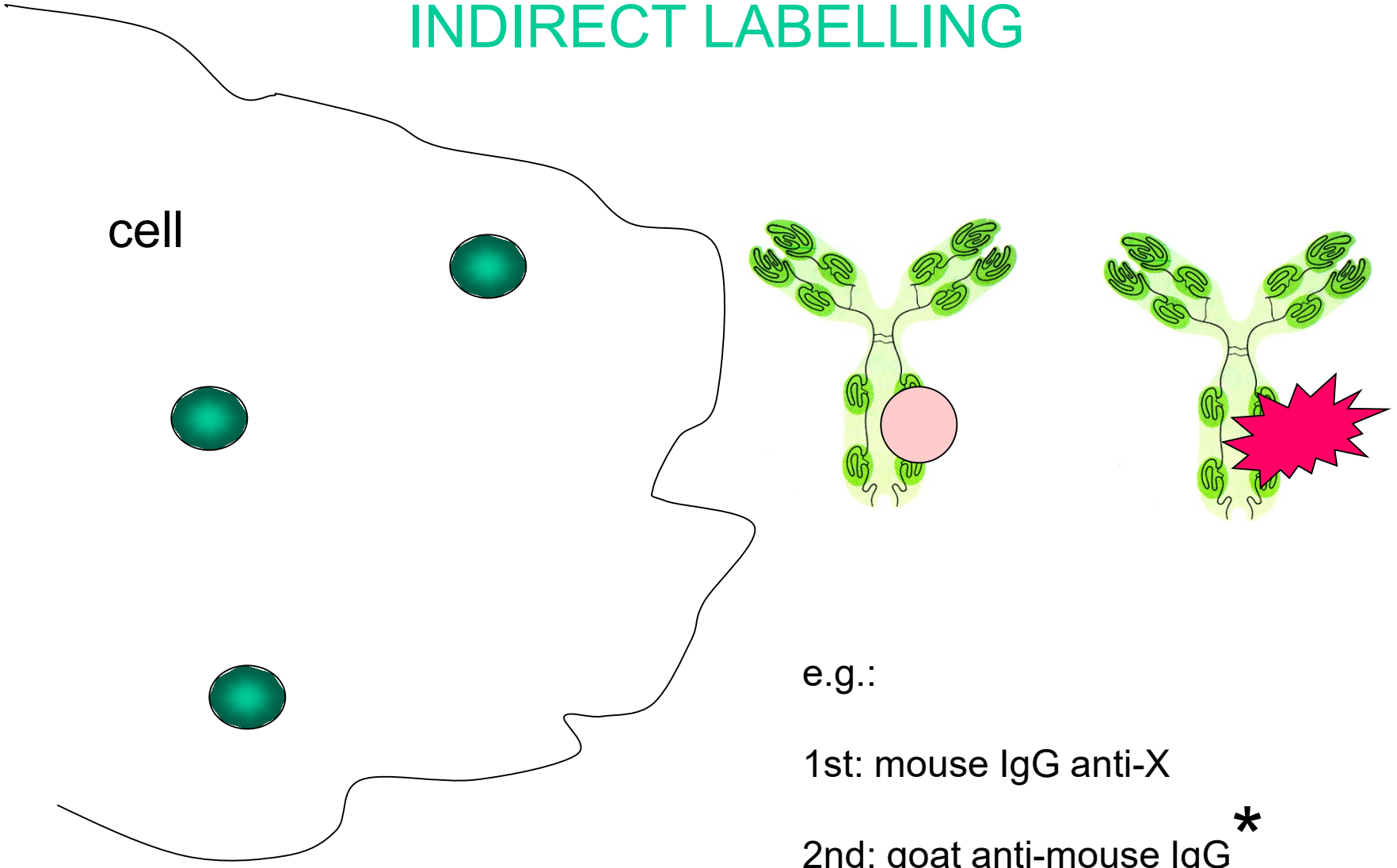
IMMUNOFLUORESCENCE MICROSCOPY

Basic terms:

- antigen
- monoclonal antibody
- polyclonal antibody
- primary antibody
- secondary antibody



INDIRECT LABELLING

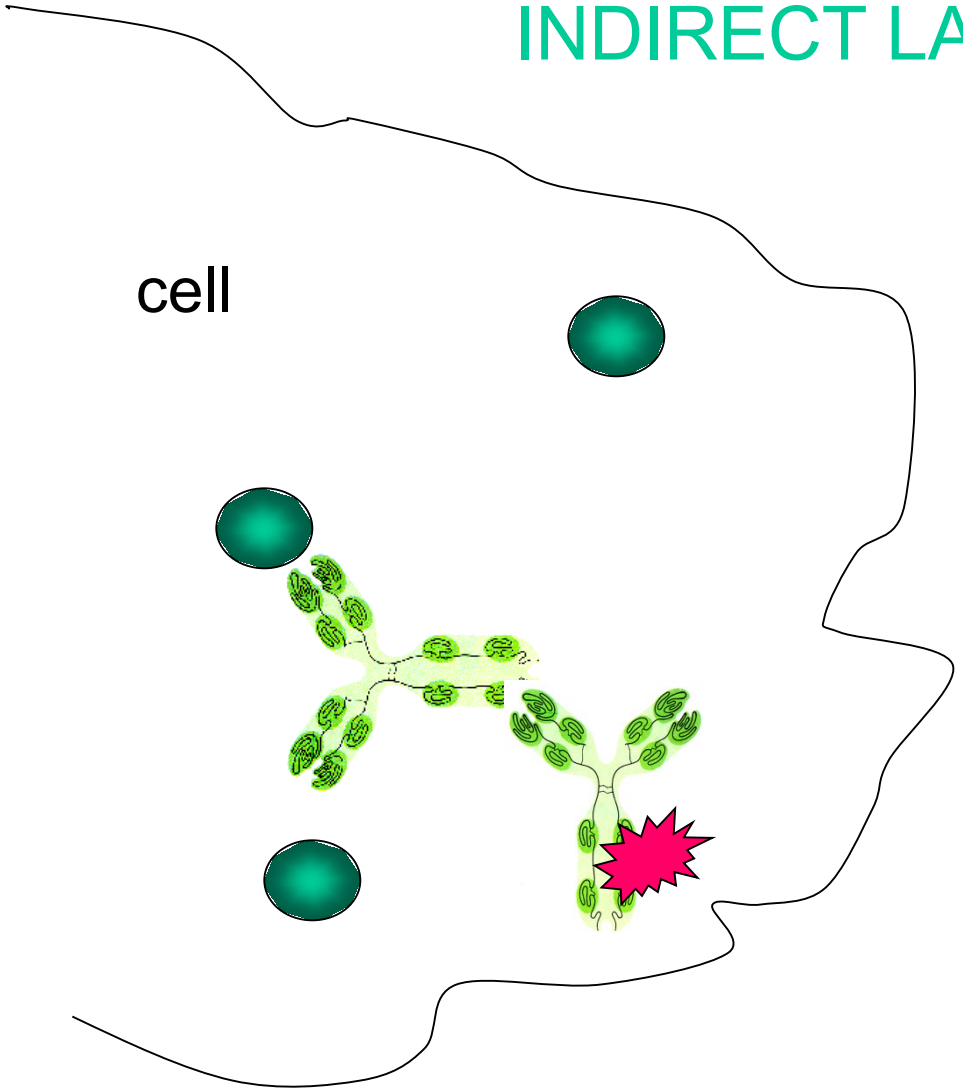


e.g.:

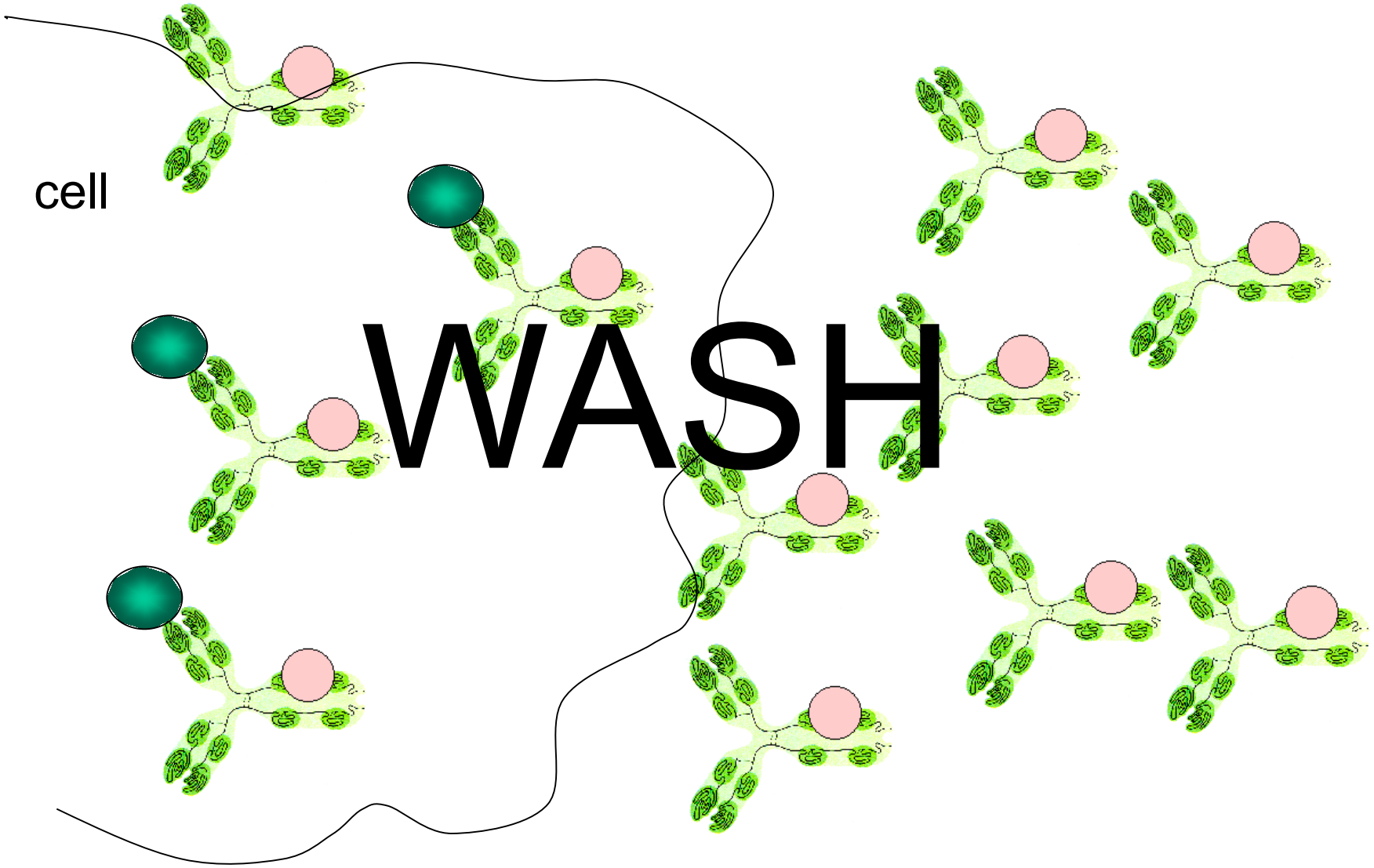
1st: mouse IgG anti-X

2nd: goat anti-mouse IgG*

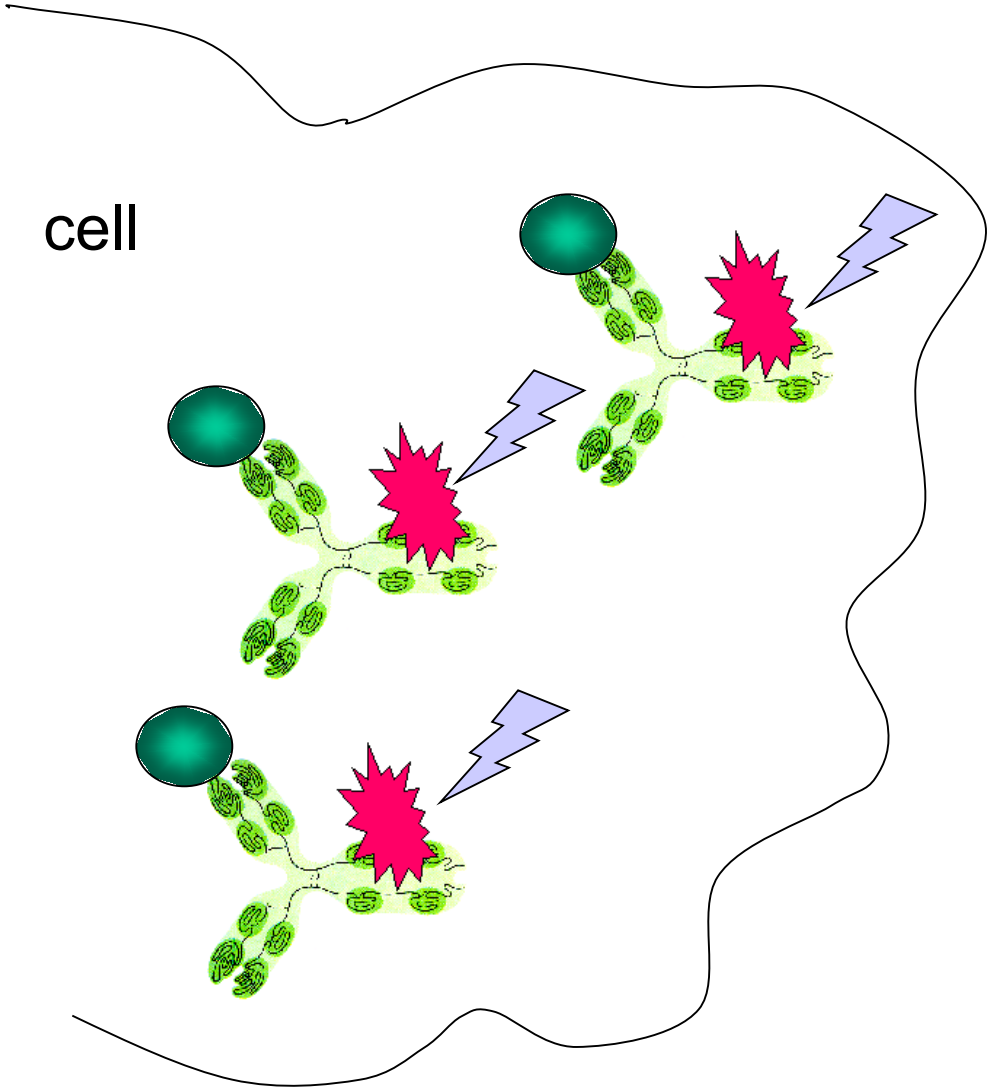
INDIRECT LABELLING



DIRECT LABELLING



DIRECT LABELLING



IMMUNOFLUORESCENT DETECTION

- fixation
- blocking with sera
- incubation with primary antibody
- wash (detergent?)
- incubation with *secondary antibody
- wash (detergent?)
- mounting

Sensitivity x Selectivity

- antibody concentration
- duration of incubation
- temperature

Controls

- autofluorescence
- primary antibody non-specific binding
- secondary antibody -“-
- cross-reactivity in multiple labellings
- optical channels

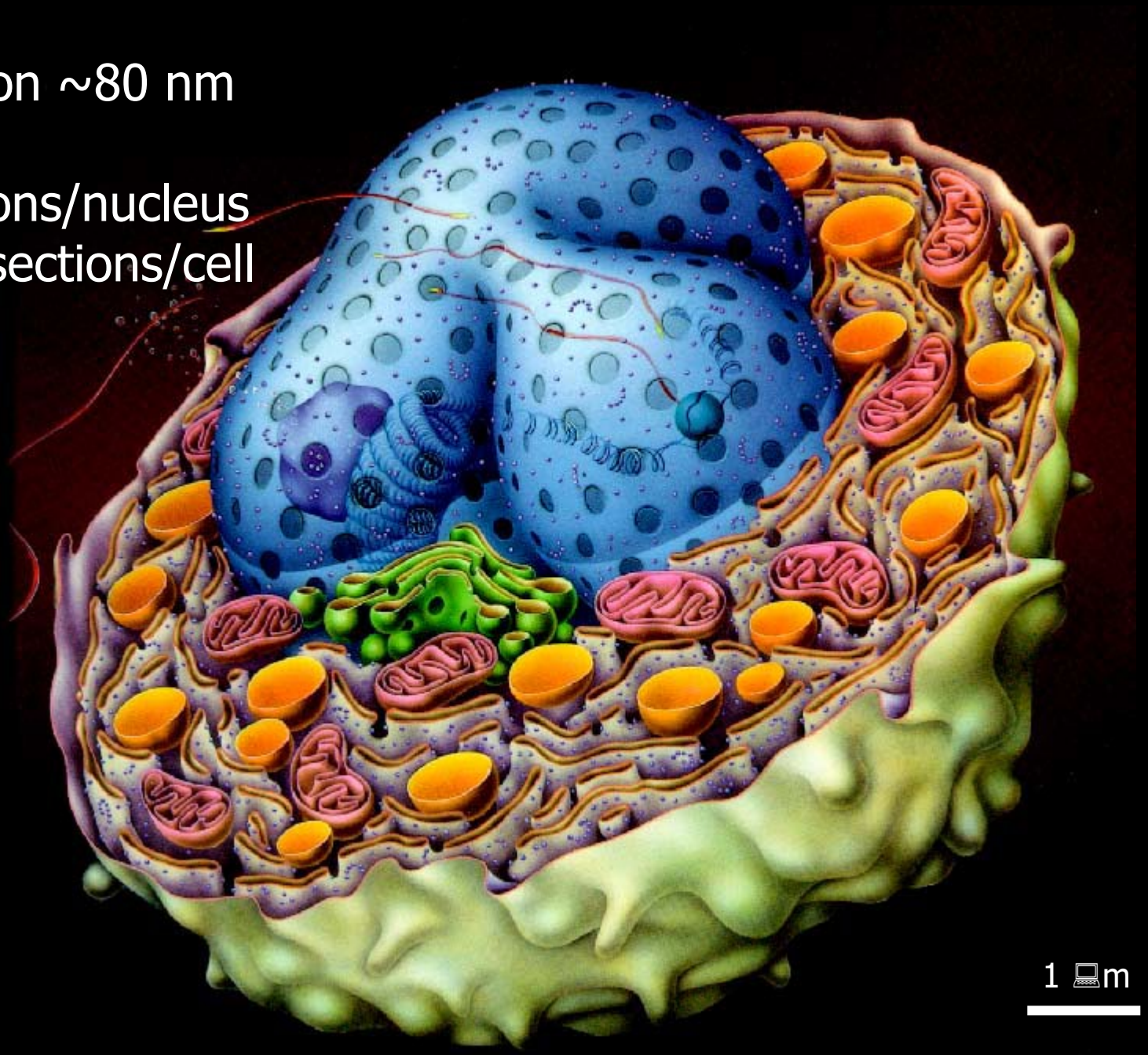
SUMMARY: CORRECT CONDITIONS OF IMMUNOFLUORESCENT DETECTION

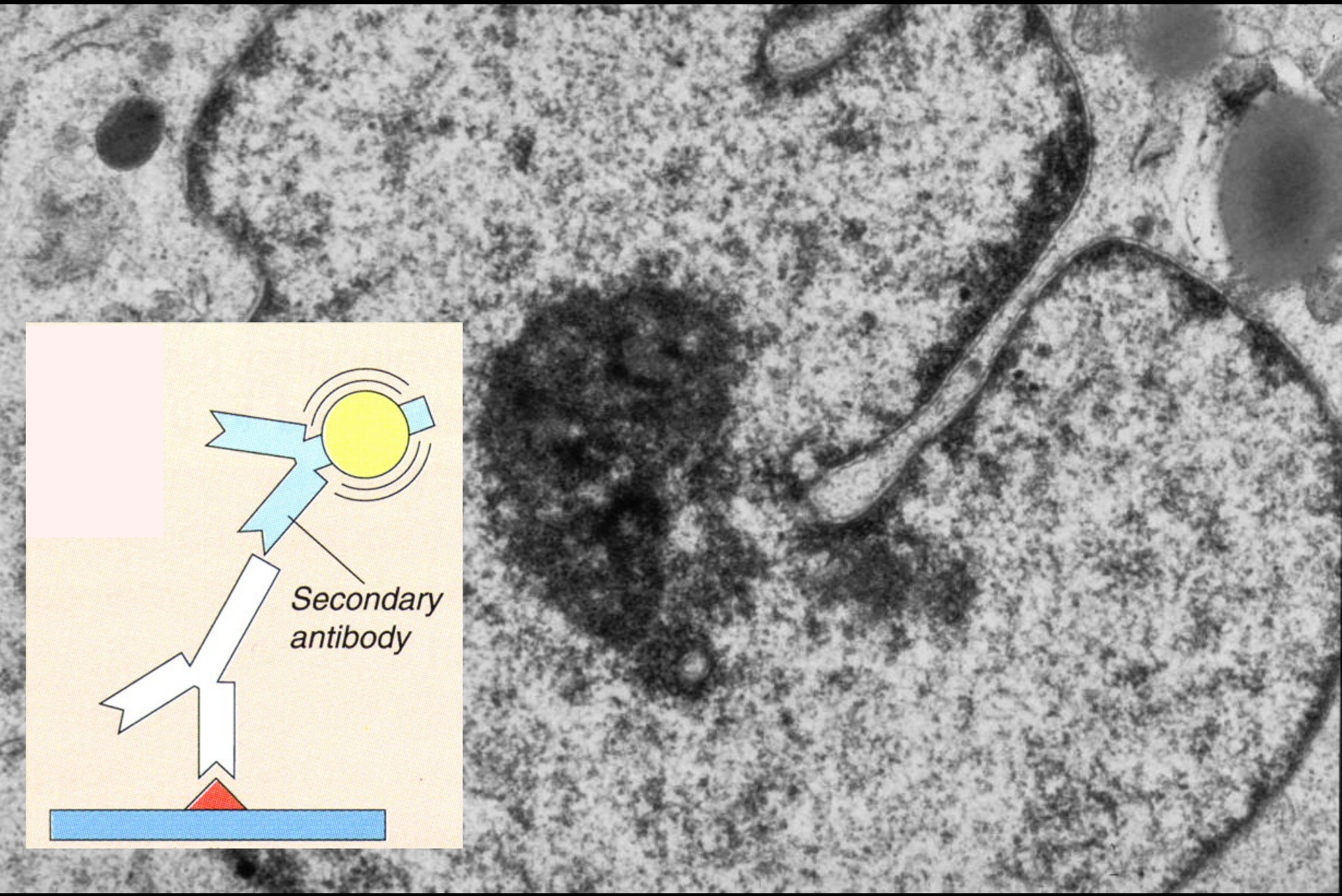
- thorough blocking
- correct selection of antibodies (isotype, non-crossreacting)
- correct concentration of antibodies (concentration x sensitivity)
- correct selection of fluorochromes and optical system
- proper control incubations
- proper embedding media

Immunogold electron microscopy

thin section ~80 nm

125 sections/nucleus
250-400 sections/cell




Secondary
antibody

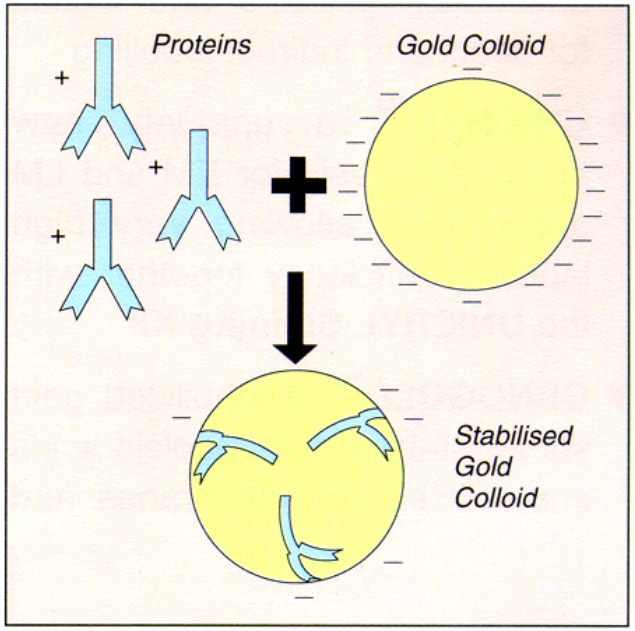
Conjugation

The conjugation of selected proteins to gold particles depends upon at least three physical phenomena:

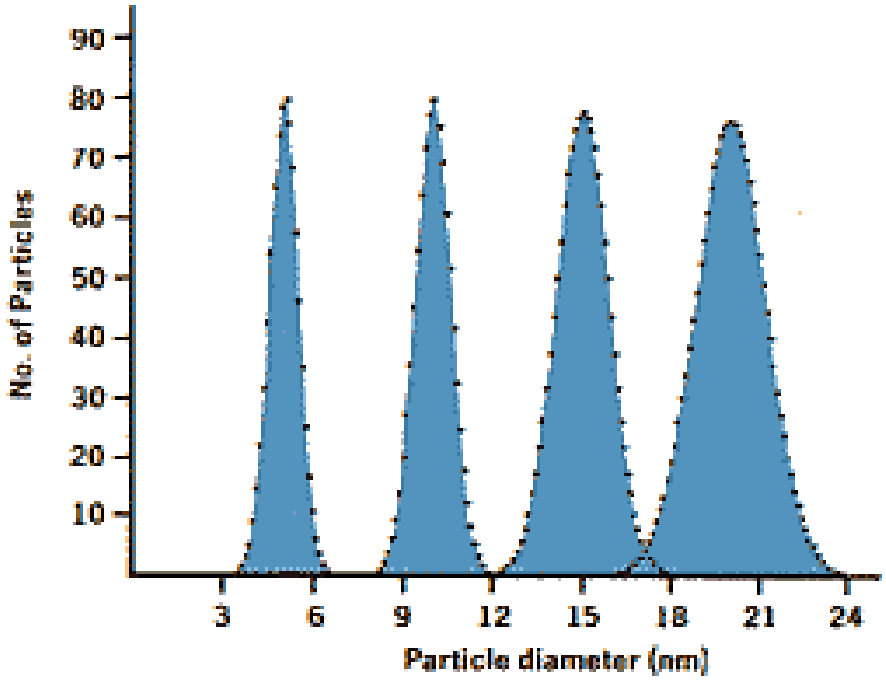
a) Charge attraction of the negative gold particle to positively charged protein

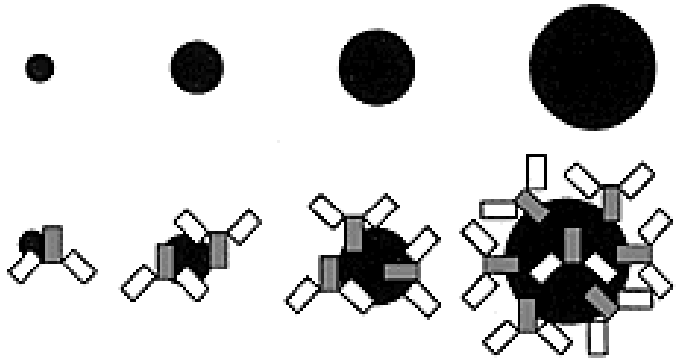
b) Hydrophobic adsorption of the protein to the gold particle surface

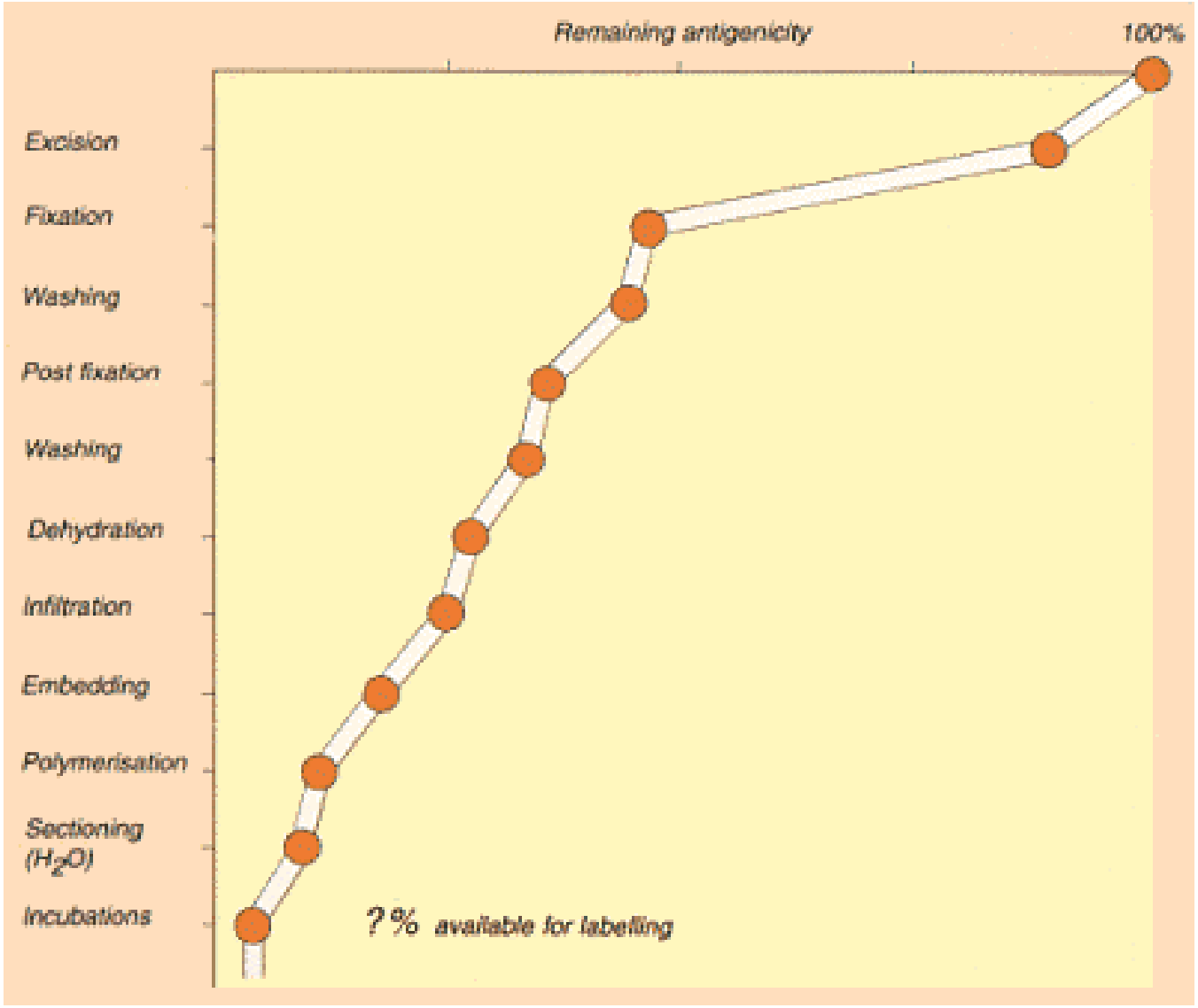
c) Binding of the gold to sulphur (dative binding) where this may exist within the structure of the macromolecule.

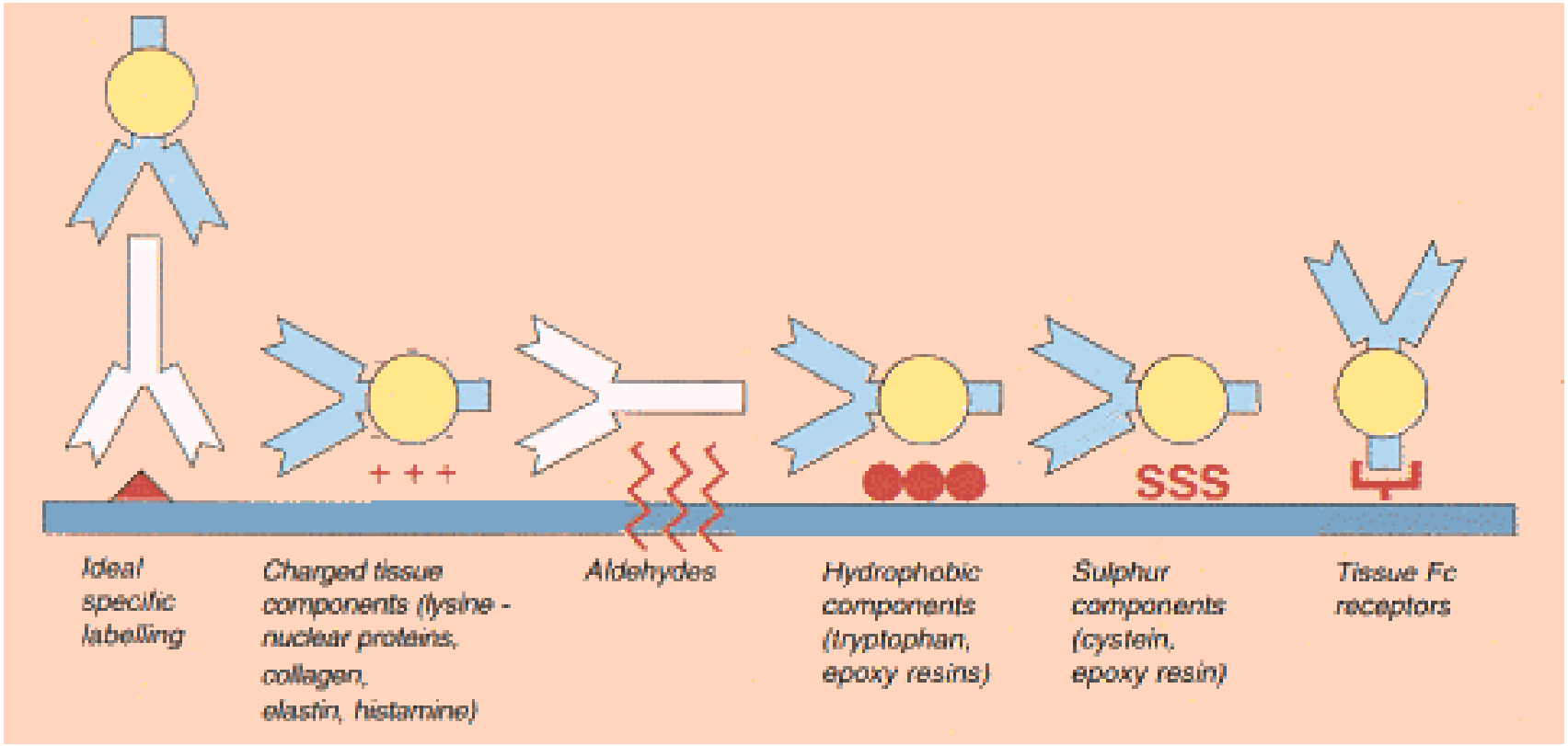


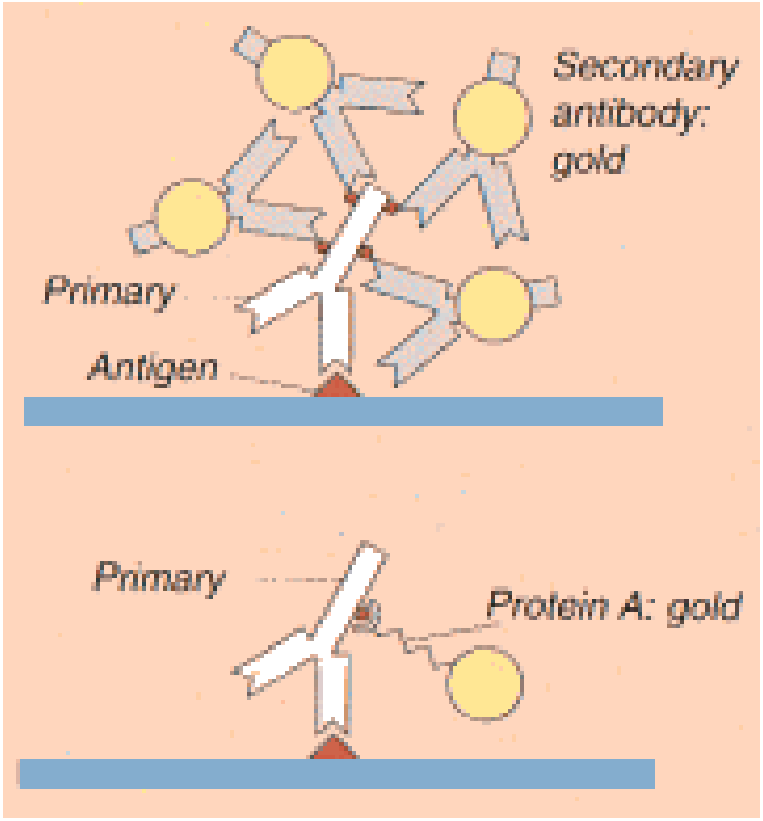
Adsorption of proteins to gold colloid

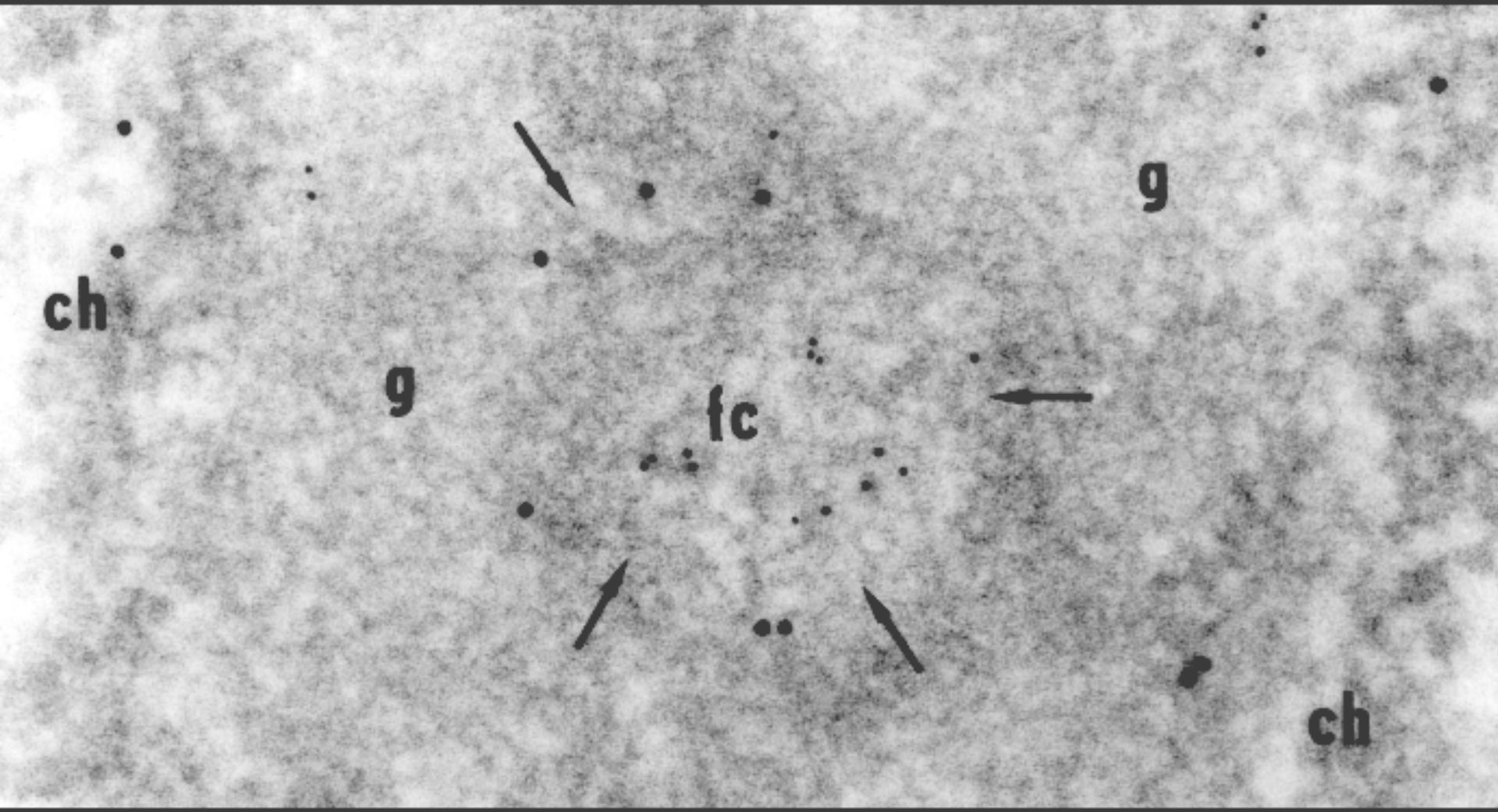

No. of Particles
90
80
70
60
50
40
30
20
10
3
6
9
12
15
18
21
24
Particle diameter (nm)




Remaining antigenicity
100%
Excision
Fixation
Washing
Post fixation
Washing
Dehydration
Infiltration
Embedding
Polymerisation
Sectioning
(H_2O)
Incubations
? % available for labelling


+ + +
SSS
Ideal specific labelling
Charged tissue components (lysine - nuclear proteins, collagen, elastin, histamine)
Aldehydes
Hydrophobic components (tryptophan, epoxy resins)
Sulphur components (cystein, epoxy resin)
Tissue Fc receptors

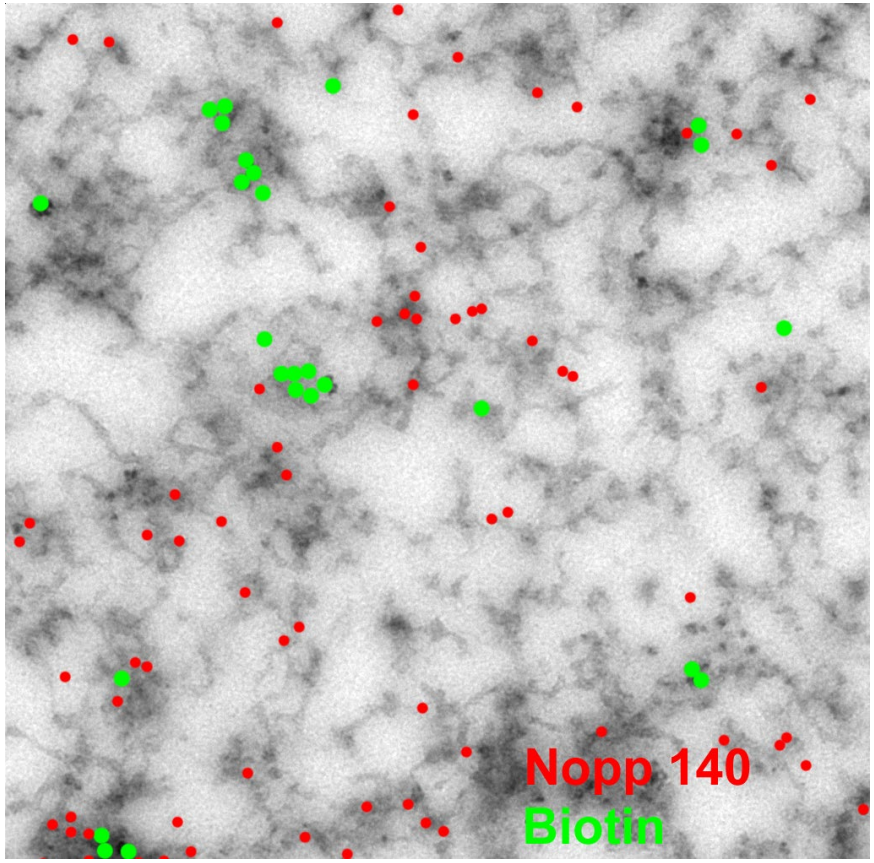

Secondary antibody: gold
Primary
Antigen
Primary
Protein A: gold

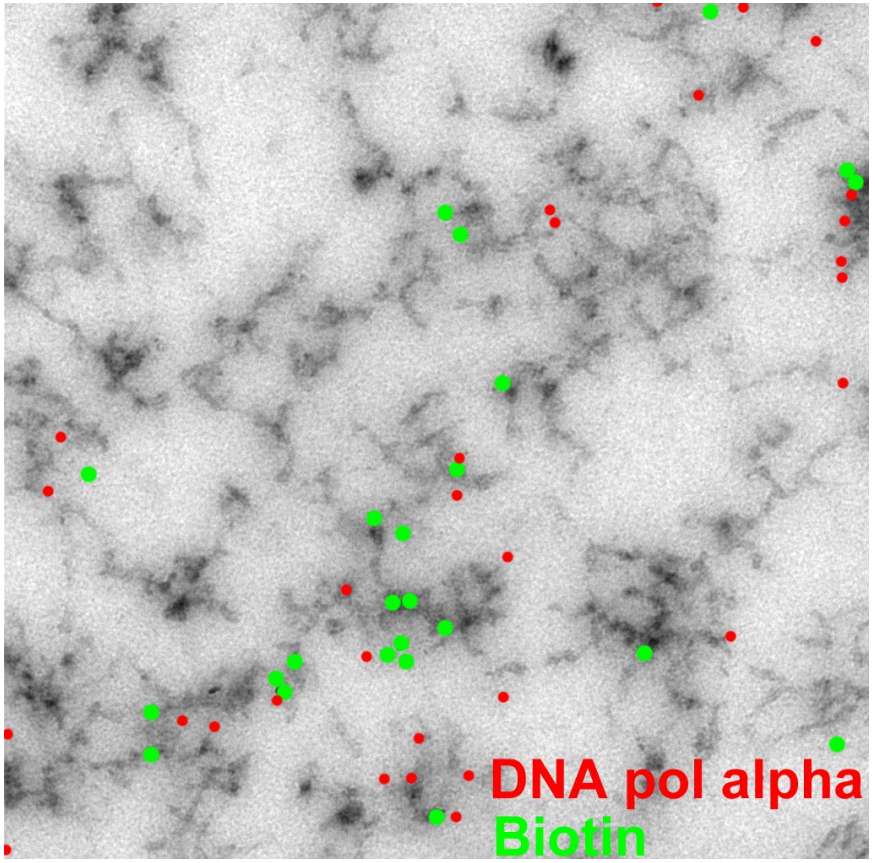


3T3 cell nucleolus

5 nm actin
10 nm myosin I

Is there a difference?





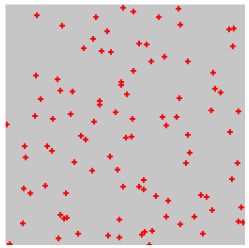
Philimonenko et al., Statistical evaluation of colocalization patterns in immunogold labeling experiments. Struct Biol. 2000, 132:201-210

Schofer et al., Mapping of cellular compartments based on ultrastructural immunogold labeling. J Struct Biol. 2004, 147:128-135.

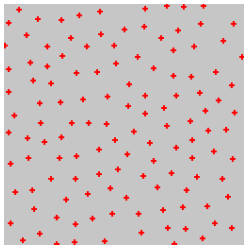
- clustering
- colocalization
- mapping

Main types of spatial point patterns with corresponding pair correlation functions.

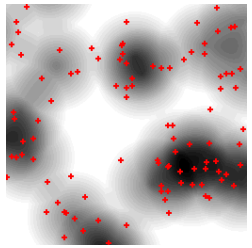
single labeling:



random

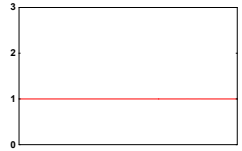


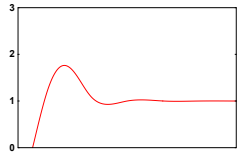
regular

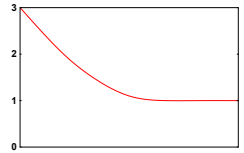


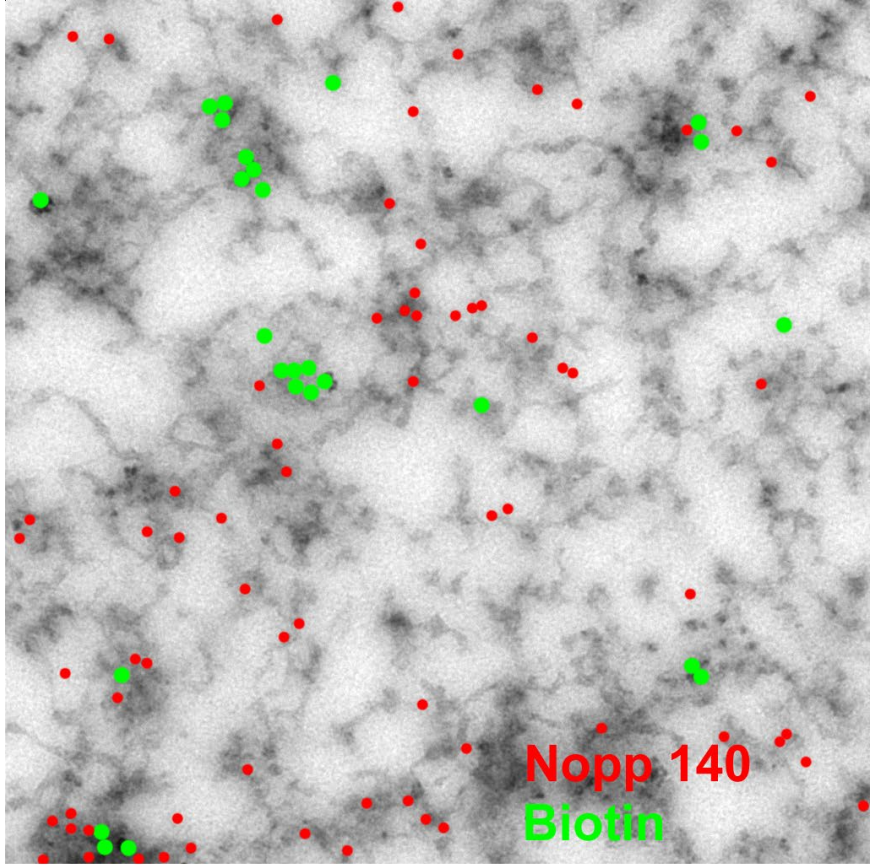
clustering

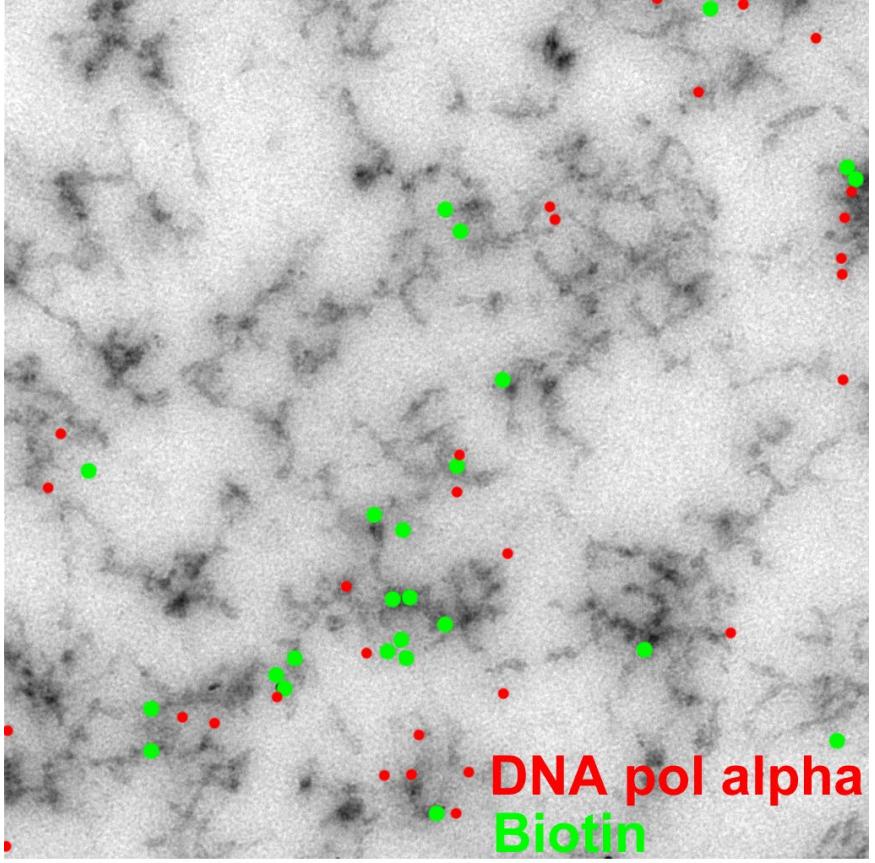
pair correlation function:

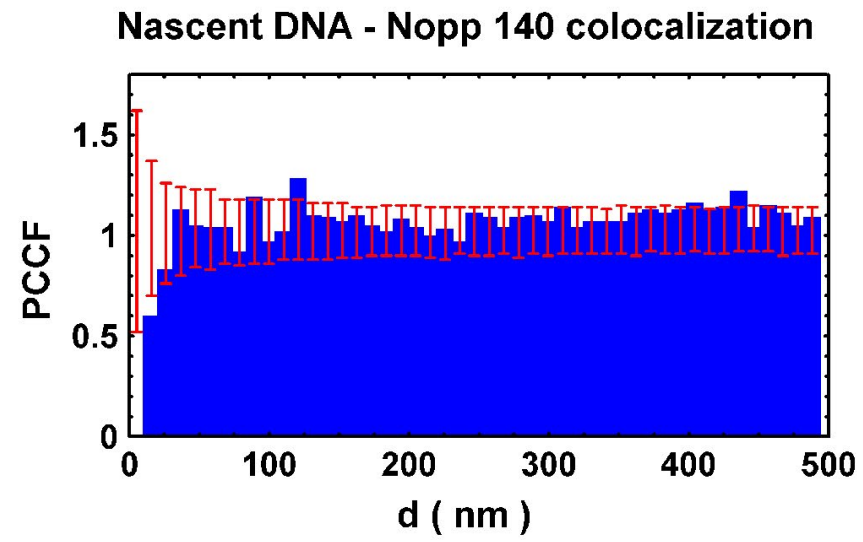


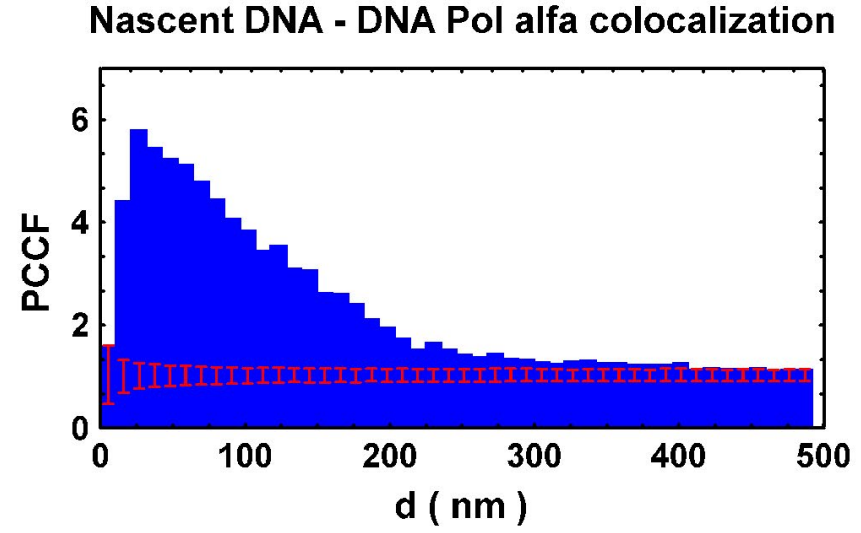





Nopp 140
Biotin


DNA pol alpha
Biotin


Nascent DNA - Nopp 140 colocalization
PCCF
1.5
1
0.5
0
0
100
200
300
400
500
d (nm)


Nascent DNA - DNA Pol alfa colocalization
PCCF
6
4
2
0
0
100
200
300
400
500
d (nm)

Minimalistic probes available?

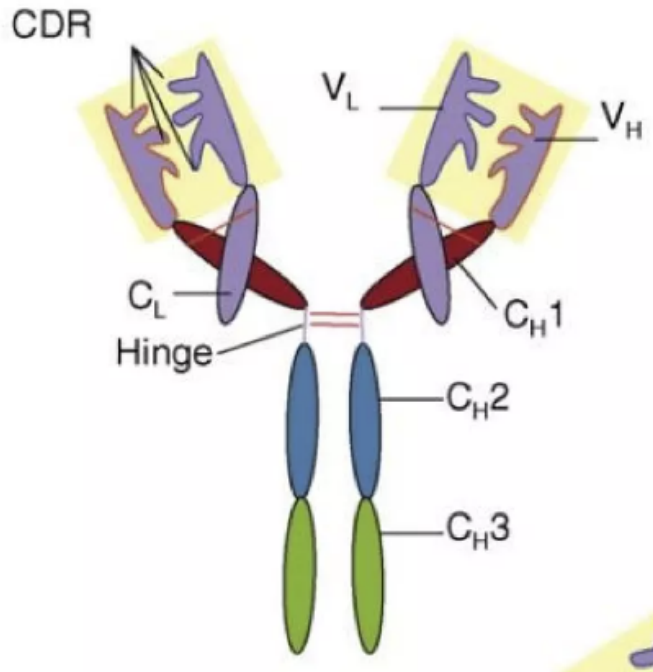
Minimalistic probes available?

IgG < IgM

multistep labelling multiplies size

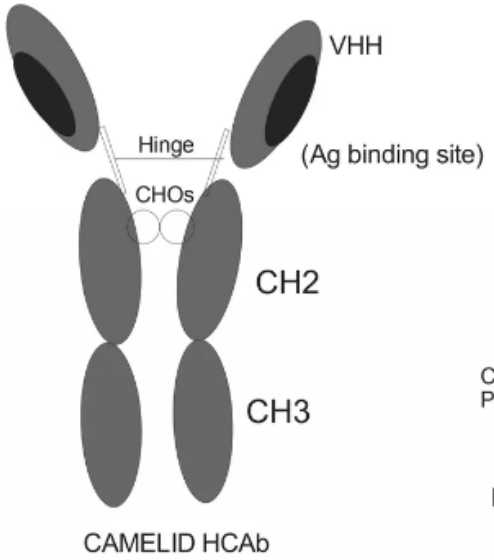
nanobodies

Conventional antibody and their engineering



IgG
150 kDa

scFv
30 kDa

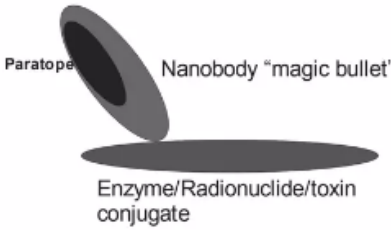


CAMELID HCAb

DOMAIN ANTIBODY
(NANOBODY)

HUMANIZED NANOBODY

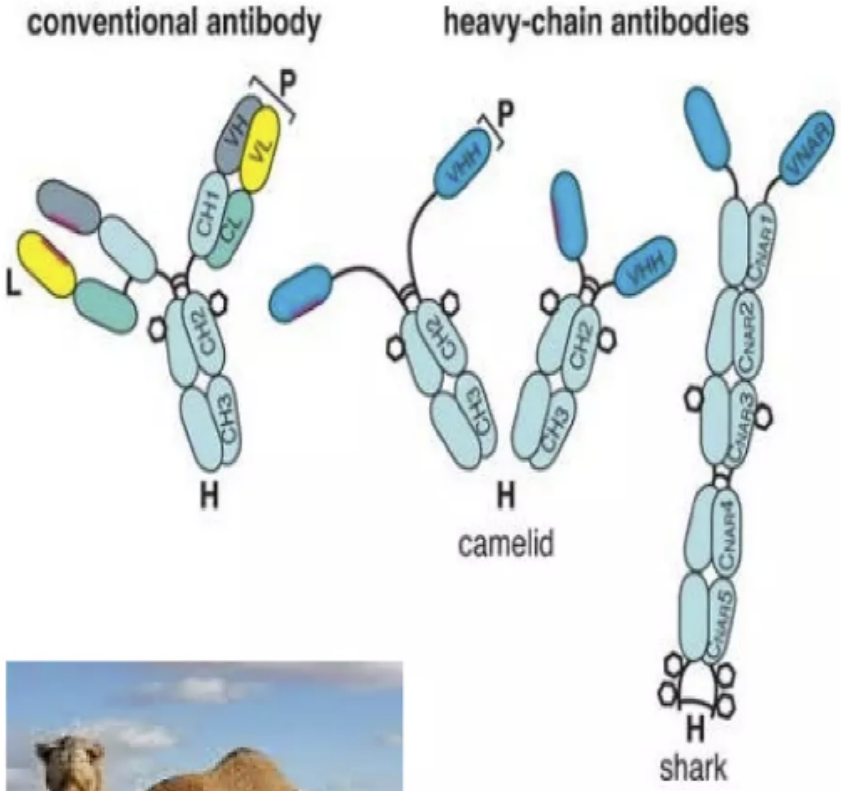
BISPECIFIC NANOBODY



BIFUNCTIONAL NANOBODY

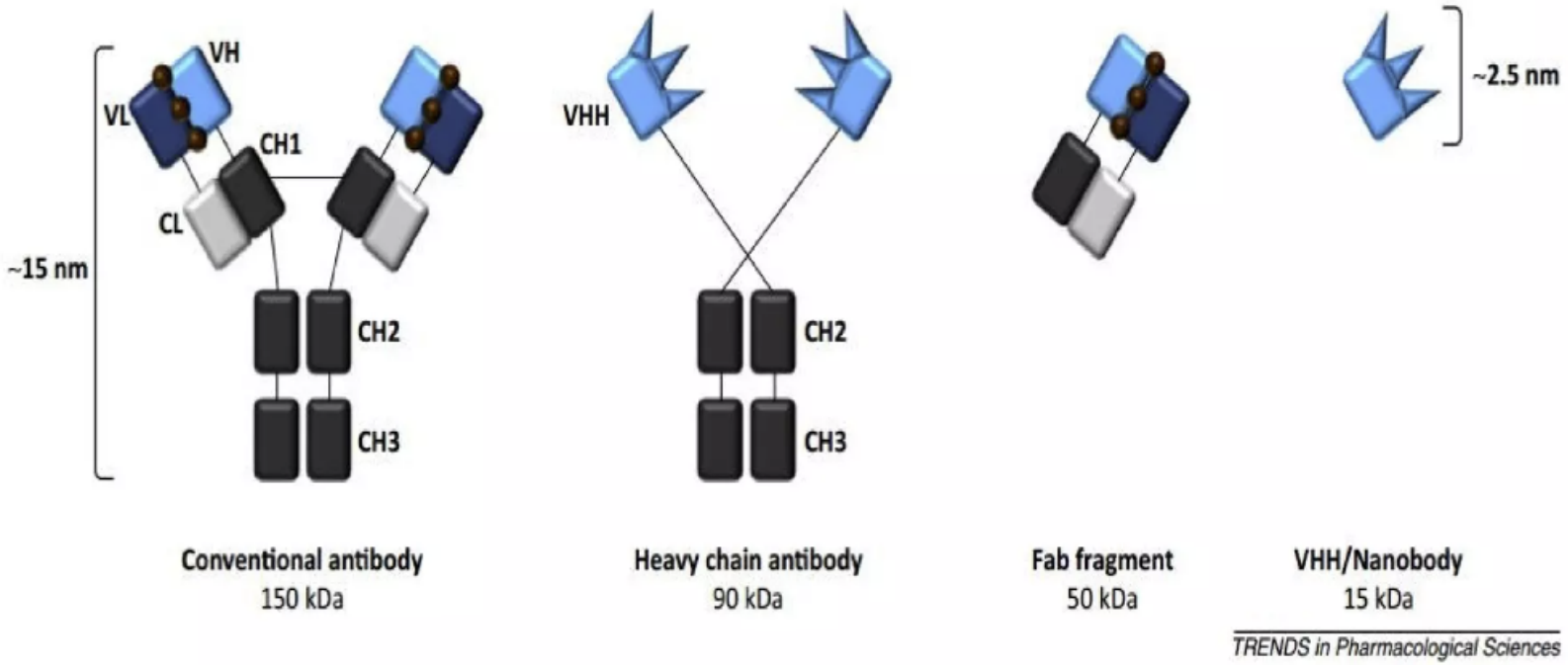
. Camelid heavy chain antibody (HCAb)and nanobody/single domair Abs & their derivatives that can be produced by rDNA techniques

Heavy chain antibodies (hcAbs)



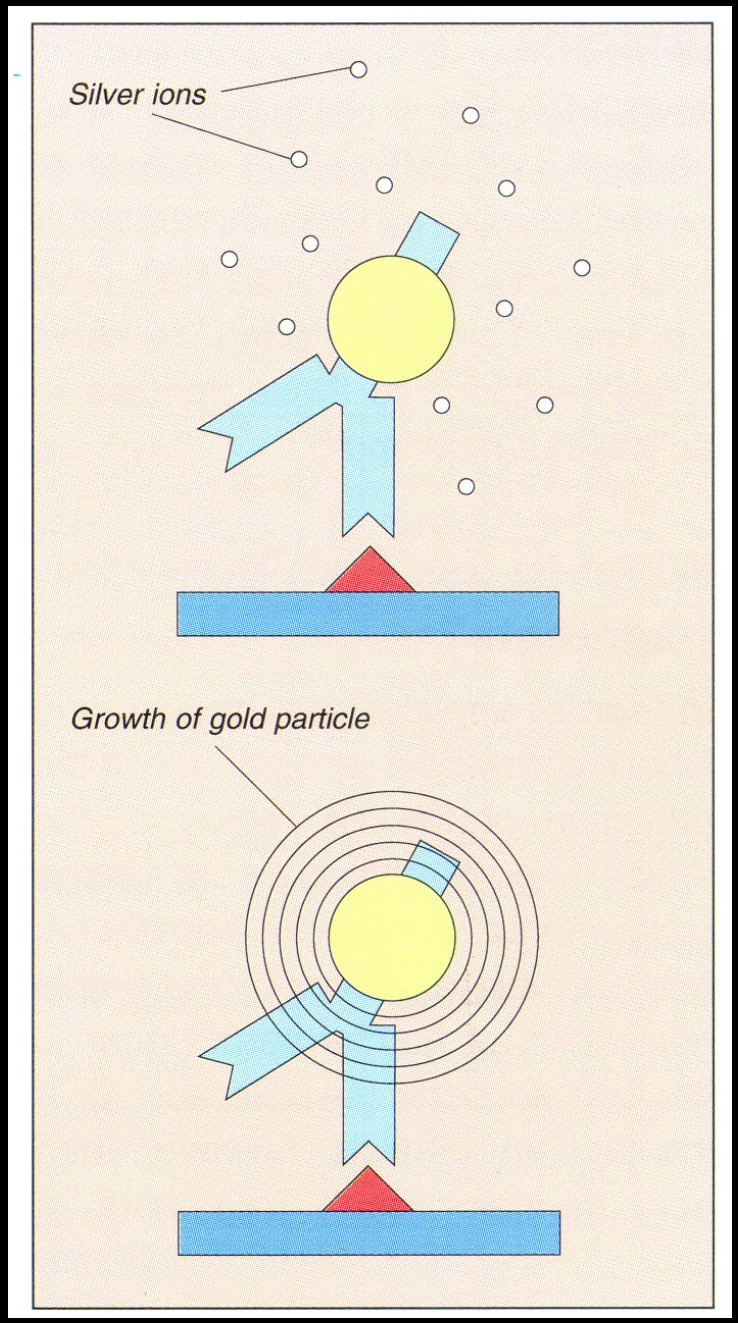
- Camel sera also contain antibodies devoid of light chains and CH1 (Hamers-Casterman et al. 1993)
- 90 KDa
- Also in Camelidae family (e.g., llamas and alpacas)
- Immunoglobulin new antigen receptor, (Ig-NAR) discovered in cartilaginous fish (Greenberg et al.1995)

Nanobodies (Nb)

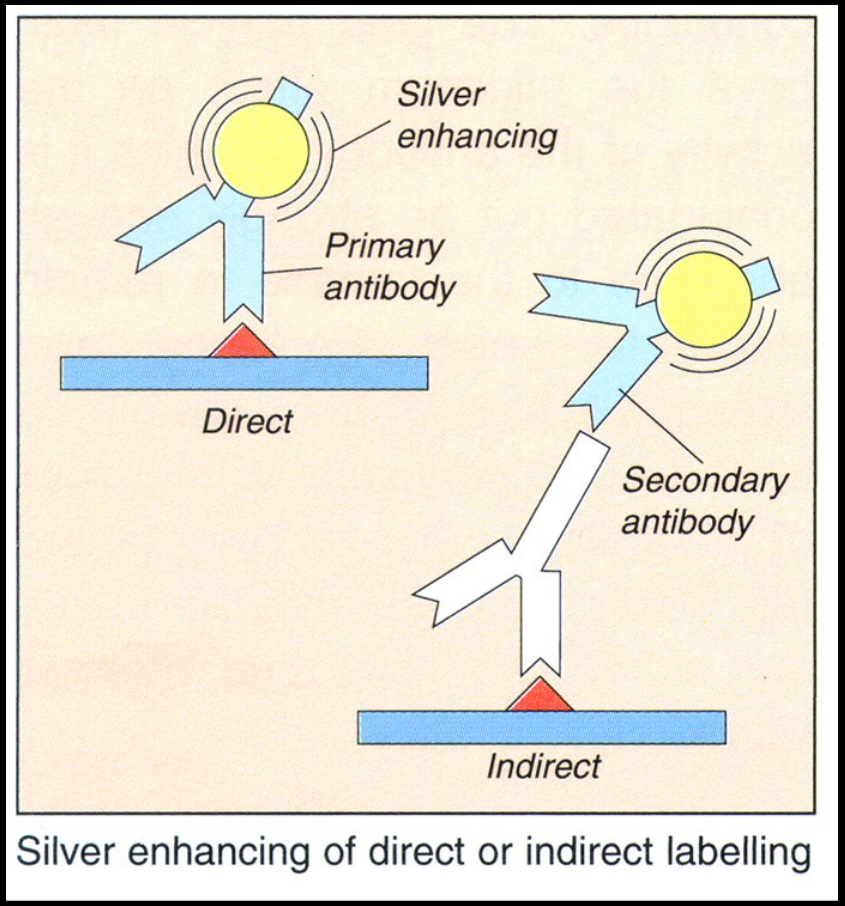


❖Single domain antibodies (SdAb)

❖ The recombinant antigen-specific, single-domain of the Heavy chain of the Heavy-chain antibody (VHH) with dimensions in the nanometer range.



SILVER ENHANCEMENT



Silver enhancing of direct or indirect labelling

Control question:

3 samples: which will get the best fluorescent yield:

A: 4% formaldehyde fixation, direct labelling with mouse IgG conjugated with fluorescein

B: 4% formaldehyde fixation, indirect labelling with mouse IgG and rabbit anti-mouse IgG conjugated with fluorescein

B: 2% formaldehyde + 1% glutaraldehyde fixation, indirect labelling with mouse IgG and rabbit anti-mouse IgG conjugated with fluorescein