Basic Fluorescence Microscopy and Sample Preparation

Micron Microscopy Course
16th March 2015

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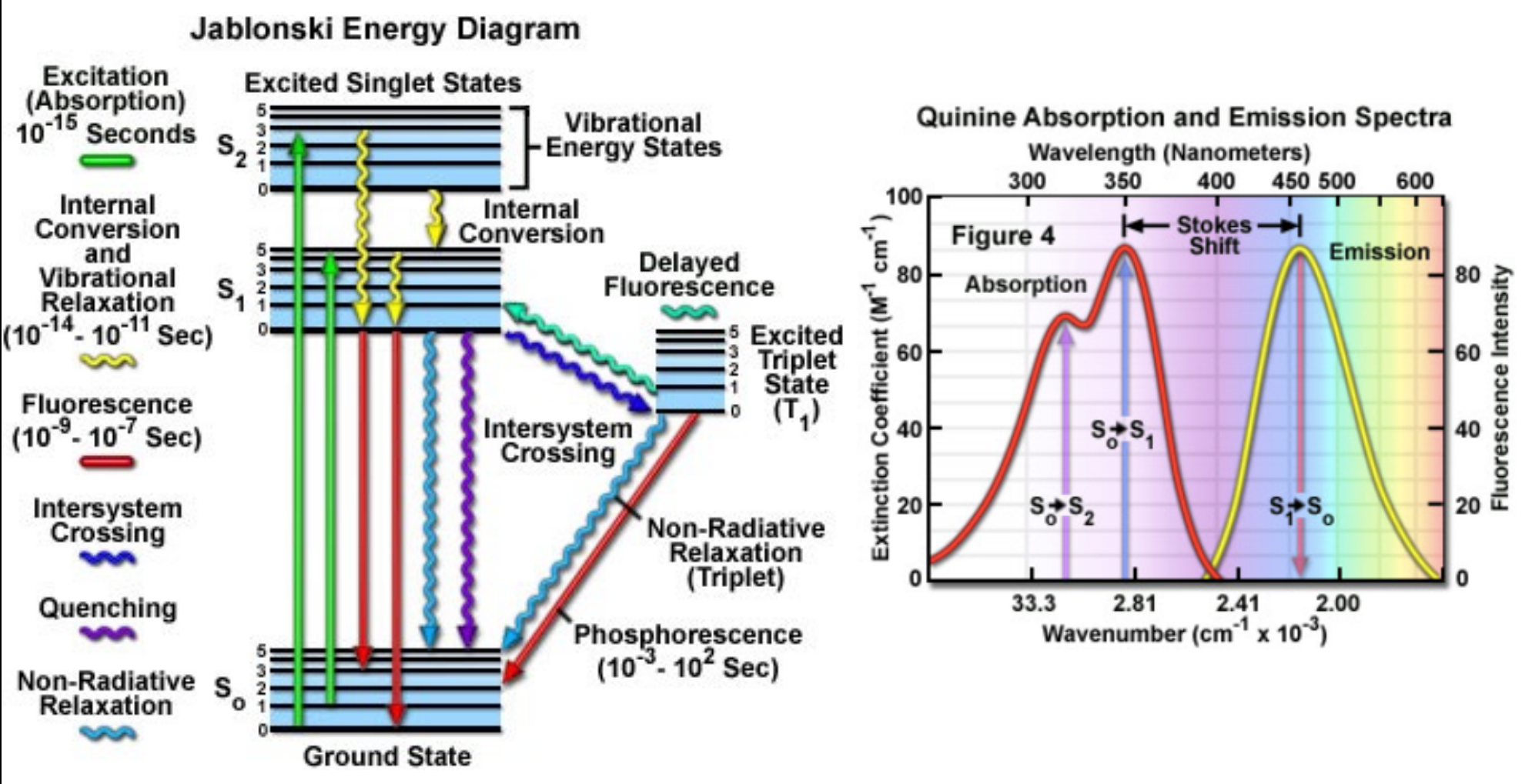
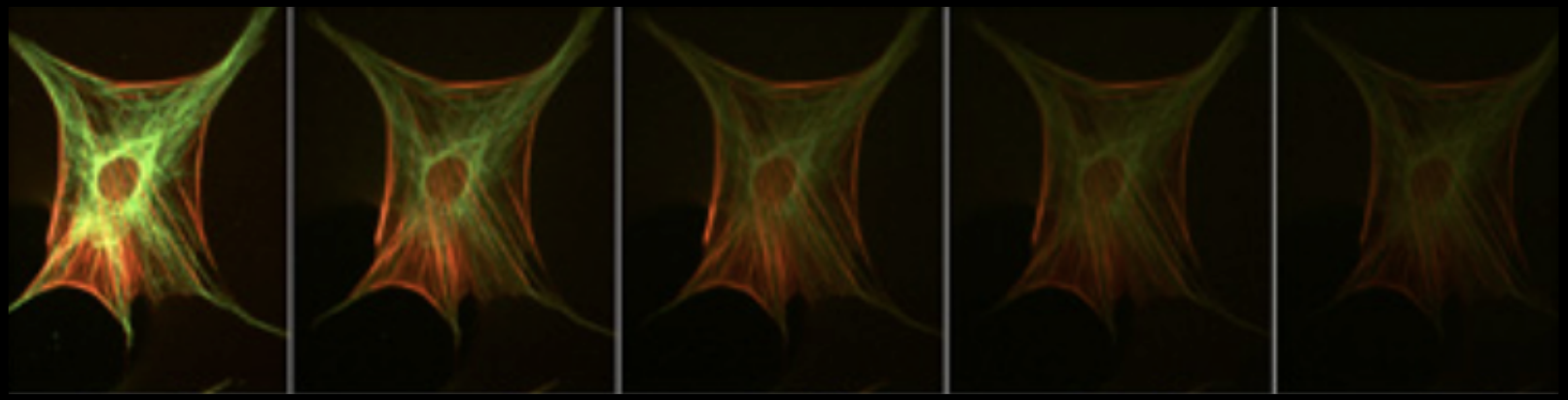
Basic Fluorescence Microscopy

Basic Concepts in Fluorescence

- **Fluorescence** is the property of some atoms and molecules to absorb light at a particular wavelength and to subsequently emit light of longer wavelength after a brief interval, termed the **fluorescence lifetime**.



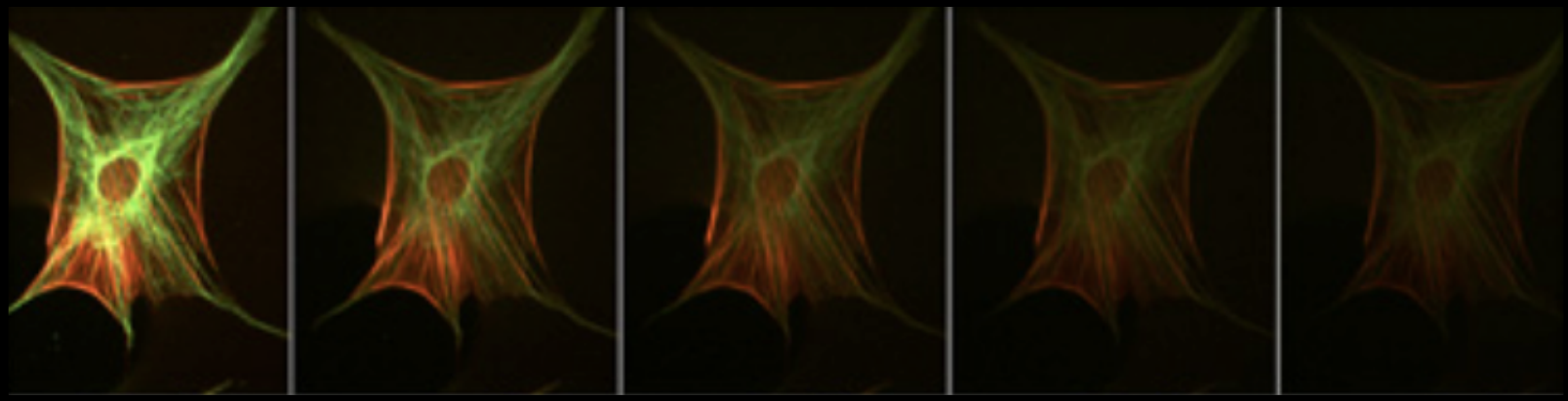
Photobleaching

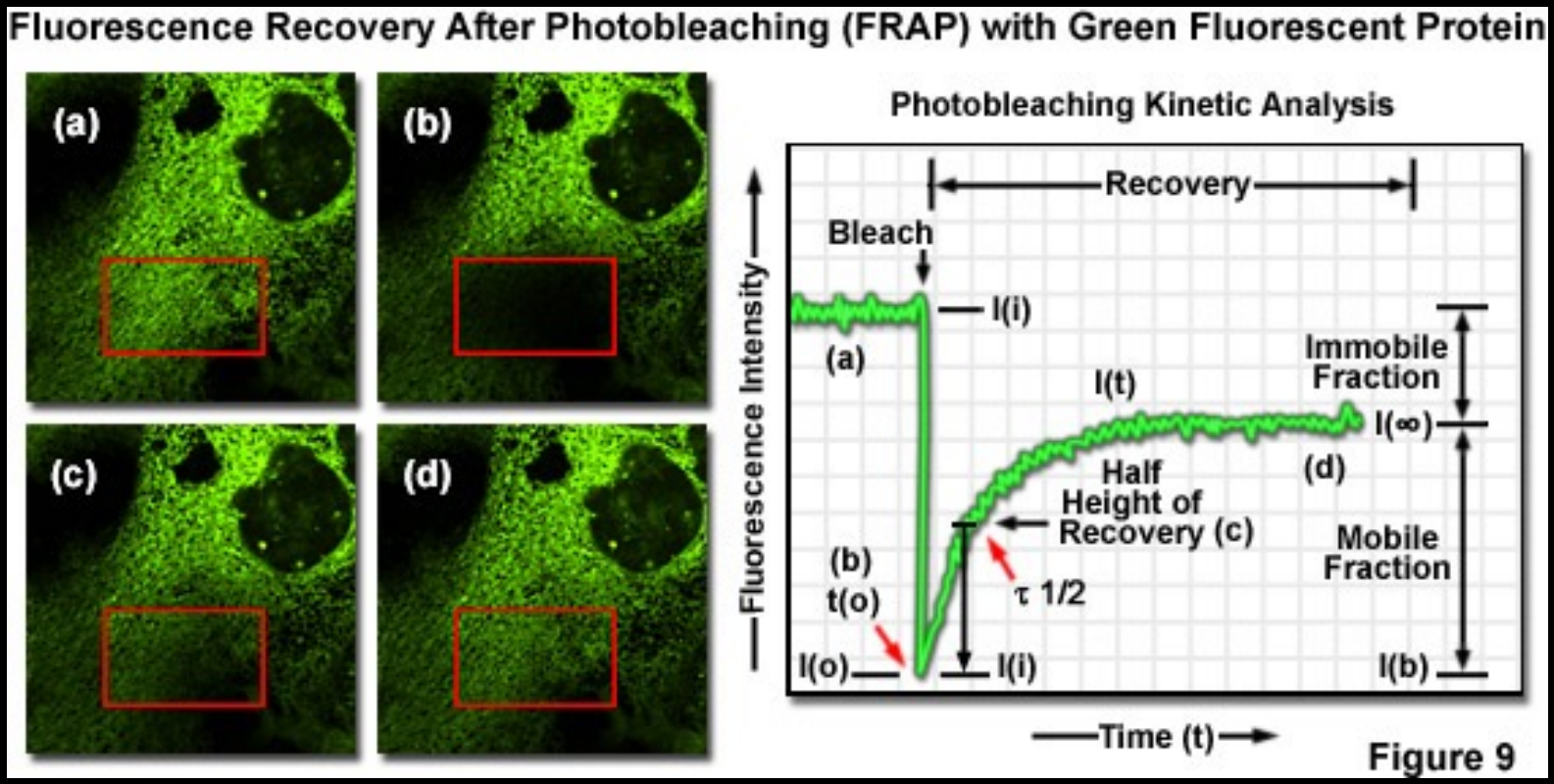


Photobleaching can be reduced by:

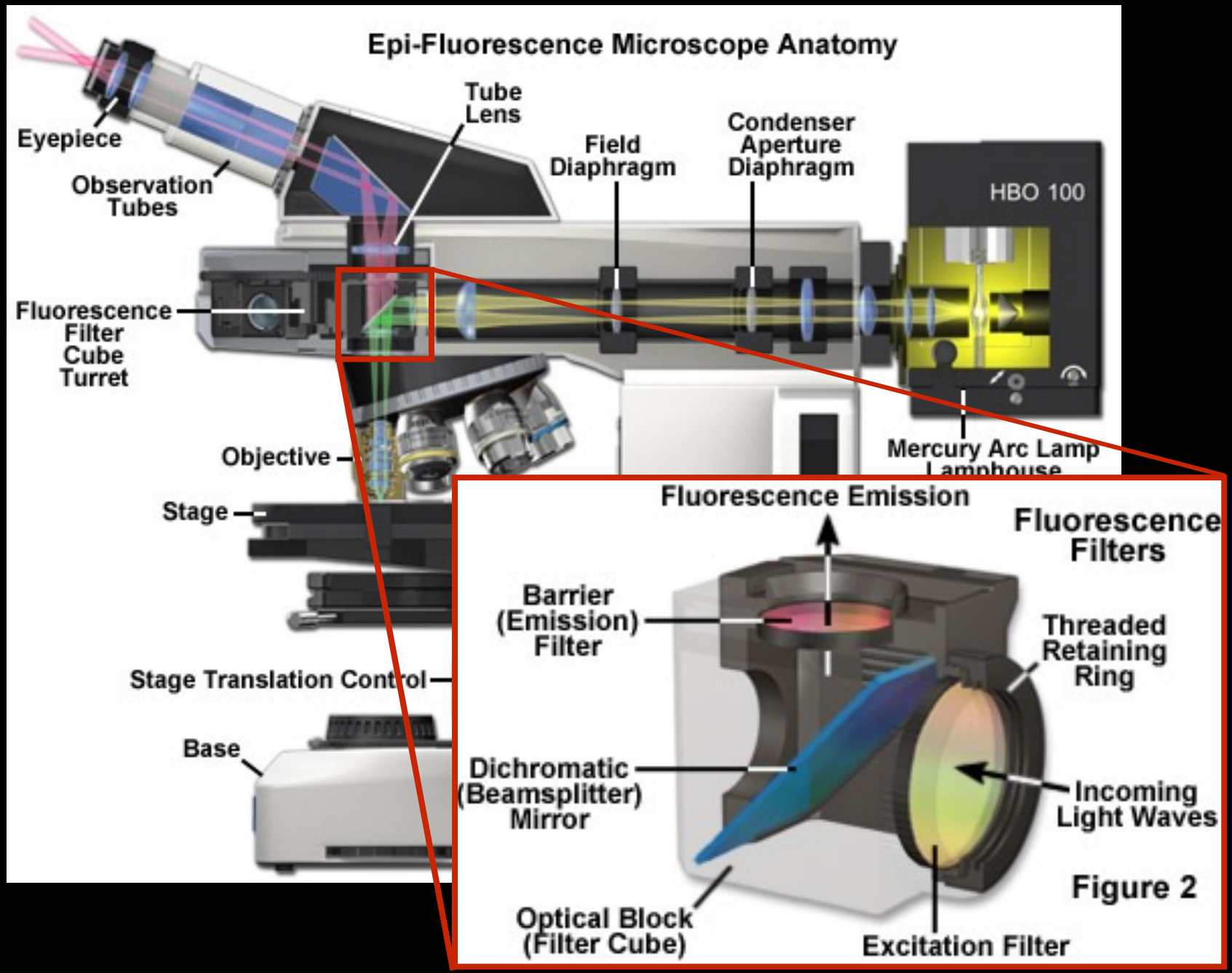
- Reducing the exposure time
- Reducing the oxygen concentration in the media
- Use of anti-fading (mounting media)

Photobleaching





The Fluorescence Microscope



Light Sources

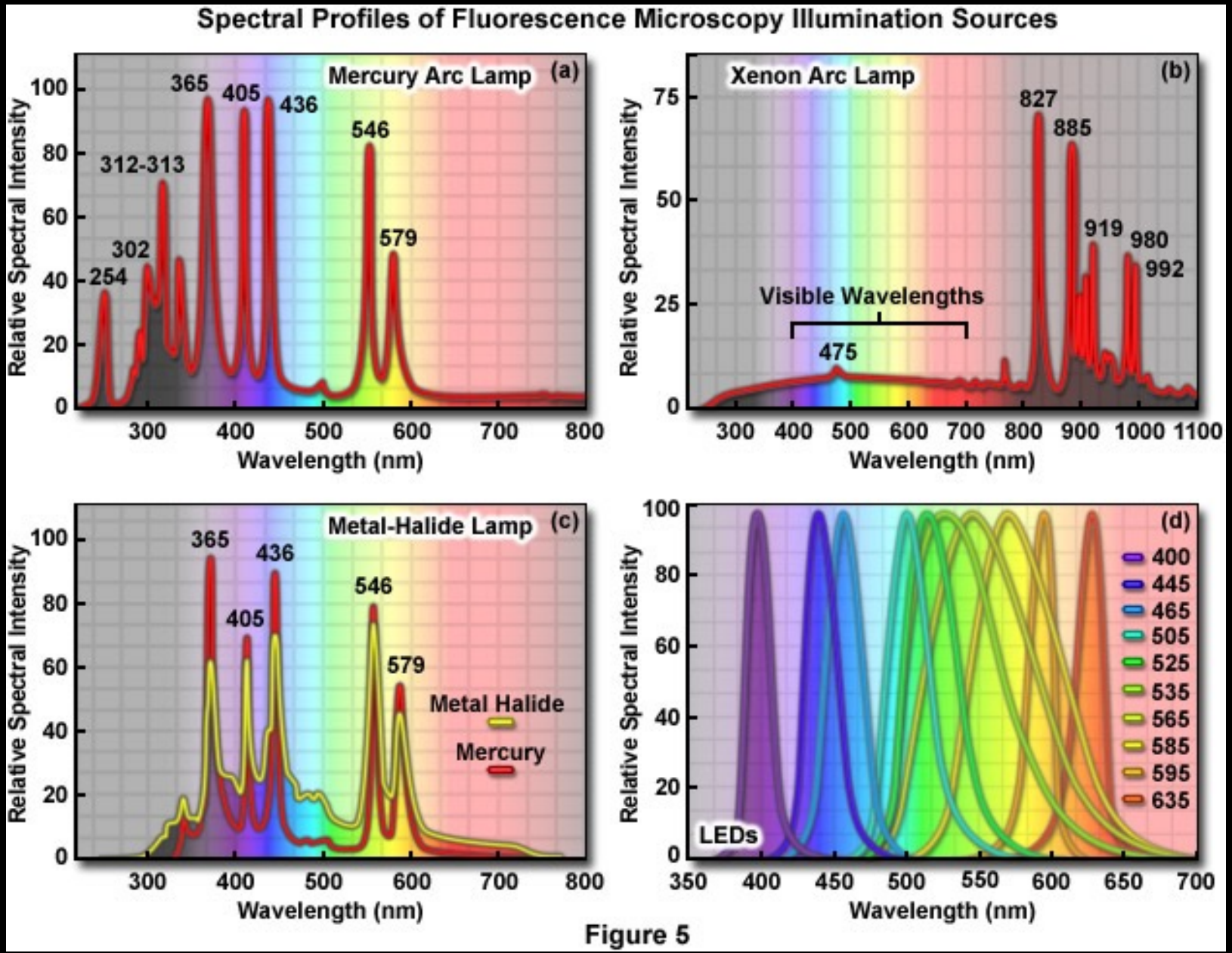
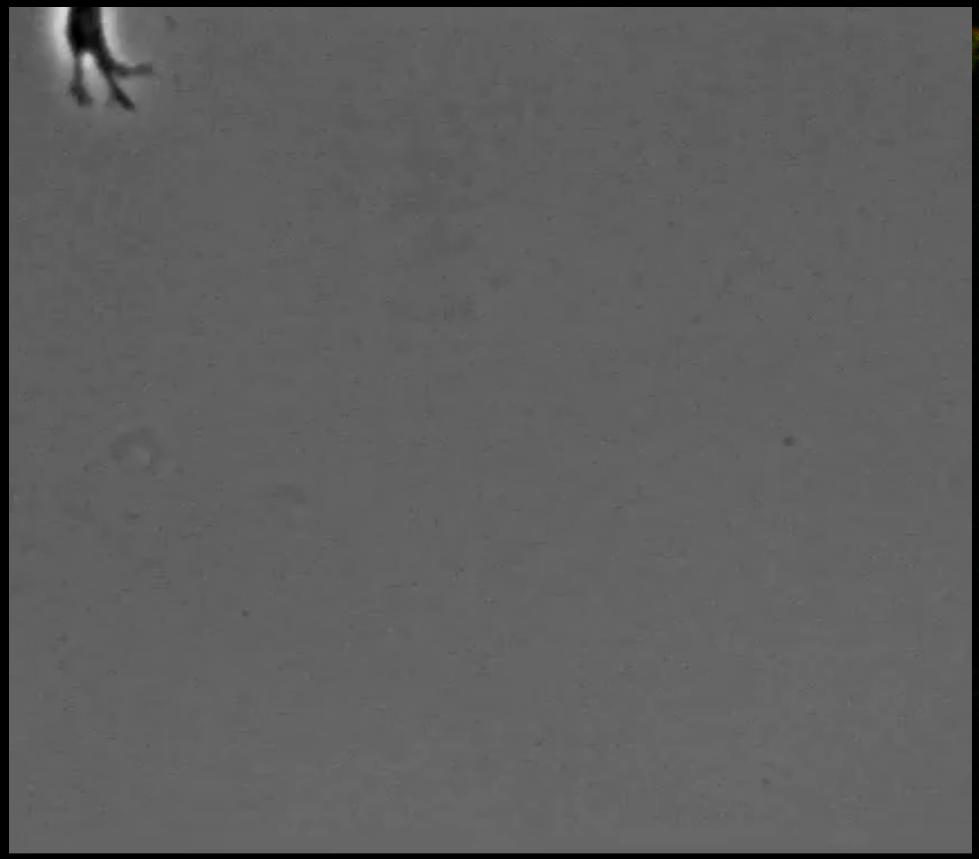
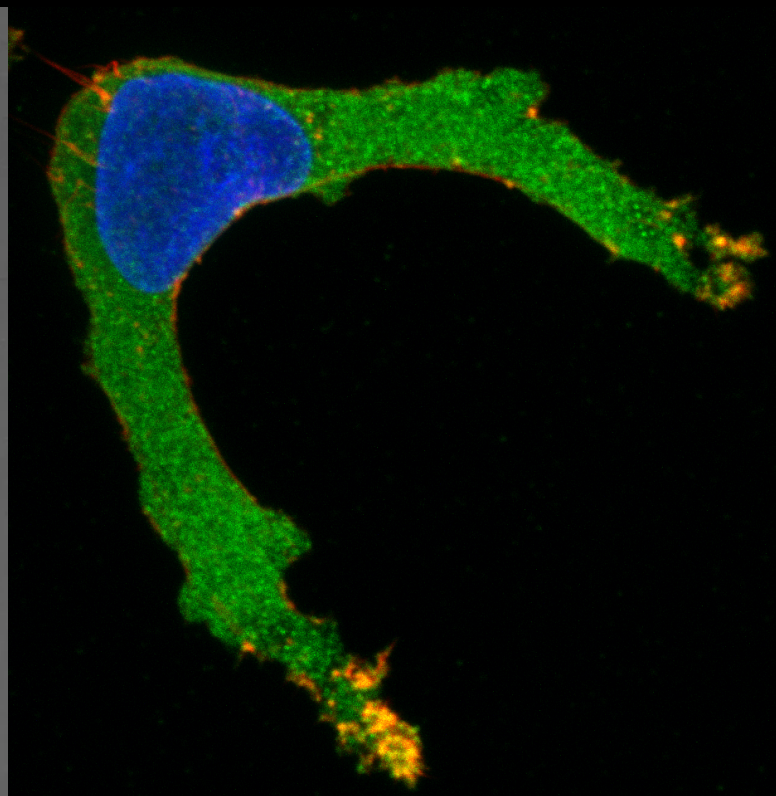


Figure 5

Preparation of fixed samples for fluorescence microscopy

Sample Preparation: live or fixed?

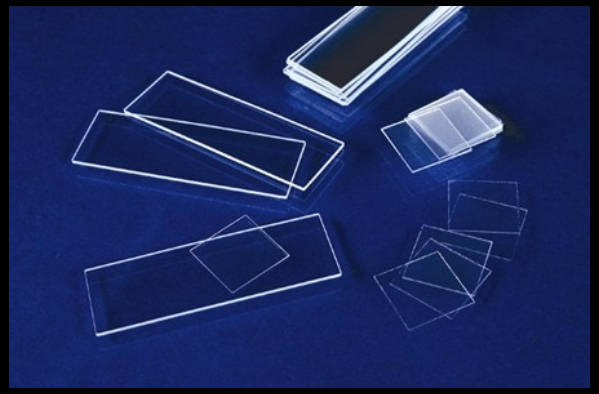


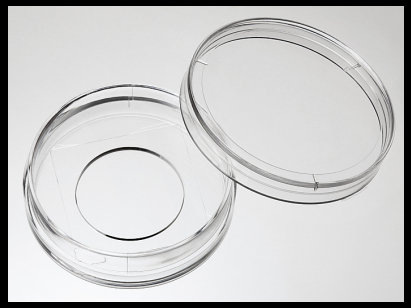


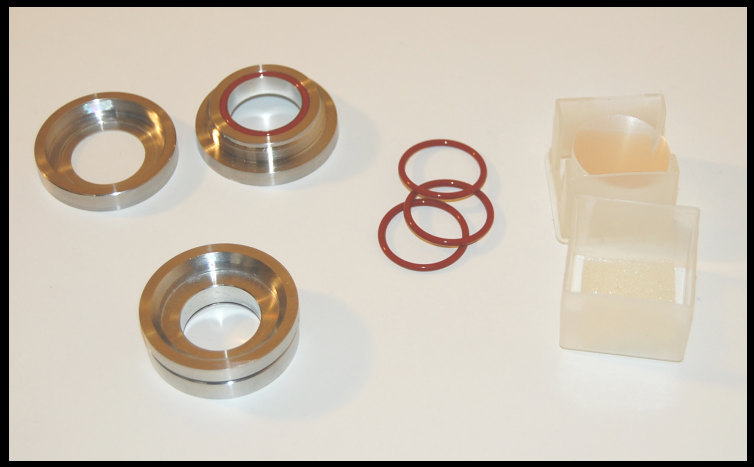
Sample Preparation: an Overview

- Inmobilising the specimen
- Fixation
- Permeabilisation
- Blocking
- Antibody/label incubation
- Mounting

Sample Preparation

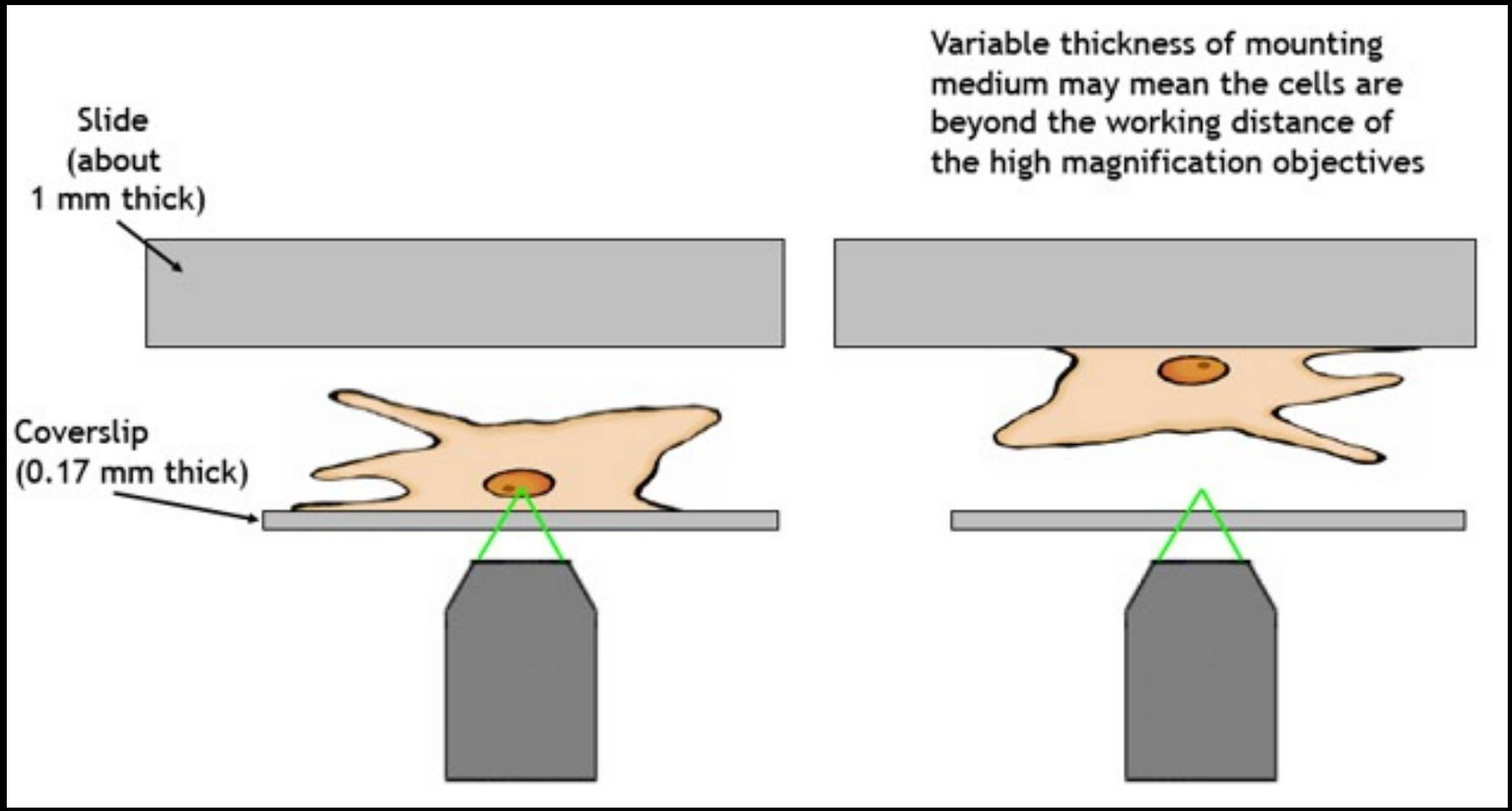






Be aware of the wide range of possibilities!

Sample Preparation

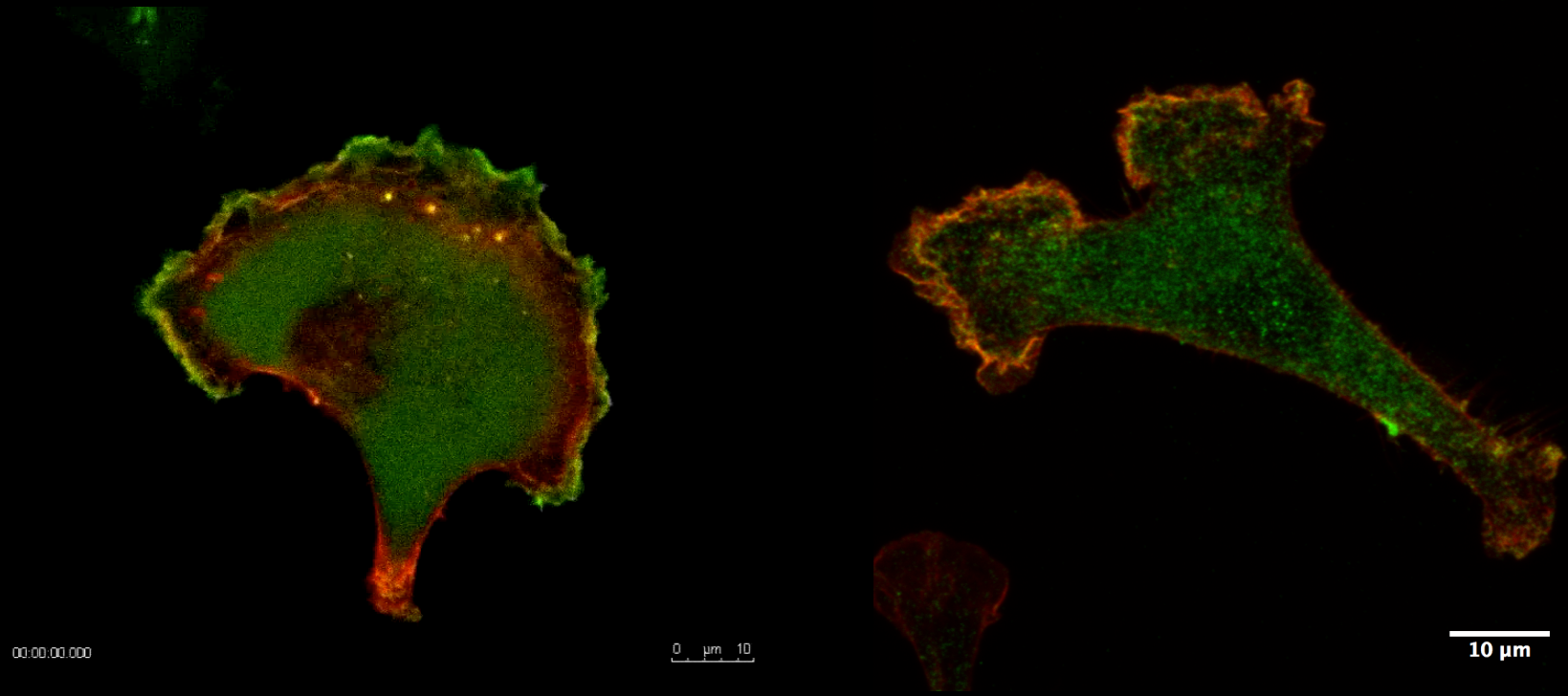


Specimen immobilisation

- **Adherent cells** directly attach to the glass or attachment might be enhanced by coating the coverslip/surface with poly-lysine, fibronectin, collagen, etc
- **Cells in suspension** require coating or centrifuging the cells onto glass slides (cytospin).
- **Tissue** and other macrostructures: might be embedded in paraffin, wax…

Key steps during Immunocytochemistry

1. Fixation
2. Permeabilisation
3. Blocking
4. Antibody incubation
5. Mounting



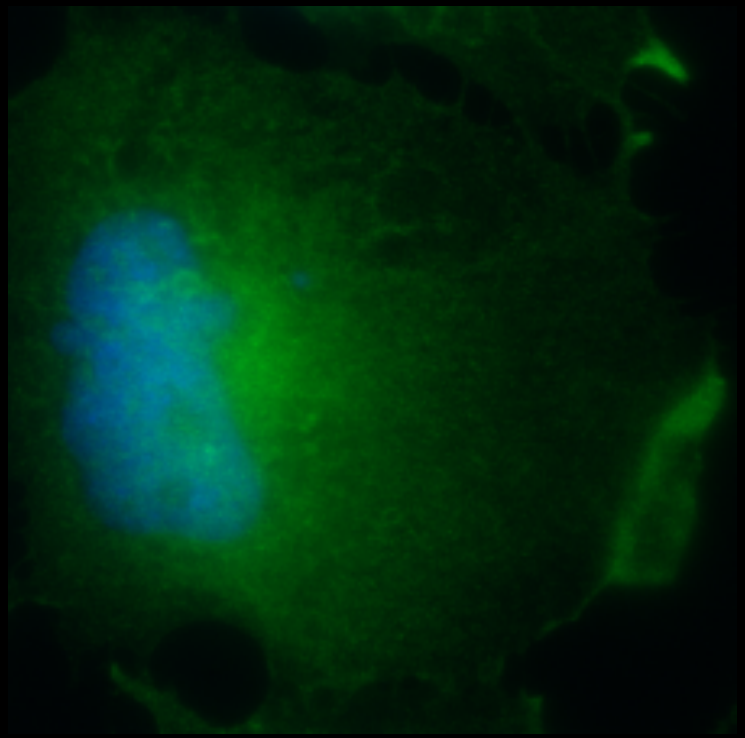
Live

Fixed

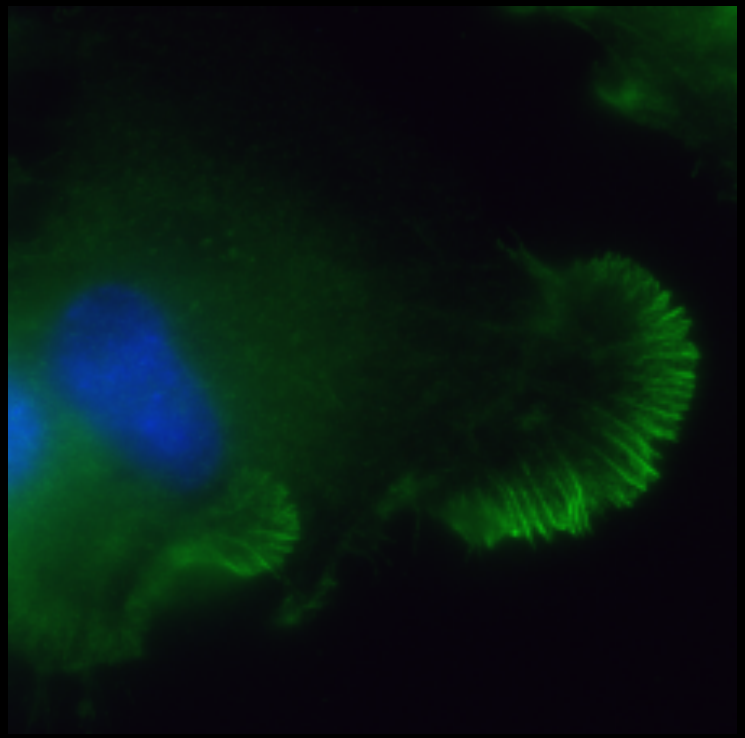
Fixation

- **Organic solvents**: methanol, ethanol, acetone, HistoChoice® (ethanedial).
  - Pros: they permeabilise the cells
  - Cons: they permeabilise the cells, can cause shrinking and disruption of organelles.

4% PFA



MetOH



Fixation

- **Organic solvents**: methanol, ethanol, acetone, HistoChoice® (ethanedial).
  - Pros: they permeabilise the cells
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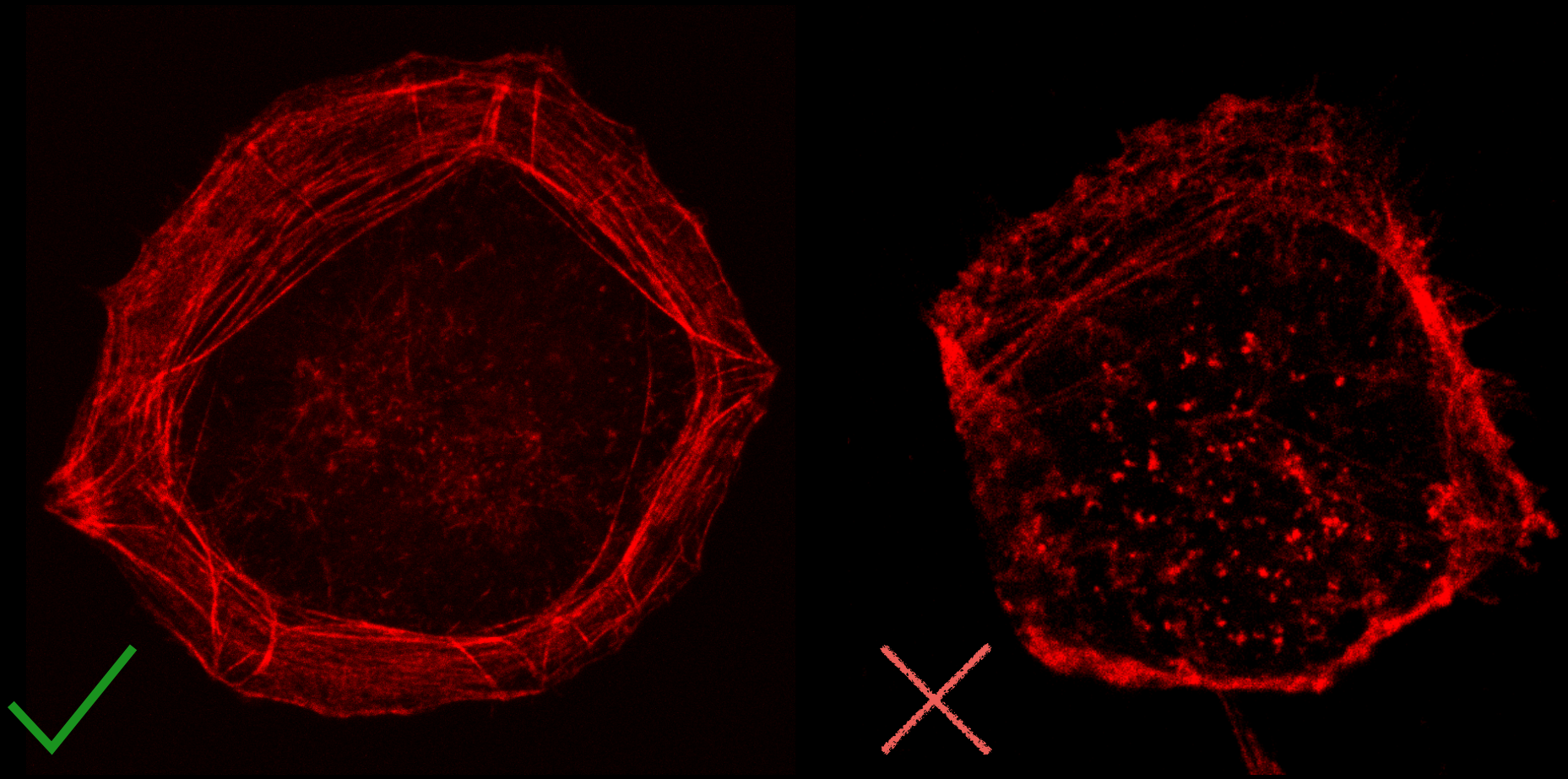
- **Aldehydes**:
  - Formaldehyde (2-4% PFA).
  - Formalin (impurities, autofluorescence, loss of some proteins).
  - Glutaraldehyde, 0.5-2% (induces autofluorescence therefore post-incubation with $NaBH_4$ is recommended for quenching and thorough washes in PBS after this).

- **Combined methods**:
  - Formaldehyde-acetone
  - Methanol-acetone
  - Methanol-glacial acetic acid

Fixation: some tips

- pH
- Temperature
- Specific buffers



MDA-MB-231 F-actin

Permeabilisation

Detergents:

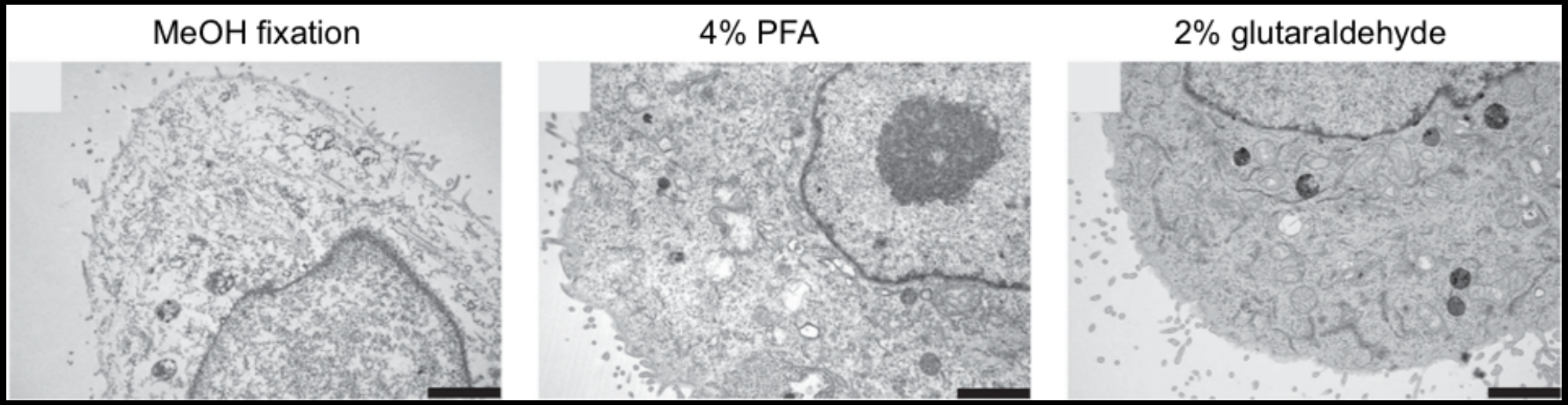
Polar lipids with a hydrophilic (water soluble) end and a hydrophobic end that binds the hydrophobic moieties of water insoluble compounds and renders them hydrophilic.

Non-ionic detergents: contain methyl groups that participate in hydrogen bonds and are able to solubilise membranes but do not destroy protein-protein interactions.

- Triton X-100: used to permeabilise unfixed or lightly fixed eukaryotic cell membranes (0.05-0.2% in PBS).
- Tween 20: milder than Triton X-100, used to reduce surface tension in blocking, antibody incubation and wash steps (0.1%).
- Nonidet P-40 (NP-40): used to permeabilise unfixed cells (0.1% in PBS for 5-10s).
- Saponins: recommended for stainning of small internal molecules, receptors and intracellular membranes (0.05-0.1% in dH2O).

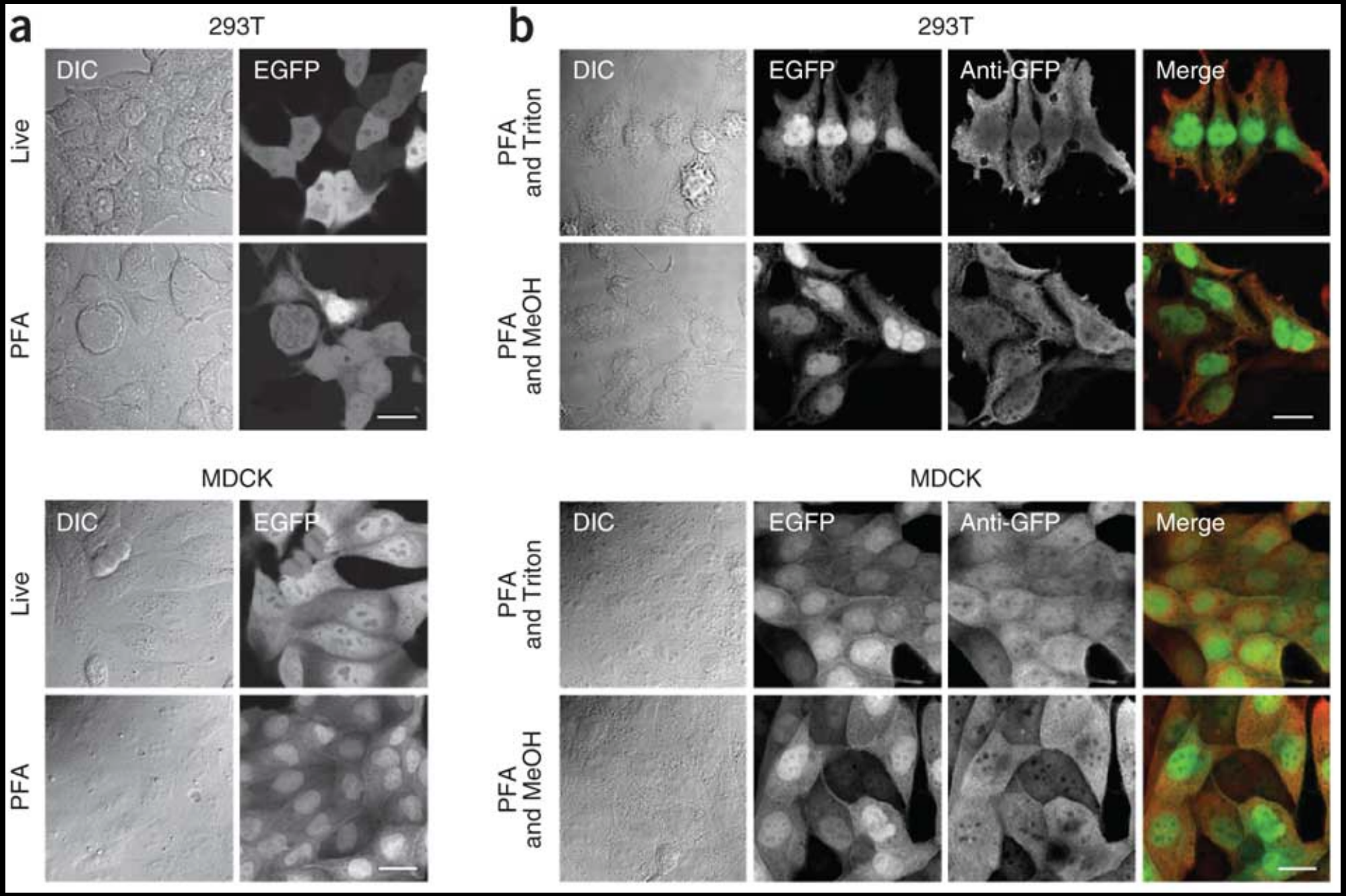
Ionic detergents: (SDS, deoxycholate, CHAPS) have highly charged hydrophilic groups and are very effective at solubilising membranes, but also destroy native three dimensional protein structures.

Effects of fixation in ultra-structure



Schnell et. al. *Nature Methods* **9**,152–158 (2012)

Effects of fixation-permeabilisation in protein extraction and antibody accessibility



Blocking

Sources of non-specific binding:

- Unreacted aldehydes may crosslink antibodies
- Highly charged or hydrophobic structures may bind antibodies
- Low affinity immunoglobulins
- Fc receptors or endogenous antibodies of some immune cells

Blocking solutions:

Dilute in PBS or PBS/low concentration detergent

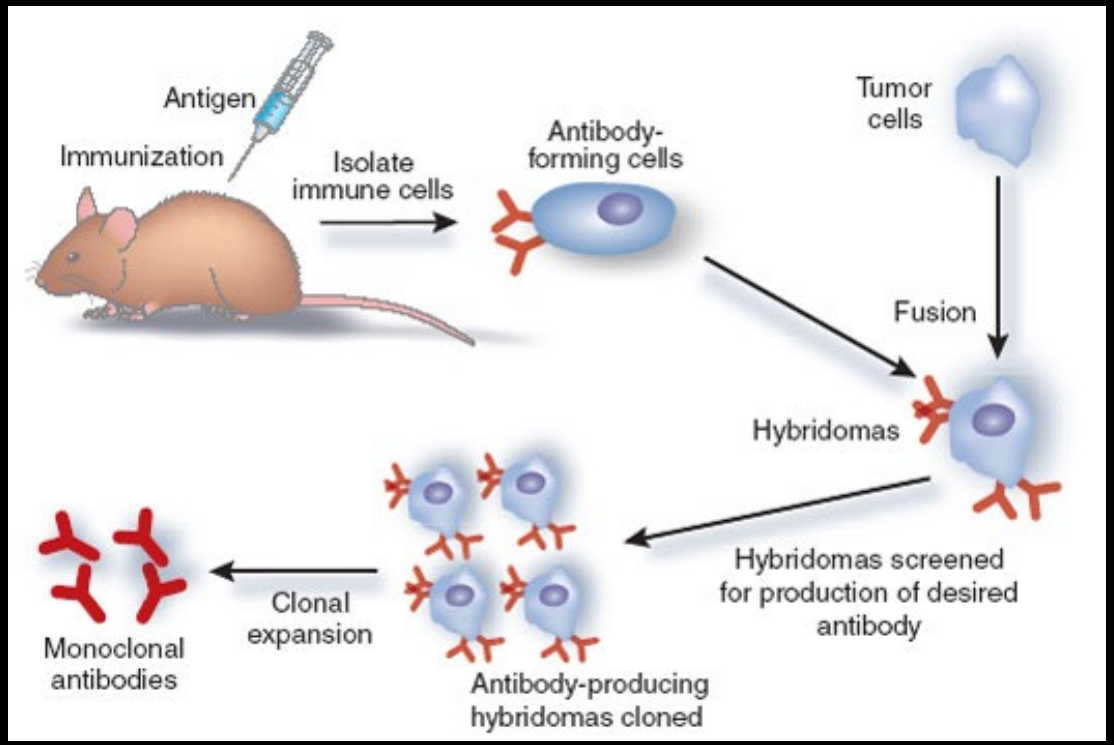
Minimum time: 30min RT

- BSA (1-10%), casein (or non-fat dry milk), gelatin
- Serum from the species of the secondary antibodies
- Fab fragments
- Commercial solutions

Antibodies

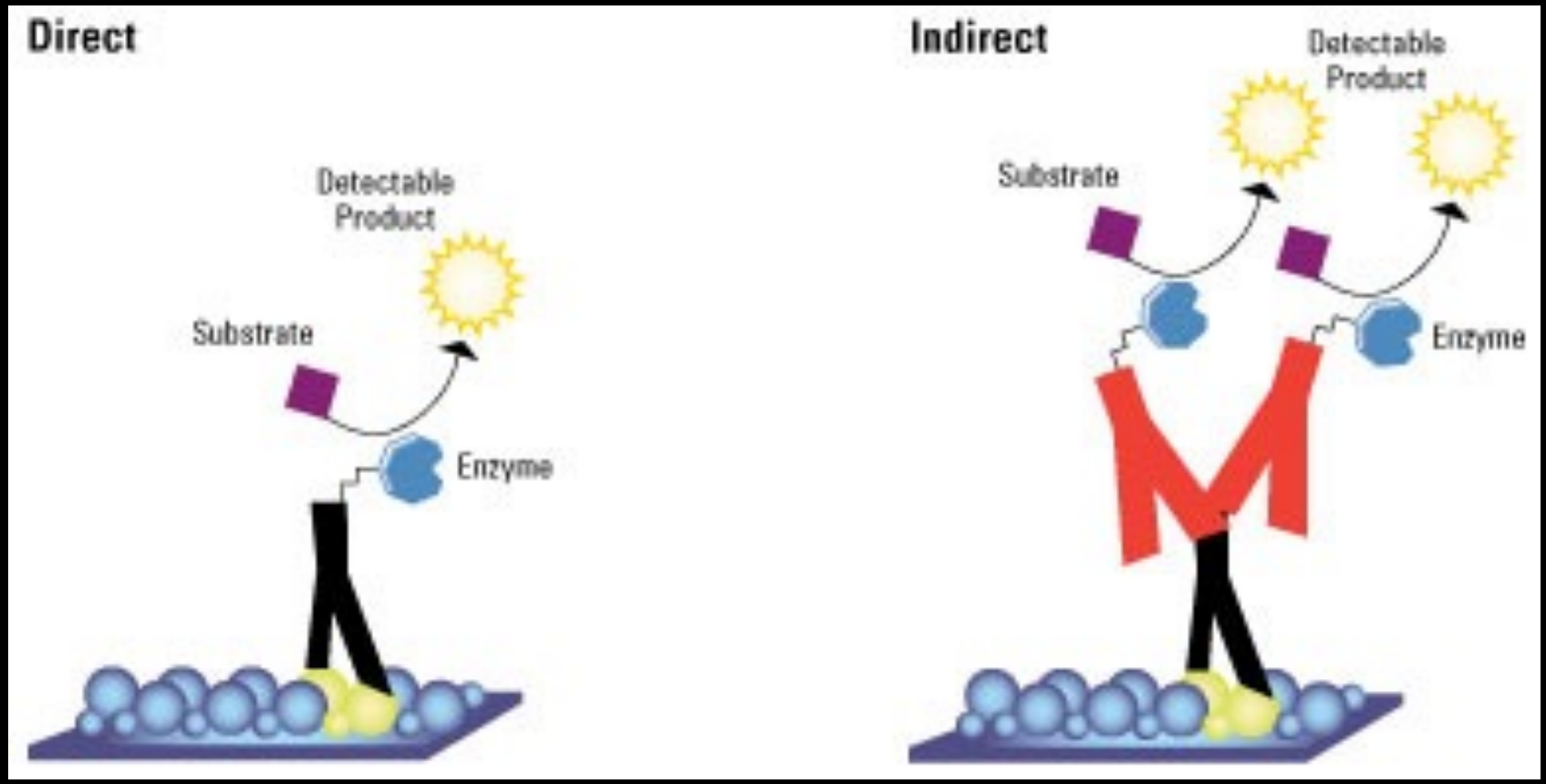
Monoclonal vs Polyclonal:

- Monoclonal abs are produced by fusing a B-cell isolated from spleen of an immunised animals (usually mouse) with an immortalised cell to obtain an hybridoma.
- Polyclonal abs are obtain from serum of immunised animals (commonly rabbit, goat, sheep or chicken). Contain a high number of different antibodies that recognise the same antigen, this introduces variability between different batches but increases signal



Goodness and drawbacks of immunoassays

- Direct immunoassay: conjugated antibodies directly bind the epitope
- Indirect immunoassay: two-step protocol, primary antibody recognises the epitope and a conjugated secondary antibody binds to the primary antibody.
  - Biotin-avidin/streptavidin



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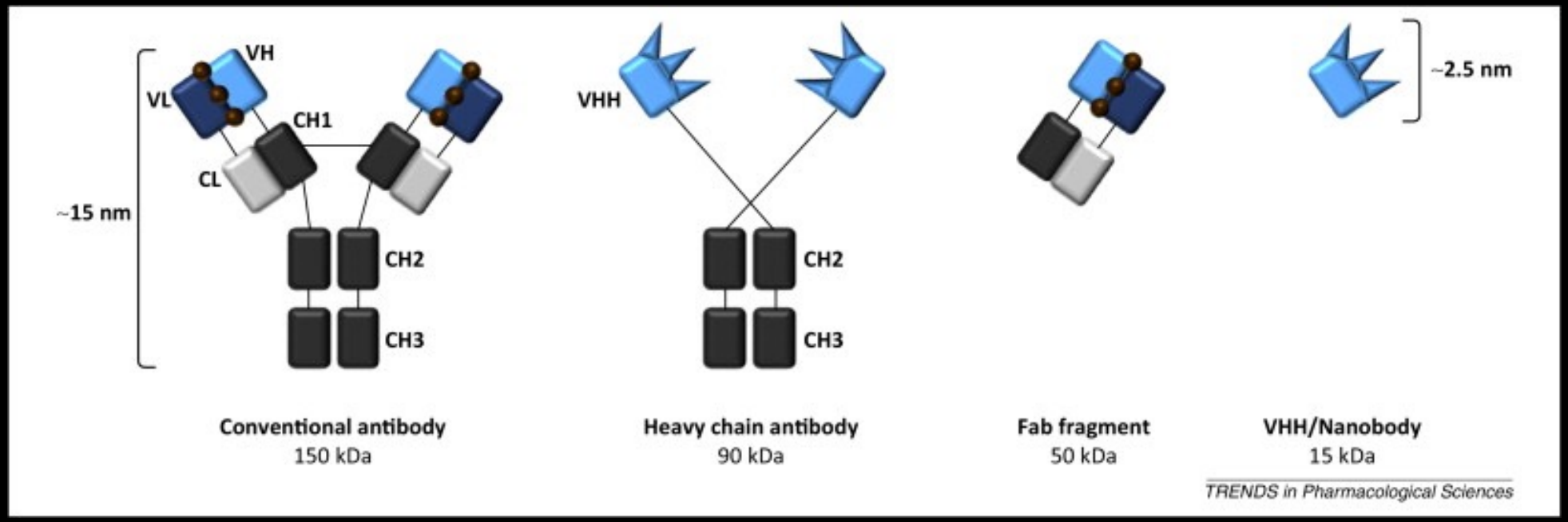
! Size of the immunocomplex and superresolution techniques



Trends Pharmacol Sci. 2014 May;35(5):247-55.

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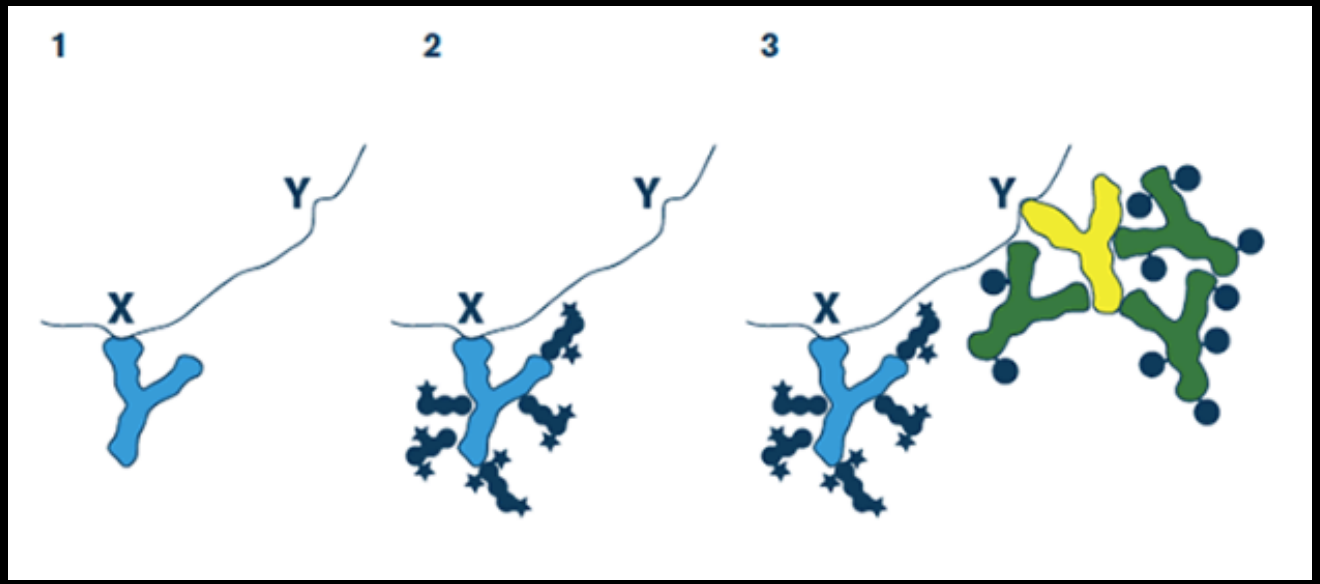
! Size of the immunocomplex

- Species limitation
  - Use of Fab fragments for blocking:



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- Species limitation
  - Use of conjugated Fab fragments for blocking
  - Use of conjugated Fab fragments for blocking to convert first primary antibody into a different specie

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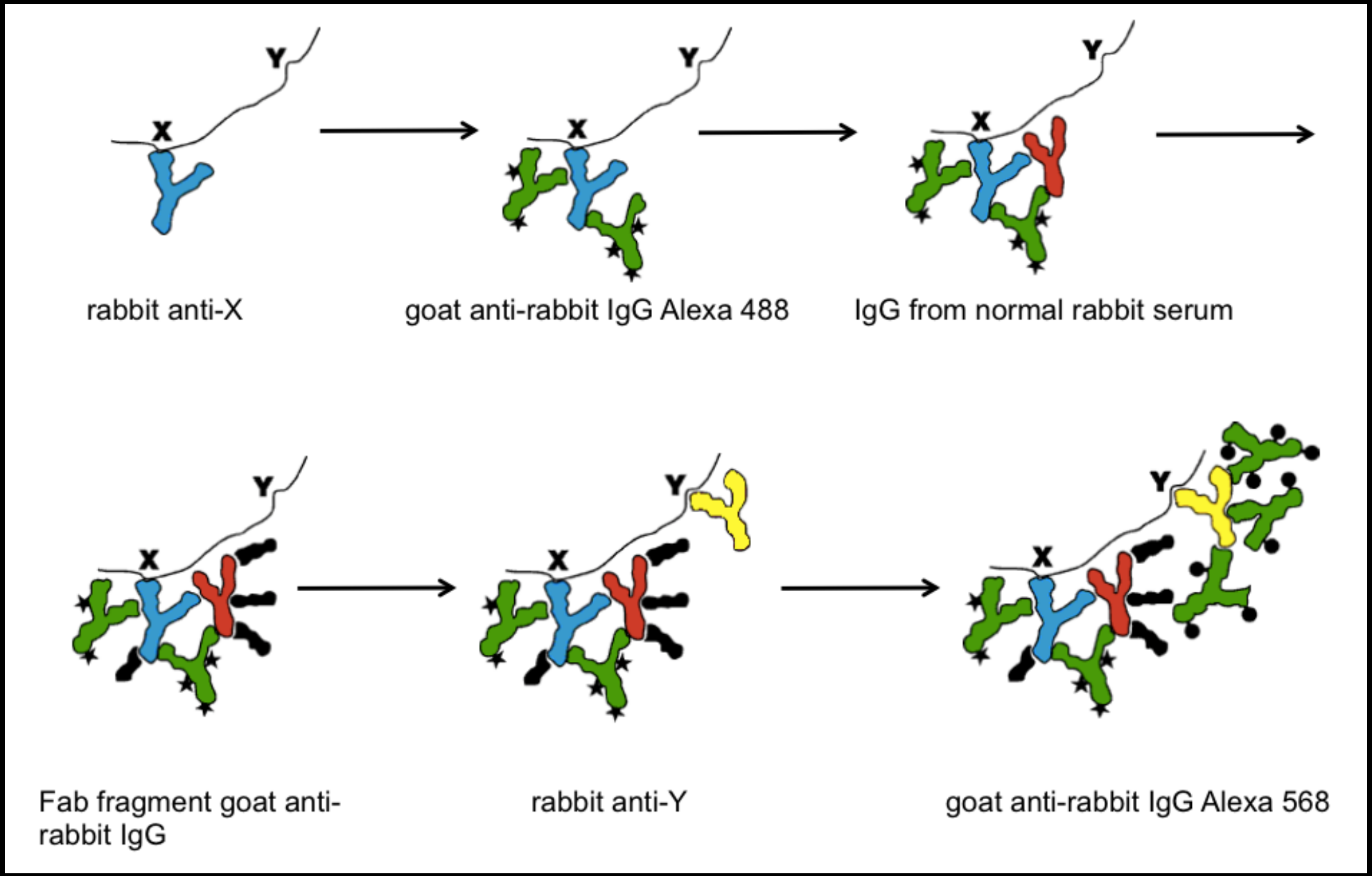
! Size of the immune-complex

- Species limitation
  - Use of conjugated Fab fragments for blocking
  - Use of unconjugated Fab fragments for blocking to convert first primary antibody into a different specie
  - Use of unconjugated Fab fragments for blocking after the first antibody step

Blocking of Fab fragments



https://www.jacksonimmuno.com

Antibody incubation

Primary antibodies:

- Dissolve in blocking solution
- Concentration depends on antibody and source (purified, supernatant, serum, ascites) - always titer concentration!
- Time of incubation: from 1h RT to overnight at 4ºC (optimise for each antibody)
- Centrifuge to get rid of immune-complexes (short spin at max speed)

Secondary antibodies:

- Dissolve in blocking solution
- Concentration as indicated by supplier, generally 1:500
- Time of incubation: 45-1h RT in darkness (key step!)
- Centrifuge to get rid of immune-complexes (short spin at max speed)

Common probes

Other common labelling agents:

- Nuclear markers: DAPI, Hoescht, TOPRO (5-10min RT in darkness)
- Phalloidins: bind F-actin, incubate for 45min RT in darkness preferably in PBS (blocking solution OK)
- Membrane markers: WGA, CellMask; permeable (live or fixed cells), available with different dyes (5-10min in darkness)

Experimental controls: the key for reliable results

Controls for immunofluorescence:

- Autofluorescence: No primary or secondary antibody
- Secondary controls:
  - Incubate with secondary but not primary antibody
  - Prepare samples for each primary antibody individually:
    A. Test cross-talk of the different fluorophores
    B. Test cross-reactivity of secondary antibodies

Experimental controls:

- Compare localisation in live and fixed cells
- Compare antibody reactivity with other known antibodies against the same epitope or target
- Test specificity in knock-out/knock-down cells

Mounting

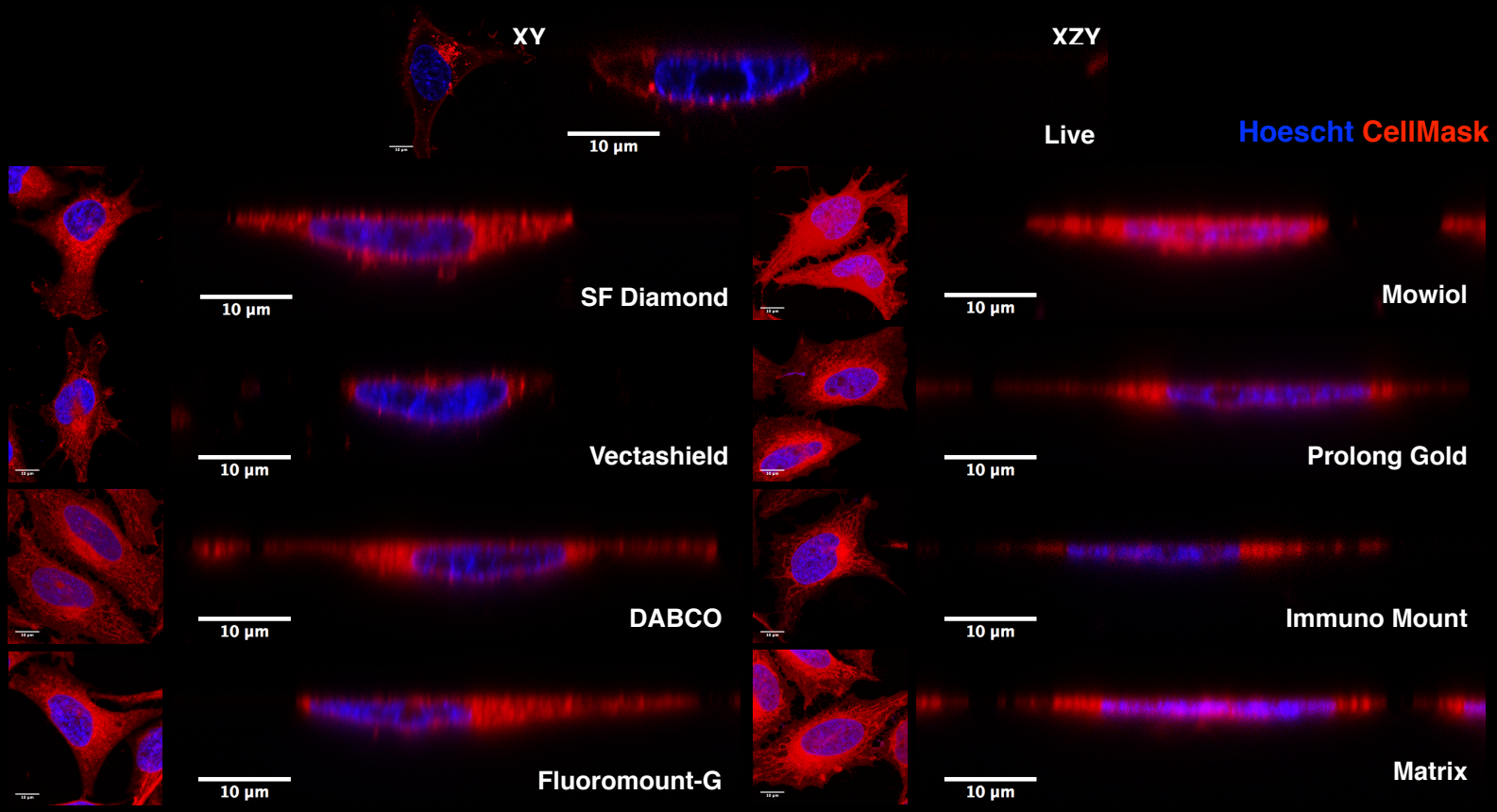
Non-hardening:

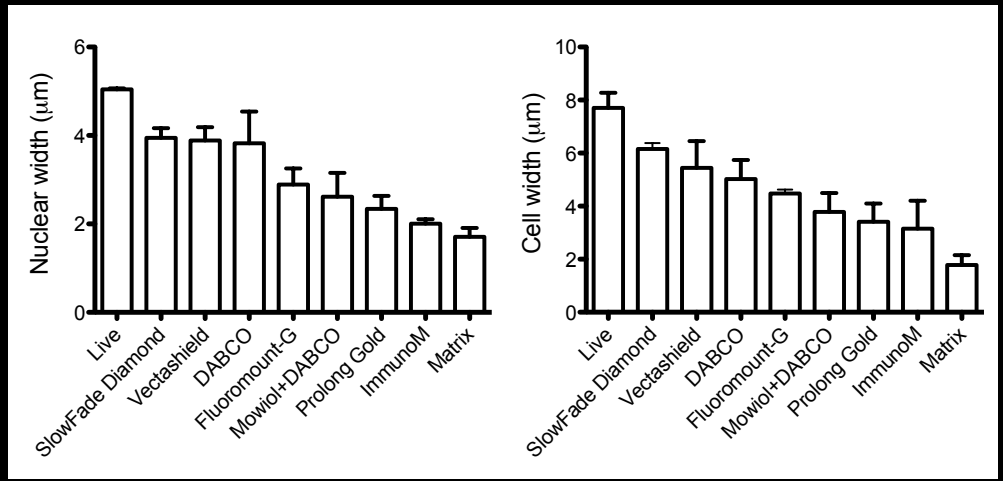
- Short-term storage (days-few weeks)
- Imaging directly after mounting
- DABCO, SlowFade, Vectashield

Hardening:

- Long-term storage (months)
- Leave polymerise (harden) before imaging (about 24h)
- Mowiol, Prolong, Vectashield Hardset, Fluoromount-G…

Artefacts due to mounting media





Further reading

http://www.olympusmicro.com http://www.microscopyu.com http://micro.magnet.fsu.edu
http://www.jacksonimmuno.com/technical
Richard W. Burry, Immunocytochemistry a practical guide for biomedical research, Springer 2010