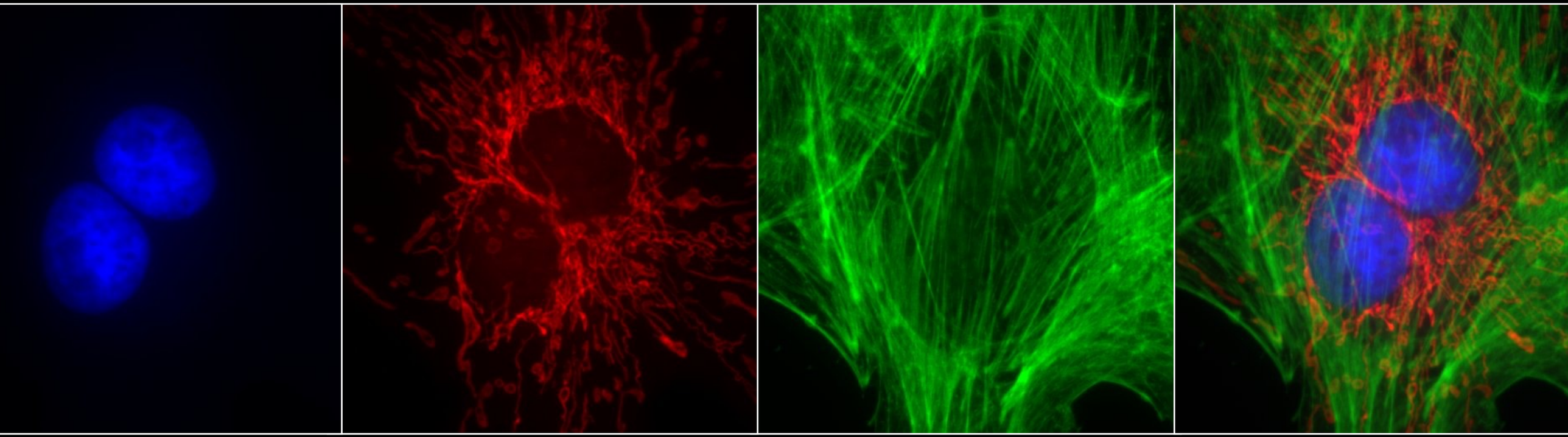
Sample preparation for fluorescence microscopy

Micron Advanced Microscopy Course - November 2020



Laser scanning confocal microscopy of BPAE cells stained with DAPI, Alexa 488 phalloidin, Mitotracker Red

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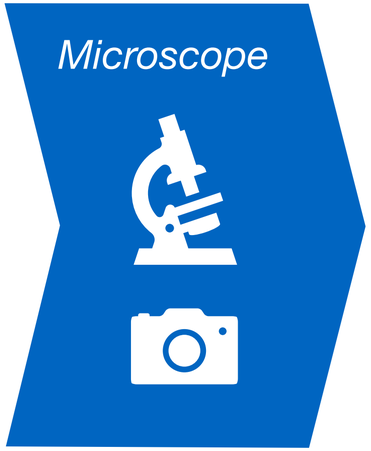
Imaging is more than microscopy

Biological question

?



~~***Answer***~~

Biological imaging: a multi-step process

Biological question

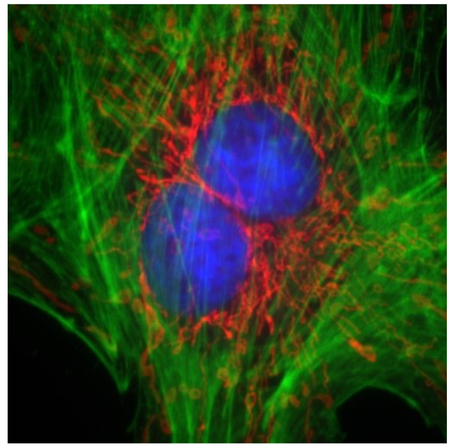
?

1 Experiment design

2 Labelling

3 Sample

4 Microscope



~~Answer~~

“Garbage in, garbage out!”

Biological imaging: a multi-step & muliti-disciplinary process

Biology → *Chemistry* → *Physics / Optics* → *Data science* → *Biology*

Biological question

?

1 *Experiment design*

2 *Labelling*

3 *Sample*

4 *Microscope*

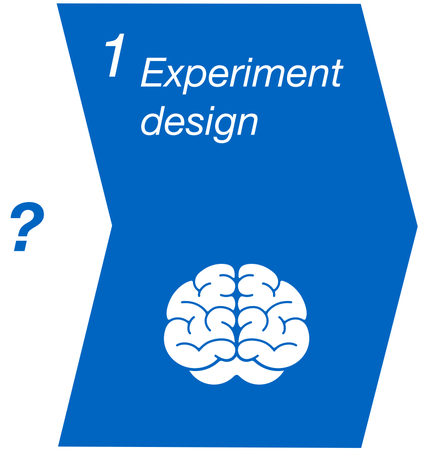
5 *Post-processing*

6 *Quantitation, Visualisation, Data storage*

Biological interpretation

"Garbage in, garbage out!"

Biological question informs the experiment design - first think hard, then work hard



What question do I want to address using imaging?

- (Sub-)cellular localisation of molecules of interest (proteins, nucleic acids, lipids, …)?
- Identify cells, sub-cellular structures, macromolecular complexes, single molecules?
- Analyse spatial relationships, co-localisation?
- Dynamic properties, interaction kinetics?
- Analyse biological response over a time period?
- Pretty pictures?

What do I need to answer this question?

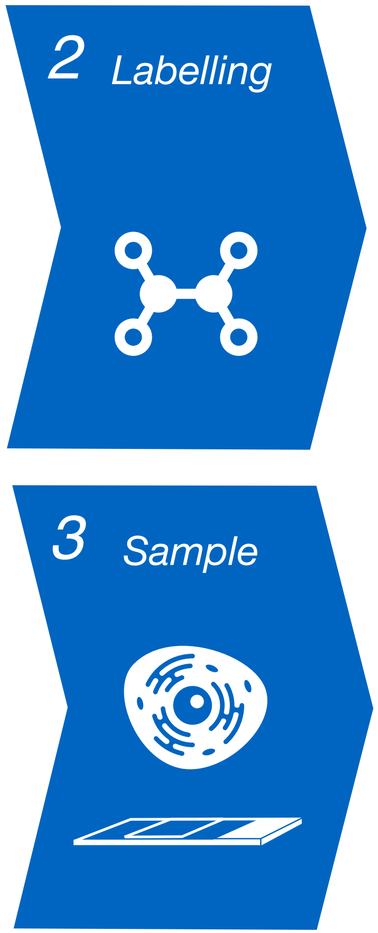
- Live or fixed specimen?
- Cultured cells, primary cells, tissue, or whole animal?
- Label-fee or fluorescence?
- What level of labelling specificity is sufficient?
- One or multiple targets? How many colours do I need?
- 2D plane or 3D sectioning, out-of-focus blur suppression?
- What level of resolution in xy, and z is necessary?
- Which microscope should I use?

Why work with fixed material?

2 Labelling

- Convenience & throughput
- Variety of molecular labelling techniques
  - Immunofluorescence (IF)
  - Fluorescence in situ hybridisation (FISH)
  - Small molecules (e.g. Phalloidin, DAPI)
  - Click-chemistry labelling
  - Protein-tags
- Ease of multiplexing with bright and photostable organic dyes
  (Cy3/5/7, AlexaFluor 488/568/647, ATTO488,590,647N, etc.)
- Sample storage

Considerations for for a good sample preparation



Structure preservation

Labeling specificity

Contrast (SNR)

Aberration

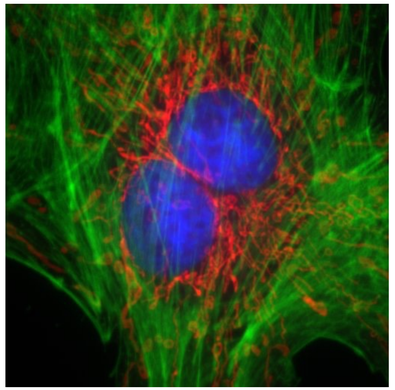
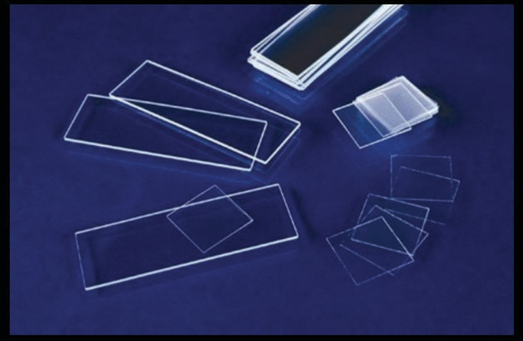
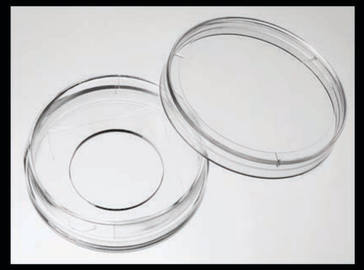
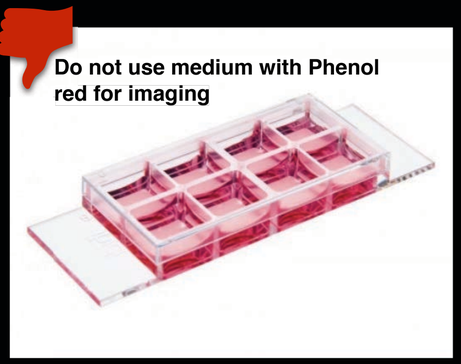


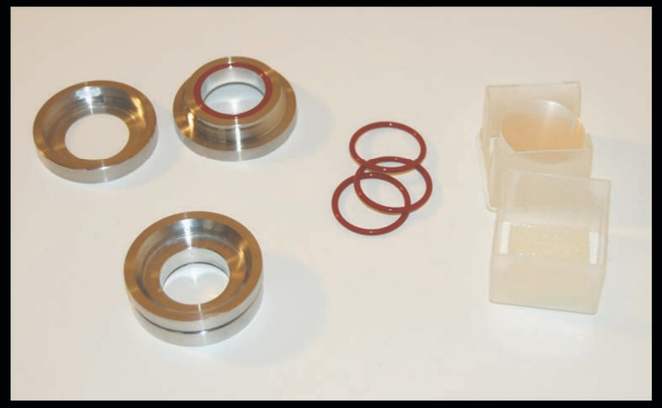
Image (data)

Immobilising cells/specimen on glass/plastic







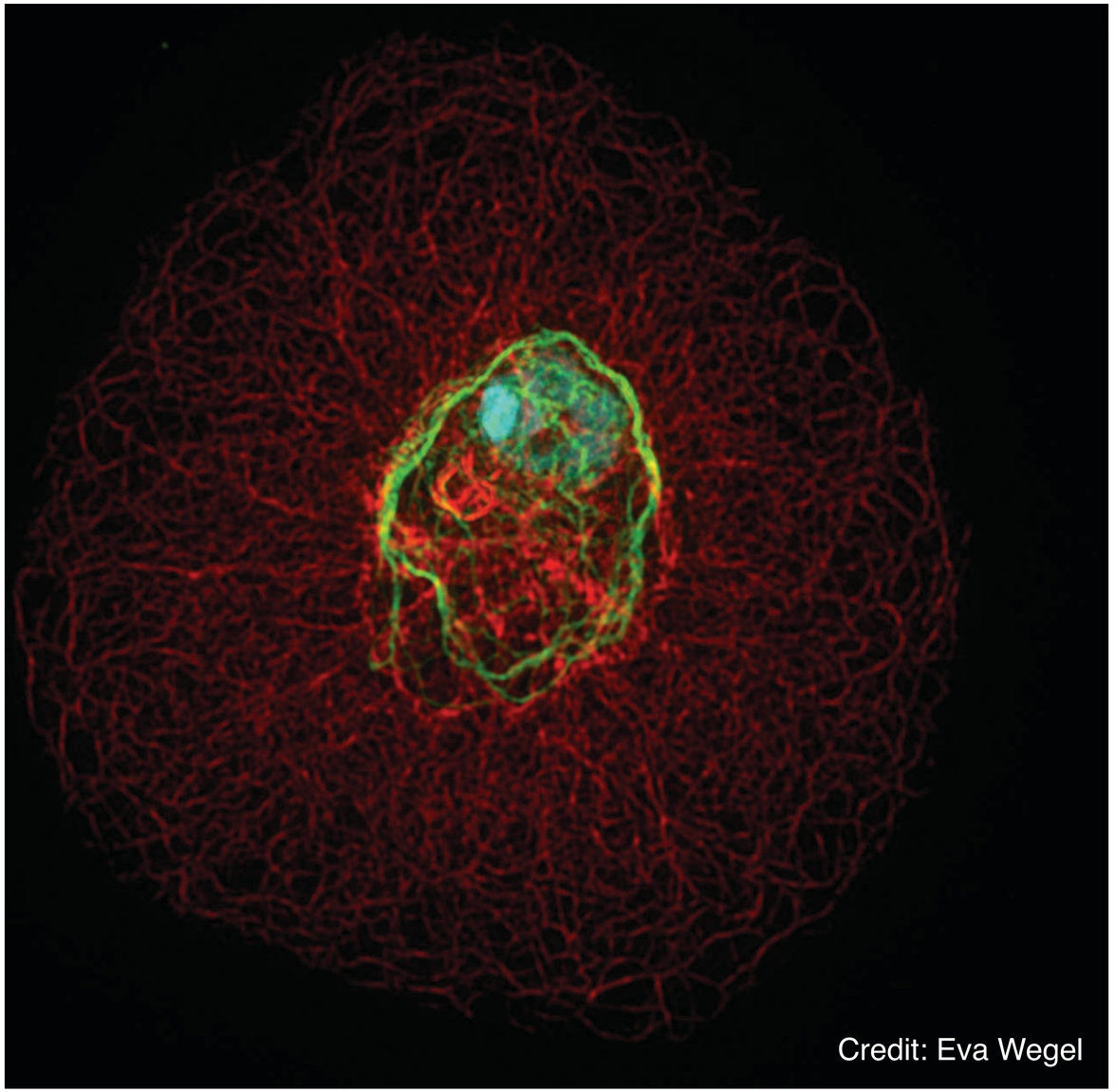


- Do specimen adhere?
  - ➡ coating (Poly-L-lysin, Fibronectin)
- Are specimen happy?
  - ➡ check growth rate and morphology
- Plastic bottom polarises light!
  - ➡ certain imaging modalities may not work
- Coverglass thickness!
  - ➡ High NA objectives are corrected for 0.170 mm (#1.5); Super-resolution requires low tolerance ± 0.005 mm

Typical immunofluorescence (IF) labelling protocol

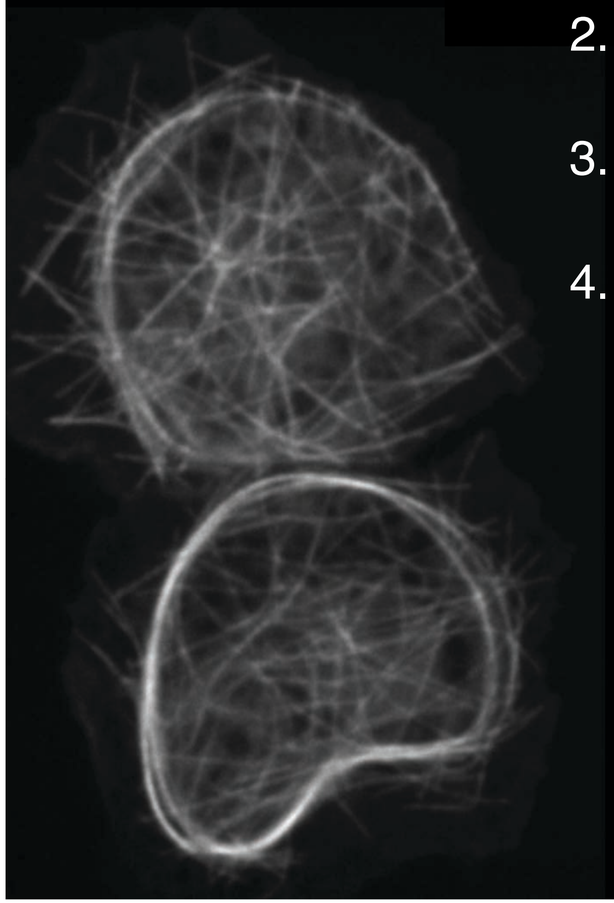
Most are variants of the following basic steps:

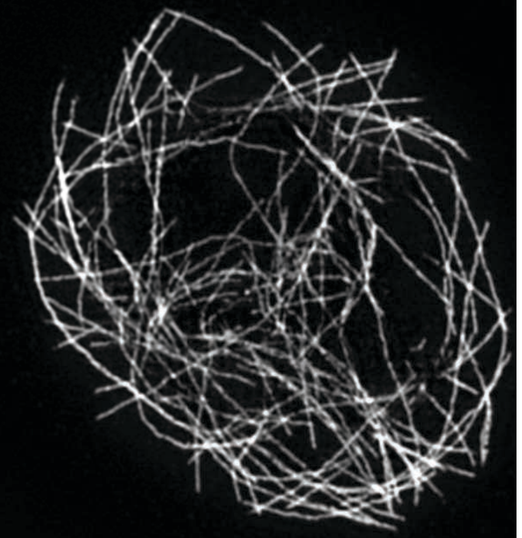
1. Fixation: formaldehyde (PFA), methanol, …
2. Permeabilisation: detergent (Triton-X), methanol
3. Washes: PBS(T), with mild detergent (Tween-20)
4. Blocking: BSA, fish skin gelatine, milk, …
5. Primary antibody labelling (1h @RT or o.n. @4°C)
6. Washes: PBS(T)
7. Secondary antibody labelling (1h @RT)
8. Washes: PBS(T)
9. Optional: post-fixation (formaldehyde)
10. Optional: counterstain (DAPI, Hoechst, SYTOX, …)
11. Mounting: (e.g. Vectashield, ProLong Diamond, …)



Fixation: preservation of cells or tissue in a life-like state

1. Preserve structural features,
   No swelling or shrinkage of cells or organells,
   no loss of essential proteins or other molecules
2. Uniform fixation throughout the sample
3. Enable dye labeling
4. Minimise background fluorescence





Microtubules in *Drosophila* macrophages

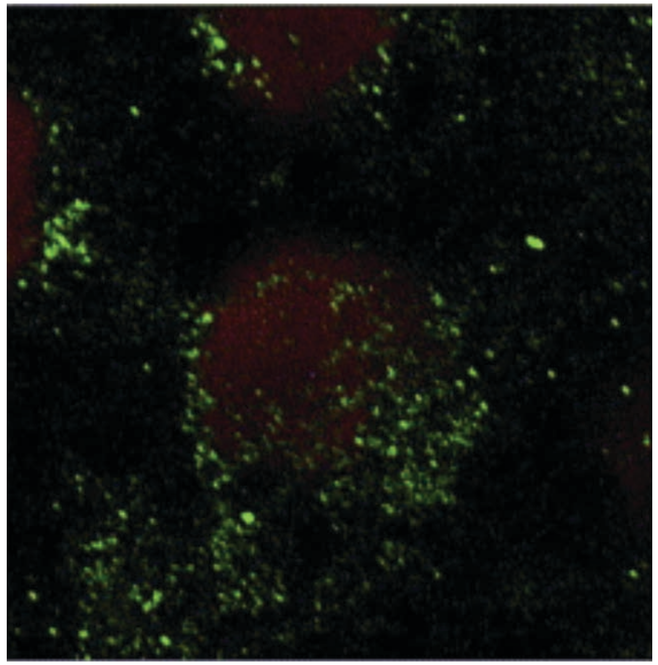
Left: Live cells expressing Jupiter-GFP

Right: PFA fixed and stained with anti-tubulin primary and AlexaFluor 488 conjugated secondary antibodies

Two types of fixation for fluorescence microscopy

Dehydration

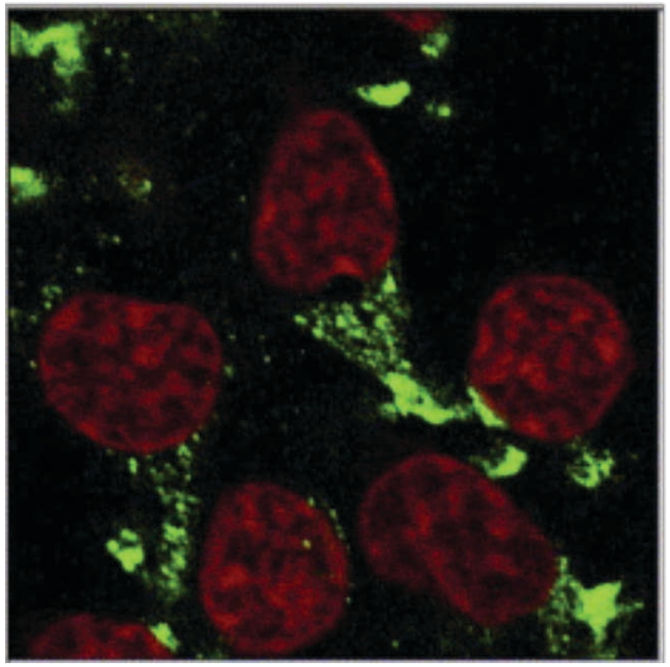
Methanol (100%, -20°C, 5 min)



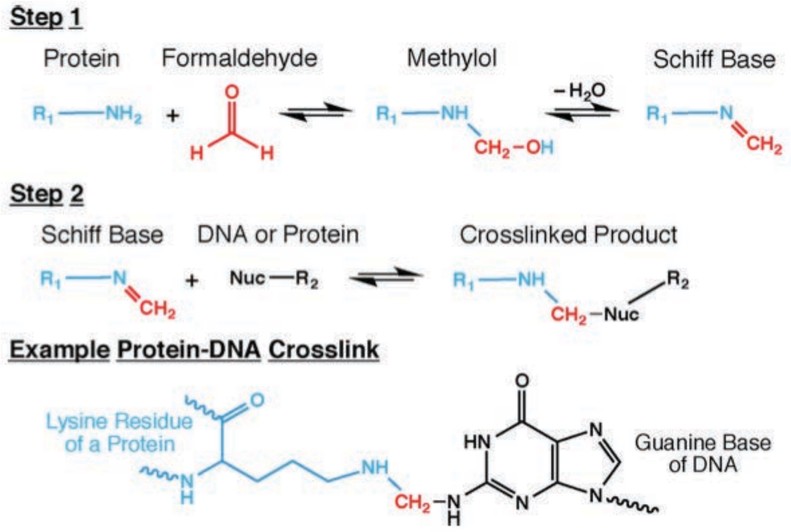
- Dissolves lipids into micelles
- Precipitates proteins and affect 3D structure, but leaves epitopes intact
- No additional permeabilisation
- Fast

Cross-linking

Formaldehyde (2-4% in PBS, 10-15 min)

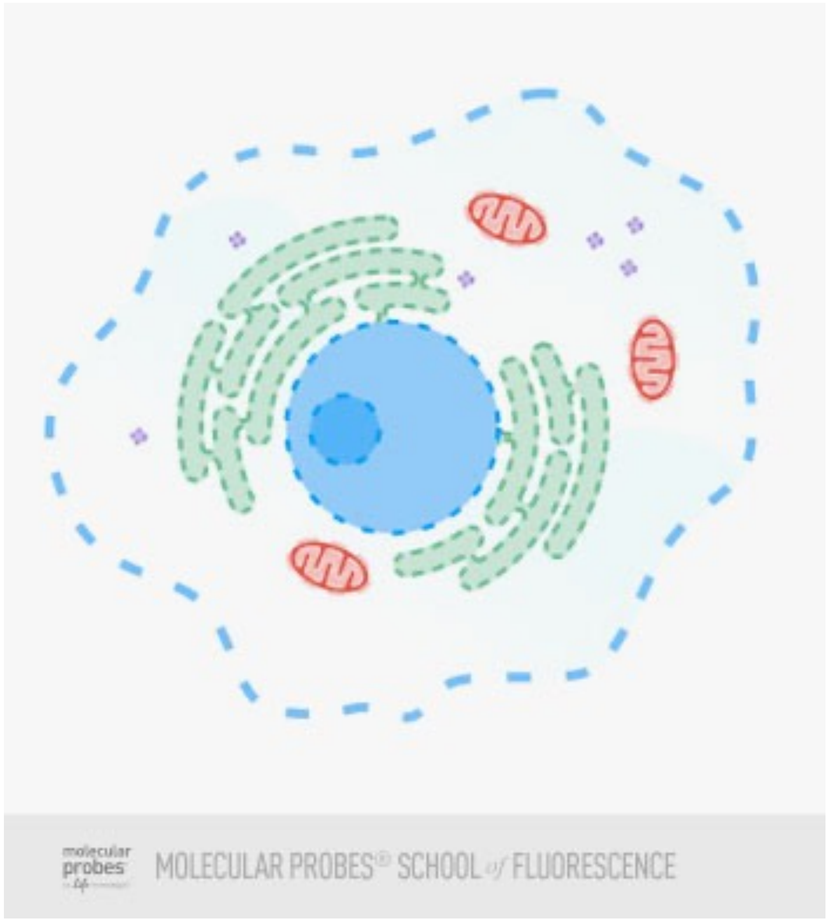


- Crosslinks proteins, DNA and lipids, but leaves membranes intact;
- Cell morphology is well maintained
- Epitopes may be modified
- Relatively slow



Hoffman et al. 2015, JBC

Permeabilisation

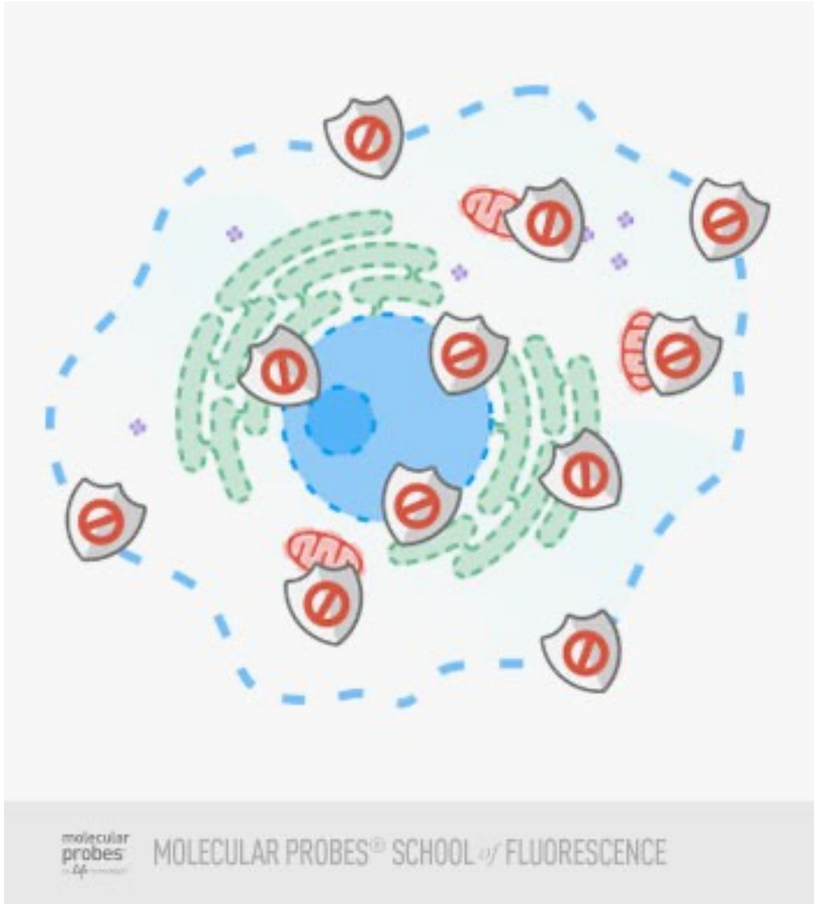


0.1-0.5% **Triton X-100** (Tween 20, Nonidet P-40)

↓

Removal of lipids; access of antibodies/dyes into fixed cells & tissues; reduce surface tension

Blocking



10% **BSA** (serum, fish skin gelatine, casein, milk)

↓

Reduction of non-specific antibody binding by excess of protein

Permeabilisation

Aim: to allow fixative to enter the cells/tissue more quickly if necessary
to allow antibodies to penetrate fixed cells/tissue
done by removing lipids with detergents

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

Nonionic detergents:

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

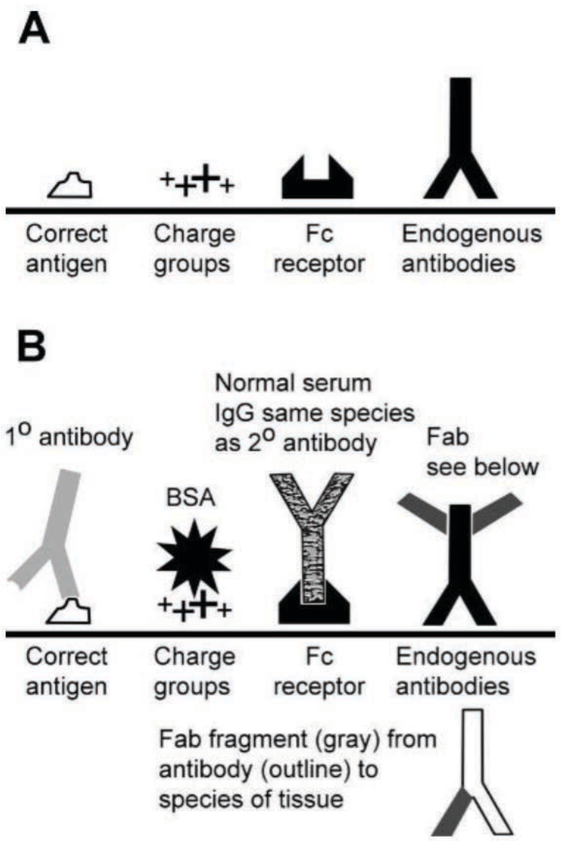
Triton X-100: used to permeabilise unfixed or lightly fixed eukaryotic cell membranes (0.1% in PBS)
Tween 20: milder than Triton X-100, used to reduce surface tension in blocking, antibody incubation and wash steps (0.1%)
Nonidet P-40 (Igepal Ca-630 from Sigma-Aldrich): used to permeabilise unfixed cells (0.1% in PBS for 5-10s)

Ionic detergents:

- have highly charged hydrophilic groups and are very effective at solubilising membranes, but also destroy native three dimensional protein structures

SDS, deoxycholate, CHAPS
Not used for immunocytochemistry

Blocking



Aim: to allow binding of antibodies only to appropriate sites

Sources of nonspecific binding:

Charged groups

Occur on proteins (esp. histones) or lipids

Also generated by fixation in formalin or glutaraldehyde

To block use bovine serum albumin at 10-30mg/mL (fraction V)

Fc receptors

On macrophages and other immune cells, which bind any antibody

To block whole IgG 1° and 2° antibodies from binding to Fc receptors, incubate cells in buffer containing 5-10% normal serum from the host species of the 2° antibody

Endogenous antibodies

Only a problem for 2° antibodies recognising the same species as your tissue/ cells and only at inflammation sites or in cell cultures of immune system cell types

To block use Fab fragments raised in the same species as the 2° antibody that recognise the species of your tissue/cells as part of the blocking procedure

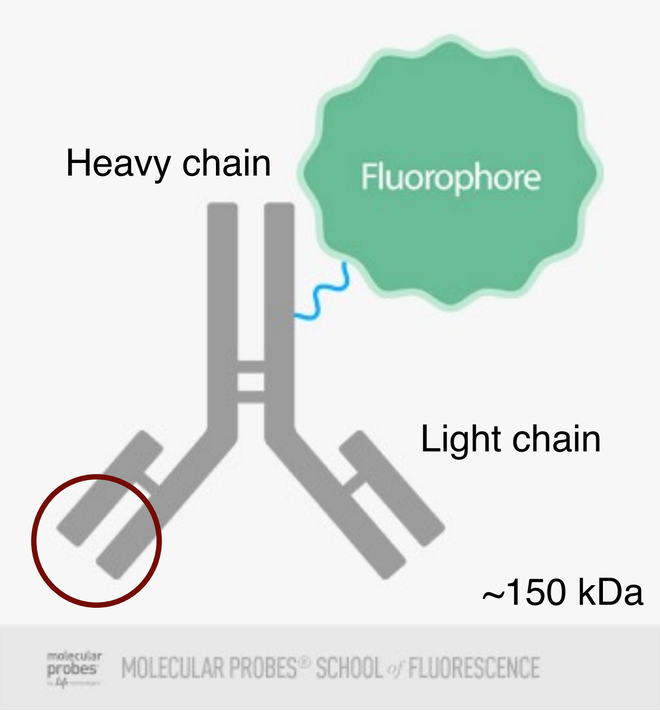
For general blocking can also try MAXblock (Active Motif): protein based, non-mammalian blocking agent, no cross-reactivity with 2° antibodies

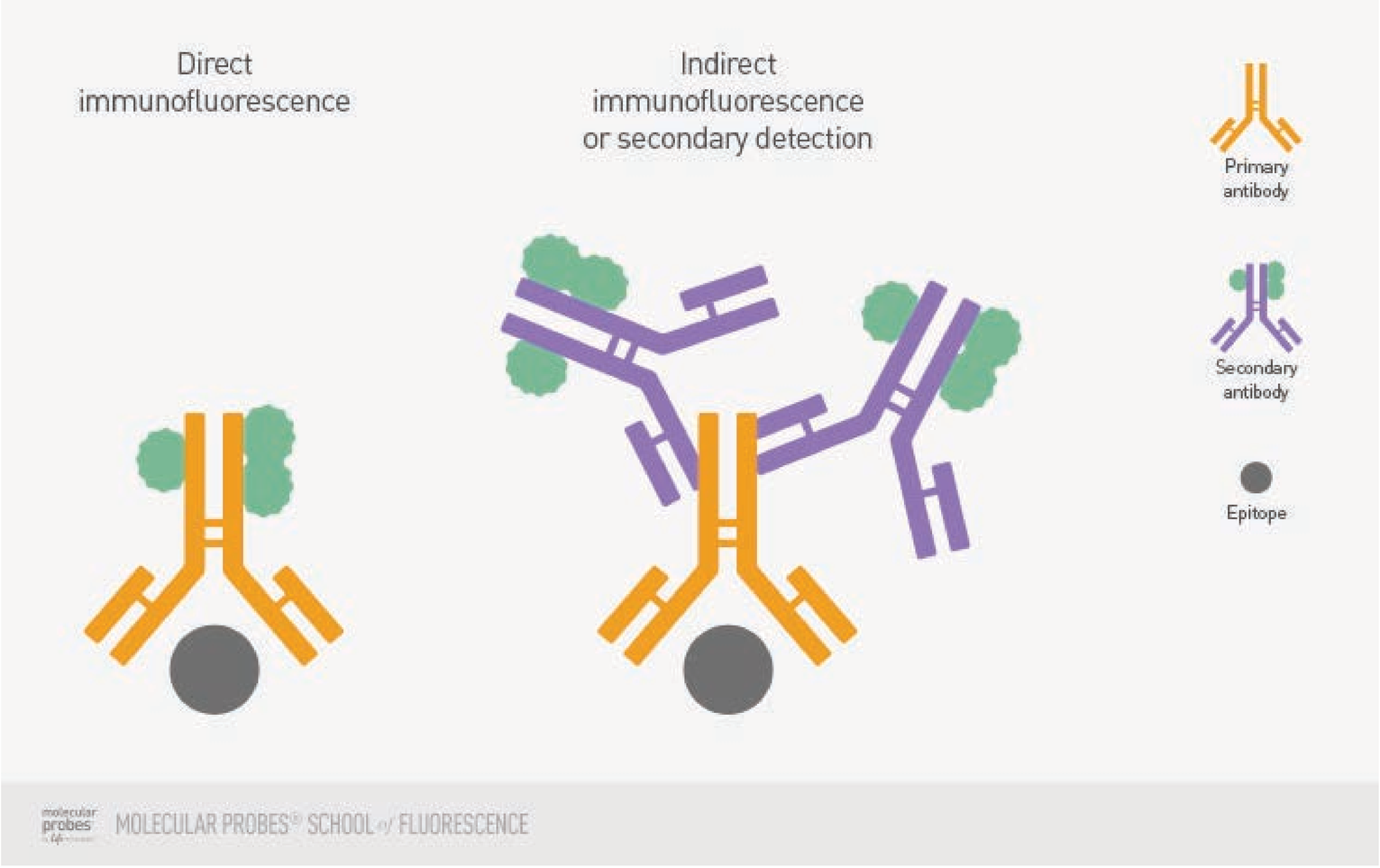
Immunofluorescence detection

Antibodies (class IgGs) recognise unique target sites (**epitope**).

Constant region (conserved)

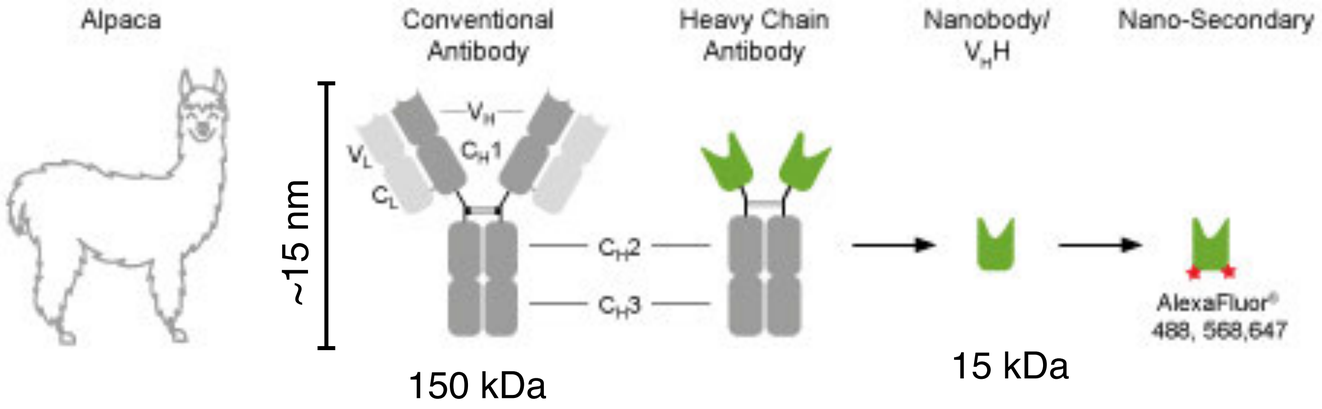
Variable antigen binding region



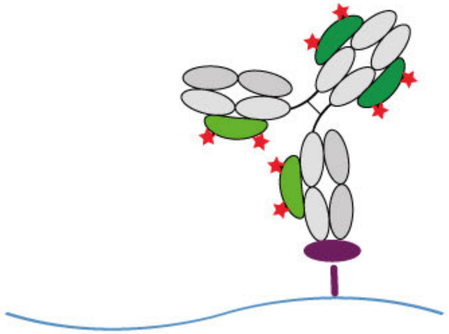


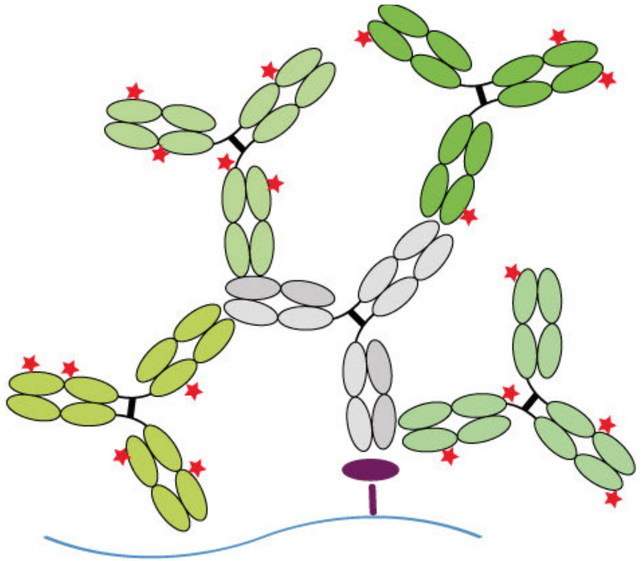
Antibody detection with secondary nanobodies

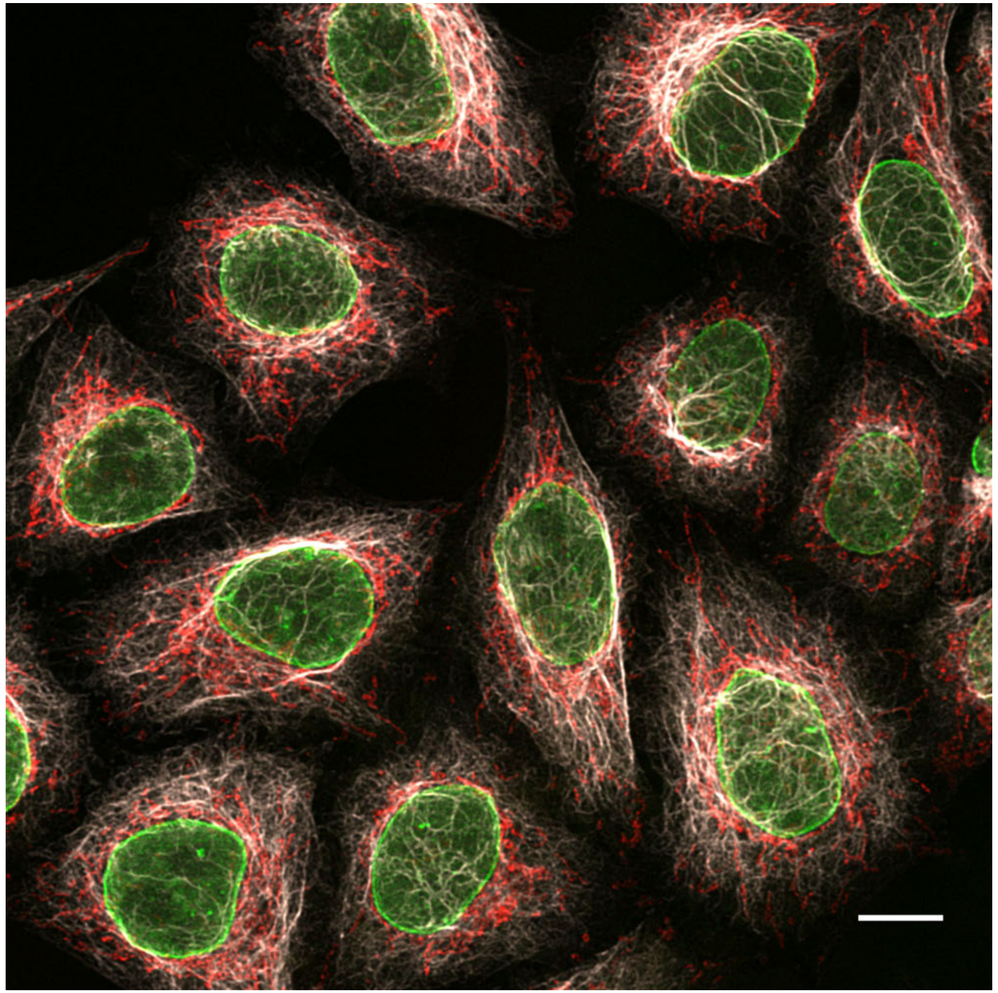
chromotek
new tools for better research
part of Proteintech Group



(Size comparison)







HeLa cells with 3 subclass-specific alpaca anti-mouse Nano-Secondaries.
Grey: Mouse IgG1 anti-Vimentin + alpaca anti-mouse IgG1 VHH Alexa Fluor® 647

Four generations of fluorescent dyes

1st Generation 1942 onwards: Fluorescein, fluorescein isothyocyanate (FITC) and rhodamine
- Bleach very quickly and are quenched when bound to antibodies

2nd Generation 1993: Cy fluorophores, cyanine dyes Cy2, Cy3, Cy5 (Jackson Immuno Research), AMCA, Texas Red
- More photostable and high quantum yields

3rd Generation 1999: Alexa Fluor dyes (Life Technologies, Molecular Probes), ATTO dyes (Sigma, ATTO-Tech), DyLight Fluor (Thermo Scientific, Pierce)
- High photostability and high quantum yields
- Very wide range of excitation wavelengths

4th Generation 2003: Quantum dots, heavy metal nanocrystals (Life Technologies, Molecular Probes)
- 8 – 30 nm in size (IgG with Alexa Fluor 4 nm)
- Excited at low wavelengths and emitting at high wavelengths
- Do not photobleach
- Penetration problems because of their size

Increase specific signal, reduce background

- Important for accurate biological interpretation & to improve signal-to-background.
- Brighter is not automatically better, if the background is also increased!
- Mind potential auto-fluorescence of your embedding medium!
- Unspecific labelling reduces contrast and generate false positive signals.
- Cross validation, e.g. antibody vs. genetic fusion!
- Thoroughly wash your samples (immunofluorescence labelling)!

- Wash with agitation (unless your cells dislodge easily) for 5-10 min for each wash step
- Wash 7 times leaving 10-20% of the buffer each time to prevent drying of your cells/tissue
- Or wash 3 times removing all buffer and replacing it immediately
- If cells/tissue dry out in between washes background is increased and cannot be removed

Washes after the 1° antibody

- Incomplete removal of the 1° antibody does not increase background but lowers the amount of specific labelling because the 2° antibody reacts with the 1° in solution decreasing its conc.

Washes after the 2° antibody

- Incomplete removal of the 2° antibody increases background

Mounting matters - importance of refractive indices (RI)

Slide

Mounting medium

n=1.33 - 1.49

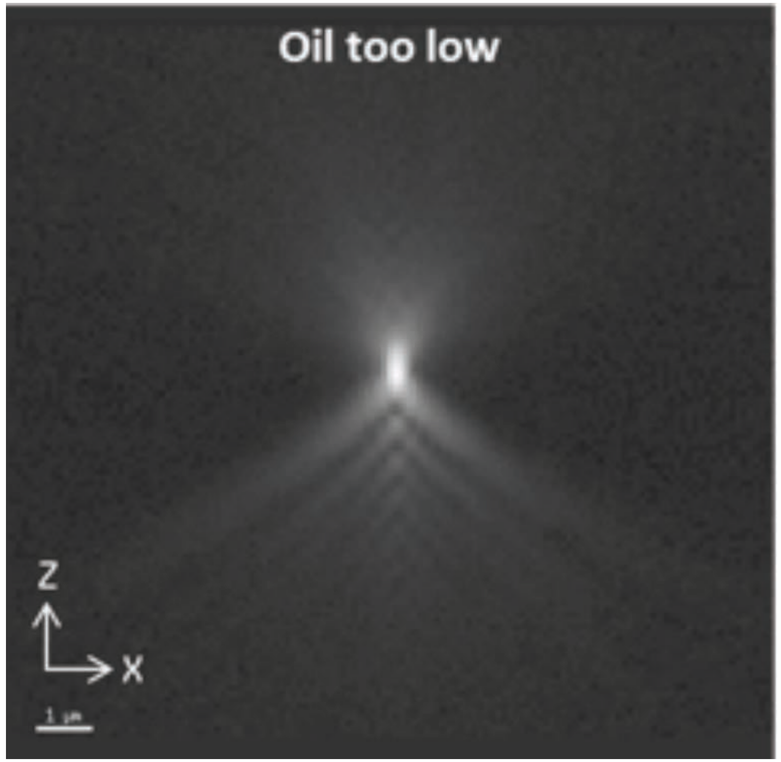
Cover glass

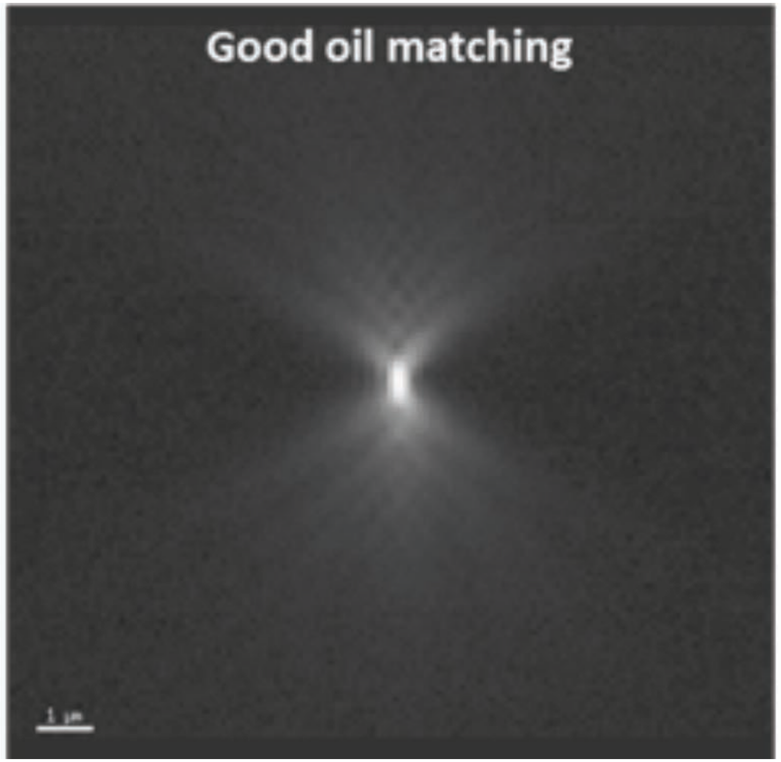
n=1.518

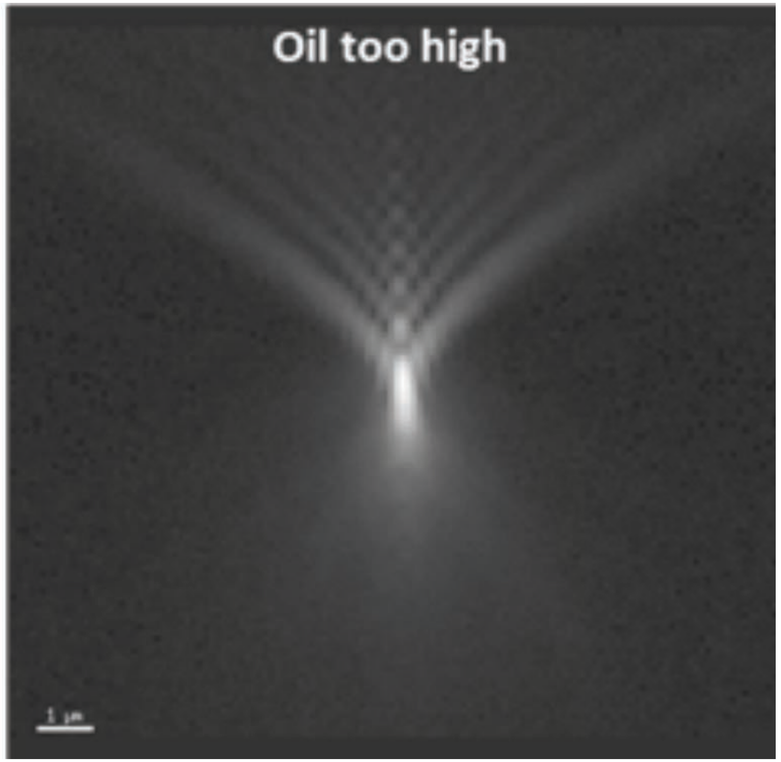
Immersion oil

n=1.510-1.518

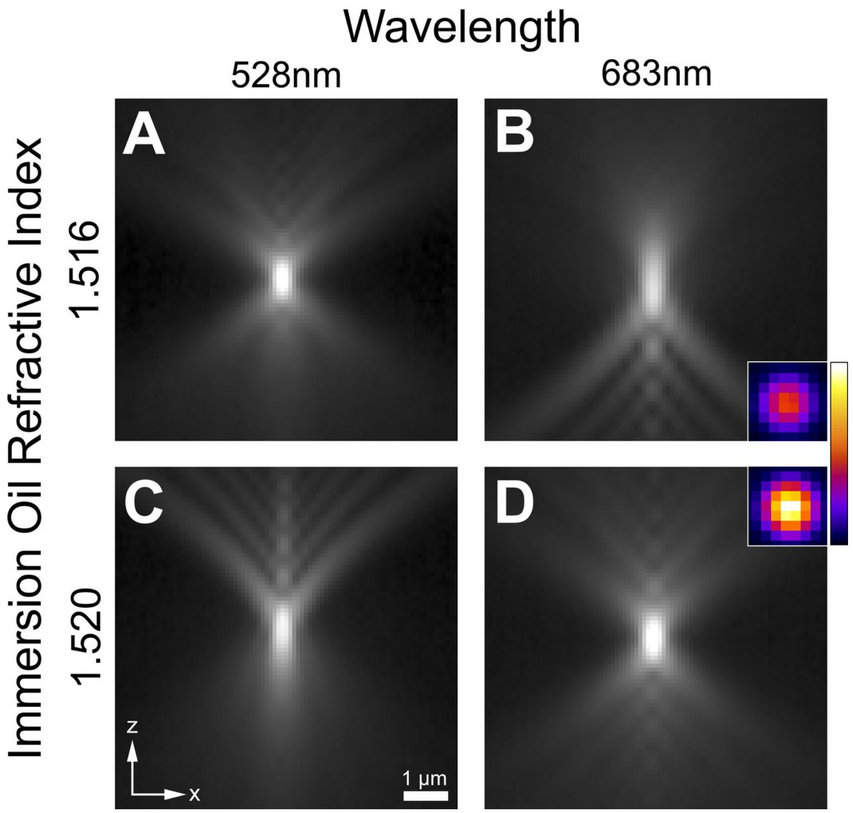
Spherical aberration - fixed by immersion oil



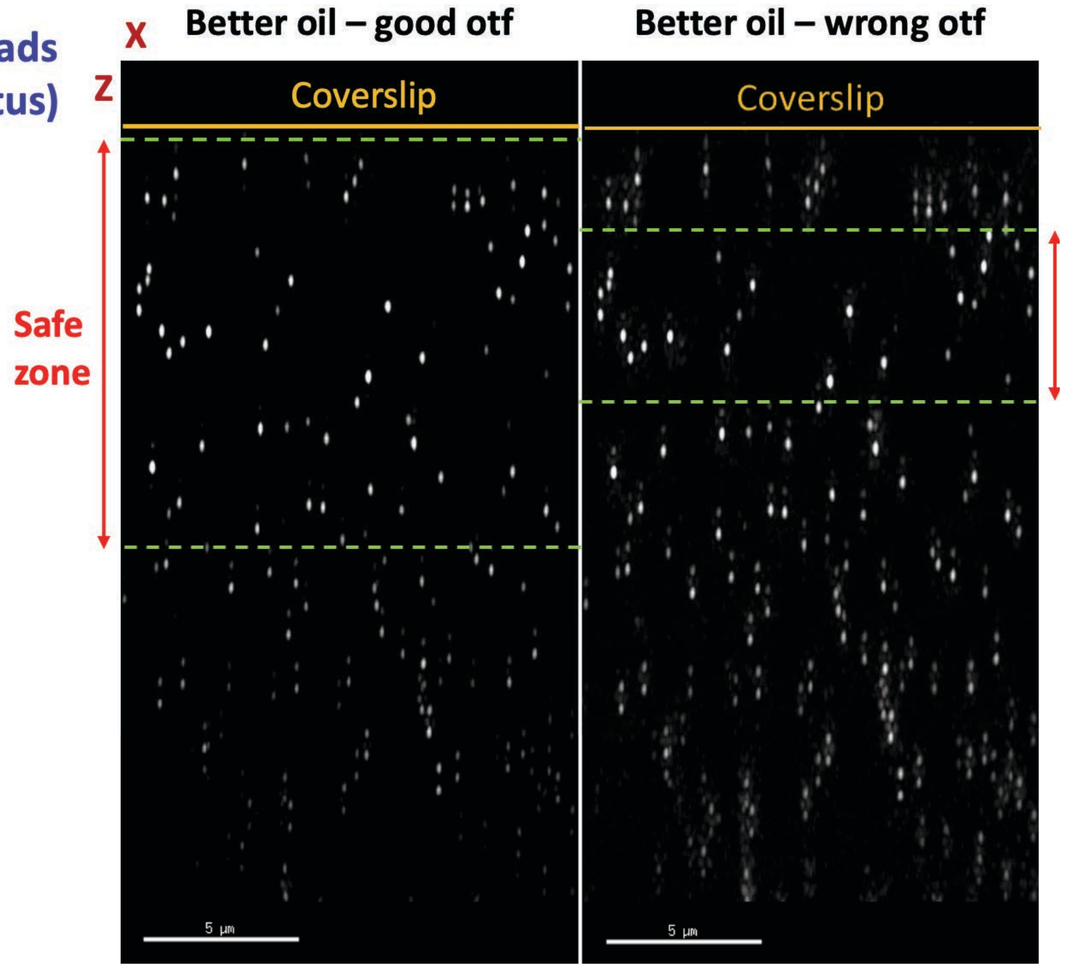




Wavelength and depth dependent spherical aberration - fixed by immersion oil



100 nm sub-resolution beads mounted in CyGel (Biostatus)

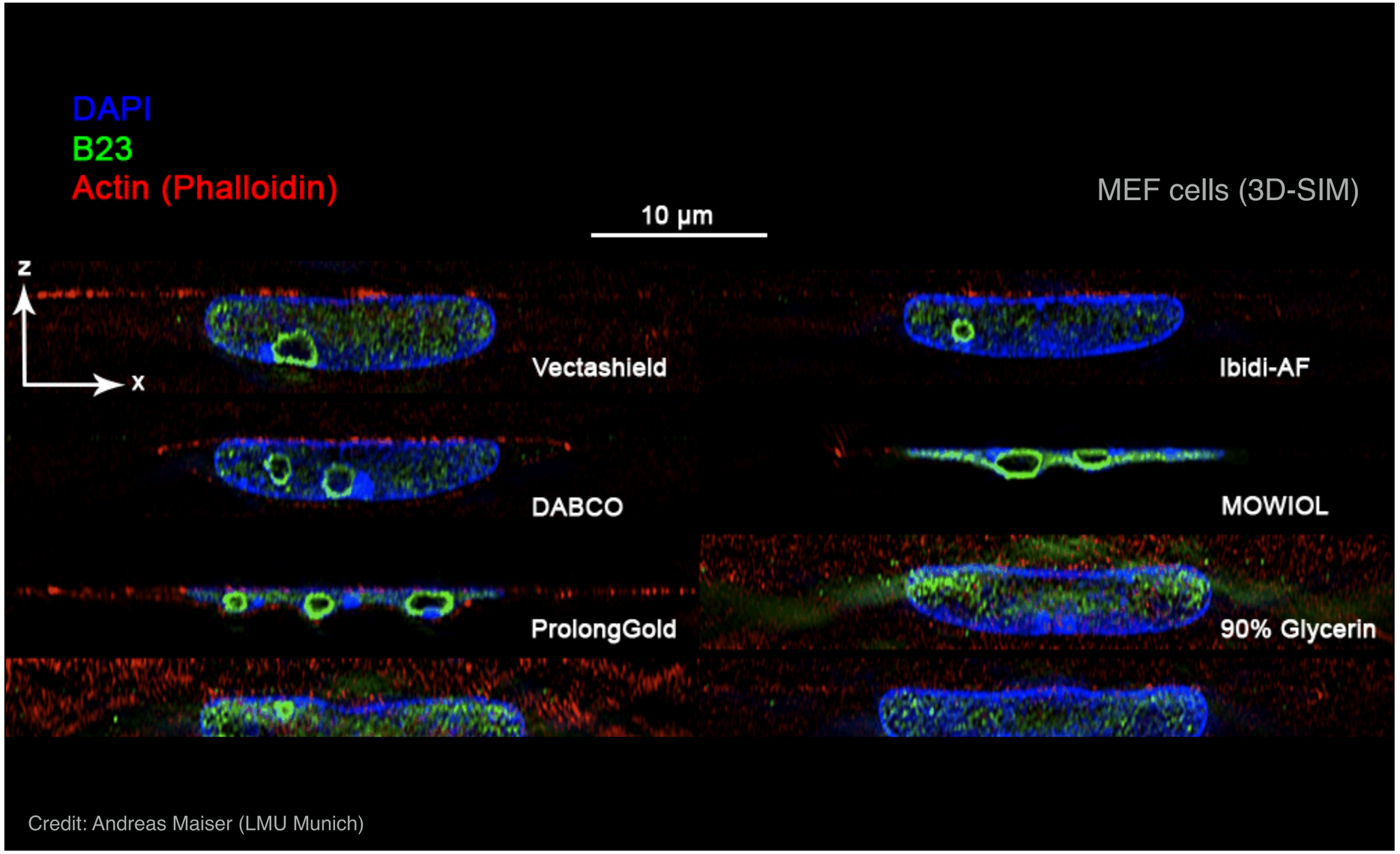


(3D-SIM)

Talley et al. 2016, JCB

Alison North

Mounting matters - 3D morphology preservation



Credit: Andreas Maiser (LMU Munich)

Criteria:

- Buffer (Tris-HCl pH 8-8.5)
- High refractive index (1.4-1.5)
- Antifading/reducing agents
- No auto-fluorescence

Non hardening MM:

Glycerol + buffer + antifade (Vectashield, Slowfade, homemade w/ DABCO)

- Needs sealing
- Storage for a few weeks (4°C)

Hardening MM:

PVA + buffer + antifade (ProLong Gold/Diamond/Glass, Vectashield hardset, homemade w/ DABCO)

- Can flatten cells/specimen
- Storage for many months (RT)

Experimental controls are key for reliable results

- ☑ No primary or secondary antibody (autofluorescence)
- ☑ Incubate with secondary but not primary antibody
- ☑ Check cross-talk between dyes and microscope filterset
- ☑ Test specificity in knock-out /knock-down cells

Sample preparation - Summary

Biological question

?

1 *Experiment design*

- Dyes (spectra, photo-stability)
- Labelling method (FPs, IF, FISH,....)
- Labelling specificity (antibodies)
- Signal-to-noise / background

2 *Labelling*

- Optical quality (coverslip, cleanness)
- Refractive index mismatch
- Embedding medium, RI immersion
- Imaging depth

3 *Sample*

4 *Microscope*

- Mechanical stability
- Photon efficiency
- Modulation contrast / calibration
- Camera: (EM)CCD / sCMOS

5 *Post-processing*

- PSF/OTF (λ-, depth-, RI-dependent)
- Channel alignment
- Quality control

6 *Quantitation, Visualisation, Data storage*

- Quality control
- Co-localisation
- Segmentation
- Tracking
- Distances
- 3D-rendering

Biological interpretation

Quality is paramount at every step!

Every little helps

Sample preparation - further reading

http://www.olympusmicro.com/
Very comprehensive and well written

http://micro.magnet.fsu.edu/primer/anatomy/anatomy.html
Very comprehensive

Immunocytochemistry a practical guide for biomedical research
Richard W. Burry, Springer 2010
http://www.jacksonimmuno.com/technical

Molecular Biology of the Cell, fifth edition.
Alberts et al. Chapter 9: Visualizing cells, page 579-616

North AJ. Seeing is believing? A beginners' guide to practical pitfalls in image acquisition. J Cell Biol. 2006 Jan 2;172(1):9-18. doi: 10.1083/jcb.200507103. PMID: 16390995; PMCID: PMC2063524.

Lambert TJ, Waters JC. Navigating challenges in the application of superresolution microscopy. J Cell Biol. 2017 Jan 2;216(1):53-63. doi: 10.1083/jcb.201610011. Epub 2016 Dec 5. PMID: 27920217; PMCID: PMC5223610.

Kraus et al. Quantitative 3D structured illumination microscopy of nuclear structures. Nat Protoc. 2017 May;12(5):1011-1028. doi: 10.1038/nprot.2017.020. Epub 2017 Apr 13. PMID: 28406495.