Basic Fluorescence Microscopy and Sample Preparation

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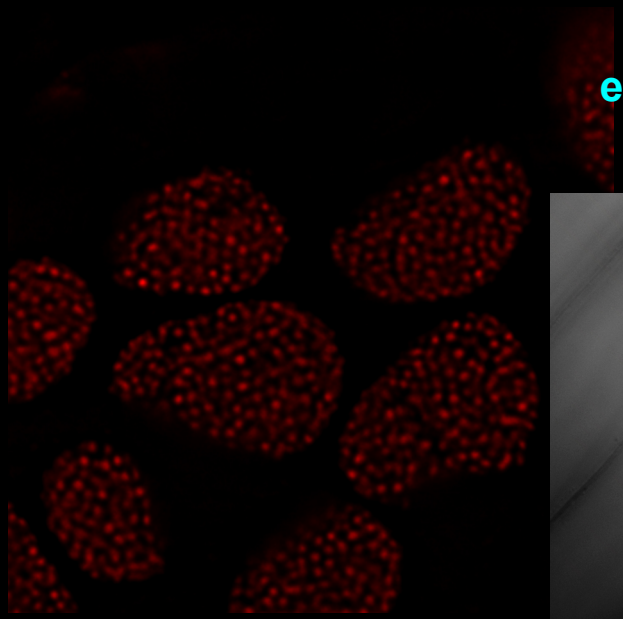
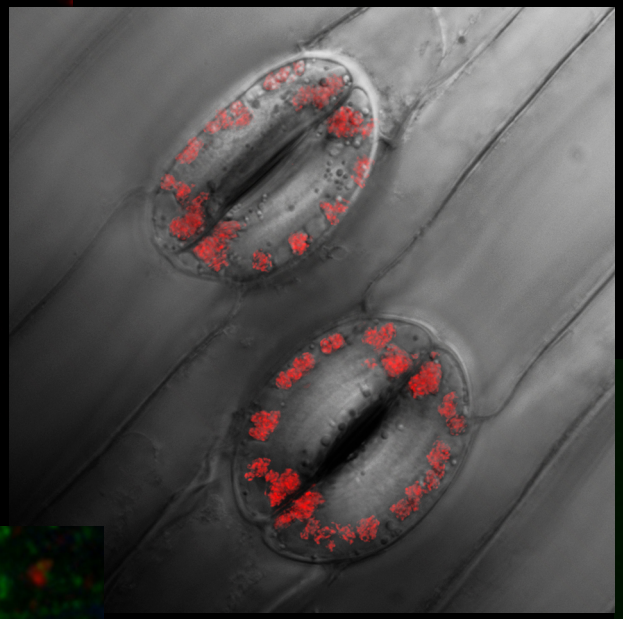
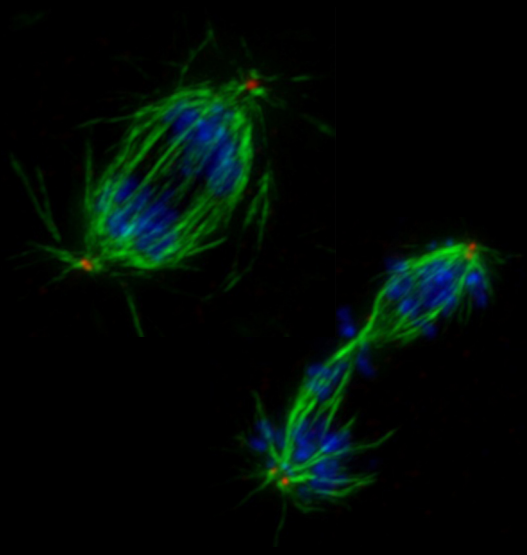
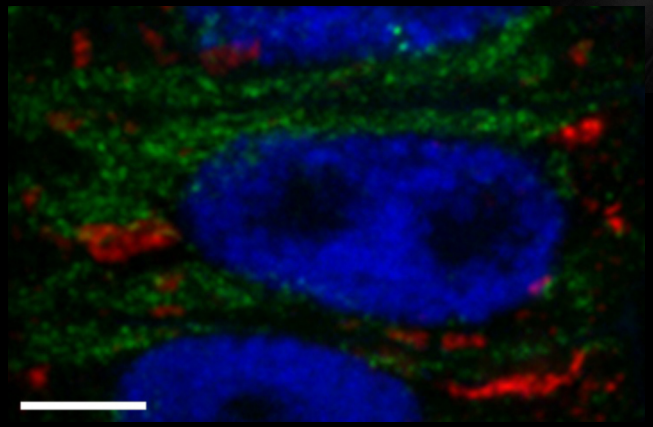
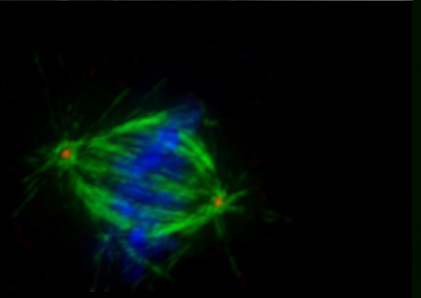
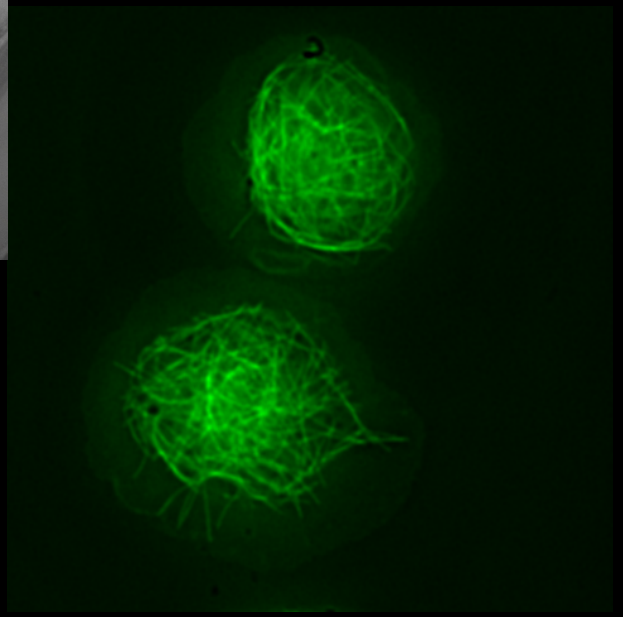




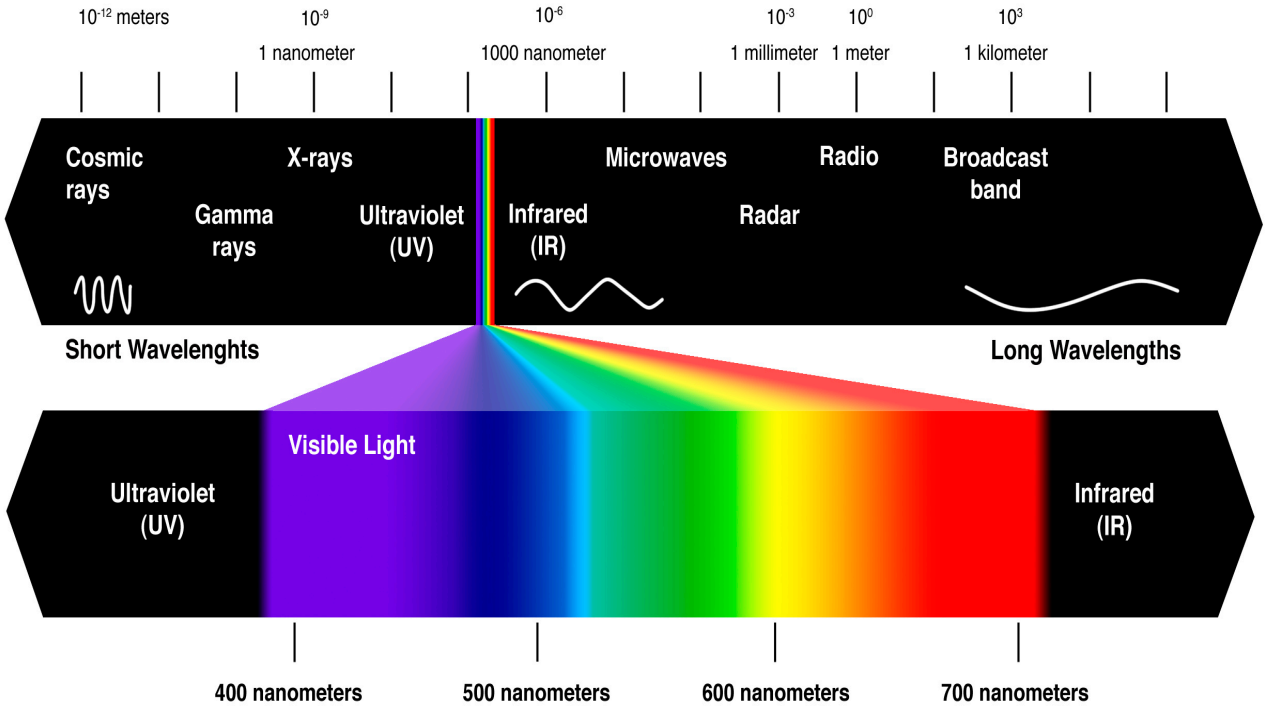








Visible Light

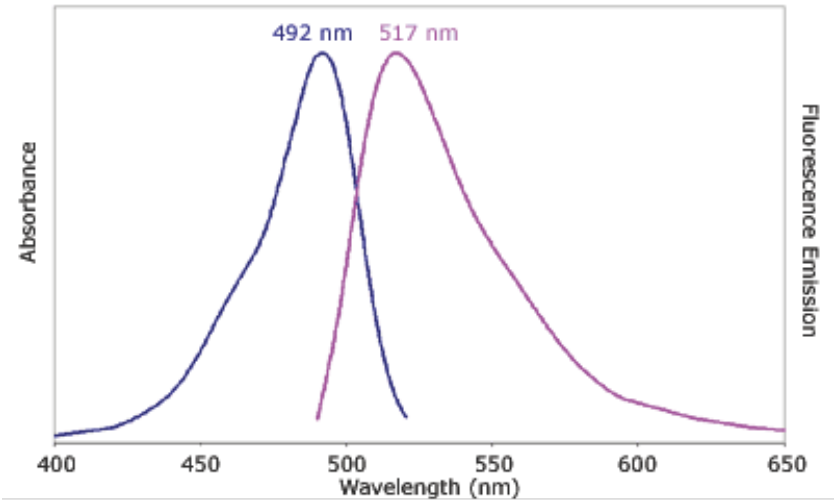


390 – 700 nm visible to the human eye
White light is split into its components through a prism
Reason: different λ refract at different angles

What is fluorescence?

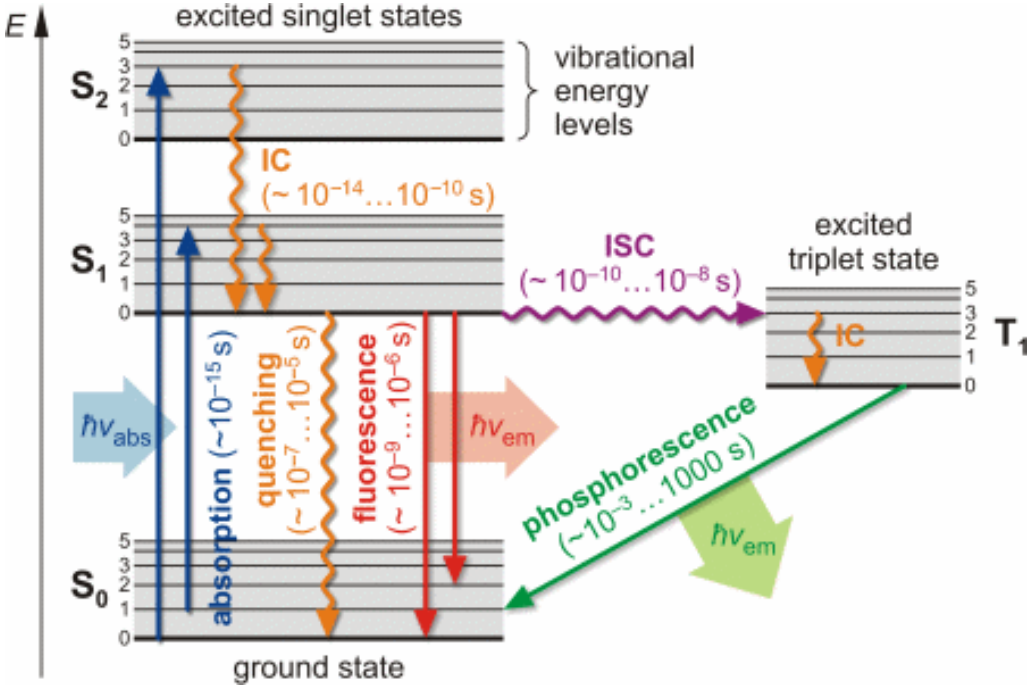
George Gabriel Stokes (1819-1903)

Stokes Shift



Alexa Fluor 488

Jablonski diagram



Photoluminescence:

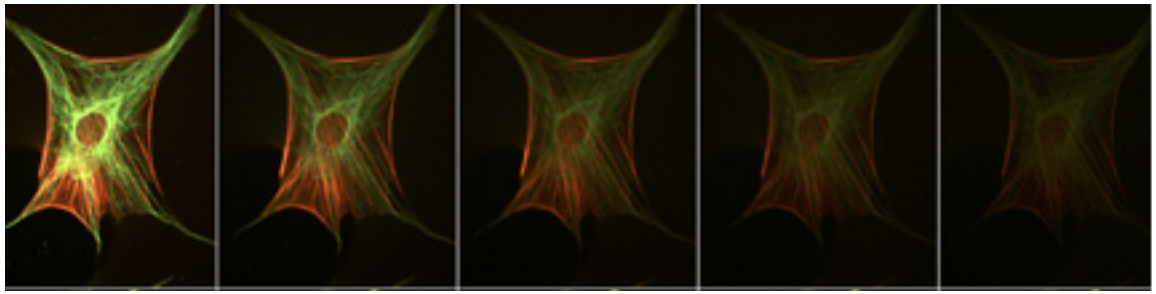
Fluorescence - spontaneous emission of light during transition of the system from its lowest vibrational energy level of an excited singlet state S_1 back to the ground state S_0 (10^{-9} to 10^{-6} s)

Phosphorescence – a non-radiative transition into an isoenergetic vibrational level of a triplet state T_1 , which lasts for 10^{-3} to 1000 s before it decays to the ground state

IC: internal conversion
ISC: intersystem crossing

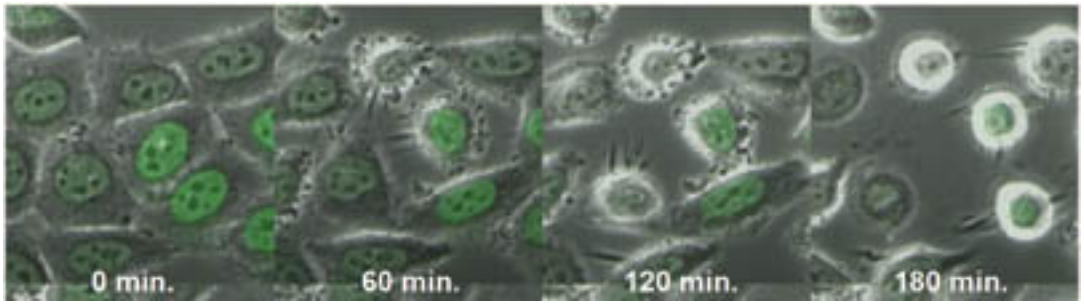
Photobleaching and Phototoxicity

Photobleaching: photochemical destruction of the fluorophore
In an excited triplet state, fluorophores may interact with another molecule to produce irreversible covalent modifications



Phototoxicity: illumination of a fluorophore causes damage to the cell expressing it, eventually leading to cell death
Common situation: the excited dye molecule passes its excess energy on to O_2, creating reactive oxygen species (ROS):

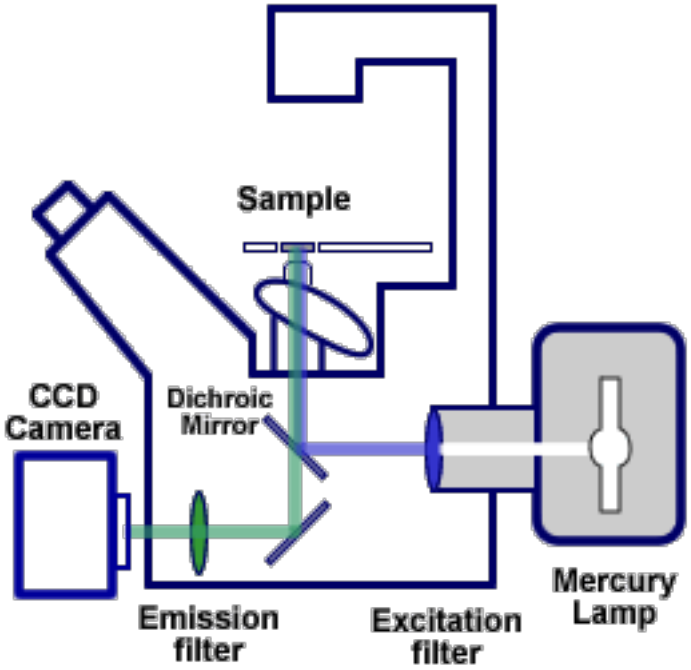
- ROS reacts with dye ⟶ dye bleaches
- ROS diffuses away and reacts with other dyes or cell components

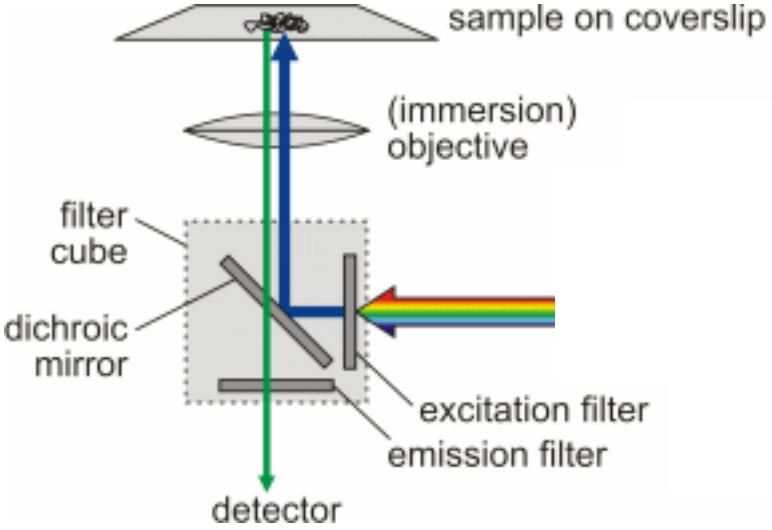


Solution:

- Reduce the intensity of the excitation light and frequency of illumination
- Close down the field aperture in order to restrict the illuminated area

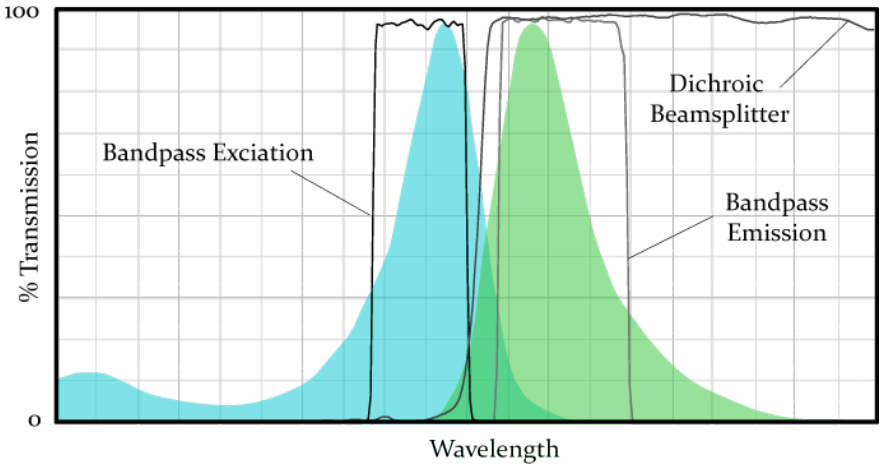
Basic Principle of an (Inverted) Fluorescence Microscope





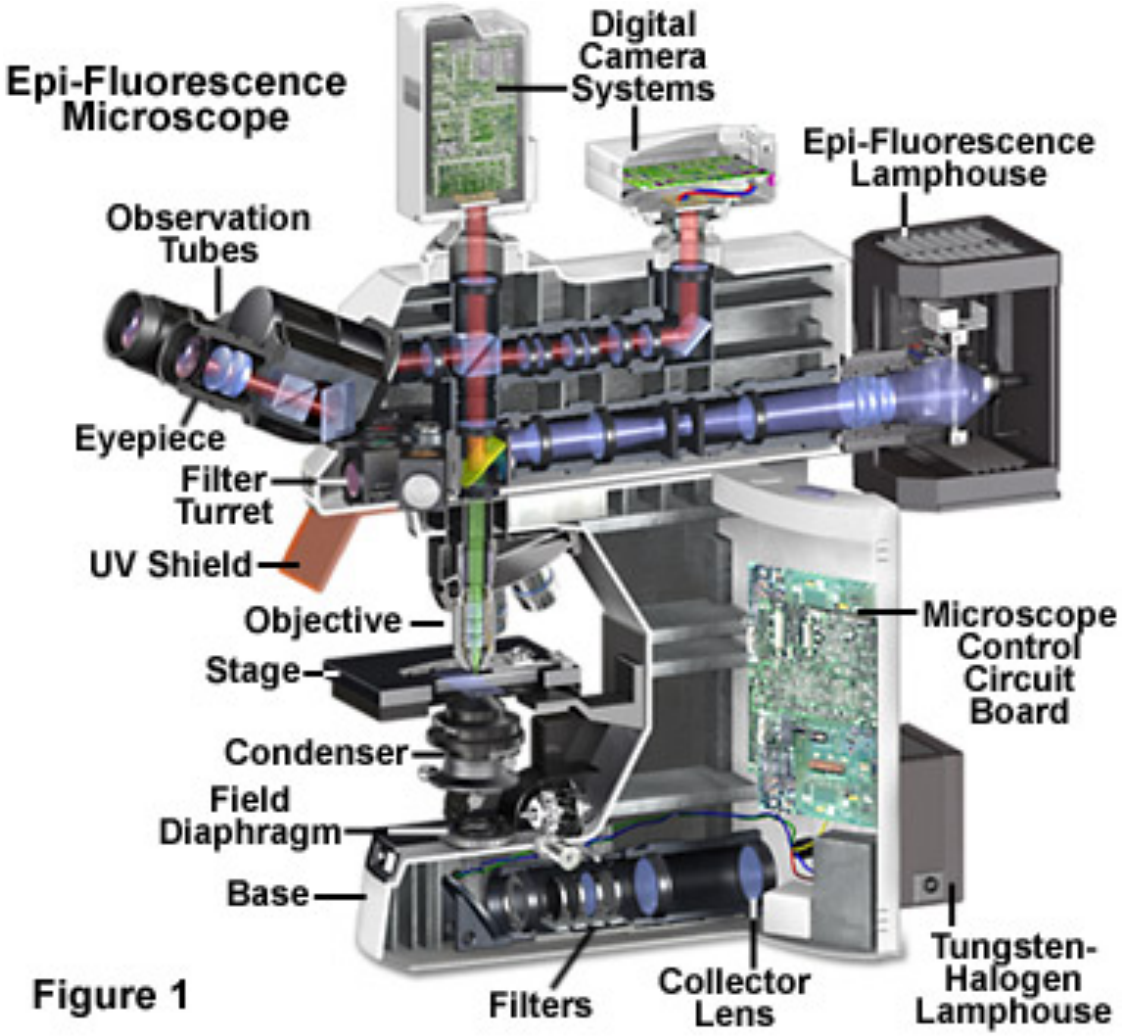
Fluorescence microscope:
Fluorescence light source
Excitation filter
Dichroic mirror
Objective
Emission filter
Camera/eye pieces

Ideal filter cube properties



Excitation
Dichroic
Emission

Upright Fluorescence Microscope



Used for fixed samples on slides and for live imaging where the objective is immersed in the medium

Inverted Fluorescence Microscope

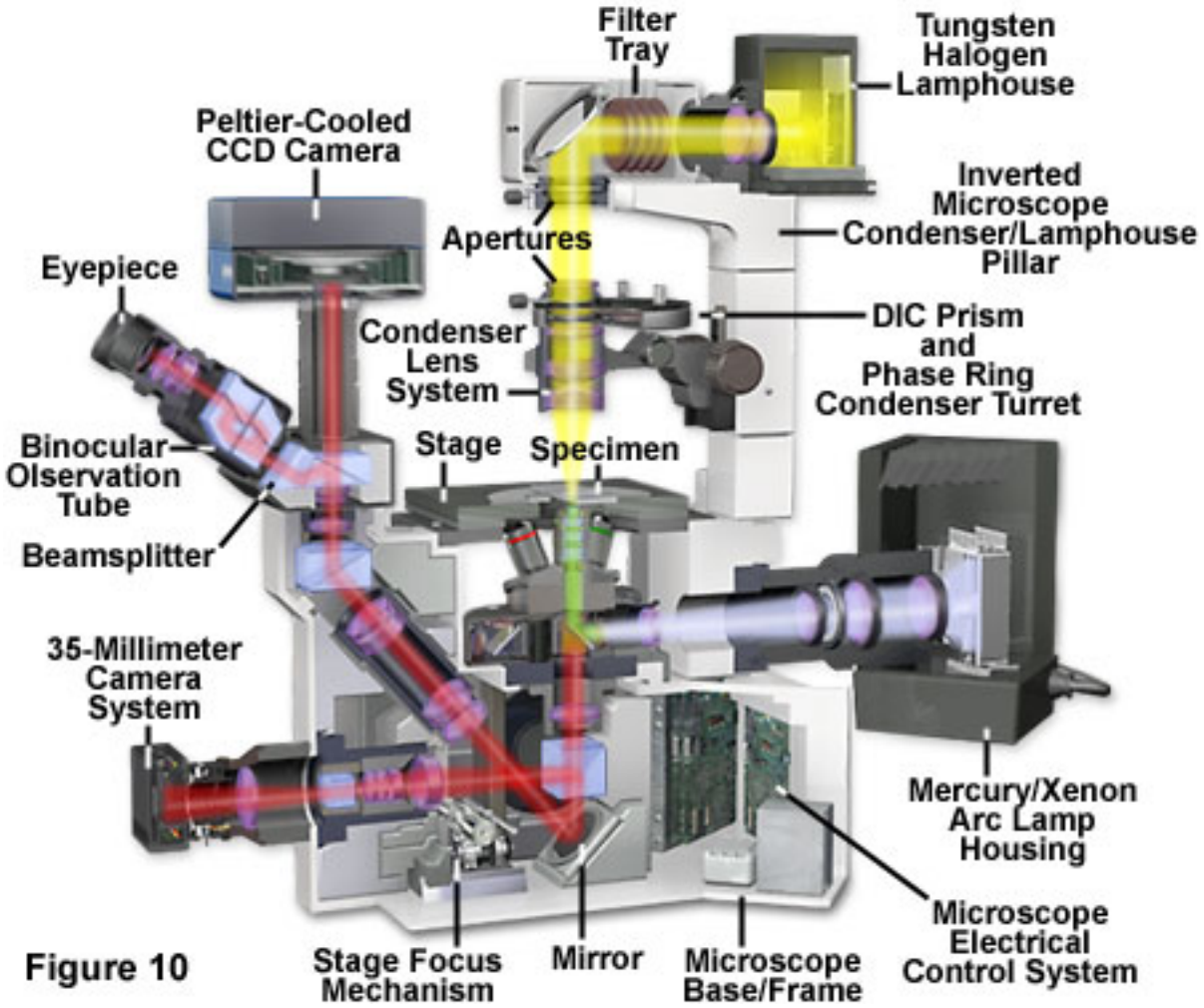


Figure 10

Used for live imaging through a coverslip and for fixed samples on slides
More versatile but danger of oil running down the objective

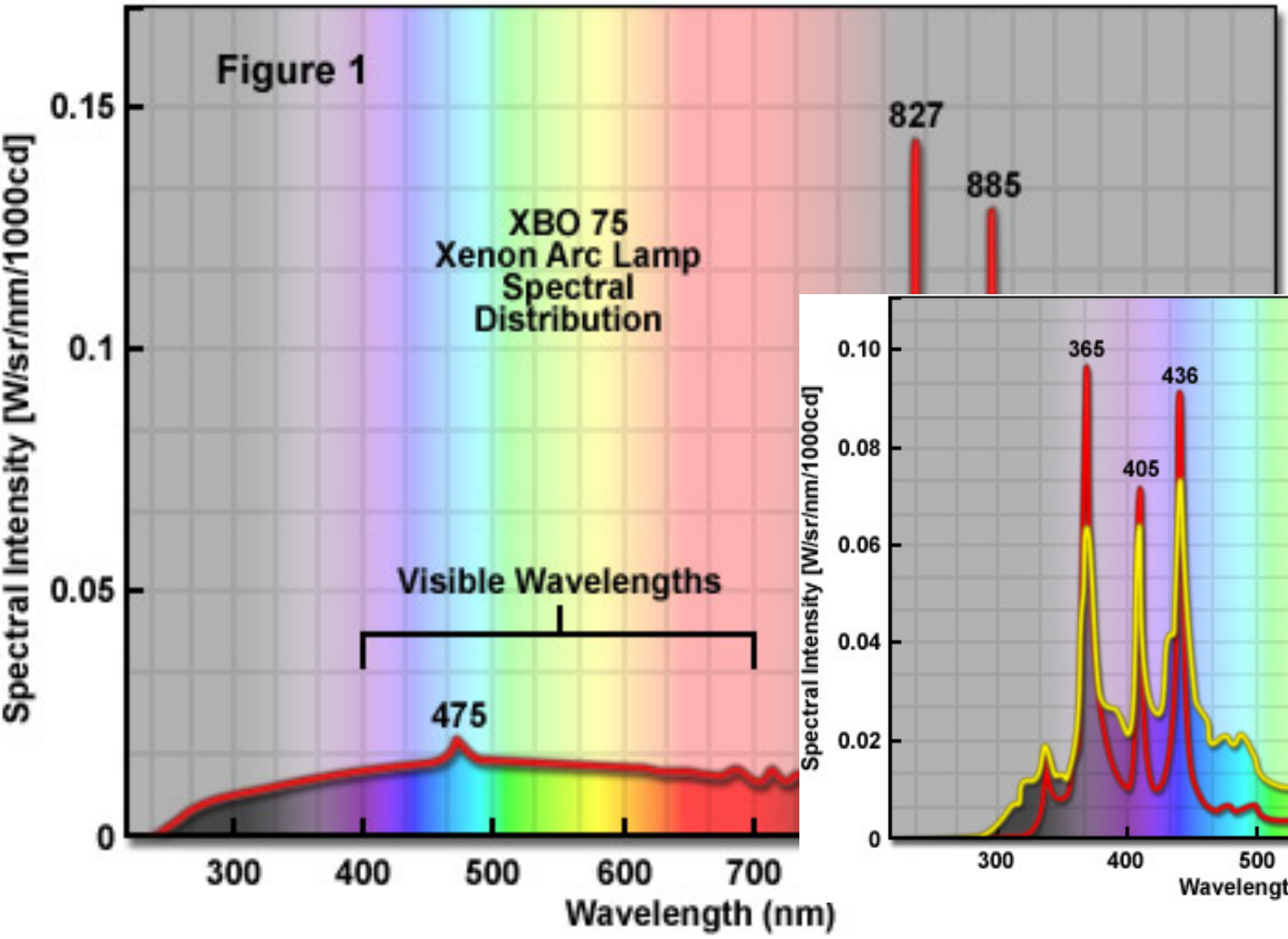
Fluorescence Light Sources

Mercury lamps (old-fashioned): 400 h life time, manual adjustment necessary

Xenon lamps: 800 h life time

Metal halide lamps: 2000 h life time, easy to fit

Spectra of Mercury, Metal Halide and Xenon lamps



Fluorescence light sources cont.

LED lights (state of the art):
bandwidth ≥ 10 nm, λ above 360 nm, 5000 h life time

Lasers (specialised wide-field fluorescence applications, confocal, multiphoton):
Narrow beams of highly monochromatic, coherent and collimated light

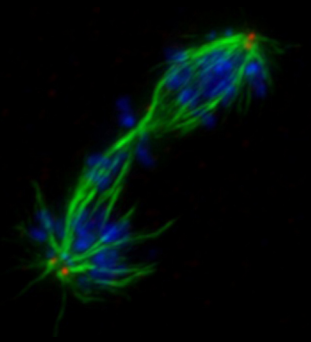
Fluorescence detectors

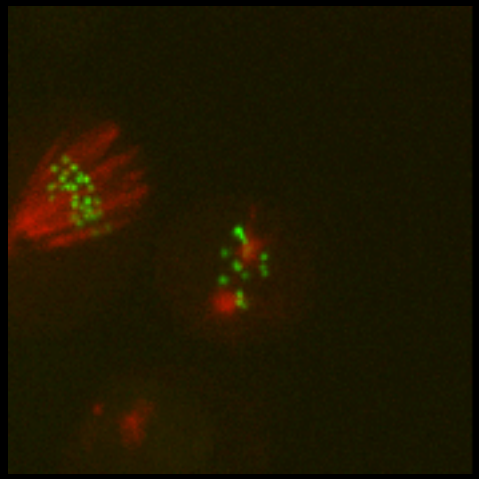
Cooled CCD cameras (Lecture 10)
EMCCD cameras (Lecture 10)
sCMOS cameras (Lecture 10)
PMTs (Lecture 8)

Preparation of Fixed Samples

Why use fixed samples at all?

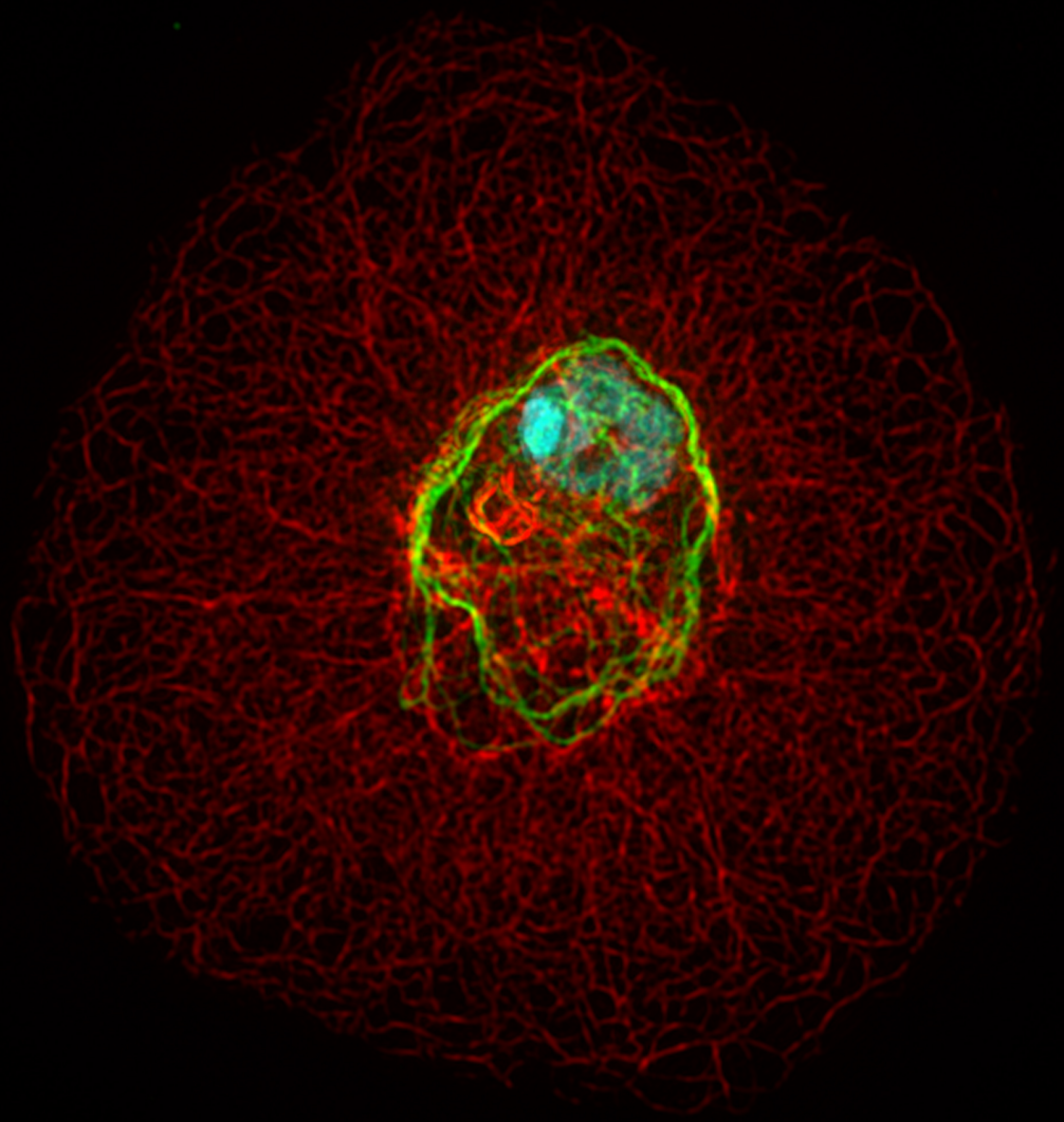
- Primary cells cannot easily be transfected and transgenic animals are time-consuming to produce and not always possible
- Brighter than fluorescent fusion proteins
- Injection of antibodies only possible with big cells (e.g. oocytes)
- Can detect four different labels or even more at the same time
- High-throughput screening





Typical Immunocytochemistry Protocol

Fixation
Permeabilisation
Washes
Blocking
1° antibody
Washes
2° antibody
Washes
Mounting



Antibodies for Immunocytochemistry

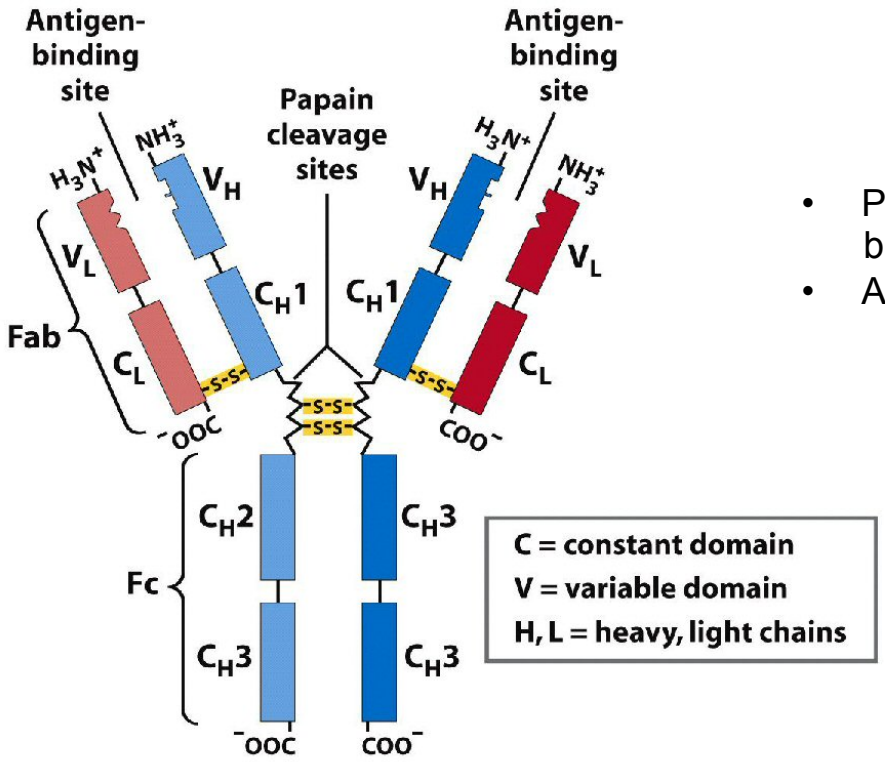


Figure 5-21a
Lehninger Principles of Biochemistry, Fifth Edition

- Preferred: IgG isotype, more consistent generation and binding
- All constant domains are recognised by 2° Abs

Polycolonal antibodies contain multiple clones of antibodies produced to different epitopes of the antigen

Monoclonal antibodies, originally from one mouse, contain a single antibody from one clone of B-cells to a single epitope on the antigen

Affinity-purified Abs best in theory because they have bound to the antigen, but some of the strongest binding Abs cannot be eluted from the affinity columns and are lost.

Polyclonal antibodies

Advantage:
- High levels of labelling because they bind several epitopes on the same protein

Disadvantages:
- Can label multiple proteins that share epitopes
- Different batches have different antibodies

Monoclonal antibodies

Advantages:
- Single epitope selected for high specificity
- Different clones can be generated to different epitopes on the same antigen
- Single clone can recognise post-transcriptionally modified protein (e.g. phosphorylation)
- Same clone can be generated indefinitely

Disadvantages:
- Low levels of labelling possible
- Mostly from mice

How to choose primary antibodies:

1. Published literature recommendation
2. Product recommended for immunocytochemistry
3. High specificity for the antigen of interest in your species
4. Species the Ab was raised in compatible with other Abs in your experiment

How to store antibodies:

10 µL aliquots in -70 freezer, after defrosting: in fridge for short-term

Four generations of fluorescent labels

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

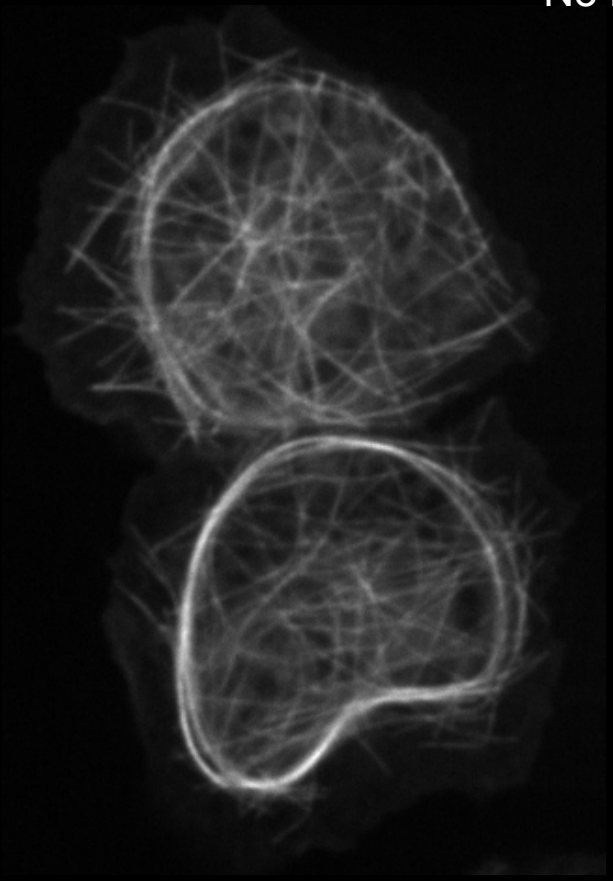
2nd Generation 1993: Cy fluorophores, cyanine dyes Cy2, Cy3, Cy5 (Jackson Immuno Research), AMCA, Texas Red
- More photostable and high quantum yields

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

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

- Fixed sample should appear similar to living sample
- Uniform fixation throughout the sample
- Cells and organelles not swollen or shrunken
- No loss of proteins, lipids and other molecules



Microtubules in Drosophila macrophages
Left :
Live cells expressing Jupiter-GFP
Right:
PFA fixed cell stained with anti-tubulin antibody and Alexa Fluor 488

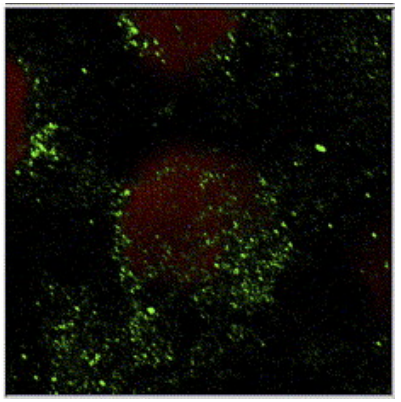
Two Types of Fixation

Denaturing fixation:

Cold methanol or cold acetone stored at -20 °C, samples submerged at -20 °C for 5 to 10 min

- destroys 3D protein structure
- dissolves lipids into micelles
- poor morphological preservation and poor protein retention
- makes some epitopes accessible
- best used after cross-linking fixation

MeOH

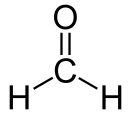


Cross-linking fixation:

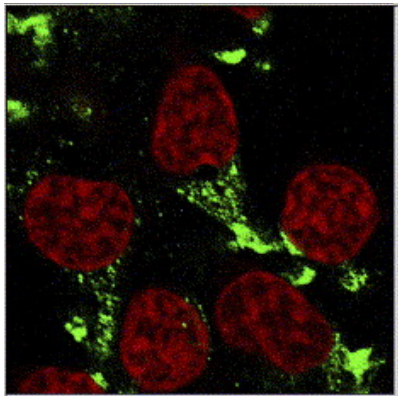
- aldehyde groups cross-link molecules in cells and tissues
- extensive cross-linking prevents antibody penetration

Formaldehyde used for immunocytochemistry in light microscopy

- cross-links 1° amines of Lys and Arg, sulfhydryl groups of Cys, OH groups, double bonds
- binds to amino acids, peptides, proteins and some lipids, but not RNA, DNA or most sugars
- retention of DNA and RNA due to protein cross-linking
- for cultured cells fixation usually for 20 min in 2 - 4% formaldehyde



PFA



Fixation cont.

Sources of formaldehyde:

Formalin

Produced by oxidation of methanol, contains 37% formaldehyde and impurities including 14% methanol

- Some proteins are washed out during fixation
- Causes high levels of autofluorescence
- DO NOT USE except for human clinical pathology

Paraformaldehyde (PFA)

Powder of polymerised formaldehyde, converted into soluble monomers by heating and adding NaOH

- Buy as 16% stock solution from Polysciences, freeze aliquots at -20 °C

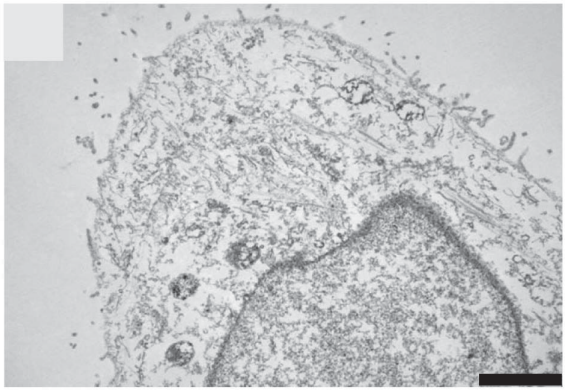
Glutaraldehyde Fixation

Can form long polymers, single most effective cross-linking chemical fixative

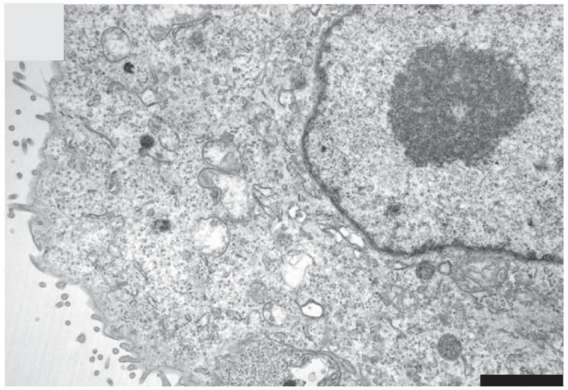
- Inhibits diffusion of antibodies into cells and tissues
- Generates autofluorescence
- Used for electron microscopy

Effects of Fixatives on Ultrastructure

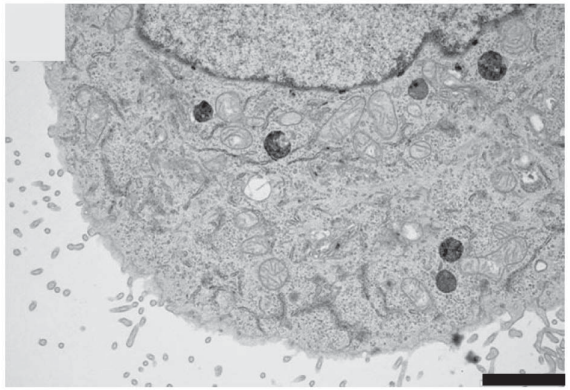
MeOH fixation



4% PFA



2% glutaraldehyde



Schnell U et al, Nature Methods, Vol 9 (2), 2012,152-158

Buffers for fixation

- pK range must be 7.0-7.3
- Maintain stable pH and have to have the same tonicity as the cells (same conc. of solutes)
- Usually phosphate buffer but specialist buffers possible: MOPS, TES, HEPES, PIPES

How to prepare cells for fixation

- Grow adherent cells on coverslips for fixation in multiwell plates
- Fix non-adherent cells in suspension after pelleting and resuspending or fix on poly-lysine coated coverslips (0.1mg/mL)

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

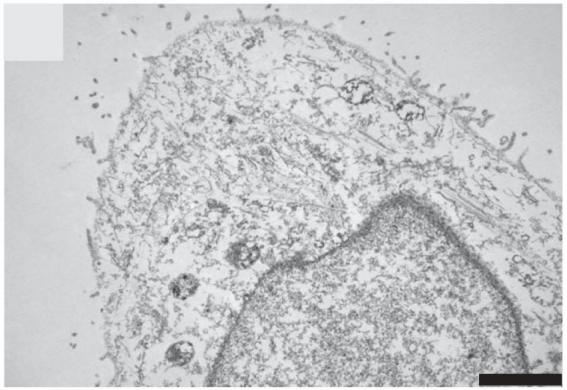
Permeabilisation

Saponins:

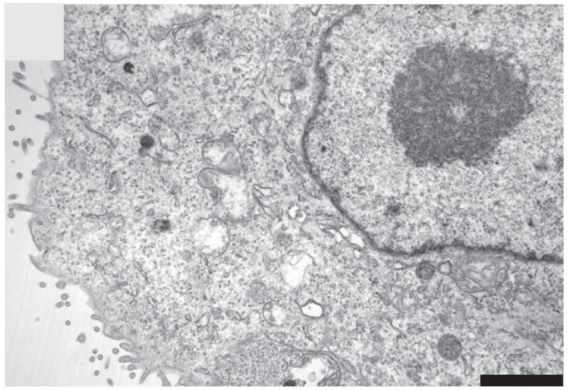
- mild detergents that preserve ultrastructural integrity
- permeabilise membranes by reversibly forming complexes with cell membrane cholesterol leading to pore formation
- sufficient for the detection of cytoplasmic antigens
- not effective for permeabilisation of cholesterol-poor membranes such as the inner mitochondrial membrane and the nuclear envelope
- add at conc. of 0.05 – 0.1% to blocking solution (block for 1h) and in antibody incubations, some protocols also include saponin in wash steps

Effects of Fixatives and Permeabilisation on Ultrastructure

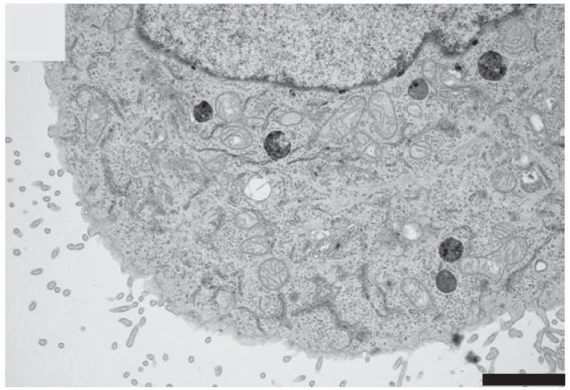
MeOH fixation



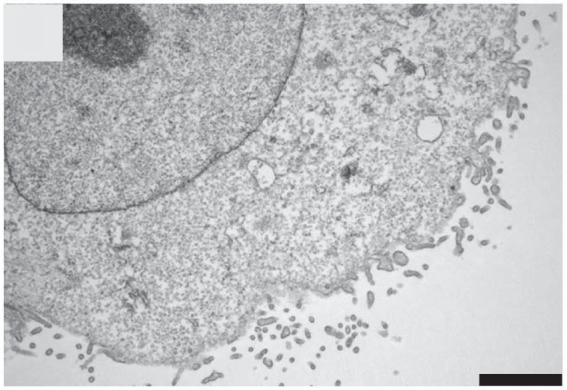
4% PFA



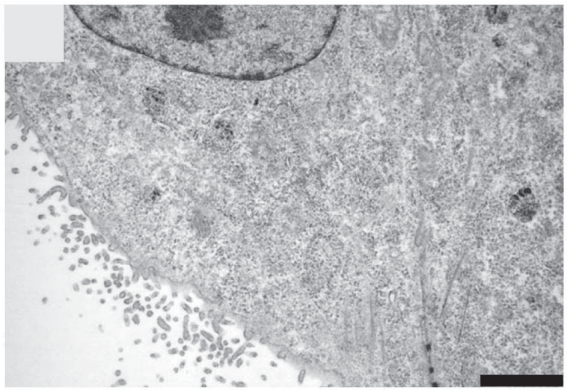
2% glutaraldehyde



4% PFA + MeOH



4% PFA + Triton X-100



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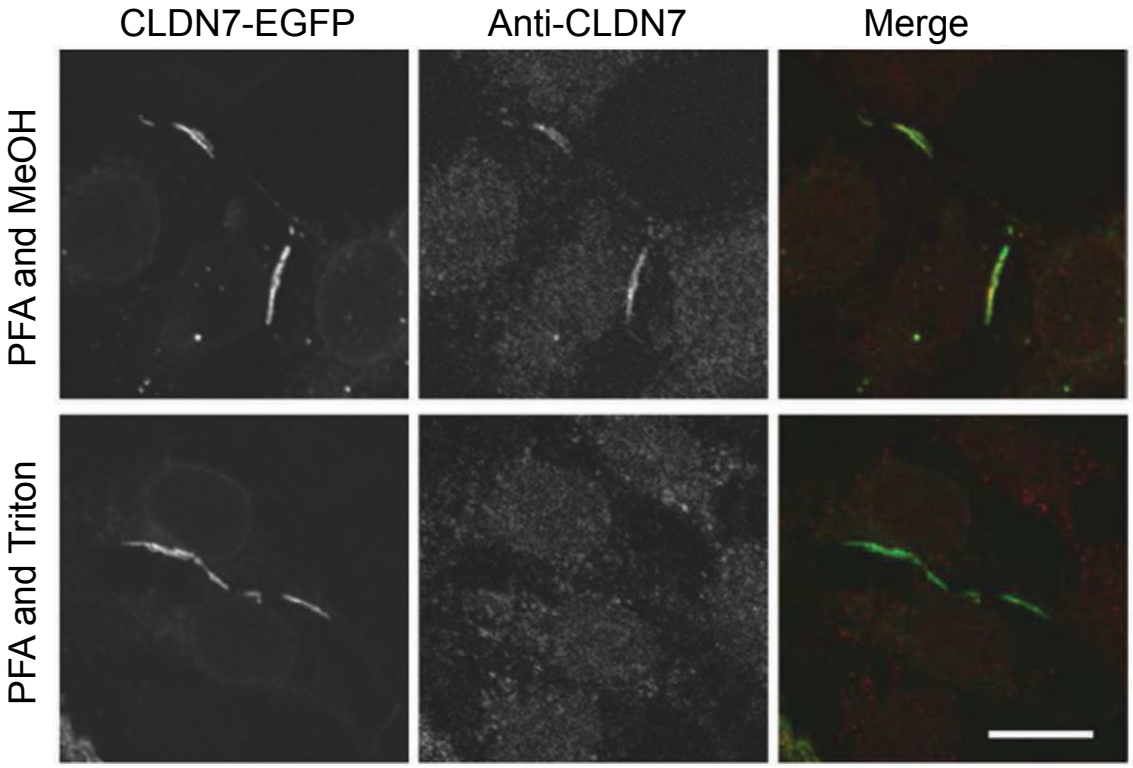
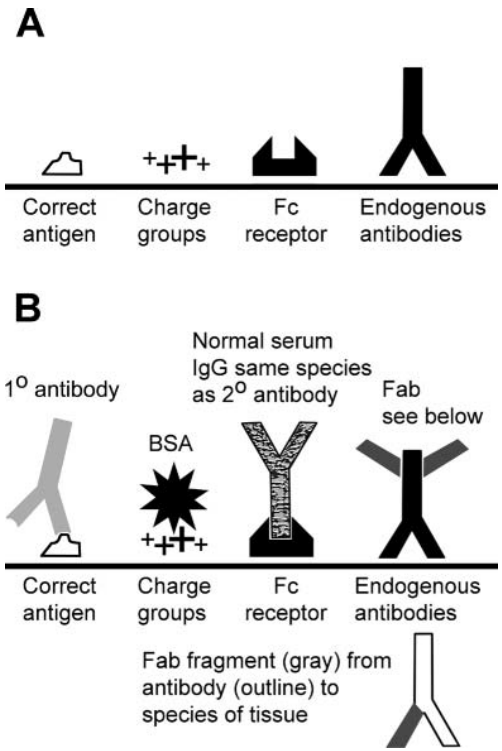
Effects of different types of permeabilisation



Images of 293T cells expressing tight junction protein Claudin-7–EGFP (CLDN7-EGFP) immunostained with an antibody to Claudin-7 (anti-CLDN7) after fixation with 4% PFA (30 min) and permeabilized with either methanol (MeOH; −20 °C) for 1 min or 0.1% Triton X-100 (Triton) for 15 min at room temperature. Scale bar, 10 µm.

Schnell U et al, Nature Methods, Vol 9 (2), 2012, 152-158

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

Washes

- 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

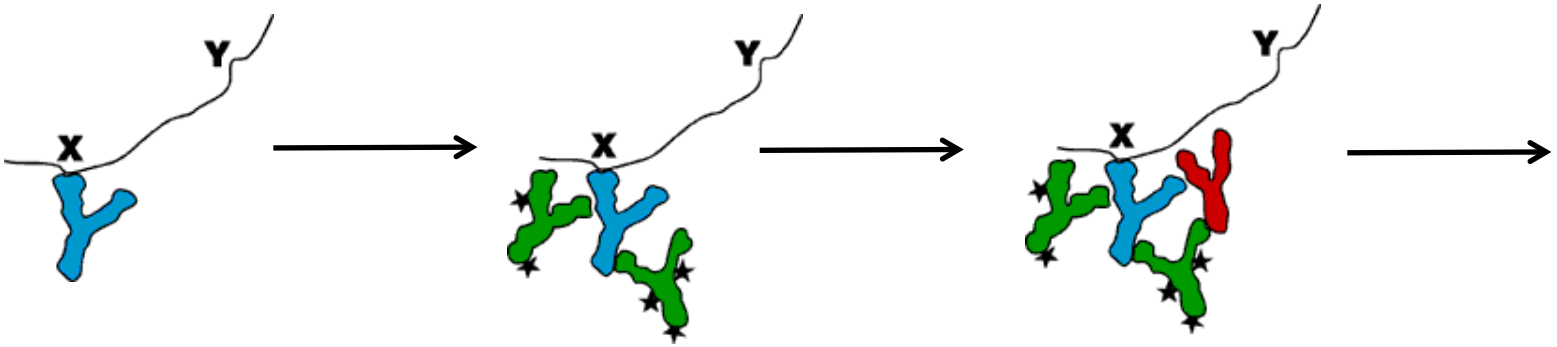
- Incomplete removal of the 2° antibody increases background

Antibody incubation

1° antibody:
Use a series of antibody dilutions: 1:50, 1:500, 1:5000
High concs. ⟶ background or very little label

2° antibody:
Low conc. of antibody usually sufficient: 1: 500, 1:1000
High concs. ⟶ background

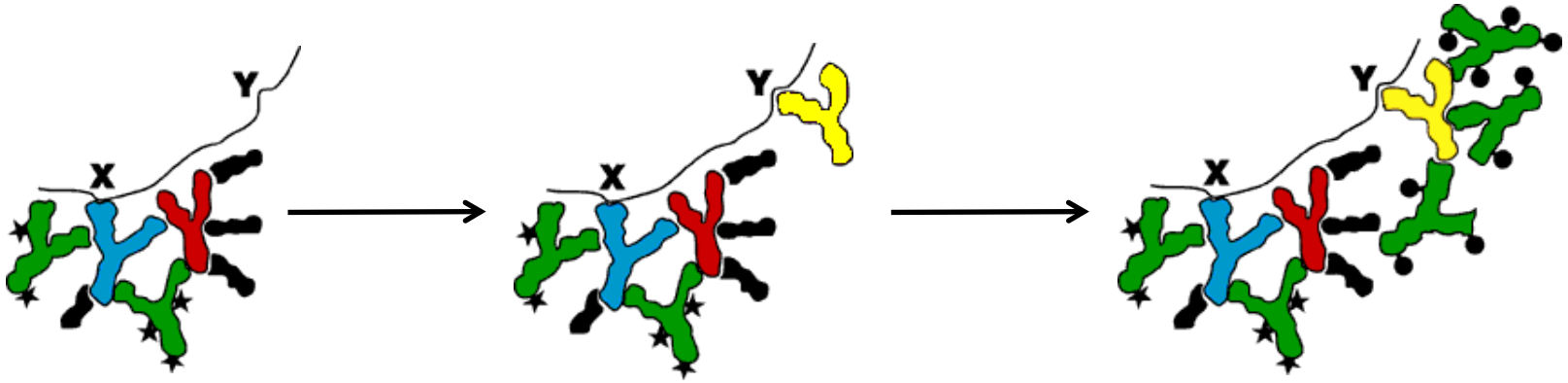
Two 1° antibodies from the same species



rabbit anti-X

goat anti-rabbit IgG Alexa 488

IgG from normal rabbit serum



Fab fragment goat anti-rabbit IgG

rabbit anti-Y

goat anti-rabbit IgG Alexa 568

Mounting media

Prolong Gold (Life Technologies)
curing anti-fade mountant, sets over 24h to RI 1.47 (glass RI 1.52)
Slides can be stored at 4 °C for several months

Vectashield (Vector Labs)
Non-setting anti-fade mountant, RI 1.44
Slides need to be sealed with nail polish and can be stored for a month at 4 °C

Do not buy either with added DAPI, stain with 1-5 µg/mL for 5-10 min in a separate step

1° Antibody Controls

- check localisation of fluorescent fusion proteins in live imaging
- compare tissue sections from a normal animal/cells and a knockout animal/cells – not often possible, knockout might not be complete
- single band on western blot or better immunoprecipitation followed by gel and silver staining
- immunocytochemical comparison with known antibody against same target or fluorescent fusion protein

2° Antibody Controls

- omit the 1° antibody and block with normal serum if you see background
- purchase 2° antibodies from reliable manufacturers
- when choosing a 2° antibody for a 1° mouse antibody the 2° frequently needs to be able to bind to the subclass of the IgG used as the 1° antibody

Labelling Controls

- omit all antibodies – all fluorescence seen is autofluorescence
- irregular or particulate autofluorescence: elastin, lipofuscin, NADH, flavins, chlorophyll, haemoglobin etc.
  ⟶ increase signal from 1° antibody or shift label to far red
- diffuse or uniform autofluorescence: aldehydes from formalin or glutaraldehyde fixation
  ⟶ reduce by blocking with glycine or sodium borohydride

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