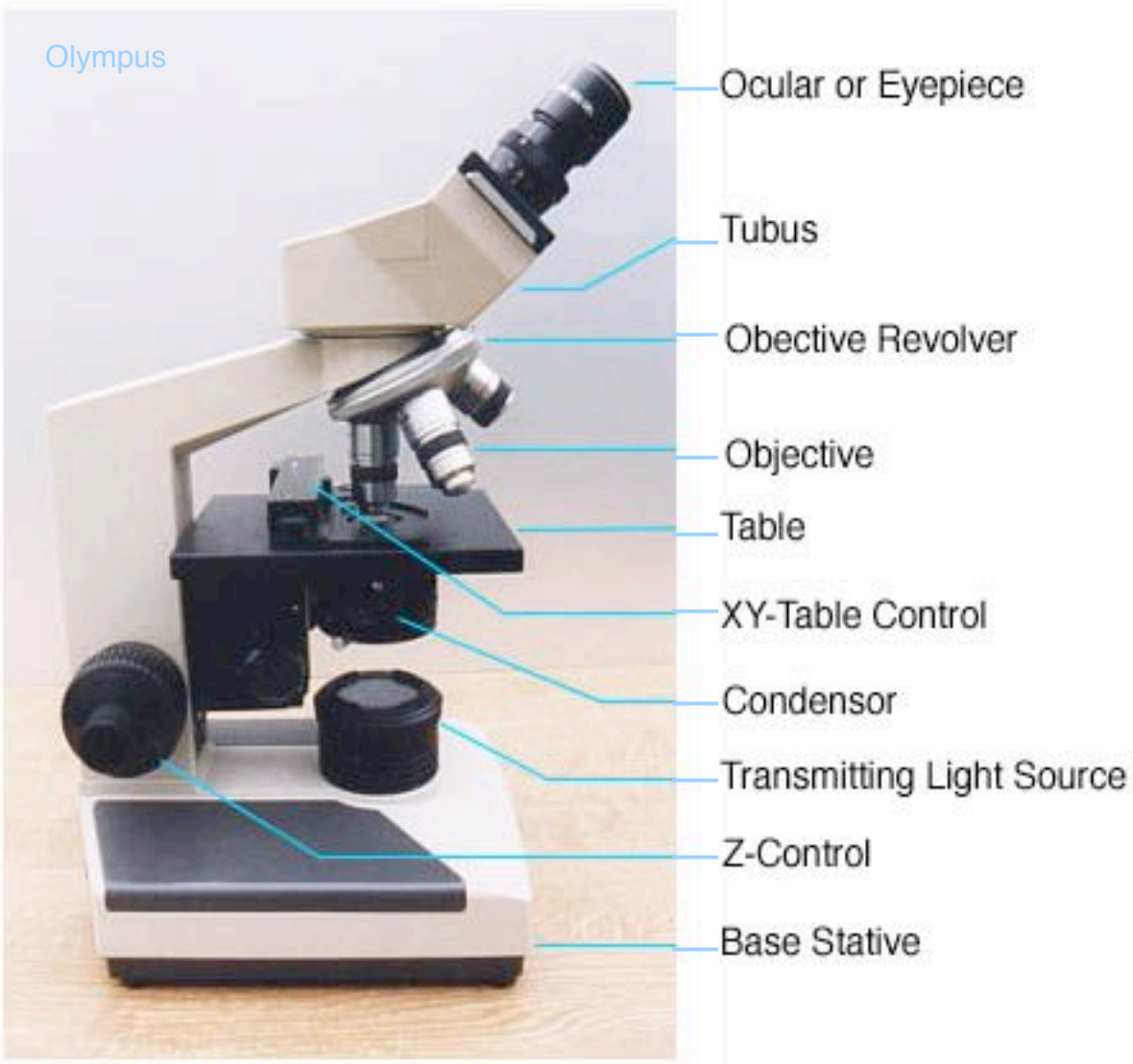
ZMB

Practical Introduction to Light Microscopy

This presentation has been put together as a common effort of Urs Ziegler, Anne Greet Bittermann, Mathias Hoechli. Many pages are copied from Internet web pages or from presentations given by Leica, Zeiss and other companies. Please browse the internet to learn interactively all about optics (i.e. microscopy.fsu.edu/primer/). For questions & registration please contact www.zmb.unizh.ch .

Up-right Microscope



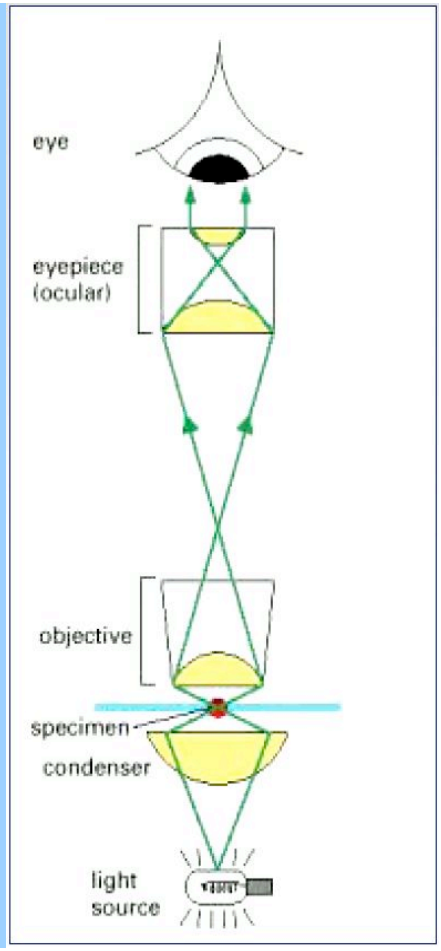
Inverted Microscope

The inverted microscope stand allows the observation of living cells in culture dishes from underneath.

An environmental box can be mounted to maintain temperature and CO_2 concentration.

The parts of the microscope

- Detector (PMT, CCD)
- Objective (±Immersion medium)
- Specimen (Cover glas)
- Light conditioning system
  - Köhler illumination
  - Phase ring
  - Wollaston prism and polarizers
  - Filter cubes (for fluorescence)
- Light source (Halogen, XBO, ...)



specimen types

- *Bulk specimens* (autofluorescence, total reflection) in any kind of holder (CLSM only). i.e. teeth, casts, scaffolds, bone
- *Thin specimens:* tissue sections, cell cultures (fluorescence-labeled, autofluorescent) on slides
- *Live cell* imaging of native (i.e. DIC, Ph2) or transfected (i.e. GFP) cell layers in suitable chamber environments

Fixation in light microscopy

Fresh Paraformaldehyde solution

- stopps cellular dynamics
- preserve cells during preparation

by crosslinking of cellular components.

Solvents (Methanol, Acetone, ...) do not cross link anything but make holes into membranes instead
-> no good structure preservation.

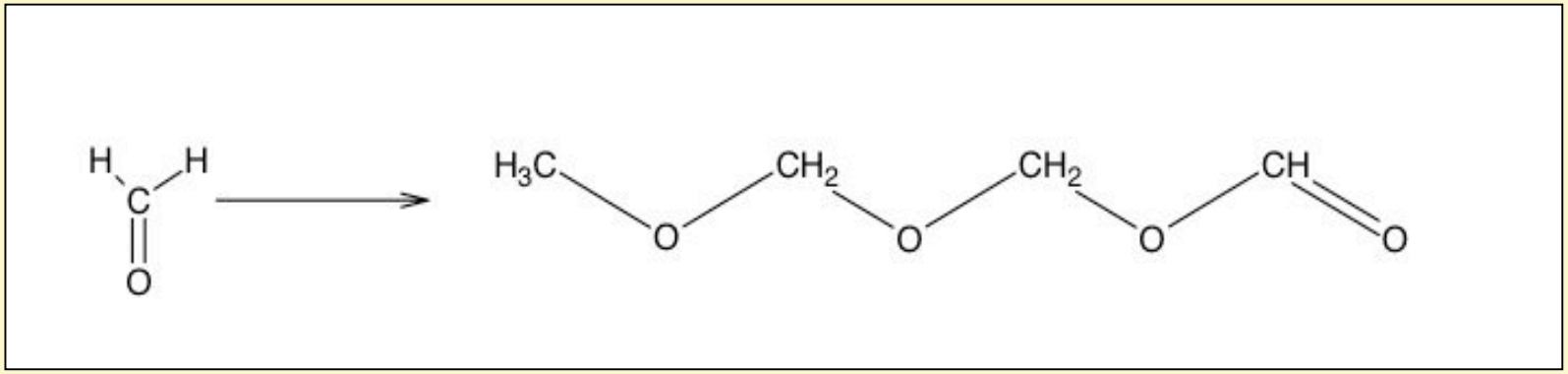
Glutaraldehyde shows autofluorescence.

Formaldehyde preparations

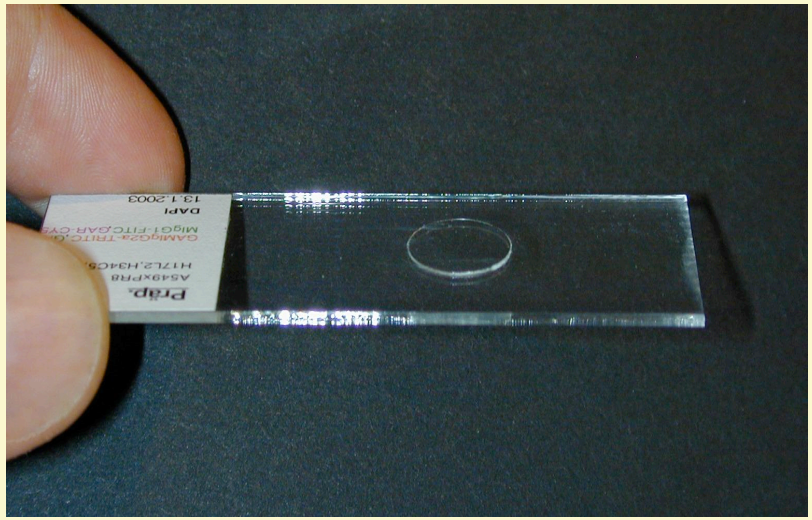
Formalin = 37% formaldehyde plus ca. 10% methanol

Commercially available formaldehyde solutions without methanol is unstable in time: forms polymers especially at 4°C

Paraformaldehyde is the solid polymer -> use this to prepare fresh formaldehyde solutions and use them right away



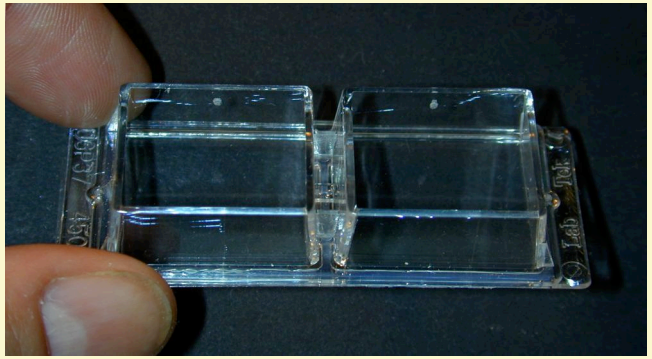
the light microscopic specimen

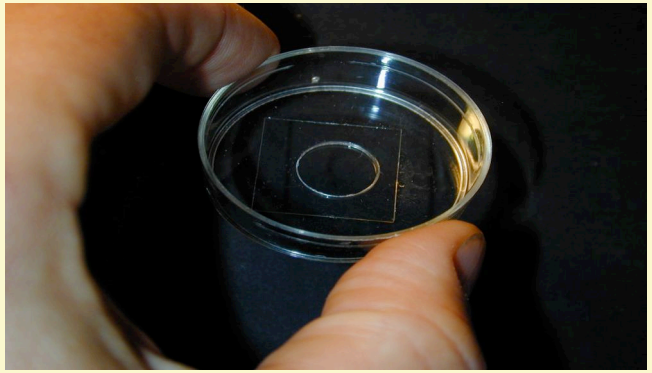


° cover glas (0,17 mm)

° sample surrounded by embedding medium (might contain anti-bleach agent)

° glas slide





specimen devices for inverted light microscopes

-> cover glass bottom allows also use of DIC and high resolution immersion objectives

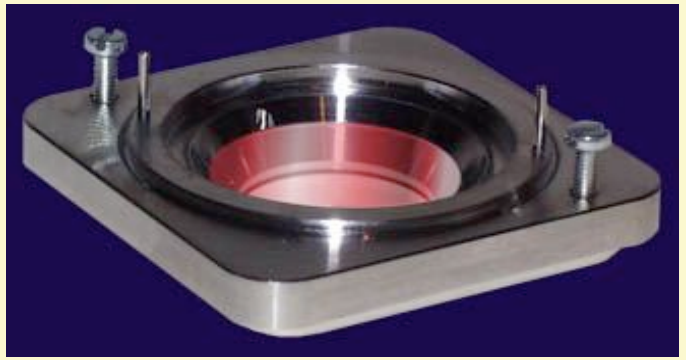
- coverslip chamber (with 1-4 chambers)
- full glass bottom dish
- culture dish with cover glass insert (w & w/o finder)

specimen devices for inverted light microscopes

-> plastic bottom dishes or plated allows use of long distance air objectives only, DIC is not possible. Some plastics show autofloures-cence.

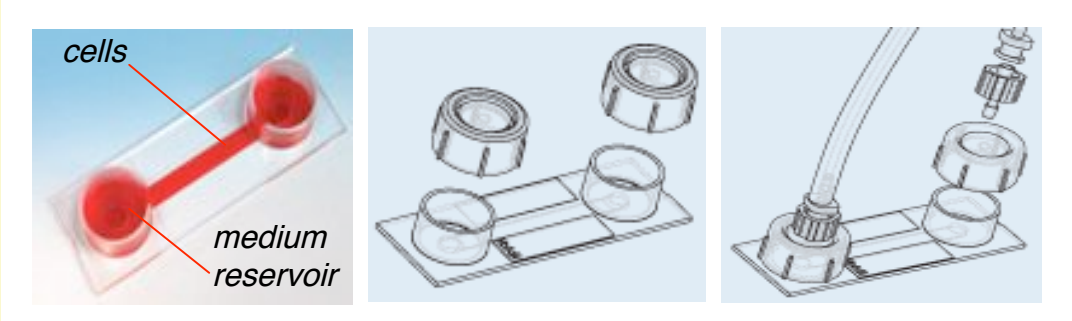
-> multi-well plates: multipe table positions can be programmed to follow cells under different conditions during the same time intervall

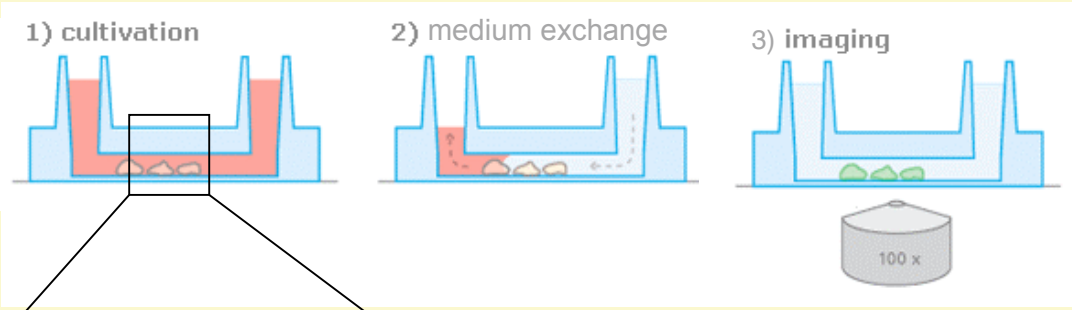
systems for live cell microscopy: the Ludin chamber

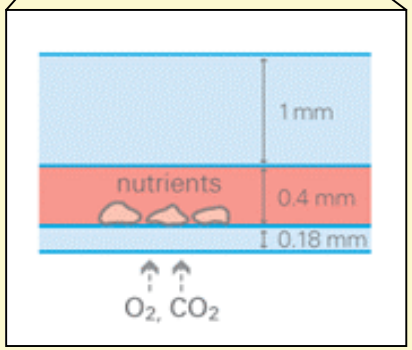


- fast transfer and mounting of the cultured coverslip
- bottom cover glass allows for immersion objectives!
- built-in liquid and gas perfusion lines
- closed and open/covered configurations
- 16 mm diameter viewable area.
- designed for easy access also for micromanipulation, microinjection and electrophysiology

systems for short term live cell microscopy: ibidi technology







-> sterile plastic devices for cell culture

-> plastic bottom foil has the same optical properties as 0,17mm cover glass

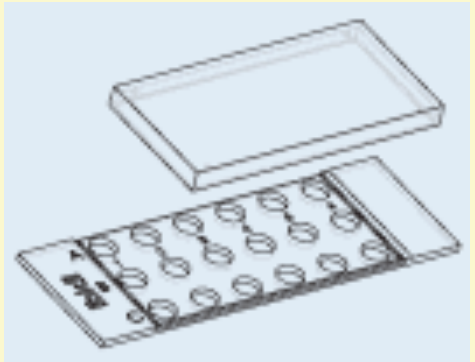
-> perfusion designs possible

-> gas exchange via bottom foil possible

-> small volume tunnel (100 µl or 30 µl)

-> multi tunnel slides available (6-tunnel-slides)

specimen devices for low volume analysis (inverted LM)



features:

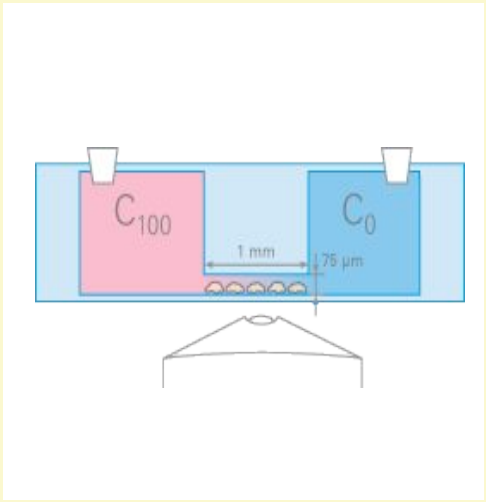
-> 18 micro-well design
(5 mm diameter & 30 μl volume)

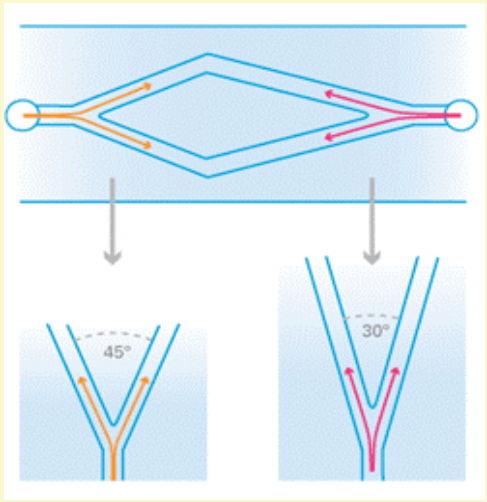
-> sterile plastic devices

-> avaliable uncoated, tissue culture treated as well as coated with collagen, fibronectin or poly-lysine

-> ibidi plastic bottom foil has the same optical properties as 0,17mm cover glass and allows for high resolution LM

special ibidi-designs for live cell studies

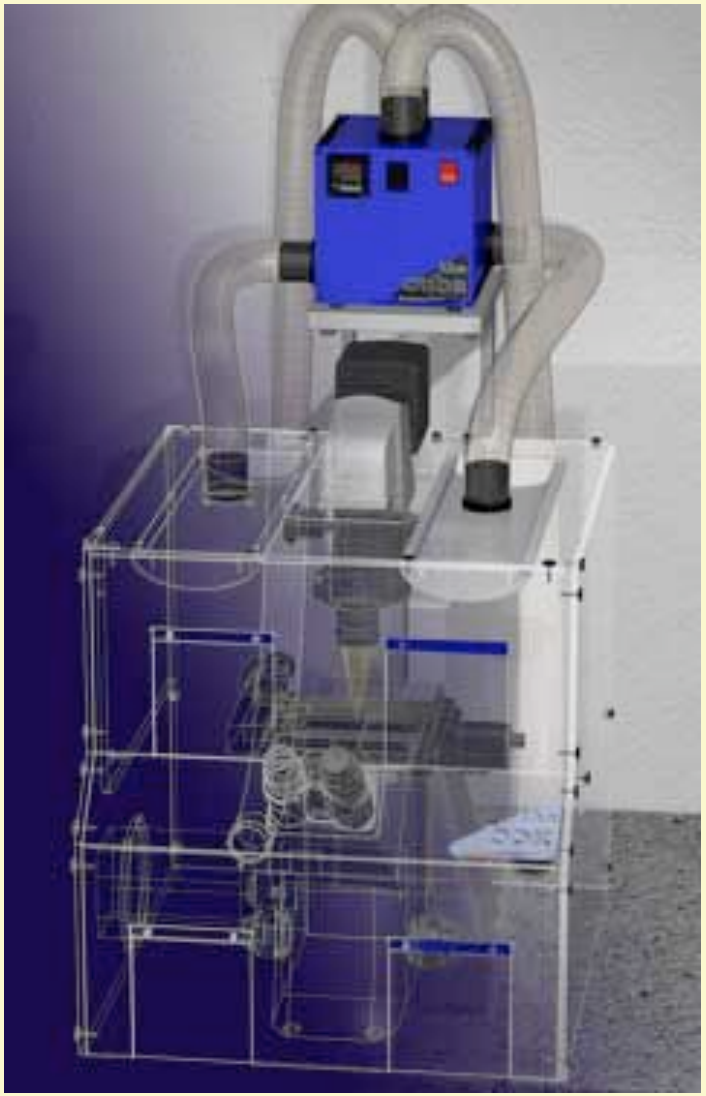




ibidi-slides with designs for:

- flow analysis (shear force effects)
- gradient environments (migration studies)

life imaging - The Box



Preventing focus instability.

Even small changes in ambient temperature lead to thermal extension or contraction in the microscope stand, stage and objective, thereby changing the plane of focus.

-> *A incubator box combined with a precision air heater ensures that the temperature of specimen and microscope remain equilibrated and tightly controlled.*

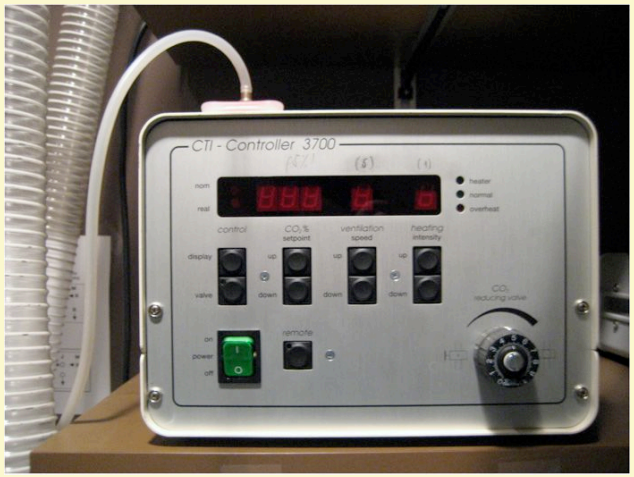
THE BOX

Custom design for the individual microscopy setup. Intricate system of openings and doors for comfortable access to microscope controls and specimen.

THE CUBE

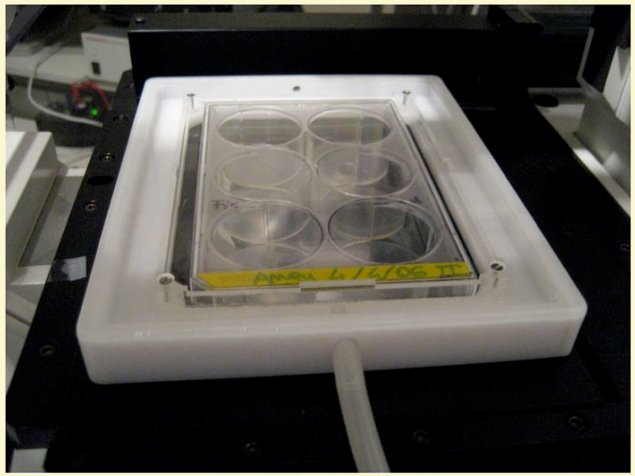
highest quality fan; controller cube with external, low-vibration & low-noise design.

Maintenance of CO_2 atmosphere

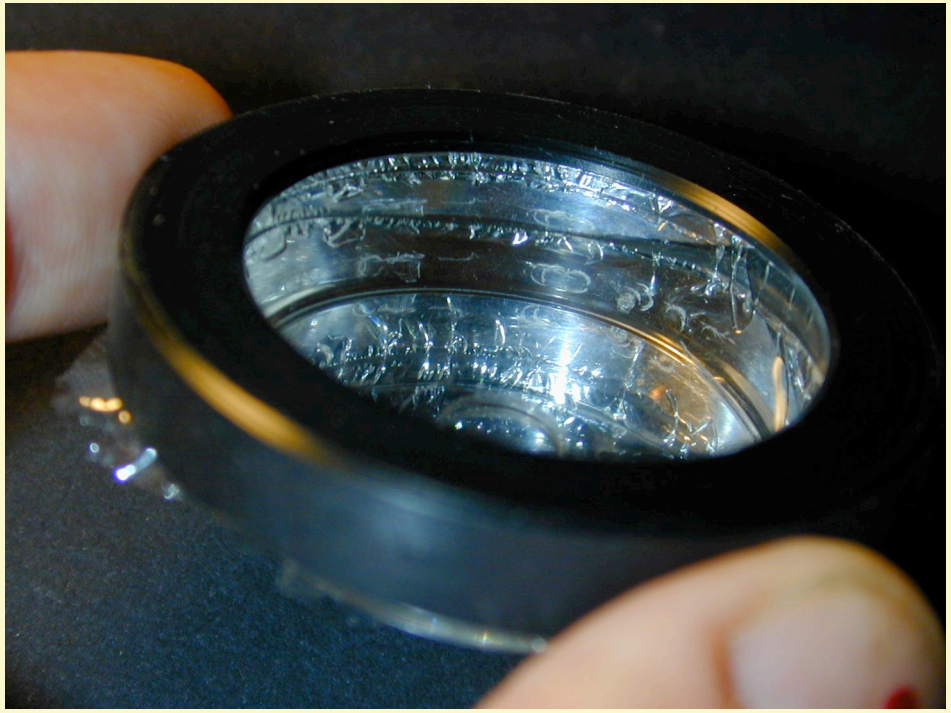


-> a controler allows to adjust air flow and the %CO_2

(there is a possibility of guiding the gas stream through a bottle of water in order to diminish loss of humidity)



-> a air tight table top encloses the live cell culture device



systems for live cell microscopy: the foil

Evaporation free cell culture system:

35 mm cell culture dish with glass bottom can be covered with a petriperm foil, which allows full access of CO_2 while blocking the water vapour transfer; the system can be sterile snap-closed.

cell culture specimens

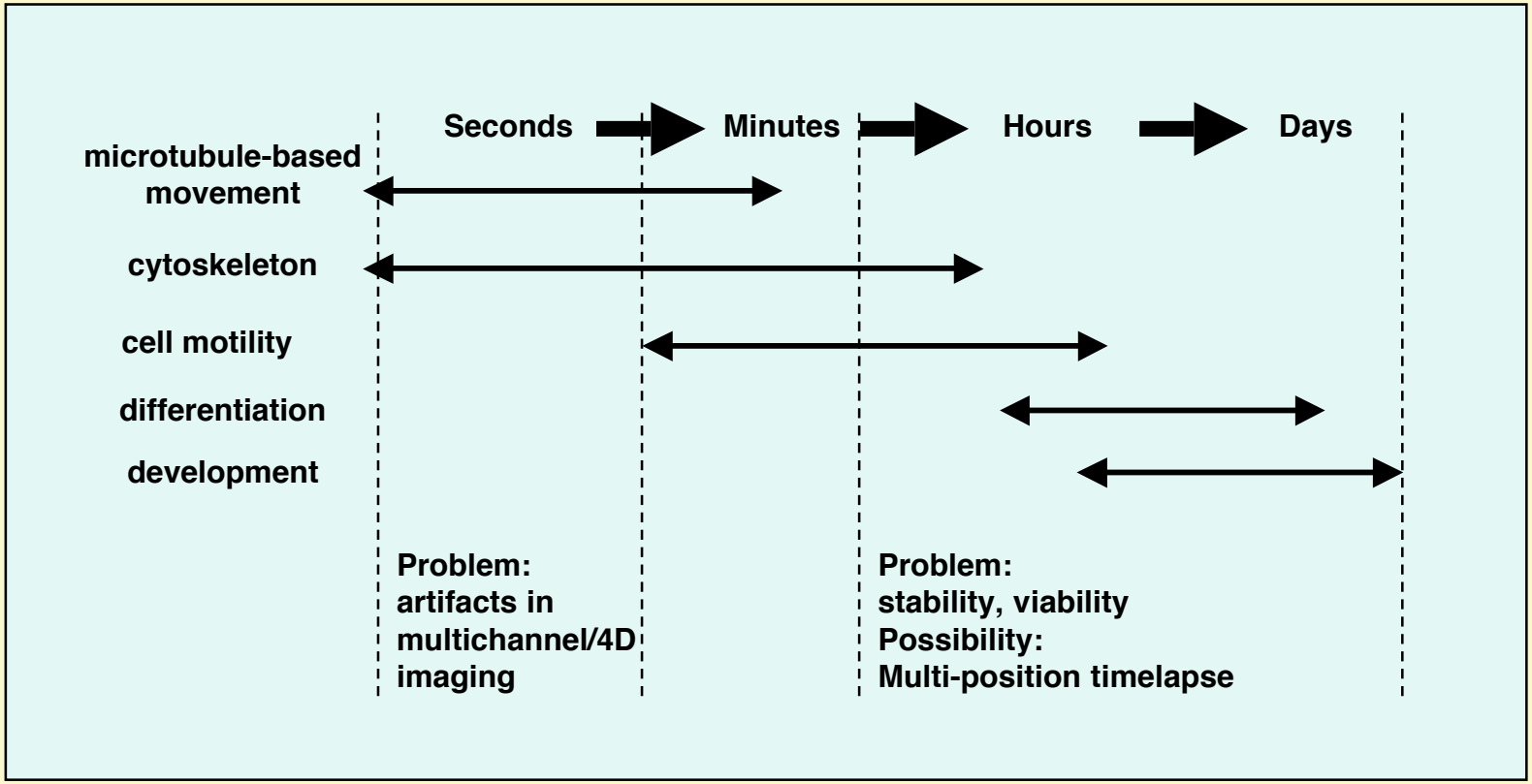
A perfect specimen is the base for good results. Therefore…

- Check the cultures regularly for mycoplasma.
- Check the culture by phase microscopy for its health before the experiment.
- Use sub-confluent cell cultures for microscopy.
- For fluorescence preps use whenever possible cover glass cultures.
- Fixation with fresh buffered 3 % Paraformaldehyde (PFA)
- Immobilize suspension cells on the cover glass i.e. via Poly-L-lysin.
- Fluorochromes have to be chosen according to the LASER lines or filter sets of the microscope and to other involved markers.
- Don`t forget to make apropriate controls (also for autofluorescence!)
- Embedding in polymerizing medium, containing anti-bleach for standard slide preparation

for live cell study:

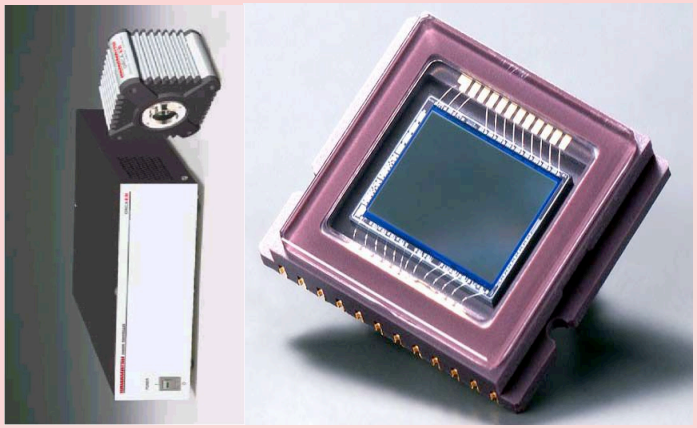
- clima chamber: temperature control, CO_2-control
- inverted microscope: use cell culture dishes i.e. with glas bottom insert; use water immersion or long distance objectives
- up-right microscope: use cell cuture dishes and a dip-in objective

Experimental timescales



Detectors in Light Microscopy

Images in widefield-light microscopes are captured with CCD-cameras



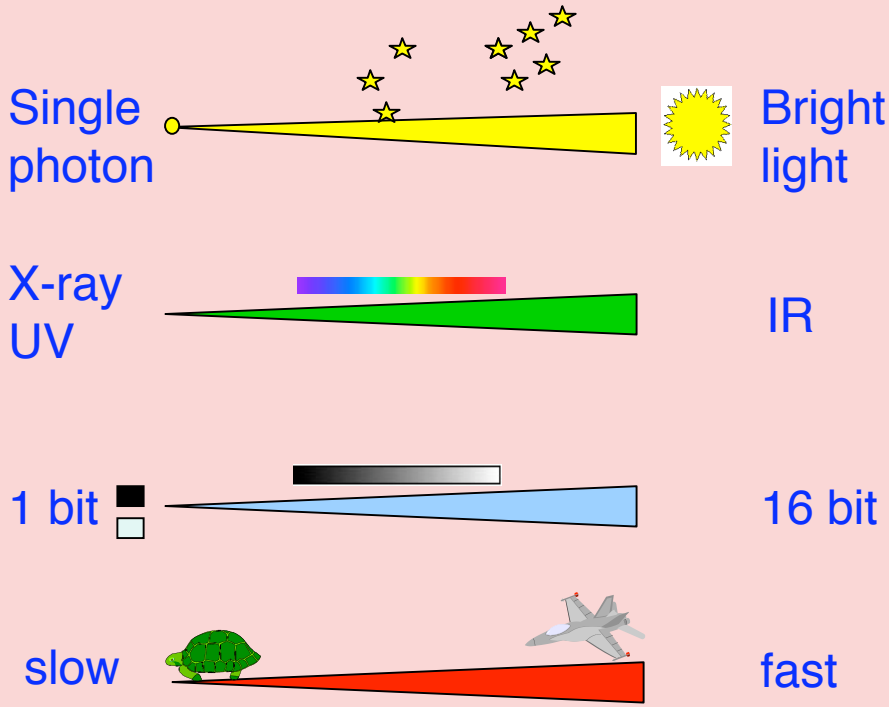
Images in scanning light microscopes are captured with PMTs

The different products of CCDs as well as PMTs have their own specific spectral sensitivity.

electronic imaging detectors

advantages of imaging detectors compared with the human eye

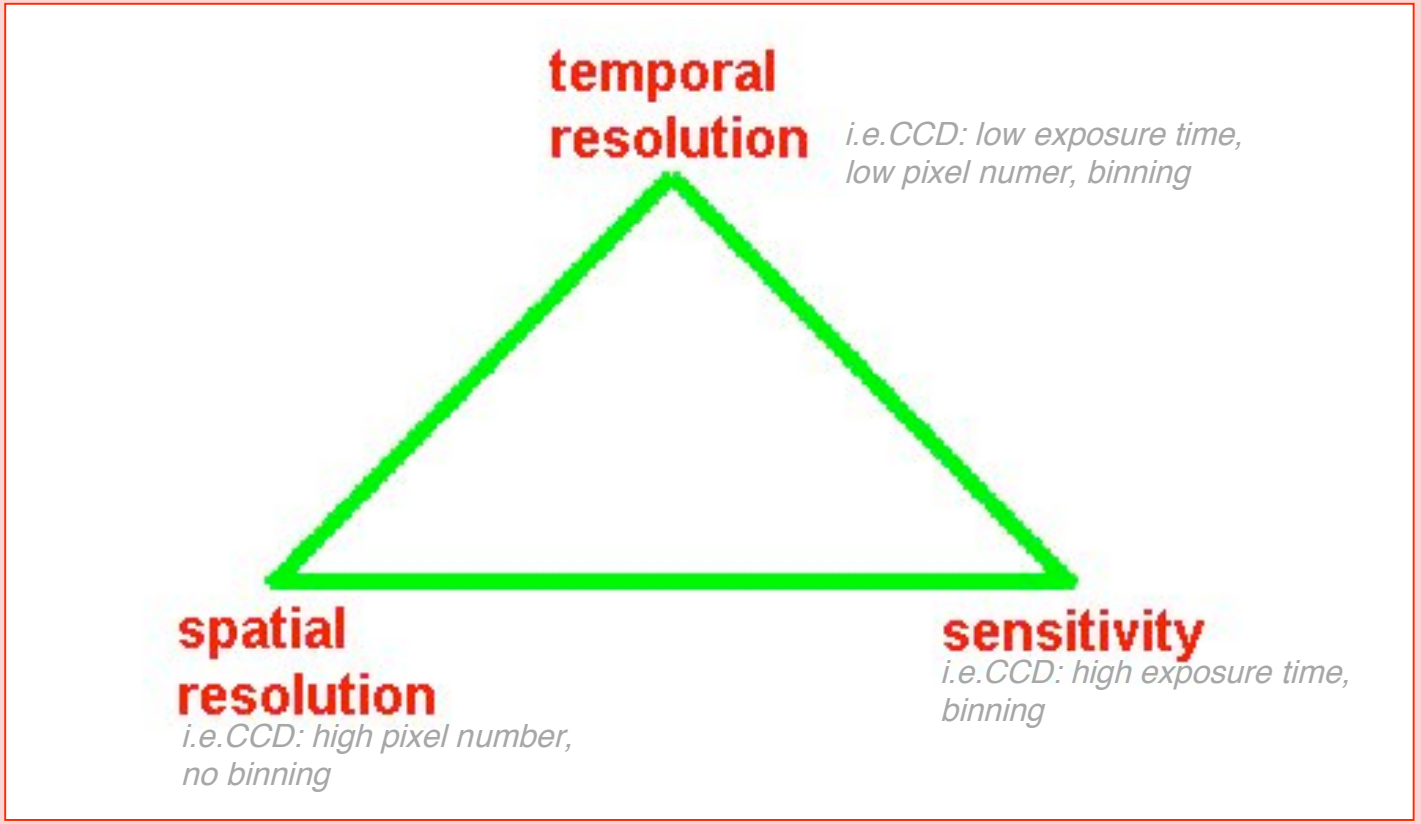
- Sensitivity
- Spectrum sensitivity
- Dynamic range
- Speed
- Image Processing



- **Analysis** ⇒ quantitative, morphological
- **Correction / Modification** ⇒ background subtraction, contrast enhancement, pseudo color,...

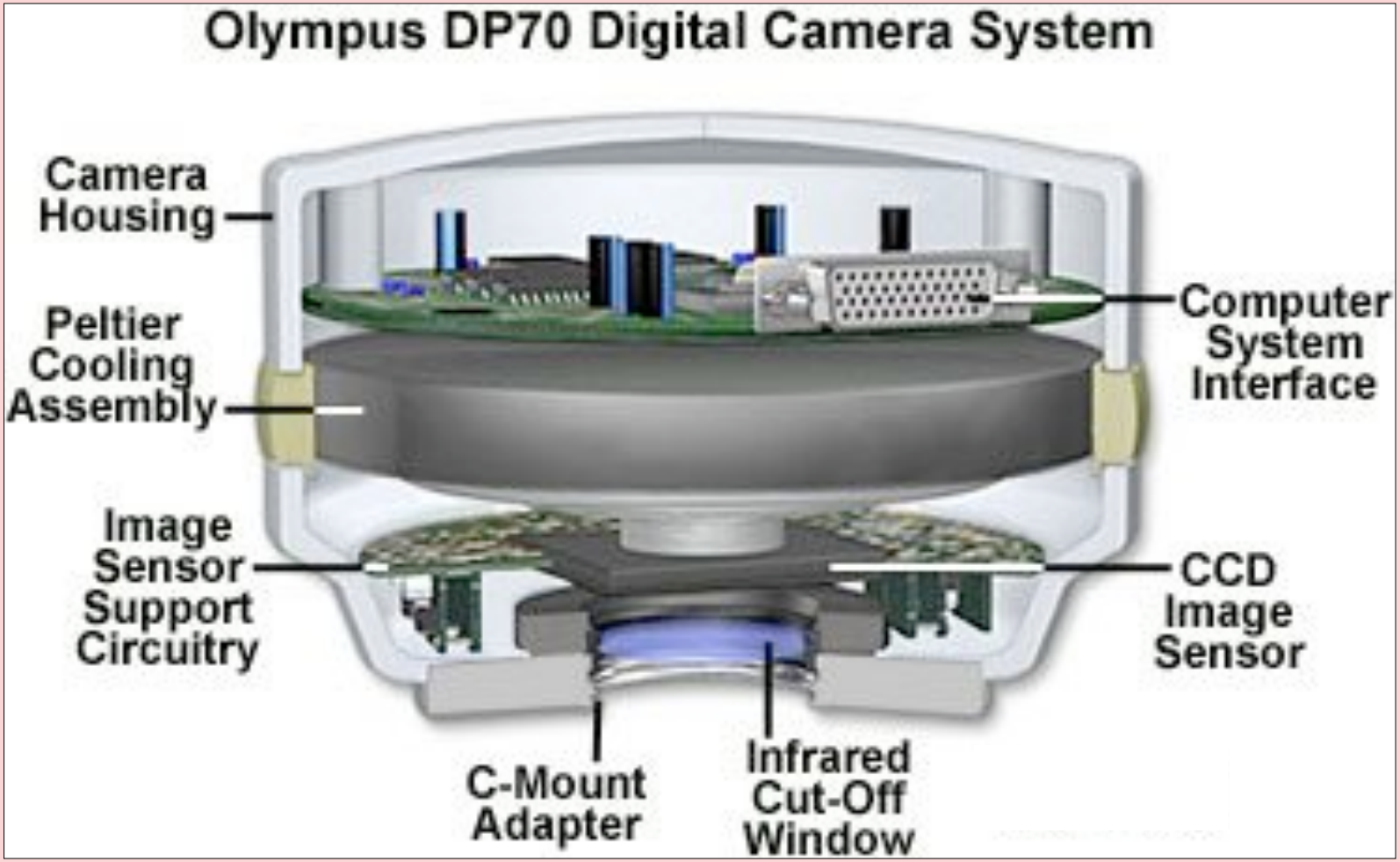
signal detection

“triangle of frustration”



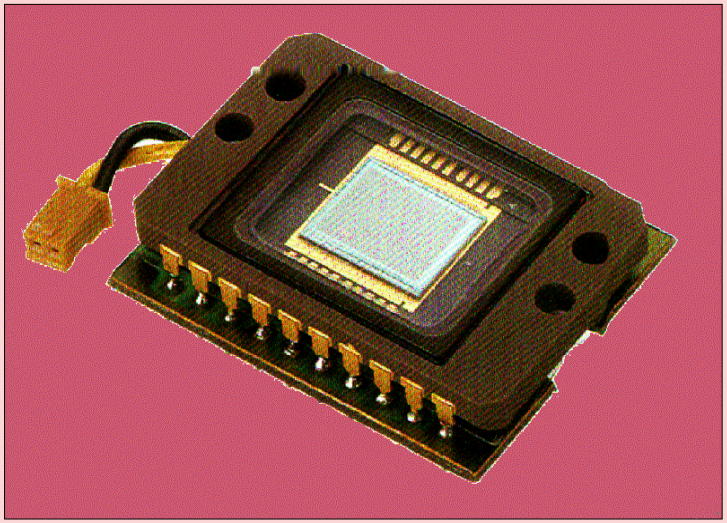
All detections have their benefits and limitations. What is best, depends on the application requirements.

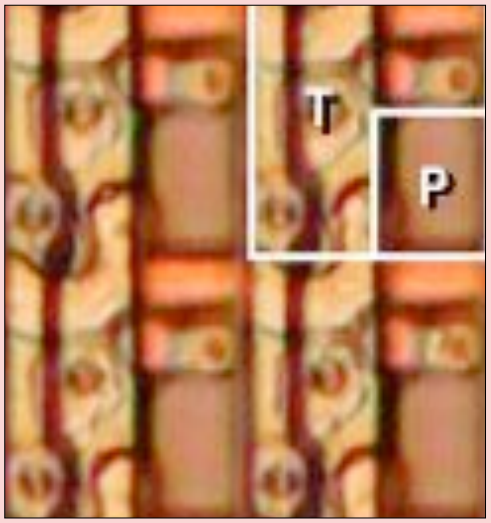
structure of a cooled CCD for widefield light microscopy

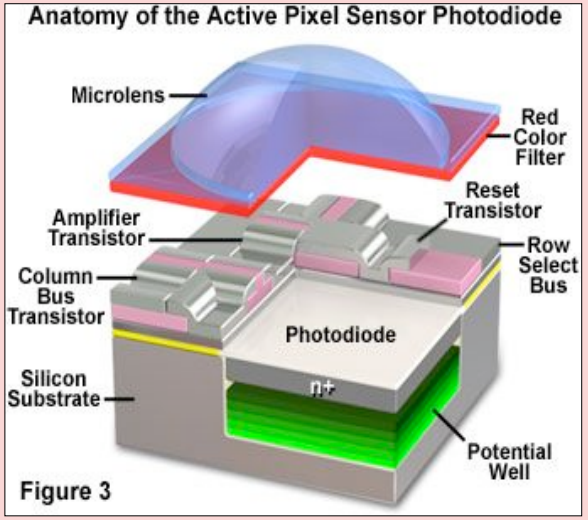


Charge Coupled Device CCD

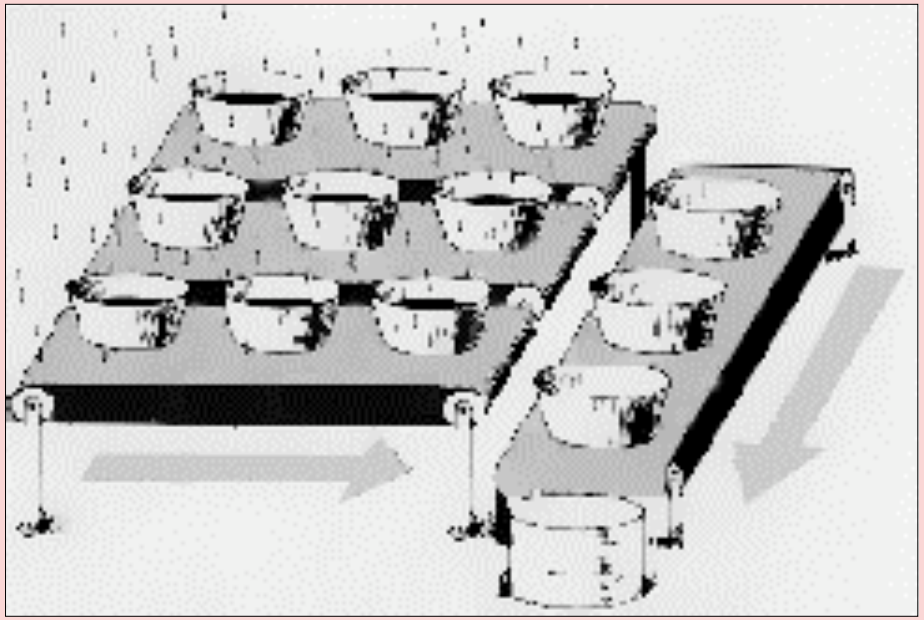
- the CCD-chip as an array of photodiodes

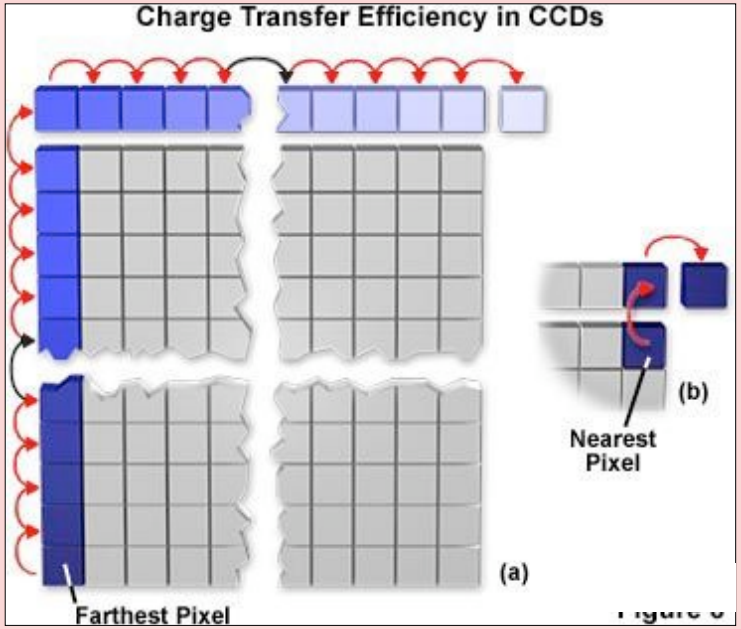






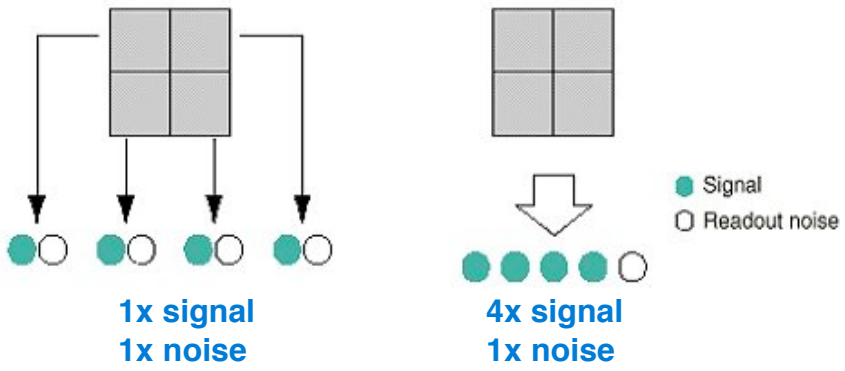
CCD: chip read out by charge transfer





CCD camera features

Binning



- Spatial integration
- Data reduction
- Increased S/N Ratio
- Reduced number of larger pixel

-> High sensitivity
-> High image frequency
-> Larger viewing area
-> Reduced spatial resolution

CCD camera features

Adjustments on a CCD:

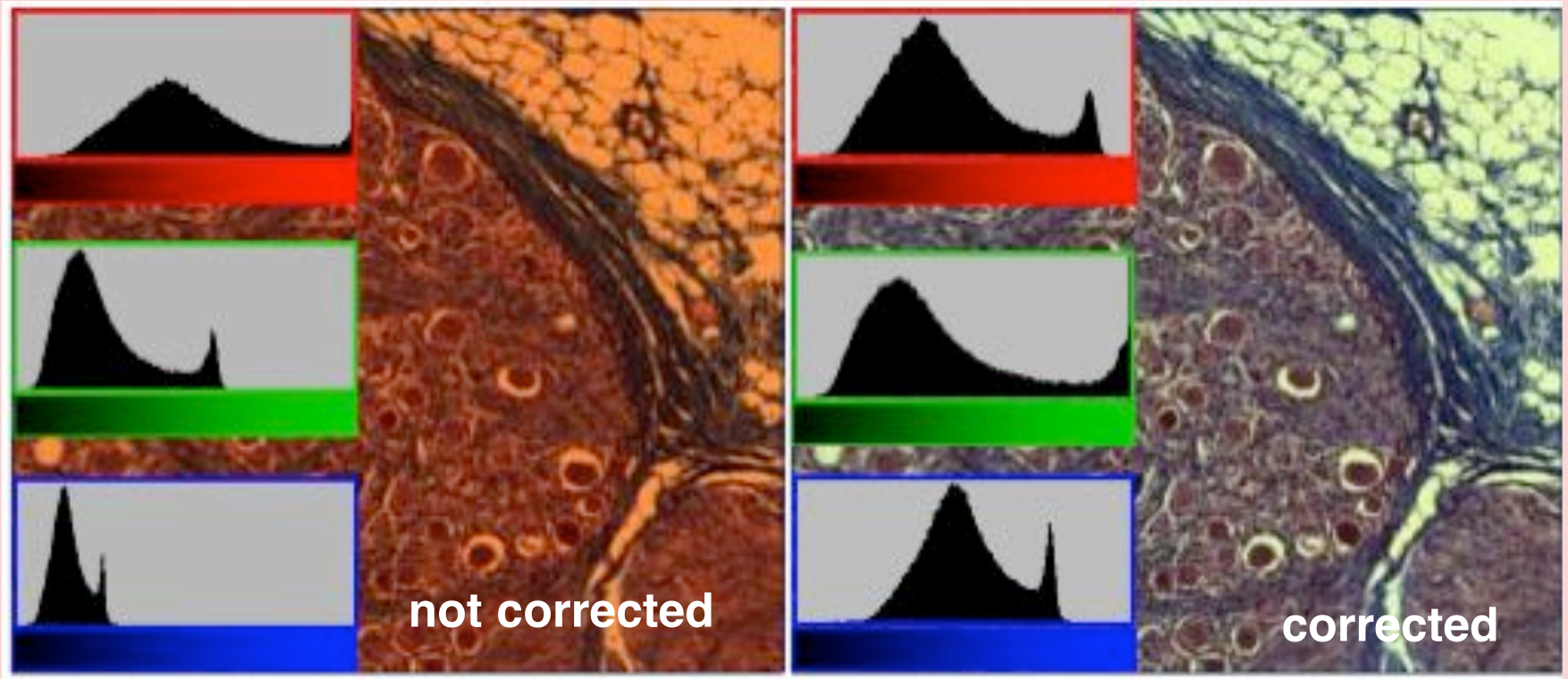
Exposure time -> Aquisition speed, Brightness, Noise
Gain -> Brightness, Noise
Offset -> Background subpression: best to leave unchanged „0“

Additional Adjustments for color CCD:

(i.e. for samples with natural colors or histo-stain
in bright field mode)
Correction for the „temperature“ (color) of the light
-> set bright background to white (white balance).

Color balance

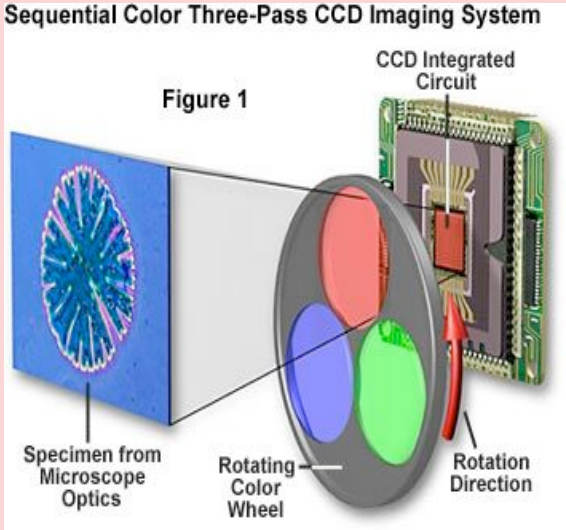
digital adjustment (white balance)



The lamp power influences light intensity & color. Digital cameras allow to correct color deviations by digital adjustment.

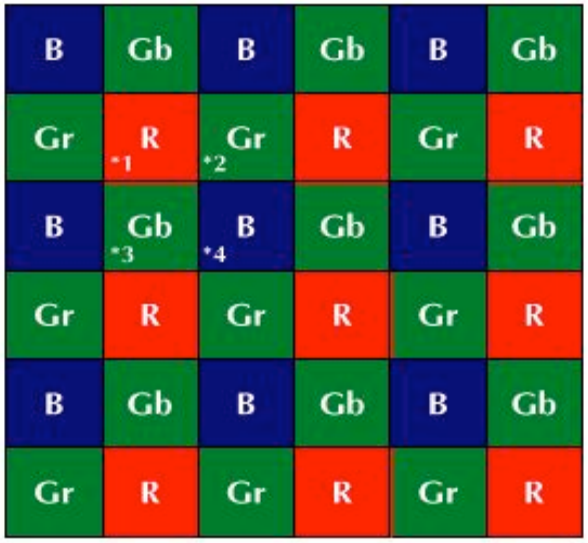
Colour cameras

1CCD b/w camera with filters



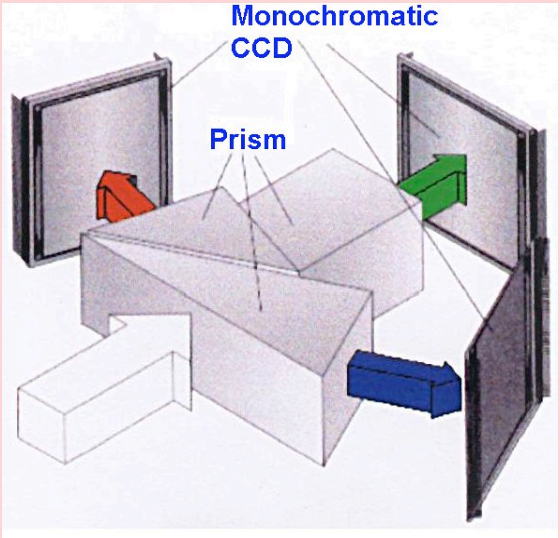
Conversion of a gray scale image into a colour scale image by rotating filters
-> seqential image aquisition

1CCD color camera



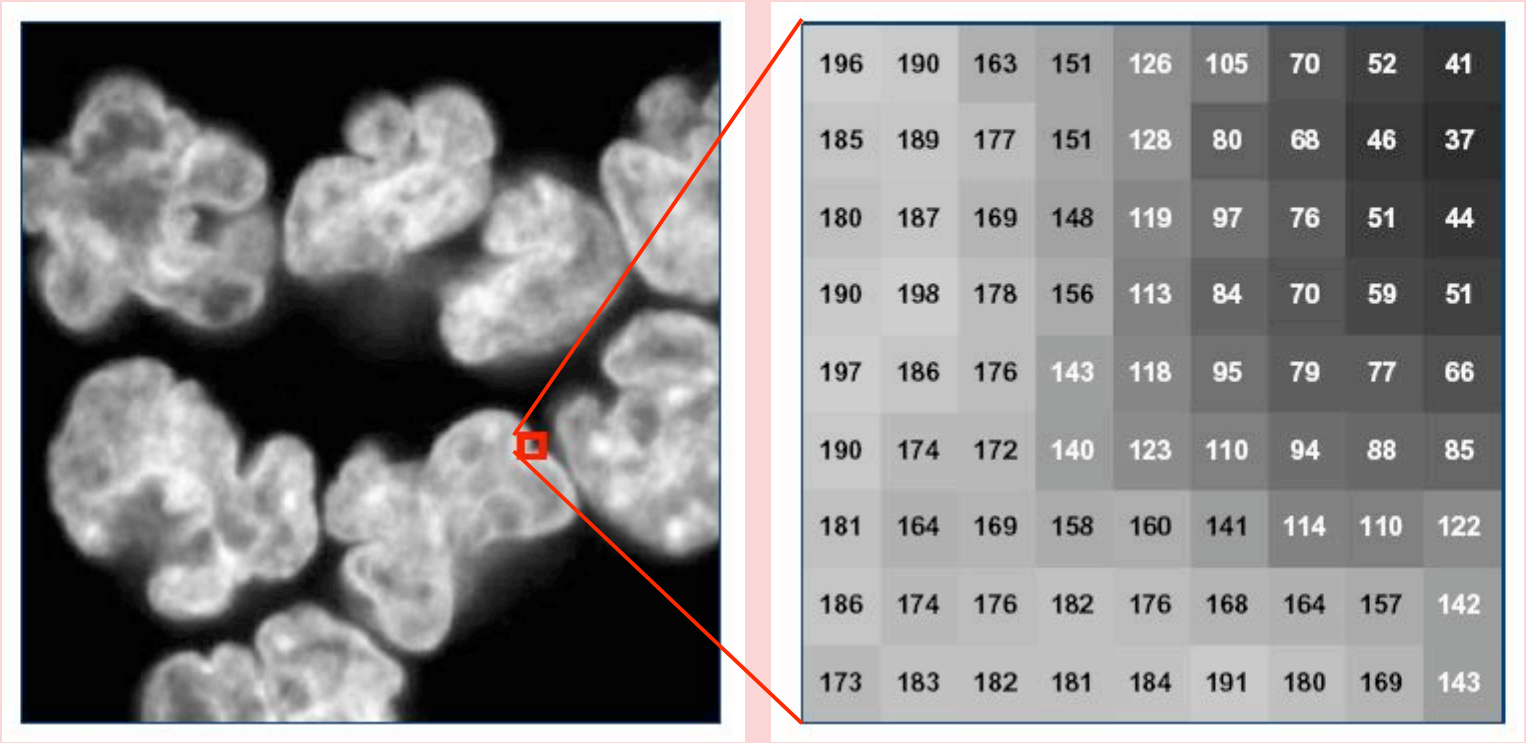
Matrix filter. Every pixel contains spatial information for one colour only -> reduced spatial resolution.

3CCD colour camera



Prisms distribute the incoming light to three separate CCD (R/G/B)
-> Real time imaging
-> Full spatial resolution for every colour

Electronic grayscale image



Each pixel (picture element) has ist coordinates and intensity values.

Resolution in digital images

dimensional resolution:

Pixel: 2D image element (i.e.image with 512 x 512 pixels)

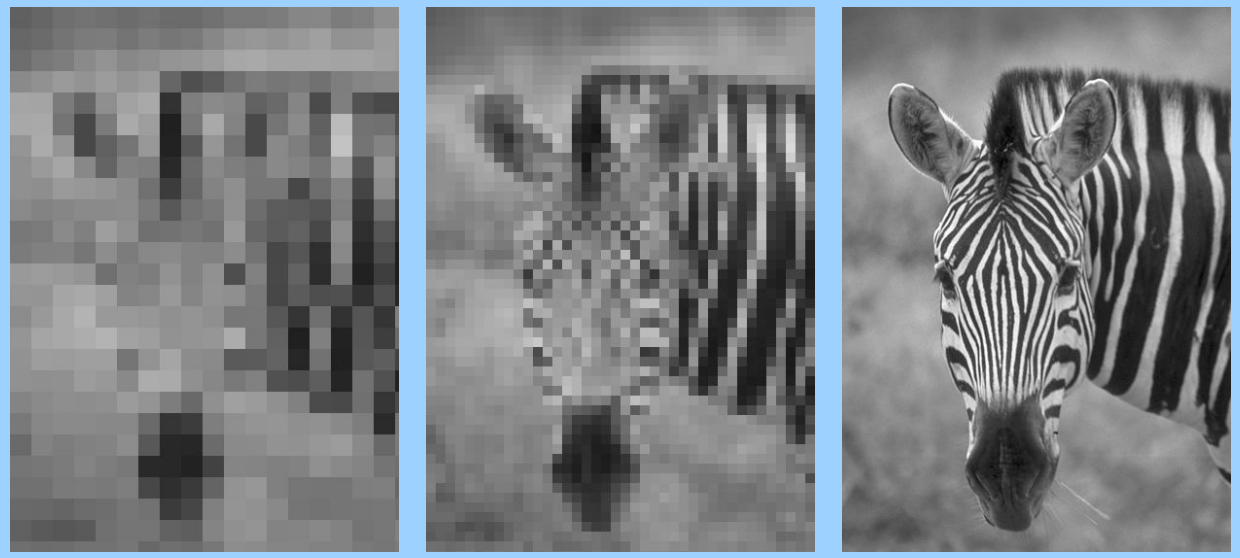
information depth:

A computer monitor can visualize 8 bit. 8 bit correlate to 256 gray values.

The bare human eye can distinguish about 60 gray levels.

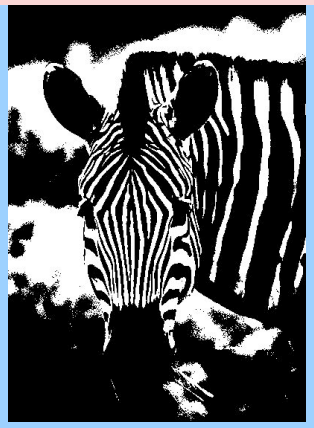
12 to 16 bit pictures allow for special image processing and quantification.

Digital Image Feature: Spatial Resolution



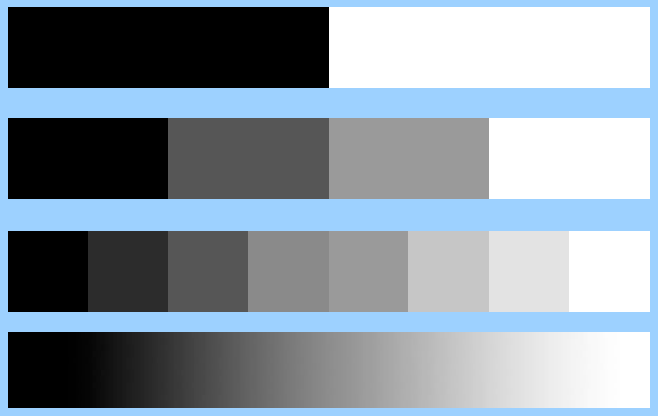
pixel area:	big	medium	small
resolution:	low	medium	high

Digital Image Feature: Intensity Resolution



1 bit

8 bit



1 bit (2^1 = 2 intensity levels)

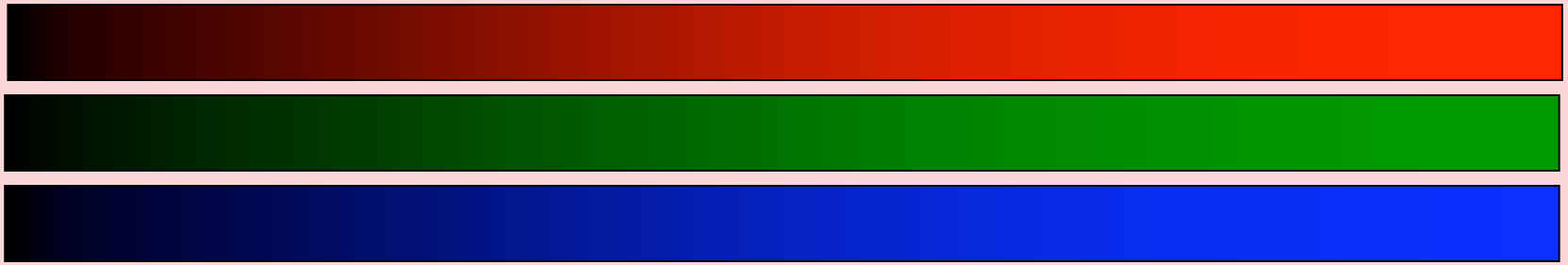
2 bits (2^2 = 4 intensity levels)

3 bits (2^3 = 8 intensity levels)

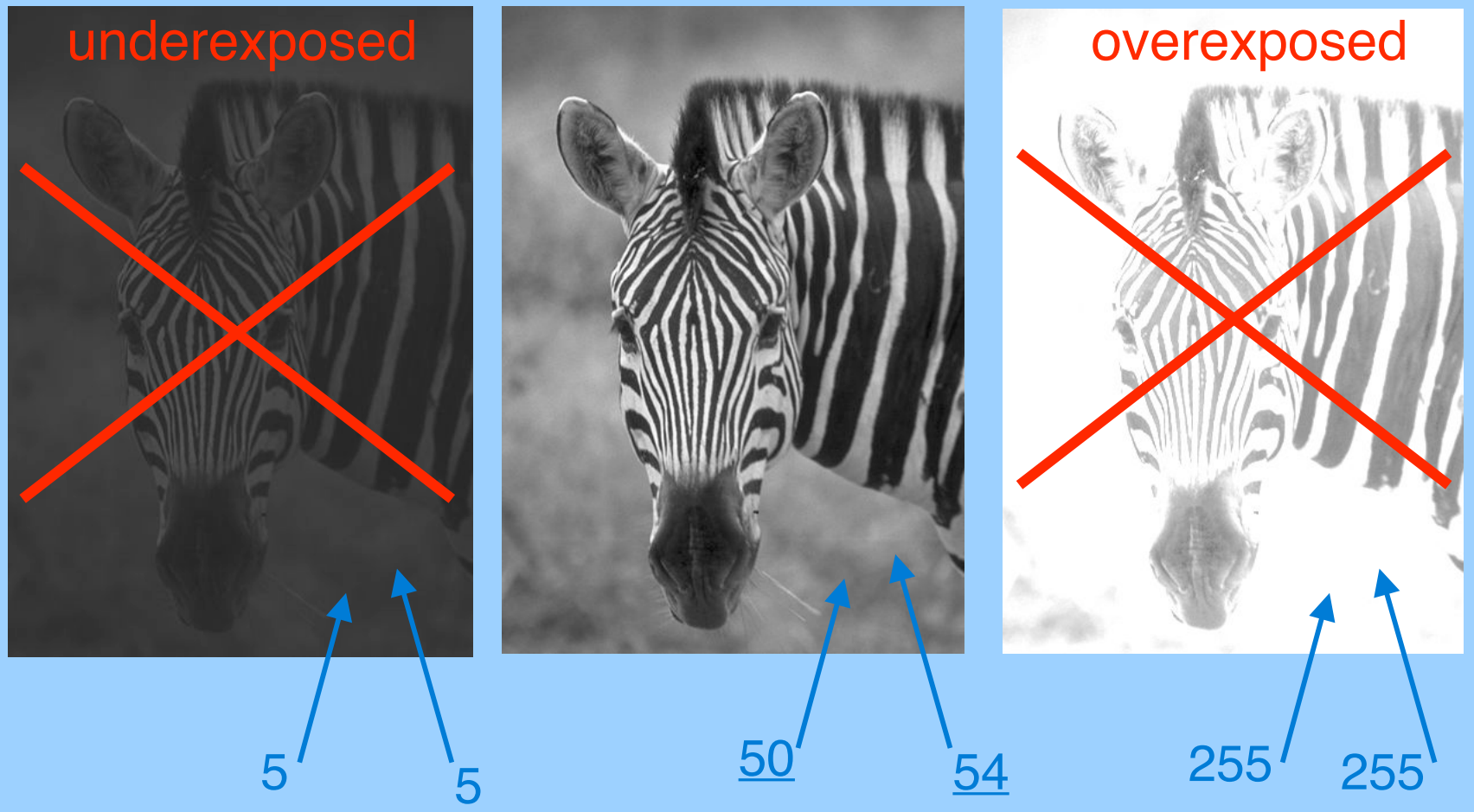
8 bits (2^8 = 256 intensity levels)

look up table (LTU)

Detected intensity values are displayed as gray levels. The display range of a typical 8-bit monitor covers 256 gray levels. The full range of the LUT is utilized if an image shows all shades of gray between black (=0) and white (=255). The gray levels might be presented in pseudo-colors.



the dynamic range of a digital image



Gain & Offset

gain and offset are used to adjust the detector signal (input) in a way that a maximal number of grey levels is included in the resulting image (output).

gain

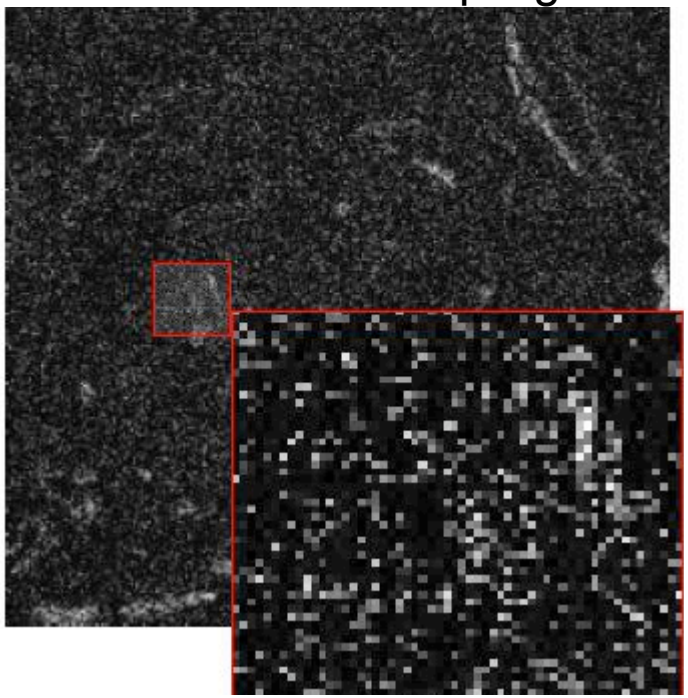
amplifies the input signal by multiplication, which results in a higher gray level value; bright features are brought closer to saturation, general image brightness is increased.

offset

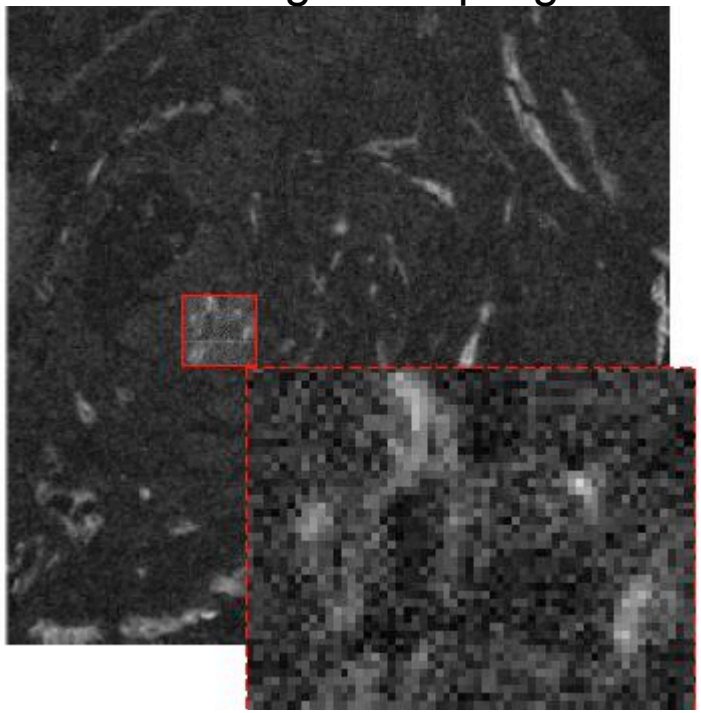
sets the gray level of a selected background to zero; adjust the darkest features in the image to black.

signal-to-noise ratio

short sampling time



longer sampling time

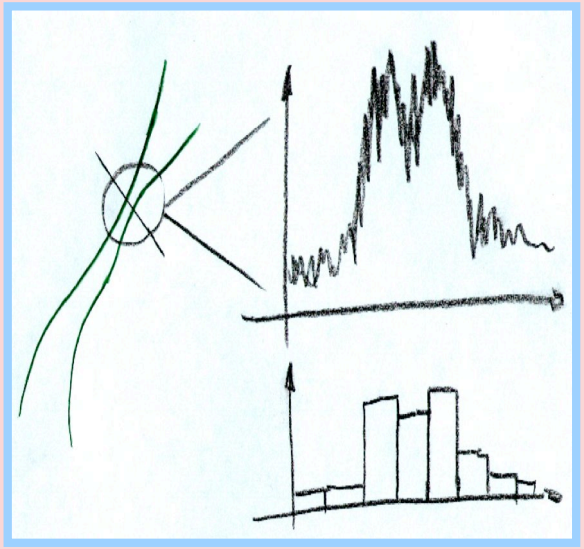


increase in sampling time improves S/N:

CCD: adjusting image brightness via „exposure time“ and „gain“

* *longer exposure time* -> less noise
* *higher gain* -> more noise (but faster aquisition!)

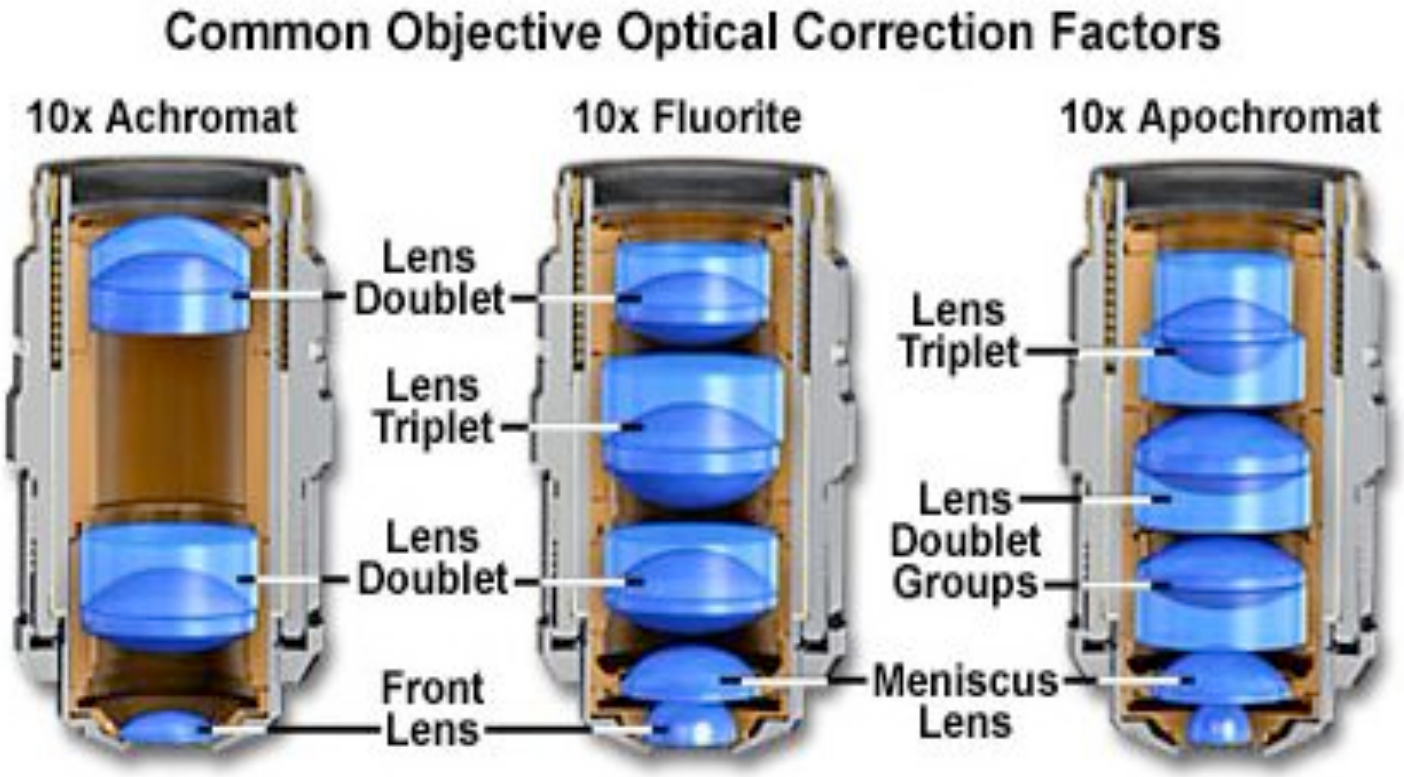
Digital image adjustments for hightest spatial resolution (xy)



Highest useful digital image resolution in light microscopy is acieved, if the minimal distance between distiguishable 2 points (= optical resolution of the objectiv) gets detected by 3 pixels.

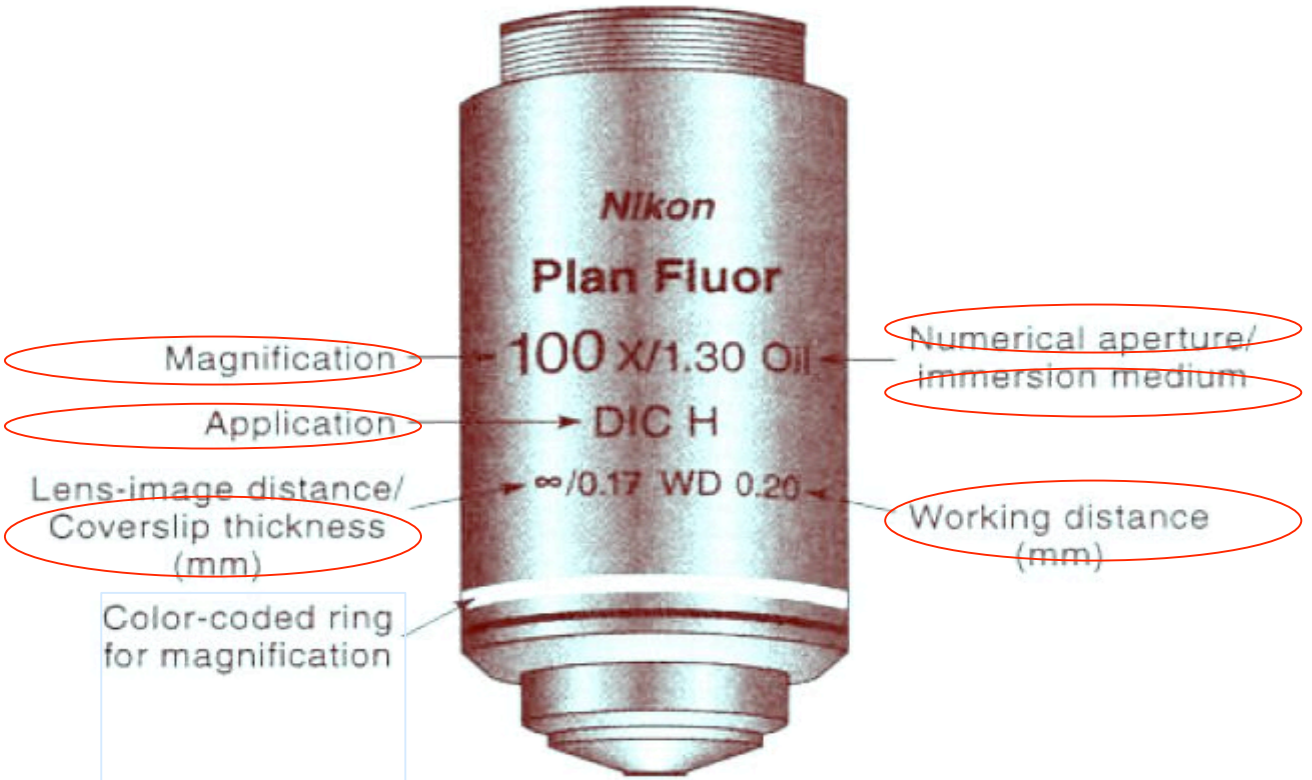
Adjustment of gain & offset can improve resolution.There has to be at least a 20%-difference in intensity between these points.

Types of Objectives



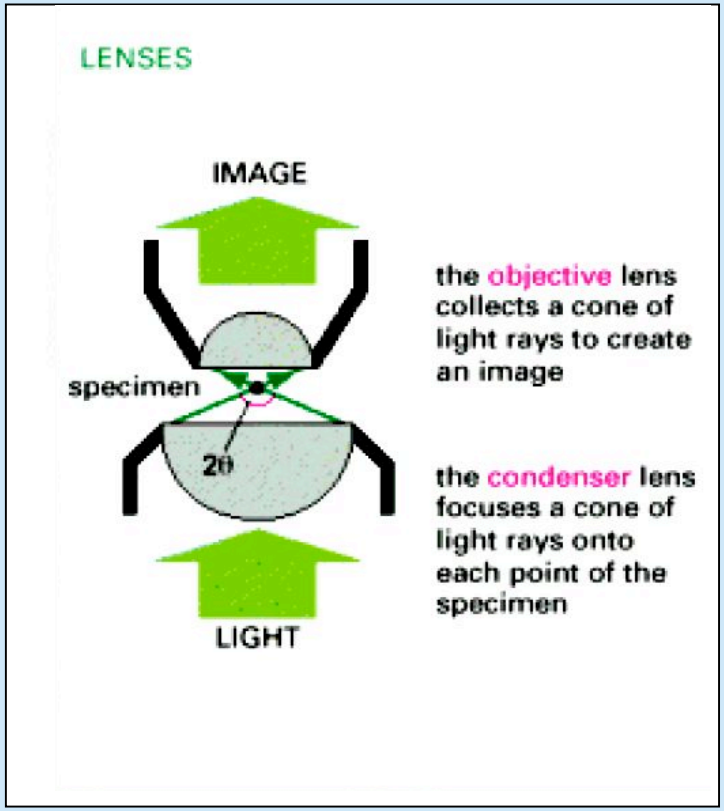
Objectives are complexe assemblies of several lenses and even other optical elements (i.e. appertures, phase rings). Depending on the inner components objectives have quite different performances. The specifications of a particular objective is encoded on the outer cylinder.

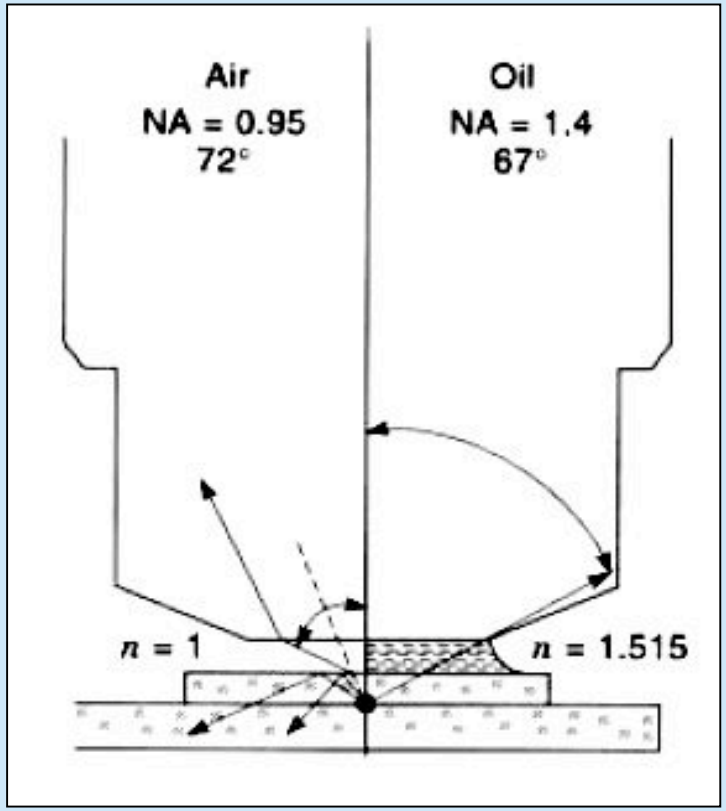
Markings on Objectives:



Mag.	1X	2X	4X	10X	20X	40X	50X	60X	100X
Color code	Black	Gray	Red	Yellow	Green	Light blue	Light blue	Dark blue	White

Numerical Aperture & resolution



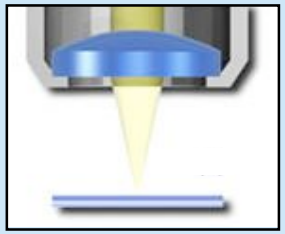


NA describes the light collecting ability of a lens.

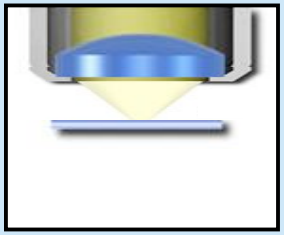
It is a function of the opening angle of the cone of ligth entering the lens and the refractive index of the immersion medium.

Resolution depends on the color of light and the NA of the objective (1/NA x 0,61 x λ). A higher numerical aperture objectiv has 1) a greater resolution power and 2) a brighter image. Dry lenses have a NA up to 1. For immersion lenses the NA might be as high as 1,4.

Numerical aperture and resolution power

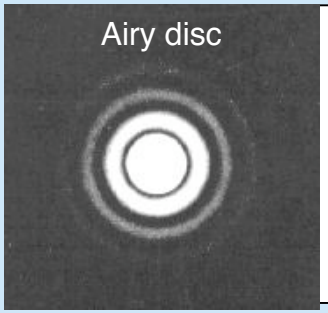


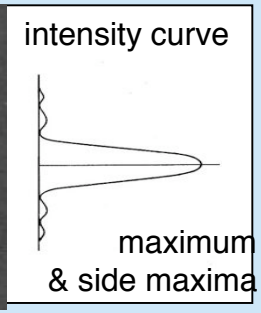
low NA

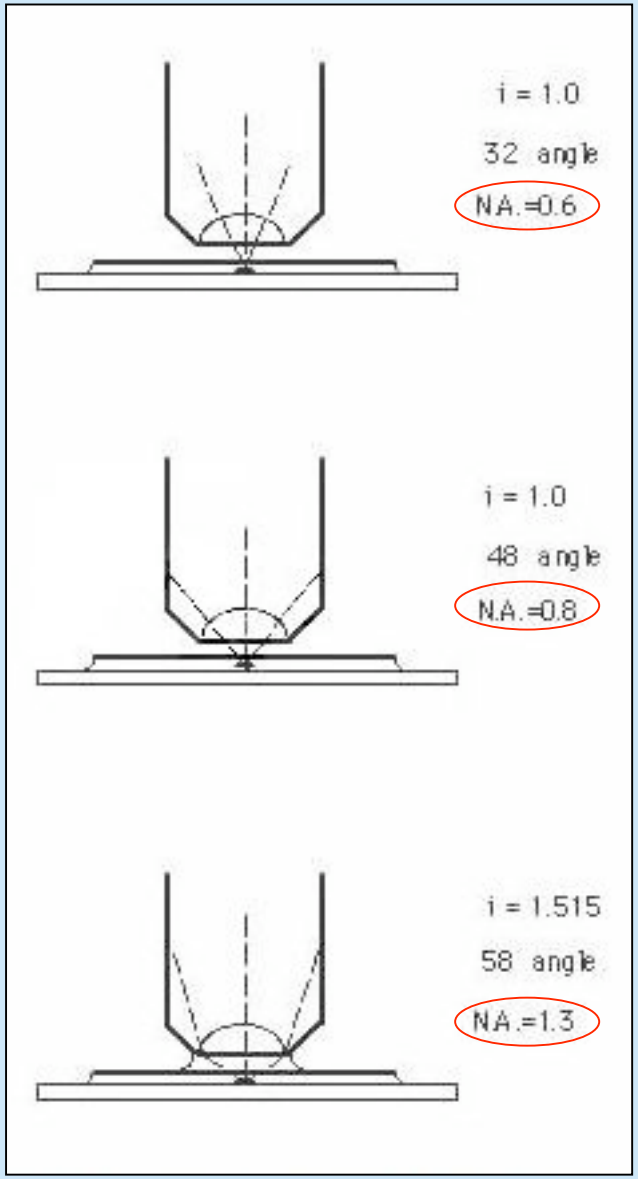


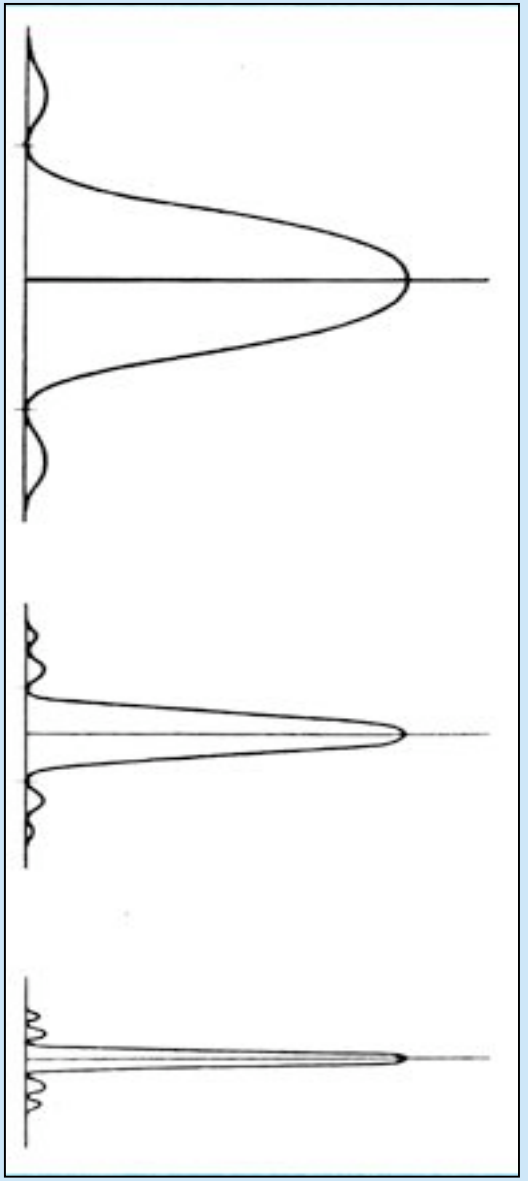
high NA

- Light cone
- Aperture angle
- Working distance
- Immersion media
- Airy disc

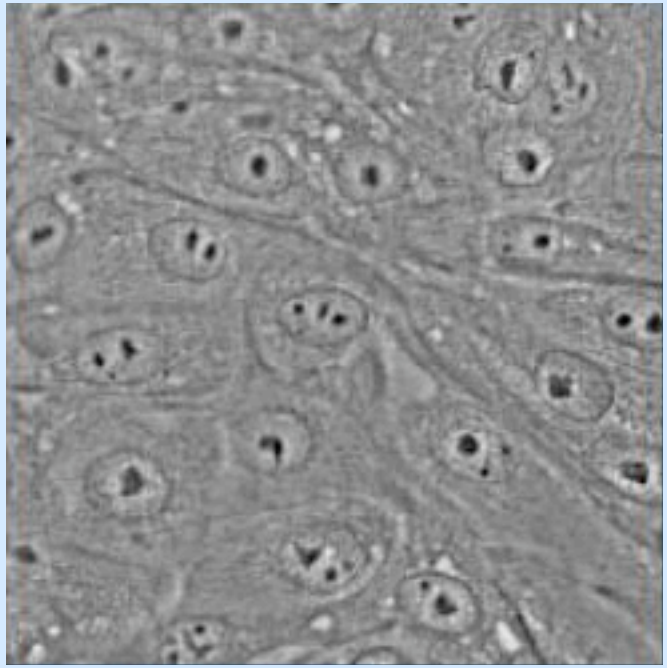




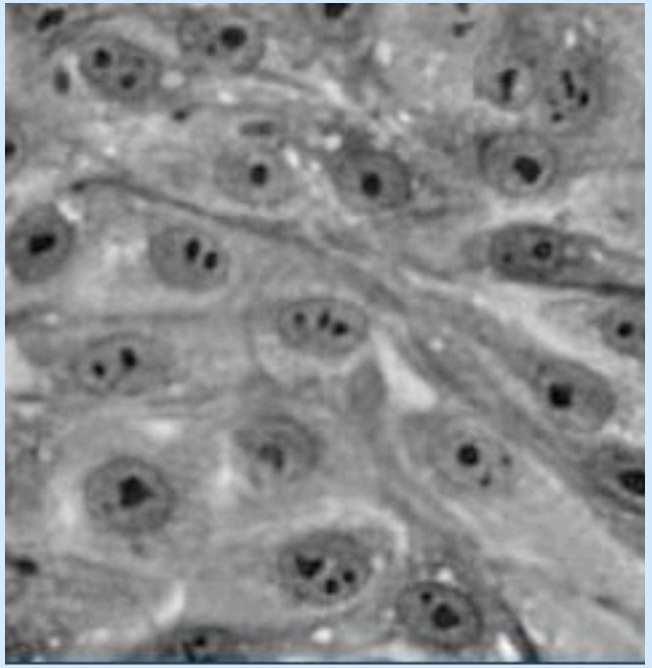




Objective resolution power



NA 1.25



NA 0.3

The aperture of the objective determines the resolution. As higher the numerical aperture as better the resolution power of the objective. (resolution ≠ magnification)

Quantum Efficiency

100 Photons/molecule generated

↓ Objective 1.4 NA, 130° opening angle

30 Photons/molecule

↓ 10% lost in objective

27 Photons/molecule

↓ 10% lost in tube lens and mirror

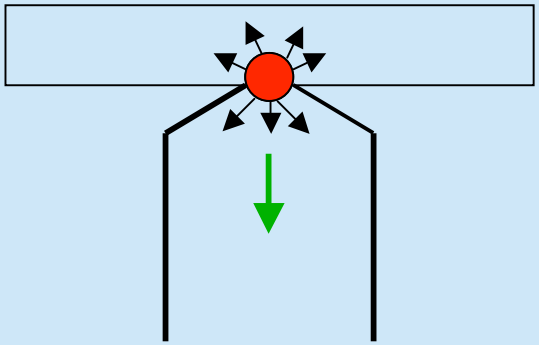
24 Photons/molecule

↓ 20% lost in filters

20 Photons/molecule

↓ Quantum efficiency of detector

ca. 6 Photons/molecule detected



The microscope is a „light loss machine“. It is important to keep the losses as small as possible by choosing the right optical elements (objective, fluorescence filters) and fine adjustment of the microscope (köhlern, polarisation filters out, ...) .

i.e.: engravings on the 20 x objective for various immersion media

Order Number

Walloston prisma
C requested for DIC

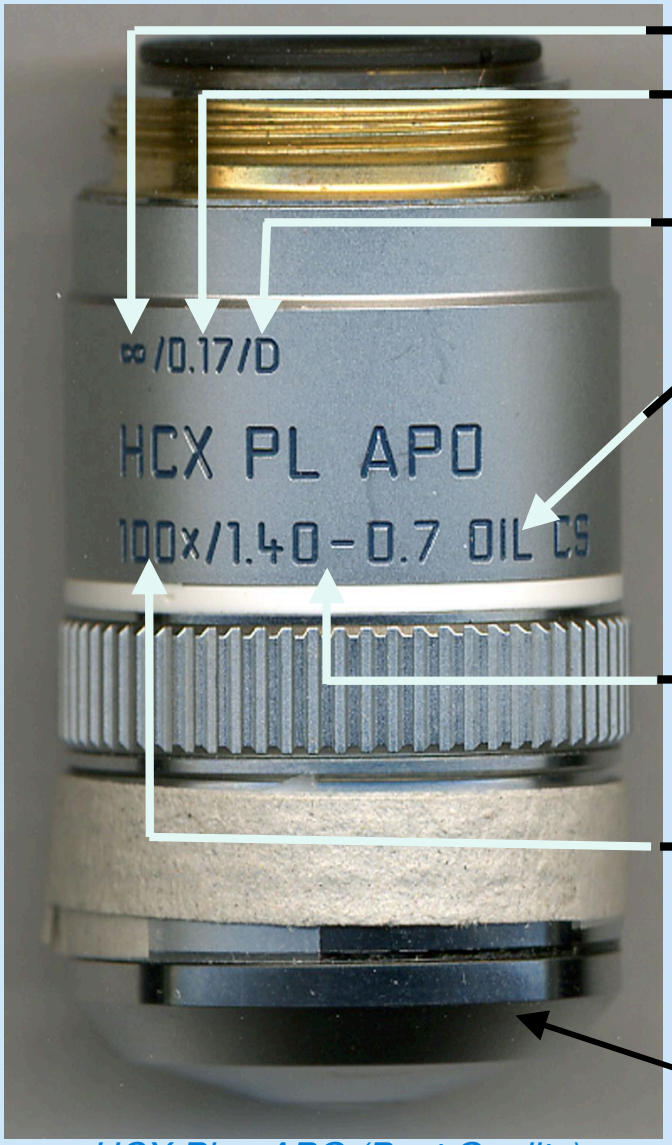
Objective corrected
for Blue - Green

Water immersion with
or without coverslip

Point set for the
type of immersion:
oil or glycerol or water
Caution: Clean objective before usage!

The front piece of all
immersion objectives
can be retracted.
Check the position!

i.e.: engravings on 100 x objectives



HCX Plan APO (Best Quality)

Infinity Optics

Coverslip thickness:
0.17 mm

Wollaston prisma
D required for DIC

Oil immersion

PH 3 condensor
Position required
for Phase contrast

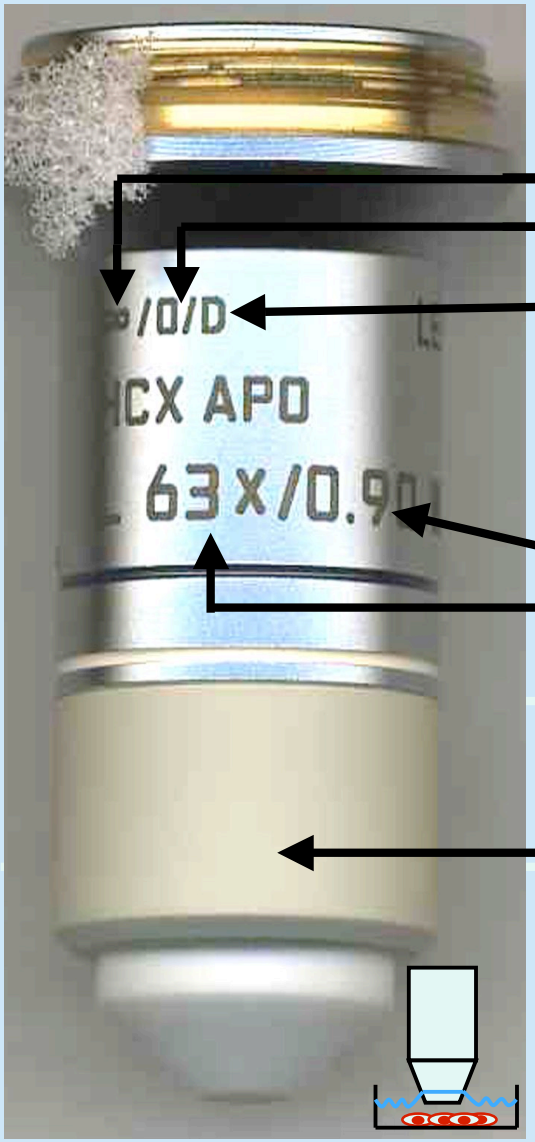
Numerical aperture
Adjustable between
1.4 - 0.7

Magnification 100x

The front piece of all
immersion objectives
can be retracted.
Check the position!

PL Fluotar (Medium Quality)

i.e.: engravings on special objectives



infinity optics

no coverslip -> *“dip in”*

Wollaston prisma
D required for DIC

Correction for temperature and coverslip thickness

Numerical aperture 0.9

Magnification 63 x

refracting Index:
1.46 for 80%Glycerol

The front is ceramic coated to allow the objective to be dipped into aggressive solut-ions. Free working distance 2.2 mm

HCX APO L (Dip In)

HCX PL APO (Glycerol Immersion)

List of Engravings on Leica objectives

Type of Objective

N PLAN	distortion corrected lens
N PLAN L	Plan long free working distance
PL FLOUTAR	Plan Fluotar (suited for fluorescence work)
PL FLOUTAR L	Plan Fluotar long free working distance
HCX PL APO	Plan Apochromat especially well suited for Confocal work
HCX PL APO L U-V-I	Plan Apochromat long free working distance UV – I R

C PLAN achromats are only recommended for fluorescence under certain conditions

Immersions:

Oil	= standard immersion oil
IMM	= either water, glycrine or oil
W	= water

Techniques:
(transmitted light brightfield, transmitted light darkfield, fluorescence & DIC contrast are not explicitly mentioned)

BD	= for brightfield/incident light darkfield
PH	= phase contrast objective
L	= long free working distance
∞	= Objective made for infinite width
RC	= refection contrast objective
P, POL	= low strain, for quantitative polarization
LMC	= Modulation contrast objective (only with DM IRB)
/	= not for incident light, except fluorescence

List of Engravings on Leica objectives, cont.

DIC prisms for interference contrast:

Condenser Prisms:

K_{1a}	only DM R with condensers UCR/UCPR, condenser head swung out
K_2-K_5+K_{11}	only with condenser top 0.90 S1 or P 0.90 S1 (upright)
K_9,K_{12},K_{15}	only with condenser top P 1.40 Oil S1

Objective prisms: A – E:

Prisms	B_2/D wide shearing = higher contrast
	B_1/D_1 narrow shearing = higher resolution

Engraving in one line separated by /

Magnification / Numerical Aperture / Techniques, CORR = Correcting Ring

(whenever there is a possibility for correction -> CORRECTION HAS TO BE DONE ! !)

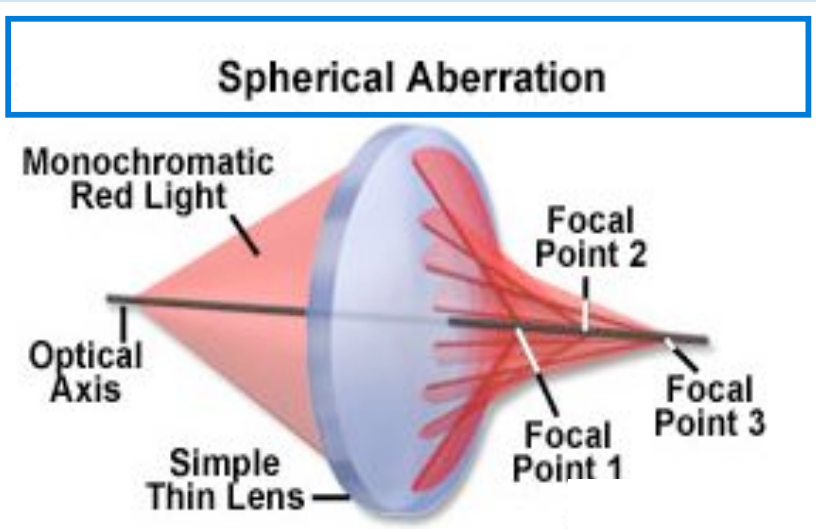
Coverglass specification:

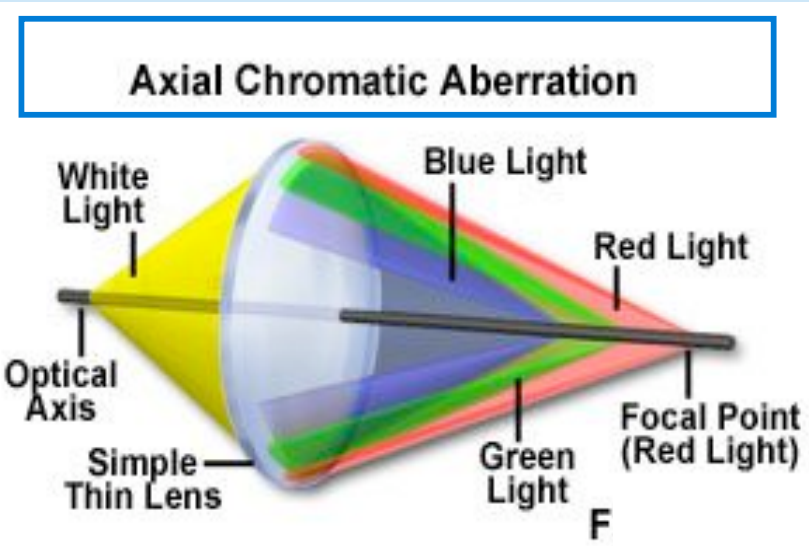
-	for use with and without coverglass
0	for use without a coverglass
0.17	for use with a 0.17 mm coverglass (DIN/ISO)
1.80	for use with 1.8 mm quartz glass window on heating stages
0 – 2	for use with coverglasses of 0 – 2 mm thickness

Rules for objective usage

- Check if the objective of your choice is mounted.
- Check if the acquisition software recognizes the objective.
- For immersion lenses: the front part has to be released out of the retracted position.
- Use the correct immersion medium. For inverted microscopes: Use immersion medium very sparingly to prevent spilling over and entering the optics.
- If you need a special objective, look up the list of the objectives available.
  Hold and support the objectives with two hands while mounting them.
  Do not forget to activate the new objective in the program -> select the objective according to the unique order Number.
  User of inverted microscopes: Keep the mounting holes for objectives on the revolver always covered!!!
- Cleaning of the objectives: clean the metal body around the lens with a soft tissue; the glas part might be cleaned with lens paper.
  The cleaning is especially necessary for objectives which can be used with either oil, water or glycerol as immersion medias.
- In case of heavy dirt: clean the lens with cotton wool tips with the help of a stereo microscope and use solvents according to the dirt or immersion media:
  * ethanol/ether=1:1 or 100% ethanol to remove oil
  * distilled water to remove glycerol or dirt such as Mowiol remains

Imaging Errors of Objectives





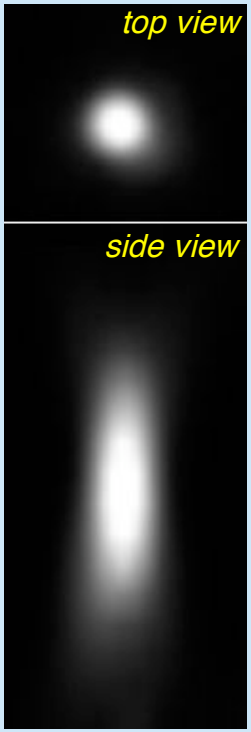
Objectives with a corrective inner life:

Achromates	the least corrected objectives, but cheap, used for routine work.
Planchromates	corrected for spherical aberration, but not for chromatic aberrations.
Plan Apochromate	well corrected for chromatic and spherical aberration
Fluorites	well corrected for chromatic and spherical aberration and well suited for UV and fluorescence applications. Cheaper than Plan apochromates.

Aberration effects in the data

3D dataset of multifluorescent beads.

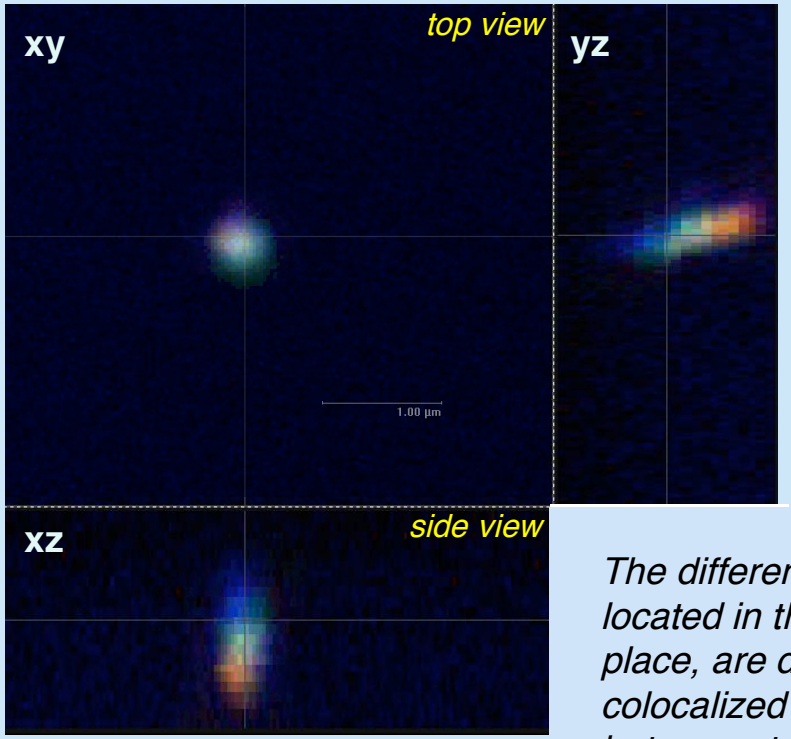
sperical aberration



The 3D round object looks perfectly concentric in xy, but iselongated in z

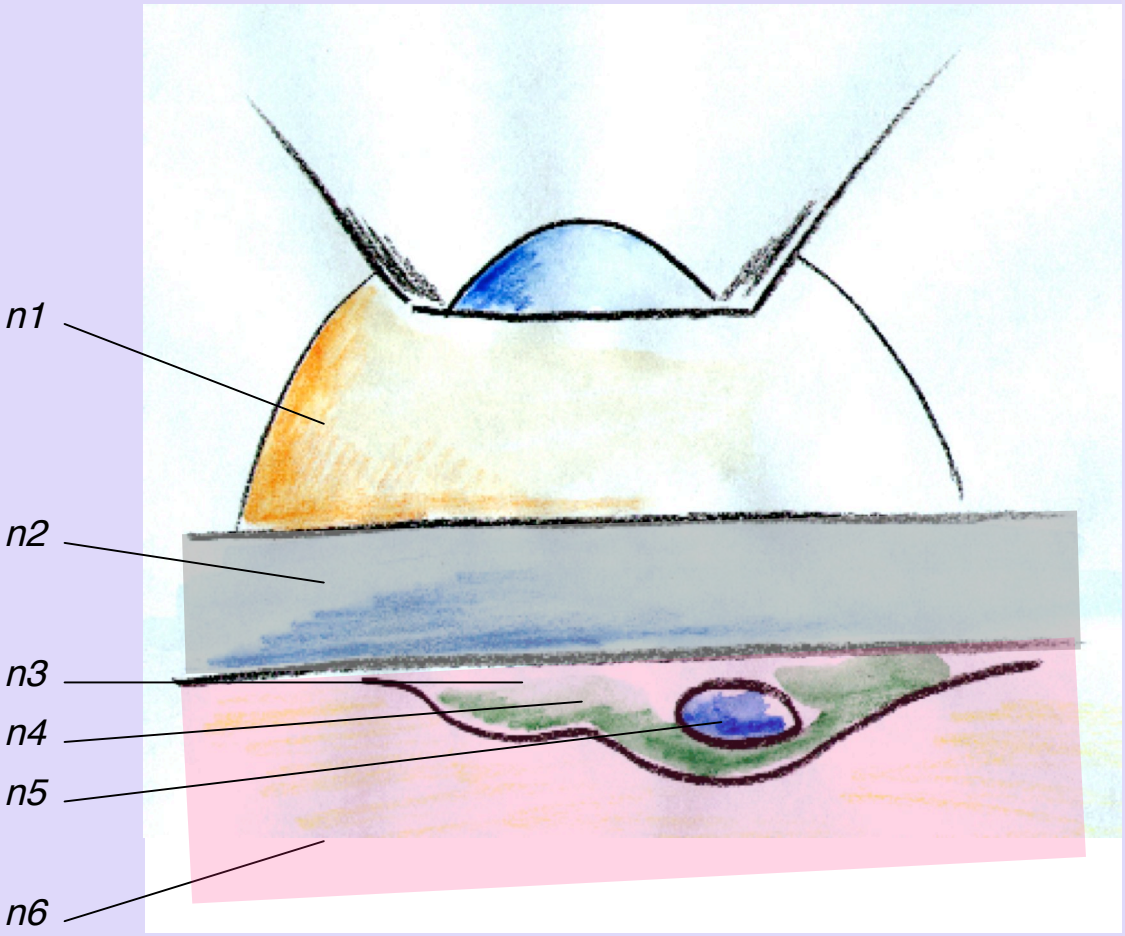
-> point spread function PSF.

axial chromatic aberration



The different colors, located in the same place, are depicted colocalized in xy , but seem to be shifted apart in z.

Optical components at the sample plane



Objective

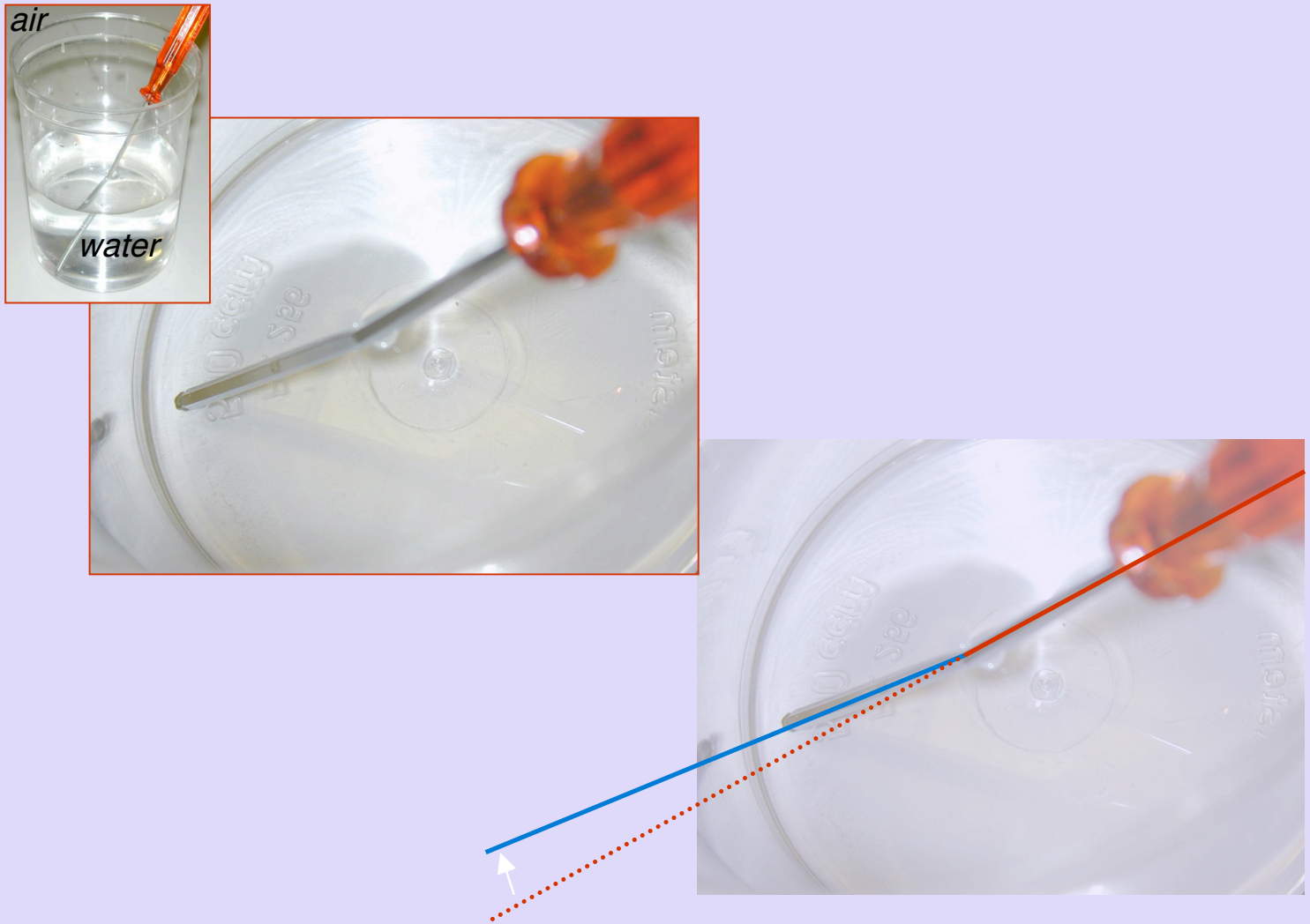
Immersion medium
Oil: n = 1.518
80% Gly: n = 1.45
Water: n = 1.33
Refractive Index & Temperature

Cover slip
glass: n =1.51
Refractive Index & Thicknes & Planarity & Tilt

Sample
Cell structures (Cytoplasma, organells, etc.) -> n = 1.35+/-
Medium: buffer n = 1.33
Moviol mount: n = 1.46
Refractive index & depth

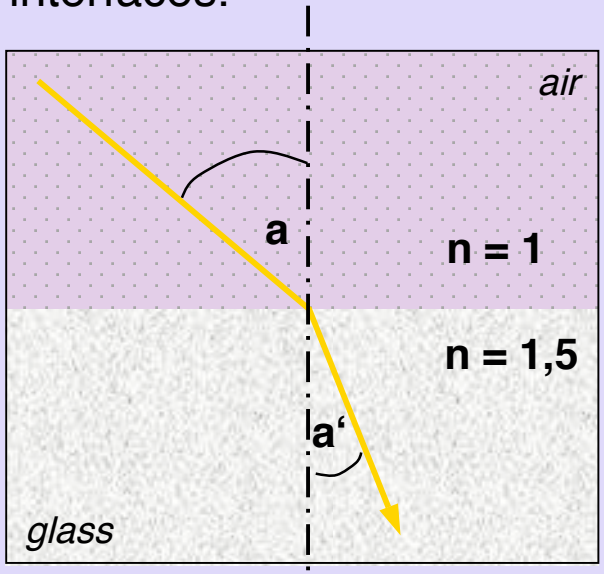
As the user is adding immersion medium, cover slip and sample to the micro-scope s/he has to exercise care in using the appropriate objective lens fitting to these optical components in order to get quality images.

Refractive Index – what's this?



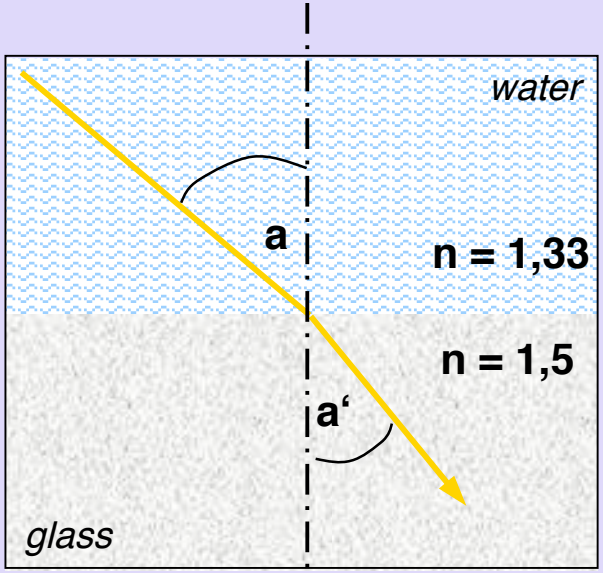
Refractive Index – what's this?

-> describes the speed of propagation of light in a medium. The difference in speed between a medium and vacuum is given by a factor which is called the refractive index *n*. Refraction effects occurs at optical interfaces.



Light ray coming from air passing glass

„strong refraction“



Light ray coming from water passing glass

„weaker refraction“

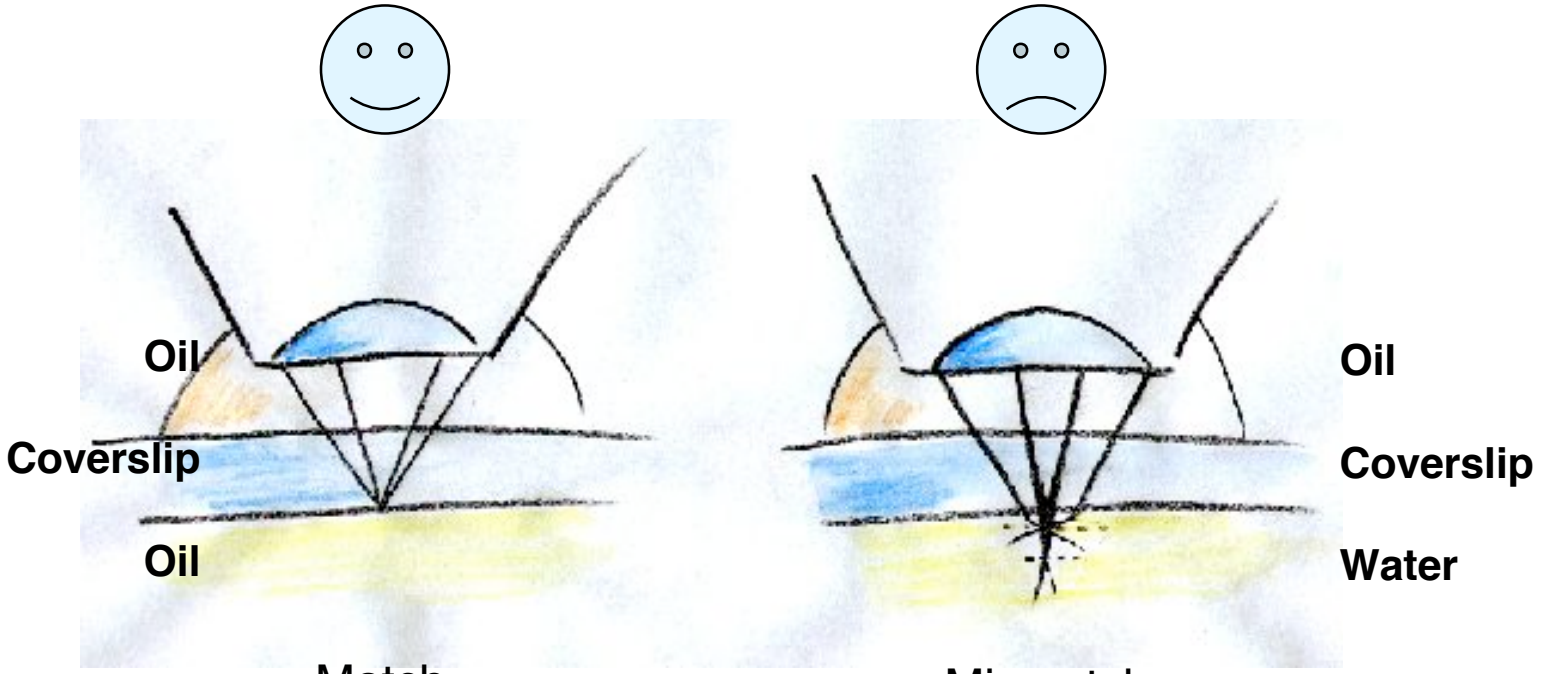
a to a‘ -> the light changes its direction = is refracted!

Refractive indices of different immersion and mounting media

Material	refractive index
Glass	1,51
Oil	1,518
100 % Glycerol	1,46
80% Glycerol / 20% water	1,451
Water	1,333
PBS	1,334
50% PBS / 50% glycerol	1,406
Vectashield	1,4523
Canada balm	1,5225
Moviol	1,46

The refractice indices of sample and immersion medium have should equal each other. Use immersion media accordingly to the engravings of the objectives!

Refractive Index Match & Mismatch



Match

Mismatch

Refractive index mismatch of the sample and the immersion medium drastically reduces resolution. Even worse in fluorescence it leads to significant loss of intensity! It is responsible for aberrations and distortions.

Requirements for good image resolution

- Immersion medium

-> Use objectives for either oil, glycerol or water (or immersion-adjustable ones) and choose the right immersion media in order to minimize refractive index mismatch with the sample.

  - **Air** -> long distance objectives, no good refractive index match -> limited resolution and brightness. Ideal for plastic culture dishes, thick samples
  - **Water** -> ideal for samples in buffer or culture medium (fixed or alive)
  - **Glycerol** -> ideal for long term live cell studies, mounted fixed samples, very thick samples. Best resolution and depth penetration.
  - **Oil** -> ideal for very thin mounted fixed samples, Best brightness and resolution.

- Coverslip thickness

-> Use the right cover slips (**0,17** mm, Grade “1.5”) or objectives which can be adjusted for the thickness of cover slips.

Objectives are designed for cover slips measuring 0.17 mm in thickness. 15μm cover slip deviation approximately kills half of the z-resolution and signal intensity.

- Temperature

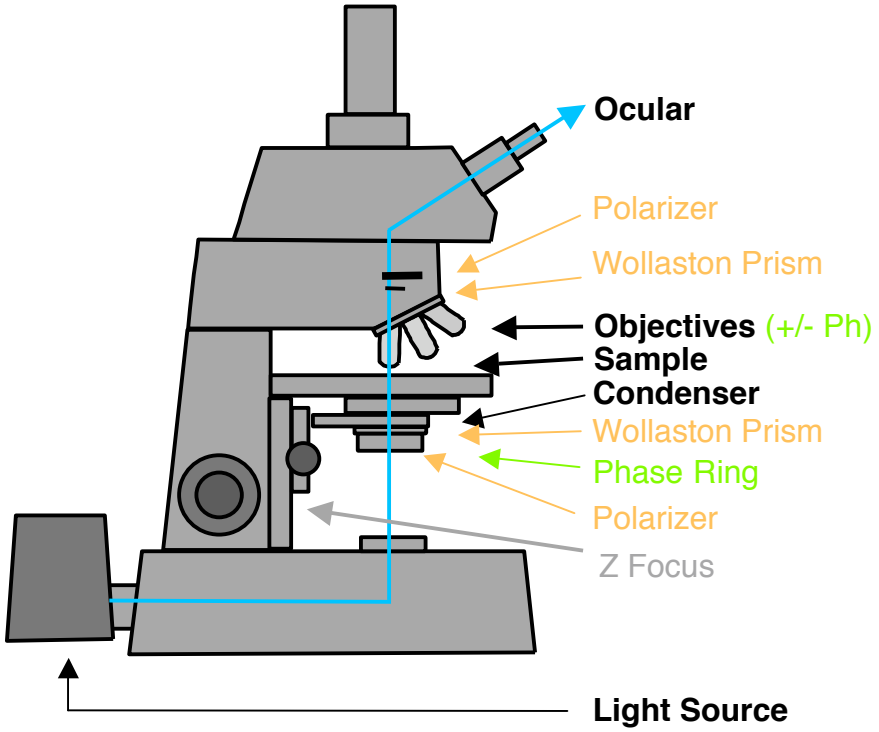
! Objectives are (usually) designed for **21°C**. There are special objectives designed for 37°C. Temperature has an influence on refractive index and the position of lenses!

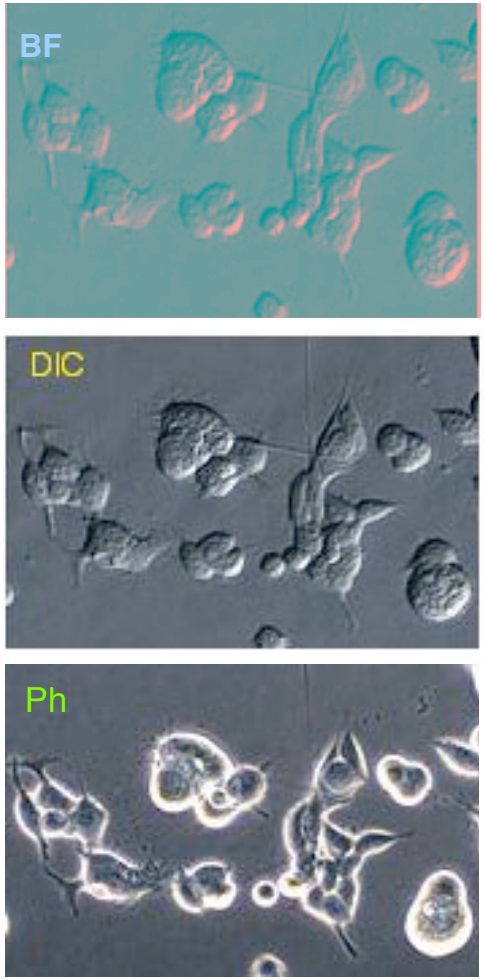
The parts of the microscope

- Light sources (Halogen, XBO, ...)
- Light conditioning
  - Köhler illumination
  - Phase ring
  - Wollaston prism and polarizers
  - Filter cubes (for fluorescence)
- Specimen (Cover glas)
- Objective (±Immersion medium)
- Detector (PMT, CCD)

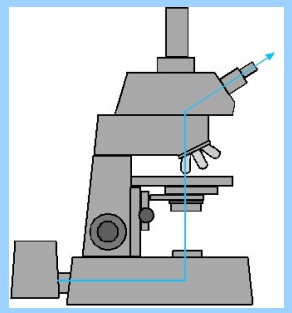
Fundamental Setup of Light Microscopes:

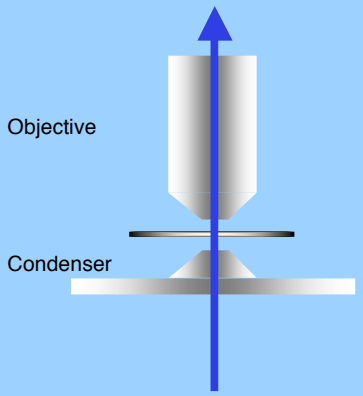
Bright field (DIC, Ph)





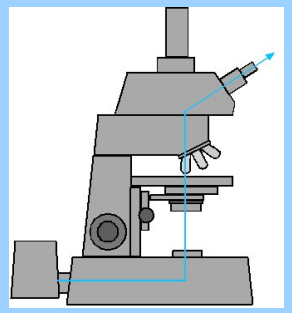
Bright Field Microscopy

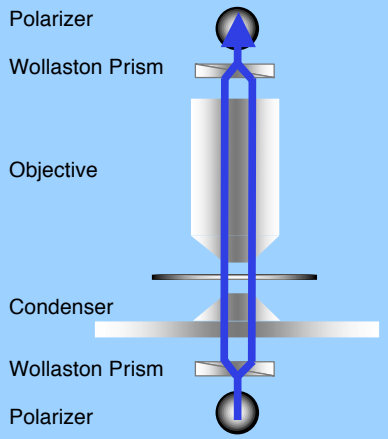




Alignment:
Köhler illumination
Condenser aperture: close max 20%
Field aperture: illuminaton of field of view

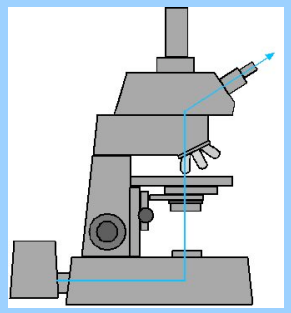
Differential Interference Microscopy

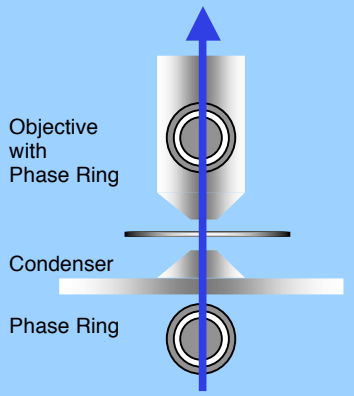




Alignment:
Köhler illumination
Condenser aperture: close max 20%
Field aperture: illuminaton of field of view
Adjust polarizers and wollaston prisms

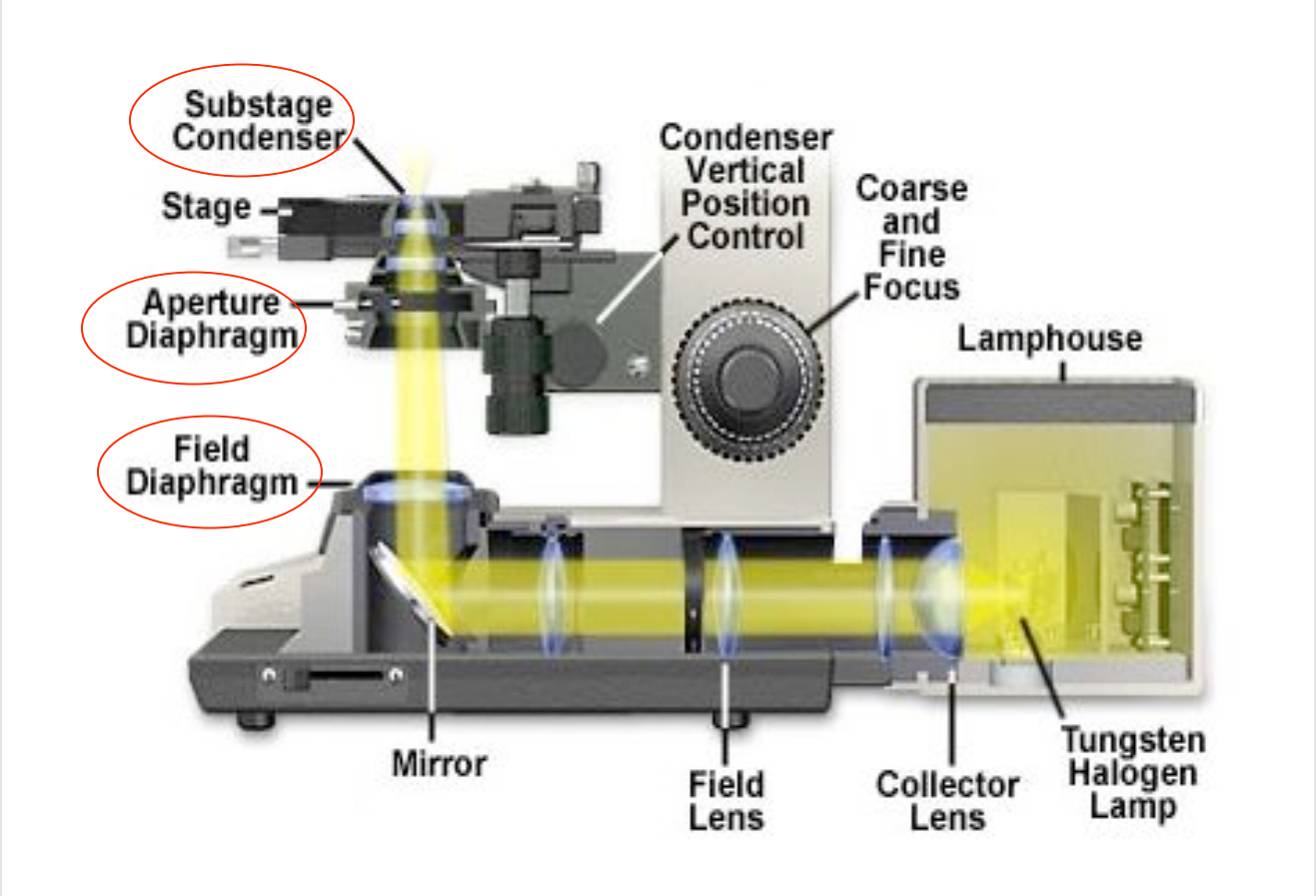
Phase Contrast Microscopy



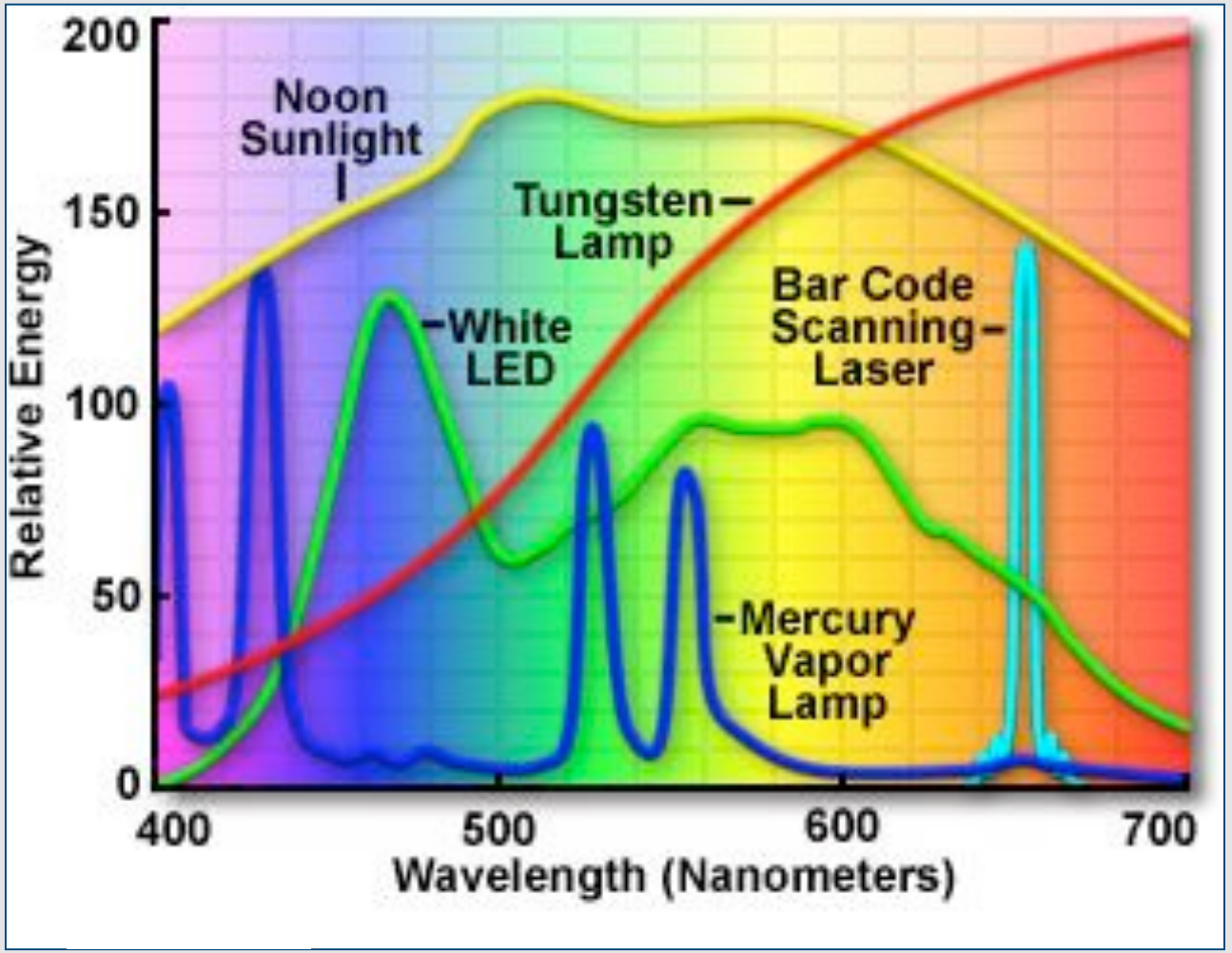


Alignment:
Köhler illumination
Condenser aperture fully open
Field aperture: illuminaton of field of view
Adjust correct phase rings

Light Path for Transmitted Light

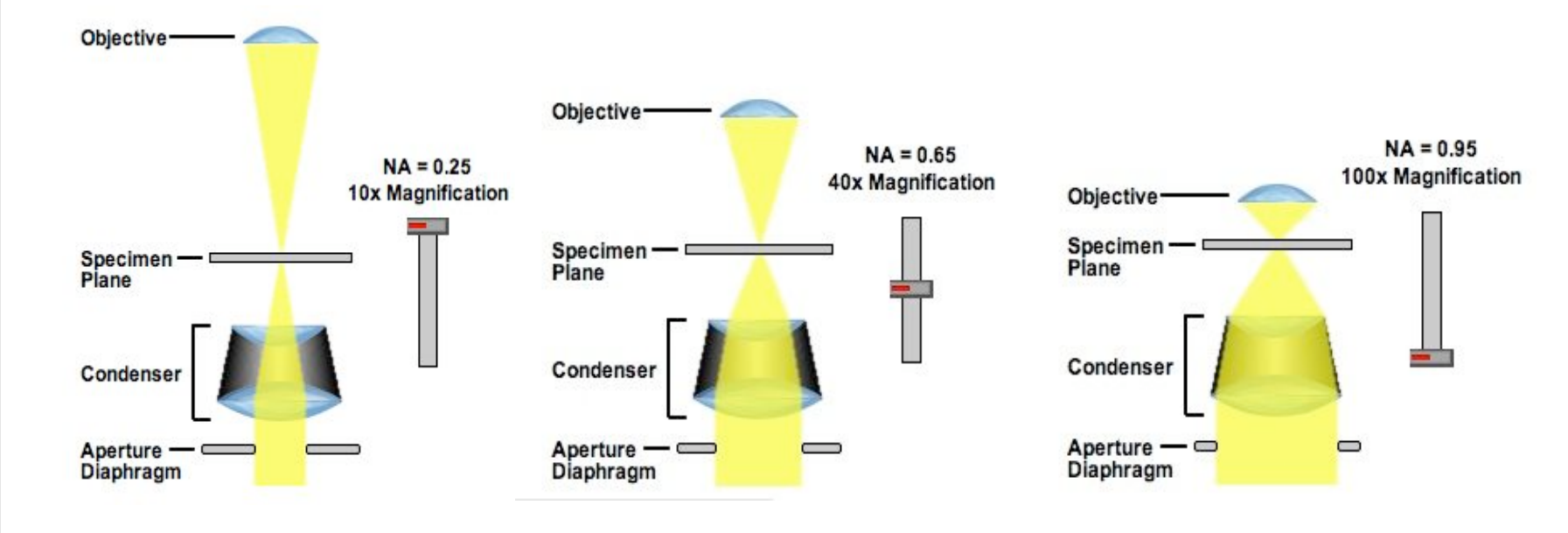


Lamp Spectras in comparision



Tungsten lamp	-> bright field illumination
XBO, Mercury Vapor, LED	-> Fluorescence exitation
Laser	-> CLSM

Condenser



The main purpose of the condenser is

1. To produce an evenly illuminated field of view
2. To light the specimen with a cone of radiation as wide as possible in order to achieve maximum resolution of fine details.
3. The condenser is a carrier for special contrast enhancing systems such as ring apertures for phase contrast, Wallostone prism for DIC,…

Five Steps to Align the Light (Köhler-Illumination)

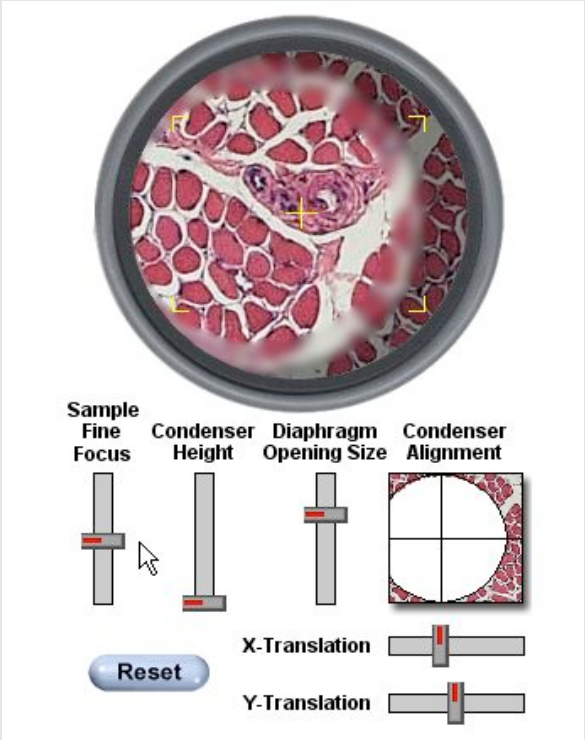
1. *Focus onto the structure of interest in transmitted light mode (bright field or phase).*

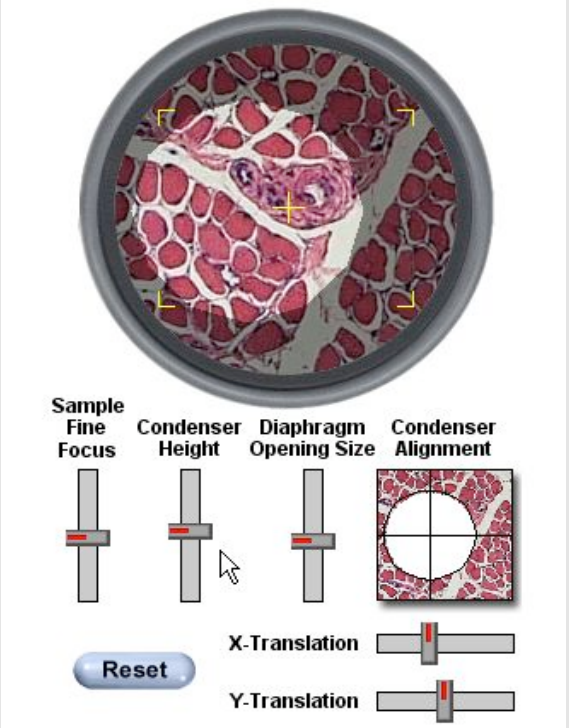
2. *Close the field diaphragm.*

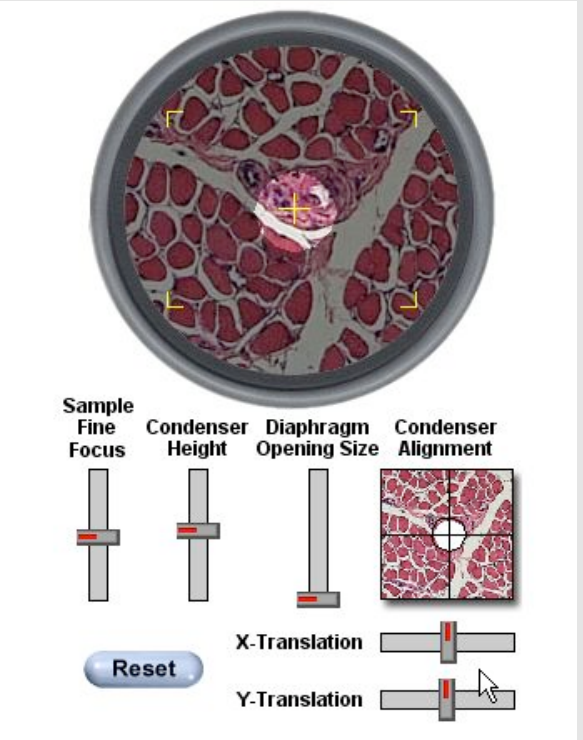
3. *Adjust condenser height to create a sharp image of the field diaphragm (-> a bright hexagon).*

4. *Center the hexagon.*

5. *Open the field diaphragm just a bit larger than the field of view.*

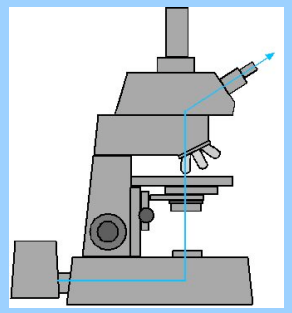


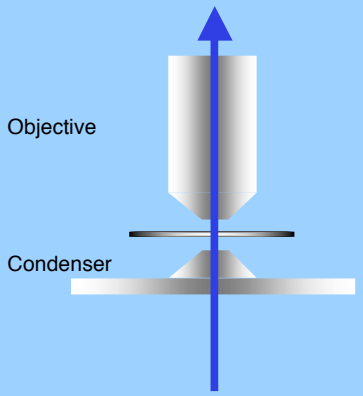




Light Path & Optical Elements in Different Microscopic Techniques

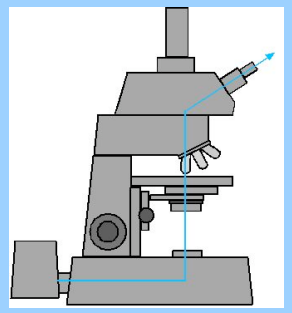
Bright Field Microscopy

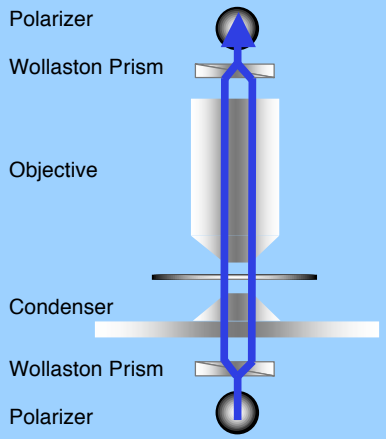




Alignment:
Köhler illumination
Condenser aperture: close max 20%
Field aperture: illuminaton of field of view

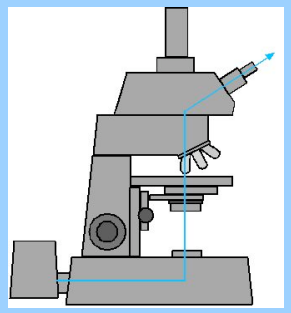
Differential Interference Microscopy

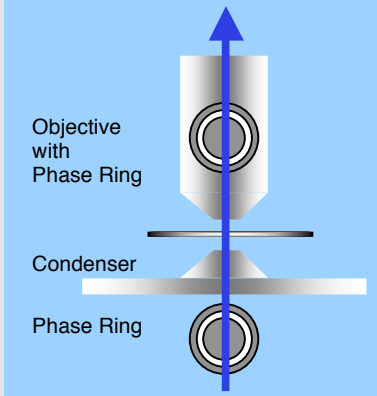




Alignment:
Köhler illumination
Condenser aperture: close max 20%
Field aperture: illuminaton of field of view
Adjust polarizers and wollaston prisms

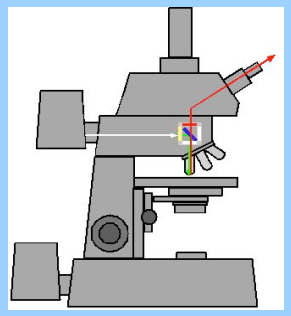
Phase Contrast Microscopy

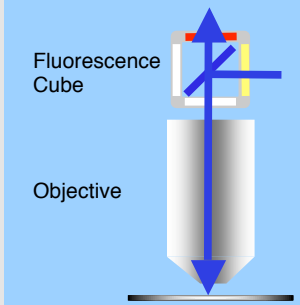




Alignment:
Köhler illumination
Condenser aperture fully open
Field aperture: illuminaton of field of view
Adjust correct phase rings

Fluorescence Microscopy





Alignment:
Correct alignment of fluorescence lamp

tips & tricks at the mic

First choose your objectiv. Then check the microscope settings: they should correspond to the informations on the objective (i.e. condensor position, immersion media, wollaston prism, polarisator). Find the focus, then köhler the condensor, optimize the transmitted light contrast, then adjust eventual objective correction rings.

In fluorescence mode the Köhler-adjustment of the condensor might be omitted. Check that all Polarizers are out of the fluorescent beam path. Avoid phase contrast optics.

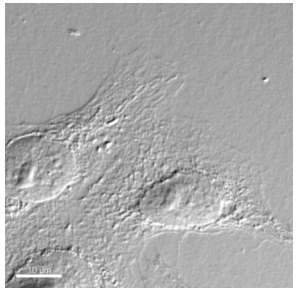
The easiest way to find a focus is in the fluorescence mode: just turn the focus wheel in the direction of increasing brightness.

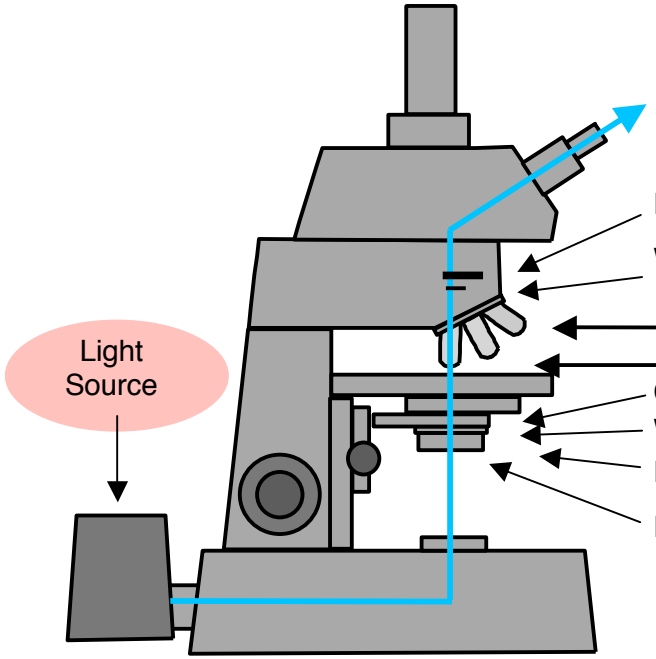
The border of the cover glass or a dot with a marker pen on the slide allow to focus quickly on a non-fluorecent sample.

Fundamental Setup of Light Microscopes:

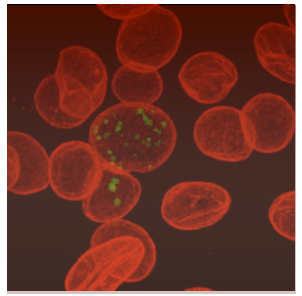
Bright Field vs. Fluorescence Mode

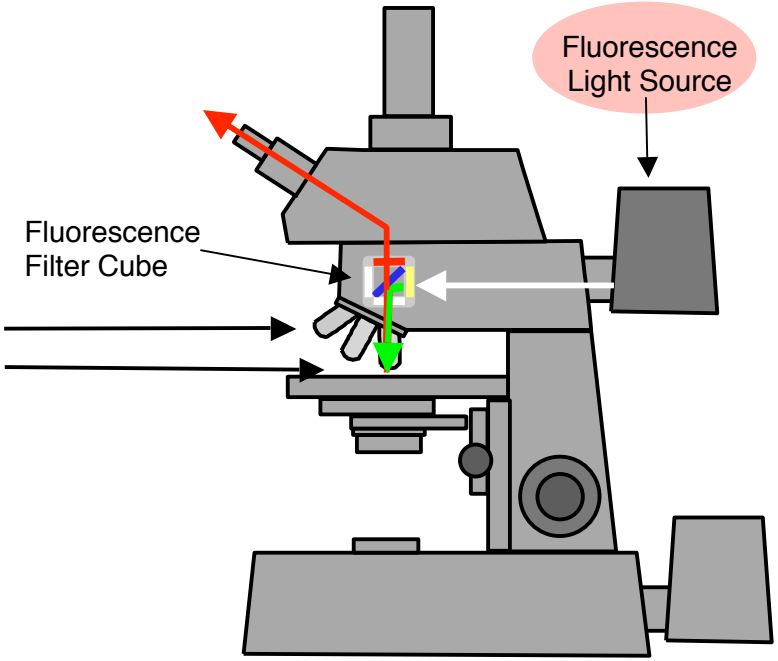
Bright Field Microscopy



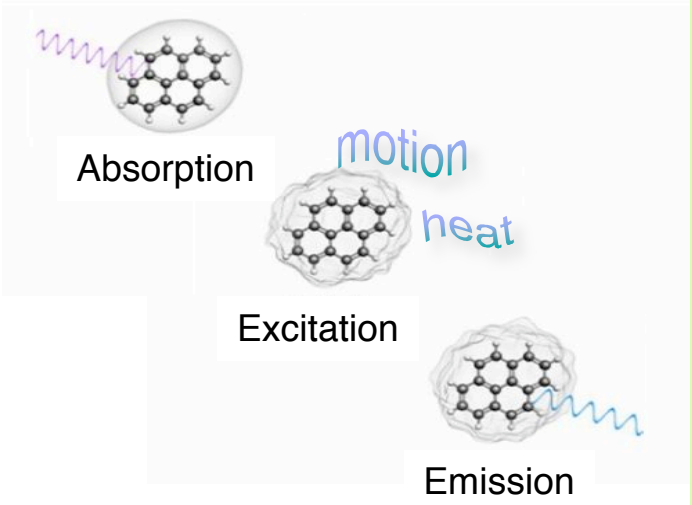


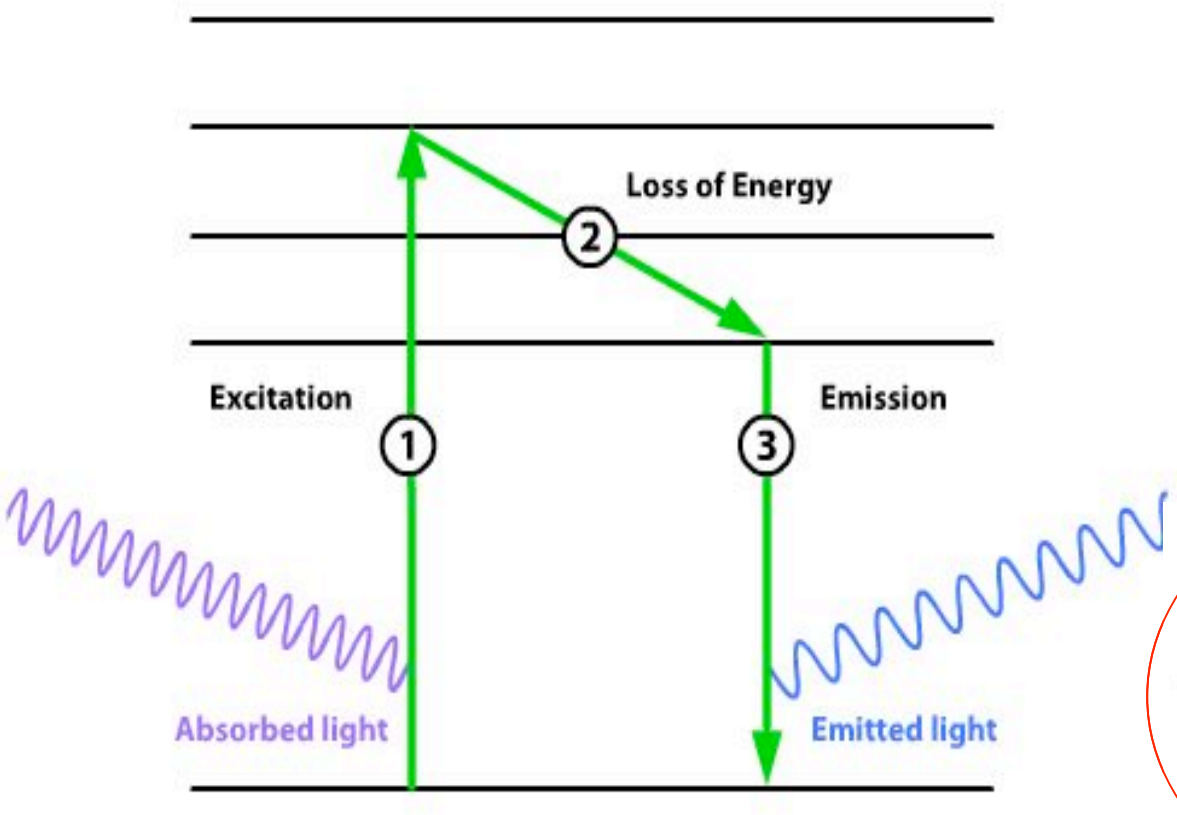
Fluorescence Microscopy

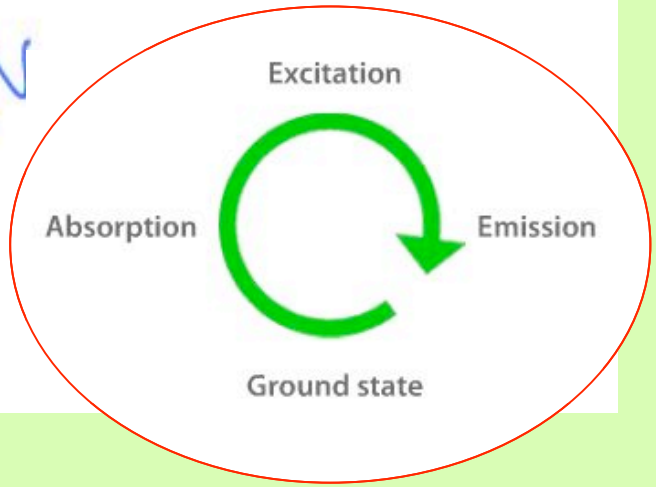




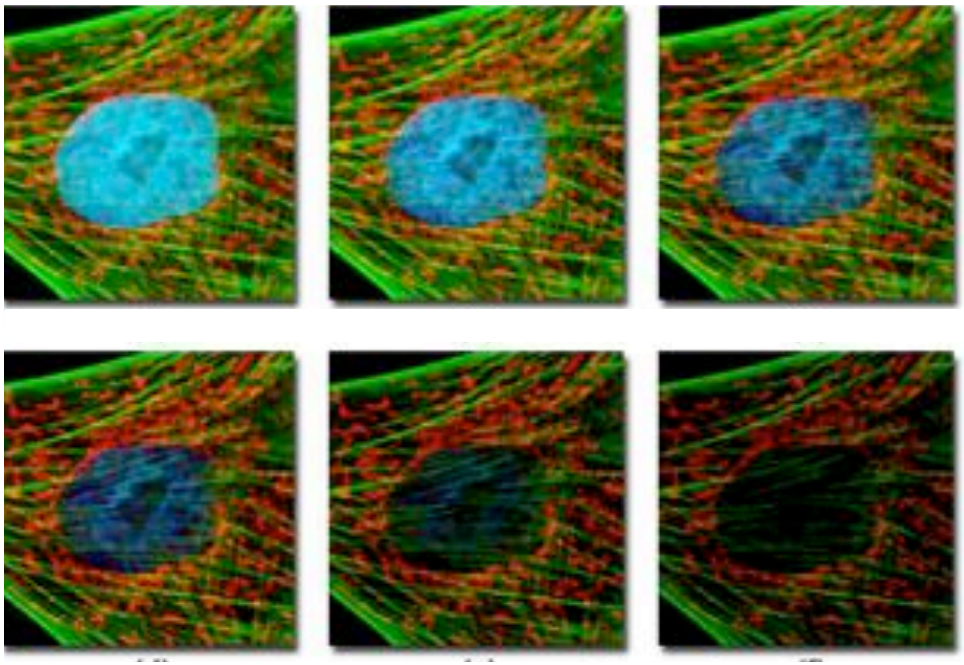
Flourescence: absorption & emission

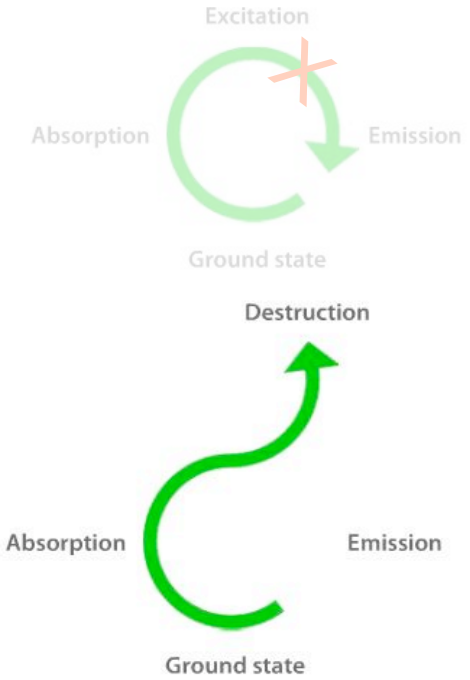






Photobleaching



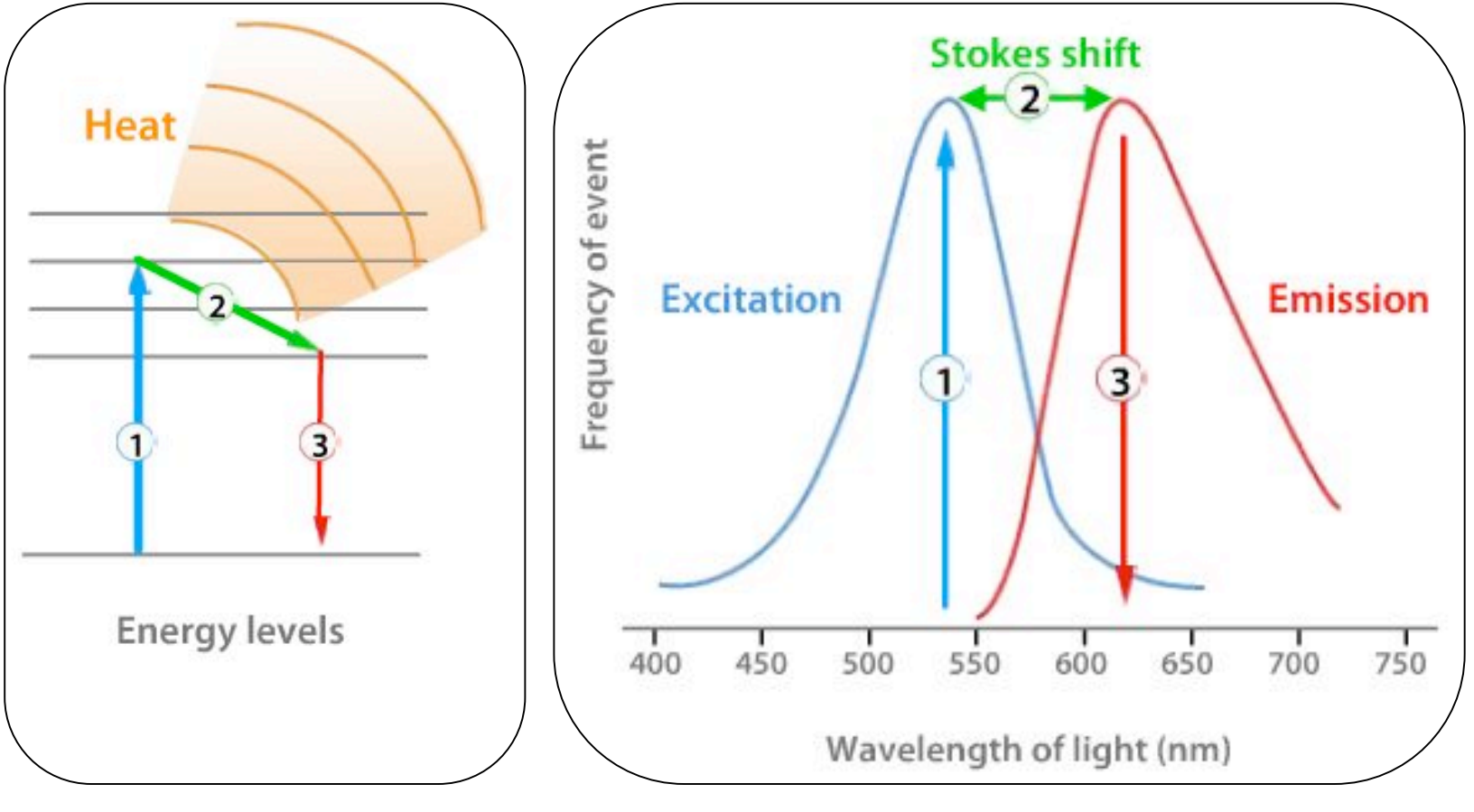


Bleaching of fluorochromes: due to high intensity illumination the fluorophores might loose permanently their ability to emit light.

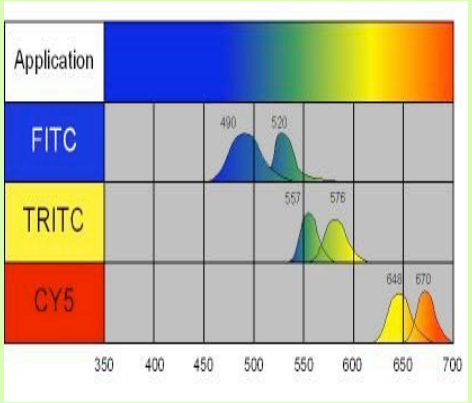
-> work with reduced excitation light intensities or gray filters, use shorter exposure times/higher gain settings and longer intervals during time lapse studies; use anti-bleach in your mounting media

Stokes shift

Due to energy loss the emitted light is shifted to longer wavelength relative to the excitation light.



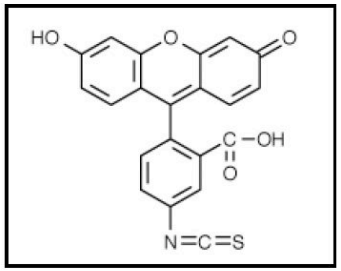
Common fluorochromes in light microscopy

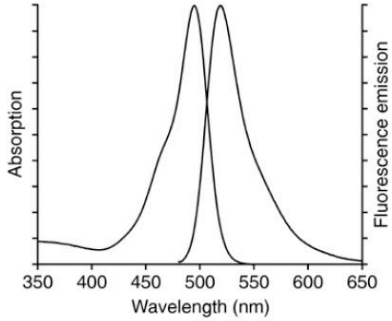


Fluorochrome Name	*Absorbtion Maximun* (nm)	*Emission Maximum* (nm)	ex/em
DAPI	358	461	ultraviolet/blue
FITC	490	520	blue/green
GFP	488	507	
Alexa 488	495	519	
Cy2	489	506	
TRITC	547	572	green/red
Cy3	550	570	
Alexa 546	556	573	
Teaxas red	595	615	
Cy5	649	670	red/infrared

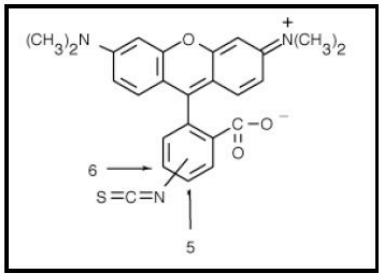
Structure of common fluorescent dyes

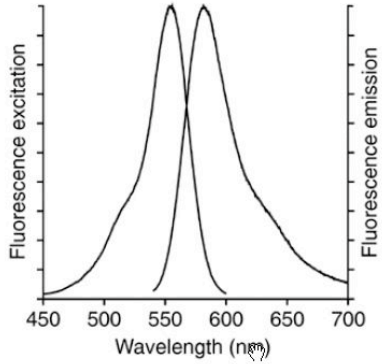
FITC



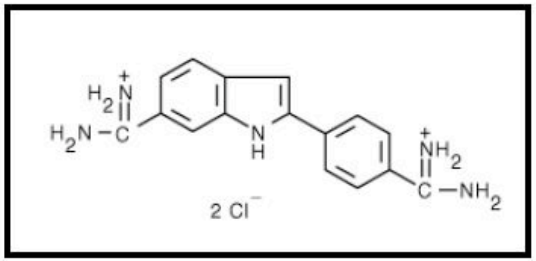


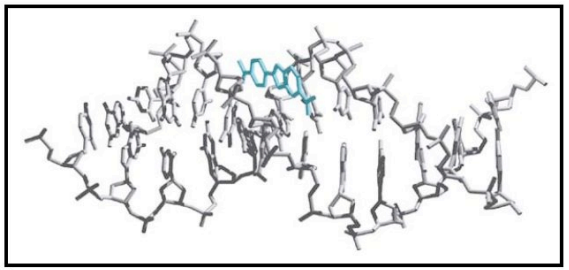
TRITC





DAPI

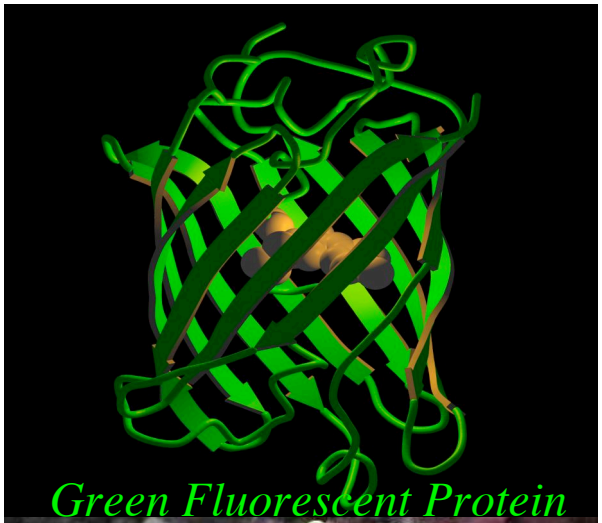




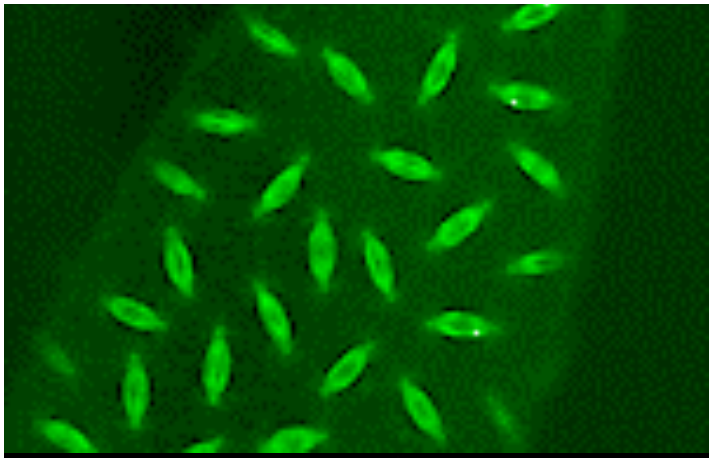
DAPI binds to the minor groove of DNA

=> Fluorochromes show ring-like chemical Structure

Fluorescent proteins: i.e. GFP

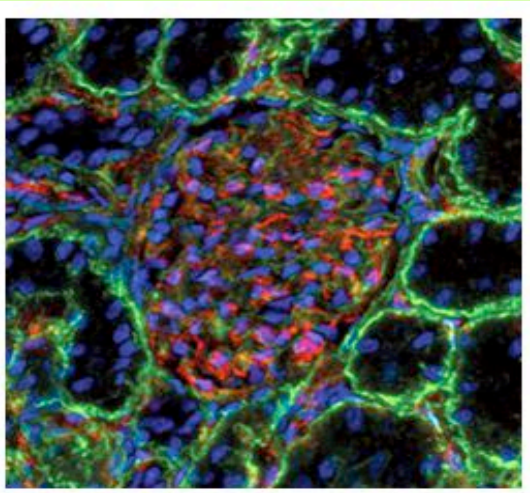


These proteins are naturally found in light-producing cells of cnidarians



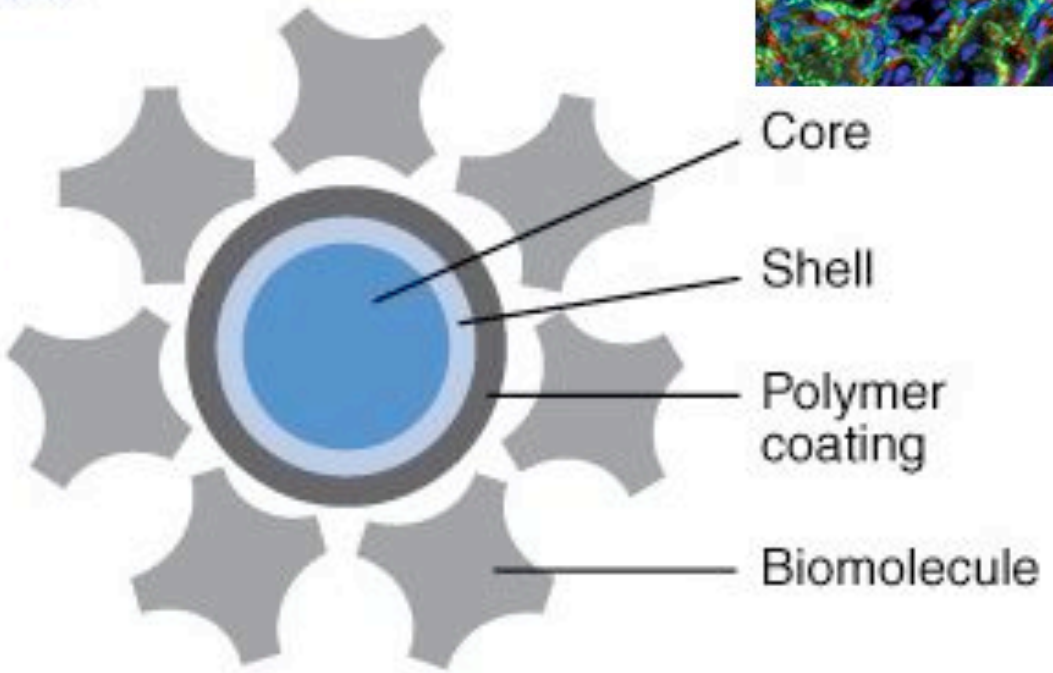
Fluorescent proteins can be fused with other proteins and introduced in cells via transfection. This allows live study of fluorescent tags in living cells/organisms.

Quantum dots (Q-dots)



Structure of a Qdot nanocrystal

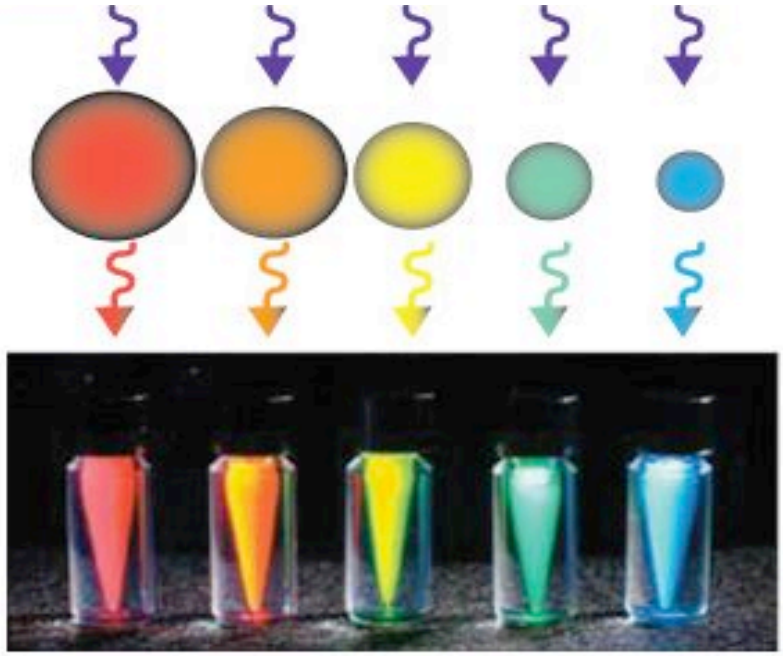
Schematic of the overall structure of a Qdot nanocrystal conjugate. The layers represent the distinct structural elements, and are drawn roughly to scale.

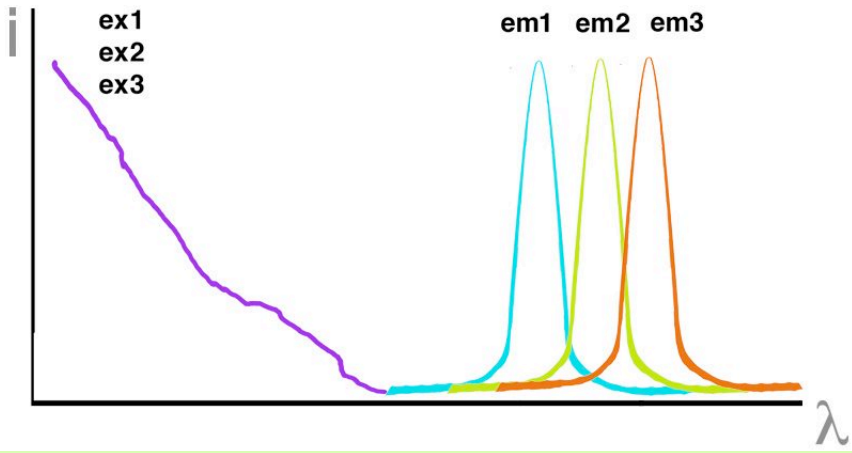


The nanocristals made from semiconductor materials might be coupled to proteins (i.e. antibodies, lectins, ...) - several biomolecules can conjugate to each bead and amplify thereby the signal.

Quantum dots (Q-dots)

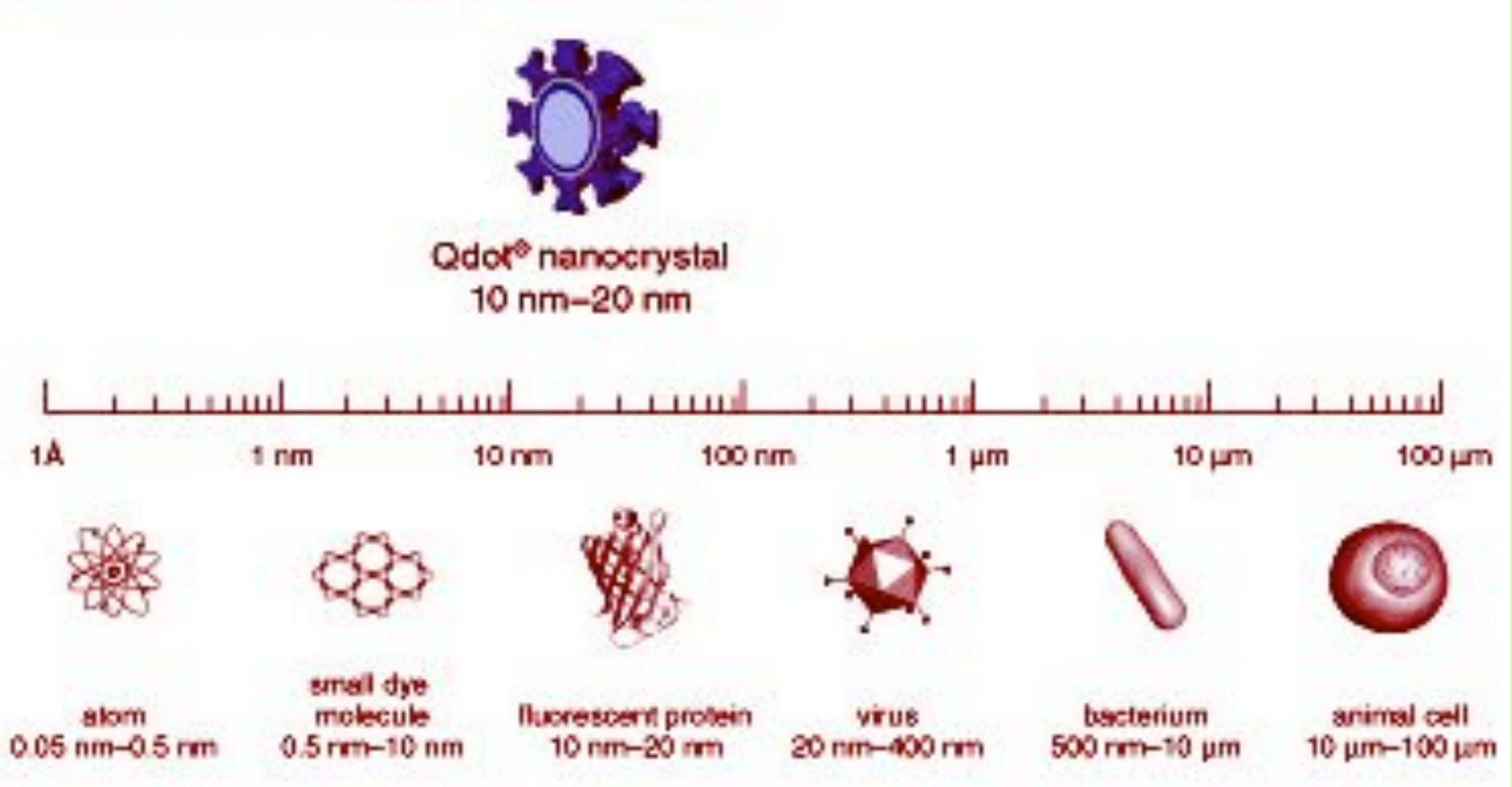
Five different nanocrystal solutions are shown excited with the same long-wavelength UV lamp; the size of the nanocrystal determines the color.





Q-dots have a wide excitation spectrum in the UV-range and narrow emission spectra in ithe visible range. They are very bright and show excellent photostability

Dimensions of different fluorescent agents



Sizes of conventional fluorochromes, fluorescent proteins and Quantum-dots.

fluorescence labeling

Fluorochromes have to be chosen according to the LASER -lines or filter sets of the microscopes !!!!!!!!!!!!!!!!!!!

For multicolor-labeling choose fluorochromes with non-overlaping emission-spectra in order to avoid cross-talk.

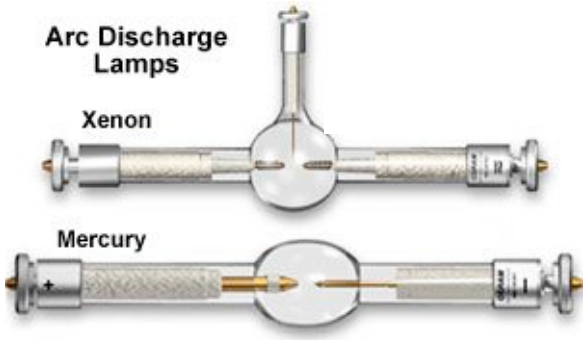
For colocalization-studies use fluorochrome-pairs with emission curves in the visible region of the spectrum in order to avoid z-level-mismatch.

A typical fluorochrome set might be: FITC & Rhodamine & Cy5.

Labeling procedure: Initial fixation with fresh 3% PFA, permeablization, blocking unspecific binding, adding specific 1rst antibody, washing, adding fluorochrome labeled 2nd antibody, washing, eventually mounting in i.e. Moviol/dabco (see also the detailed protocols on the ZMB-homepage)

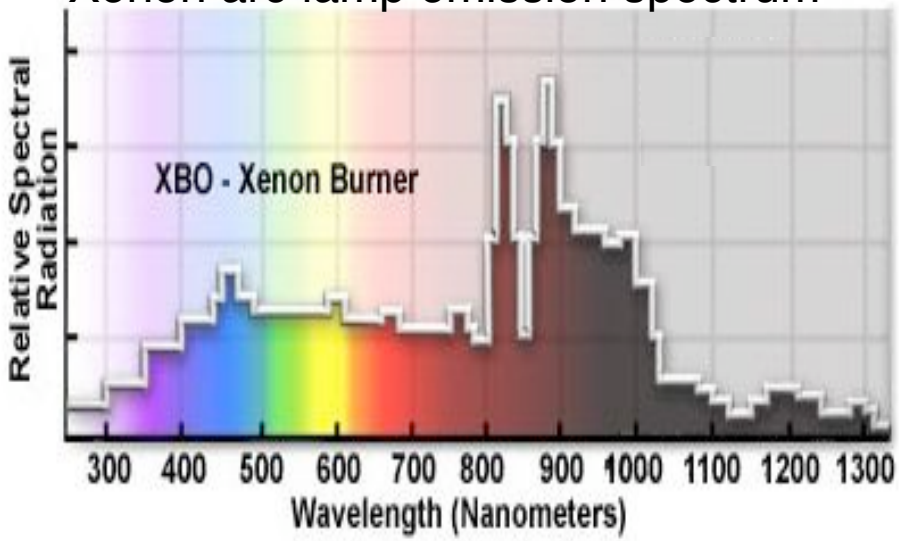
The parts of the microscope:

Fluorescence excitation

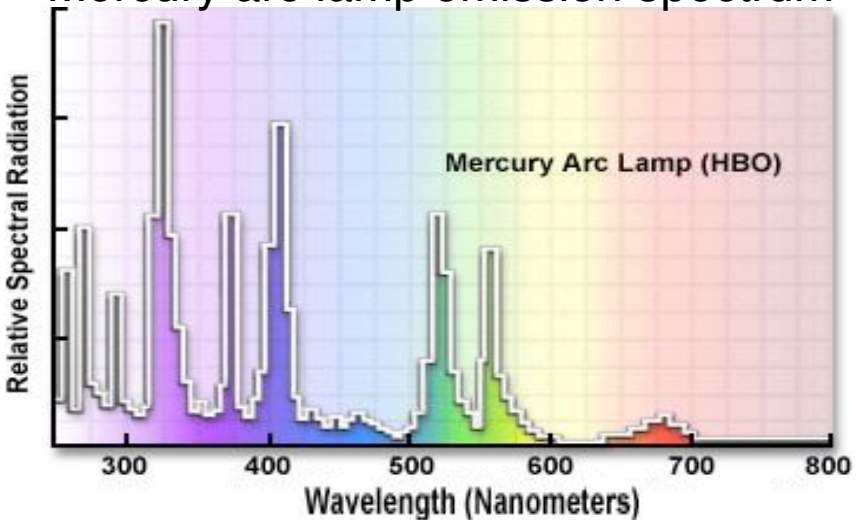


The arc discharge lamps have a limited time span of usage (300 to 400 h) and are expensive.
Restarting needs a cooling time of at least 30 minutes!!!!!

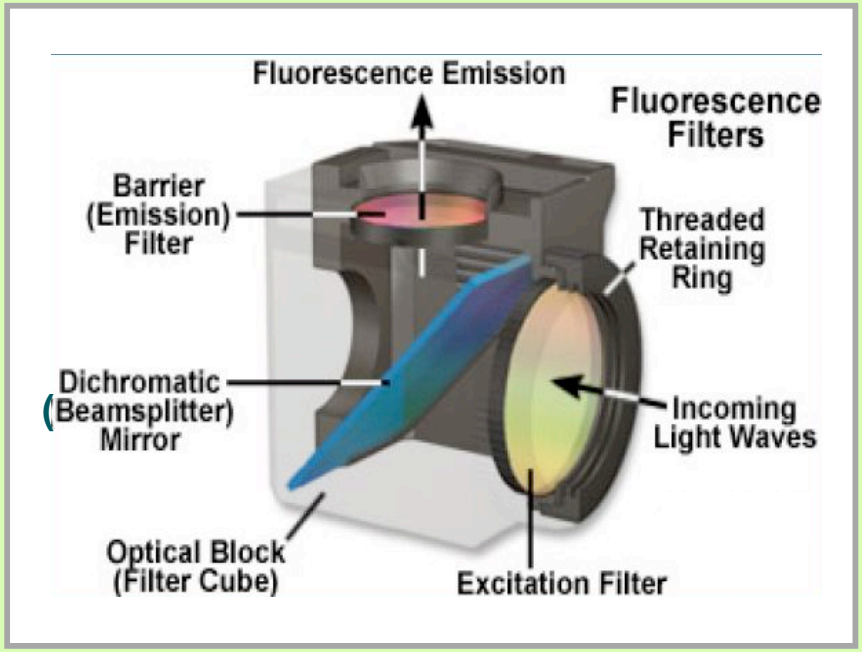
Xenon arc lamp emission spectrum

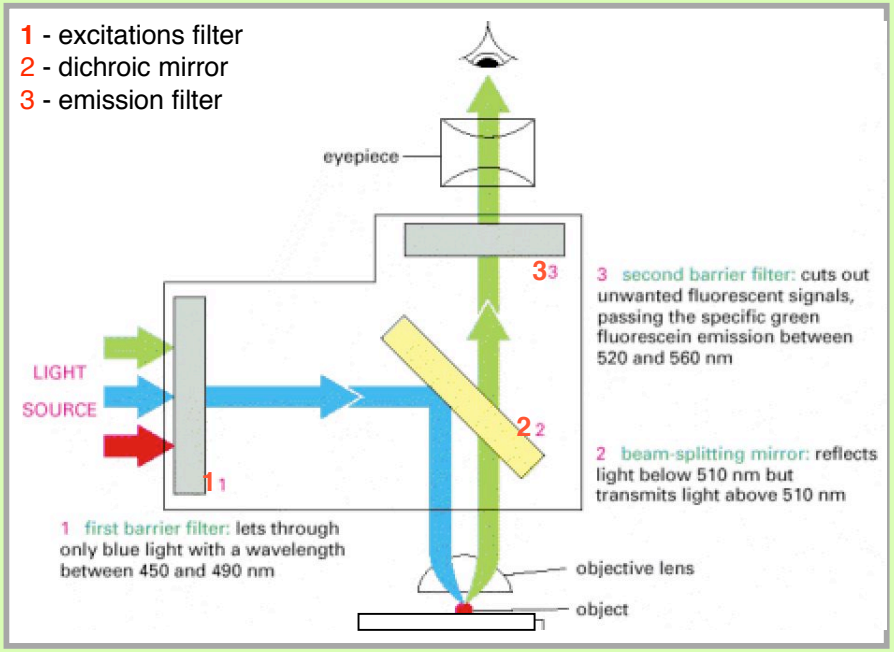


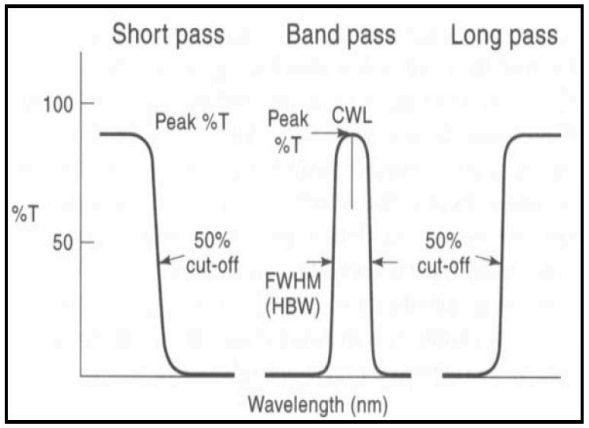
Mercury arc lamp emission spectrum

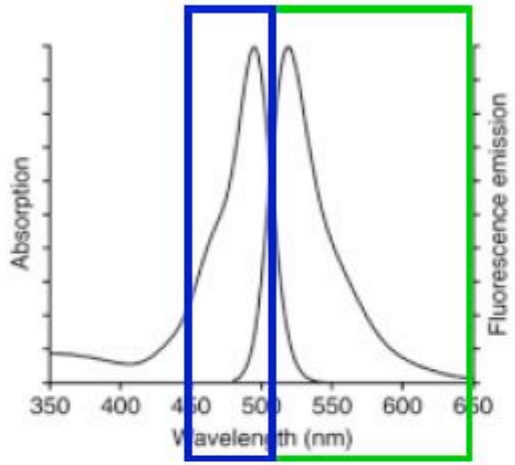


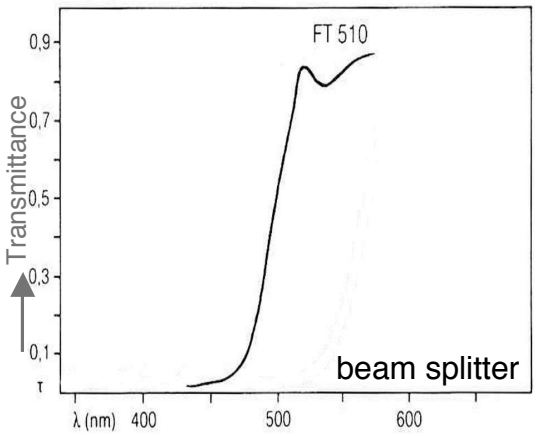
conventional fluorescence filters



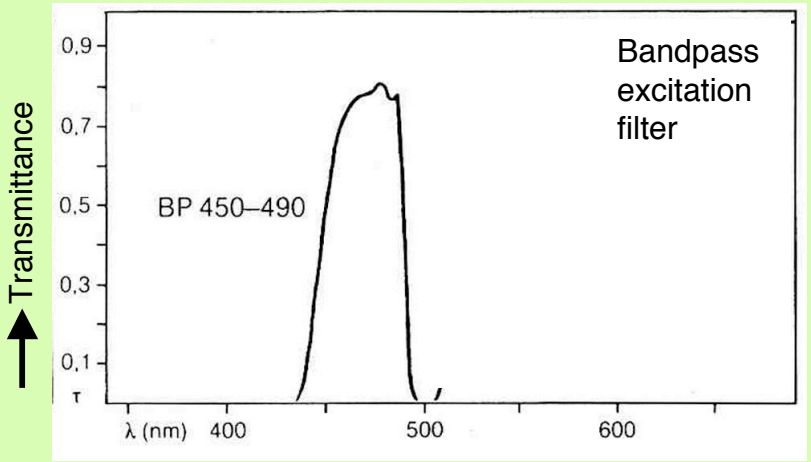


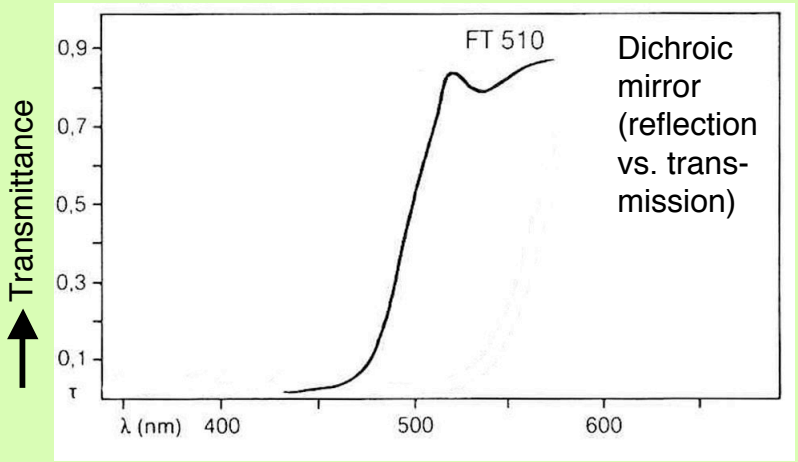


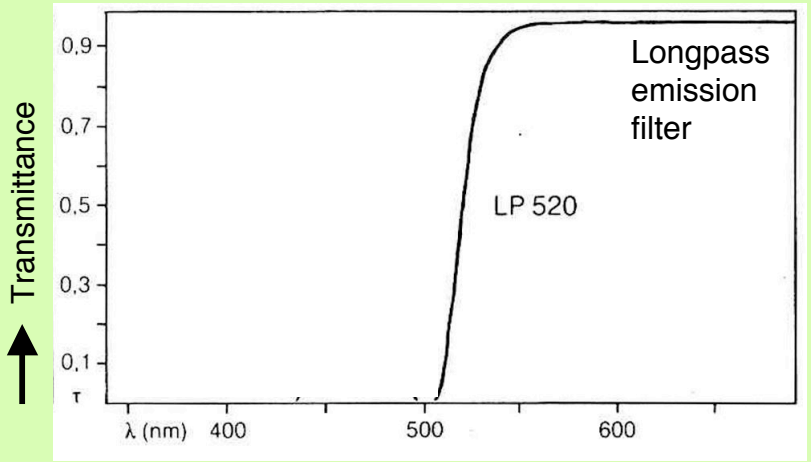


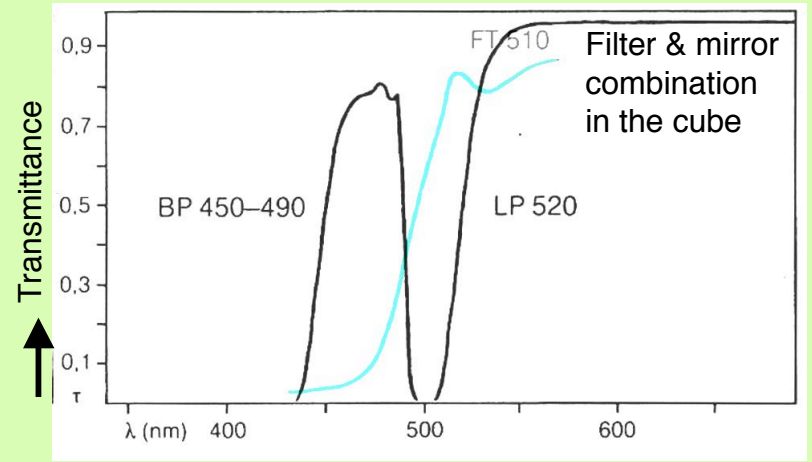


Composition of FITC filter cubes (i.e.)

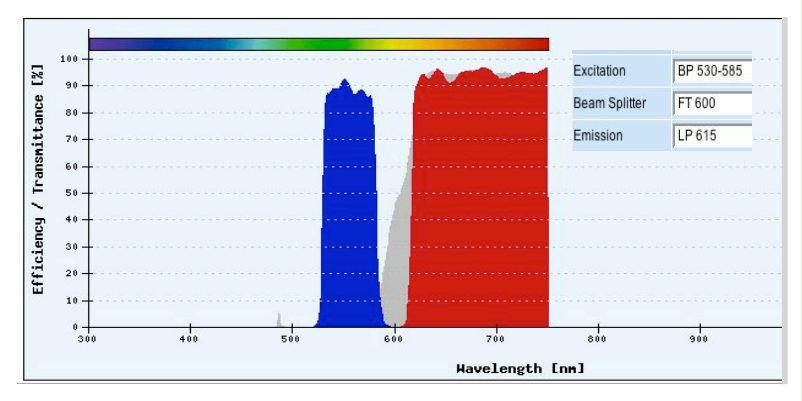




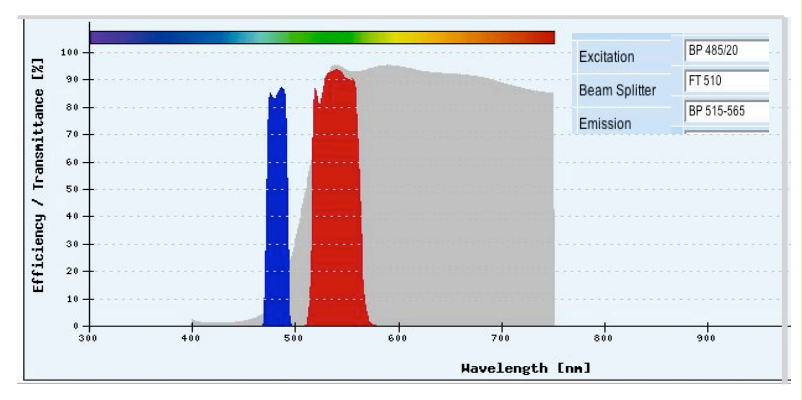




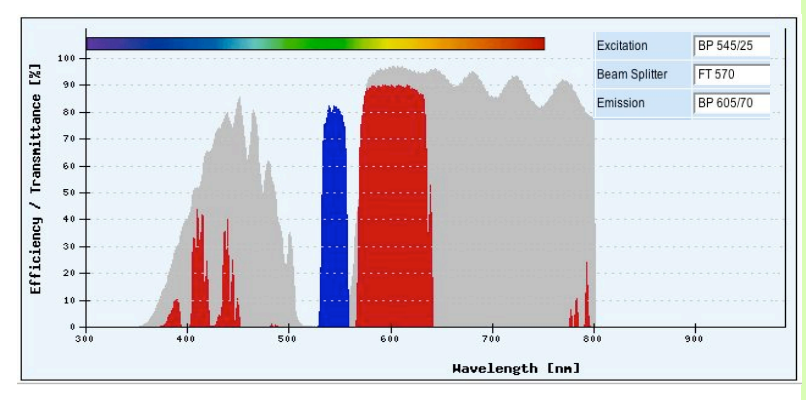
Different filter characteristics



long pass filter
for emission & beam splitter

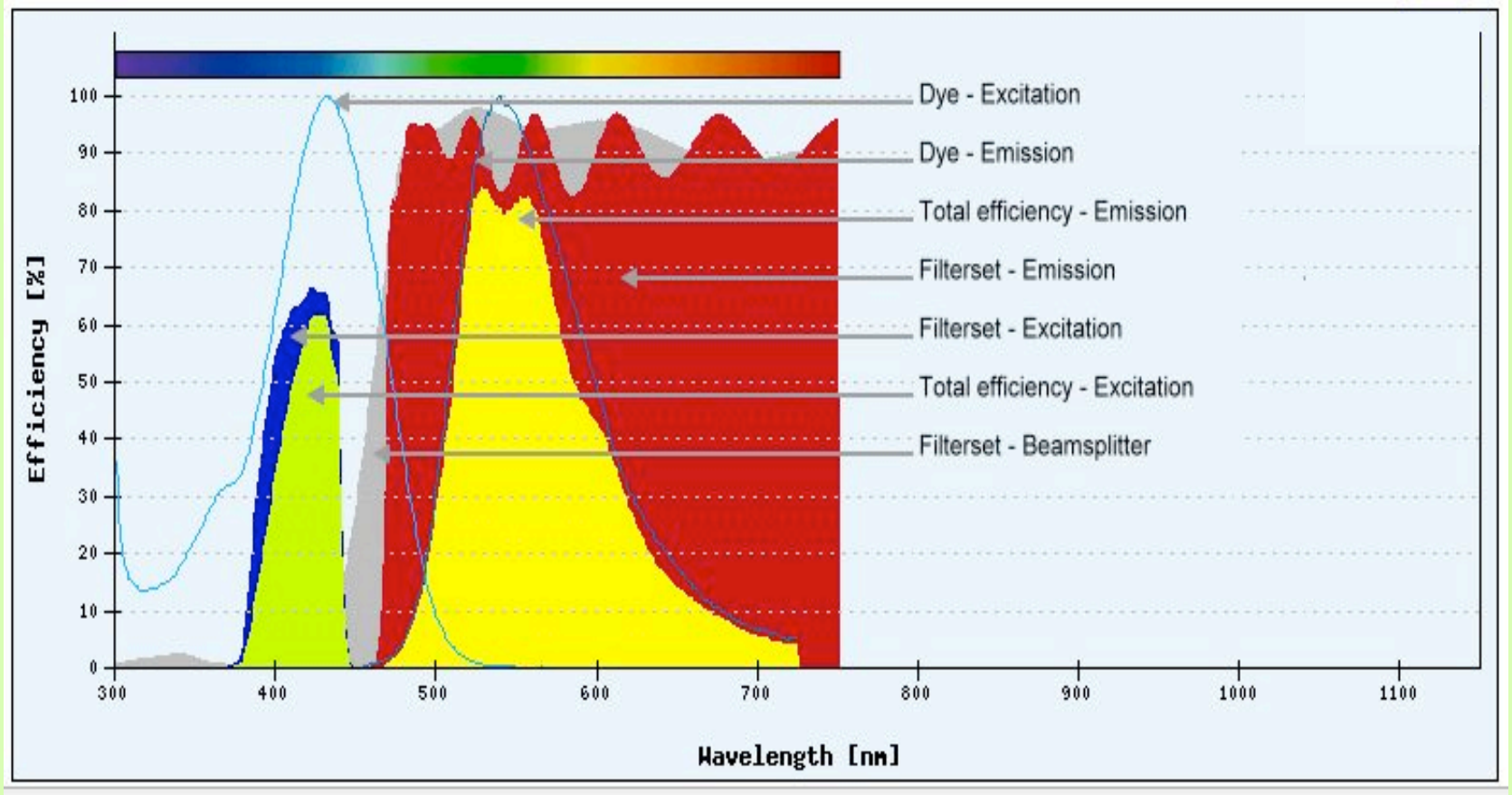


band pass filter
for emission



complex transmission
characteristics of filters

Transmittance of fluorescence filters

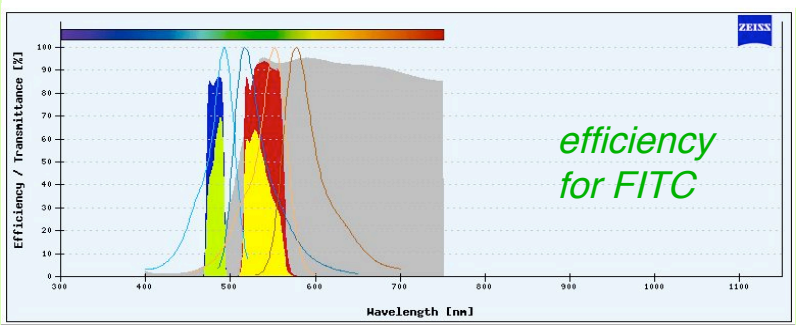


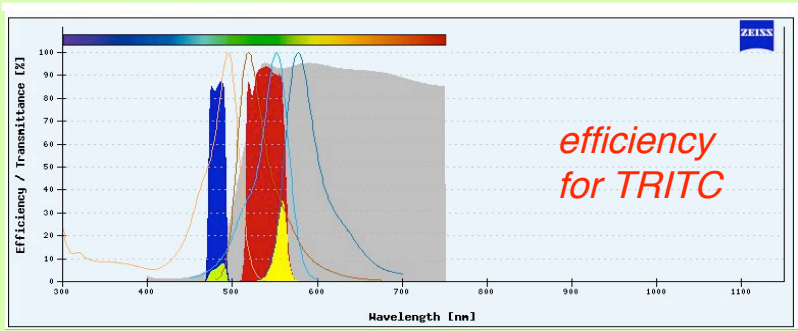
Transmittance of fluorescence filters is never 100 %
-> light loss depends on individual filter properties

Filtersets, fluorochromes & cross talk

example: FITC & TRITC

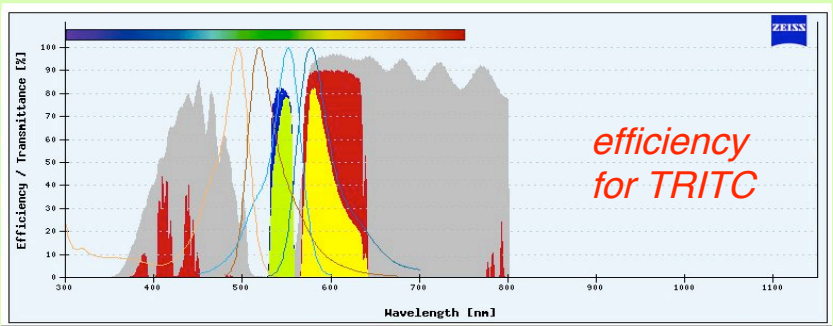
Filterset „17“
(*ex* BP 485/20, *mirror* FT 510, *em* BP 515-565)

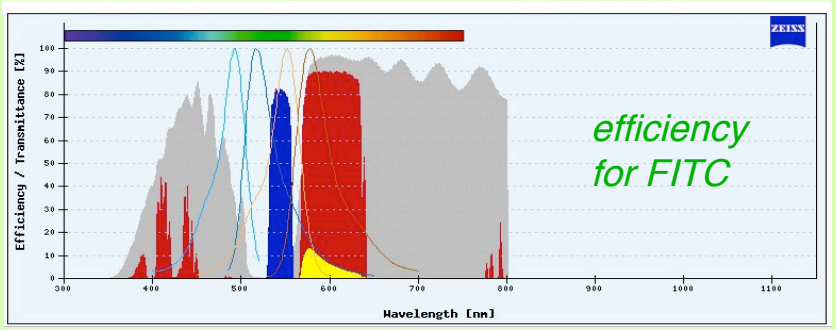




red signal bleeds
into the green channel
-> better use different filter set !!

Filterset „43“
(*ex* BP 545/25, *mirror* FT 570, *em* BP 605/70)

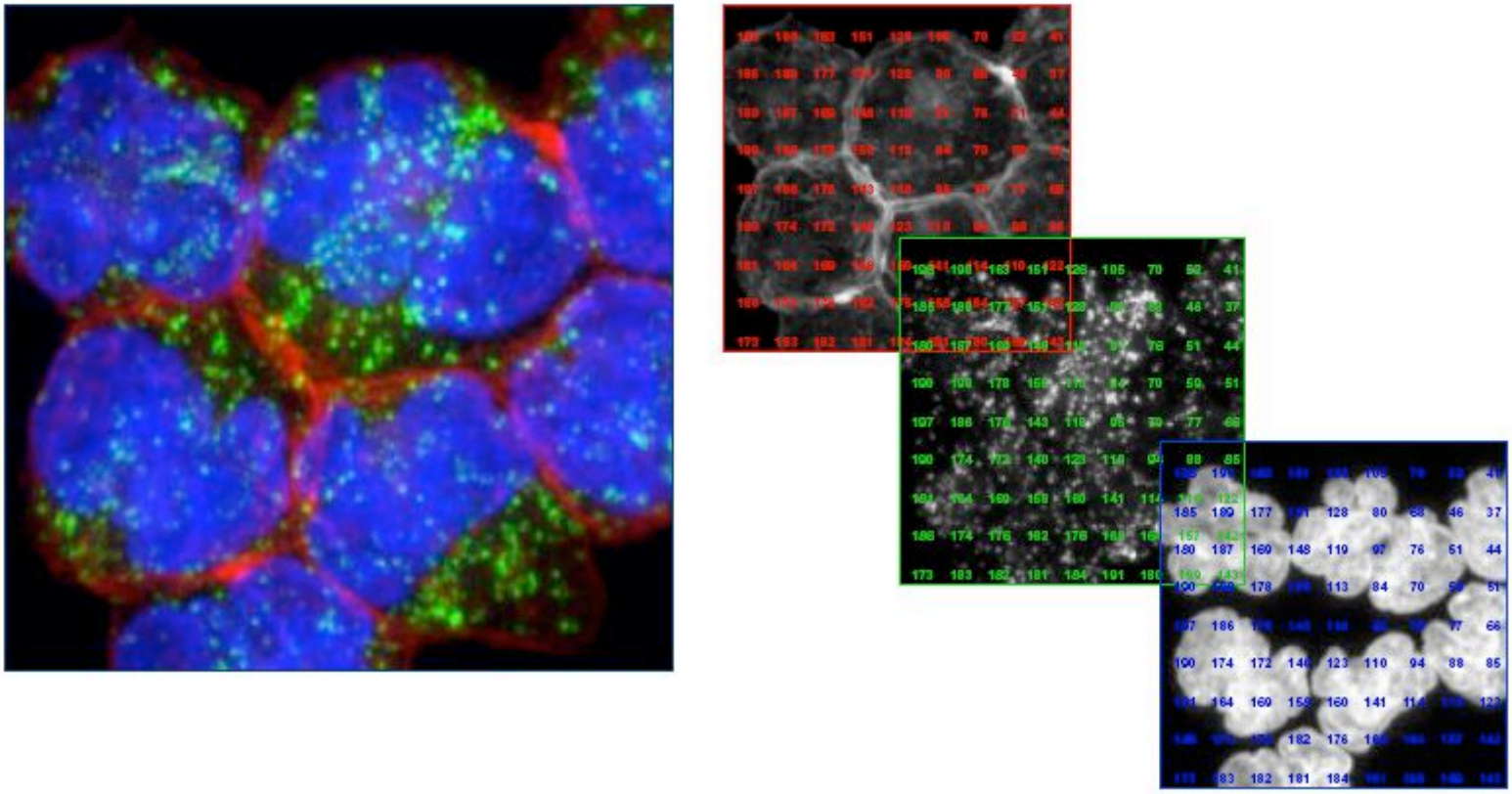




no bleeding for this filterset
& fluorochrome combination
(no cross excitation, therefore
no false emission)

Cross talk phenomena depend on fluorochrome combination AND filterset characteristics.

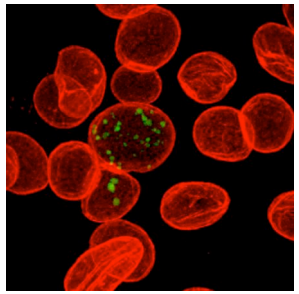
Electronic pseudocolor images



Multilabeled samples are imaged under different fluorescence conditions by black&white-detector -> overlay of pseudocolor-indexed grayscale images

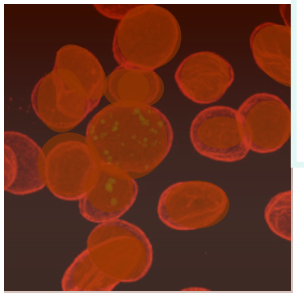
Fundamental Set-up of Fluorescence Microscopes:

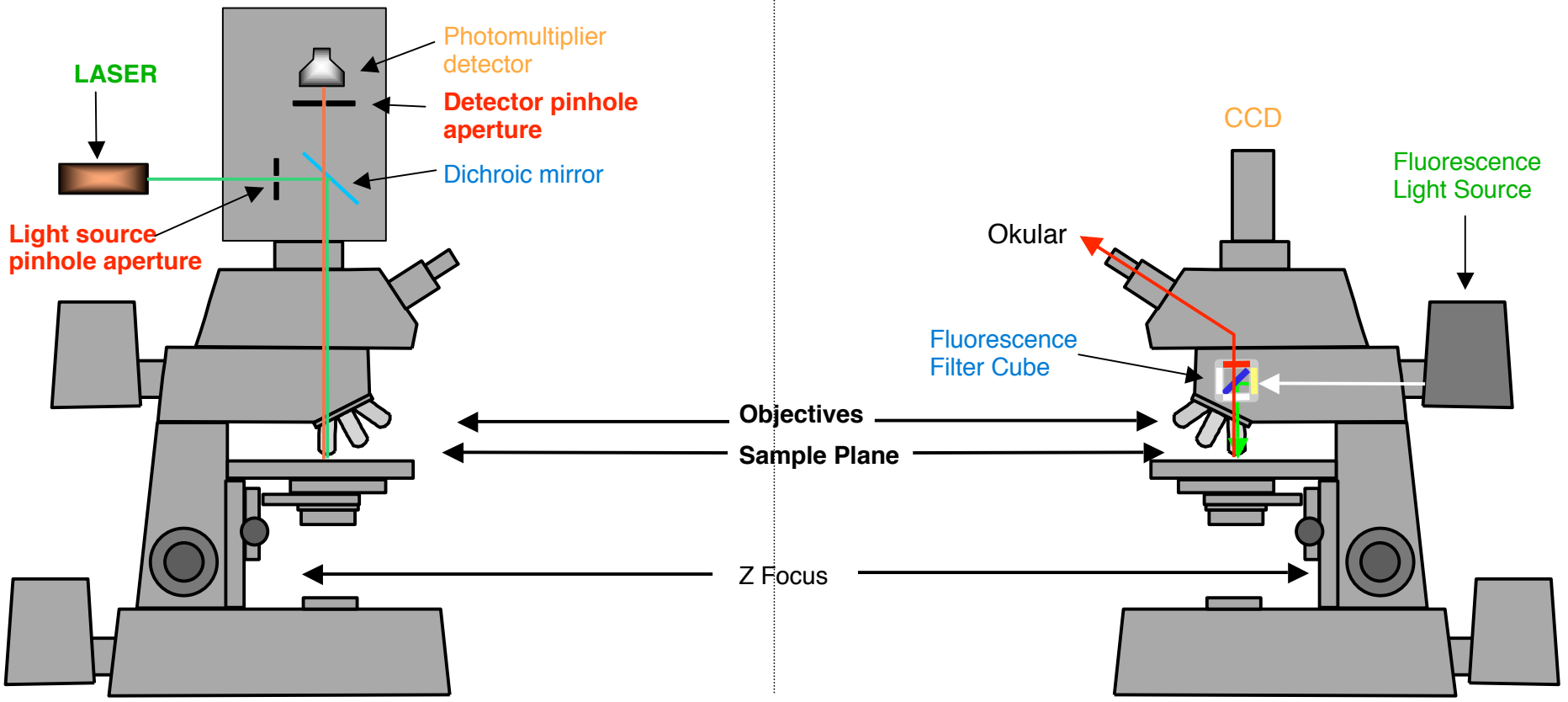
confocal vs. widefield



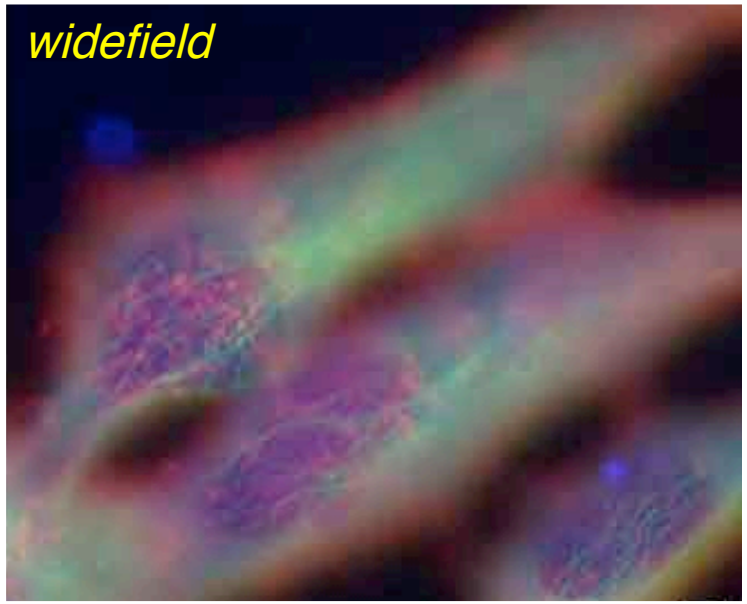
Confocal Fluorescence Microscopy

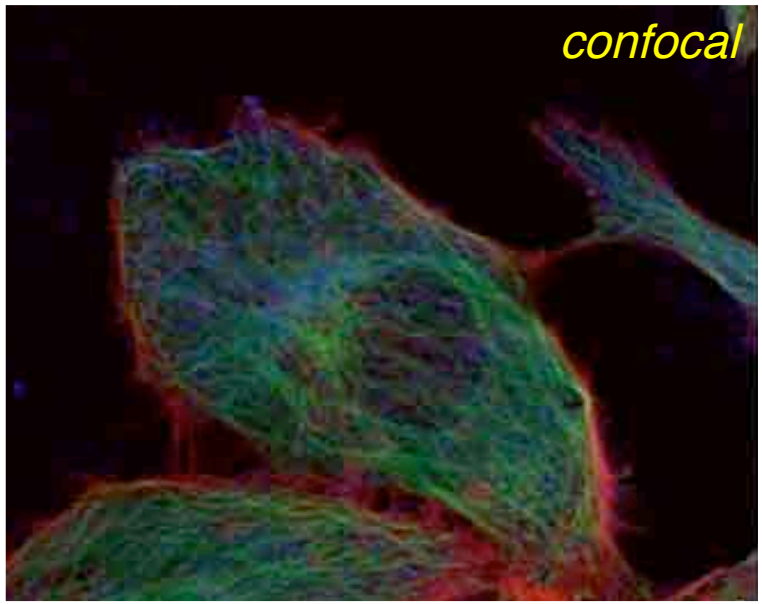
Widefield Fluorescence Microscopy





Comparison: Widefield - Confocal





Higher z-resolution and reduced out-of-focus-blur make confocal pictures crisper and clearer.

Only a small volume can be visualized by confocal microscopes at once. Bigger volumes need time consuming sampling and image reassembling.