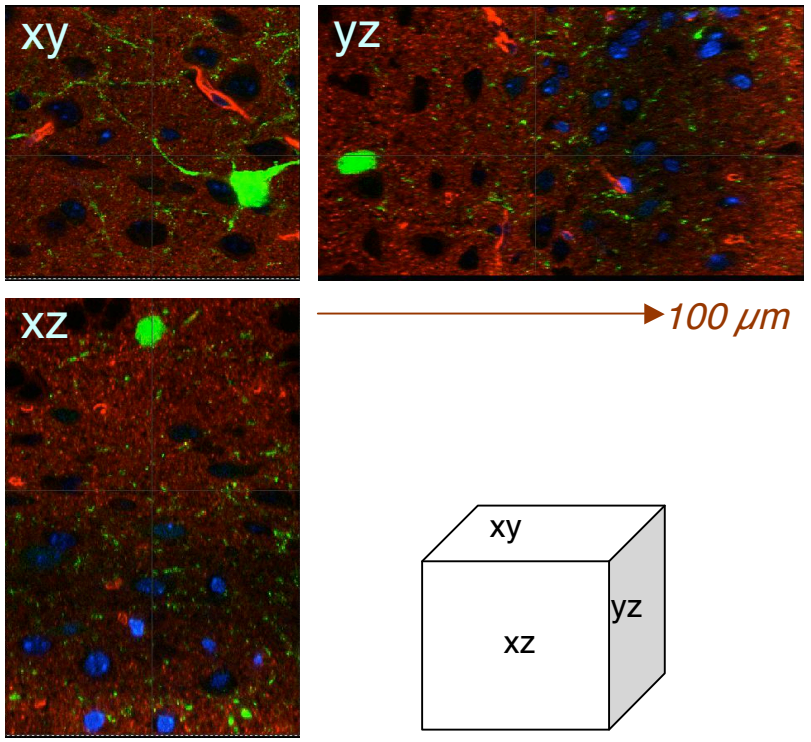
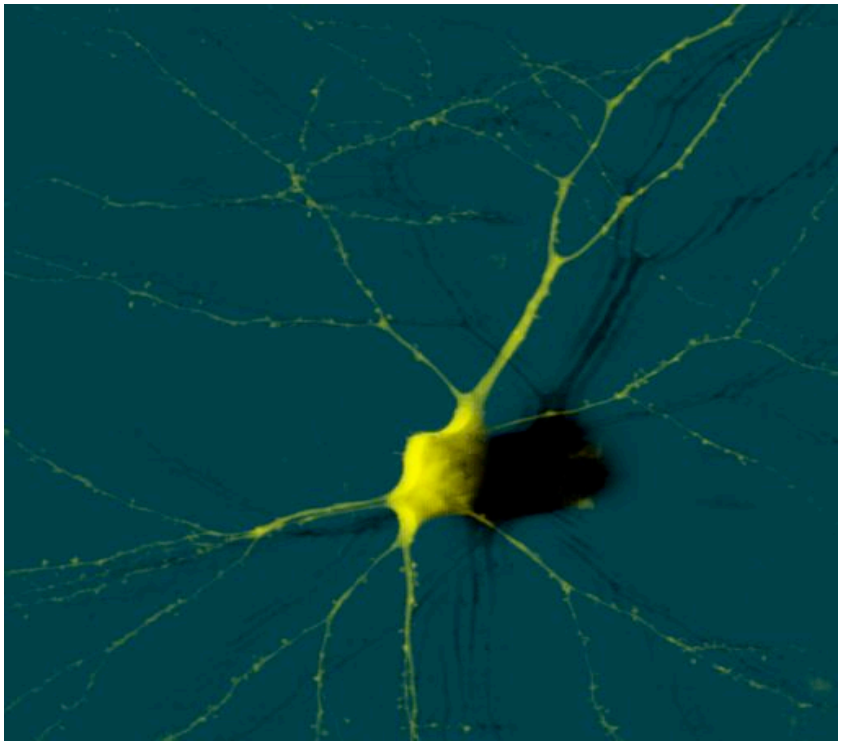
ZMB

Introduction to Confocal Laser Scanning Microscopy (LEICA)

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. For questions & registration please contact www.zmb.unizh.ch .

Confocal Laser Scanning Microscopy

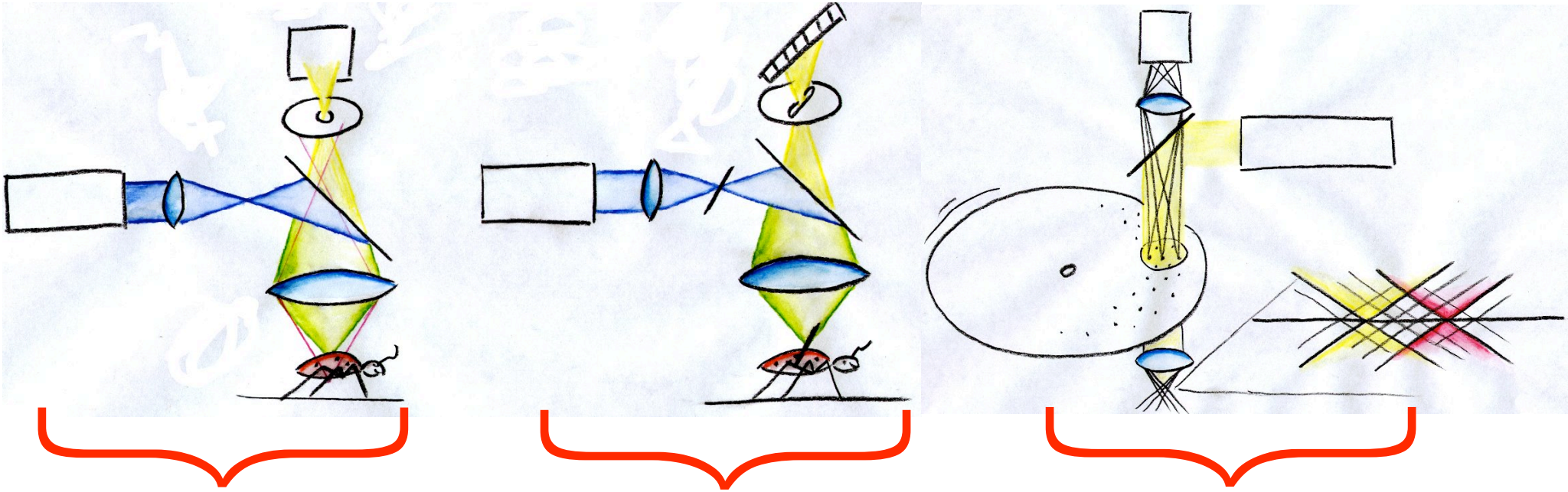




thick specimens at different depth

3D reconstruction

Types of confocal microscopes



point confocal

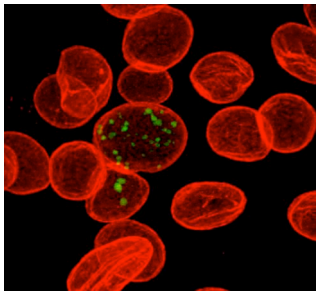
slit confocal

spinning disc confocal (Nipkov)

Best resolution and out-of-focus suppression as well as highest multispectral flexibility is achieved only by the classical single point confocal system !

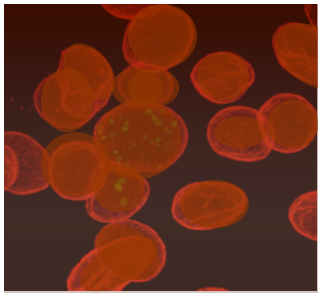
Fundamental Set-up of Fluorescence Microscopes:

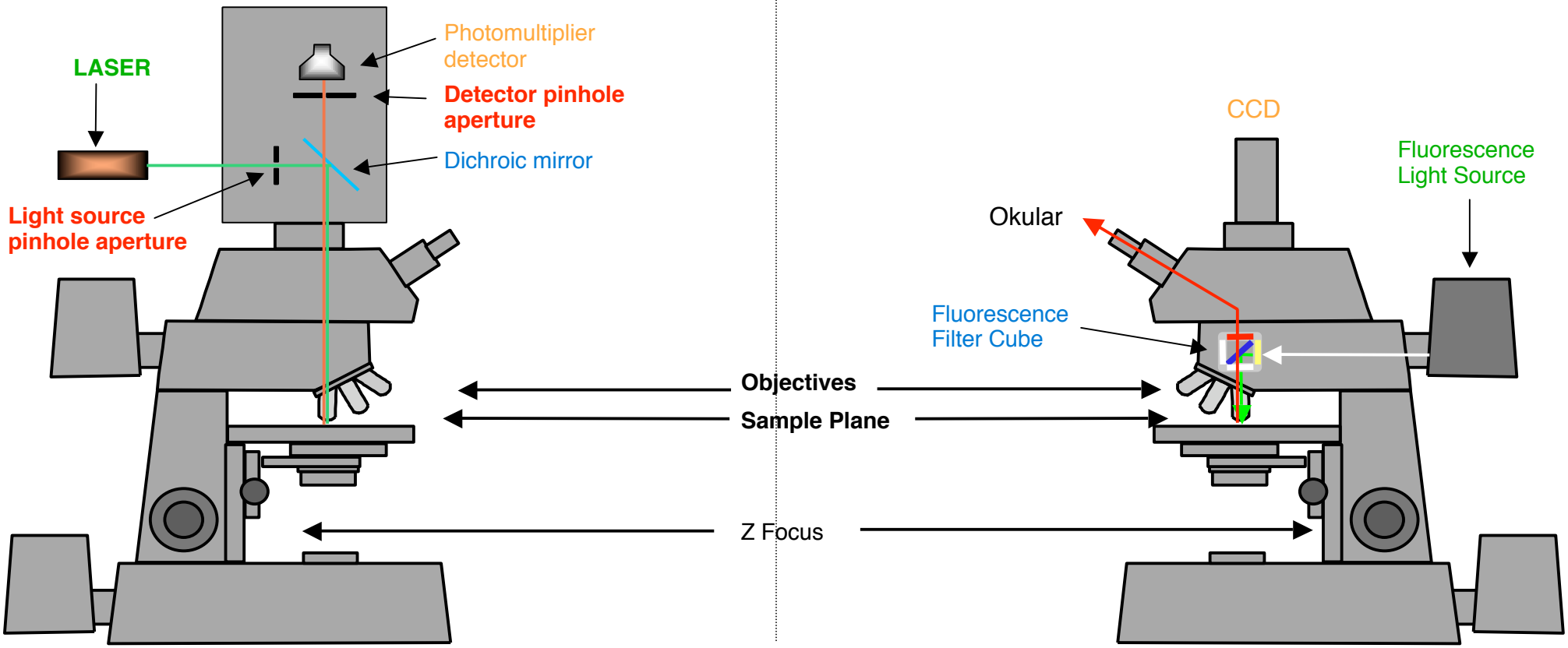
confocal vs. widefield



Confocal Fluorescence Microscopy

Widefield Fluorescence Microscopy

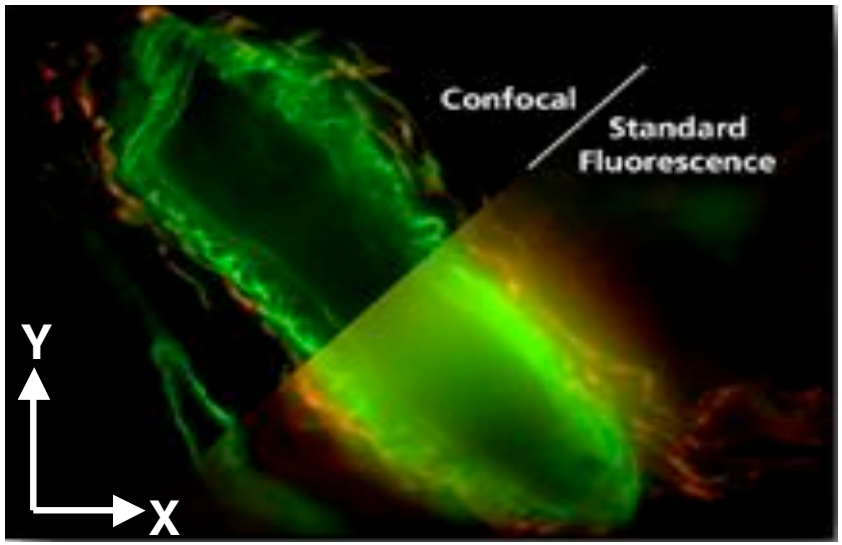


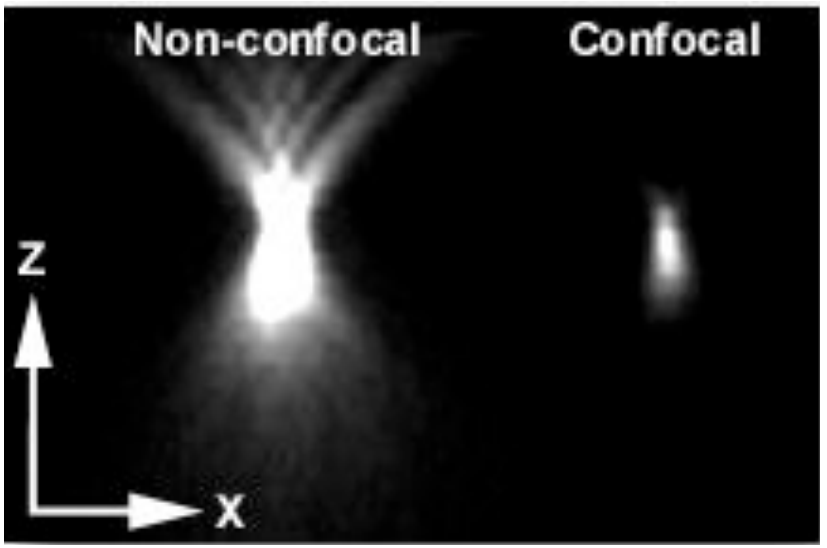


Confocal laser scanning microscope - set up:

The system is composed of a a regular florescence microscope and the confocal part, including scan head, laser optics, computer.

Comparison: Widefield - Confocal





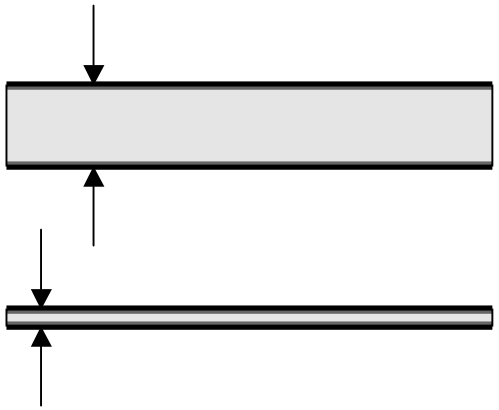
Higher z-resolution and reduced out-of-focus-blur make confocal pictures crisper and clearer.

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

Comparison: Widefield - Confocal

optical resolution in z

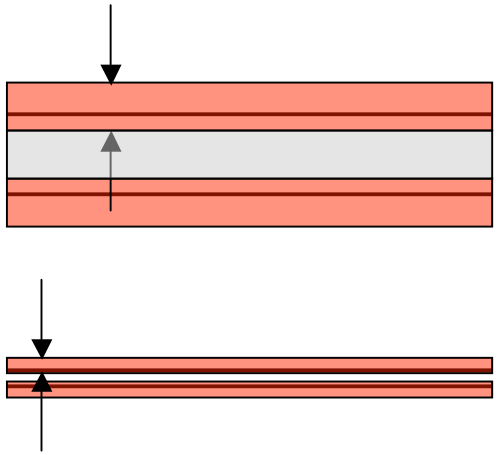
Widefield	2 - 3 μm
Confocal	0.5 μm



Comparison: Widefield - Confocal

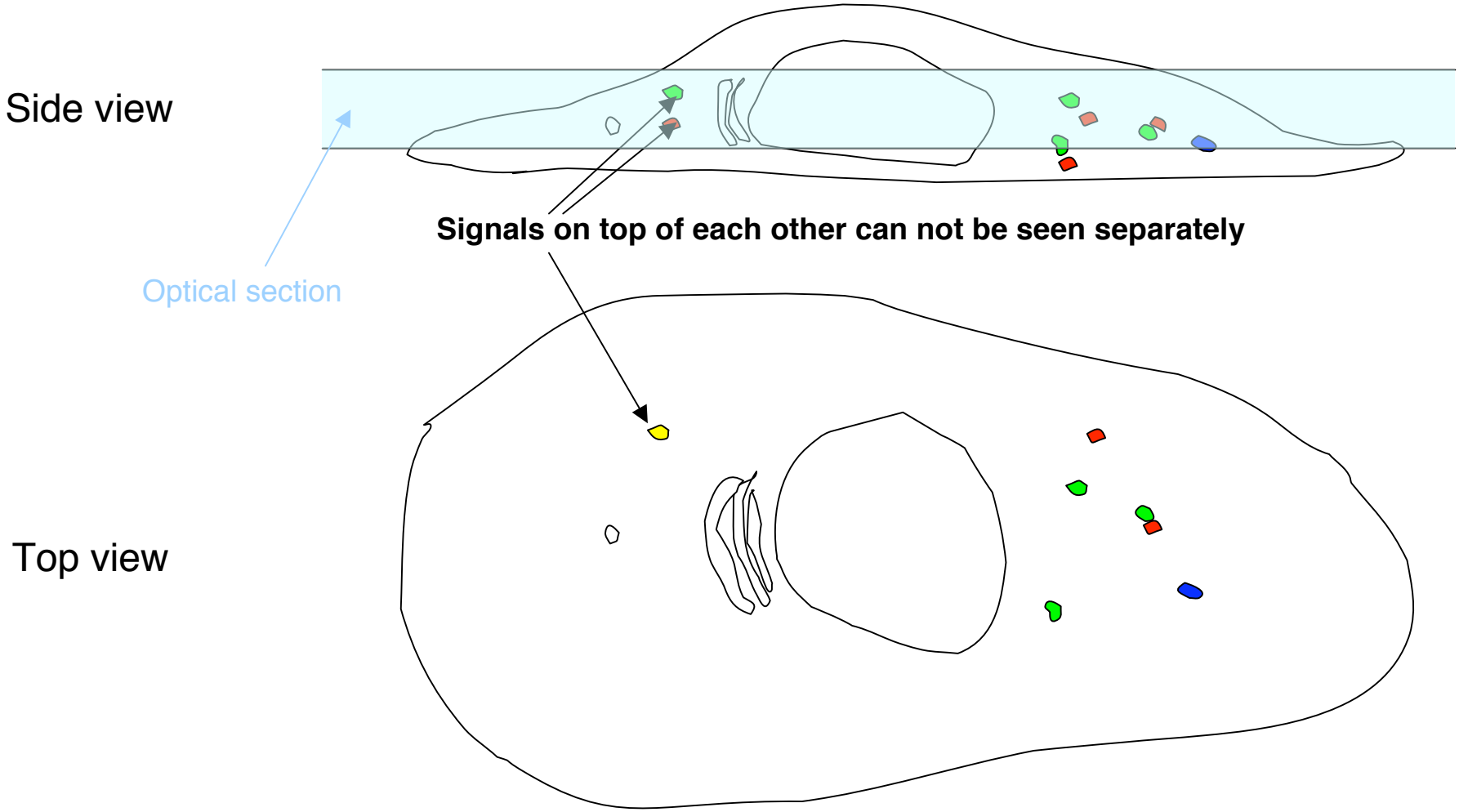
region of out-of-focus-information

Widefield	blurred & large
Confocal	very small



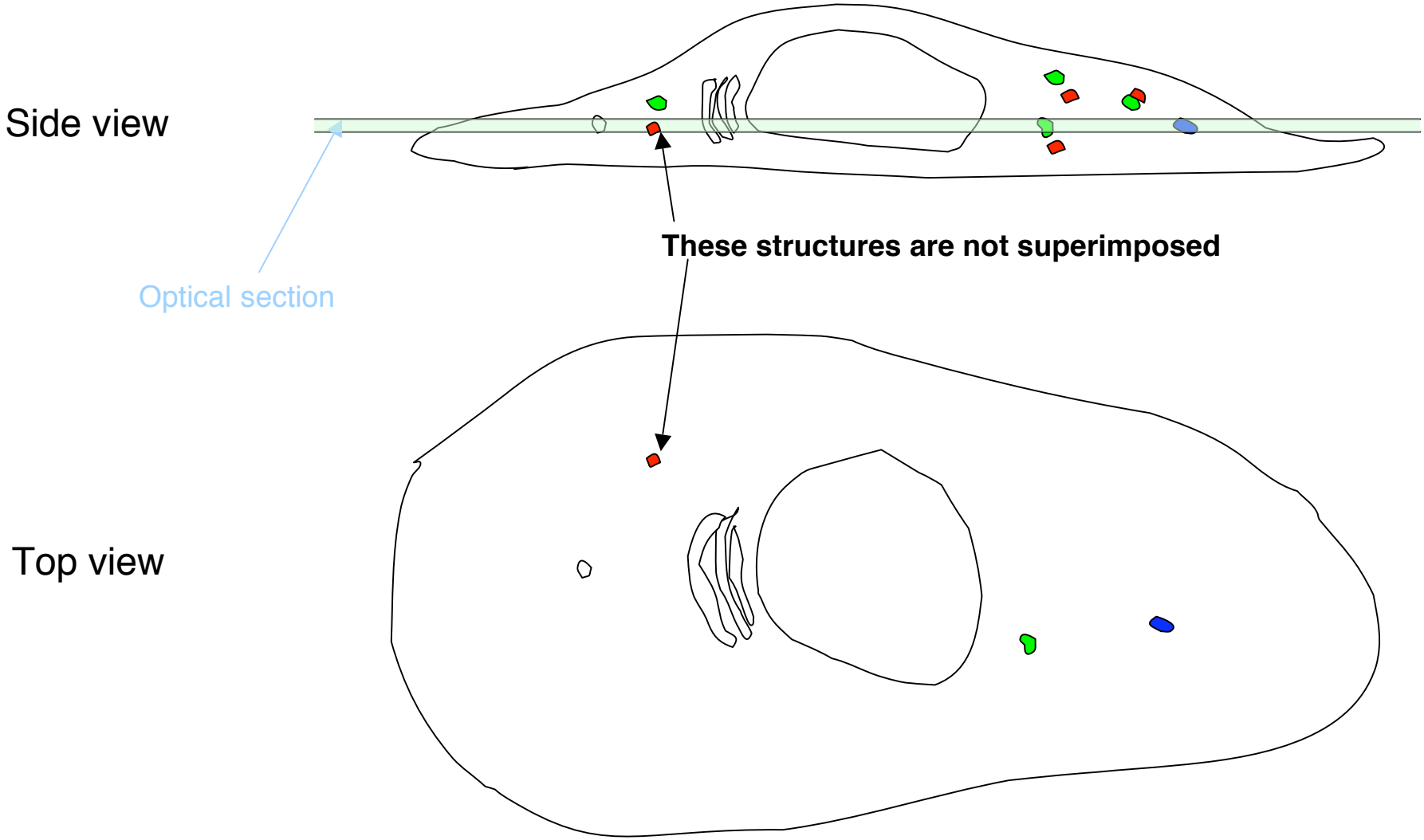
Widefield: *optical section*

Many signals can not be seen separately!



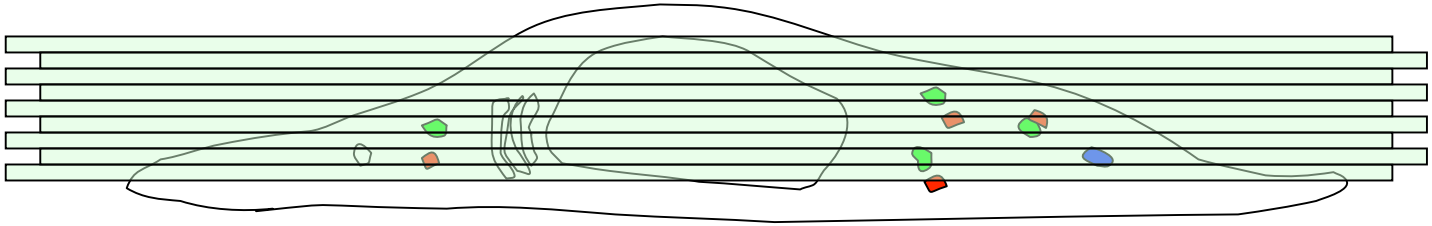
Confocal *: optical section*

Improved z-resolution allow for more accurate signal discrimination!



Confocal: *“extended focus””*

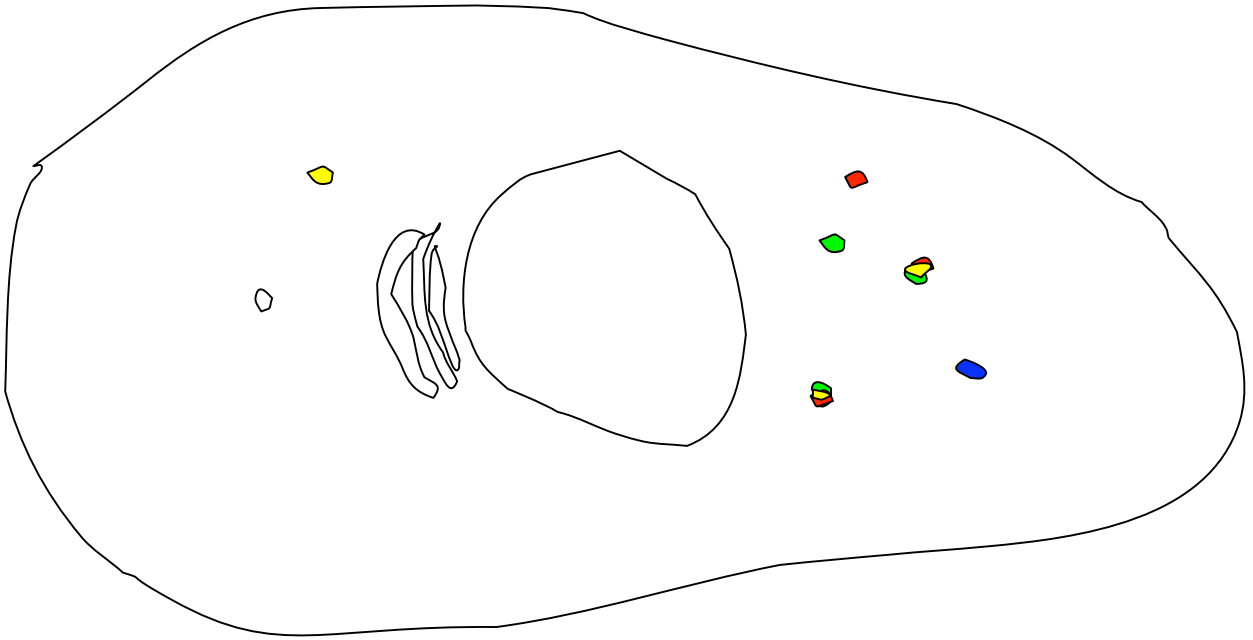
Side view



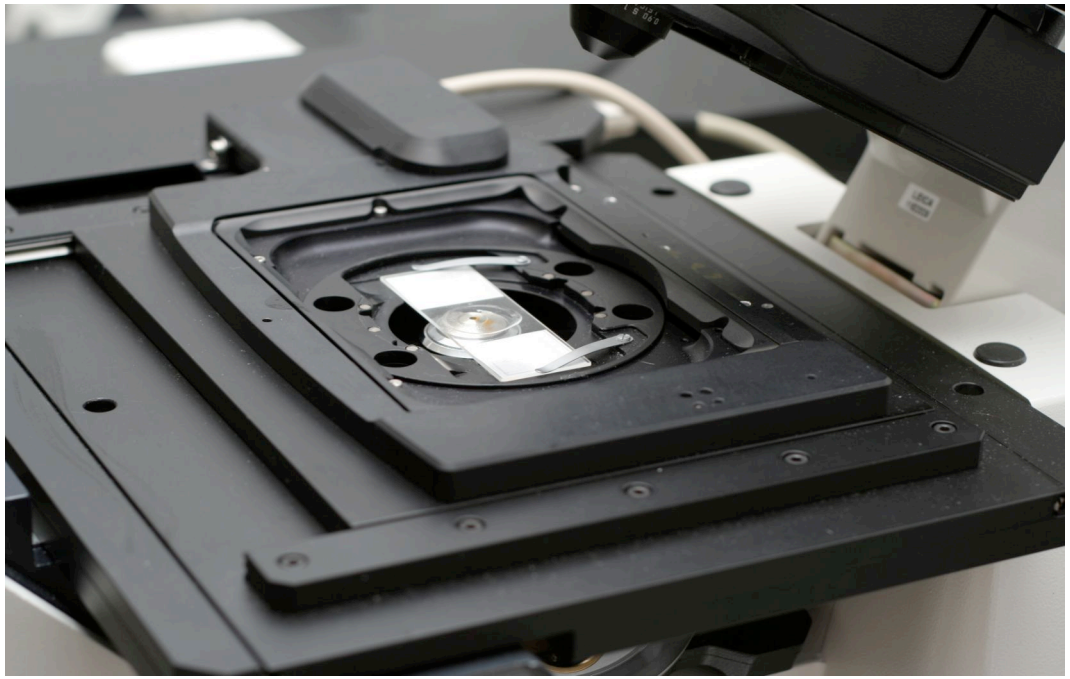
single optical sections get projected on one plane - the result is an clean image: everything is focused over the hole depth without any out-of-focus-noise.

Projection
(Top view):

Information content of all the sections is projected to one plane.

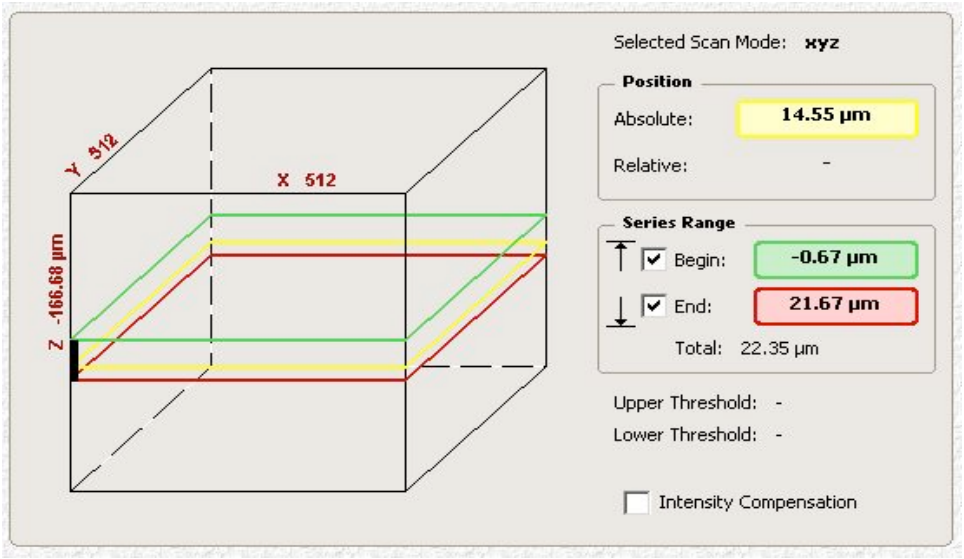


Z galvo stage provides fast z stacking



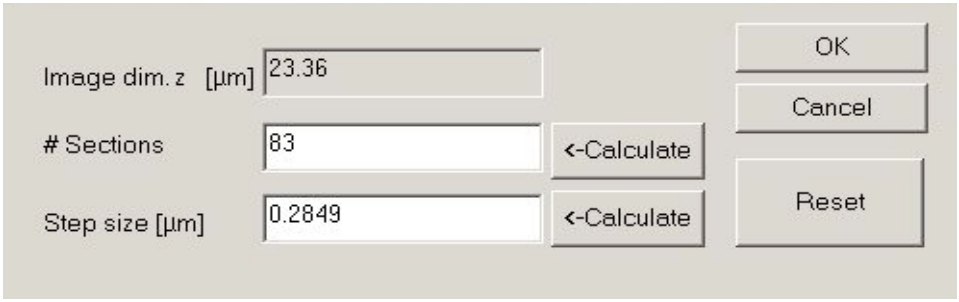
- Pivot-mounted arm with galvo motor
- 166 μm-z-range on SP2
  1.5 mm-z-range on SP5
- fast motor allows live xz-imaging
- Reproduceability 40nm
- Different inserts possible

Z-stacking



Defining a volume:

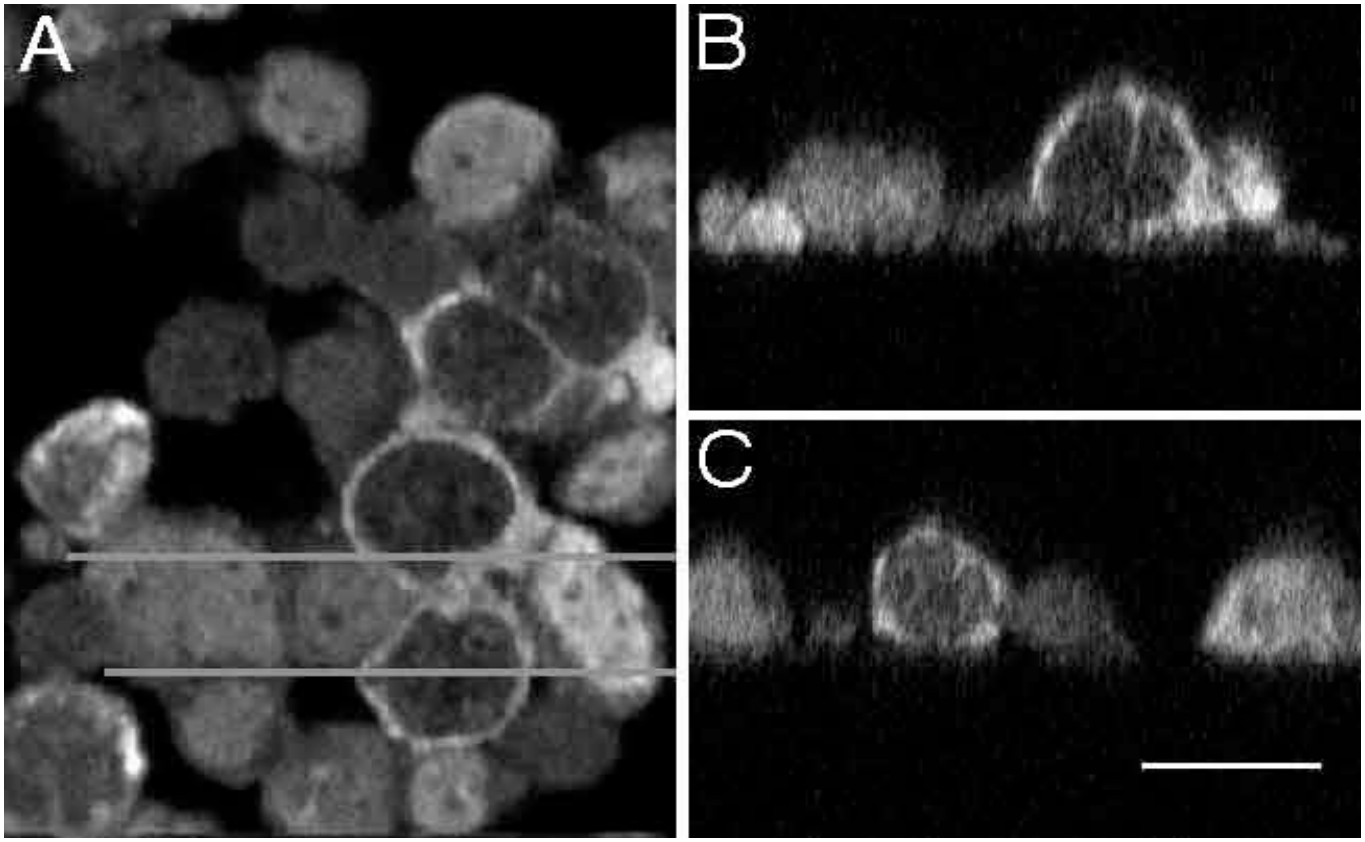
Setting the z-values for *begin* & *end* of the sampling



Defining the resolution:

defining the thickness and number of optical sections within the volume

Aquisition of 3D data sets

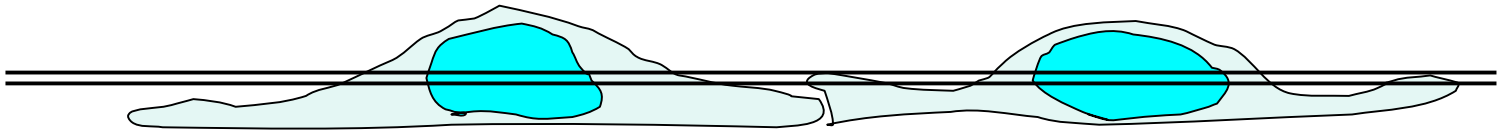


A= xy top view
B, C = xz side views
at different y-positons

The number of optical sections defines the z-resolution in the data set. The section thickness together with the xy-pixel dimension defines the „voxel“ size (voxel = volume element, the smallest unit of the sampled 3D volume).

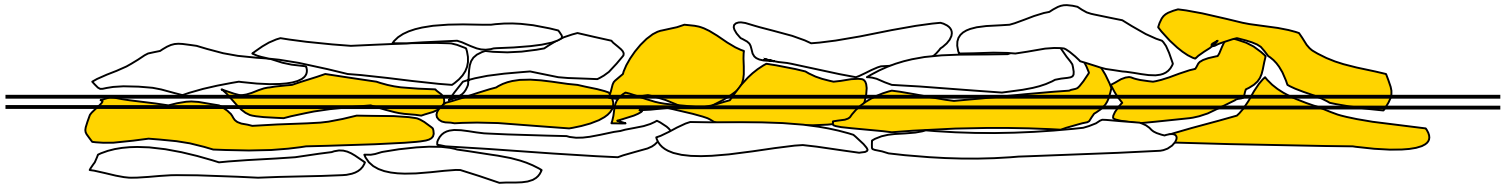
Consequences for the confocal image

Cell culture



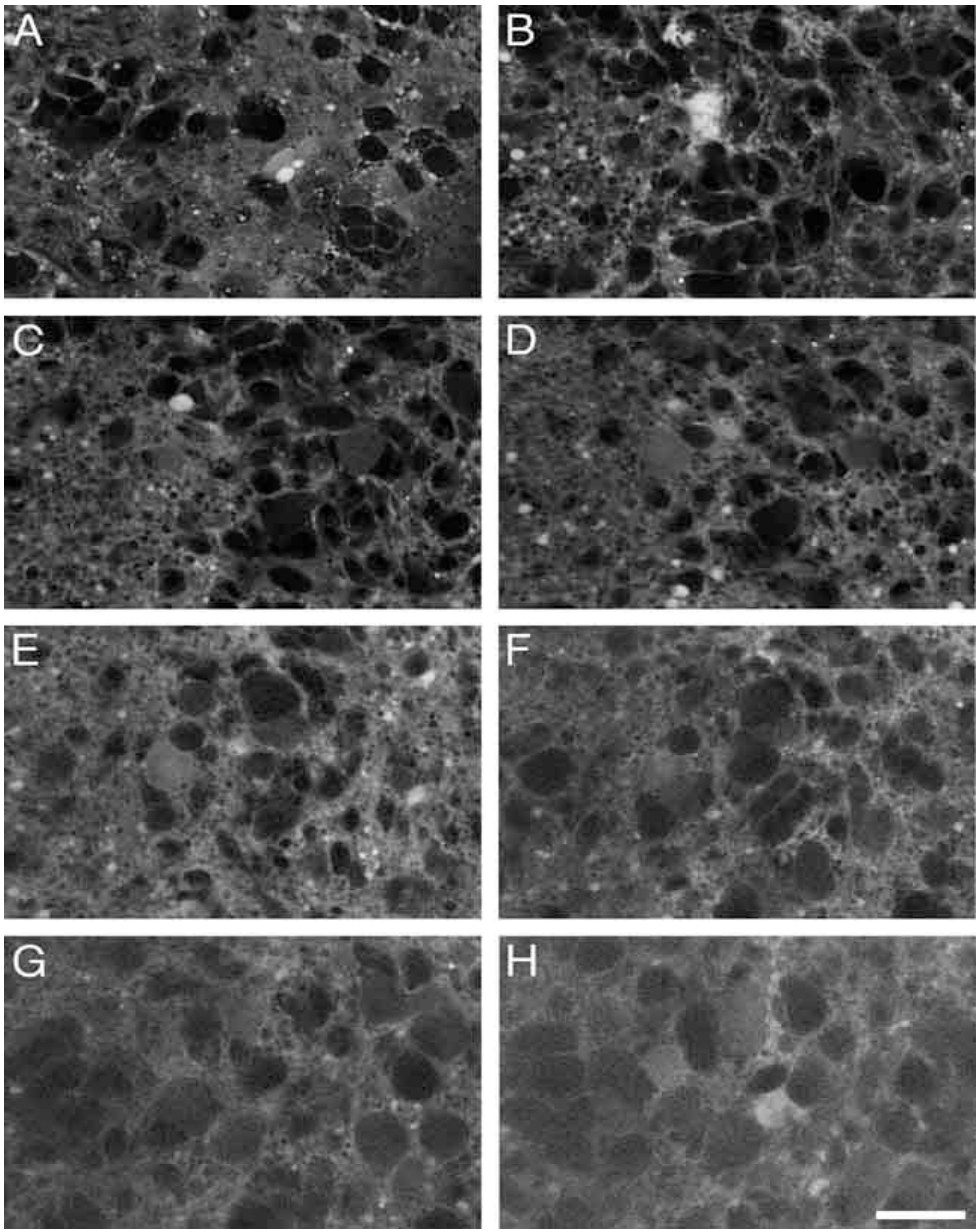
Cellular structures can be resolved due to the good resolution in z

Tissue



Only a very thin layer through the tissue is visualized.

Image aquisition from different sample depths

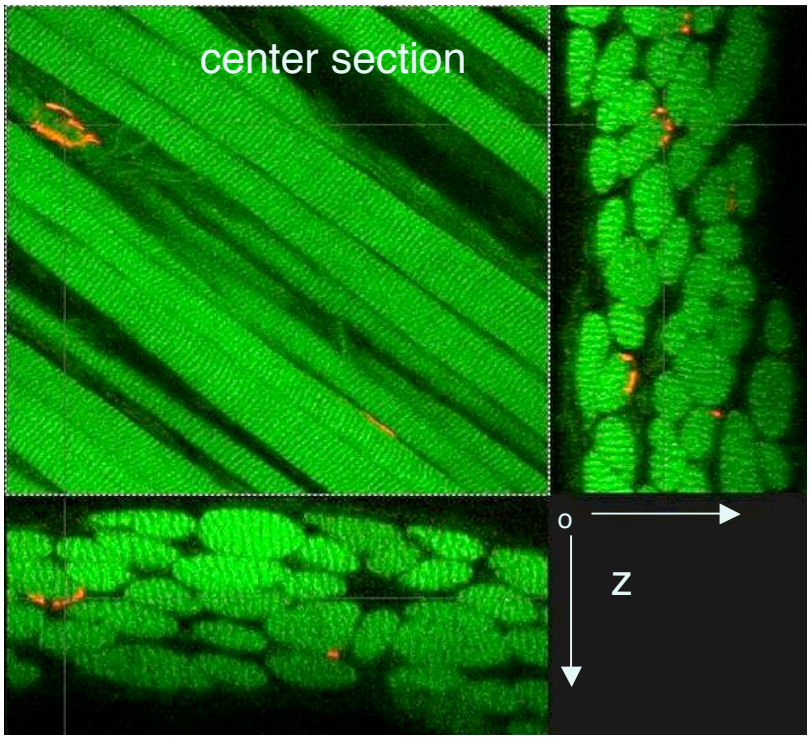


A tissue section was optically sectiones every 10 μm. On each section a different situation in the very same tissue context can be imaged.

Deep Penetration into a thick sample

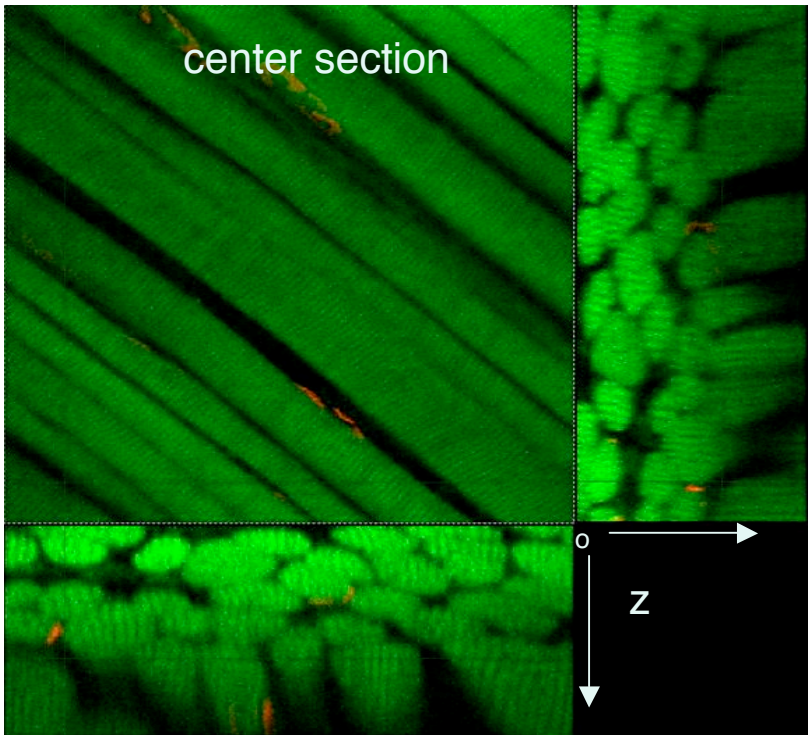
Thick specimen (100μm): GFP muscle fibers, embedded in Glycerol (80/20)

match



Glycerol Objective PL APO 63x1.3

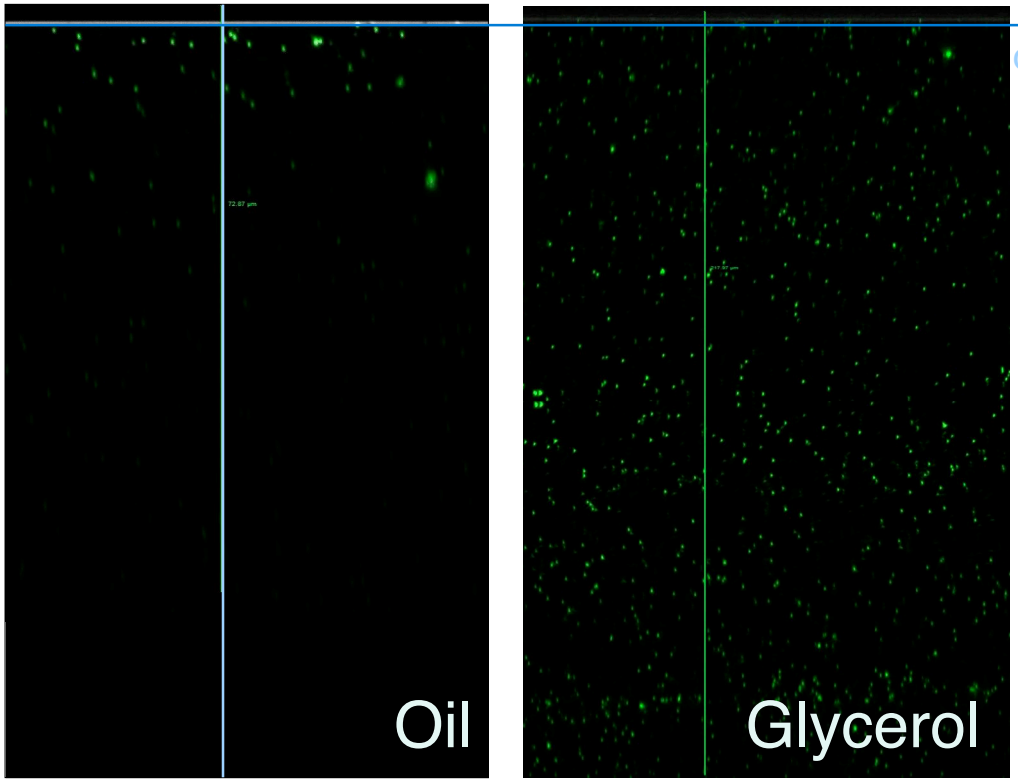
mismatch

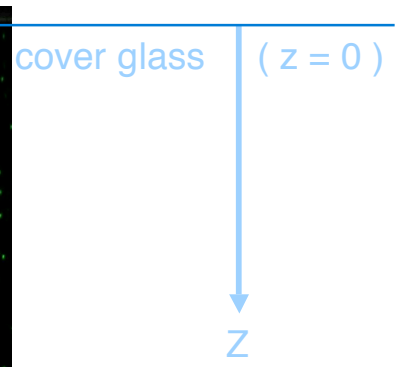


Oil Objective PL APO 63x1.32

Glycerol immersion allows deeper penetration into the sample without severe light loss or distortion. Oil immersion is ideal for imaging near the cover glass.

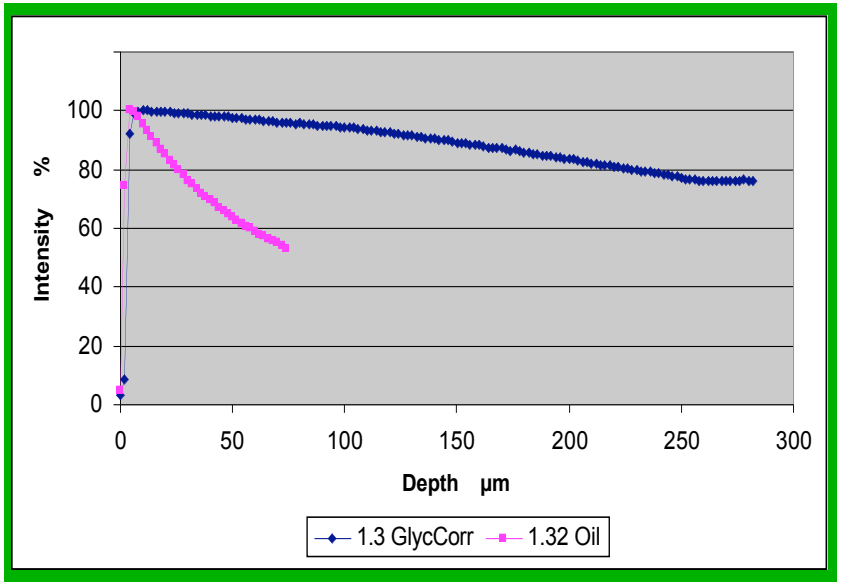
Immersion media and depth penetration





Beads 220 nm embedded in Glycergel.

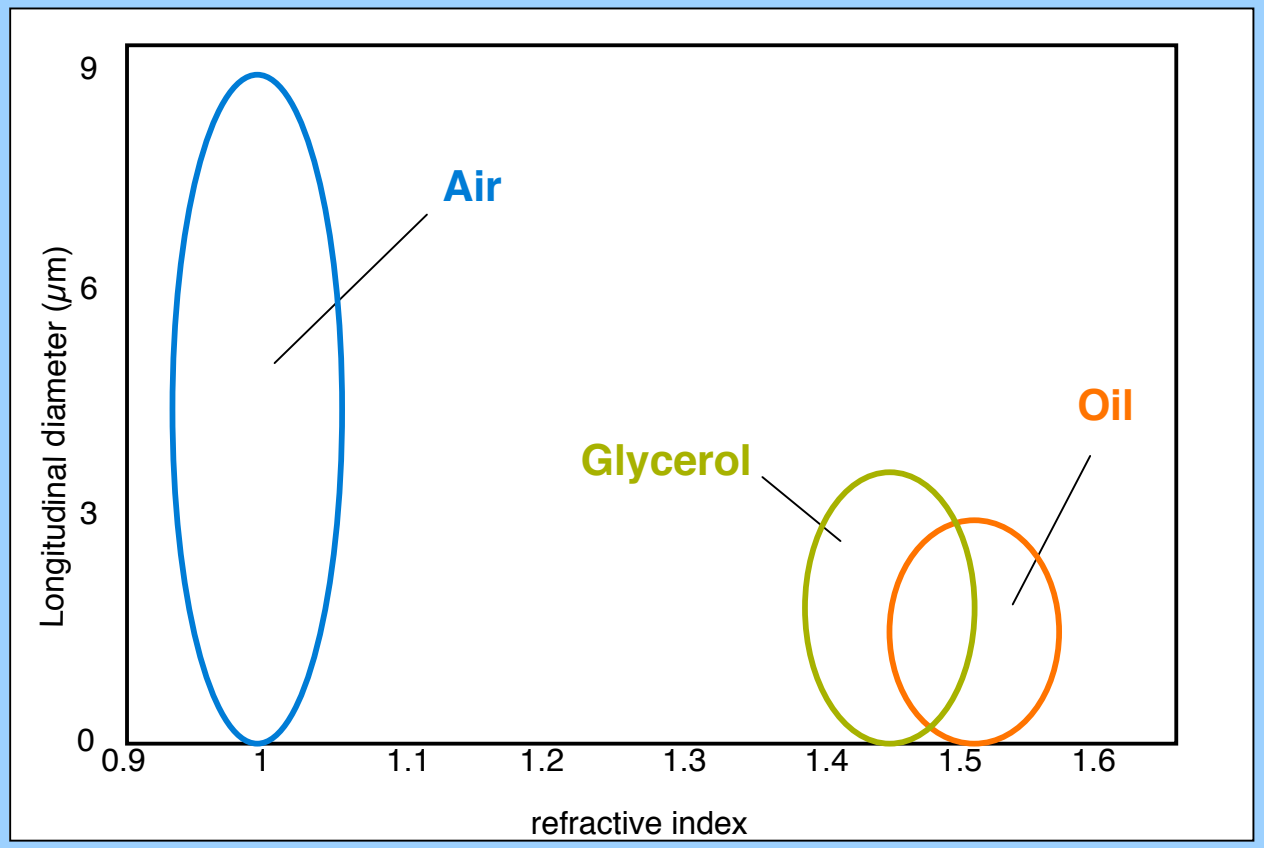
10µM FITC in Glycerol-Water (80/20)-xyz-series



Glycerol-objectivs allow for deep penetration into the embedded biological sample (distortion, brightness).

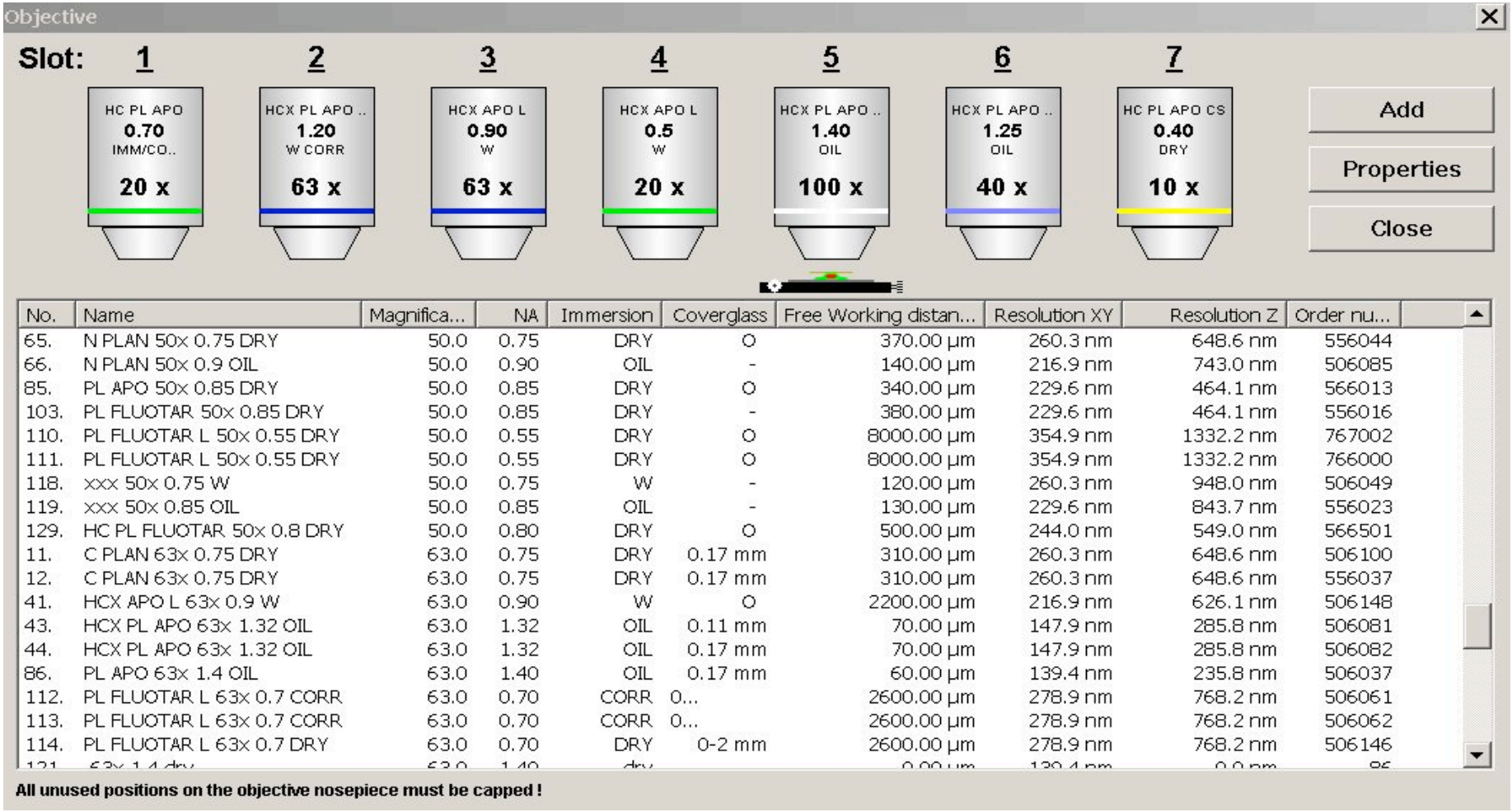
Effect of immersion media on sperical aberration

Spherical aberration is one of the most commonly observed problems in confocal microscopy !



Side view (xz plane) of the 2.76 μm fuorescent microsperes embedded in different media with known refractive indices. The speres immersed in oil appear to be more sperical than the others.

Specifications of the objective

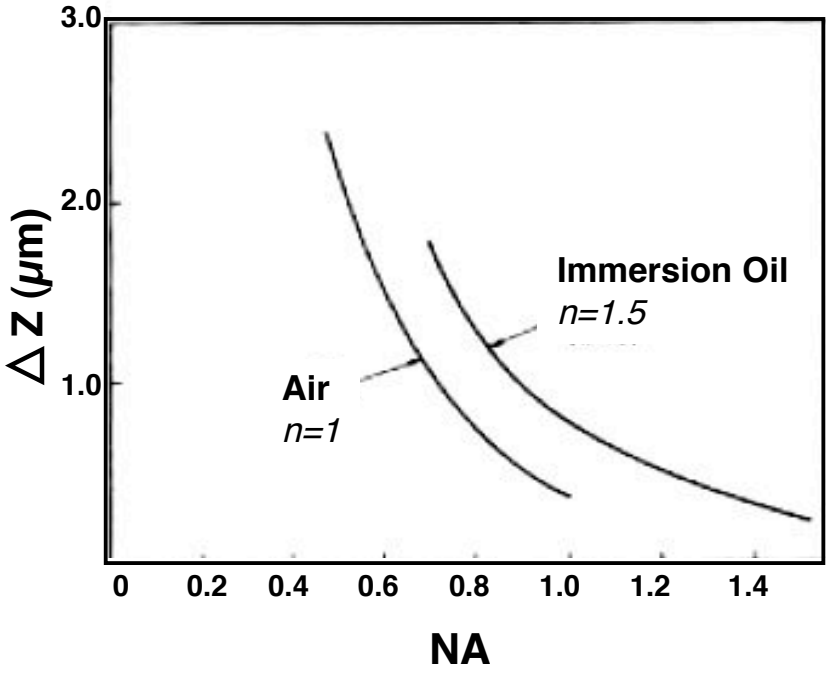


No.	Name	Magnifica...	NA	Immersion	Coverglass	Free Working distan...	Resolution XY	Resolution Z	Order nu...
65.	N PLAN 50x 0.75 DRY	50.0	0.75	DRY	O	370.00 µm	260.3 nm	648.6 nm	556044
66.	N PLAN 50x 0.9 OIL	50.0	0.90	OIL	-	140.00 µm	216.9 nm	743.0 nm	506085
85.	PL APO 50x 0.85 DRY	50.0	0.85	DRY	O	340.00 µm	229.6 nm	464.1 nm	566013
103.	PL FLUOTAR 50x 0.85 DRY	50.0	0.85	DRY	-	380.00 µm	229.6 nm	464.1 nm	556016
110.	PL FLUOTAR L 50x 0.55 DRY	50.0	0.55	DRY	O	8000.00 µm	354.9 nm	1332.2 nm	767002
111.	PL FLUOTAR L 50x 0.55 DRY	50.0	0.55	DRY	O	8000.00 µm	354.9 nm	1332.2 nm	766000
118.	xxx 50x 0.75 W	50.0	0.75	W	-	120.00 µm	260.3 nm	948.0 nm	506049
119.	xxx 50x 0.85 OIL	50.0	0.85	OIL	-	130.00 µm	229.6 nm	843.7 nm	556023
129.	HC PL FLUOTAR 50x 0.8 DRY	50.0	0.80	DRY	O	500.00 µm	244.0 nm	549.0 nm	566501
11.	C PLAN 63x 0.75 DRY	63.0	0.75	DRY	0.17 mm	310.00 µm	260.3 nm	648.6 nm	506100
12.	C PLAN 63x 0.75 DRY	63.0	0.75	DRY	0.17 mm	310.00 µm	260.3 nm	648.6 nm	556037
41.	HCX APO L 63x 0.9 W	63.0	0.90	W	O	2200.00 µm	216.9 nm	626.1 nm	506148
43.	HCX PL APO 63x 1.32 OIL	63.0	1.32	OIL	0.11 mm	70.00 µm	147.9 nm	285.8 nm	506081
44.	HCX PL APO 63x 1.32 OIL	63.0	1.32	OIL	0.17 mm	70.00 µm	147.9 nm	285.8 nm	506082
86.	PL APO 63x 1.4 OIL	63.0	1.40	OIL	0.17 mm	60.00 µm	139.4 nm	235.8 nm	506037
112.	PL FLUOTAR L 63x 0.7 CORR	63.0	0.70	CORR	0...	2600.00 µm	278.9 nm	768.2 nm	506061
113.	PL FLUOTAR L 63x 0.7 CORR	63.0	0.70	CORR	0...	2600.00 µm	278.9 nm	768.2 nm	506062
114.	PL FLUOTAR L 63x 0.7 DRY	63.0	0.70	DRY	0-2 mm	2600.00 µm	278.9 nm	768.2 nm	506146

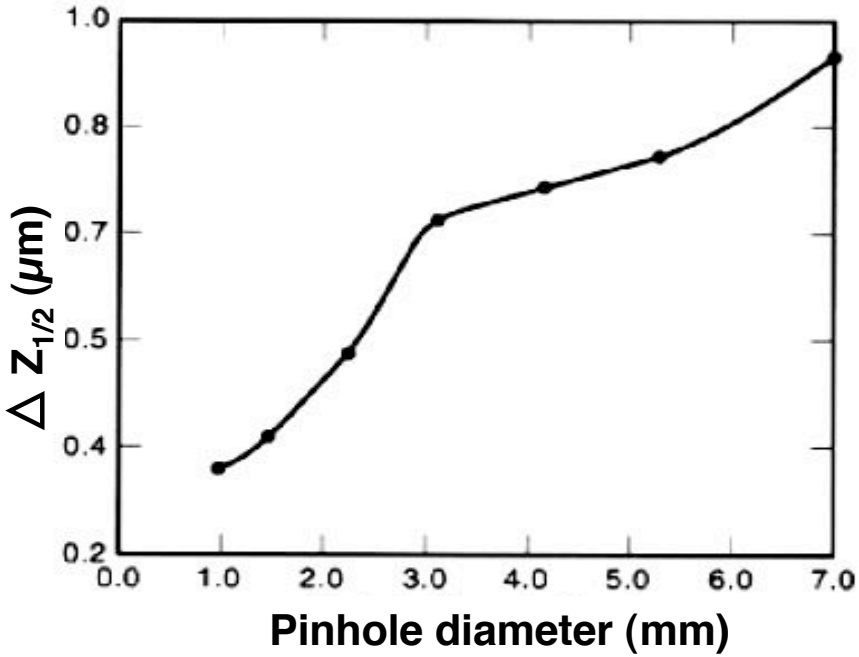
-> need for cover glass thickness (i.e. 0,17 mm), immersion medium (air, water, oil, glycerol)
-> abilities for working distance (sample thickness), NA (signal brightness), magnification (field of view)
-> resolution power in XY & Z (-> optical section thickness)

z - resolution in confocal microscopy

Optical sectioning thickness
as a function of NA of the objective

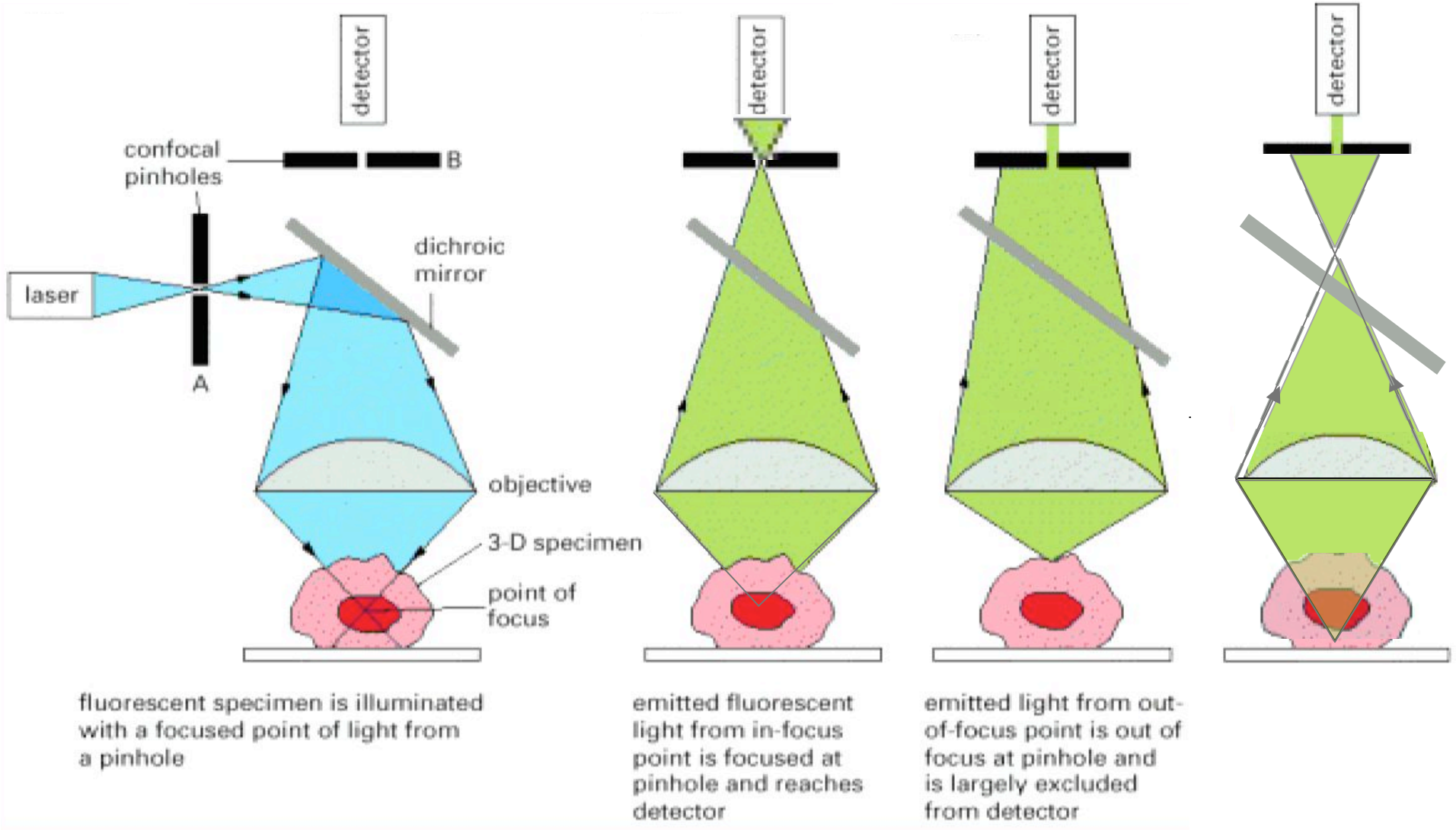


Optical sectioning thickness
versus confocal pinhole diameter



confocal imaging - in focus/out of focus

ex *em*



Pinhole diameter effects

opt. section

small pinhole diameter:

-> thin optical section

= high z-resolution possible
= low signal strength

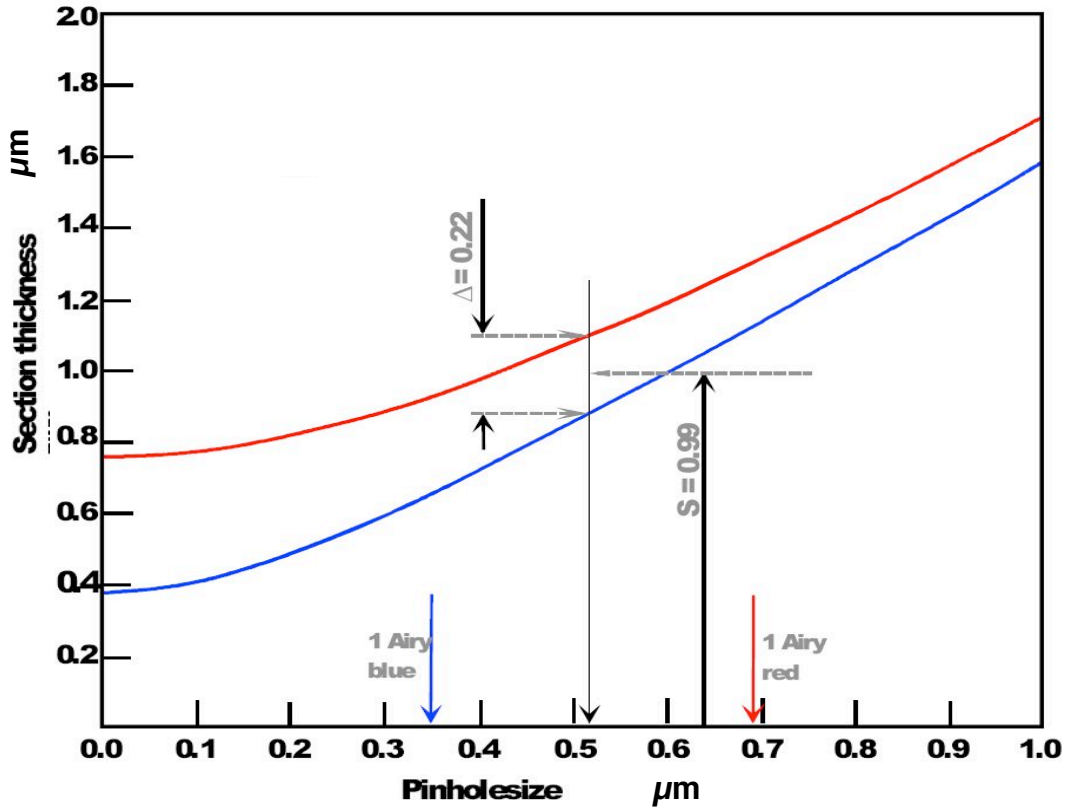
big pinhole diameter:

-> thick optical section

= low z-resolution
= brighter signal

Pinhole size, color and z-resolution

- The pinhole is optimized **for each objectiv.**
- „**Airy 1**“ is a good start, but NOT an iron rule; play with pinhole to get either more light or more resolution.
- Resolution depend also on **wavelength**; keep in mind if resolution REALLY matters. Leica pinhole values are optimized for medium wavelength.



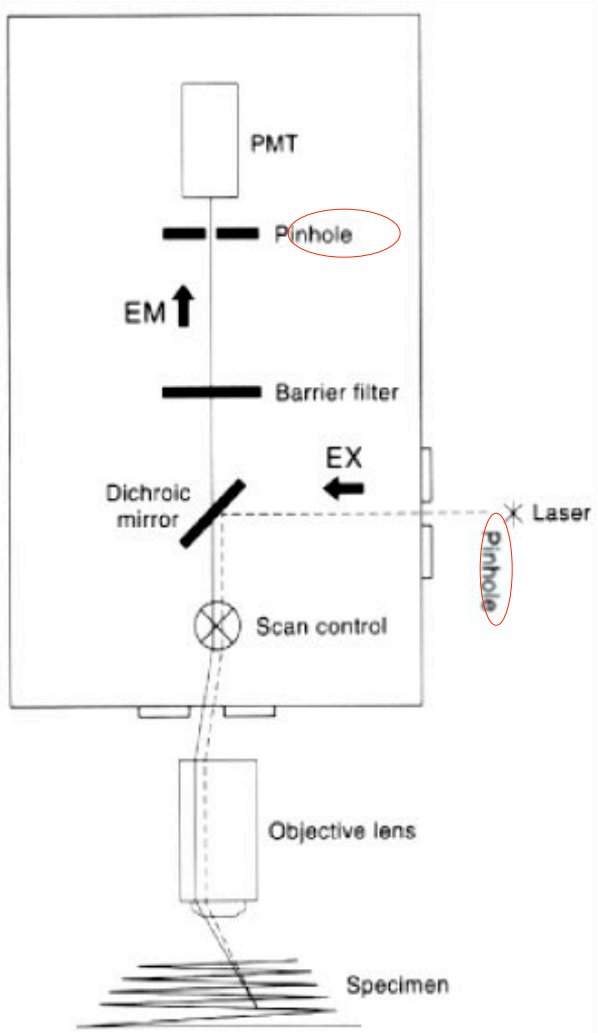
The pinhole variable determines your z-resolution.

The single point confocal system

Beam diameter is limited by a „pinhole“ aperture
-> field of illumination & detected signal are pointed !

consequences for confocal imaging:

- The illumination intensity has to be very high. (LASER light)
- Photo multipier tubes (PMT) are used for sensitive and fast single point intensity registration.
- The light source is scanned over the sample. The image has to be rebuilt from the recorded point intensities according to the xy-coordinates.
- The image is not directly visible for the eye. The image has to be electronically generated. (Sequential acquisition process)



LASER as confocal light source

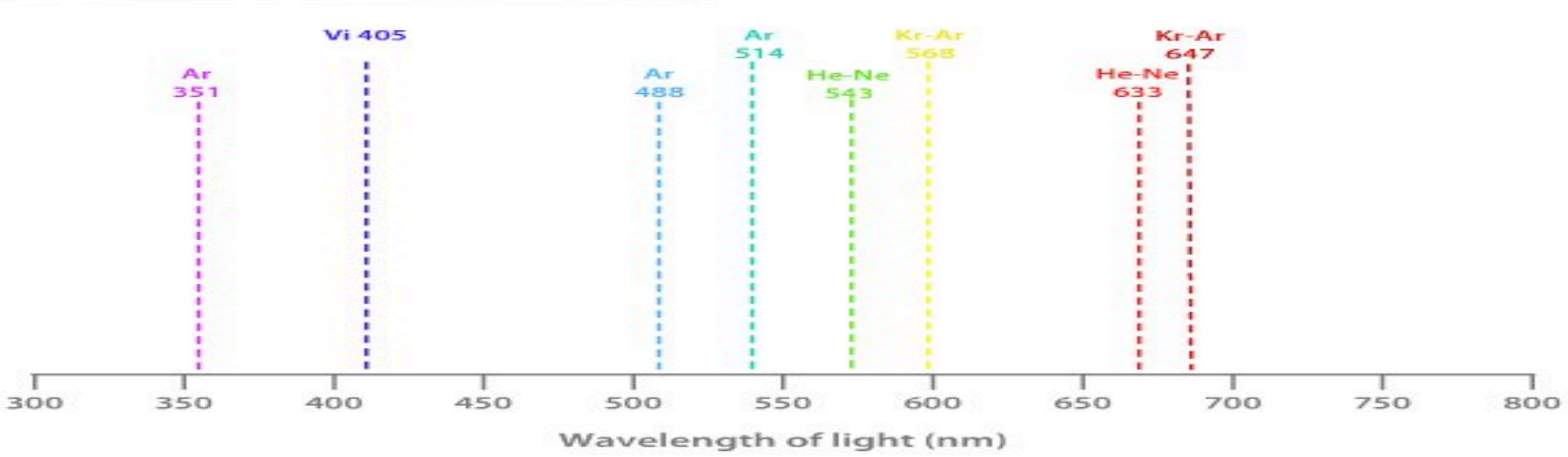
Mercury and Xenon Light sources are to week for point confocal systems. Strong bundled light is generated by LASERS.

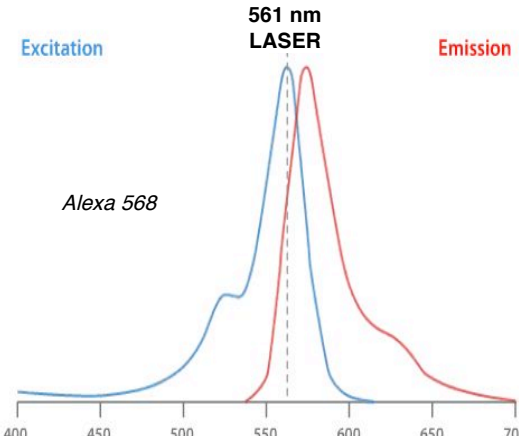
There are different types of LASERS: Argon, Argon-Krypton, Helium-Neon, etc. The coupling to the system and the alignment has to be done by trained engineers.

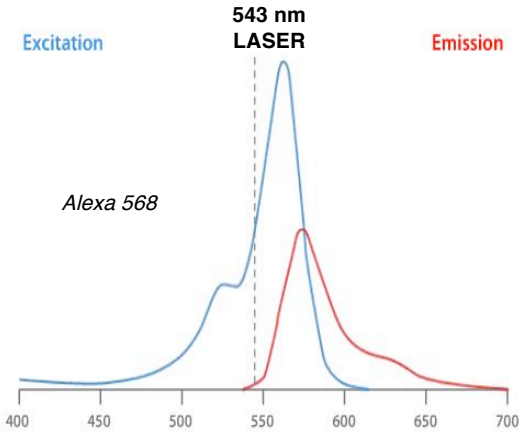
LASER sources generate **monochromatic light of a discrete wavelength** -> “LASER line”. For the spectral range are different LASERS necessary. Depending on the hardware of the microscope, some of the following lines might be available (λ in nm):

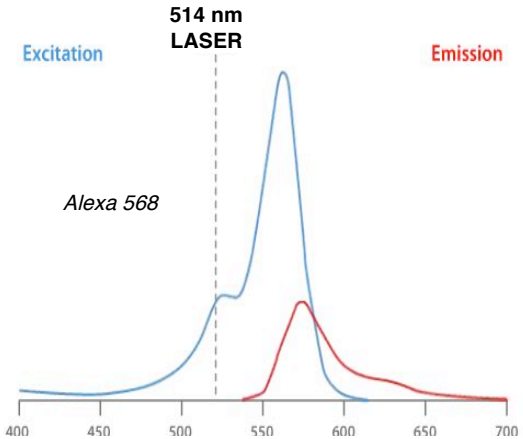
352, 364, 405, 430, 458, 476, 488, 496, 514, 543, 561, 596, 633

Laser Excitation



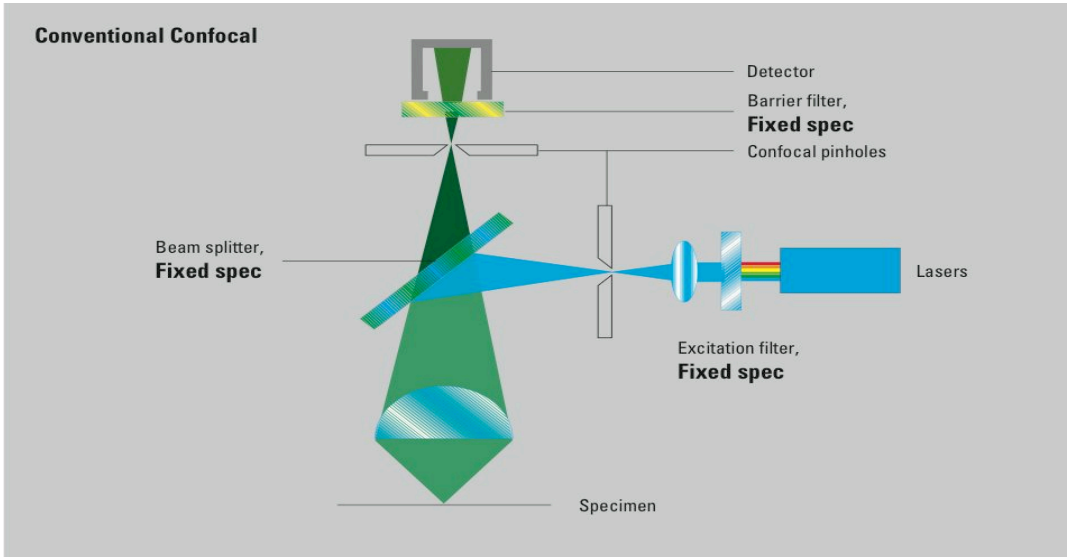






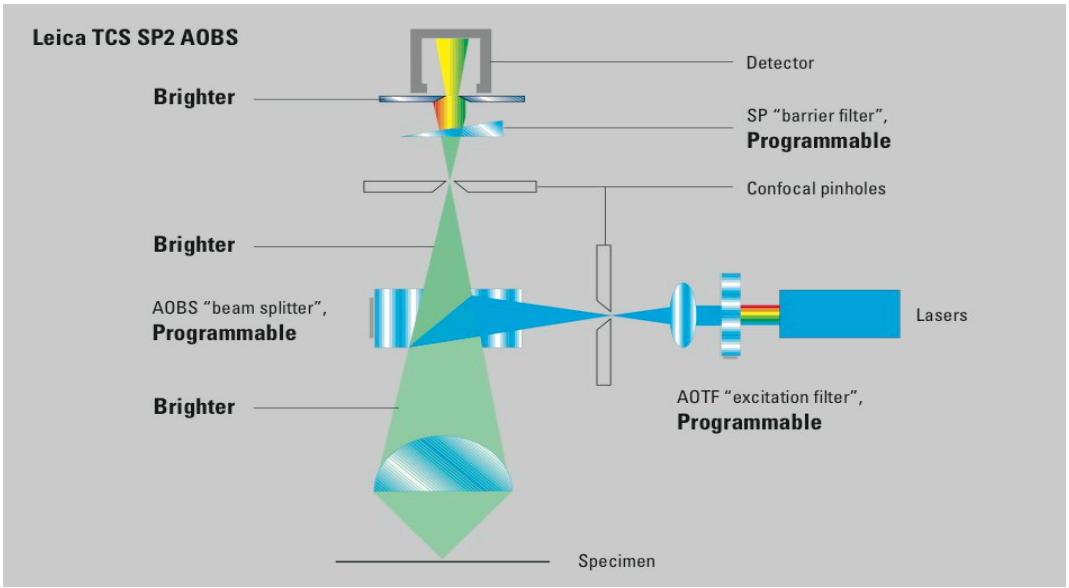
! choose florochromes accordingly to the laser lines: as further away the laser line is from the absorption maximum of a fluorochrome as weaker the emission signal gets!

The filter free CLSM: Leica confocals



Non-LEICA:

glas filters and dicroic mirrors determine the spectral detection



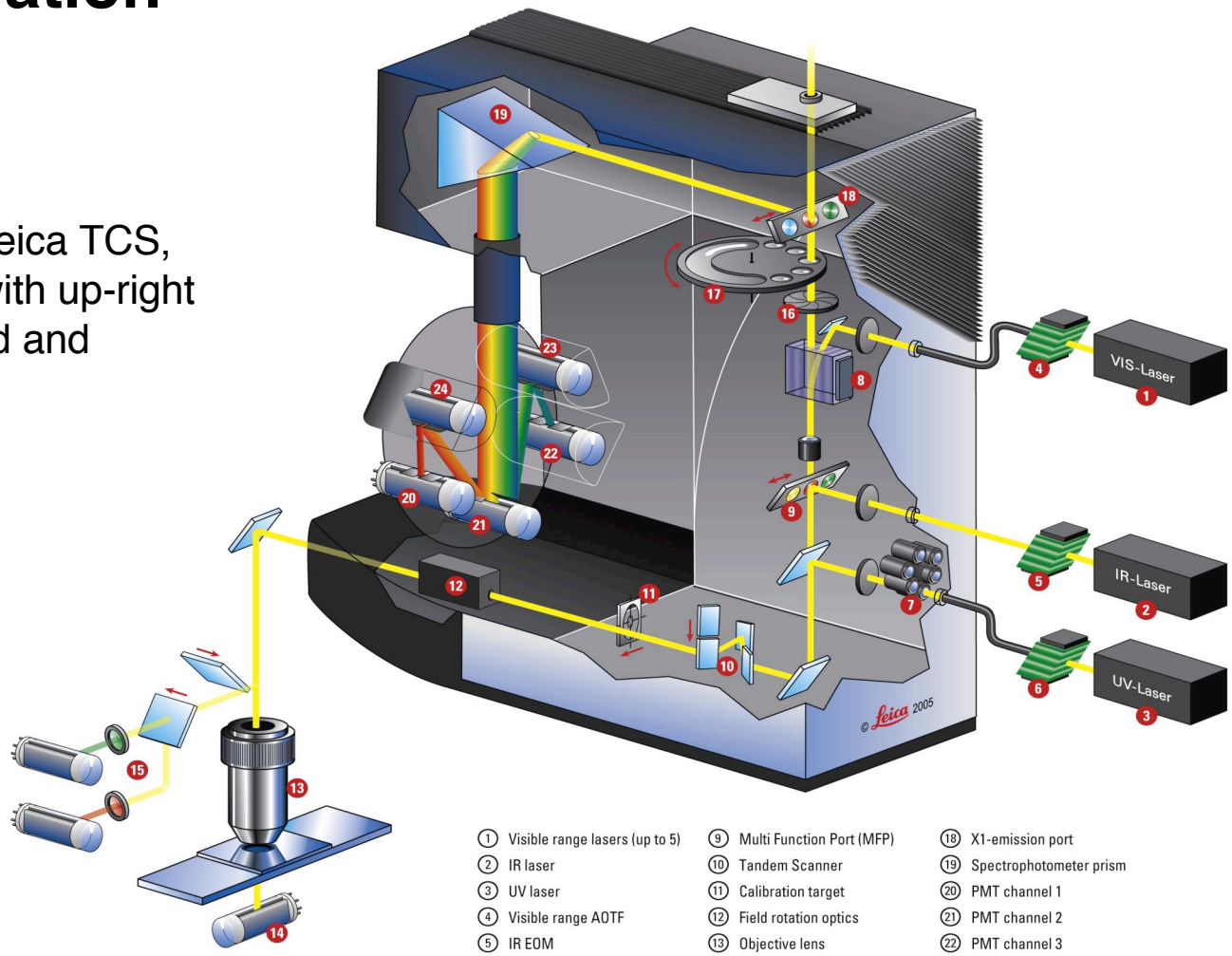
LEICA:

Prisms, free adjustable barriers and tunable quarz cristals determine the spectral detection: AOTF, AOBS, SP

Leica TCS SP5*

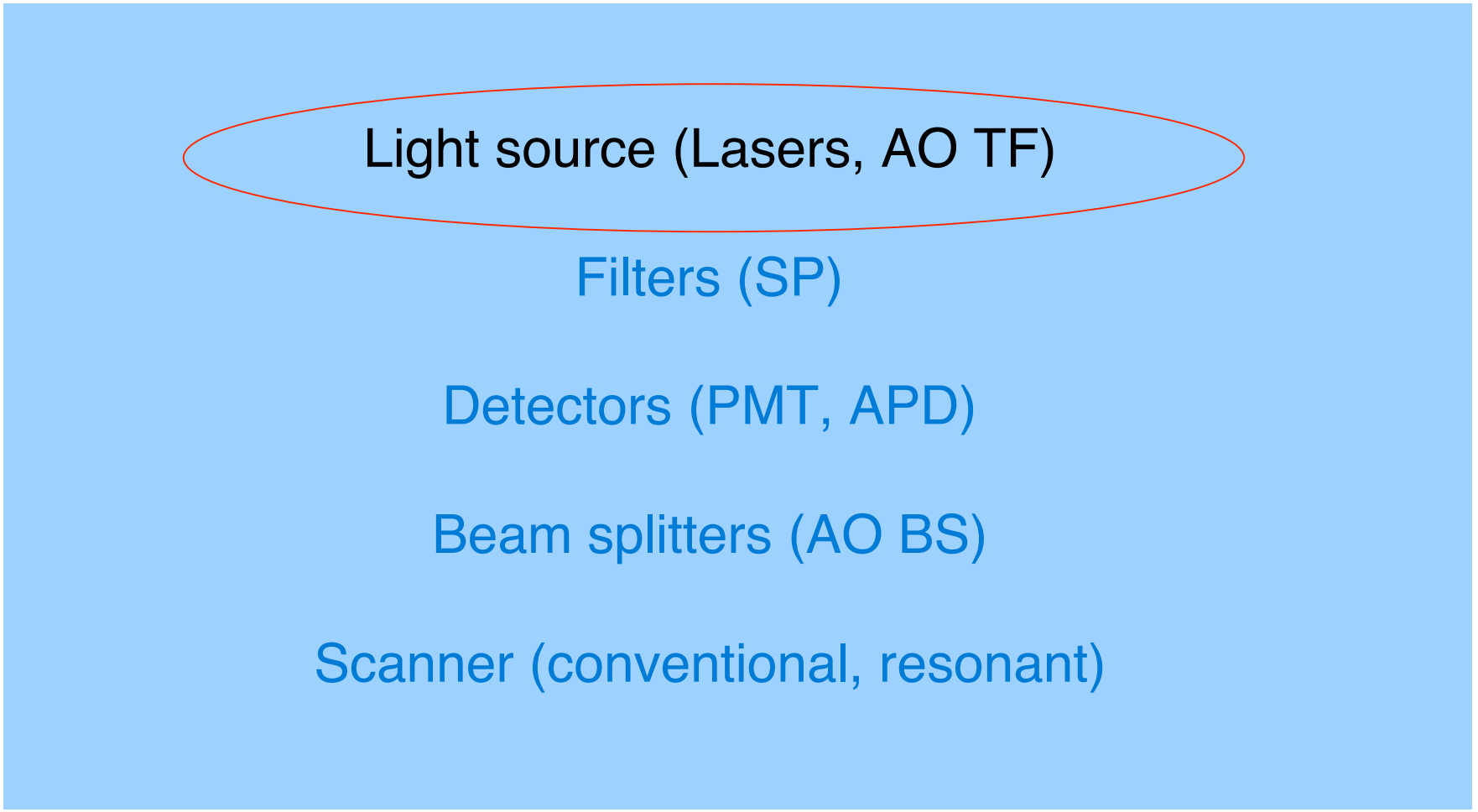
-the newest generation of Leica confocal microscopes

*The ZMB owns an inverted Leica TCS, a TSC SP2 confocal system with up-right and inverted microscope stand and an inverted Leica TCS SP5.



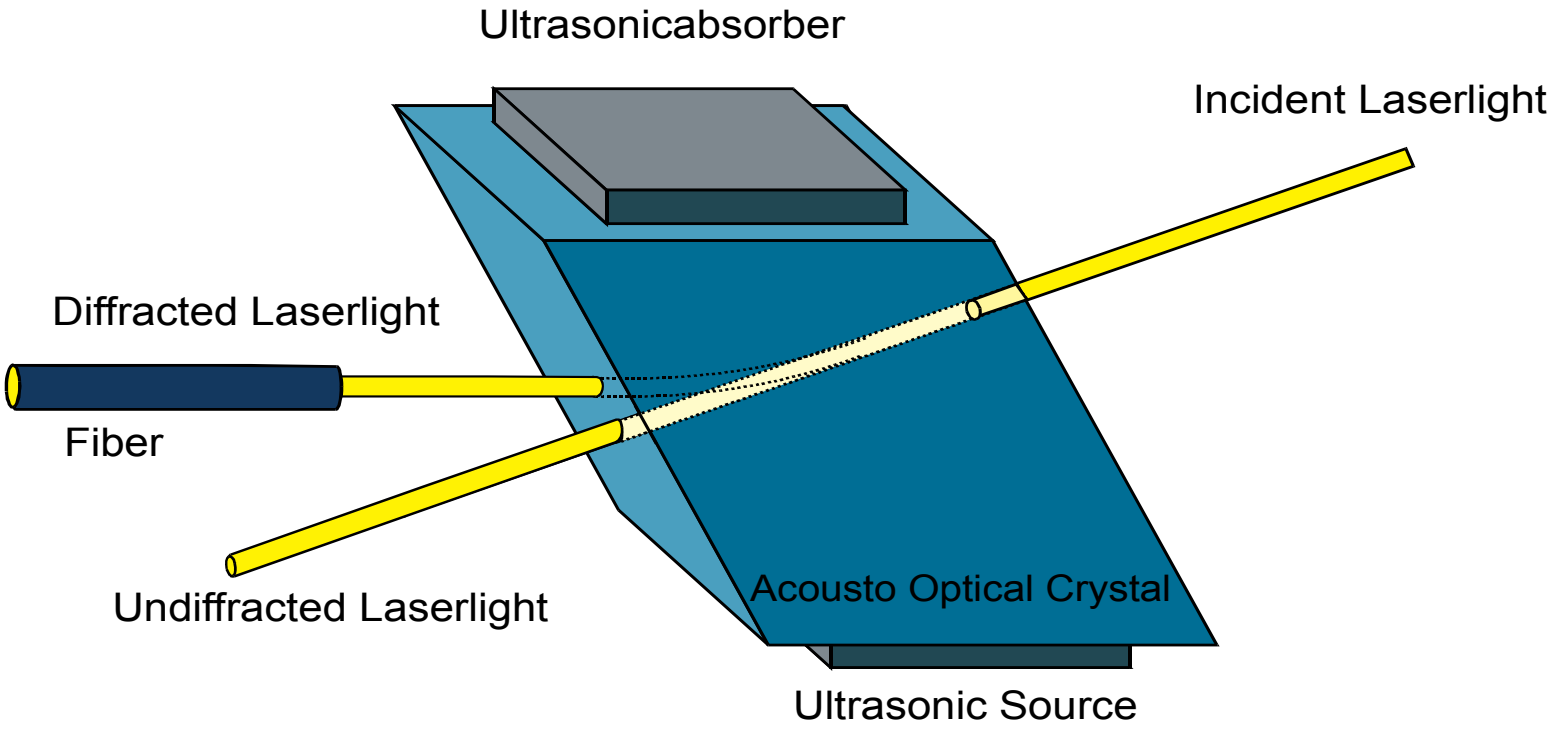
1. Visible range lasers (up to 5)
2. IR laser
3. UV laser
4. Visible range AOTF
5. IR EOM
6. UV AOTF
7. UV optics imaging
8. AOBS
   Acousto Optical Beam Splitter
9. Multi Function Port (MFP)
10. Tandem Scanner
11. Calibration target
12. Field rotation optics
13. Objective lens
14. Transmitted light dedector
15. Reflected light NDD
16. Confocal detection pinhole
17. Filter- and polarizer wheel
18. X1-emission port
19. Spectrophotometer prism
20. PMT channel 1
21. PMT channel 2
22. PMT channel 3
23. PMT channel 4 (optional FLIM)
24. PMT channel 5 (optional FLIM)

Leica confocal laser scanning microscope



AOTF

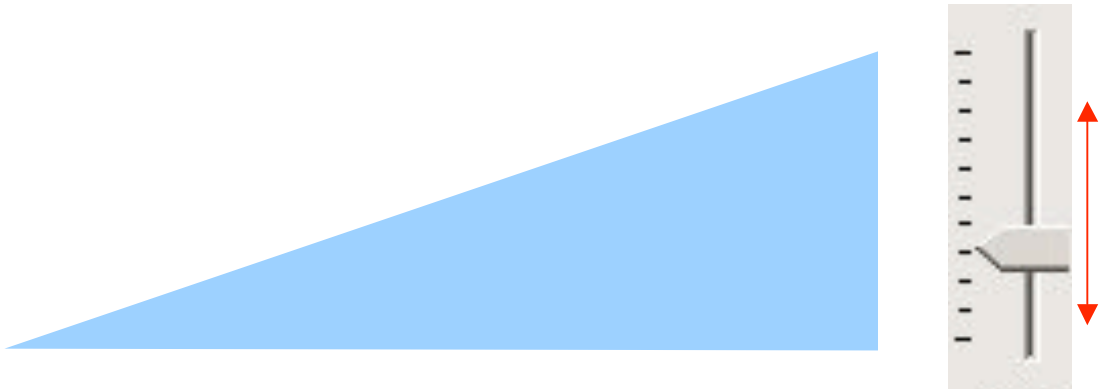
Acousto **O**ptical **T**unable **F**ilter



This adjustable quartz filter works at frequencies as high as sound, that is "Acousto-". => Light, which passes the AOTF, is diffracted depending on ist own wavelength and the wavelength of the ultrasonic wave field. The ultrasonic wave field can be modulated, so that the intensities of the different laser lines can be changed between 0% and100% by the software even during the scanning process.

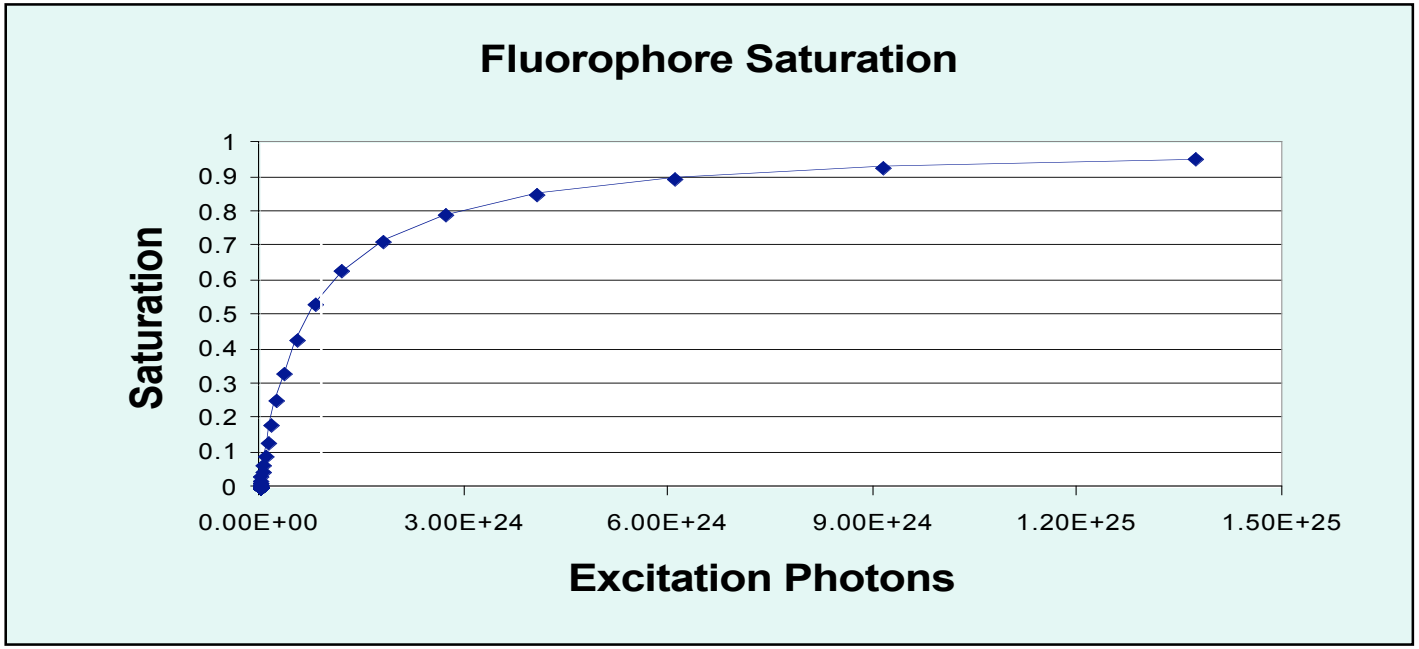
AOTF **A**cousto **O**ptical **T**unable **F**ilter

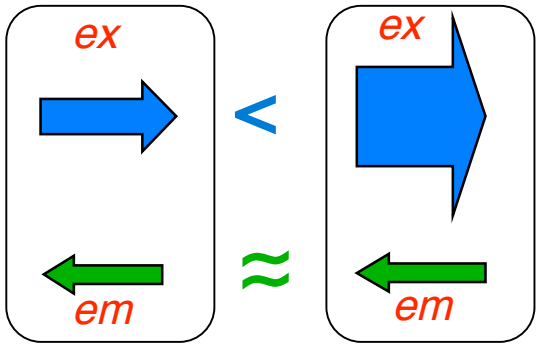
The AOTF enables you to select the wavelengths (laser lines on/off).



The AOTF enables you to control the intensity of the excitation light.

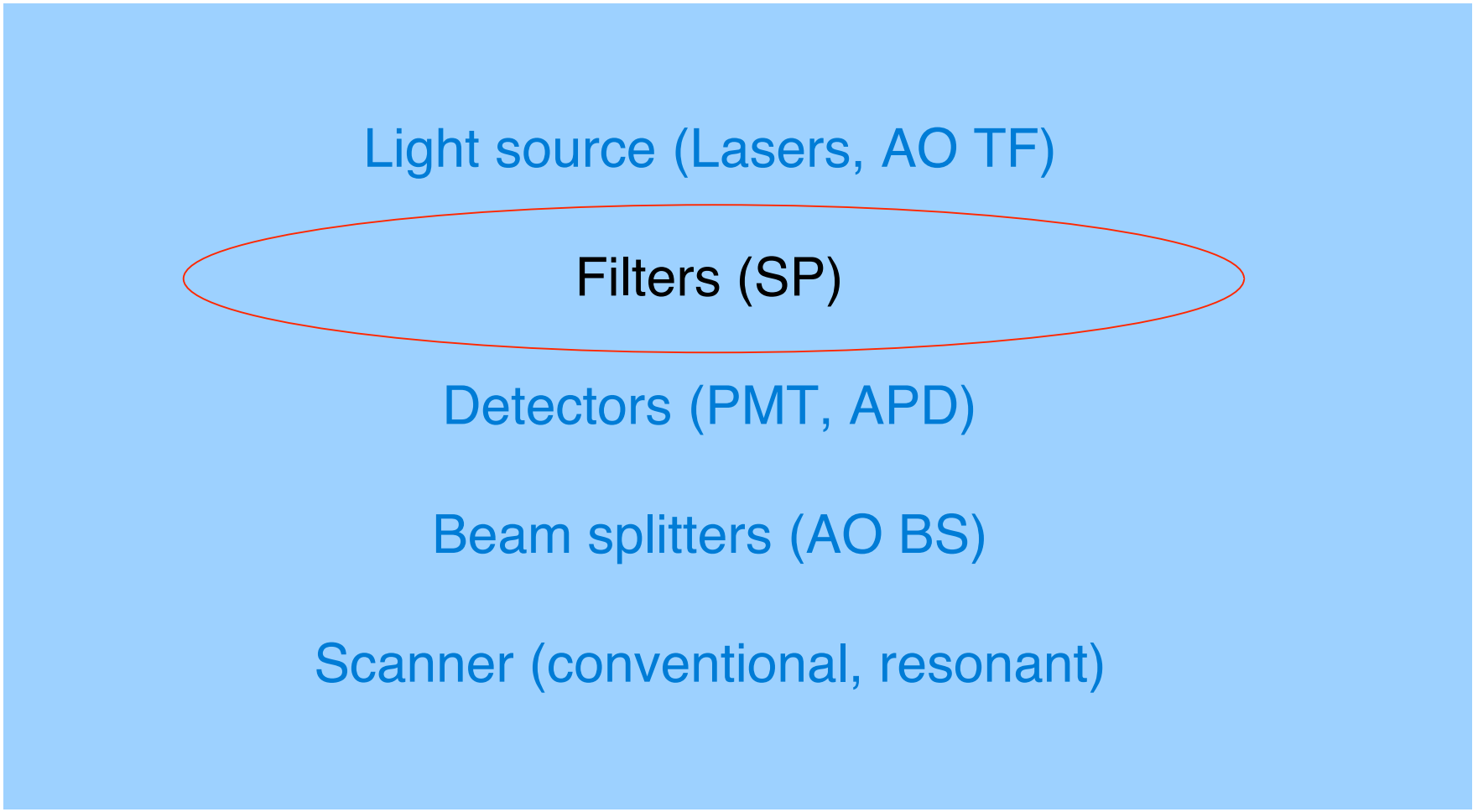
Excitation optimum



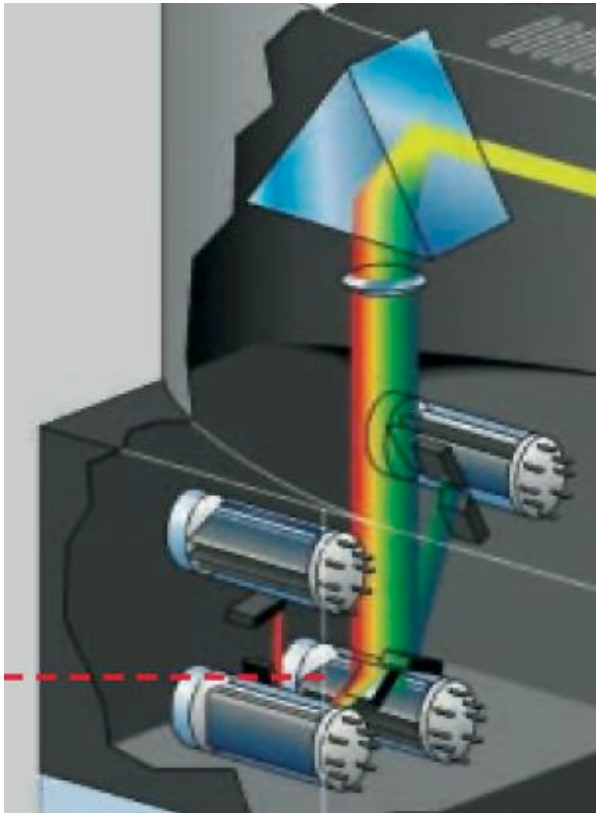


Avoid oversaturation !
-> lower light power decreases phototoxicity and bleaching.

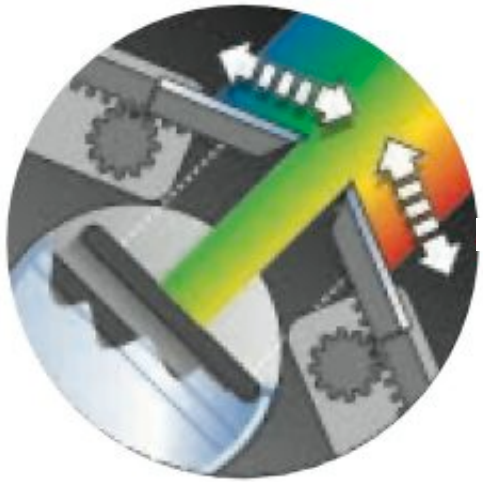
Leica confocal laser scanning microscope



spectral detection in Leica CLSM

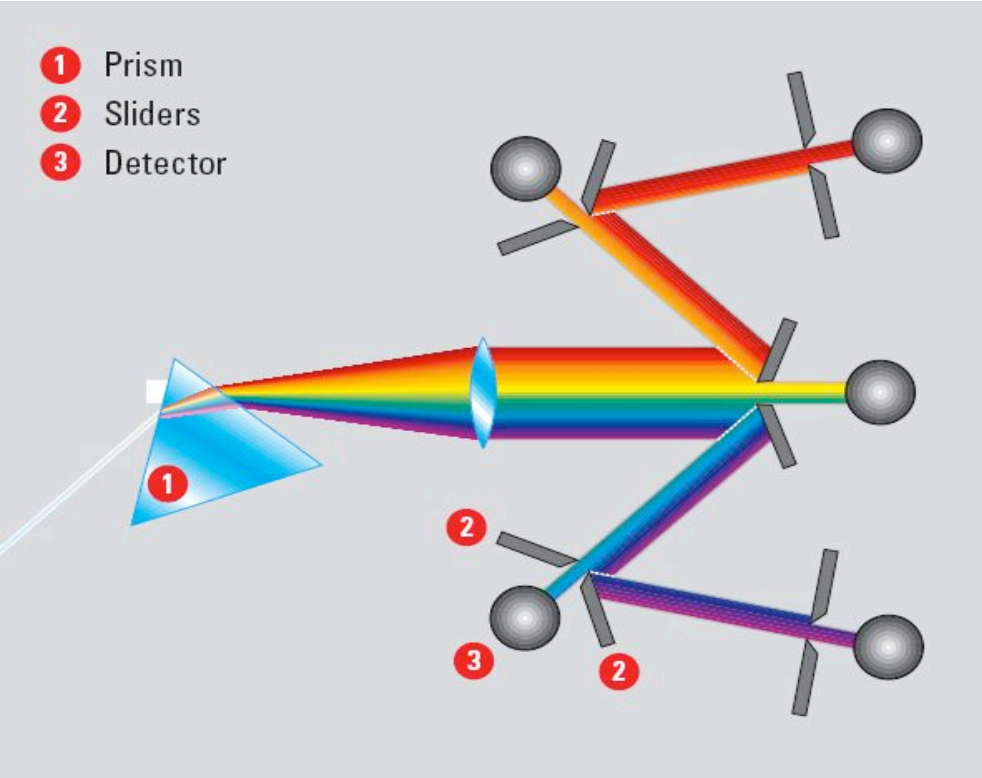


Prisma and adjustable barriers allow free choice of detection

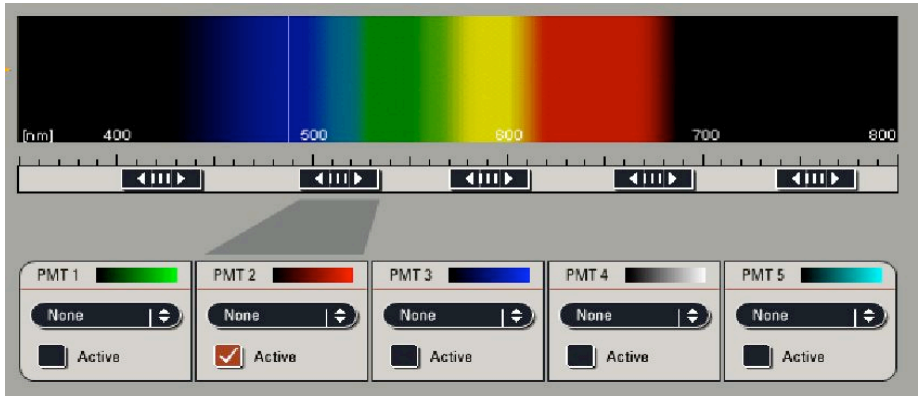


The Leica spectrophotometer detection system (SP): After passing the detection pinhole, the light emitted from the focal plane is passed through a prism, which stretchs the emitted light. The entire spectrum can be imaged onto the window of the PMT. In front of the PMT is a slit. The slit 1) can be widened or narrowed to include a larger or a smaller part of the spectrum & 2) can be moved across the spectrum. Due to the slit in front, the PMT detects only a particular bandwidth. The remainder of the spectrum is excluded by the plates on each side of the slit.The surfaces of these two plates are mirrored and angled to reflect the rejected portions of the spectrum off to other detectors.

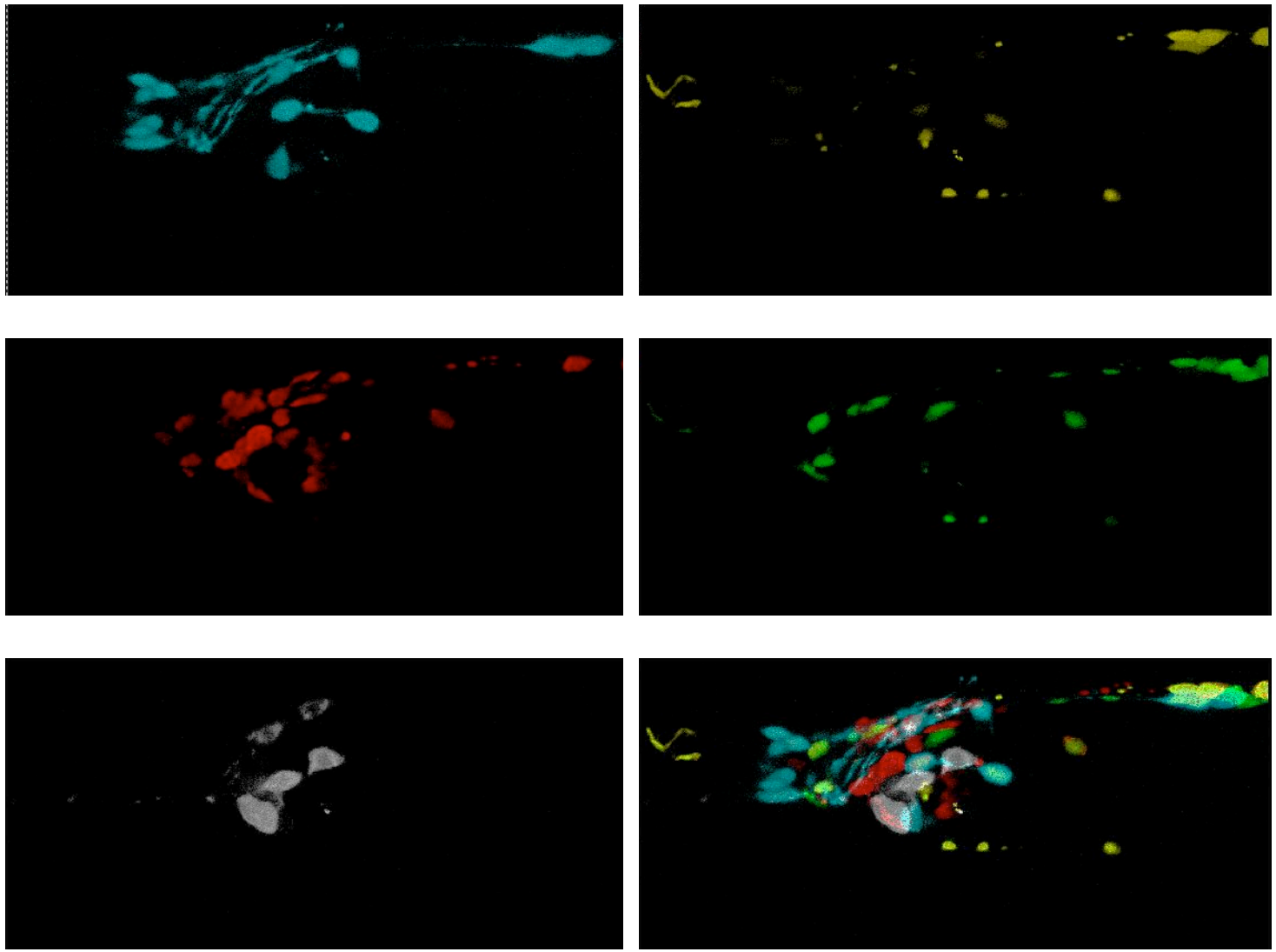
Multispectral imaging with the Leica spectrophotometer detection system (SP)



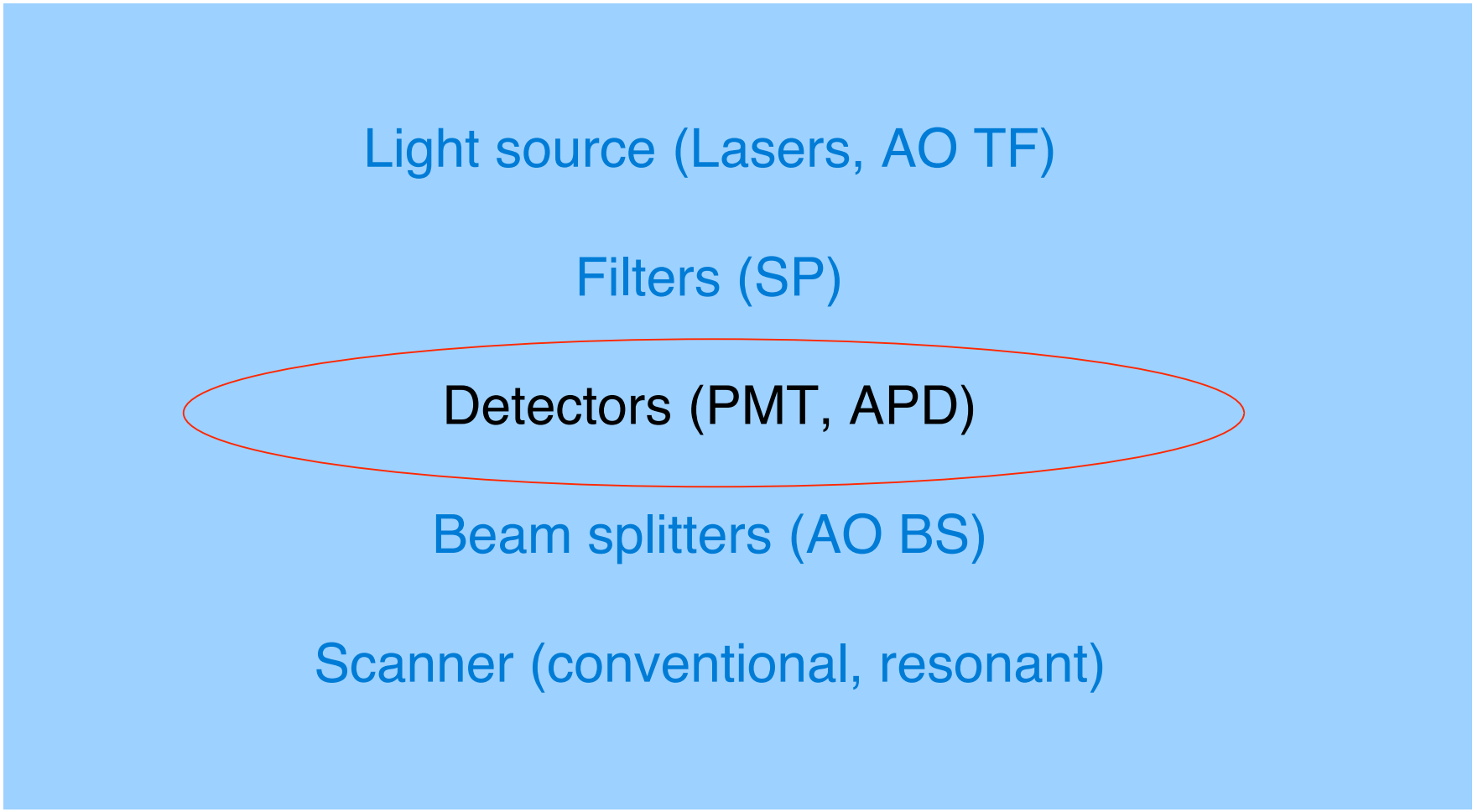
- freely tuneable emission bands allow to adjust for a variety of dyes
- Up to 5 confocal channels simultaneously (multispectral imaging)
- Recording of emission spectra via λ-scan



sprectral discrimination...

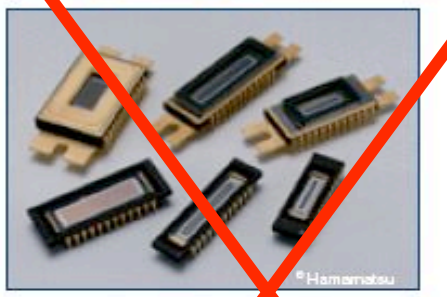


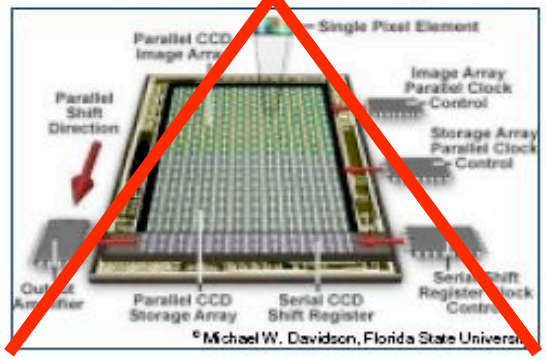
Leica confocal laser scanning microscope



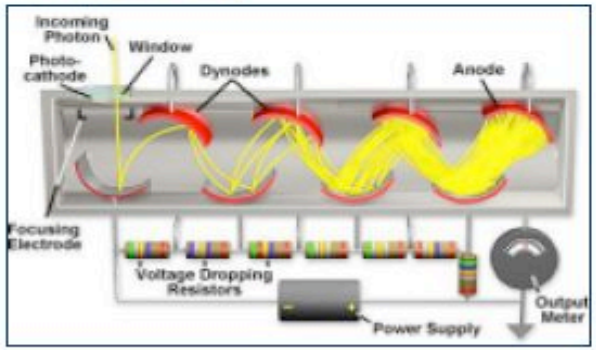
Digital image detectors in CLSM

CCD arrays

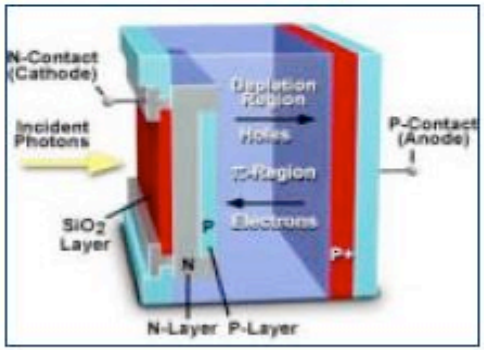




Photomultiplier



Photodiodes



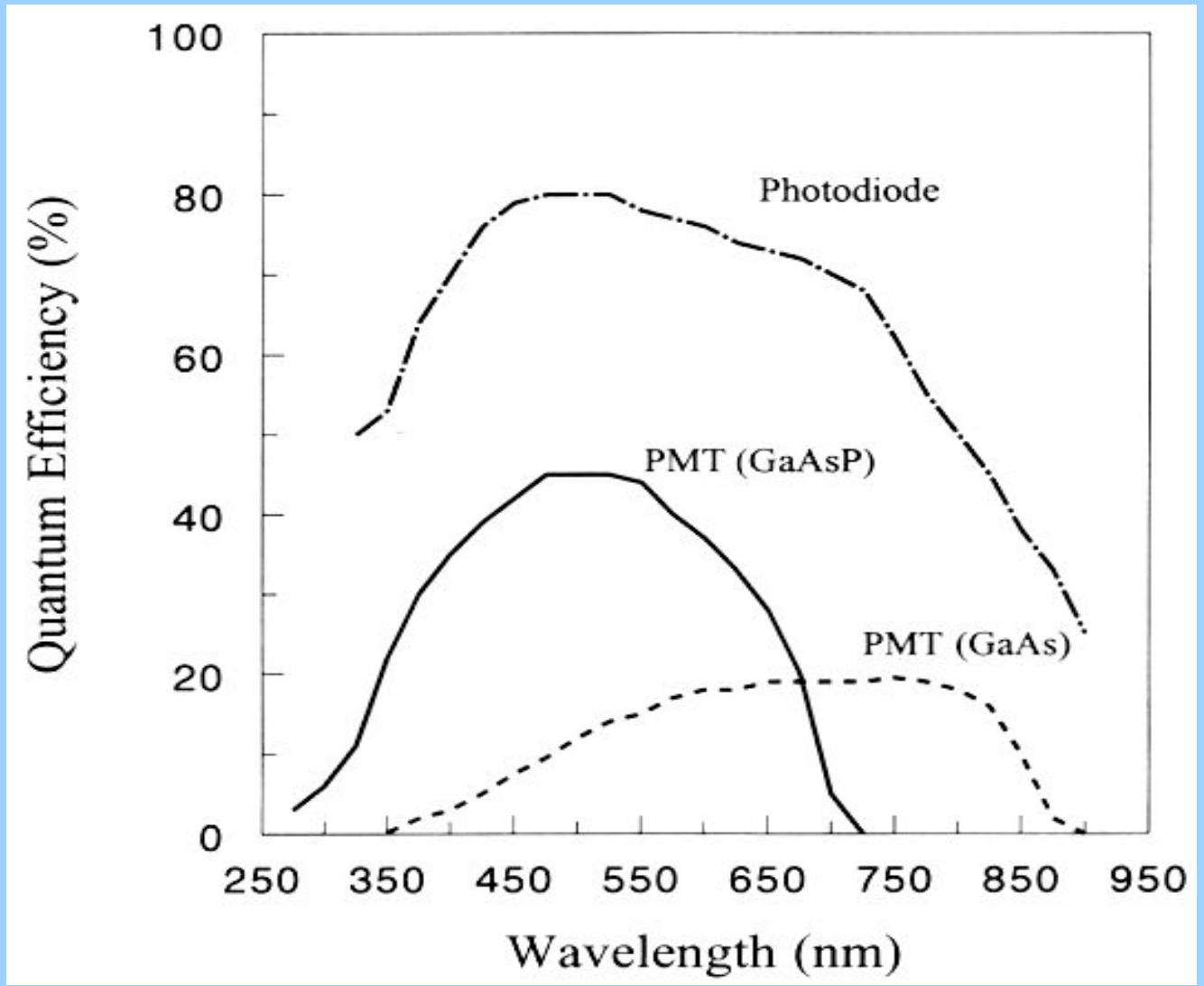
Read-out of all sensors: voltage / current

CCD cameras for point-confocal microscopes not suitable.

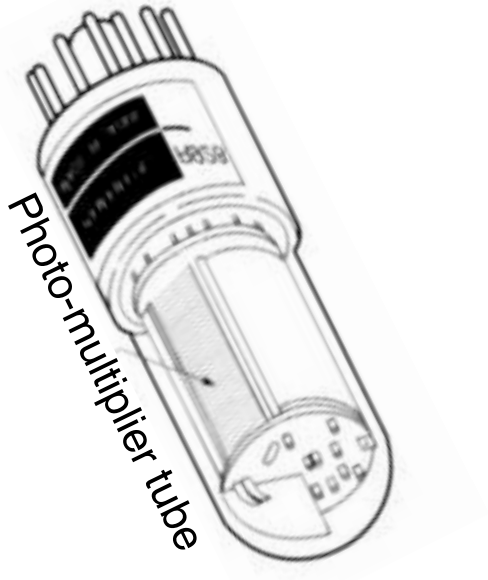
PMT`s have a high dynamic range and noise-free signal amplification.

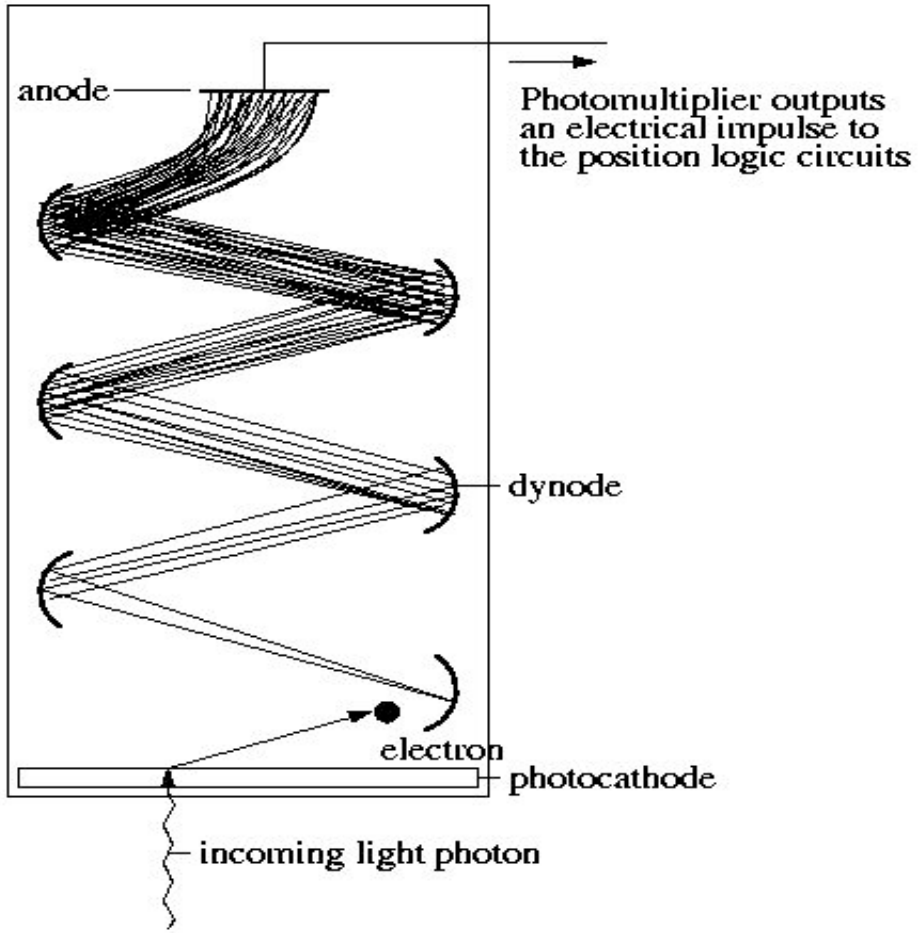
APD photodiodes have highest sensitivity and wide spectral range.

Spectral sensitivity of confocal detectors



How PMT`s work ...





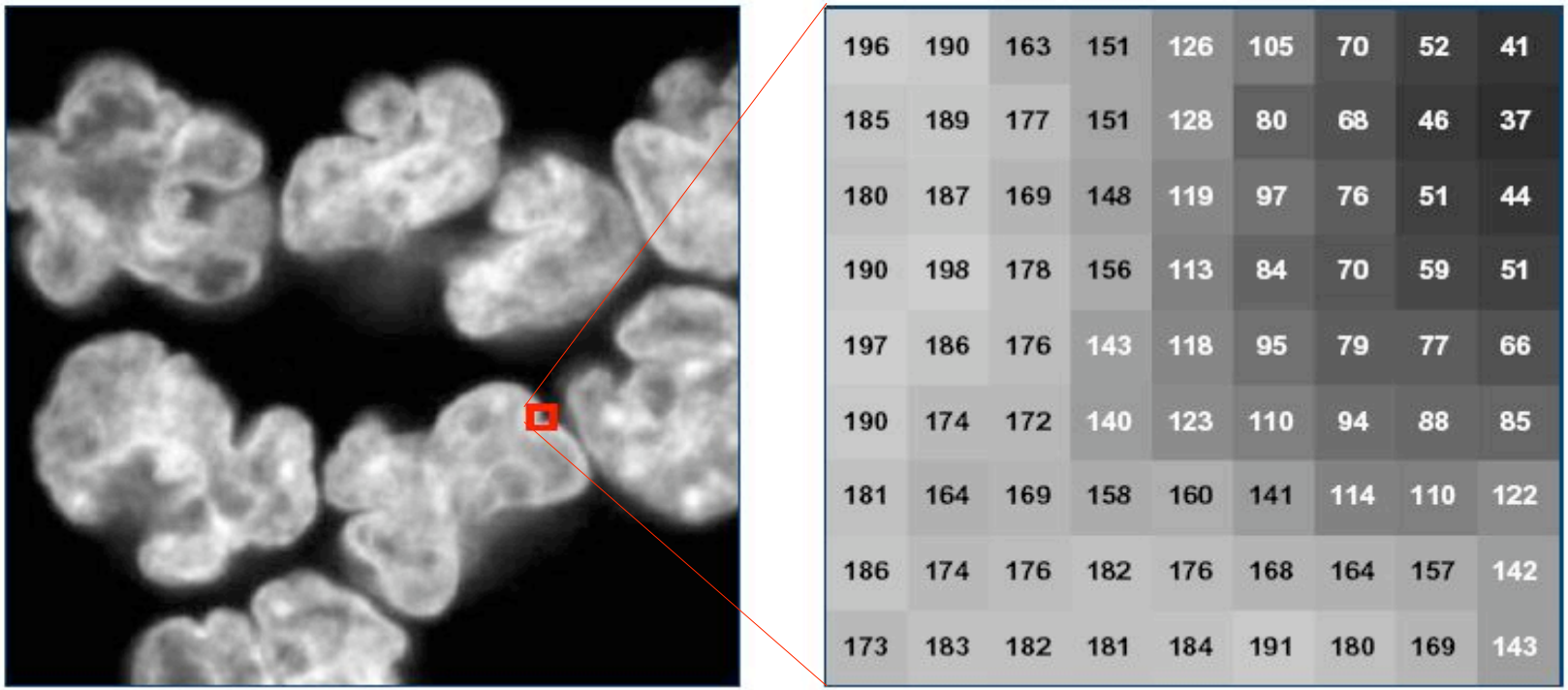
<u>Intensity measurements</u>
without spectral information (high sensitivity, pseudo colors)

<u>Sequential</u> single point measurements
-> coordinates get defined by position in the scan sequence

Principle of signal amplification

1) Conversion of photones into electrons
2) Multiplying electrons
3) Signal readout

Electronic grayscale image

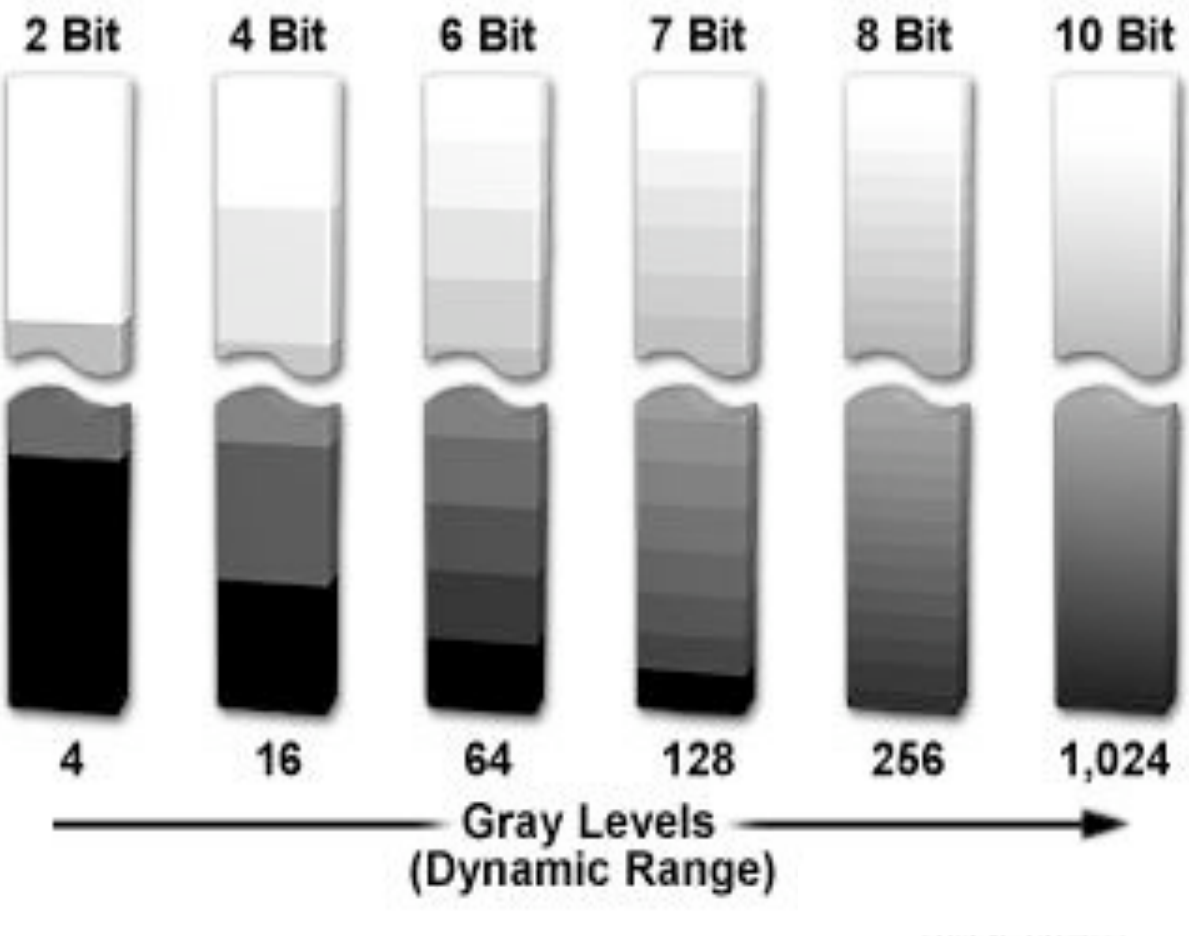


196	190	163	151	126	105	70	52	41
185	189	177	151	128	80	68	46	37
180	187	169	148	119	97	76	51	44
190	198	178	156	113	84	70	59	51
197	186	176	143	118	95	79	77	66
190	174	172	140	123	110	94	88	85
181	164	169	158	160	141	114	110	122
186	174	176	182	176	168	164	157	142
173	183	182	181	184	191	180	169	143

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

Dynamic range

information depth - number of grey levels in an image, resolution of intensity

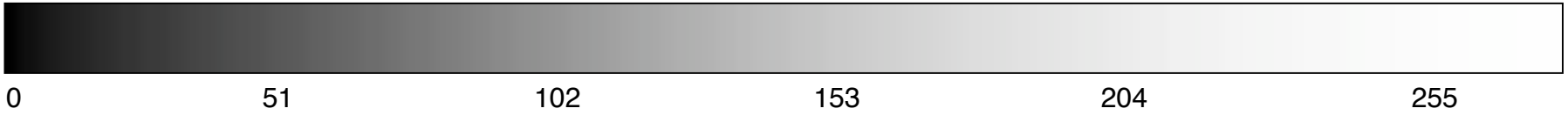


A higher dynamic range allows quantifications, image analysis.

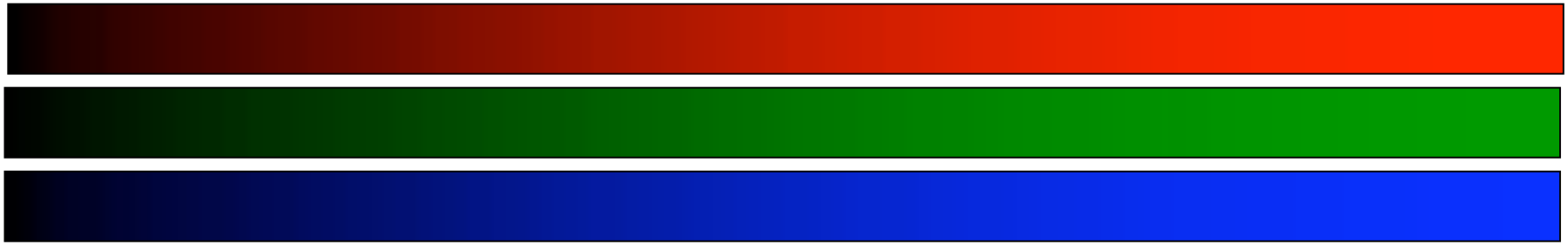
The computer monitor displays 256 grey levels. The human eye can discriminate about 60 gray levels (6 bit).

More Bits need more storage space in the computer.

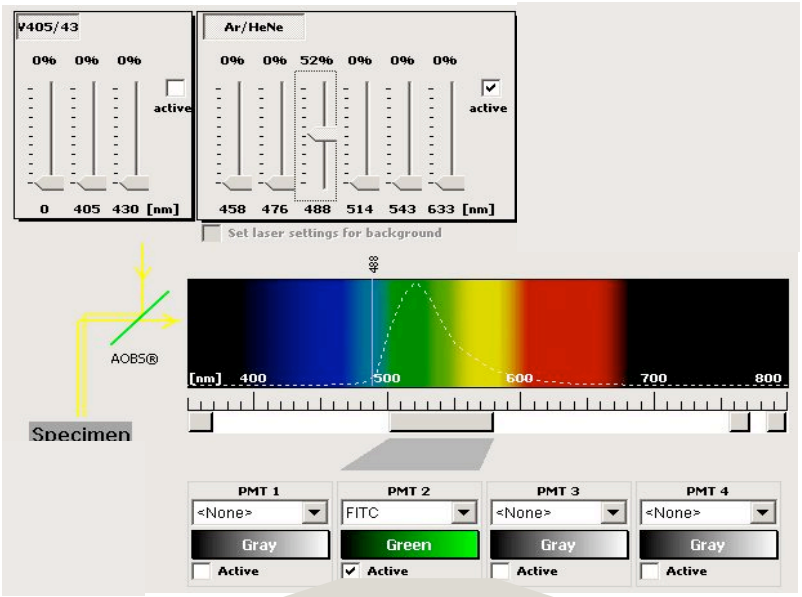
look up table (LTU)

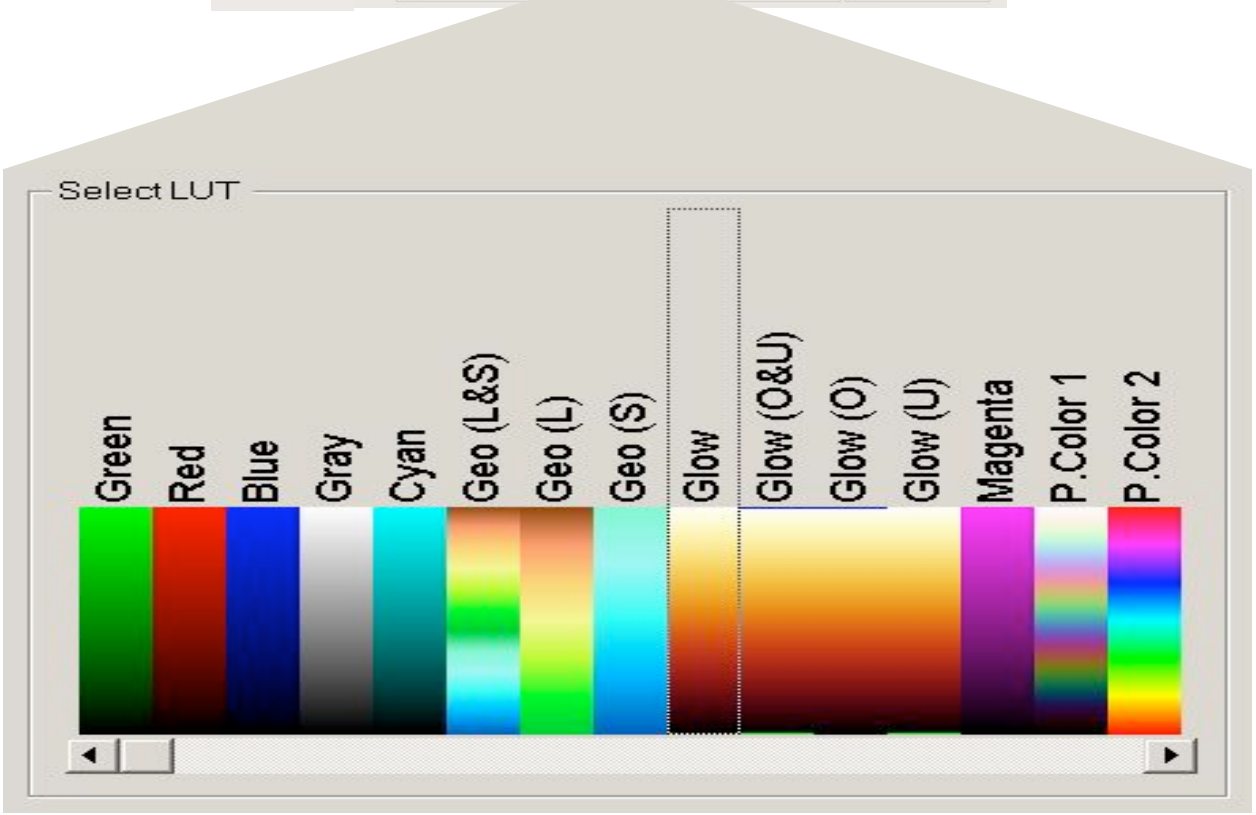


Detected intensity values are displayed as gray levels. The display range of a typical 8-bit monitor covers 256 gray levels. The full range of the LUT is utilized if an image shows all shades of gray between black (=0) and white (=255). The gray levels might be presented in pseudo-colors.



Asigning Pseudocolors

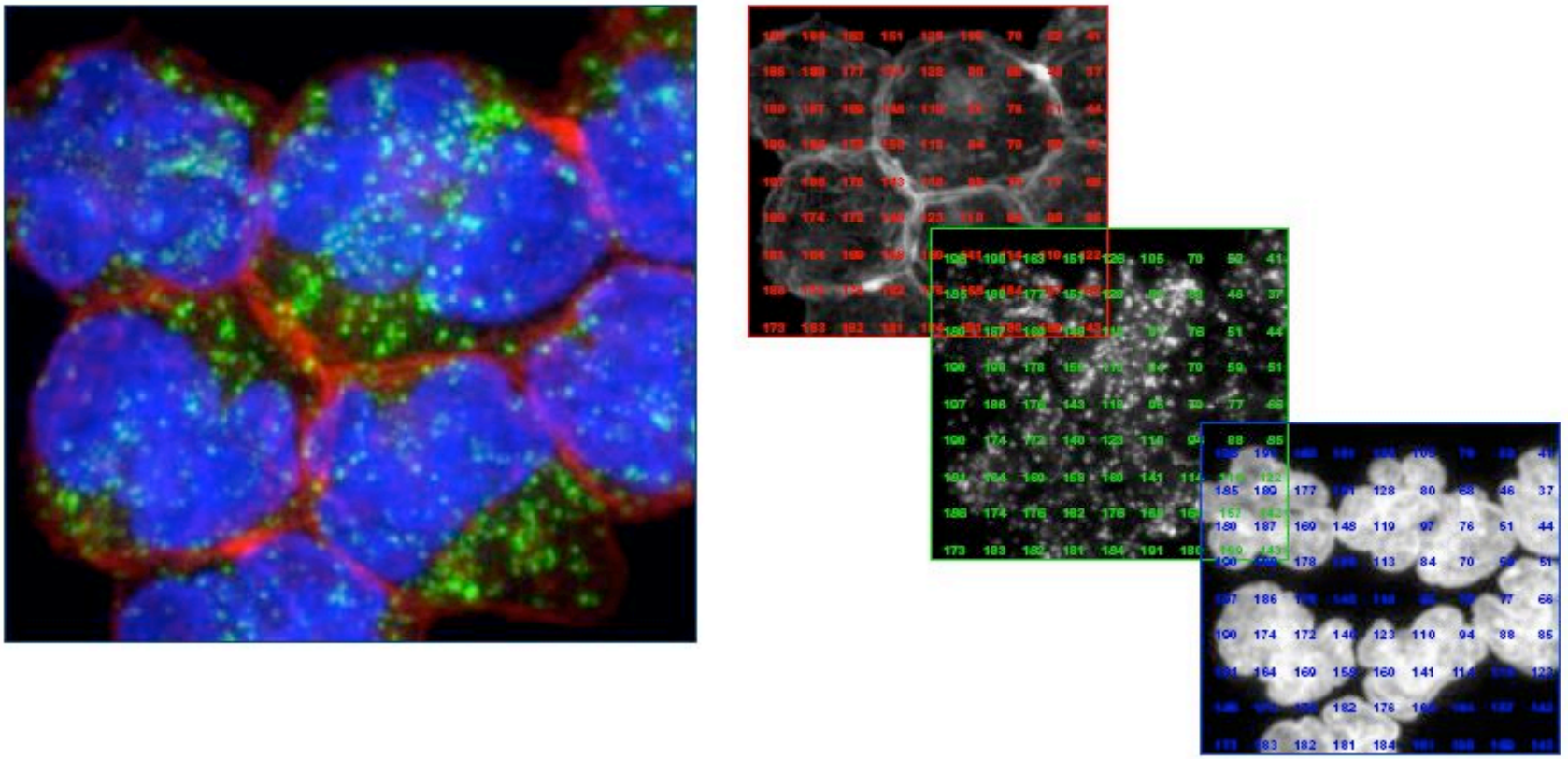




For multi-channel-aquisition it is helpful to asign indexed colors to the different gray-scale-images.

„Glow over-under“ facilitates the gain & offset-adjustments.

Electronic pseudocolor images



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

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

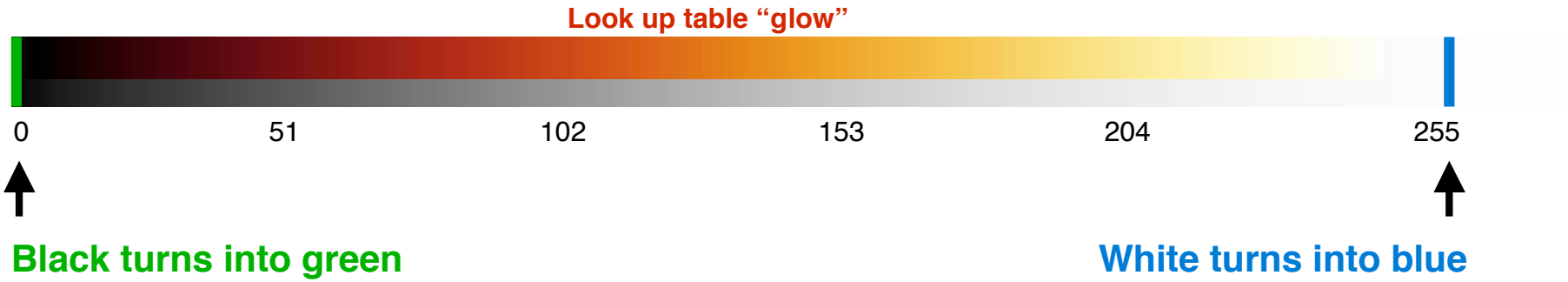
amplifies the input signal by multiplication, which results in a higher gray level value; bright features are brought closer to saturation, general image brightness is increased.

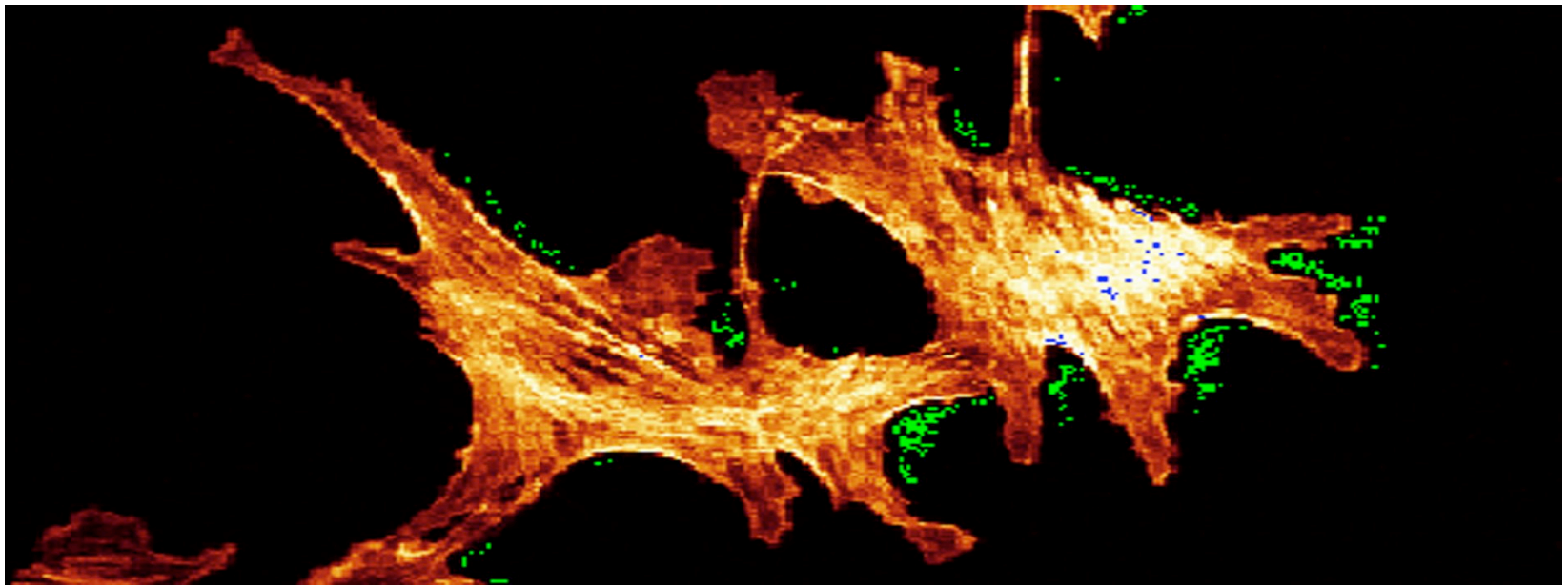
offset

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

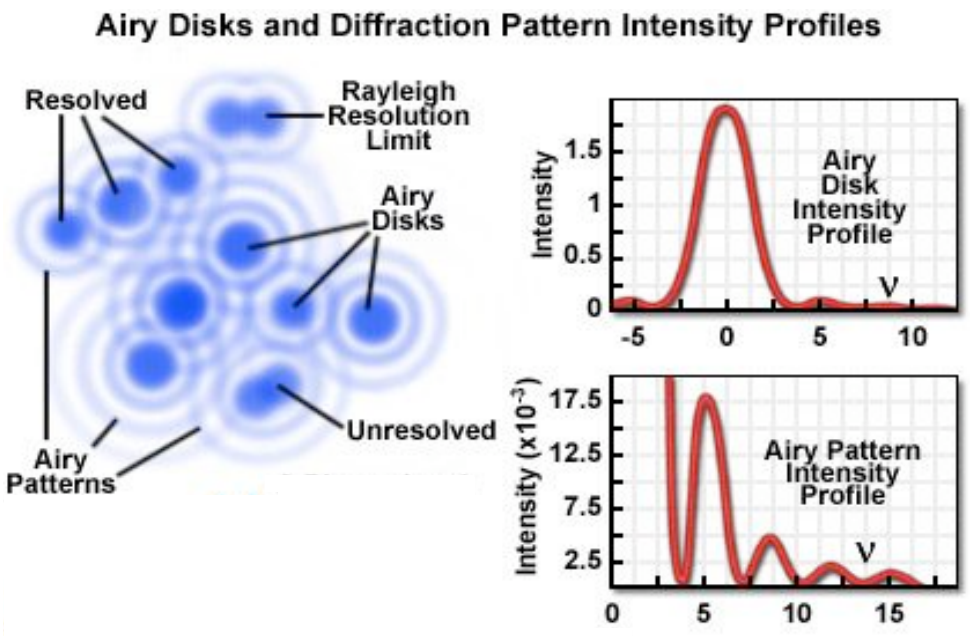
look-up table “glow over/under”

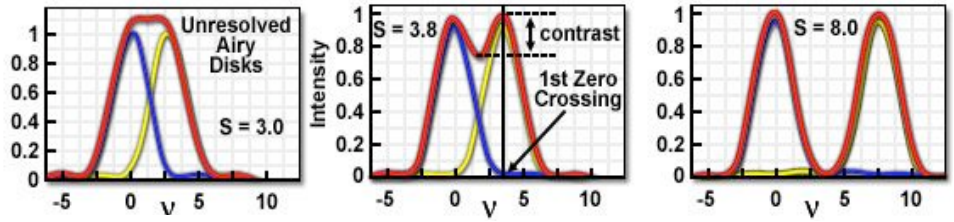
to determine underexposure and saturation of an image





Contrast and resolution





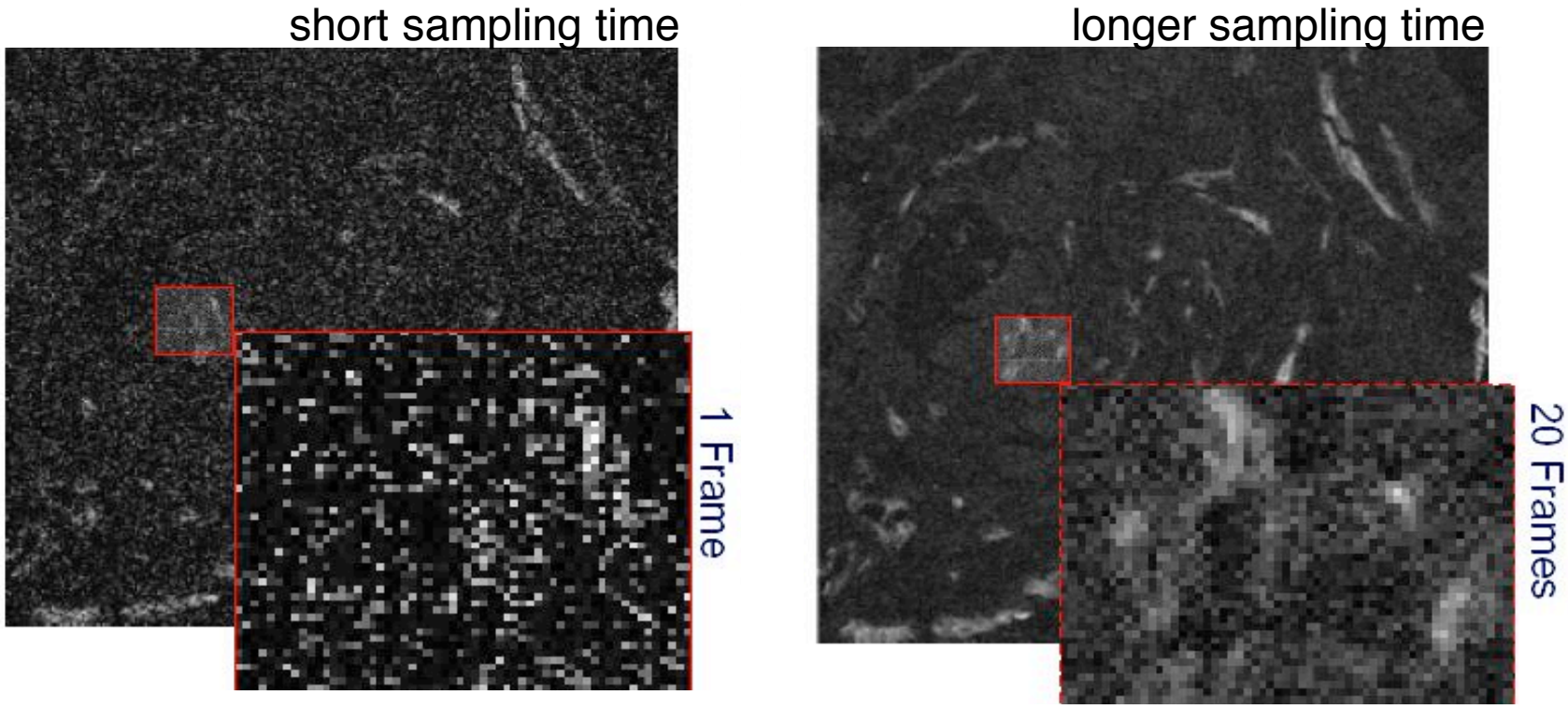
unresolved *Rayleigh limit* *resolved*

Resolution also depends on contrast!

Rayleigh criterion: The separation between two points requires a certain level of contrast between them. A 26.5% depression in brightness appearing between two maxima, is giving the sensation of twoness.

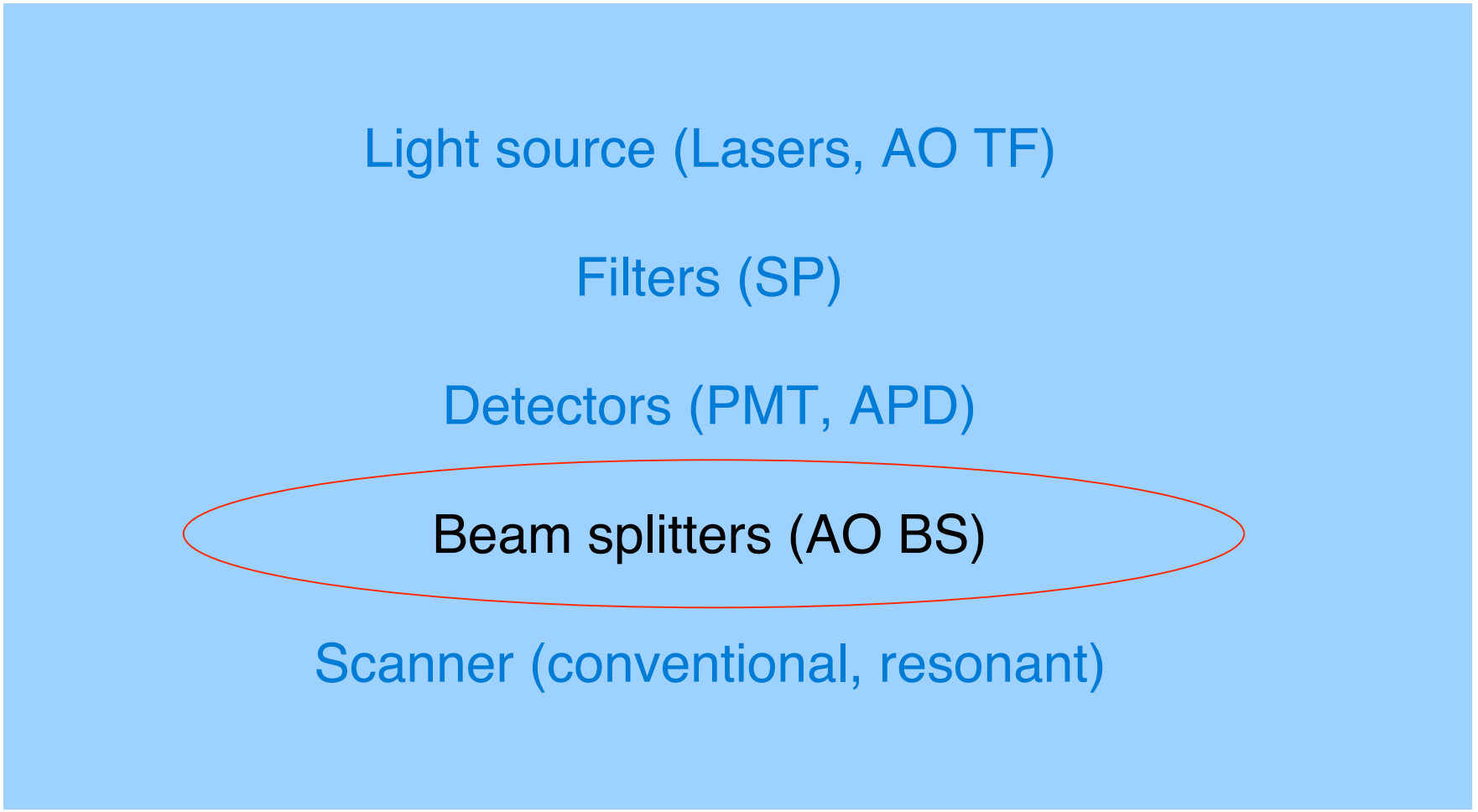
-> Adjustment of gain & offset can improve resolution!!!

signal-to-noise ratio & averaging



Several images (frames) get accumulated and averaged.
Averaging allows to reduce noise -> signal apears clearer.

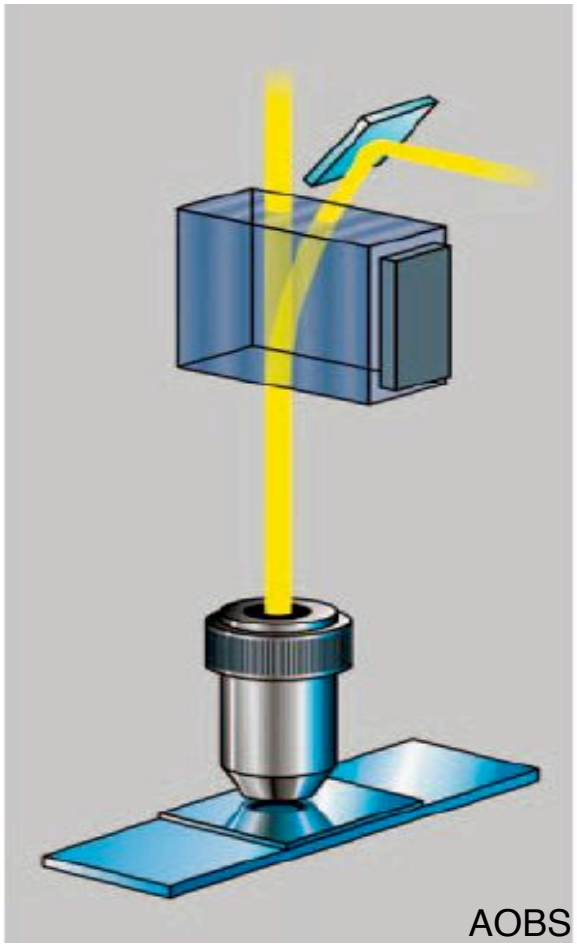
Leica confocal laser scanning microscope



AOBS **A**cousto-**O**ptical **B**eam **S**plitter

in comparison to the filter-mirror beam splitter

- Electronically tuneable
- Fixed device (no mecanical movements)
- Fast switching time
- Up to 8 Illumination lines possible simultaneously

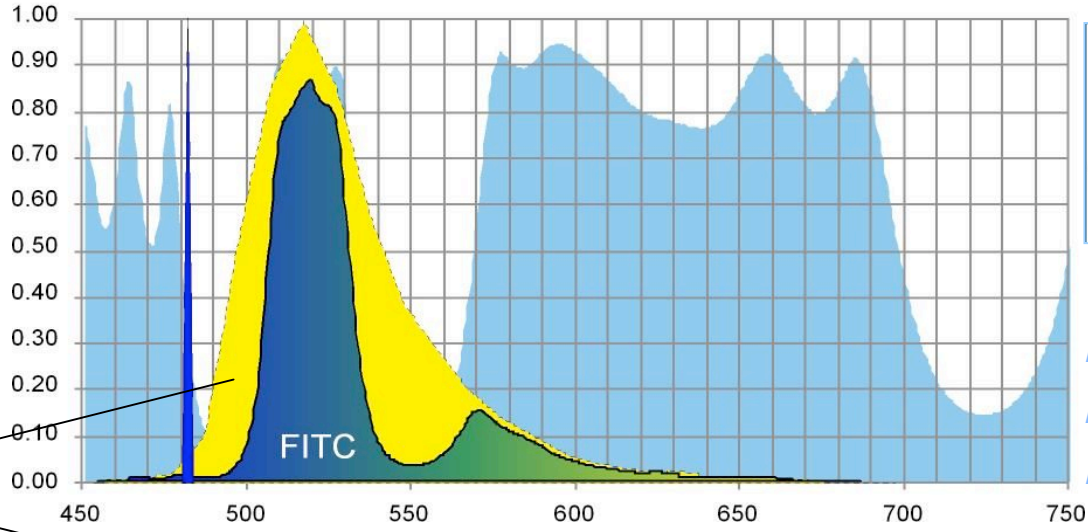


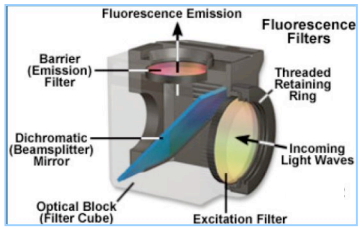
AOBS

Conventional beam splitter

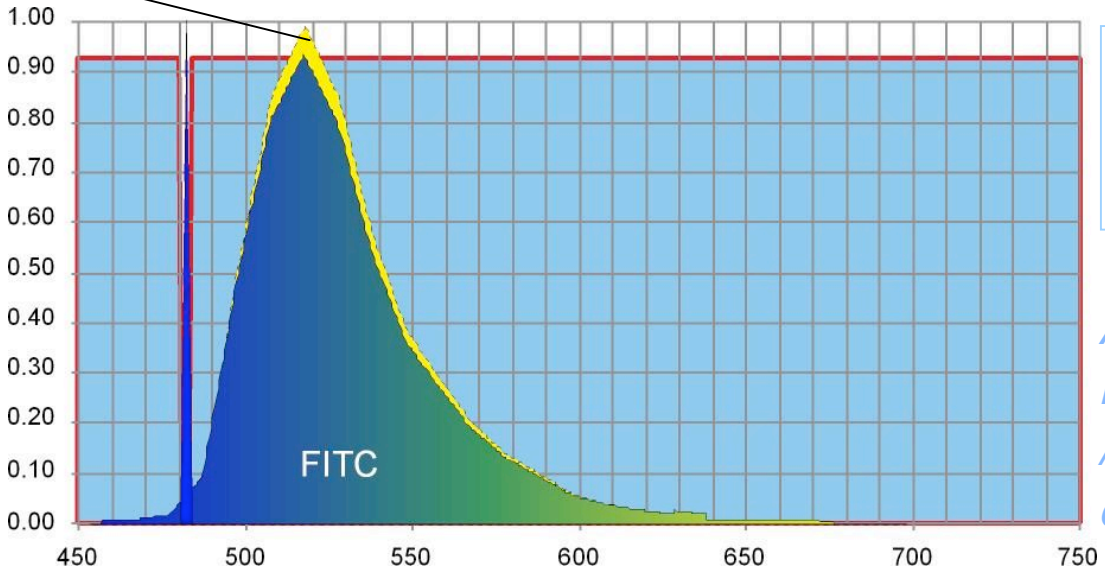
i.e. FITC - Beam splitter versus AOBS

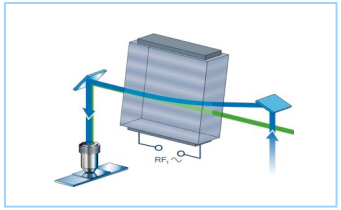
„cut off“ (lost emission signal)





Dicroic Beam splitter DD 488/543

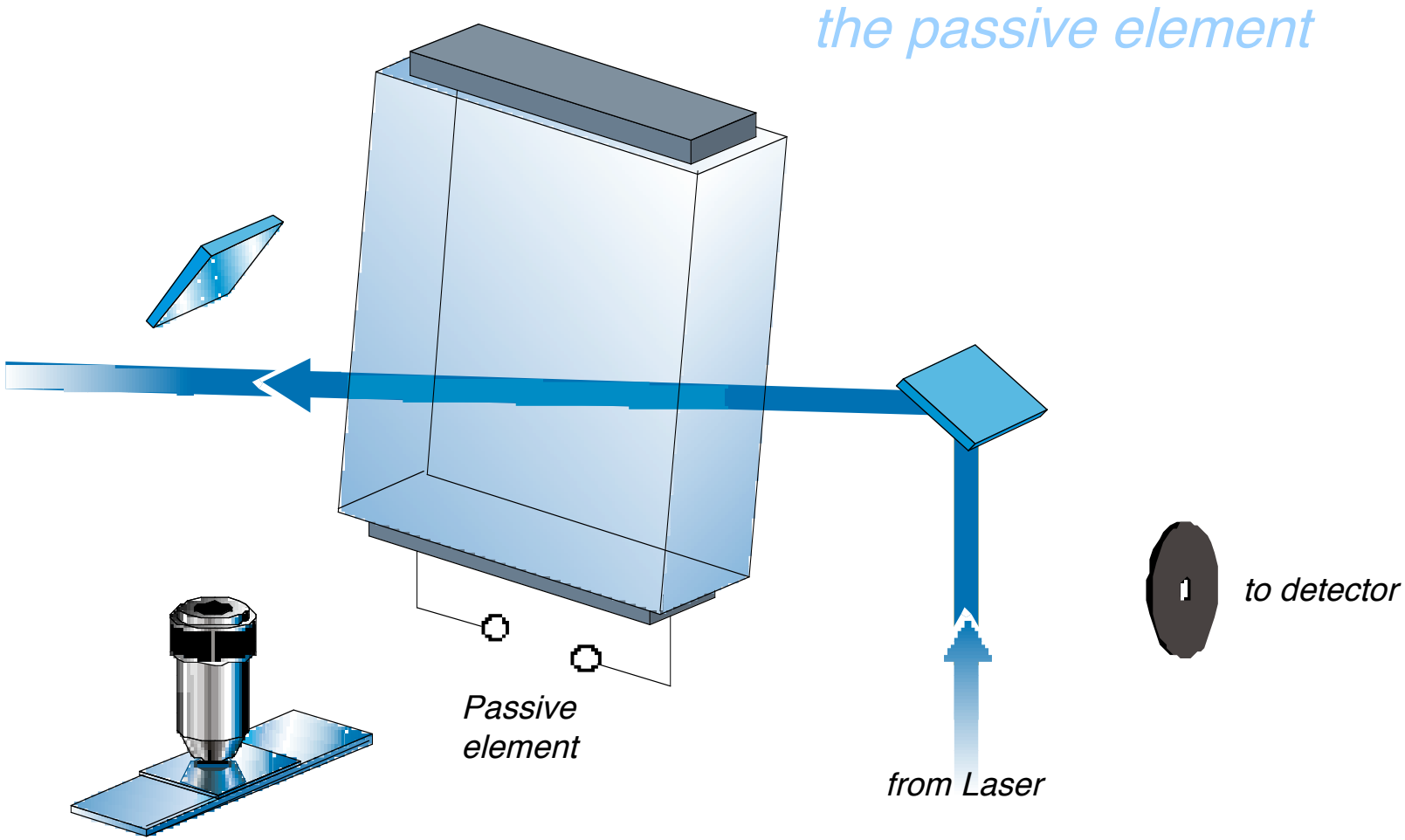




Acusto-Optical Beam splitter /flexible characteristics)

AOBS: Operation (1)

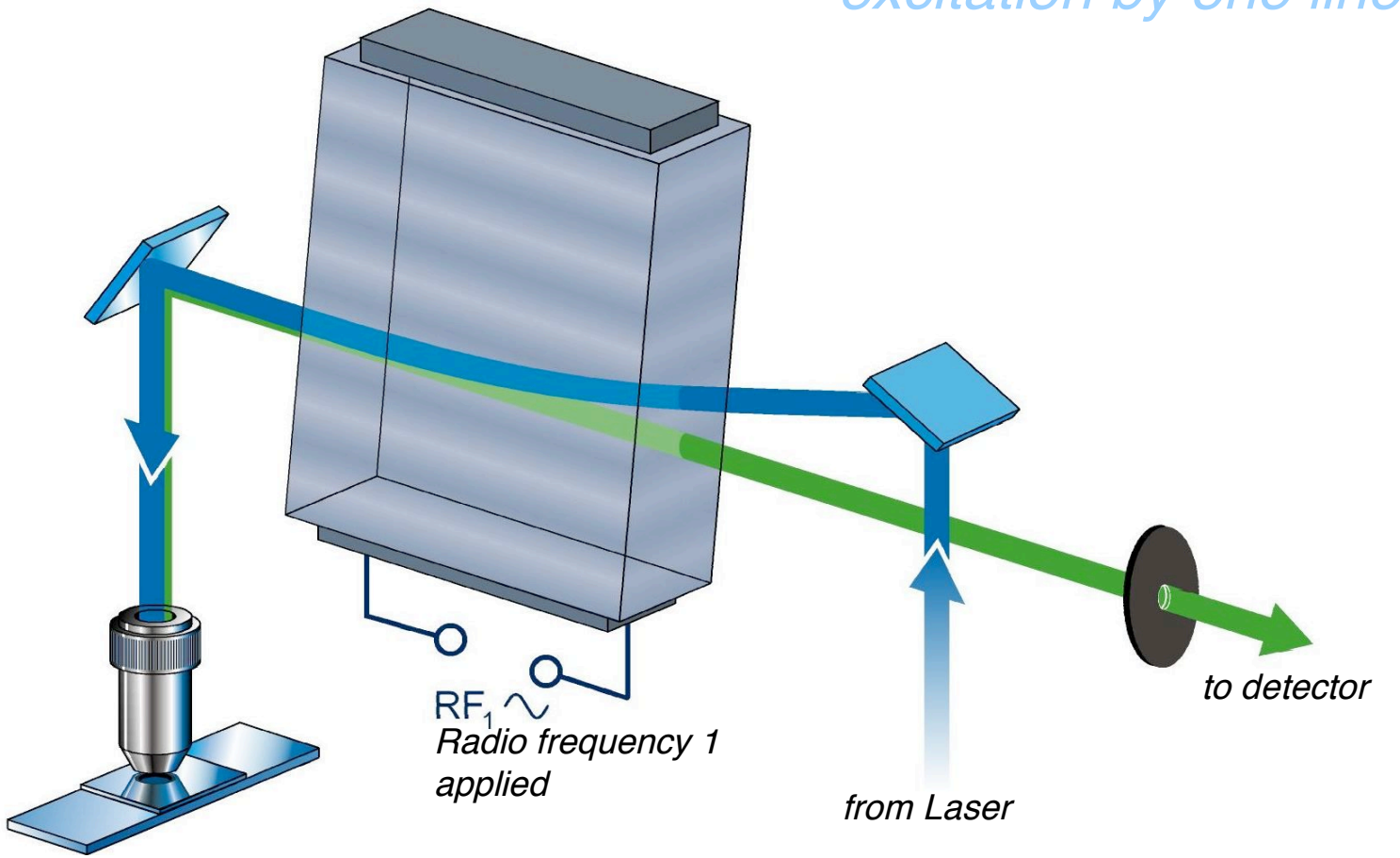
Acousto-**O**ptical **B**eam **S**plitter



AOBS: Operation (2)

Acousto-**O**ptical **B**eam **S**plitter

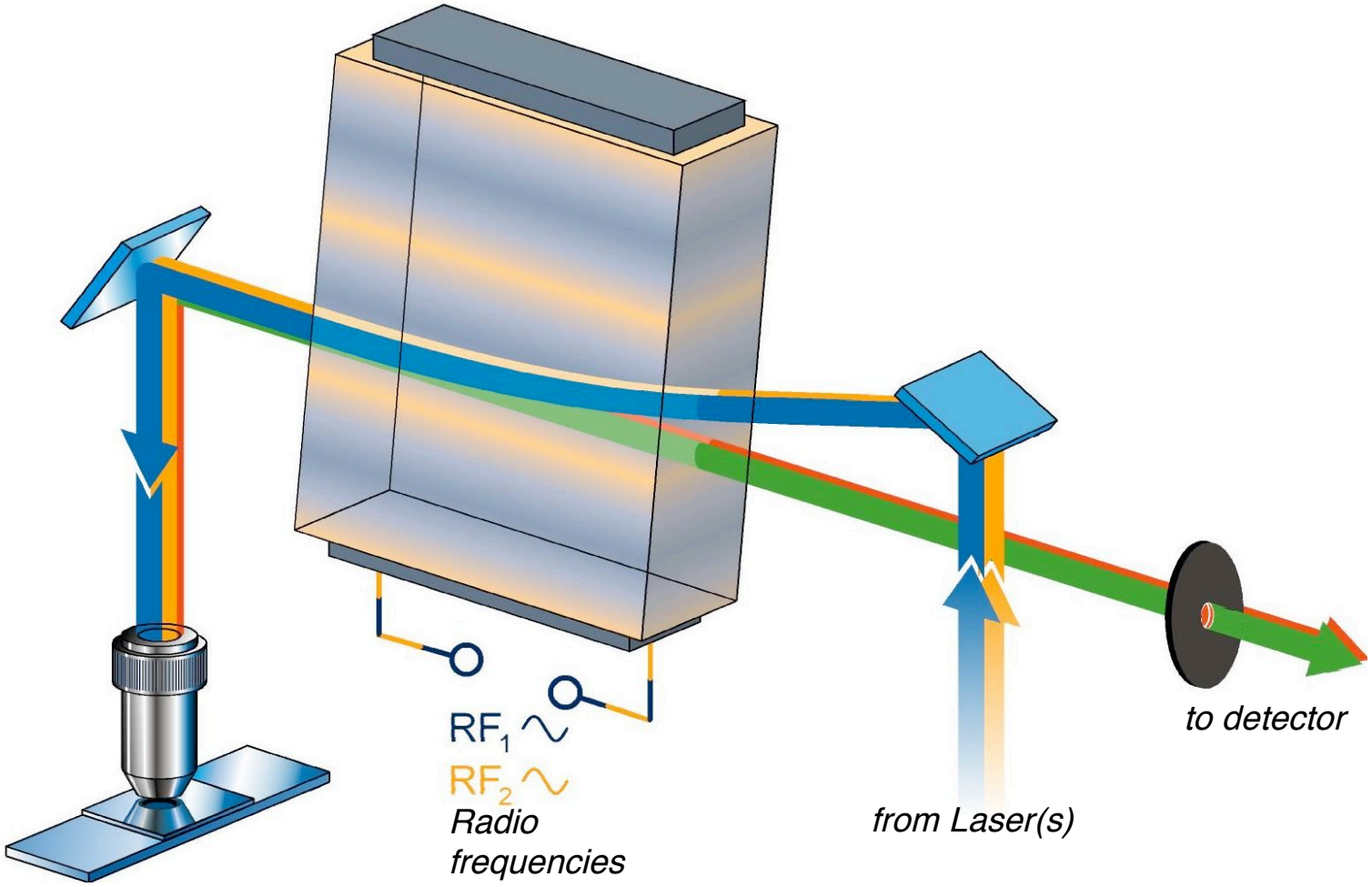
excitation by one line



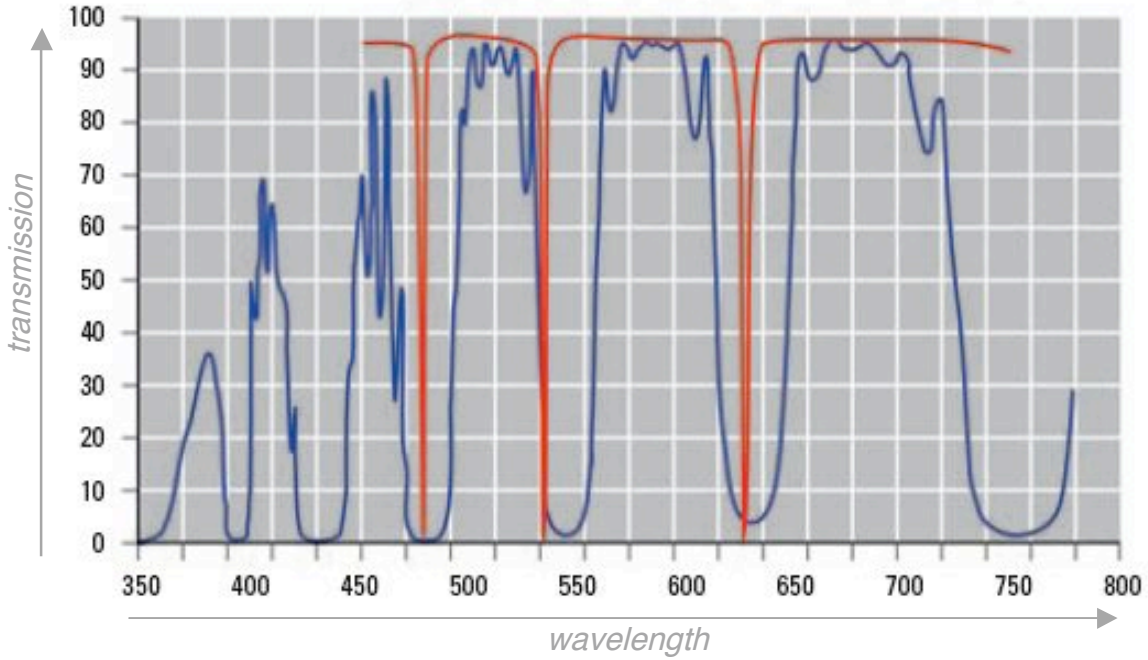
AOBS: Operation (3)

Acousto-**O**ptical **B**eam **S**plitter

two excitation lines:
….and so on…up to 8 lines!



Beam splitter transmission



Transmission curves

Blue: triple dichroic, blue, green, red

Red: AOBS® tuned to 488, 543,594, 633 nm

Higher transmission, wider bands and steeper slopes with AOBS®

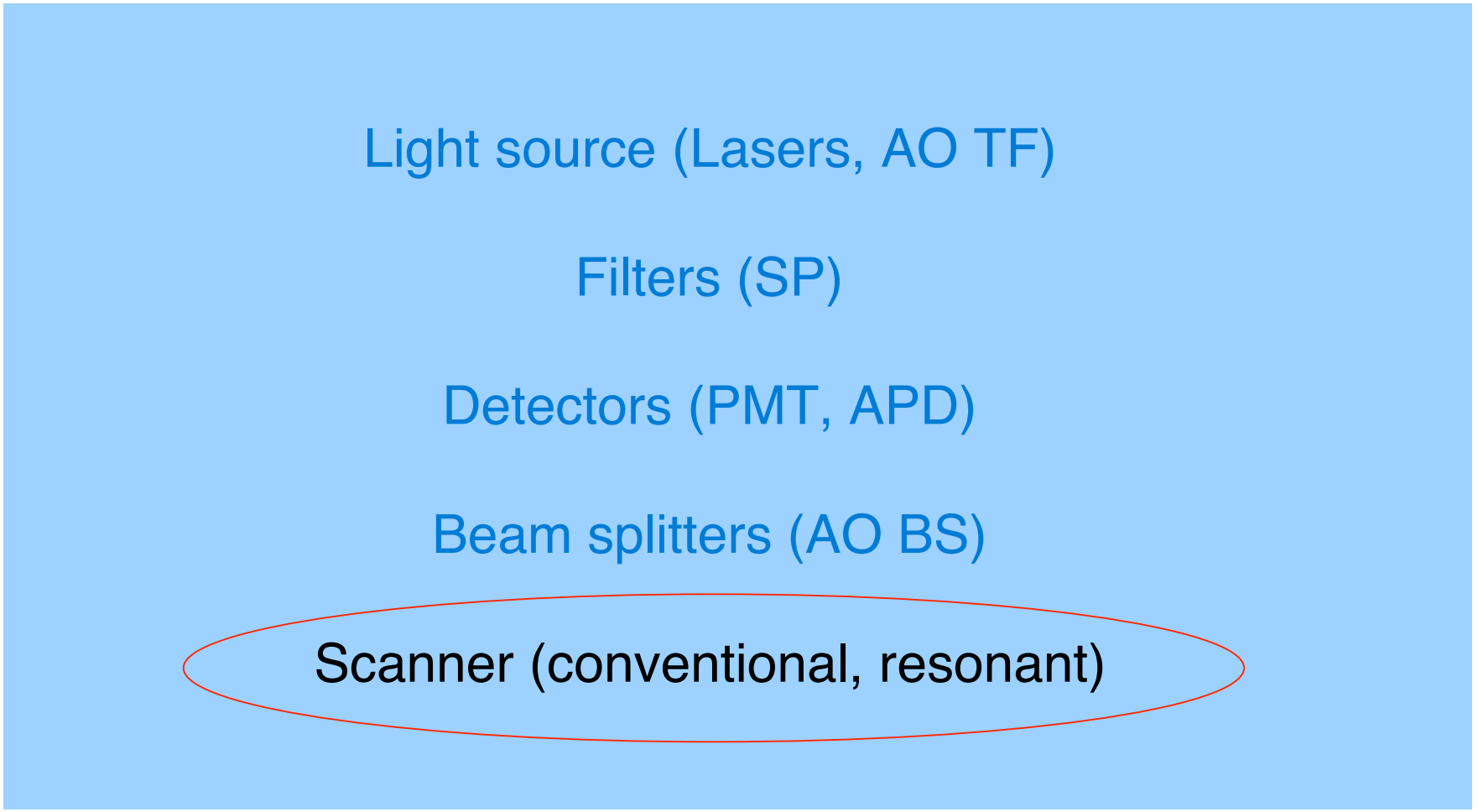
Conventional dichroic beam splitter:

- No sharp bands
- Transmission holes
- Fixed characteristics
- Non-linear transmission-distorted spectra

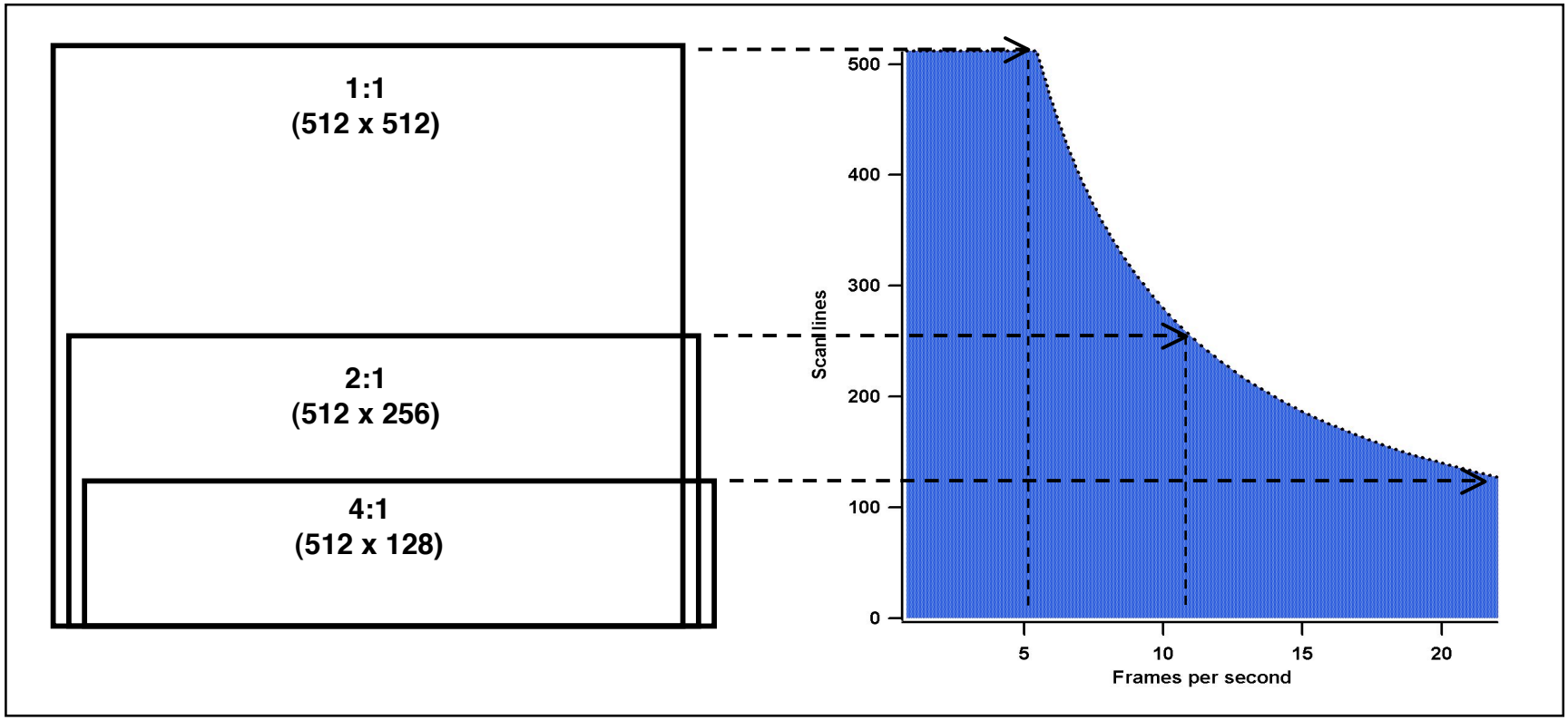
Acusto-Optical beam splitter:

- Perfect selectivity (0,6-2 nm bandwidth)
- More transparent
- More “room” to detect fluorescence
- Linear transmission, correct spectra

Leica confocal laser scanning microscope

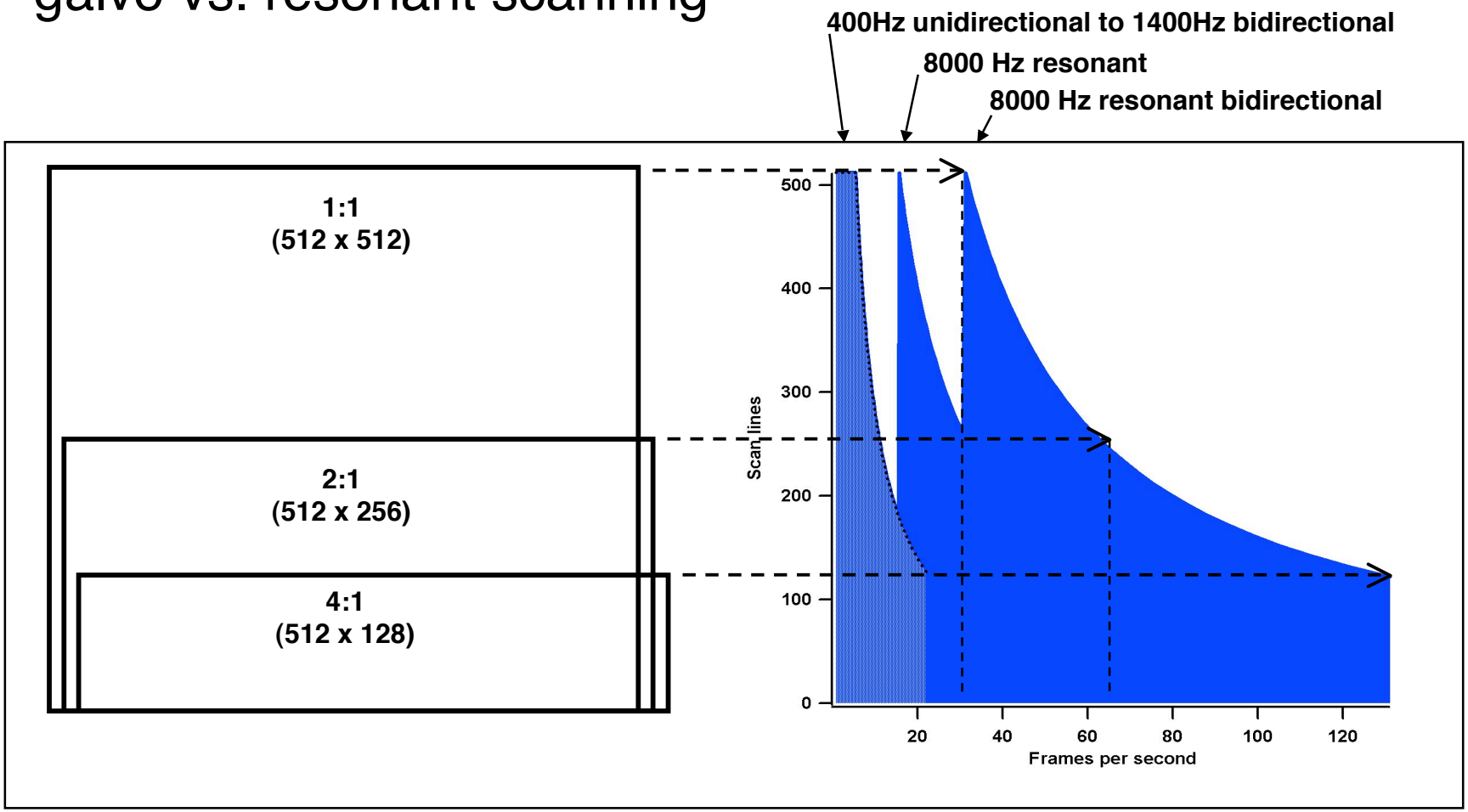


Detection timescales - image formats



Detection timescales

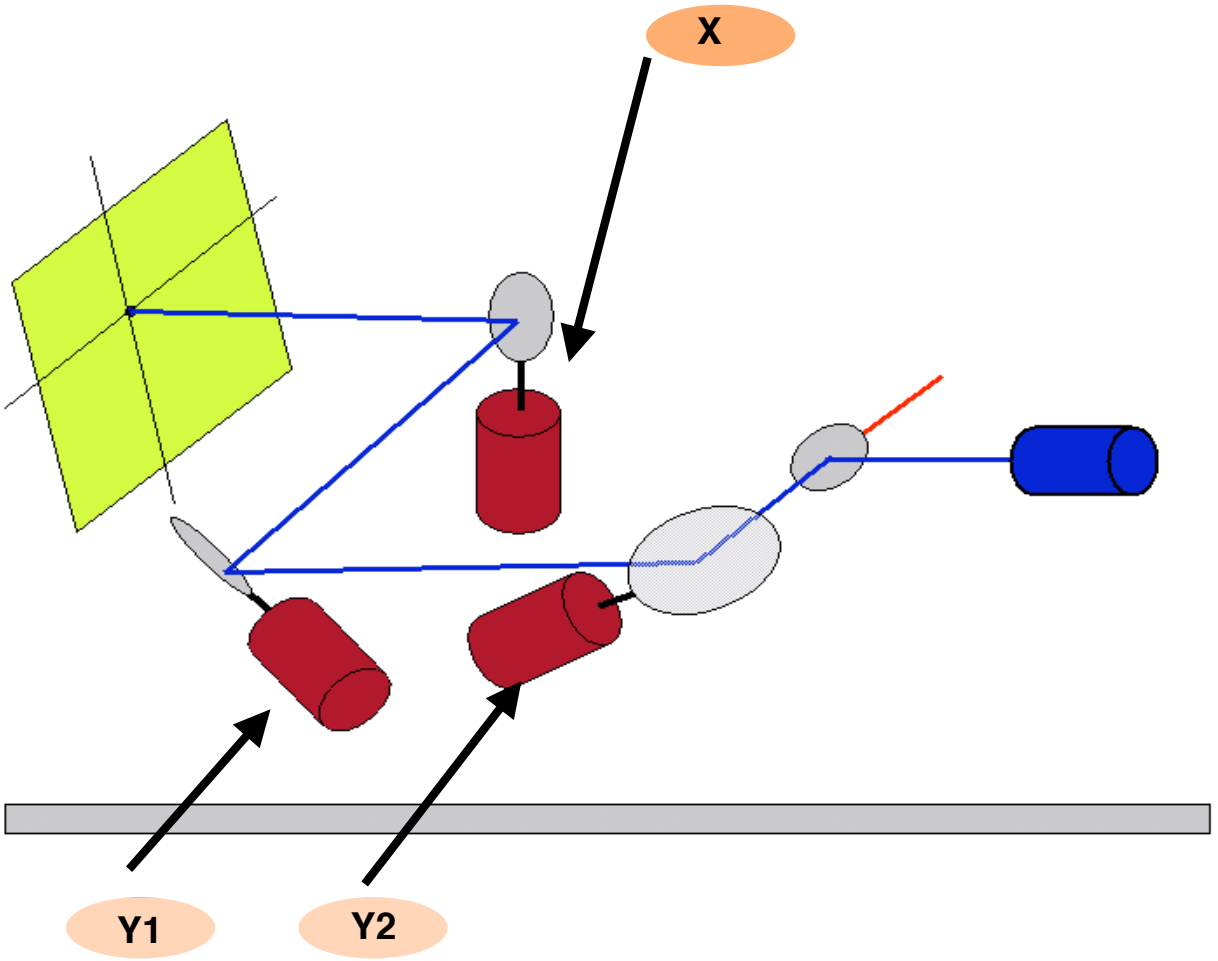
* uni- vs. bi- directional scanning
* galvo vs. resonant scanning



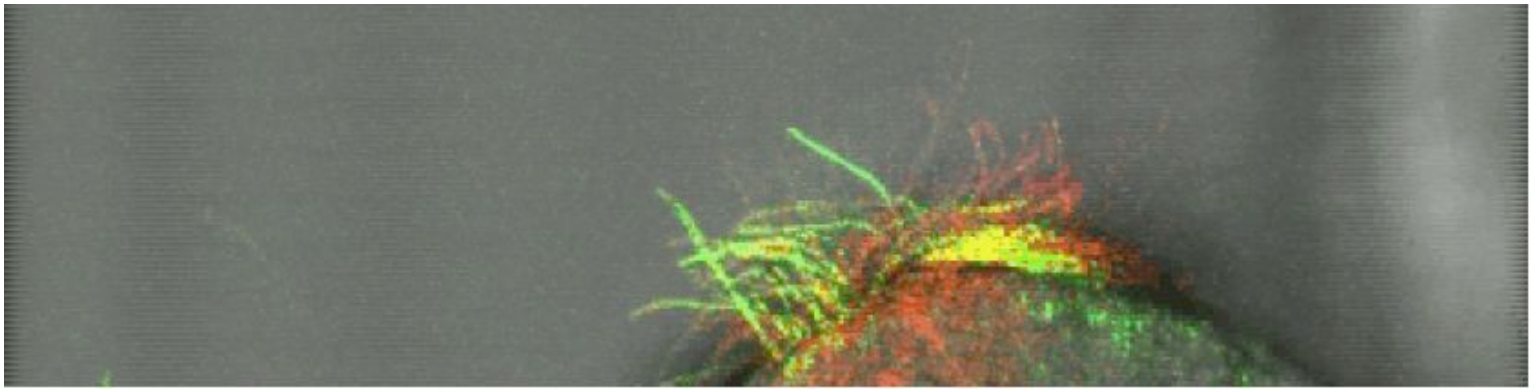
The resonant scanner:

increases speed and sensitivity

high scan speed
by coupling the
x-scanner with
two y-scanners
(x-2y scanner set)

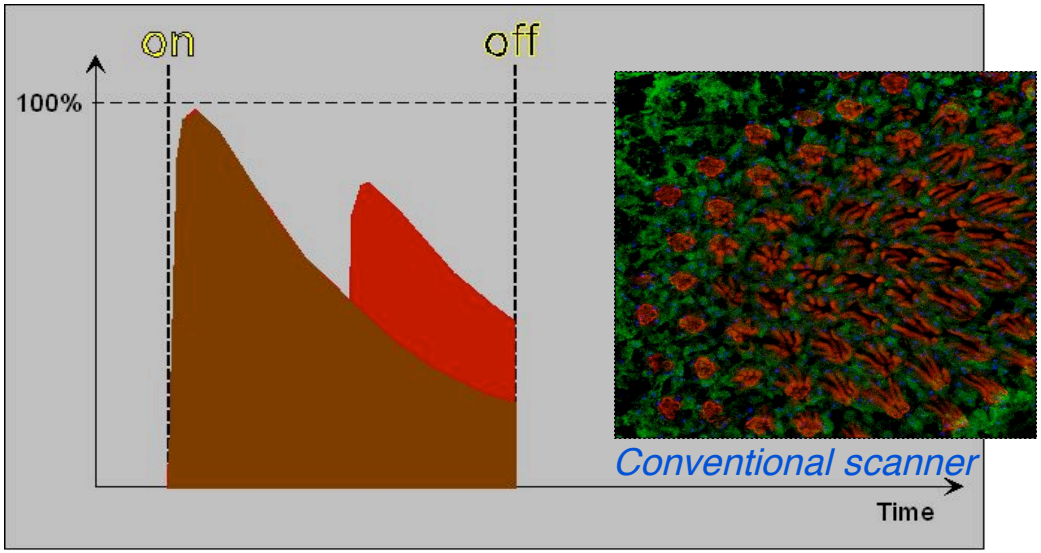


Resonant scanner (Leica TCS SP5)



- Dynamic live cell imaging and kinetic measurements
- Brighter images
- Less photobleaching
- Work with a frequency of 8000 Hz instead of the conventional 400 Hz

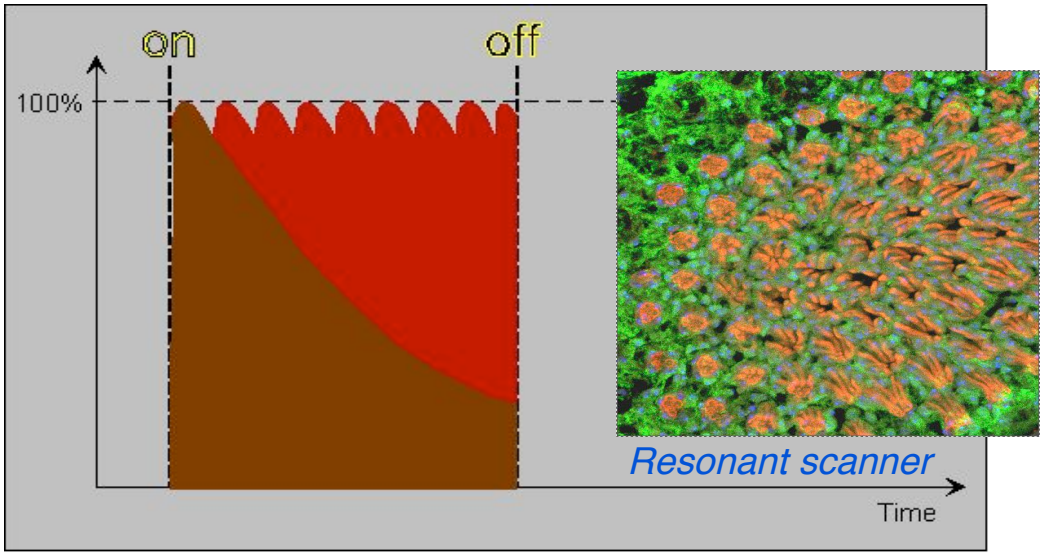
Resonant scanner delivers brighter images



Conventional scanner

Conventional scanner: from a certain location we gain an amount of fluorescence

When running the scanner at double speed (and line-accumulation resp. averaging), we gain more signal.



Resonant scanner

If illumination is short enough, we get much better signal-to-noise ratio in identical acquisition times.
Note: total illumination time stays constant.

=> Repetitive short illumination results in brighter images

confocal software

Options:

Microscope control

Multi-spectral aquisition

3D-sampling

Spectrum collection

Dye finder

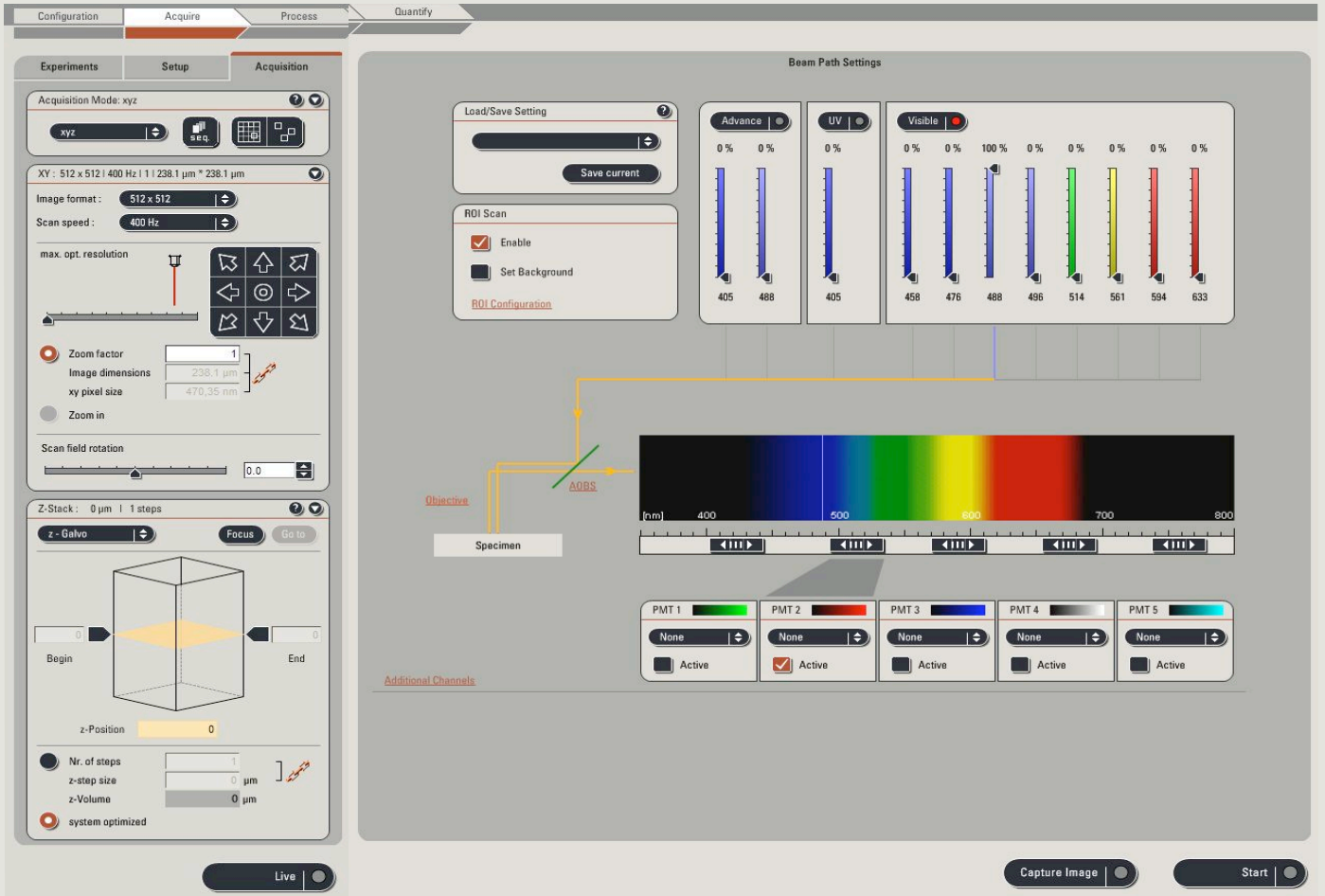
Quantification

Time laps

Image processing

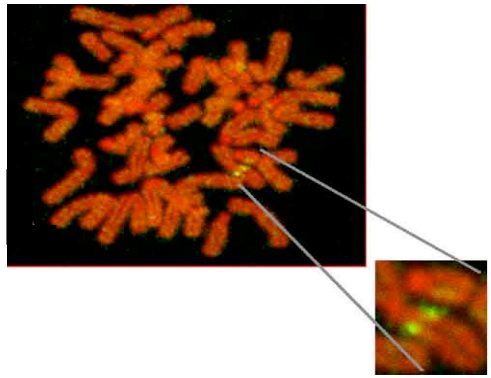
Multi-position imaging

Modules for FRET,FRAP, FLIM

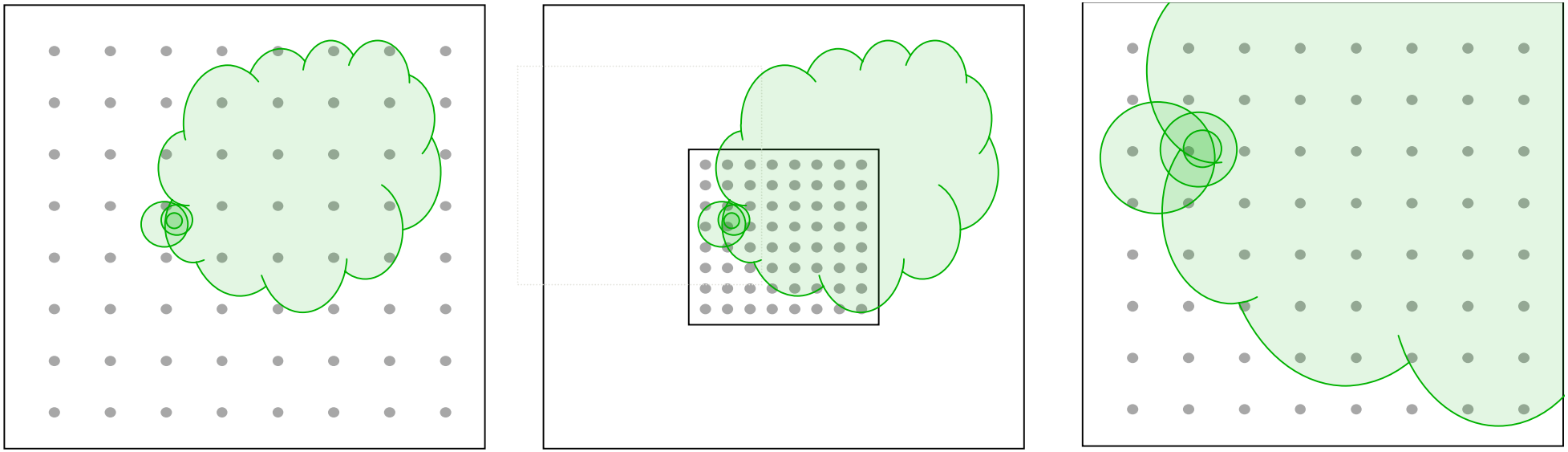


Zoom-function

allows flexible higher magnification



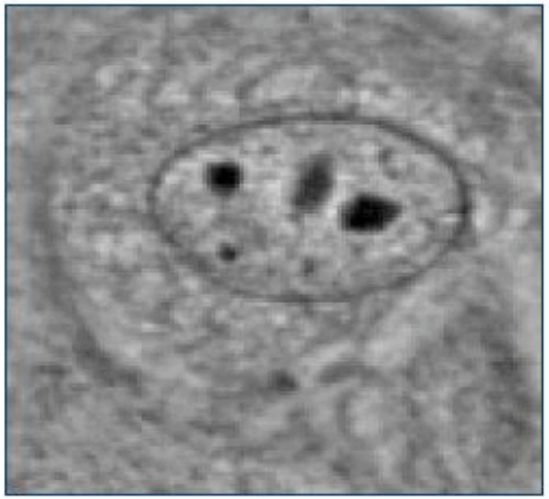
no zoom *zoom*



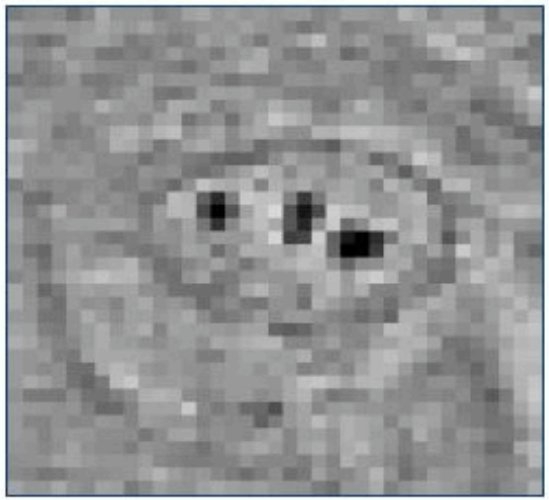
By zooming, a smaller area gets scaned with the same number of image points -> the field of view is reduced, the pixel resolution stays constant, details are shown magnified.

(The zoom up to 10-15x is real: more details get depicted. Additional zooming (20-30x) is „empty“: no informaition gain, the same detais are shown bigger by blown-up pixels.)

pixel resolution



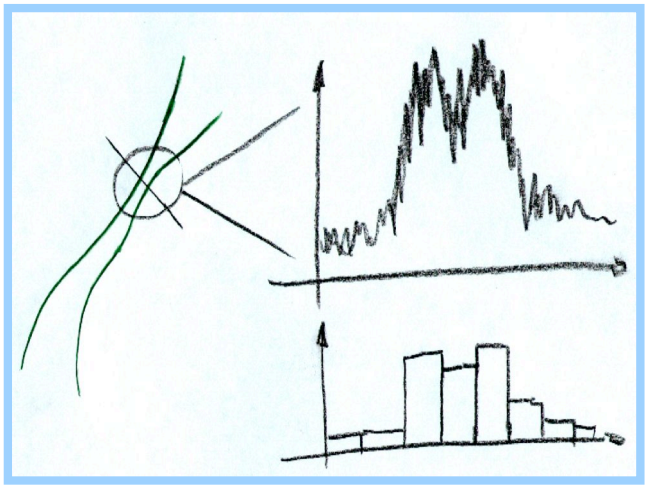
Aquired with 512 x 512 pixels



Aquired with128 x 128 pixels

How many pixels are needed to reproduce the object with the full resolution obtained by the microscope?
-> Nyquist criterion for digital resolution: smallest resolved structures should have 2,3 pixels!!

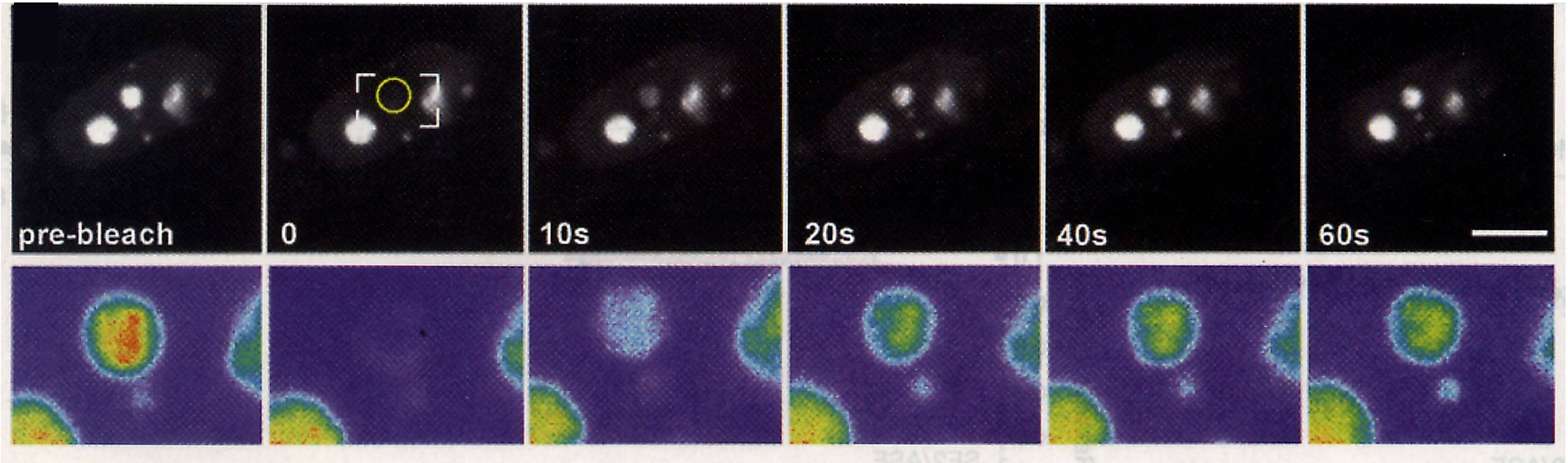
NA	λ	Resolution (nm)	Pixel size with Nyquist criterion (nm)
1.4	650.0	186	81
1.4	546.0	156	68
1.4	440.0	126	55
1.0	650.0	260	113
1.0	546.0	218	95
1.0	440.0	176	77
0.5	650.0	520	226
0.5	546.0	437	190
0.5	440.0	352	153



Resolution ($d_{x,y} \approx 0.4 \frac{\lambda}{NA}$)

Scanning options:

beam parking, regions of interest (ROI),...



Regions of interest (ROI):

- some regions in the field of view might be illuminated differently than the surrounding area
- The regions might have any shape or position

Beam parking allows:

- Spot bleaching
- Spot measurements

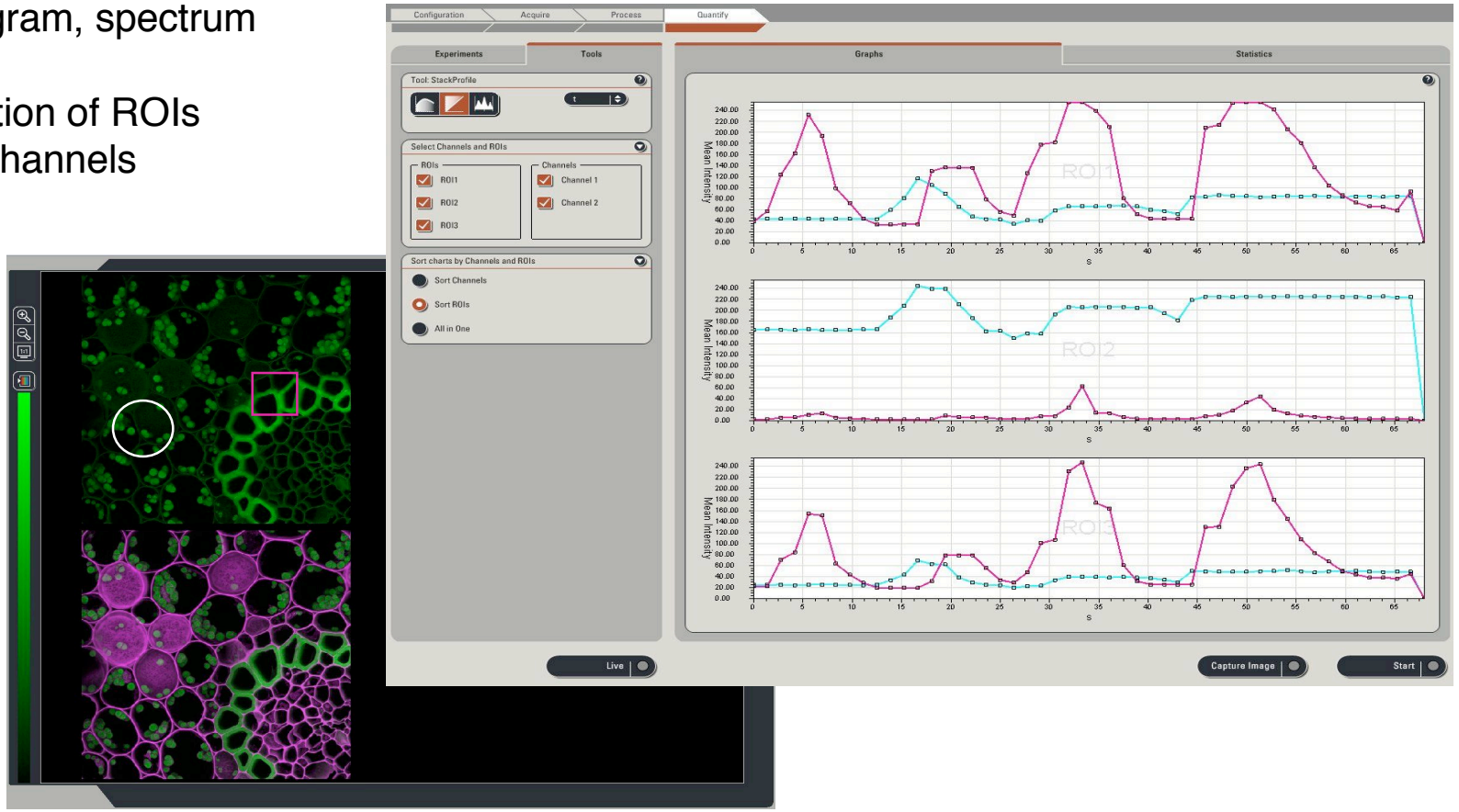
-> FRAP & FLIP -Experiments

fluorescence recovery after photopleaching

fluorescence loss in photobleaching

Quantifications

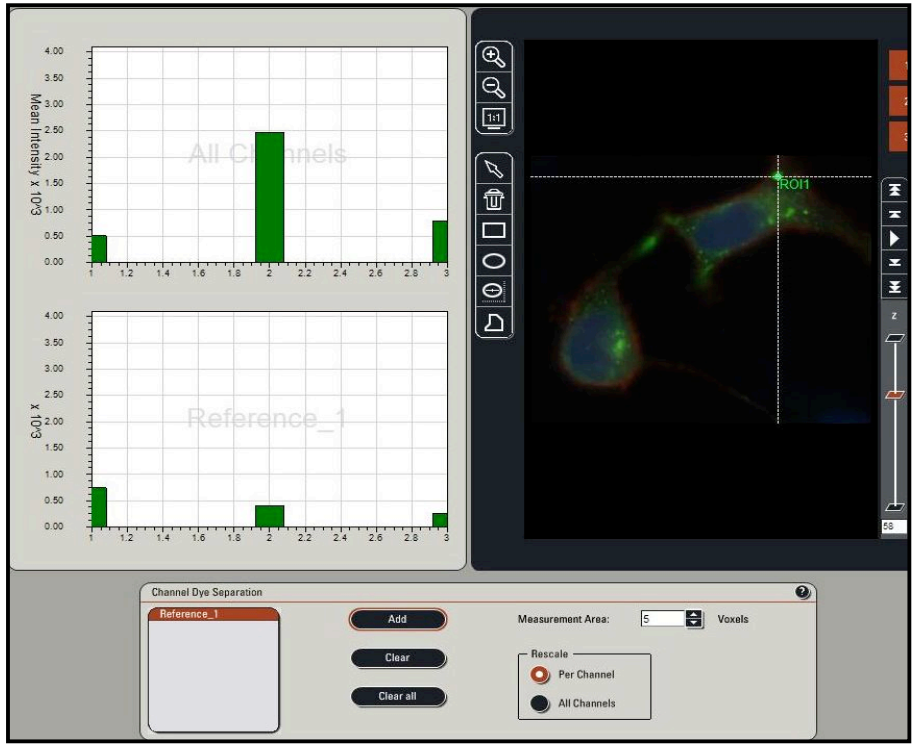
- Intensity measurements
- Histogram, spectrum
- Selection of ROIs and Channels



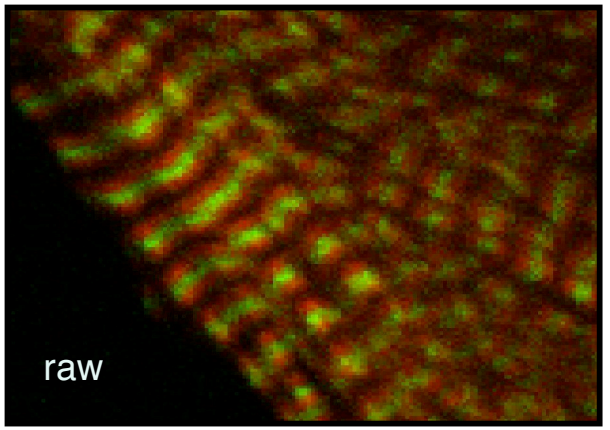
Processed DyeSeparation

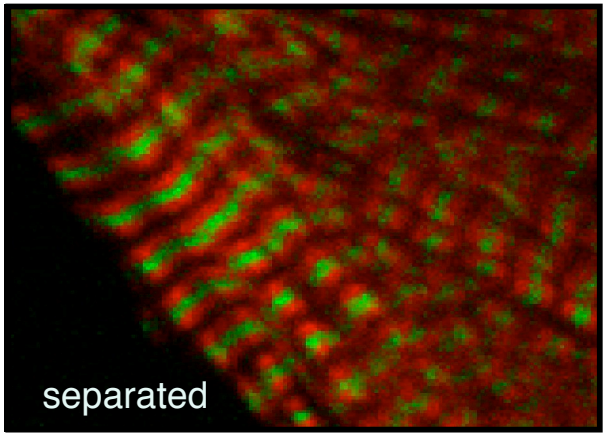
DyeFinder-tool:

- Fast elimination of crosstalk
- Use of References
- Suppression of autofluorescence



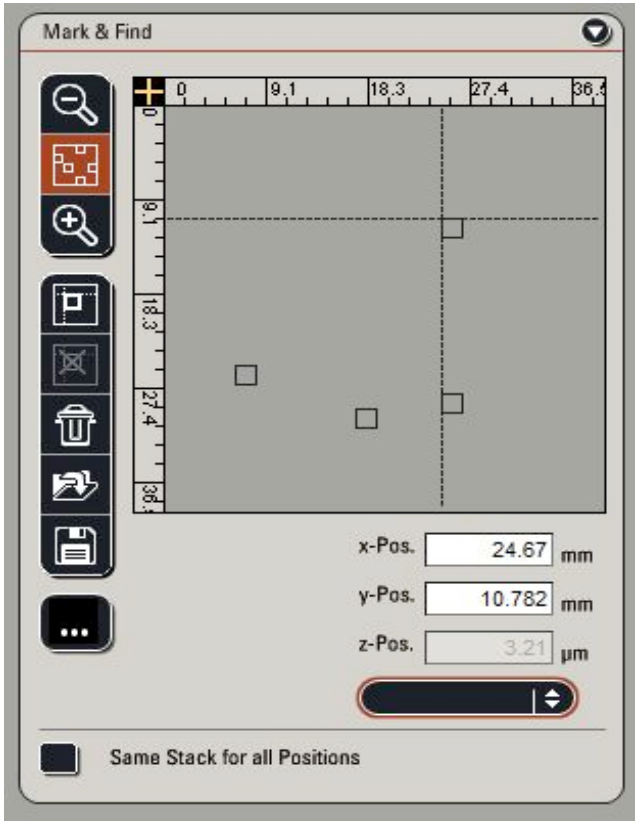
Not wanted: Crosstalk





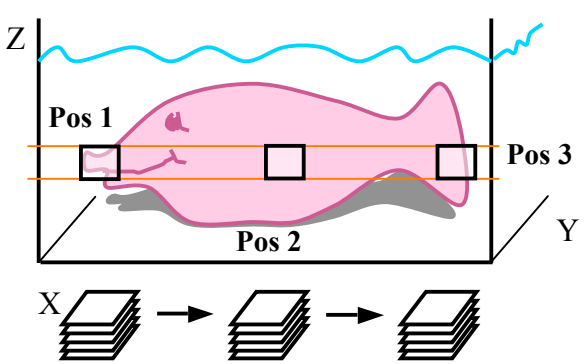
Wanted: Perfect Dye Separation

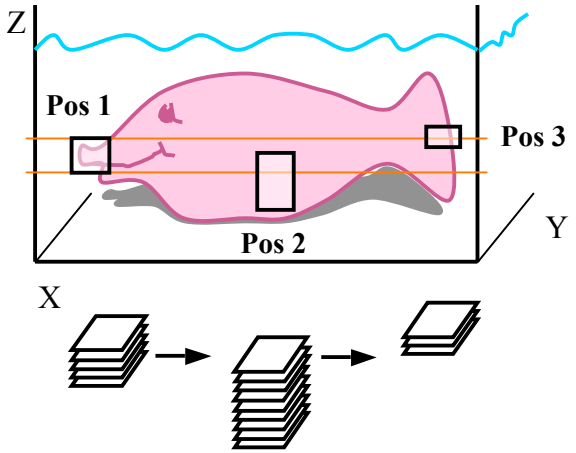
Multi position sampling



Integration of motorized xy-stage allows mark&find functions:

- Multi Positioning
- Location specific stacks
- Combination with time lapse

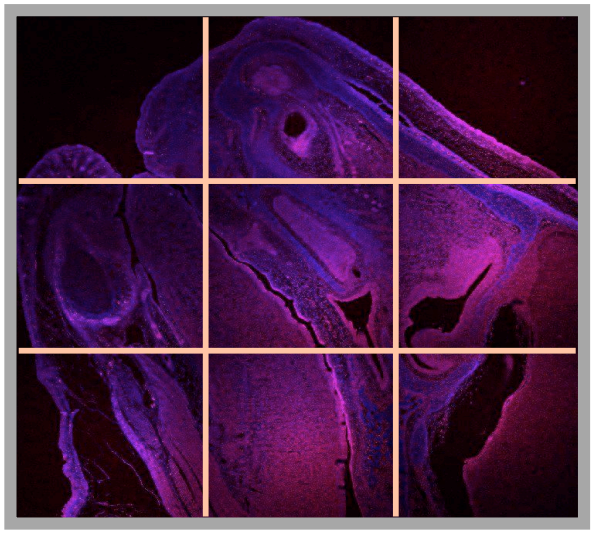


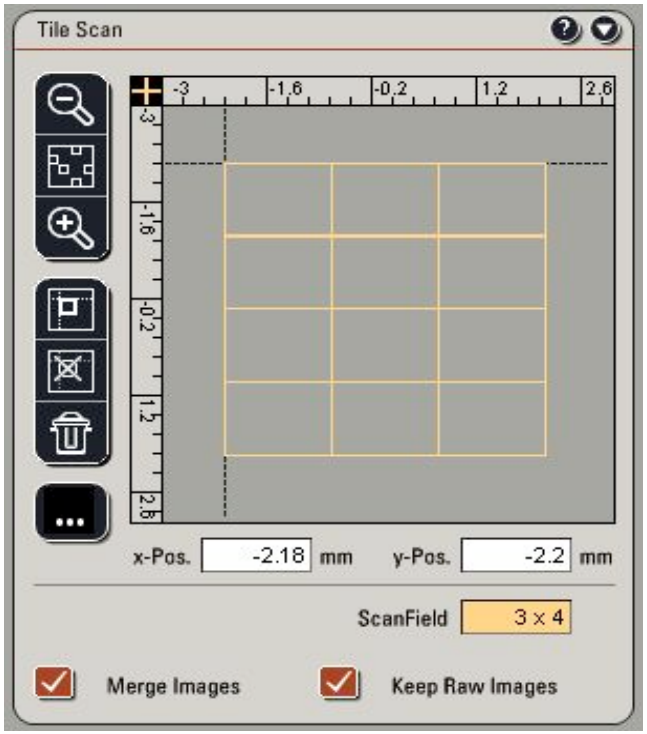


Tile Scan

High Resolution Overview

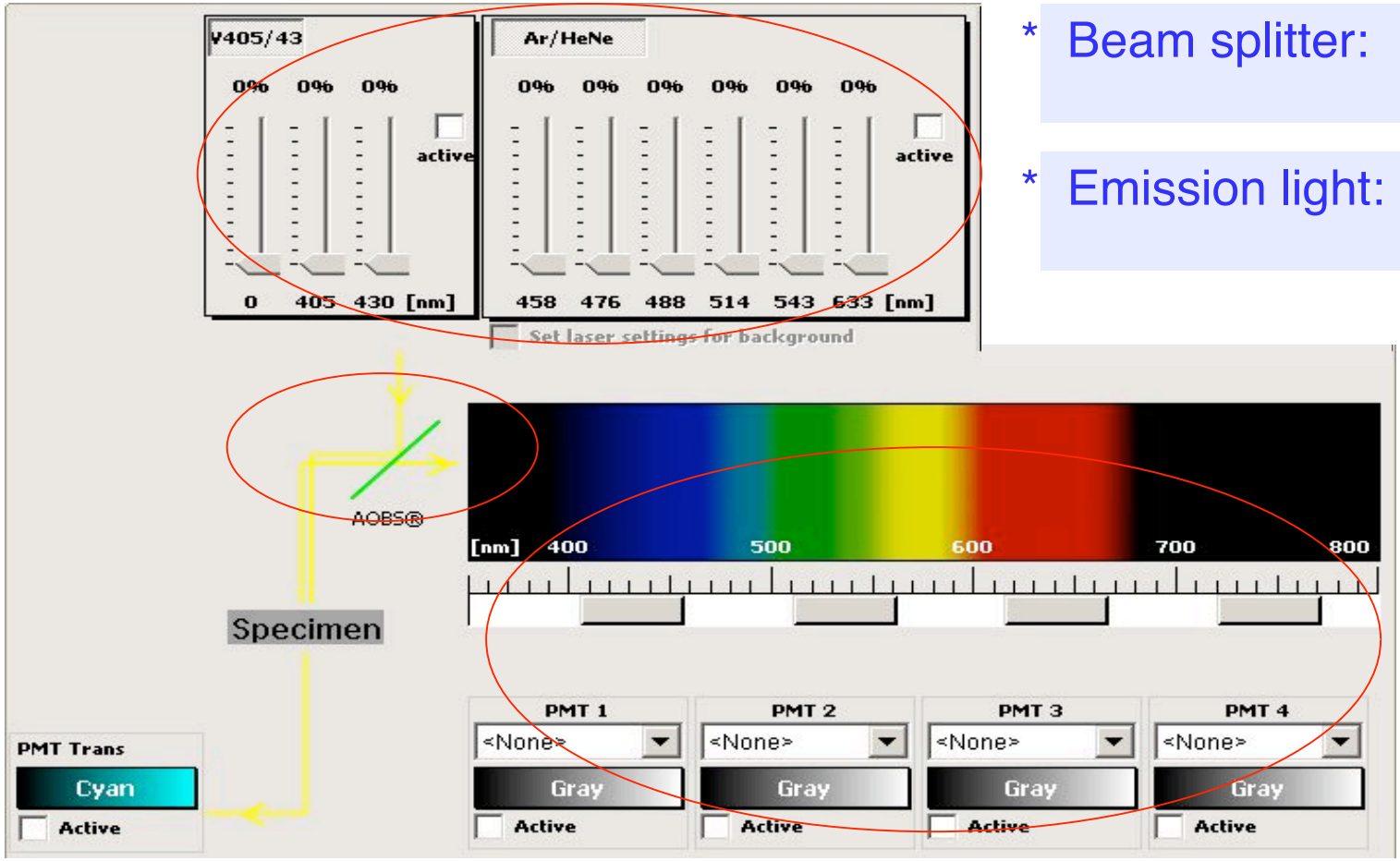
Integration of Motorized xy-stage allows stitching of neighbouring data sets -> hight magnification in a larger field of view





Beam Path Settings

* Excitation light: fixed LASER lines
* Beam splitter: auto-adjustment
* Emission light: free choice of detection windows



Visualizing fluorescent samples in Leica CLSM

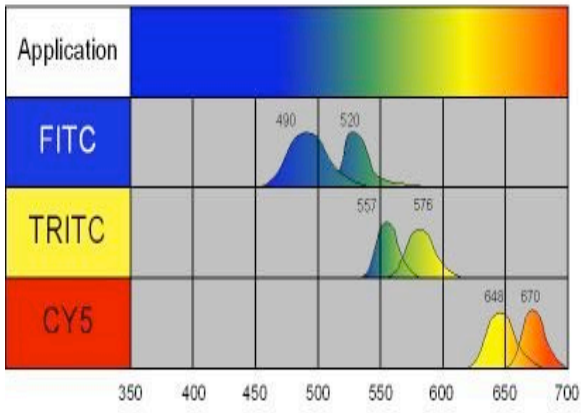
- You have to choose one of the given LASER lines accordingly to the excitation properties of your dye.

- You are totally free to choose your CLSM detection window.

  In order to do so, you must know about the emission properties of your dye. The detection window should not hit an active LASER line.

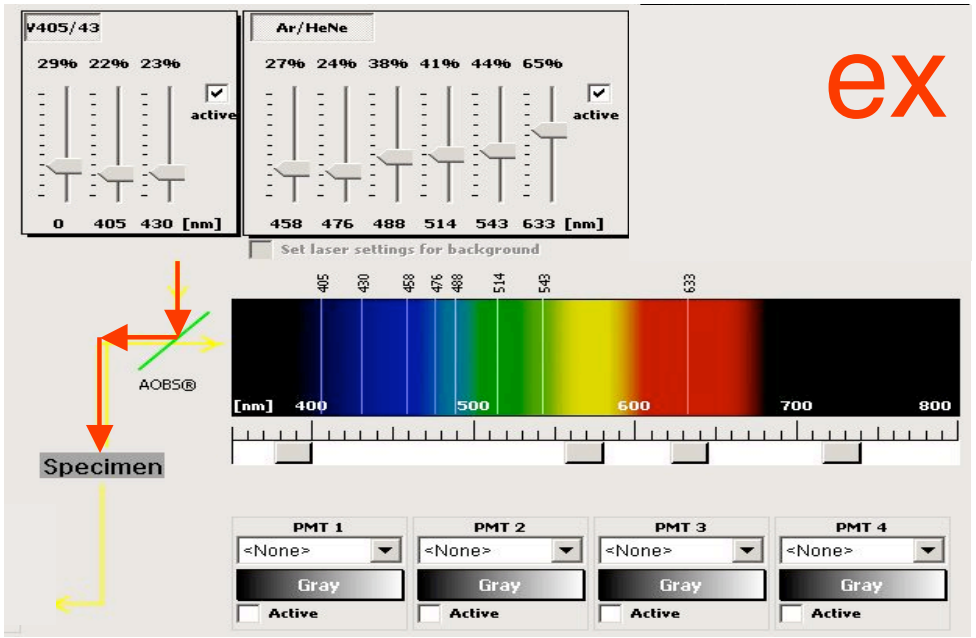
- If you don`t know anything about your fluorochrome, you have to check different laser lines for response and perform a λ–scan to determine the emission properties.

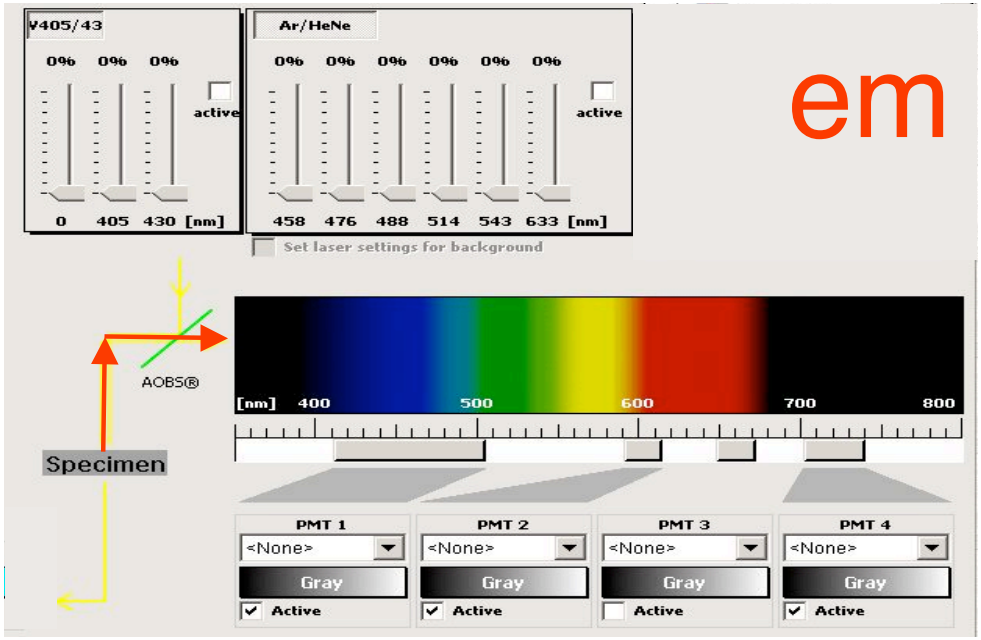
ex/em-properties of some common fluorochromes



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

Beam Path Settings

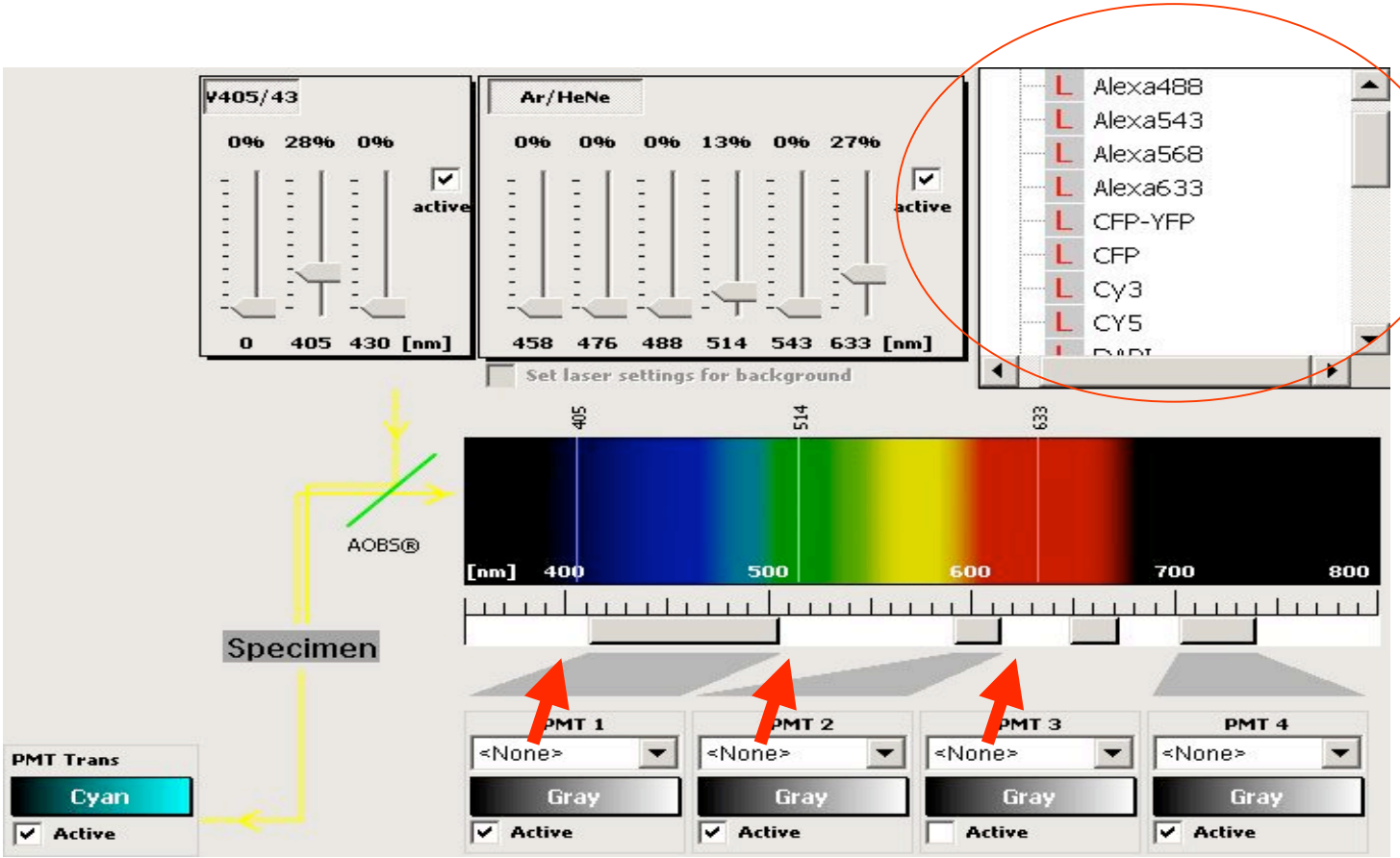




Choose appropriate laser lines and tune them to the minimal useful intensity

Place detection windows within the spectral range and adjust the band width.

Beam Path Settings

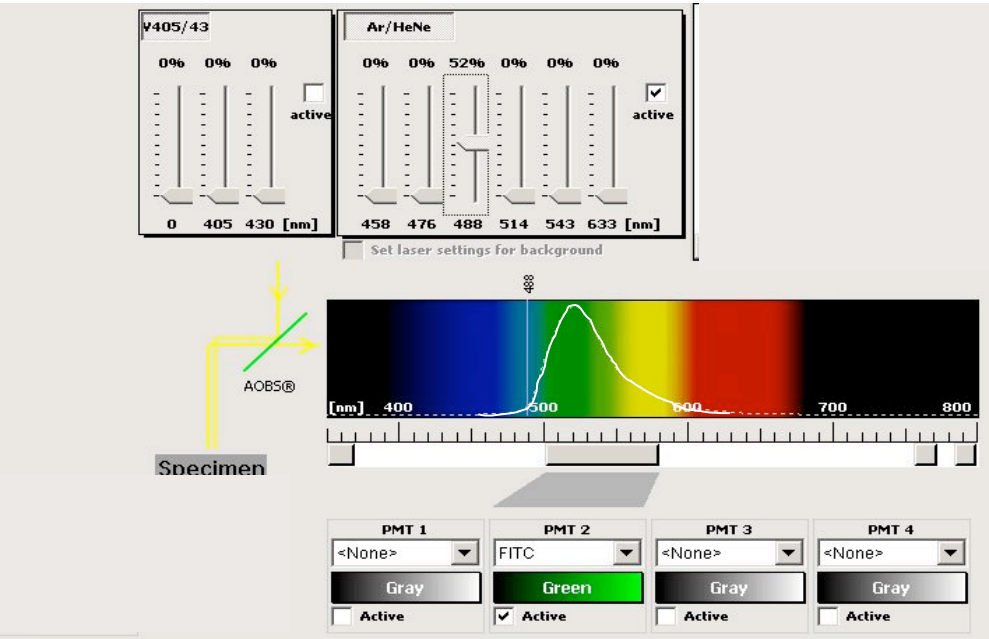


Settings might be saved

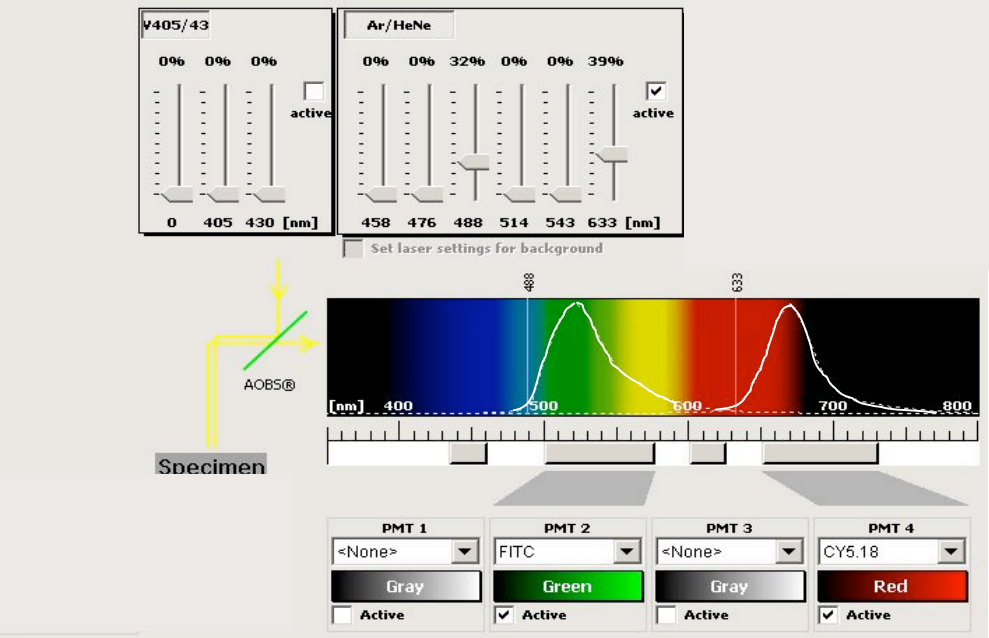
Choose settings according to ex/em-properties of your fluorochrome

*Avoid detection **on** active LASER lines (-> reflection !)*

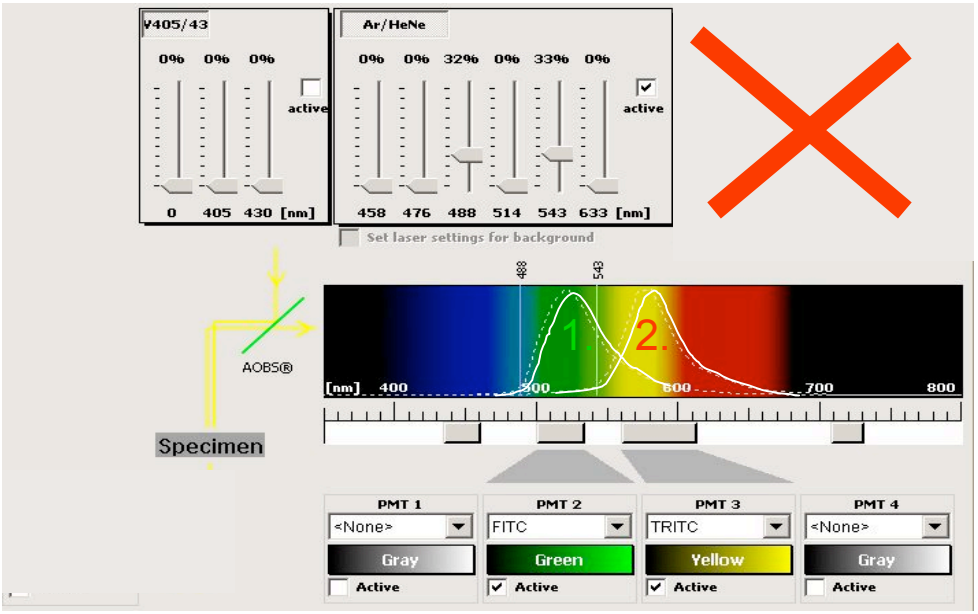
Multi-channel detection I

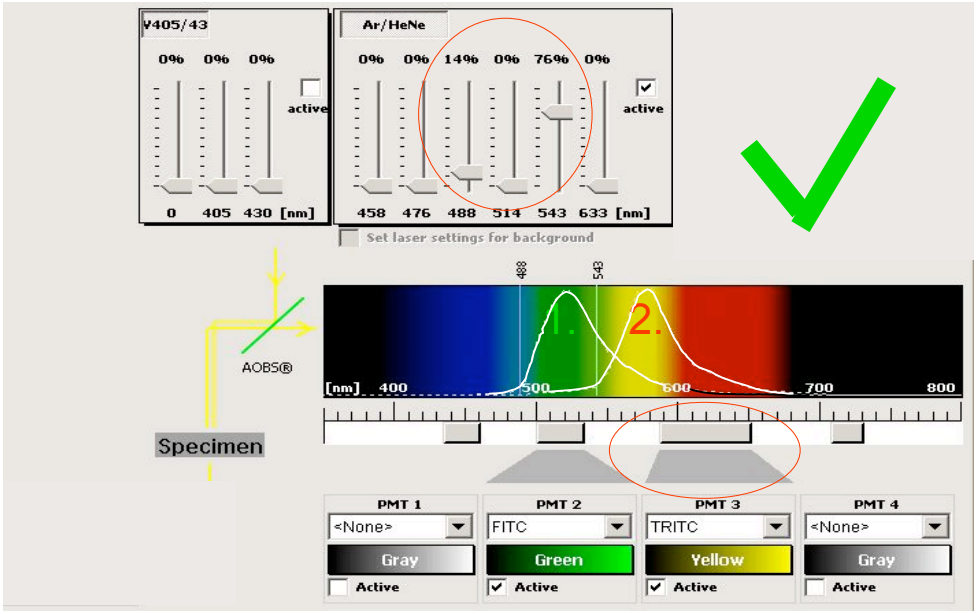


Up to 4 fluorescent channels can be captured simultaneously.



Fluorochromes with non overlapping emission spectra might be detected in <u>parallel</u>.





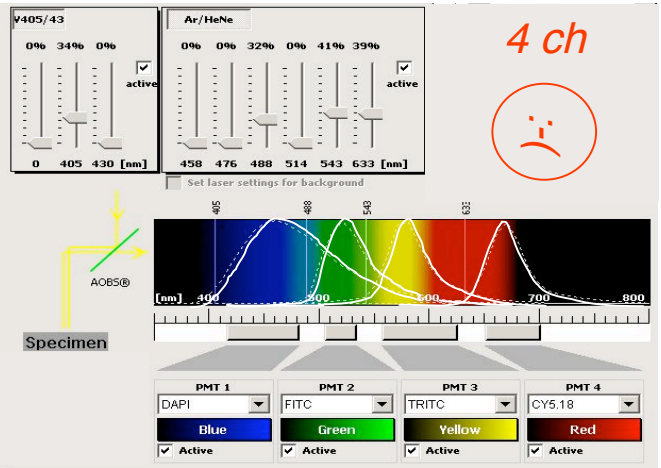
Multi-channel detection II

Fluorochromes with overlapping emission spectra might be detected in parallel if LASER power and GAIN are properly adjusted and the detection window is well choosen.

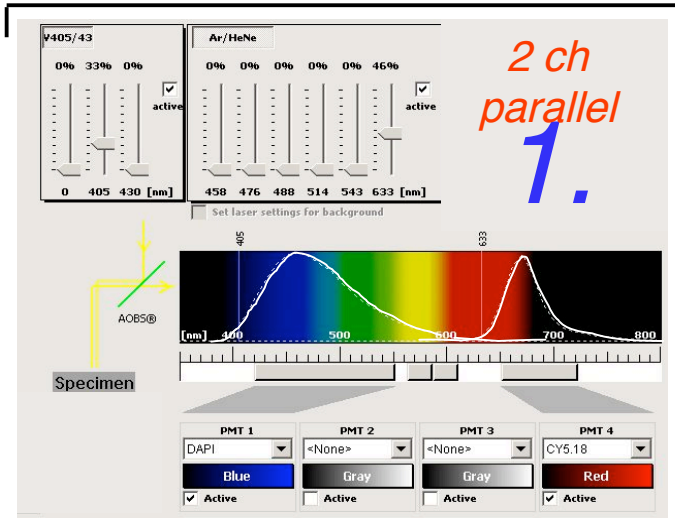
Fluorochrome 1(shorter wave lenght ex/em-spectra)
-> use as low LASER power as possible

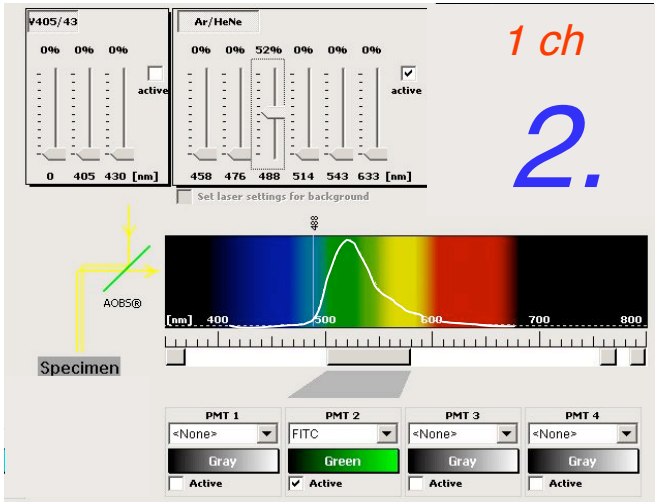
Fluorochrome 2 (longer wave lenght spectra)
-> low gain
-> high LASER power
-> detection window is shifted out of the overlapping zone as much as possble
-> check carefully for absence of cross talk !

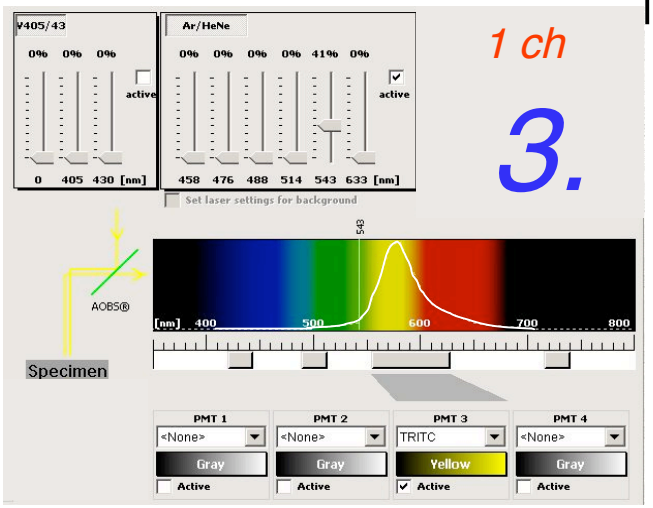
Multi-channel detection III



Fluorochromes with strongly overlapping emission spectra are best detected sequentially in order to avoid cross talk.







Sequential detection of emission channels

Multi-channel detection modes in CLSM

- *Parallel („the fast choice“)*

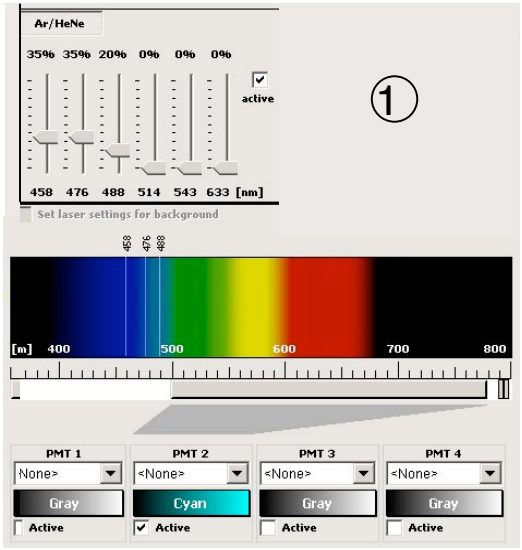
  Several laser lines excite the various fluorochromes in the sample at the same time. The multicolor emissions are collected in several channels simultaneously by several active PMTs.

- *Sequential („the safe choice“ - avoids cross talk !)*

  Only one laser line is active. Only one fluorophore is excited and emits its signal, which is collected by one active PMT. Then Laser & PMT are switched off and the next laser line and PMT are activated in order to capture the next channel.

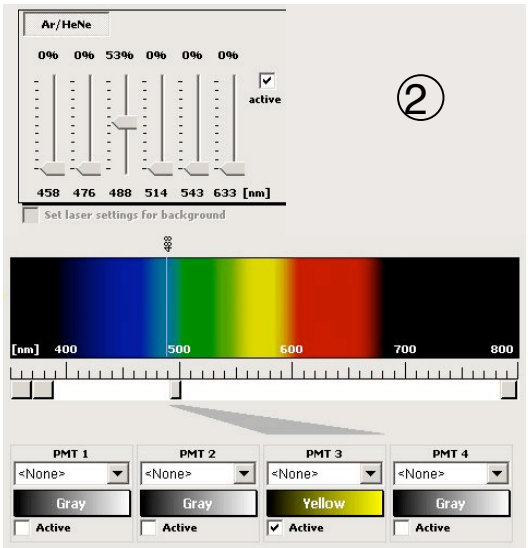
Inquiring spectral properties

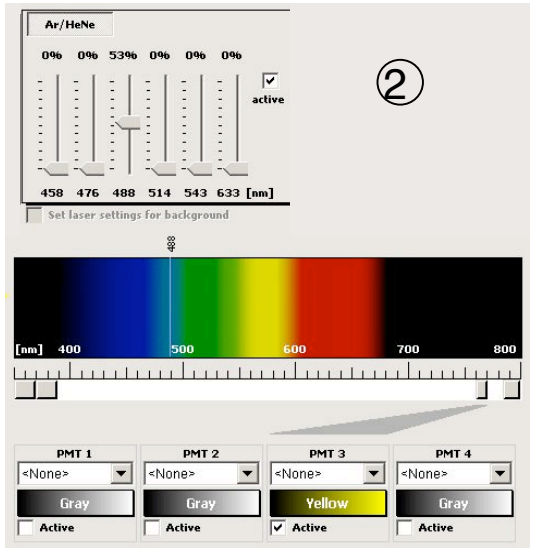
Autofluorescence (and unknown flourochromes) might need some characterization…

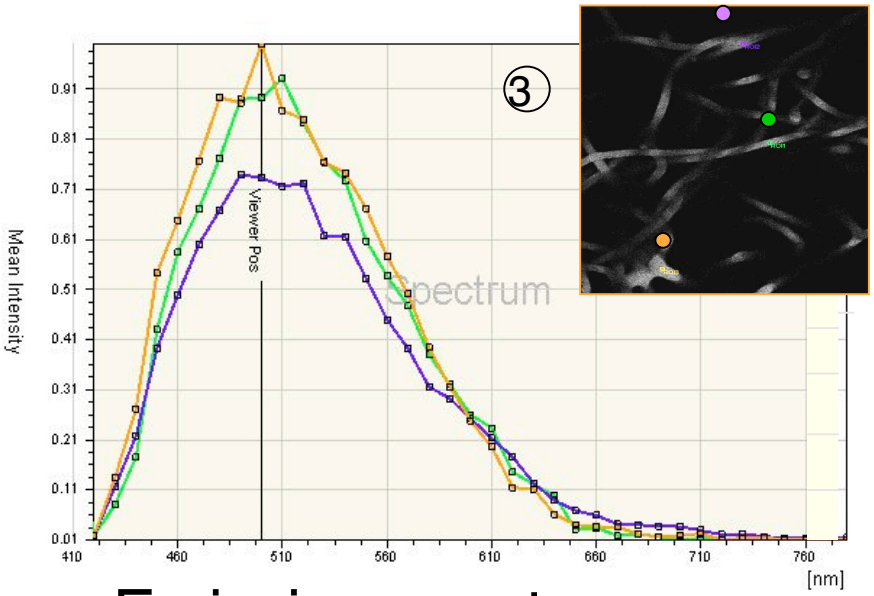


1 Which LASER line triggers the strongest emission response in the sample? -> checking different LASER lines with a wide open detecton window.

2 **Lambda-scan:** a narrow detection window is measuring the emission signal at different λ







3 **Emission spectrum:** emission signal at selectet spots: intensity versus λ

CLSM: Choosing fluorochromes

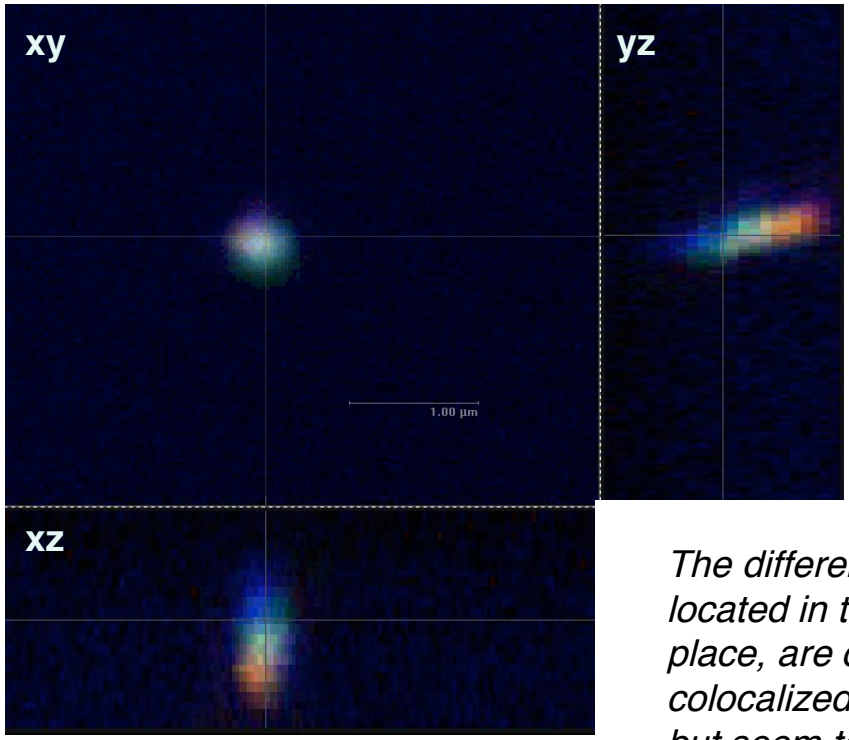
- Choose fluorochromes accordingly to the LASER lines of the system (excitation spectrum should have ist maximum close to a given LASER line)!!!
- Remember: not all suitable fluorochromes are visible by eye (i.e. Cy5)
- For multi-channel fluorescence microscopy, best use fluorophores with non-overlapping spectra.
- If your fluorophores have overlapping emission spectras, avoid cross talk by careful adjustments **OR** by detecting the channels sequentially instead of parallel
- Because of the chromatic abberation of the lenses, you best use a green/red-pair of fluorescent markers for co-localization to avoid z-mismatch of the channels

Lens aberration effects in the data

-> need for image processing: deconvolution, pixel shifts, ...

3D dataset of multifluorescent beads.
best xy-resolution
> 200 nm

The 3D round object looks perfectly conc-entric in xy, but seems elon-gated in z -> ***point spread function*** *PSF.*
best z-resolution
> 300-400 nm



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

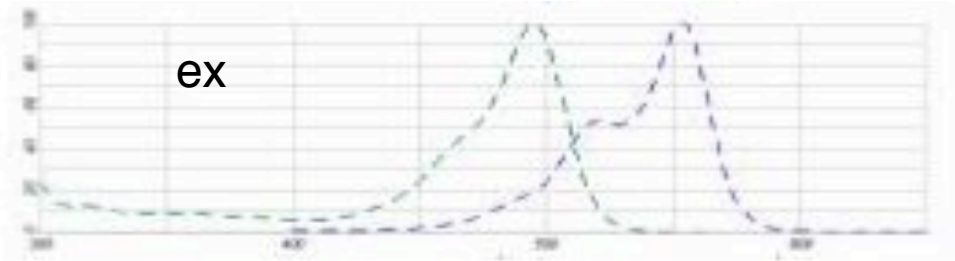
Sperical aberration

Chromatic aberration

Fluorescent dyes with overlapping spectra

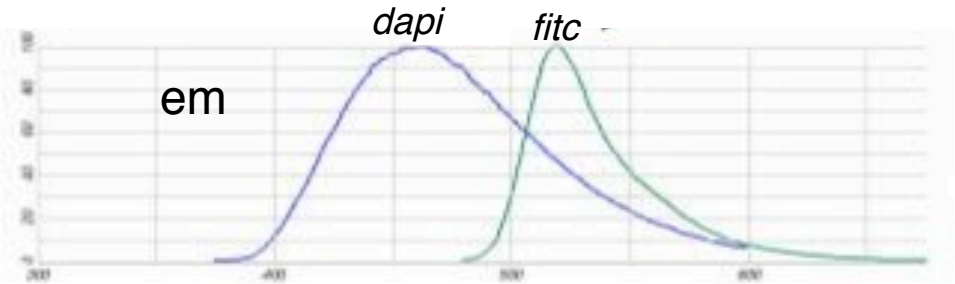
- Cross excitation

  The excitation spectra of two fluorochromes are broad and overlapping to a significant extend -> avoid this fluorochrome combination



- Bleed through

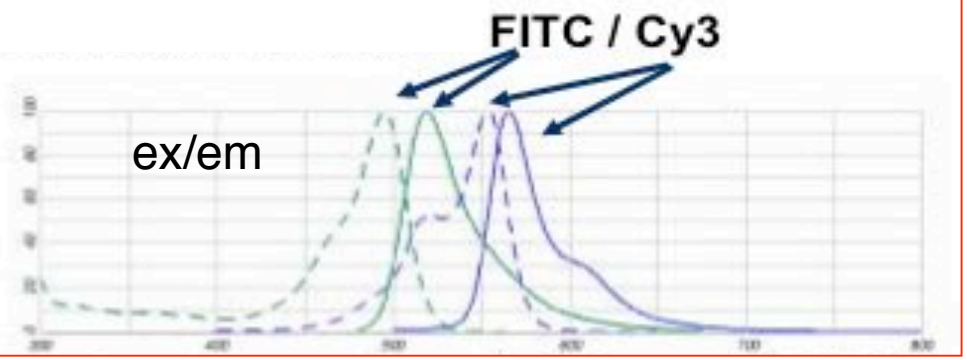
  The emission spectra of two fluorochromes are overlapping -> measure the emission sequentially



- Energy transfer

  The emission light of one dye stimulate excitation of the second dye

  (-> ideal only for FRET colocalization studies)



Preparation of confocal sampes

Confocal microscopy is an expensive and time consuming technique. Only good preparations are worth to be examamined. The higher resolution power of confocal microscopes has special demands on the sample:

-> i.e. collocation of structures in fixed cell cultures

- use freshly prepared buffered paraformaldehyde for fixation
- choose fluorochromes for optimal excitation and minimal crosstalk
- use water soluble embedding media
  which polymerizes and contains antibleach-agent.
- use cover glass- set ups (cover glass thickness of 0,17 mm)
- use immersion objectives (oil or glycerol immersion)

-> i.e. observation of living cells

- Heatable table, clima chamber with CO_2 gas control
- Inverse microscope: use glass bottom cell culture dishes
  and water- or glycerol-immersion objectives
- Upright microscope: use plastic dishes and dip-in objectives

Resolution controling factors in confocal microscopy

xy (image resolution) depends on emission wavelenght, numerical aperture of the objective, immersion medium, stability of the system, brightness/contrast-settings, pixel size

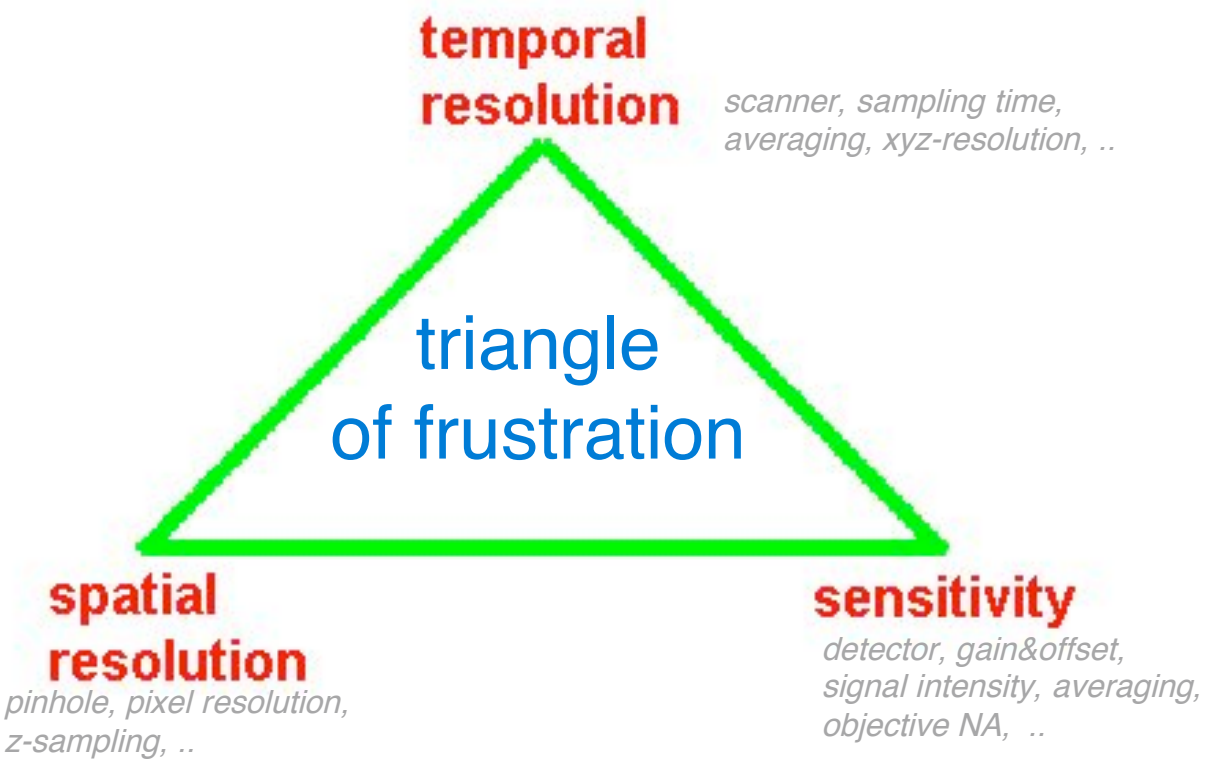
z (optical section thickness) depends on pinhole size, coverglass thickness (0,17 mm !!!), immersion medium

t (time resolution) depends on hardware parameters like scanning speed

λ (spectral resolution) depends on spectrophotometric devise (SP) and/or beam splitters and filters

i (dynamic range) depends on bit-resolution

Decisions in signal detection



You allways have to decide what is the “must have” of your experiment. All settings have their benefits and limitations! Compromises in some respects are necessary. What is best, depends on the application requirements!