

# **BENEFRI Neuroscience Workshop 2011**

## **Calcium imaging and voltage - sensitive imaging in neurons and neuronal tissues**

Thomas Berger

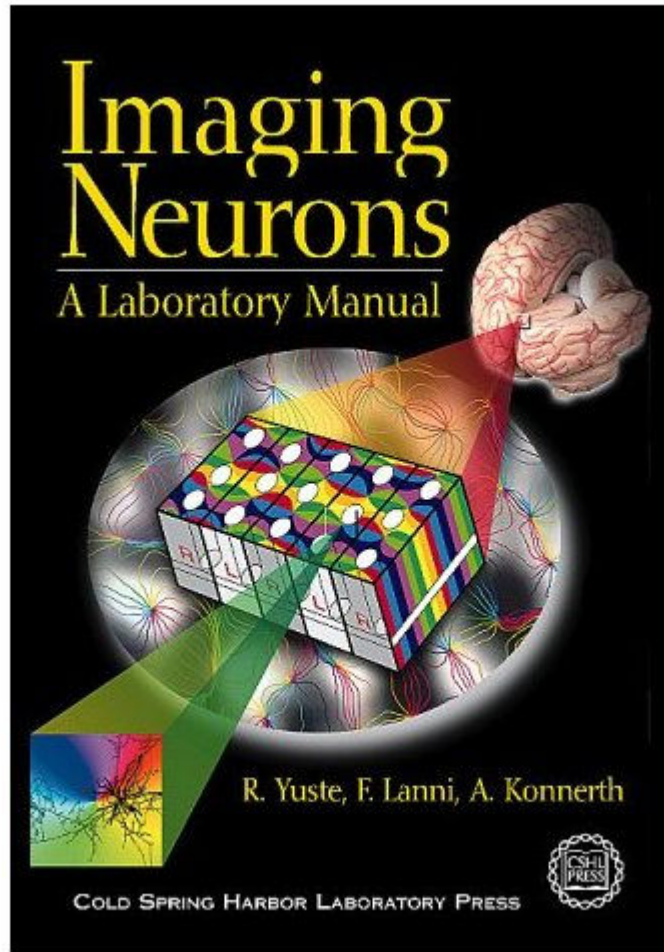
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**UNIVERSITÄT**  
**BERN**

## Literature



### **Imaging Neurons: A Laboratory Manual**

by Rafael Yuste, Frederick Lanni,  
and Arthur Konnerth

Cold Spring Harbor Laboratory Press

## Extremely useful web sources

<http://www.olympusmicro.com/> (Olympus Microscopy Source Center)

<http://www.hi.helsinki.fi/amu/> (Confocal tutorial)

# **BENEFRI Neuroscience Workshop 2011**

## **Calcium imaging and voltage-sensitive imaging in neurons and neuronal tissues**

### **Principles of imaging techniques**

Detection of cellular and subcellular activity  
with CaSD and VSD imaging techniques

Detection of network activity  
with CaSD and VSD imaging techniques

# Principles of Imaging Techniques

## Introduction: Light and Fluorescence

Imaging techniques: eyes

Imaging techniques: equipment

Epifluorescence imaging

Two-photon imaging

Confocal imaging



# Imaging: Working with light and dyes

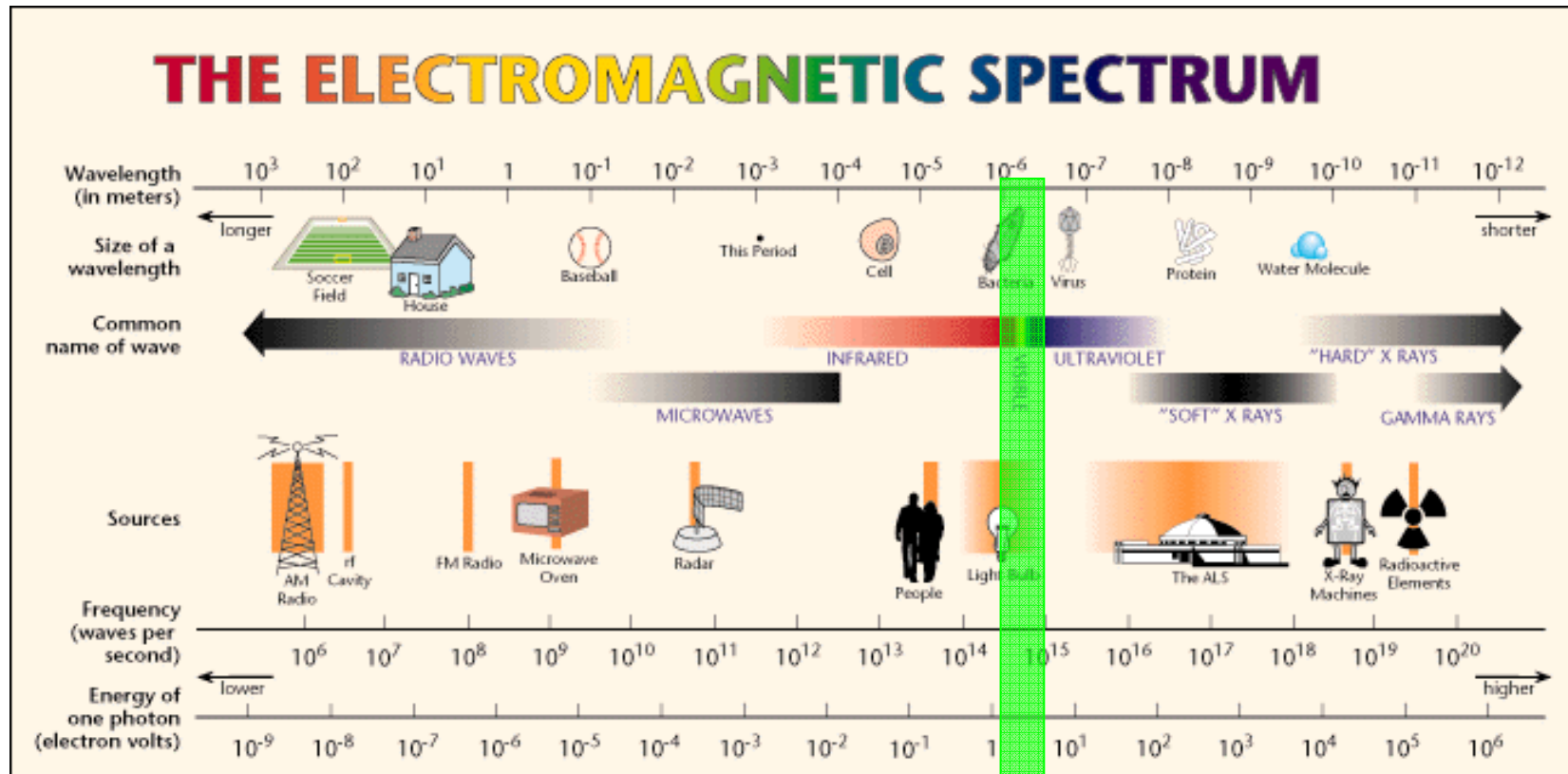
**Brad & Janet:**

**There's a light, ... light ... in the darkness of everybody's life.**

Source:



# Imaging: Working with light



Used wavelengths for imaging:

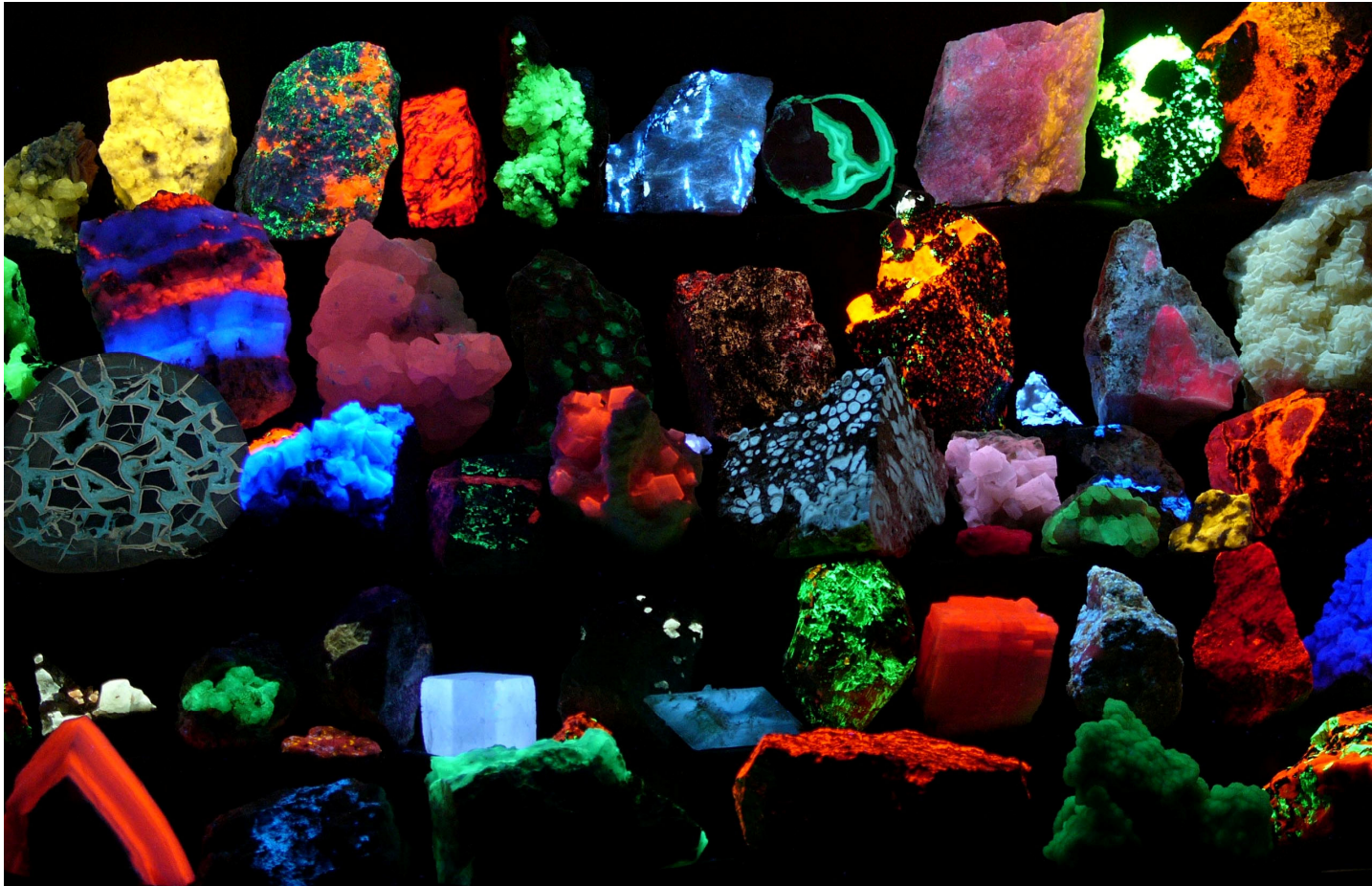
Epifluorescence: from the near-UV to the near-IR;  $\lambda \sim 300 - 750$  nm

Two-Photon: from the blue to the far-IR;  $\lambda \sim 450 - 1000$  nm



# Fluorescence: The name

Named by the salt Calcium Fluoride.



Fluorescent Minerals

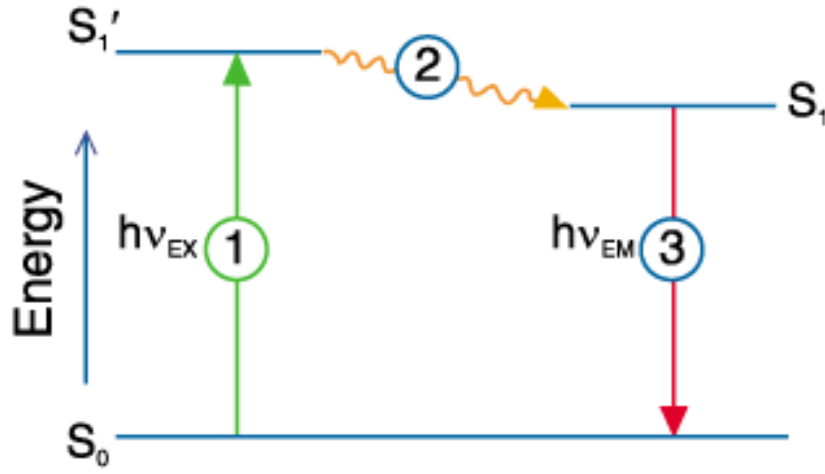


# Fluorescence: The principle

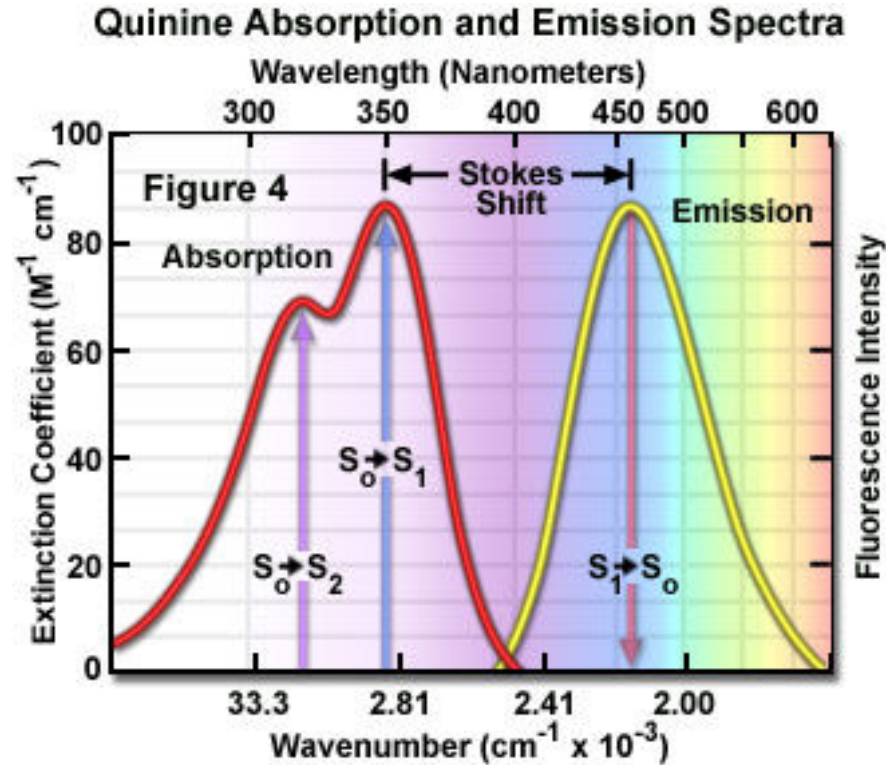
Definition : Light emitted during absorption of radiation of some other wavelength.

Principle:

1. Energy is absorbed by the atom which becomes excited.  
The electron jumps to a higher energy level ( $S_0 > S_1'$ ).
2. Vibrational relaxation to the lowest vibrational energy level of  $S_1$   
Loss of energy in the absence of light emission, heat production. Takes only psecs.
3. The electron drops back to the ground state  $S_0$ ,  
emitting a photon - the atom is fluorescing.

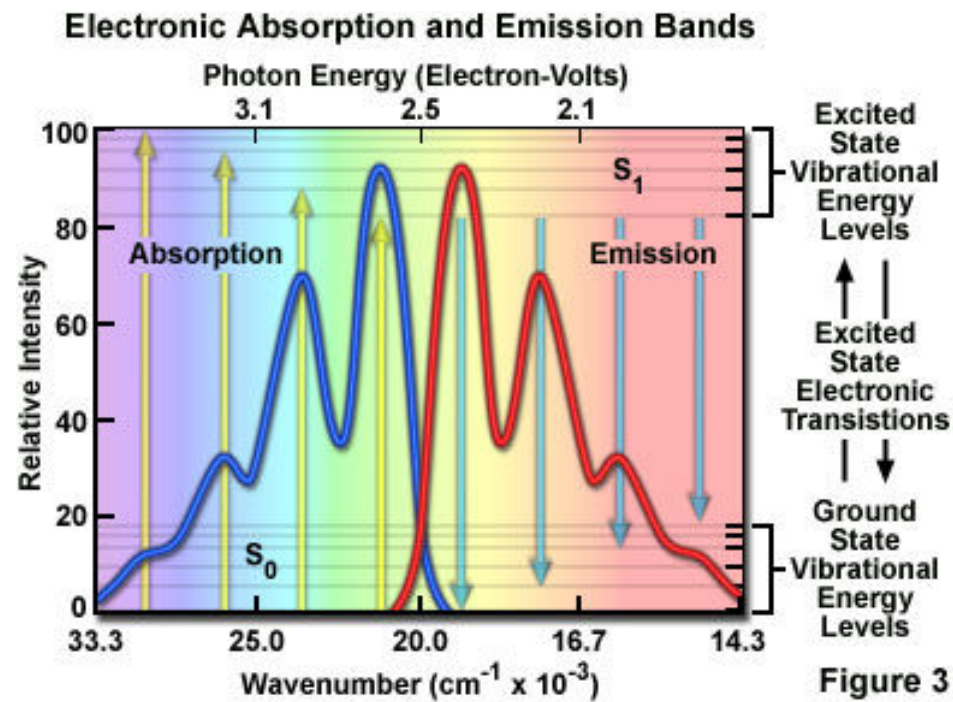


# Fluorescence: The Stokes shift



Emission has always a maximum at higher wavelength (i.e. smaller energy content) than the absorption. This so called Stokes shift is due to the vibrational relaxation and the corresponding energy loss in  $S_1$ . This shift (range of 20 – 200 nm wavelength) is essential for the distinction of absorption and emission light.

# Fluorescence: Absorption and emission spectra



**Absorption and emission spectra are mirrored.**

# Principles of Imaging Techniques

Introduction: Light and Fluorescence

Imaging techniques: dyes

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Two-photon imaging

Confocal imaging



## Advantages and disadvantages of different dyes

### Voltage-sensitive Dyes (VSDs; e.g. RH1691)

Advantages: Direct monitoring of the membrane potential changes, easy application in networks

Disadvantages: bad signal to noise ratio, possible toxicity

### Calcium-sensitive Dyes (CaSDs, e.g. Oregon Green BAPTA-1)

Advantages: Superior signal to noise ratio, no toxicity

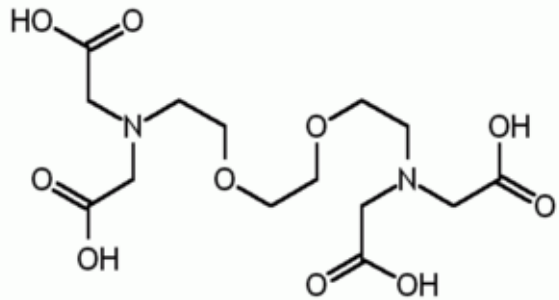
Disadvantages: Indirect signal due to calcium influx during action potentials depolarization, application into the cell interior necessary

### Sodium-sensitive Dyes (NaSDs; e.g. SBFI)

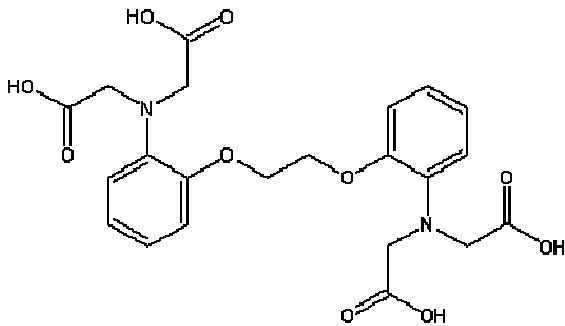
Advantages: No toxicity

Disadvantages: Indirect signal due to sodium influx during action potentials and excitatory synaptic activity; relatively bad signal to noise ratio due to bad dyes, application into the cell interior necessary

# Dyes : How do CaSDs work ?

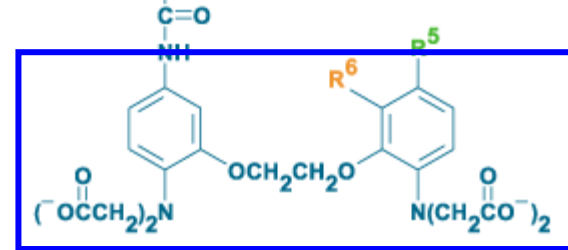
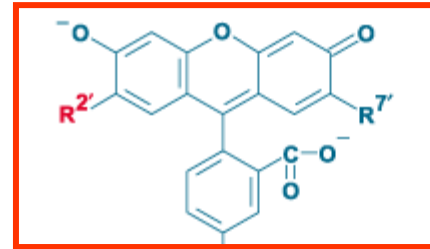


EGTA



BAPTA

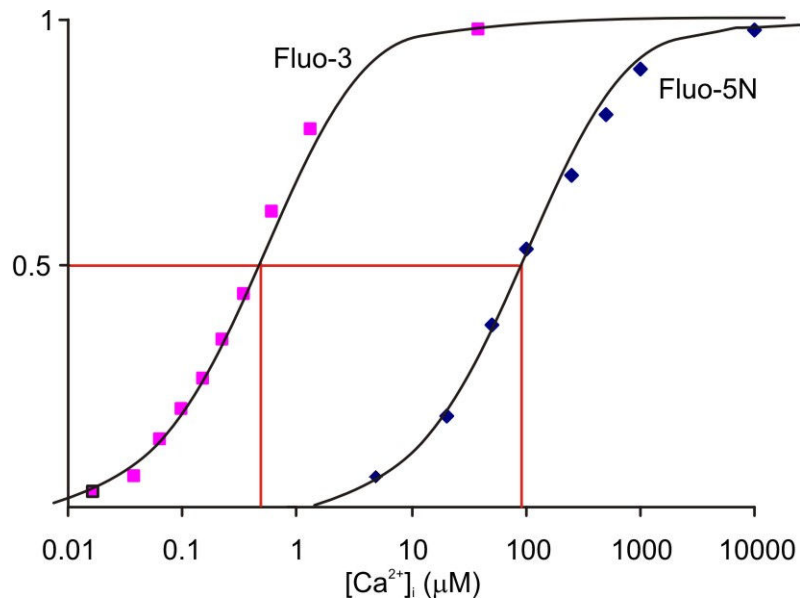
Calcium dyes are fluorescent derivatives of the calcium chelator BAPTA.



Indicator	$K_d(\text{Ca}^{2+})$	$R^{2'}$	$R^{7'}$	$R^5$	$R^6$
Calcium Green-1	0.19 $\mu\text{M}$	Cl	Cl	H	H
Calcium Green-5N	14 $\mu\text{M}$	Cl	Cl	$\text{NO}_2$	H
Oregon Green 488 BAPTA-1	0.17 $\mu\text{M}$	F	F	H	H
Oregon Green 488 BAPTA-6F	3 $\mu\text{M}$	F	F	H	F
Oregon Green 488 BAPTA-5N	20 $\mu\text{M}$	F	F	$\text{NO}_2$	H

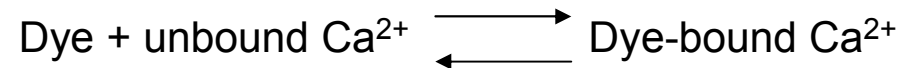
CaSDs contain in addition to the calcium chelating BAPTA component a structure which changes its fluorescence in dependence from the calcium binding. Different residues ( $R^x$ ) determine the calcium affinity which is reflected in the  $K_d$  value.

# Dyes : CaSD affinity for calcium



Affinity curves of the high and low affinity CaSDs Fluo-3 and Fluo-5N  
( $K_D$  400 nM and 90  $\mu M$ )

CaSDs are therefore nothing else but fluorescent calcium buffers. Their binding behavior to calcium can be described with simple Michaelis-Menten kinetics:

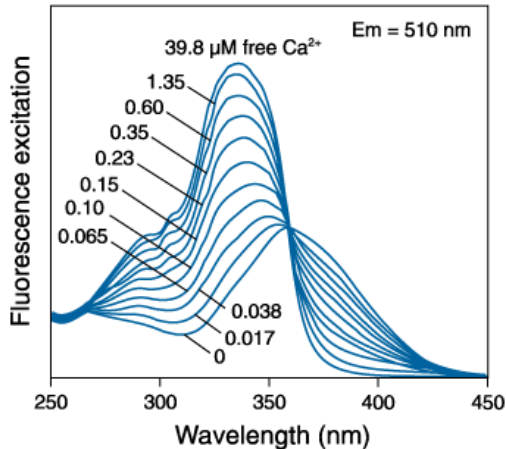


The calcium concentration where binding and unbinding of the calcium to the dye are in equilibrium is a property of the dye (its affinity) and is described by the term  $K_D$ . Is the  $K_D$  low, the dye needs only a low calcium concentration to be halfmaximally saturated, its affinity is high. With a high  $K_D$ , the affinity of the dye is low.

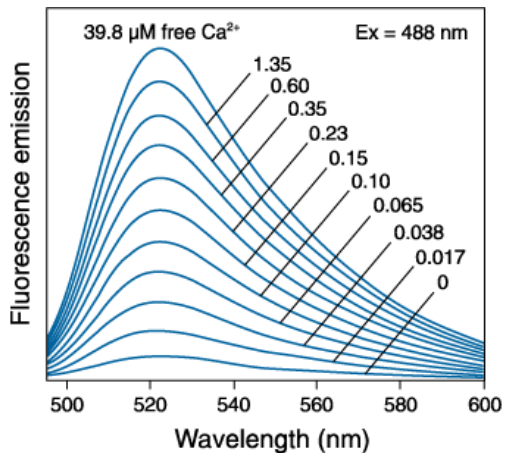
In most studies, dyes with  $K_D$  values in the range of about 200 nM are used which is also the range of the intracellular resting calcium concentration. These dyes are best suited to reflect physiological calcium concentration changes with detectable fluorescence changes.



# Dyes : Ratiometric versus non- ratiometric CaSDs



Fluorescence excitation spectra of fura-2 in solutions containing 0–39.8  $\mu\text{M}$  free  $\text{Ca}^{2+}$ . At an excitation wavelength of 340 nm fluorescence increases with increasing calcium concentration, at 380 nm the opposite is true (Increase in fluorescence and shift of the emission curve). Calcium concentration is best imaged as a ratio of the fluorescence at these wavelengths (**ratiometric dye**). The dye concentration can be imaged at the isobestic point at 360 nm (calcium-independent fluorescence)

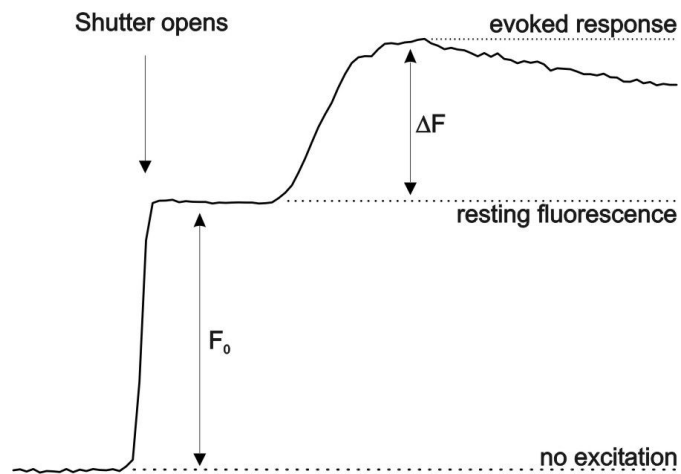


$\text{Ca}^{2+}$ -dependent fluorescence emission spectra of the Oregon Green 488 BAPTA-1 indicator. The spectral properties of OGB-1 do not change following calcium binding. Fluorescence increases with increasing calcium concentration (**non-ratiometric dye**). OGB-1 changes its fluorescence maximally in the nanomolar range. This dye is therefore best suited to detect changes in this physiologically important range.

# Dyes : What is actually imaged?

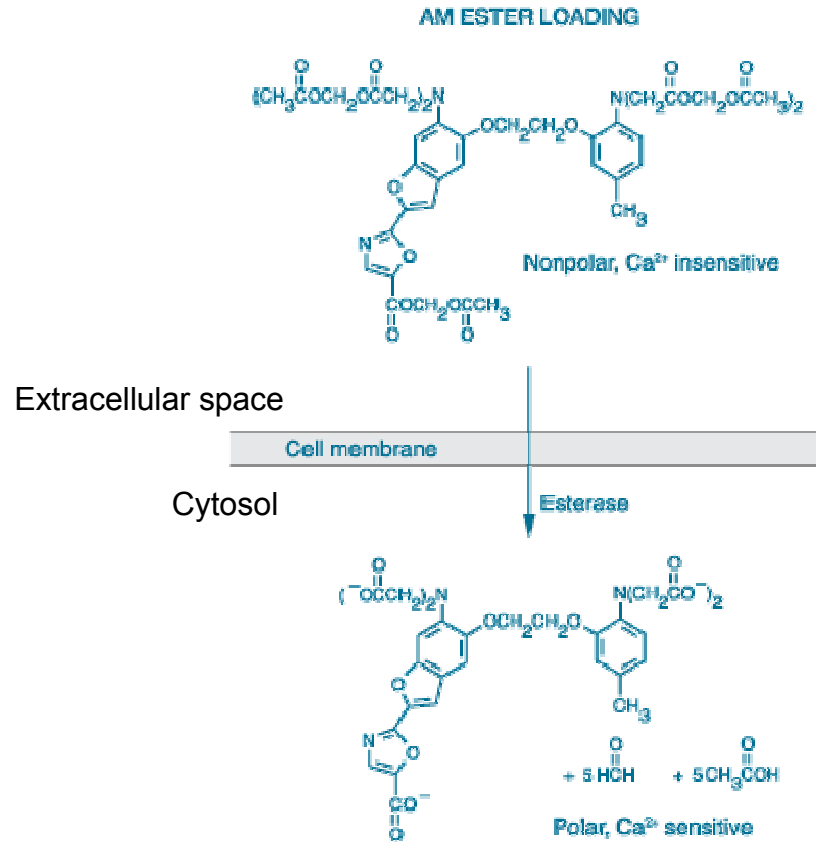
## Calcium concentrations (CaC) vs. relative changes in CaC

In order to image the actual calcium concentration (CaC) and its changes one has to calibrate the system (Cell plus dye). It does not help to take the  $K_D$  value of the dye to get a CaC because the dye acts differently in each cell. How to do it? After the imaging experiment one has to make the cell membrane permeable for calcium and one has to apply different buffered CaC. This is the titration curve for the experiment. It works only in single cells, not in networks.



This is pretty complicated and therefore one looks only for the relative changes in CaC not for the absolute CaC value. This change is given as the ratio for the ratiometric dye and as the  $\Delta F/F_0$  value for non ratiometric dyes.

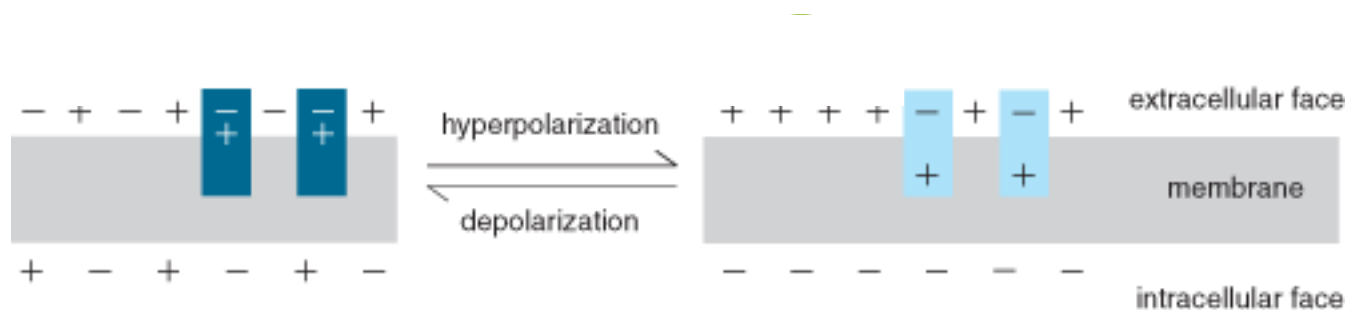
# Dyes : How do AM esters of CaSDs work ?



As already mentioned, CaSDs have to be intracellularly to detect intracellular changes in the calcium concentration. Either CaSDs have to be injected into the cell or cells have to be loaded over the cell membrane with the CaSD.

Schematic diagram of the processes involved in loading cells using membrane - permeant acetoxymethyl (AM) ester derivatives of fluorescent indicators, in this case fura-2.

# Dyes: How do VSDs work ?

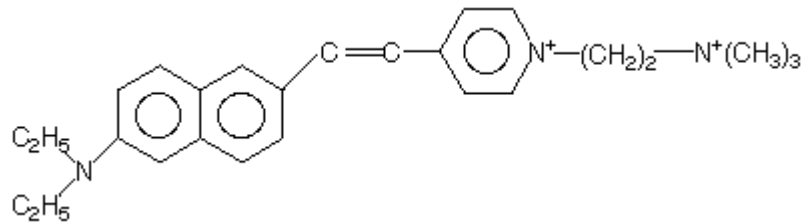


Response mechanisms of membrane potential–sensitive probes.

VSDs are situated in the membrane bilayer. **Fast-response** probes undergo electric field–driven changes of intramolecular charge distribution that produce corresponding changes in the spectral profile or intensity of their fluorescence (represented by color changes in the illustration). Thus, potentiometric response speed directly reflect the time constants of the underlying process — fast intramolecular redistribution of electrons.

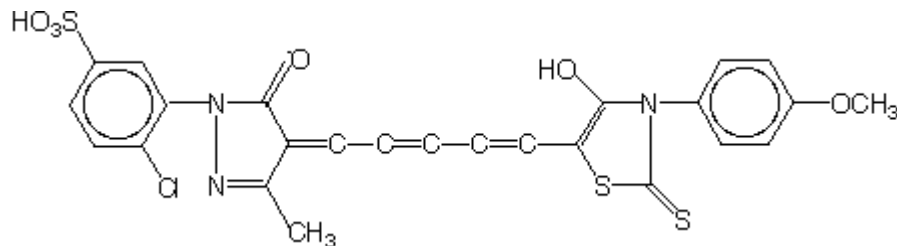
Another type of **slow** VSDs are lipophilic anions or cations that are translocated across membranes by an electrophoretic mechanism. Fluorescence changes associated with transmembrane redistribution result from sensitivity of the probe to intracellular and extracellular environments.

# VSDs acting at the inner and the outer side of the membrane : JPW1114 and RH1691



## JPW1114

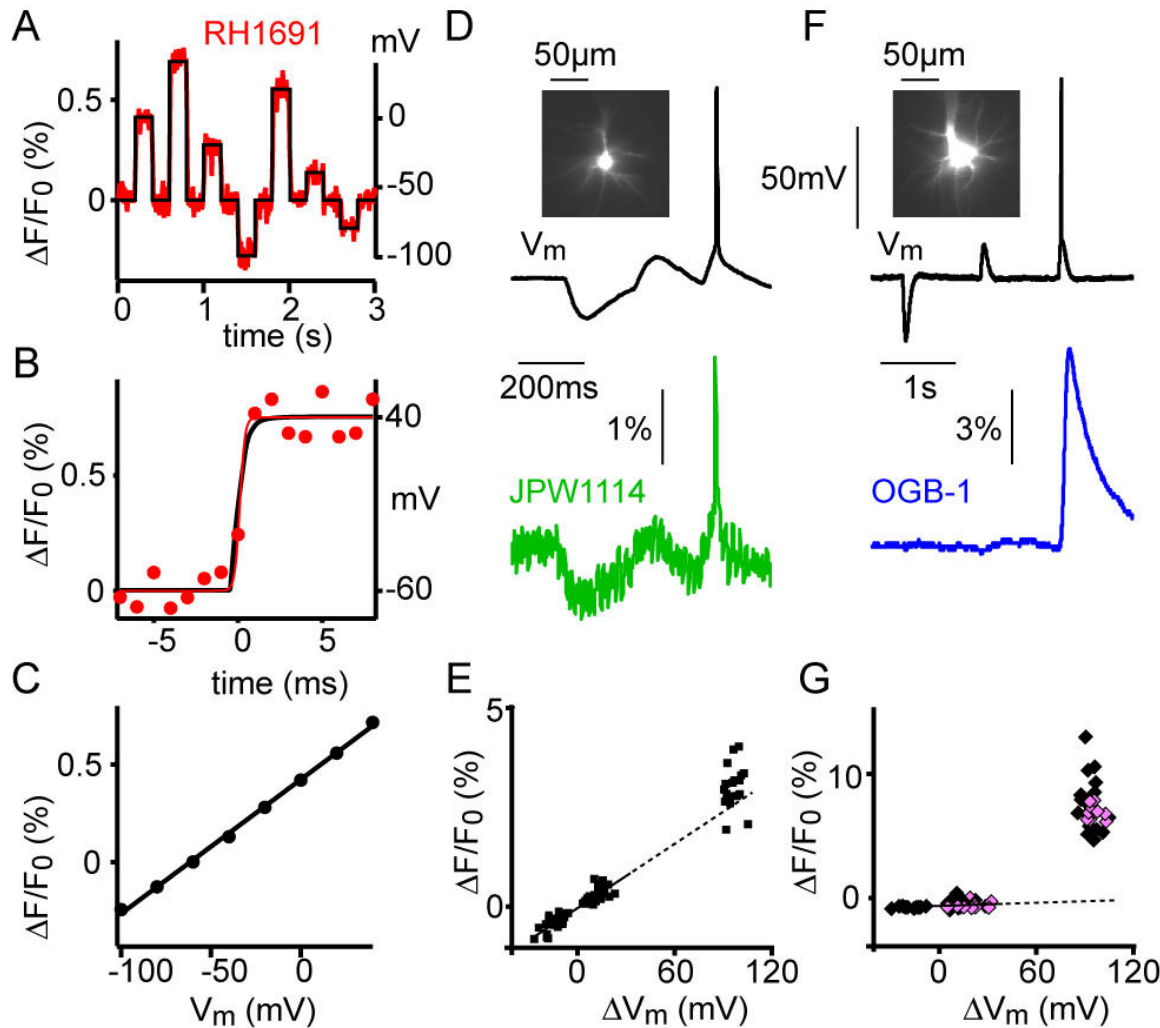
Due to its positive charges  
it can bind very well  
to the inner side of the membrane



## RH1691

Due to its charges  
it can bind only to  
to the outer side of the membrane

# VSD and CaSD signals in single cells : The activation range of the dyes



Fluorescence changes of VSDs (RH1691 and JPW1114) reflect in an analog way the change in the membrane potential. Small response!

CaSDs (OGB-1) bind only calcium and change their fluorescence only following spiking activity. This is a digital dye. Large response!

# Which dye shall I use?

Before ordering a dye one has to clarify several questions:

- Which type of neuronal activity is of interest for me? Do I want to have a 1 to 1 representation of the membrane voltage (use a VSD)? Am I only interested in spiking activity (use a CaSD)?
- Do I want to image the activity in single cells (VSDs, CaSDs applied with a patch pipette) or in whole networks (VSDs applied to the extracellular space, CaSDs applied as AM esters to the extracellular space)?
- How old are my animals ? (AM esters of CaSDs work not very well in older animals)
- How large is the area I want to study ? (large areas are difficult to fill with AM esters of CaSDs, no problem with VSDs)
- How high is the dye concentration in the cell ? (CaSDs are modified calcium buffers)
- What is the affinity of the CaSDs ? (high-affinity CaSDs won't detect additional large calcium concentration changes, low-affinity dyes won't detect small changes)
- Is the dye toxic ? (especially intracellular VSDs – if excited too long – kill cells)
- In which preparation do I want to work ? (some VSDs do only work in some preparations, not in others)
- Compartmentalization ? (Some dyes accumulate in organelles like mitochondria)
- Can I average evoked activity or do I want to study spontaneous activity ? (VSDs have a poor signal to noise ratio which needs in general to be improved by averaging)

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# Principles of Imaging Techniques

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Two-photon imaging

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# Epifluorescence

**Excitation:** The exciting light is mirrored on the preparation.

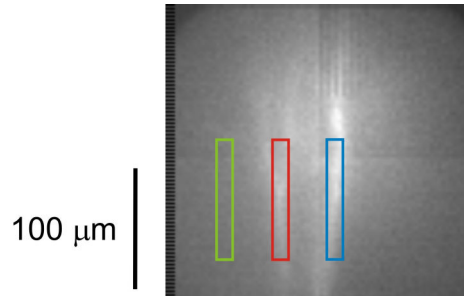
Excitation is strongest at the focus level of the optics, but also present above, below and aside.

**Emission:** If excitable dye is present, fluorescence will be emitted from the focus but also from above, below and aside.

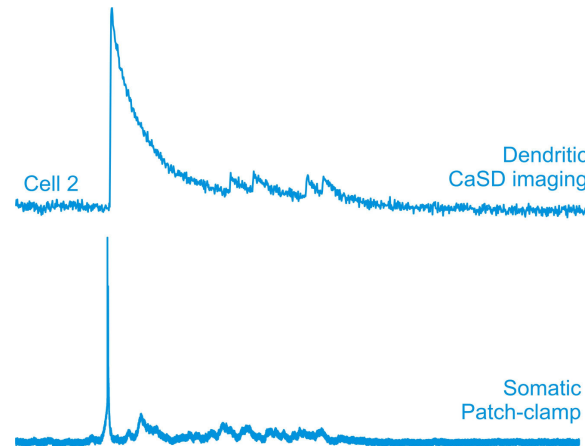
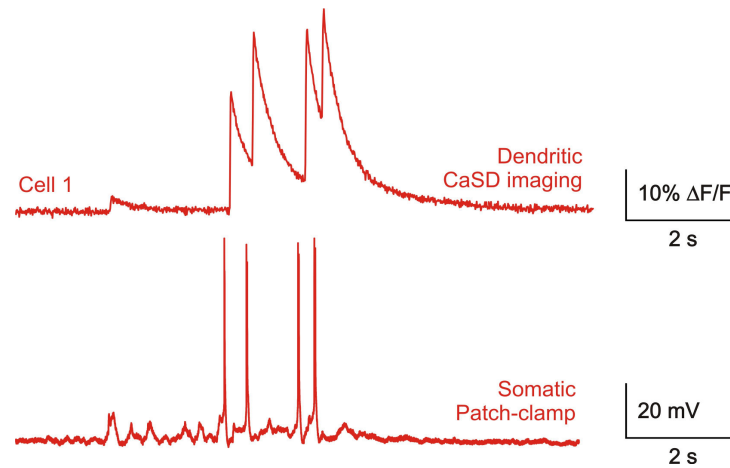
**Advantages:** No means are necessary to restrict the excitation to a narrow spot (see Two-Photon) or to collect light only from a small volume (see Confocal microscopy). More light is collected resulting in a better signal to noise ratio.

**Disadvantages:** The location of the fluorescence source is totally uncertain in Z-direction (depth of the preparation), but also not defined in X- and Y-direction, due to scattered light coming from fluorescent structures above, below and aside. Possible photodamage of the preparation.

# Epifluorescence pitfalls: From where comes the signal?

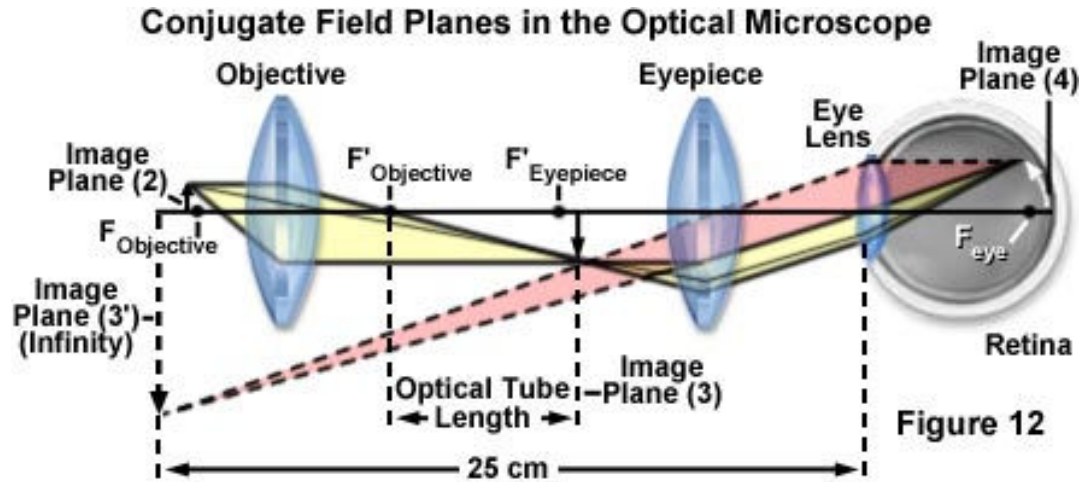


The epifluorescence picture from a given pixel does not only contain information from that particular position but also from positions within a radius of about 50  $\mu\text{m}$ .

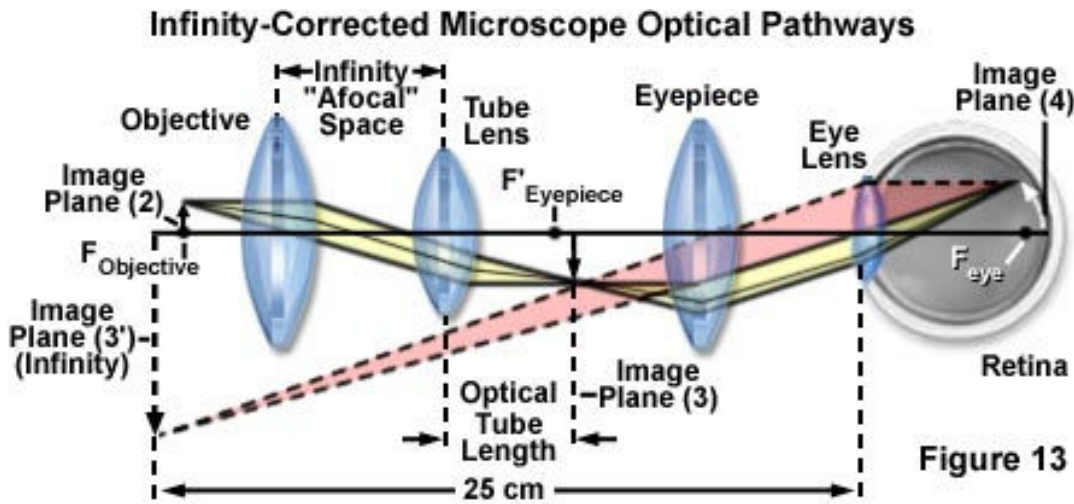


Taken from: Berger, unpublished data

# Techniques : How does a microscope work ?

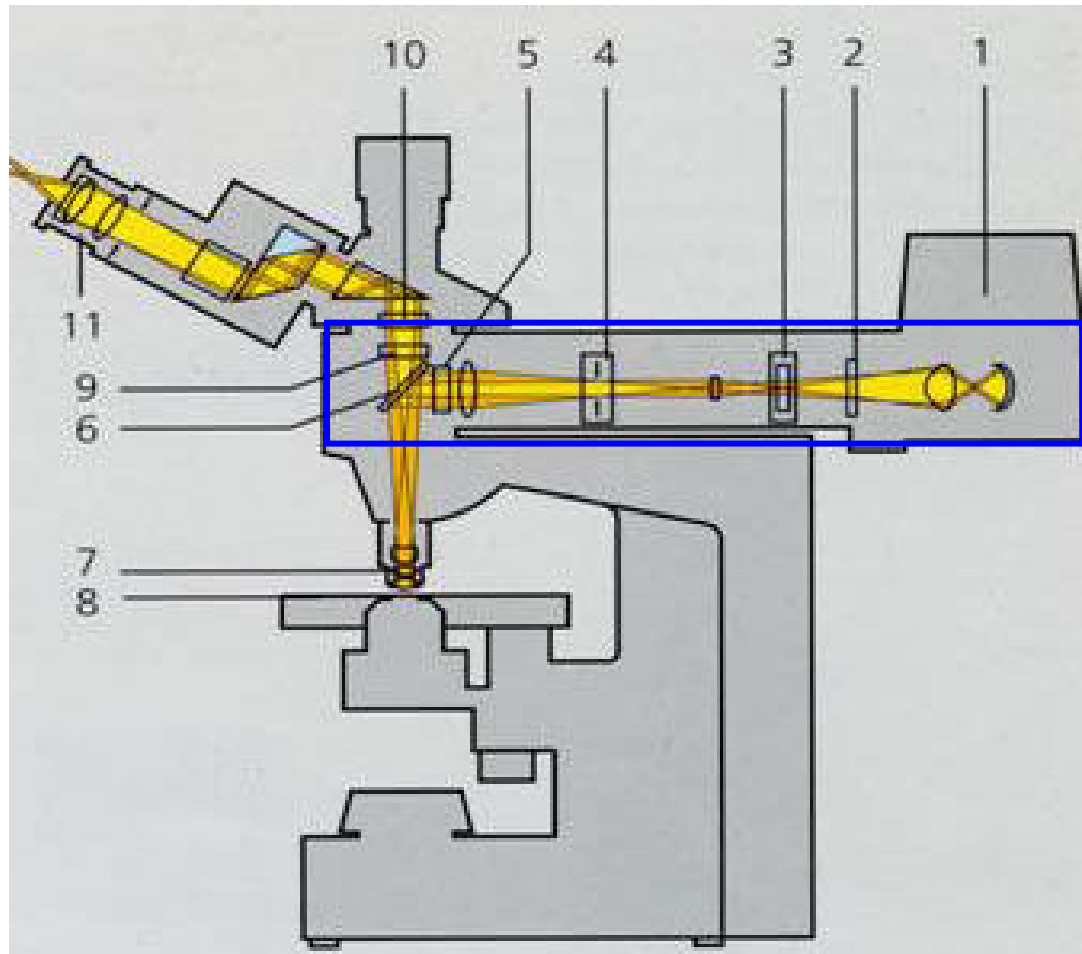


The objective generates a picture of the specimen in plane 3. The optics of the eyepieces (or comparable optics in front of a camera) focus on this picture and generate a sharp picture on the retina or the chip.



The objective of a modern “infinite” microscope produces an infinite, afocal picture of the specimen, which is focused by the tube lens. Due to this, additional optical elements (filters, analysators...) can be included between the objective and the tube without changing the magnification and other optical properties.

## Techniques : The (epi-)fluorescence microscope



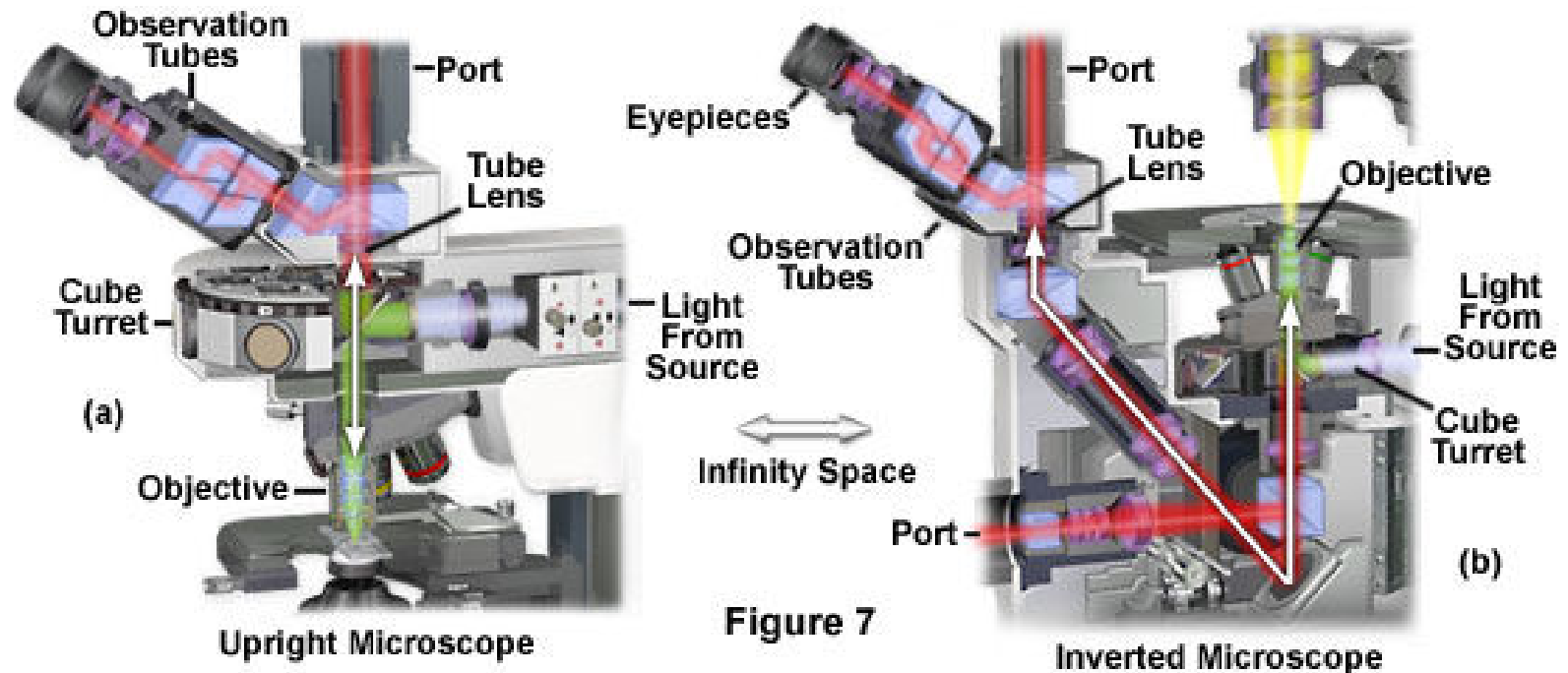
Afocal space in the microscope. Here, the whole epifluorescence device with the filter cubes can be included as an additional part.

If there would be no afocal space, the optics and the eyepieces of the microscope had to be adjusted each time such an additional device is added or withdrawn from the microscope. This space is a prerequisite for a modular microscope.

# Techniques :

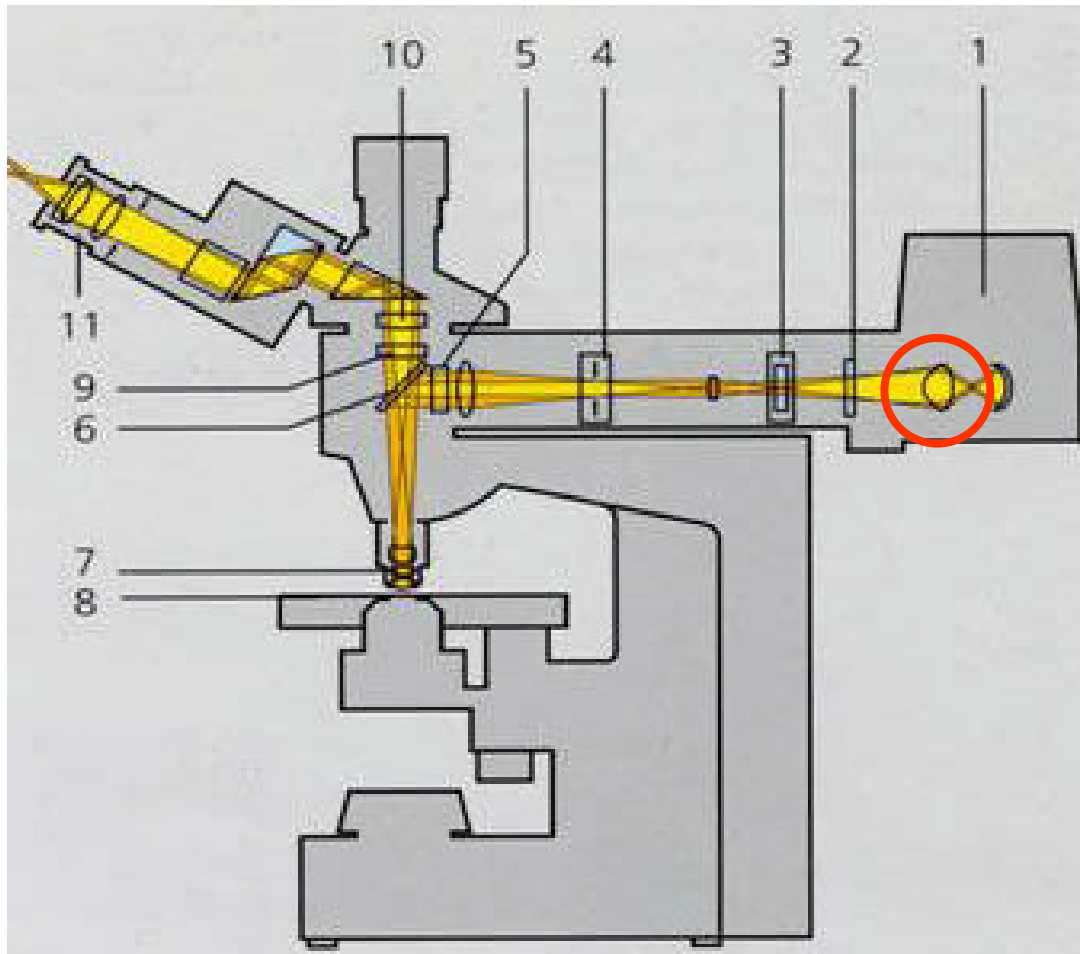
## Upright and inverted microscopes

### Parallel Infinity Space in Upright and Inverted Fluorescence Microscopes



Upright and inverted fluorescence microscopes in contrast to standard brightfield microscopes have one thing in common: Exciting light and emitted fluorescence are coming and going via the same optics. With excitation optics from below and emission collecting optics from above it would be nearly impossible to get a picture.

# Techniques : The epifluorescence microscope



- 1 : Lamp
- 3 : Shutter
- 5 : Absorption filter
- 6 : Beamsplitter
- 7 : Optics
- 8 : Specimen
- 9 : Emission filter
- 10 : Tube lens
- 11 : Eye pieces / Camera



# Light sources : Mercury and Xenon Arc Lamps



Figure 1

A voltage difference between both electrodes results in a light arc (short circuit).

Mercury Arc Lamp UV and Visible Emission Spectrum

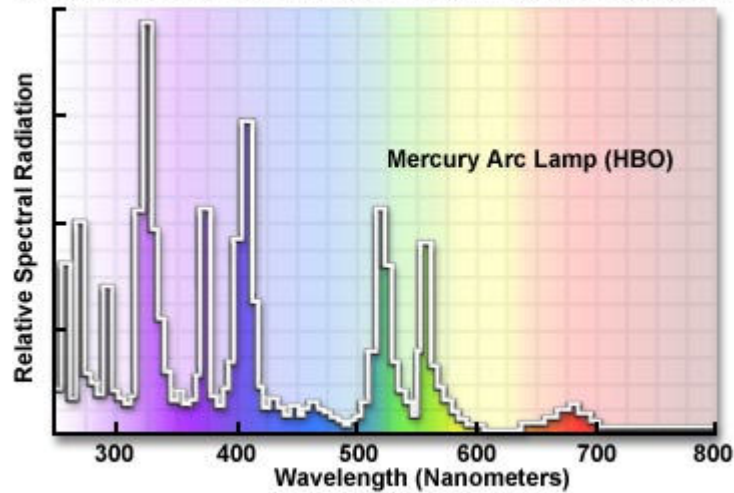


Figure 2

Advantage of Mercury Arc Lamps (HBO):  
High intensity in the UV  
Disadvantage: Uneven intensity with  
Some  $\lambda$  "spared", e.g. at 488 nm

Xenon Arc Lamp Emission Spectrum

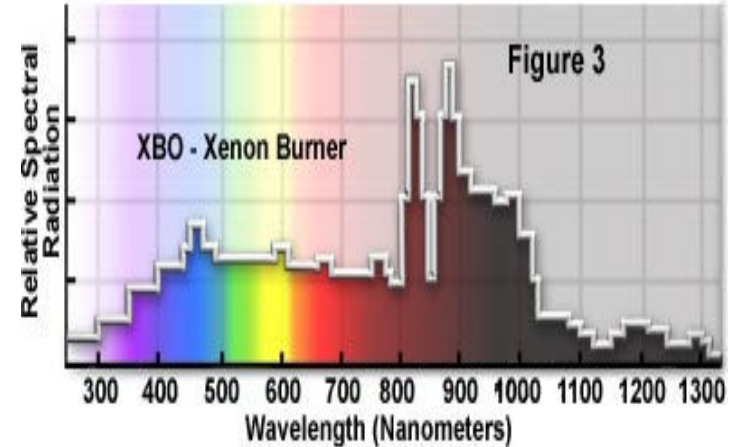
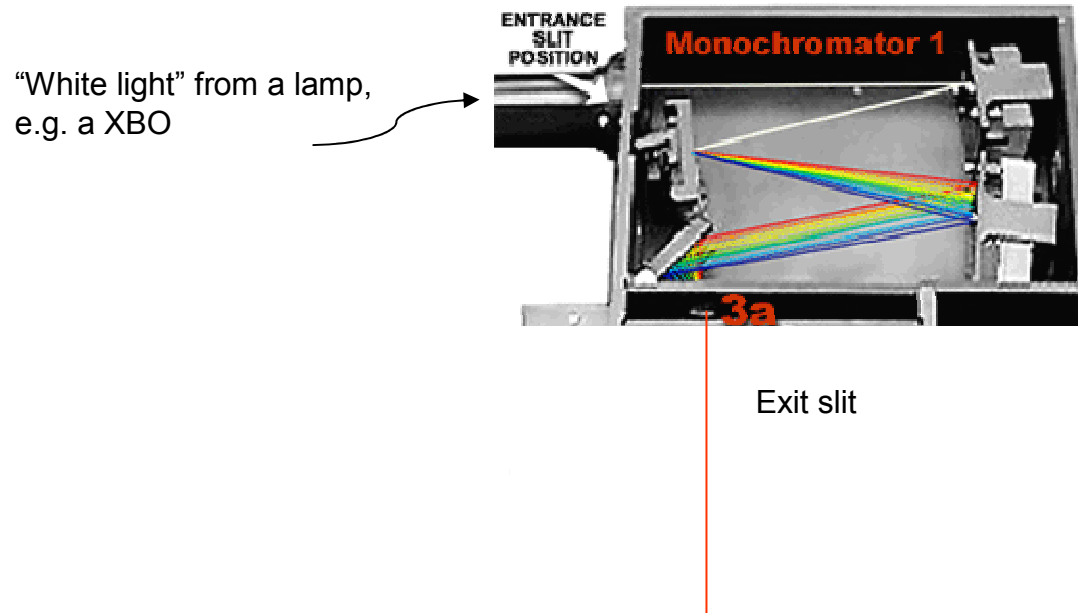


Figure 3

Advantage of Xenon Arc Lamps (XBO):  
Even intensity in the visible spectrum  
Disadvantage: Low intensity in the UV,  
high intensity in the IR (Heat!)

# Light sources : Monochromators

## They generate absorption light with a defined wavelength

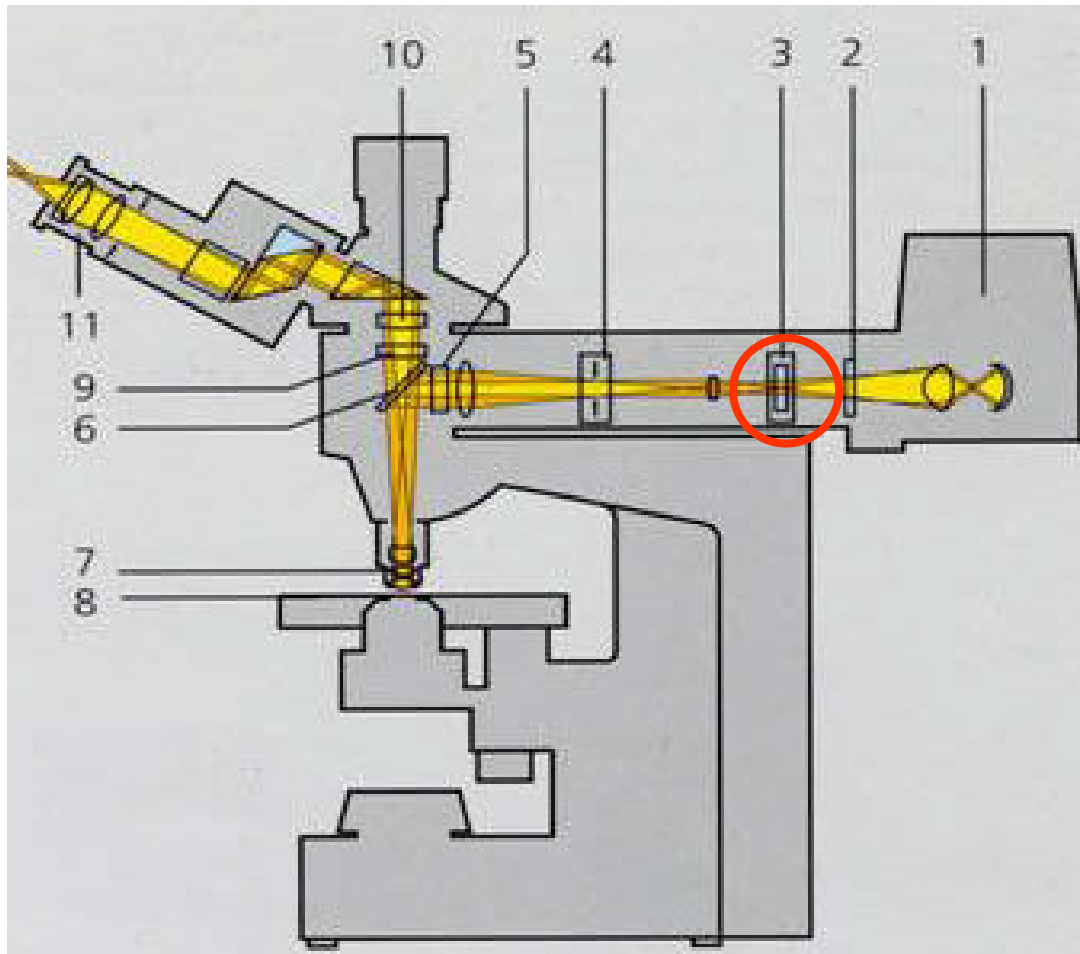


Principle: White light is separated in its spectral components using diffraction gratings.

Advantage: Depending on the positioning of the last mirror light with a defined and desired wavelength leaves the monochromator to excite the dye (high flexibility). No excitation filter necessary in the microscope.

Disadvantage : Price. Loss of light due to a narrow exit slit.

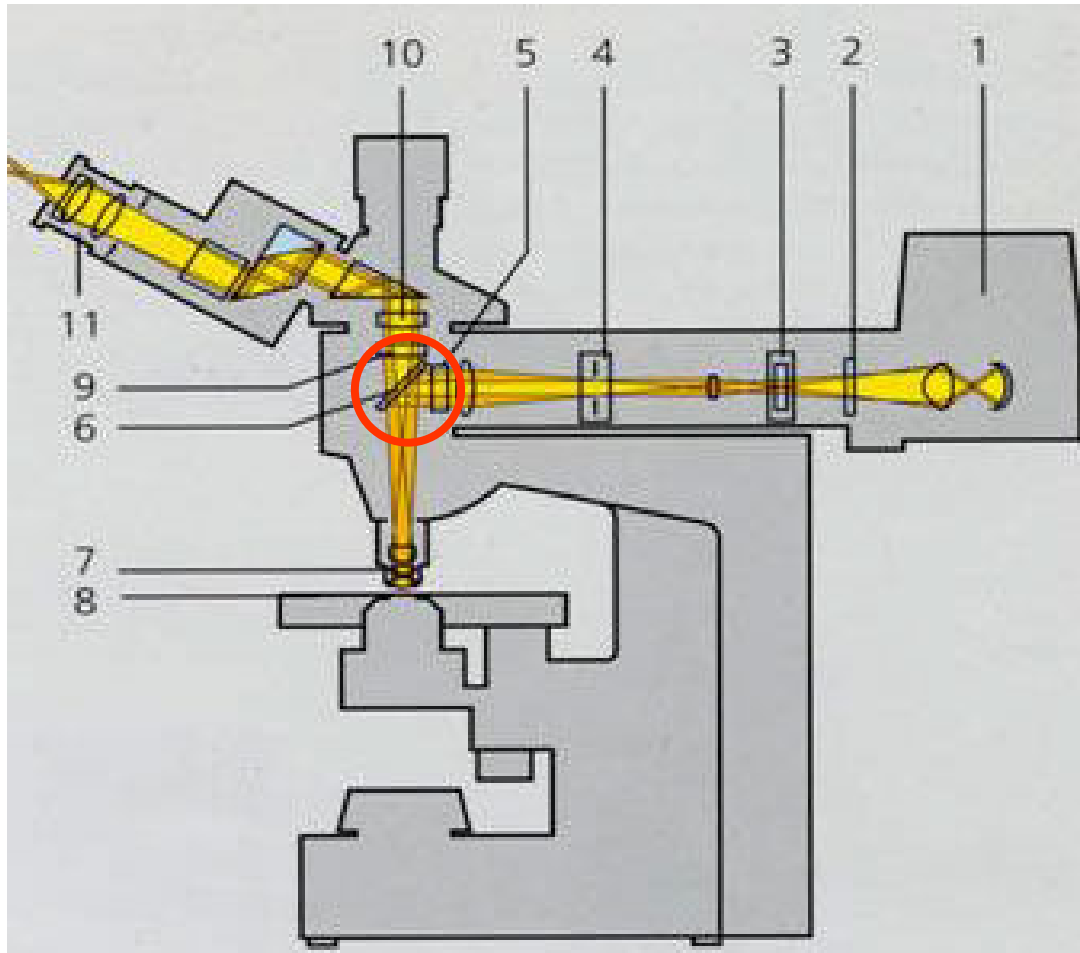
## Techniques : The shutter



- 1 : Lamp
- 3 : Shutter
- 5 : Absorption filter
- 6 : Beamsplitter
- 7 : Optics
- 8 : Specimen
- 9 : Emission filter
- 10 : Tube lens
- 11 : Eye pieces / Camera

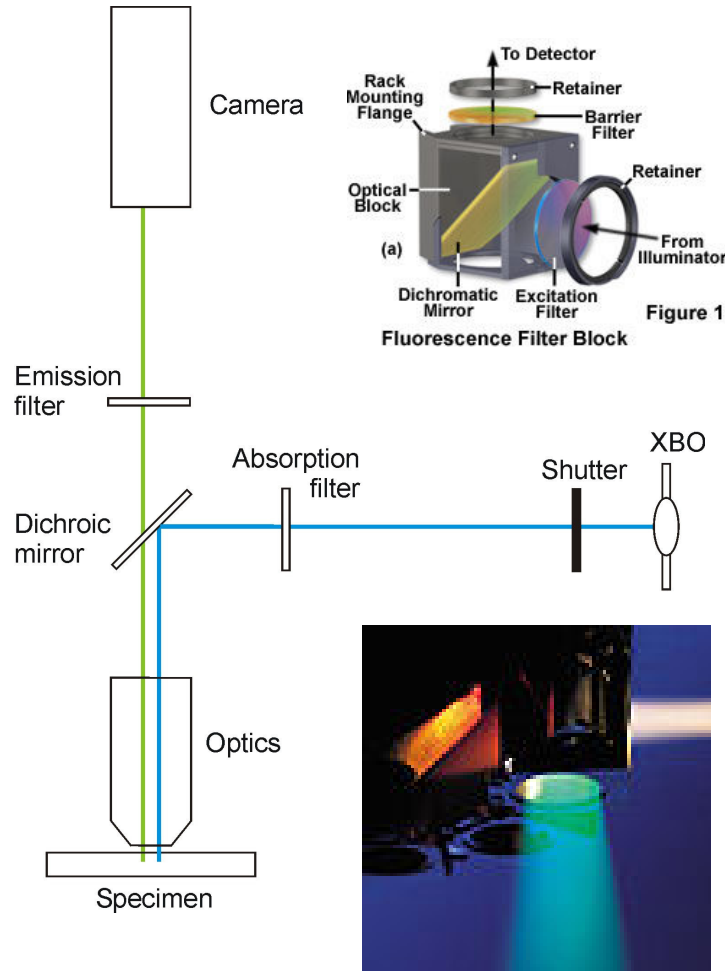
Closing of the shutter reduced illumination to an essential minimum and thereby prevents bleaching of the dye and phototoxicity.

## Techniques : The fluorescence filters



- 1 : Lamp
- 3 : Shutter
- 5 : Absorption filter
- 6 : Beamsplitter
- 7 : Optics
- 8 : Specimen
- 9 : Emission filter
- 10 : Tube lens
- 11 : Eye pieces / Camera

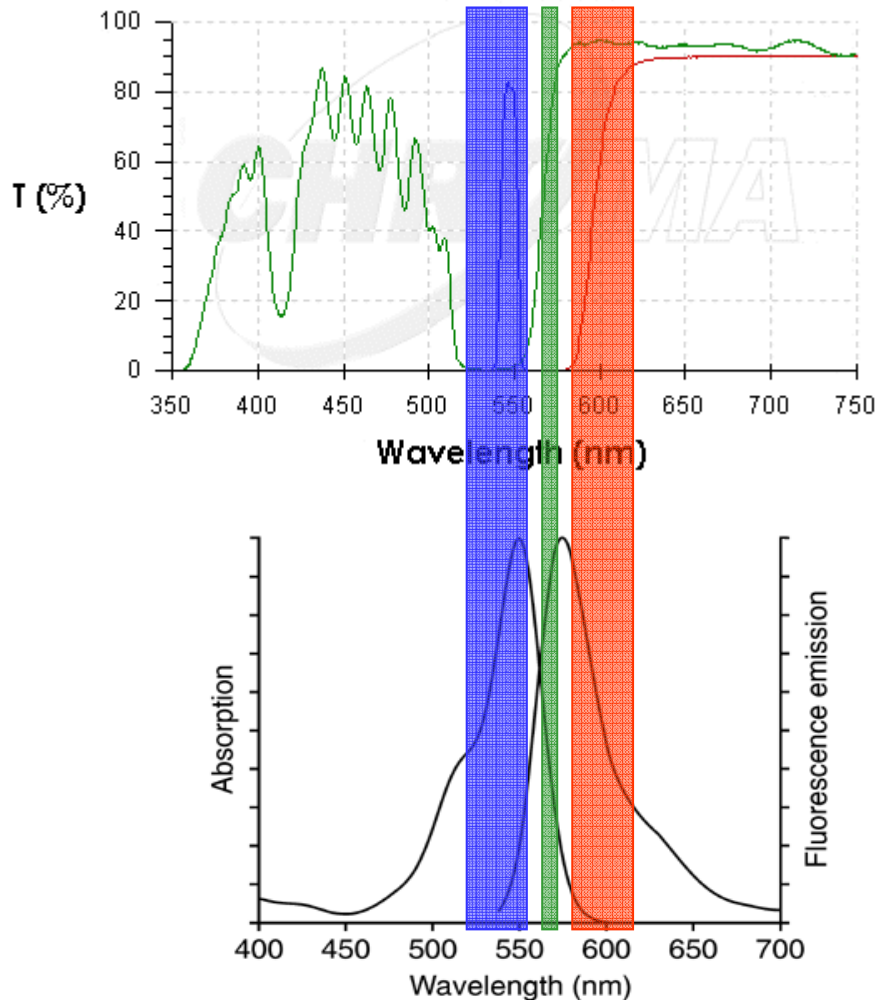
# Techniques : The filter cube



In the filter cube three filters are assembled which were selected to produce the light for the excitation of a specific dye and which let the emitted light pass to the camera / eye pieces:

1. Absorption filter. This bandpass filter blocks all light outside the absorption spectrum of the dye. Not necessary with a laser or monochromator.
2. The dichroic mirror or beamsplitter mirrors the excitation light to the specimen while the emitted light passes this mirror.
3. The emission filter constrains the wavelength of the emitted light. Essential if two dyes are used simultaneously.

# Techniques : Selection of the filters



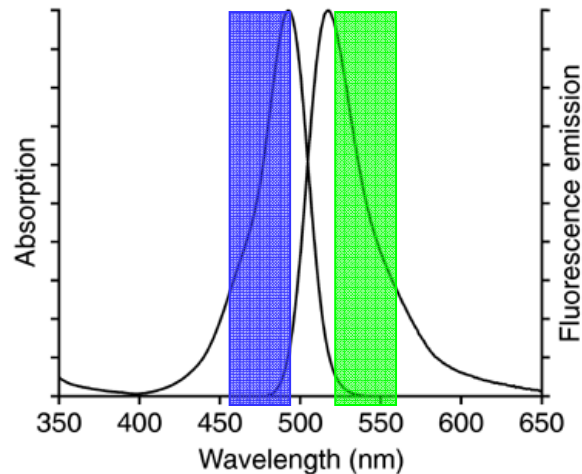
The selection of dyes and filters with matching spectral properties is an essential step for the imaging experiment.

Desired properties of the filters:

1. **Absorption filter**. This filter has a maximal transmission at the maximal absorption of the dye. Thereby the dye is maximally excited.
2. The **dichroic mirror** has a minimal transmission at the absorption wavelength and a maximal transmission at the emission wavelength. Thereby the excitation light is mirrored to the specimen while the emitted light is passing the mirror to the camera or eye pieces.
3. The **emission filter** enables transmission of the emitted light.

Filter settings and absorption / emission spectra of Calcium Orange  
Sources: Chroma Technology / Molecular Probes

# Techniques : Selection of the filters : Two dyes in the same preparation - consecutively

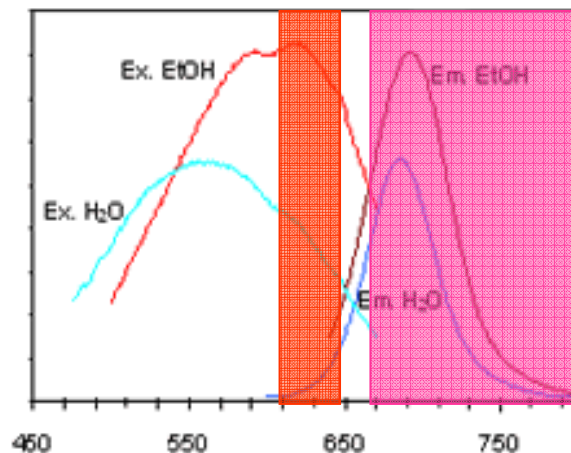


Spectra  
Oregon Green BAPTA-1  
(Molecular Probes);  
CaSD

Essential properties for the work with two or more dyes:

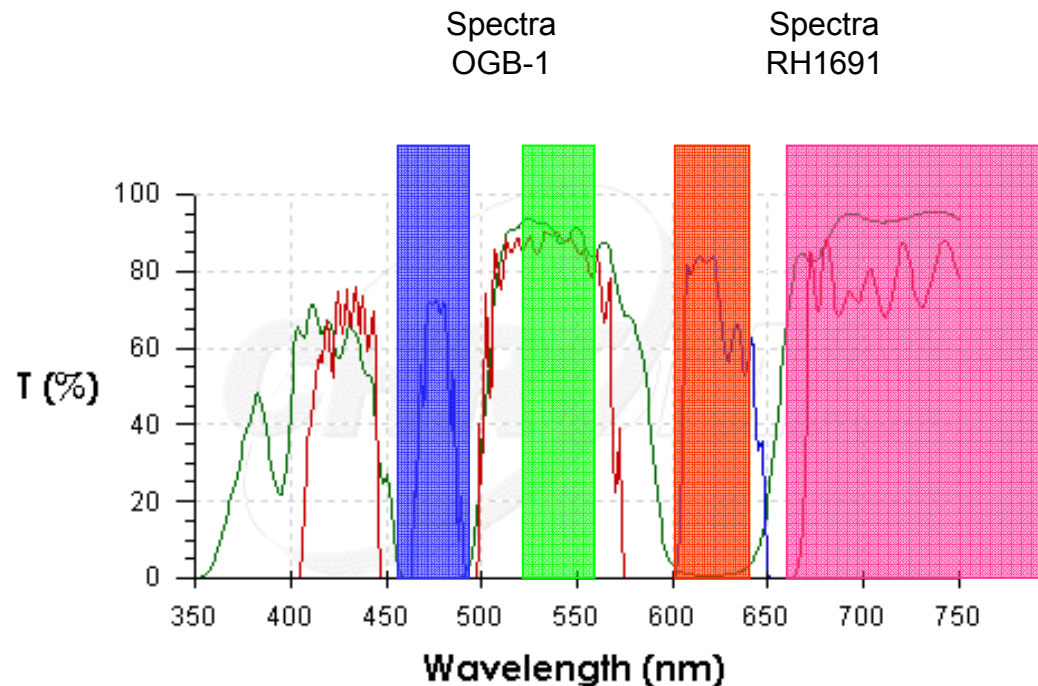
1. The absorption spectra shall not overlap. Otherwise one does not know which emission is visible (“Bleed-through”).
2. If both dyes are excited at the same wavelength, they have to have different emission spectra which are distinguished with a dichroic mirror.
3. If the emission of dye 1 is partially overlapping with the absorption of dye 2, the emission filter of dye 1 has to be a bandpass filter. With a longpass filter in that position one could also see indirect fluorescence from dye 2.

Spectra RH1691  
(Optical Imaging);  
VSD





# Techniques : Selection of the filters : Two dyes in the same preparation - simultaneously



Simultaneous Excitation and Emission  
of Oregon Green BAPTA-1 and RH1691

Filterset 51008 taken from webpage of Chroma (<http://chroma.com/>)

There are the same prerequisites as if two dyes are imaged consecutively.

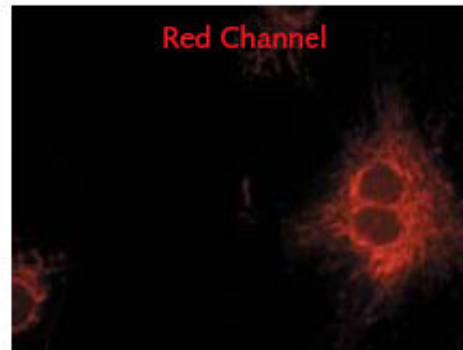
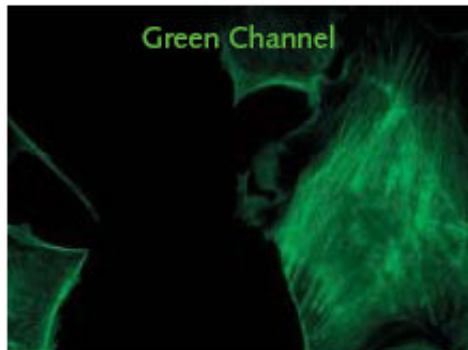
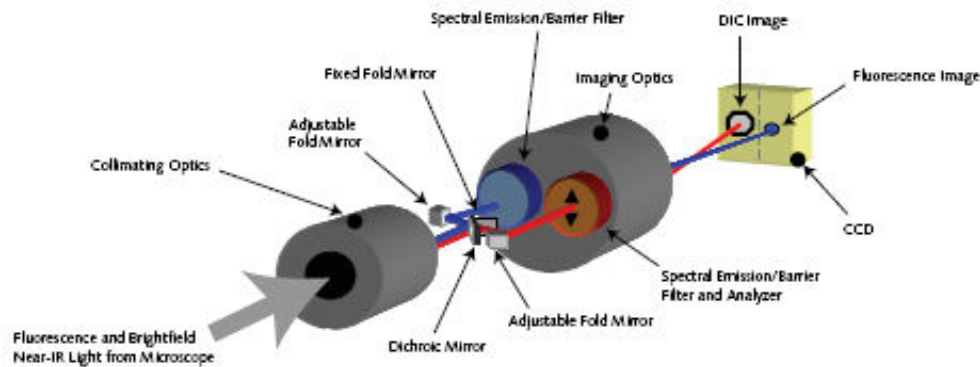
In addition, one has to work with an excitation filter which mirrors both wavelengths necessary for both dyes. Alternatively one can couple in two lasers or use a fast monochromator jumping between both excitation wavelengths (not real simultaneous).

The dichroic mirror needs two low transmission gaps for the excitation light and two transmission peaks for the emitted light.

The emission filter needs two high transmission gaps for the emitted light.

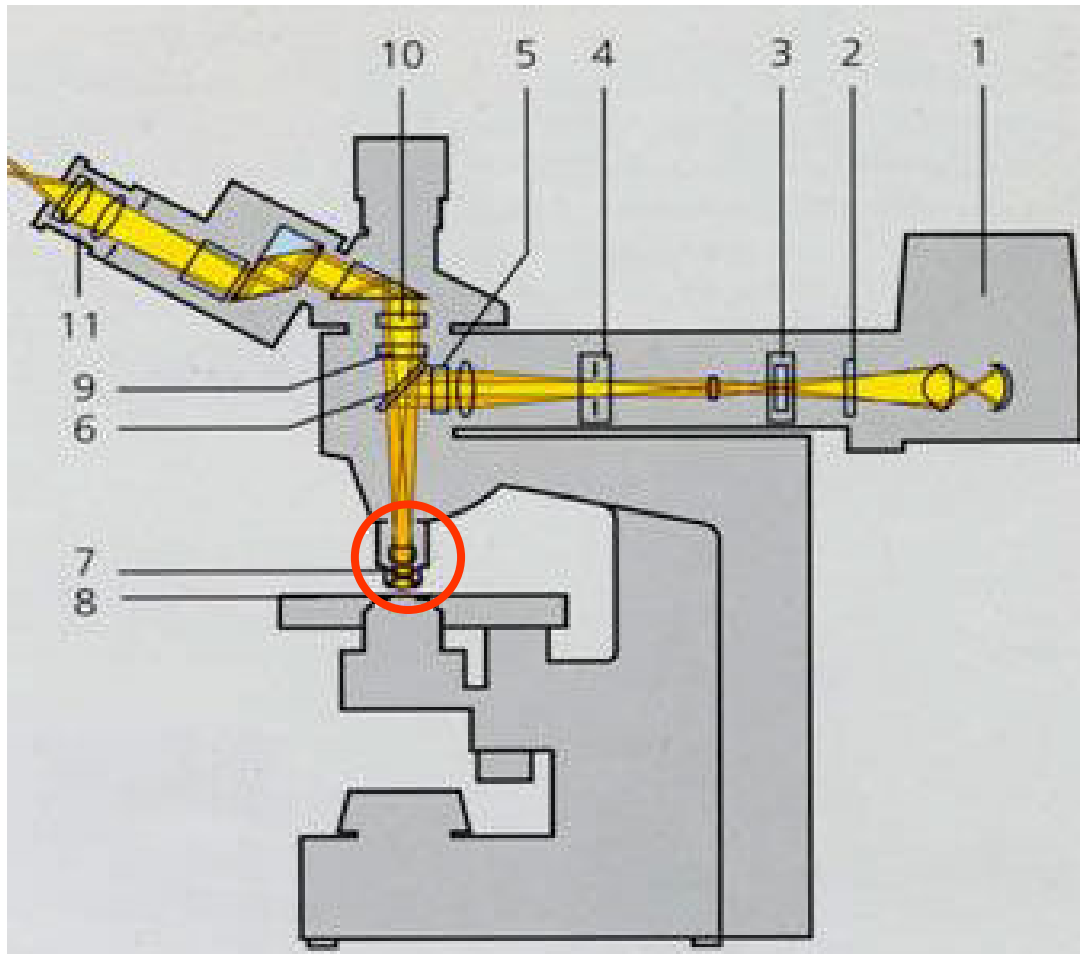
# Techniques : Visualization :

## Two dyes in the same preparation - simultaneously



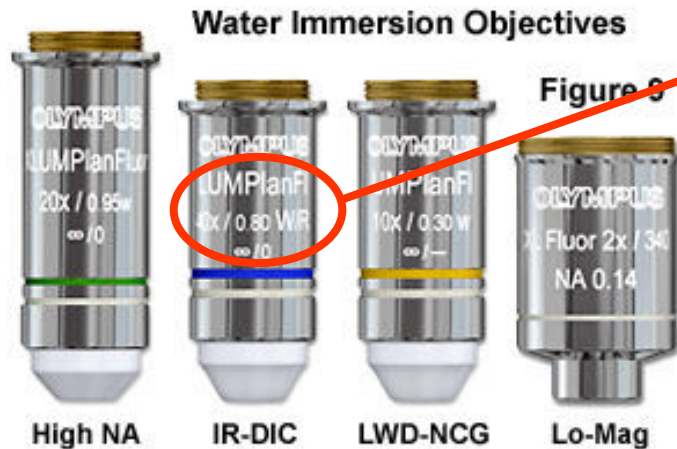
To visualize both dyes separately which have been excited and are emitting simultaneously one needs an additional beamsplitter in the optic path which takes both emitted wavelengths apart.

## Techniques : The optics



- 1 : Lamp
- 3 : Shutter
- 5 : Absorption filter
- 6 : Beamsplitter
- 7 : Optics
- 8 : Specimen
- 9 : Emission filter
- 10 : Tube lens
- 11 : Eye pieces / Camera

# Techniques : The optics



Key features of the optics:

40x / 0.8 means 40 times magnification and a numerical aperture (N.A.) of 0.8 .

Magnification determines the field of view which can be finally seen with the camera. For single cell imaging 40x or 60x optics are well suited, for network or in vivo activity 10x or even smaller optics.

Numerical aperture describes the opening angle of the optics and the diffractive properties of the medium the front lens is in contact with (maximal N.A. air 1.0, water 1.33, oil 1.56). Optics with higher magnification have higher N.A. values. The higher the N.A. the better the resolution of the optics and the brighter the picture. High N.A. values are therefore especially important for fluorescence detection.

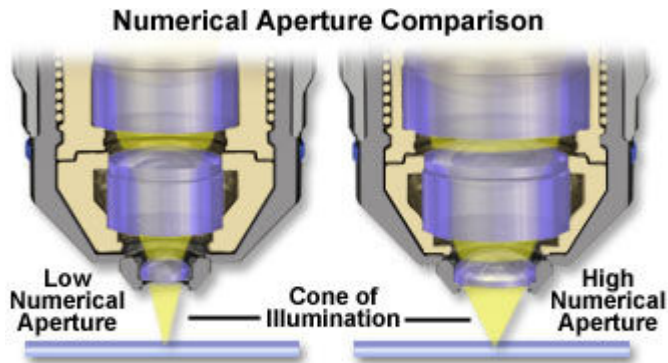
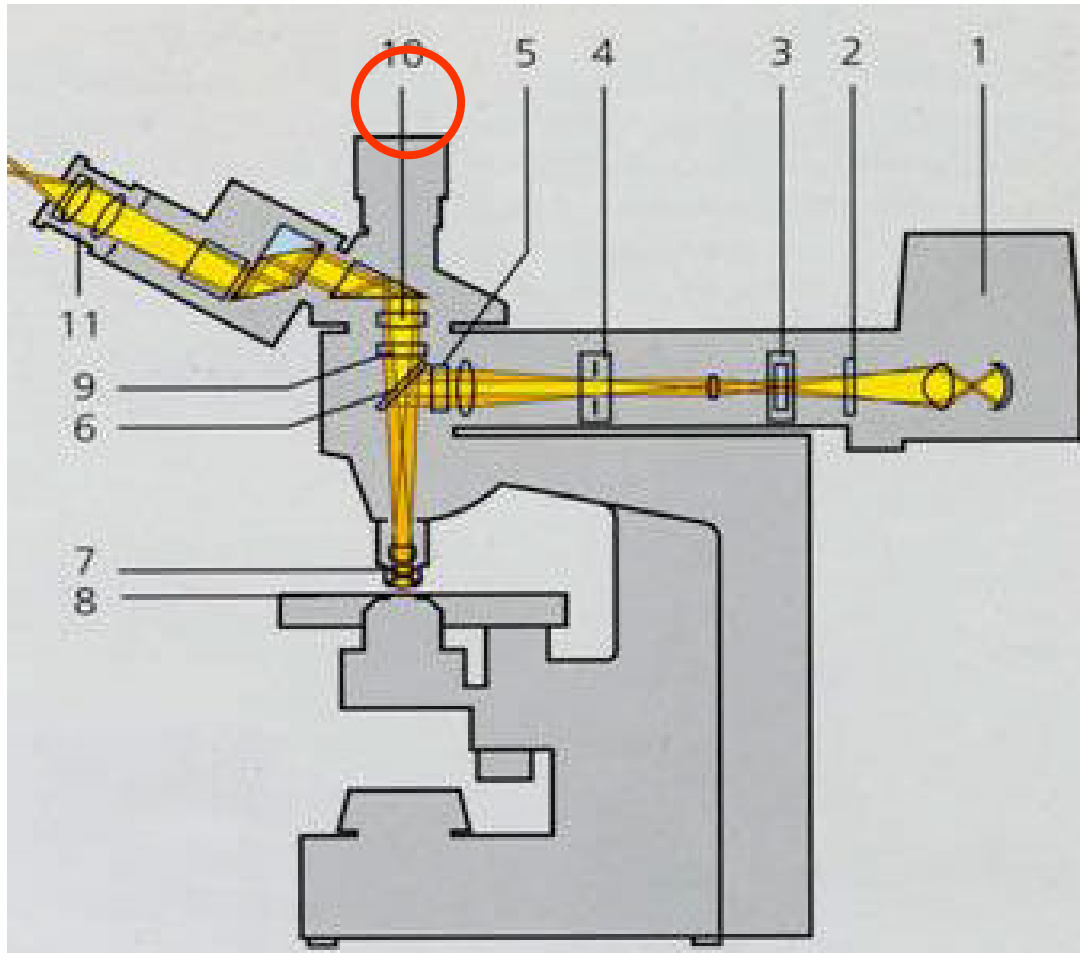


Figure 2

Discrepancy between large field of view and high N.A. for the detection of network activity !!

## Techniques : Recording devices



- 1 : Lamp
- 3 : Shutter
- 5 : Absorption filter
- 6 : Beamsplitter
- 7 : Optics
- 8 : Specimen
- 9 : Emission filter
- 10 : Tube lens
- 11 : Eye pieces / Camera

# Techniques : Recording devices

Large diversity of recording devices:

Photodiode arrays and CCD cameras are the most important ones

Choice depends on:

Object to be imaged: single cells, brain slice, in vivo

Resolution necessary (pixel size)

Field of view necessary (chip size)

Signal to noise ratio of the dye (if bad, you need to put as much light in the system as possible)

Saturation properties (well size)

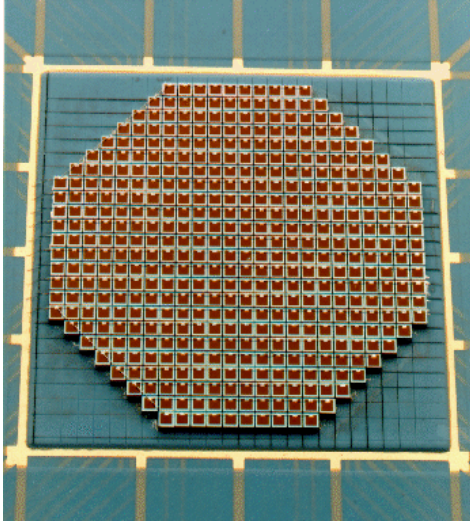
Sensitivity (quantum efficiency, back-illuminated or not)

Temporal dynamics of the processes to be imaged (readout rate)

.....

Will present three examples.

# Techniques : Photodiode array



Low spatial resolution – fast temporal resolution – a lot of light is necessary to get the signal out of the noise

To achieve this only a restricted number of detection diodes with a large surface is used (e.g. 24 x 24 diodes with a surface of 100 x 100  $\mu\text{m}$ ). These diodes are not saturated even with one billion electrons.

Dyes with a bad signal to noise ratio like voltage-sensitive dyes need as much light as possible to compensate for this disadvantage. Therefore the diodes have to be able to collect as much photons as possible. Due to the small amount of diodes which have to be read out, this process can be pretty fast (kHz range). On the other side spatial resolution is lost.

Ideal for network studies of populations of neurons which act in a temporally correlated way (e.g. epilepsy).



# Techniques : charge-coupled device (CCD) cameras

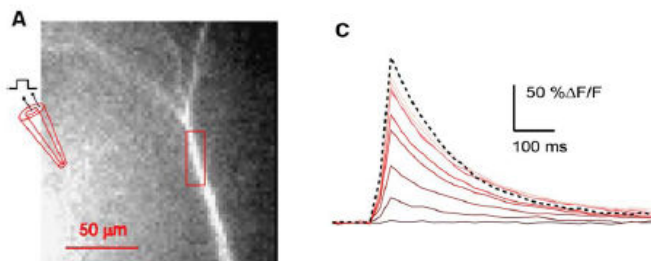


High spatial resolution – slow temporal resolution – signal to noise ratio is no problem

To achieve this charge-coupled devices with a large amount of small pixels are used (e.g. 1200 x 1200 pixels with a surface of 6 x 6  $\mu\text{m}$ ). These pixels can be saturated with e.g. 30'000 electrons.

Dyes with good signal to noise ratios like calcium-sensitive dyes do not need a lot of light. Therefore the pixels can have a small well depth and can be pretty small. Due to the large amount of pixels which have to be read out, this process can take relatively long ( $\sim 10\text{-}50$  Hz). Not suited if the signal is too fast (e.g. voltage sensitive dyes and action potentials). On the other side spatial resolution is very good.

Ideal for studies of single neurons or subcellular processes (e.g. dendrites, spines) using calcium-sensitive dyes.





# Techniques : CCD cameras with large pixels

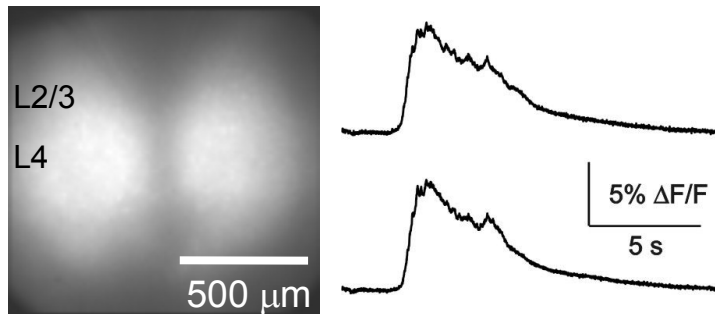


Intermediate spatial resolution – high temporal resolution – can be well used for CaSD and VSD

To achieve this an intermediate number of pixels with an intermediate surface is used (e.g. 80 x 80 diodes with a surface of 24 x 24  $\mu\text{m}$ ). These can work with relatively high amounts of light ( $\sim 1$  million electrons).

These CCD cameras can work with VSDs characterized by a bad signal to noise ratio. However it is also possible to use them for calcium imaging. Still pretty fast (kHz range). Intermediate spatial resolution.

A very good compromise between perfect spatial and temporal resolution. Can be used on the single-cell and the network level.



Lower part taken from: Neubauer & Berger, unpublished data

# Principles of Imaging Techniques

Introduction: Light and Fluorescence

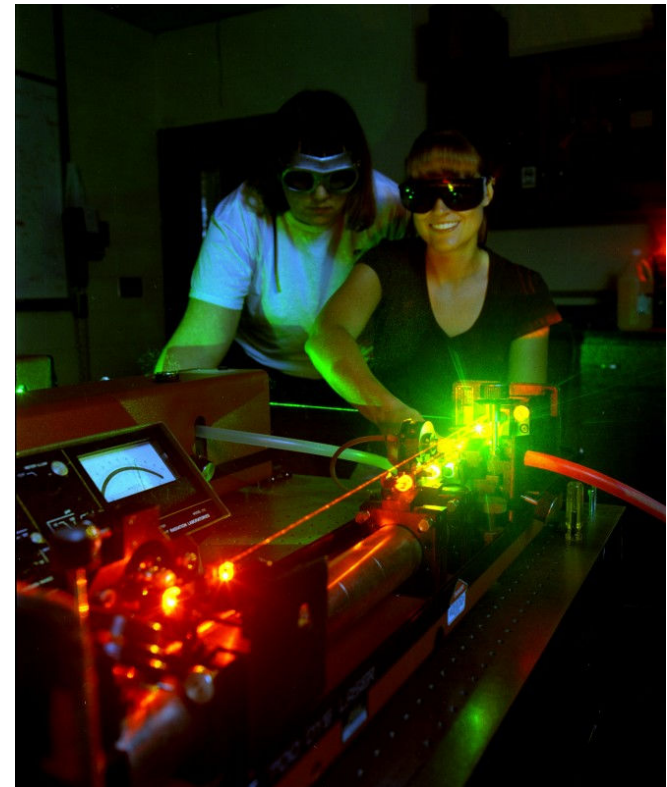
Imaging techniques: dyes

Imaging techniques: equipment

Epifluorescence imaging

Two-photon imaging

Confocal imaging



# Two-photon imaging

Excitation: The exciting light has the double wavelength, i.e. half the energy necessary to excite the dye. Absorption depends on intensity and therefore the dye is only excited in a tiny volume in the focus, not below, above or aside. The tissue is scanned with the laser beam.

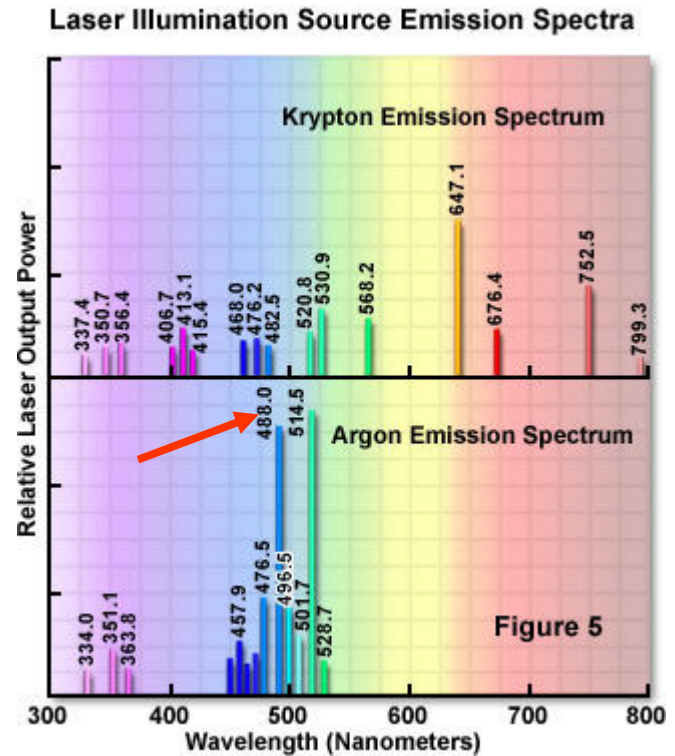
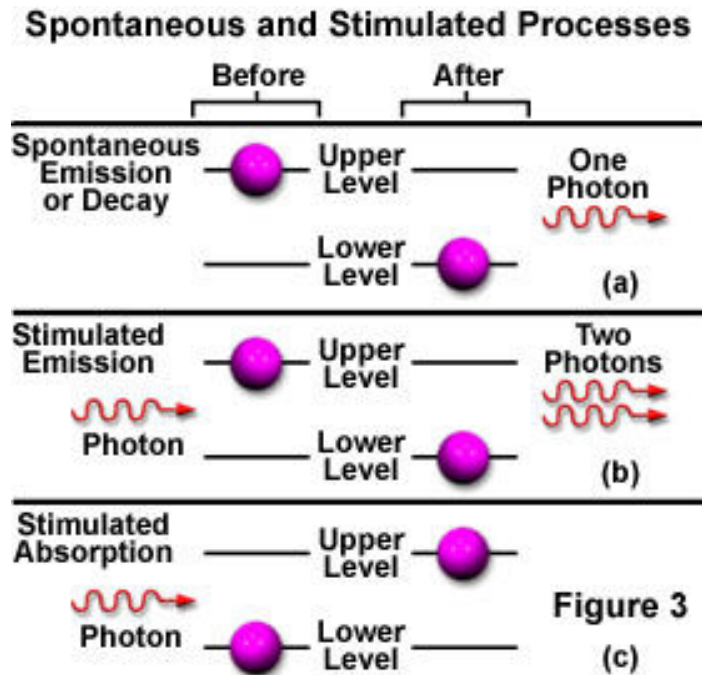
Emission: All emitted fluorescence is from the focus and is collected with photomultipliers from above and below the specimen. Spatial and temporal properties of the emitted signal have to be re-calculated.

Advantages: All fluorescence is from a defined structure. Photodamage is prevented because only a small volume gets sufficient intensity for excitation.

Disadvantages: Imaging is restricted to relatively small volumes and structures. Only optics with a high numerical aperture (and therefore high magnification) produce a sharp focus. Relatively slow. Costs!

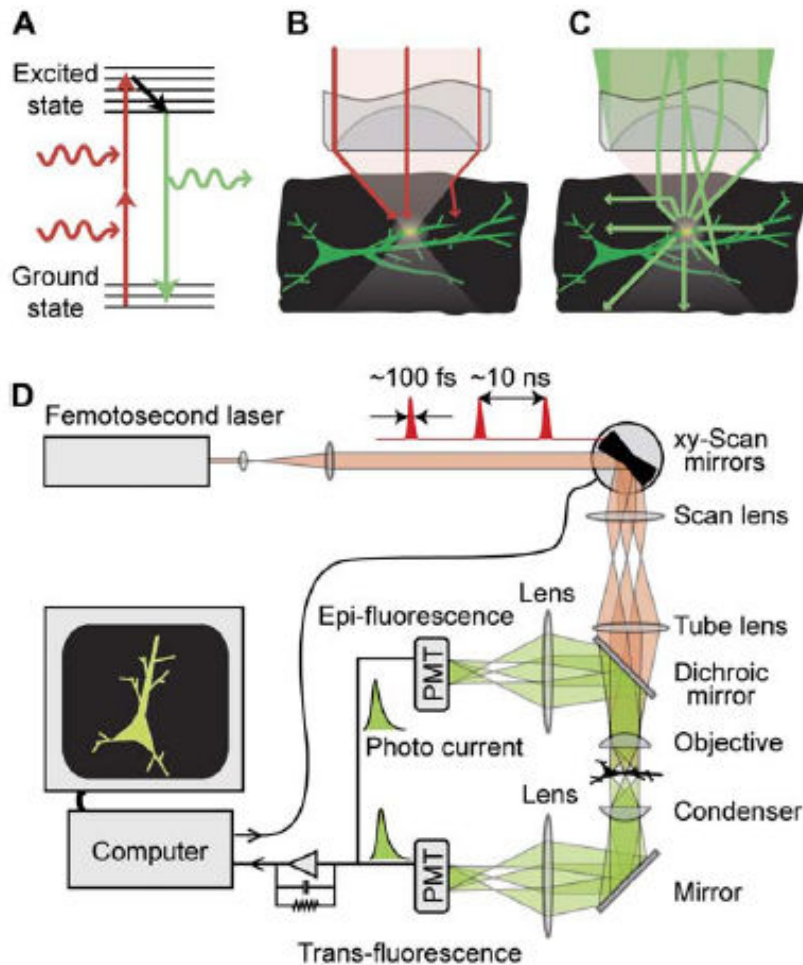
# Light sources for Two Photon Imaging : Lasers

Light **A**mplification by the **S**timulated **E**mission of **R**adiation -  
 Reversal of the stimulated absorption



Advantage of Lasers: Very high intensity at some  $\lambda$ , e.g. Argon laser at 488 nm  
 Disadvantage: Use only at these wavelengths possible, costs

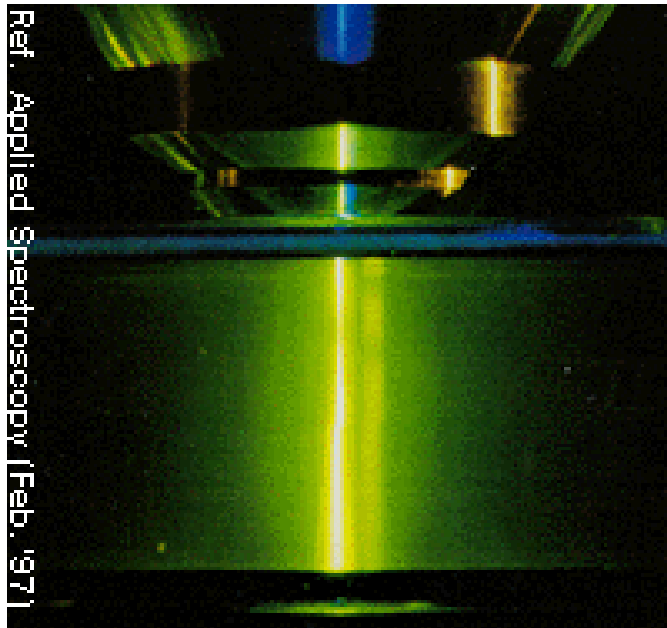
# Two-photon Imaging : Reduction of noise on the absorption side



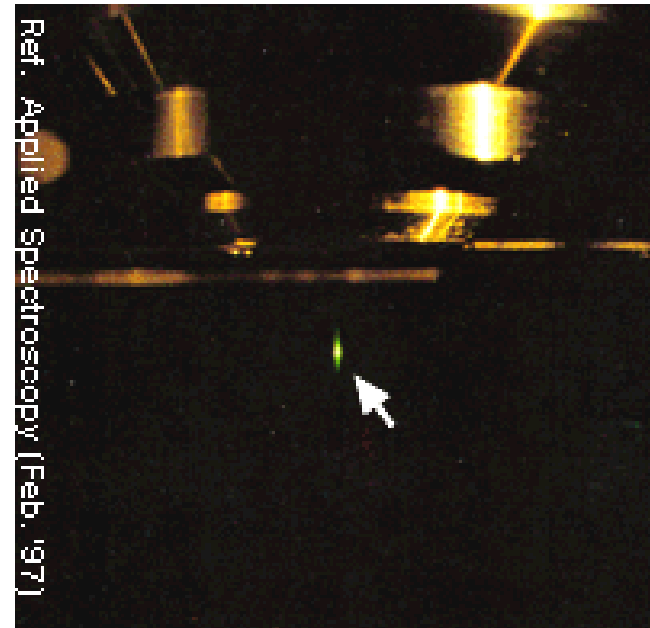
- (A) Simplified Jablonski diagram of the 2PI process.
- (B) Localization of excitation in a scattering medium (black). The excitation beam (red) is focused to a spot by an objective where it excites green fluorescence in a defined dendritic branch. The paths of two ballistic photons and one scattered photon are shown (red lines). Scattered photons are too dilute to cause off-focus excitation.
- (C) Fluorescence collection in a scattering medium. Fluorescence photons are emitted from the excitation volume. Even scattered fluorescence photons contribute to the signal if they are collected by the objective.

# Two-photon Imaging : Reduction of noise on the absorption side

One-Photon  
Fluorescence Microscopy



Two-Photon  
Fluorescence Microscopy



# Principles of Imaging Techniques

Introduction: Light and Fluorescence

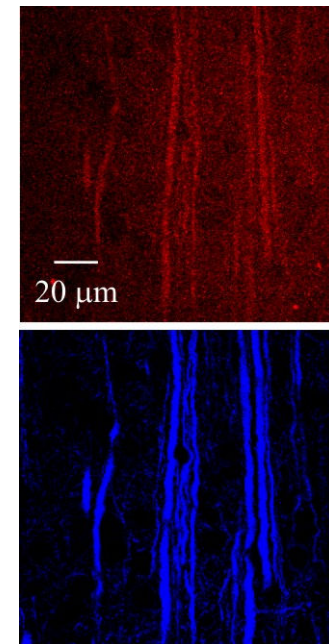
Imaging techniques: dyes

Imaging techniques: equipment

Epifluorescence imaging

Two-photon imaging

Confocal imaging



## **Confocal techniques: Laser Scanning Confocal Microscopy (LSCM)**

**Excitation:** The exciting light is produced by a laser and mirrored on the preparation. Excitation is strongest at a focal level defined by the optics and a pinhole aperture, but also present above and below. To reduce bleaching and damage the focus level is scanned (X-Y or X-t).

**Emission:** Fluorescent light will be emitted from the focus but also from above and below. The Z-level of the obtained picture is defined by a second confocal pinhole which prevents that the majority of out-of-focus light enters the photomultiplier. Descanning gives the real X-Y distribution.

**Advantages:** The signal belongs to a defined depth. Photodamage is prevented by scanning the preparation. Three-dimensional Z-stacks are possible by calculation from X-Y pictures. A relatively high speed can be obtained by linescans (X-t scans).

**Disadvantages:** Because the majority of light cannot reach the chip, one needs powerful light sources like lasers. The signal source is limited to one level. Scanning is relatively slow. Costs!

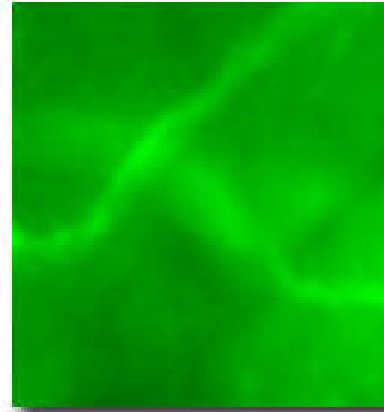


## Confocal advantages: no blurred details

Epifluorescence



(a)



(b)

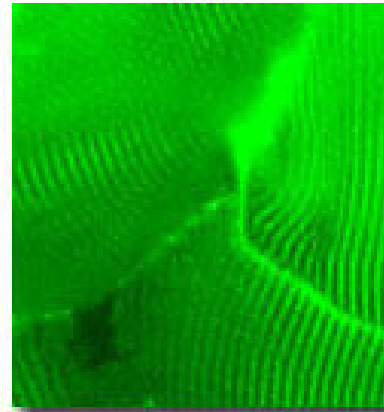


(c)

Confocal



(d)

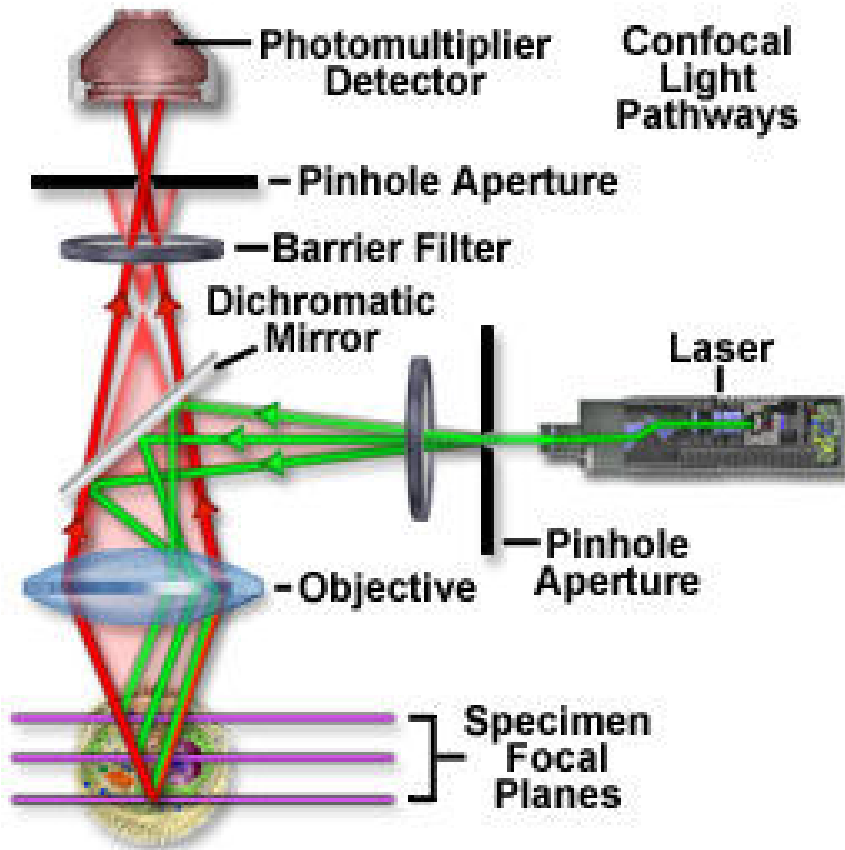


(e)



(f)

## Confocal Techniques : Reduction of noise on the emission side



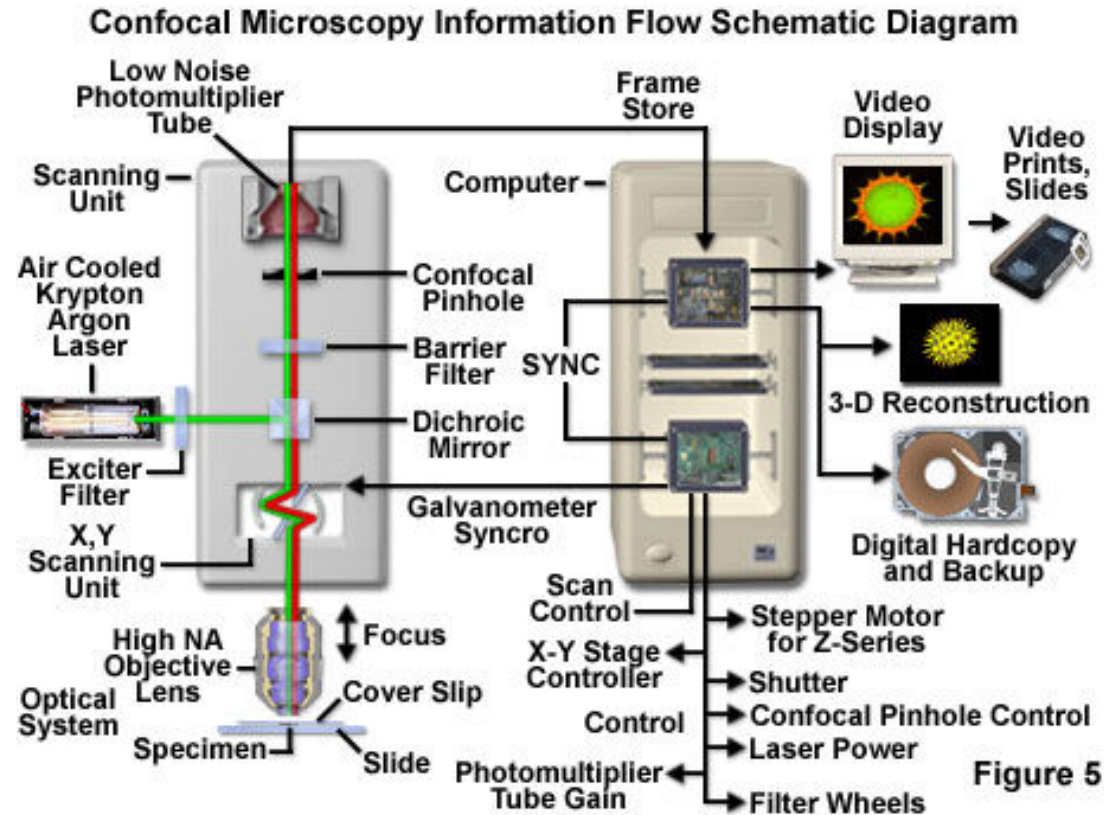
Source: Olympus Microscopy Source Center

Coherent light emitted by the laser passes through a pinhole aperture that is situated in a conjugate plane (confocal) with a scanning point on the specimen and a second pinhole aperture positioned in front of the detector.

As the laser is scanned across the specimen in a defined focal plane, fluorescence emitted from points on the specimen (in the same focal plane) are focused as a confocal point at the detector pinhole aperture.

The significant amount of emission that occurs at points above and below the objective focal plane is not confocal with the pinhole and therefore not seen by the detector.

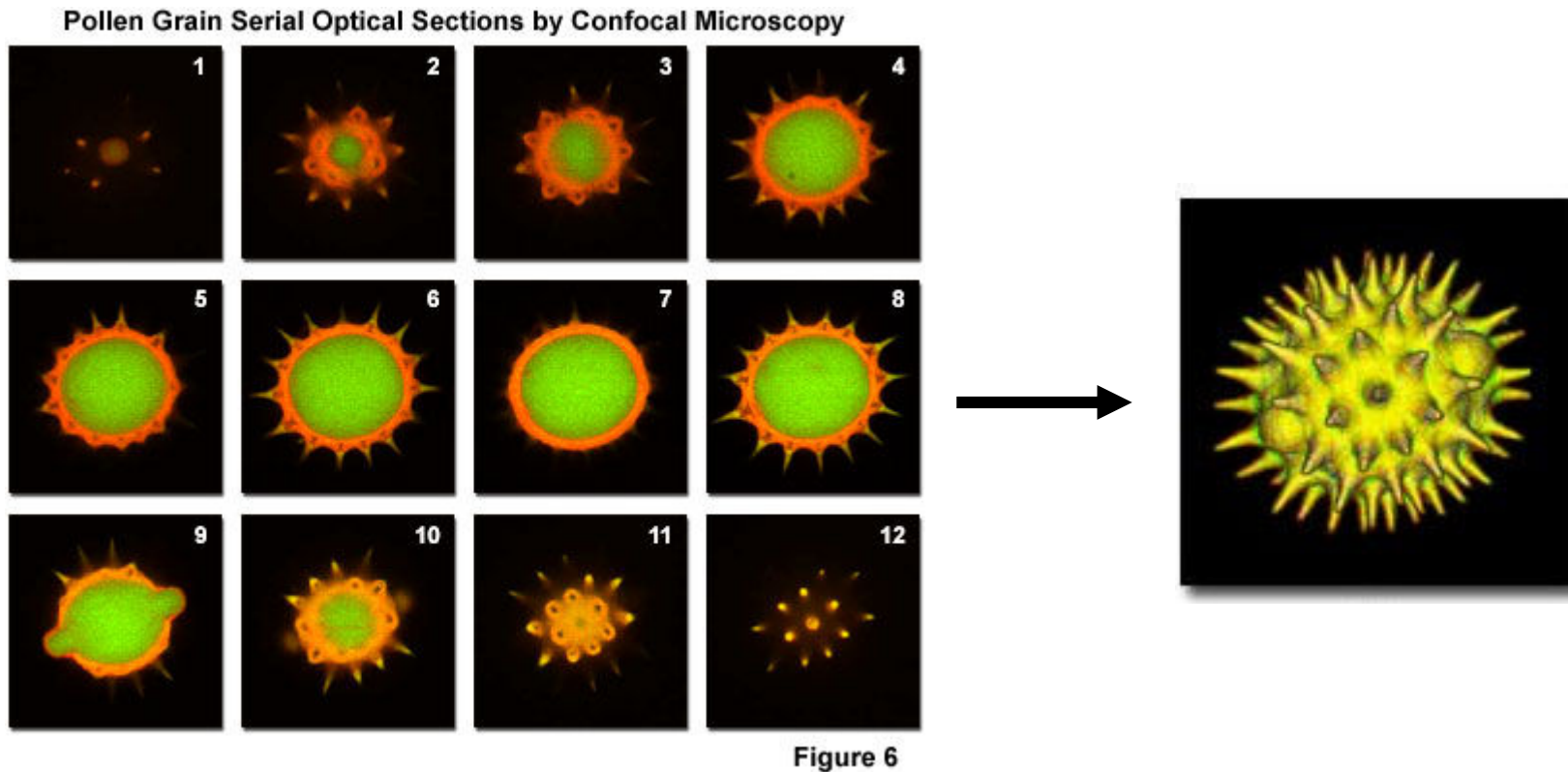
# Confocal Techniques : Recalculating the two-dimensional picture



The X-Y picture of the specimen has to be recalculated because the photomultiplier does not detect a real X-Y picture just the fluorescence at a given time point related to a given scanning position.

## Confocal Techniques :

The three-dimensional structure can be obtained from Z-stacks



The X-Y-Z picture of the specimen can be recalculated from a series of X-Y pictures (so called Z-stacks).

# Comparison Epifluorescence Imaging, Confocal Imaging and Two-Photon Imaging

Epifluorescence Imaging : High temporal and spatial resolution imaging technique in very large areas like whole brain hemispheres. The source of the signal is not completely clear due to the collection from out-of-focus fluorescence. Perfectly suited for physiological studies of fast activity.

Two-photon imaging : Good temporal and perfect spatial resolution in small volumes. Perfect definition of the signal source. Perfectly suited for physiological studies in small subcellular compartments.

Confocal Imaging : Well defined spatial resolution in small volumes. Bad temporal resolution. Better suited for morphological than for physiological studies.