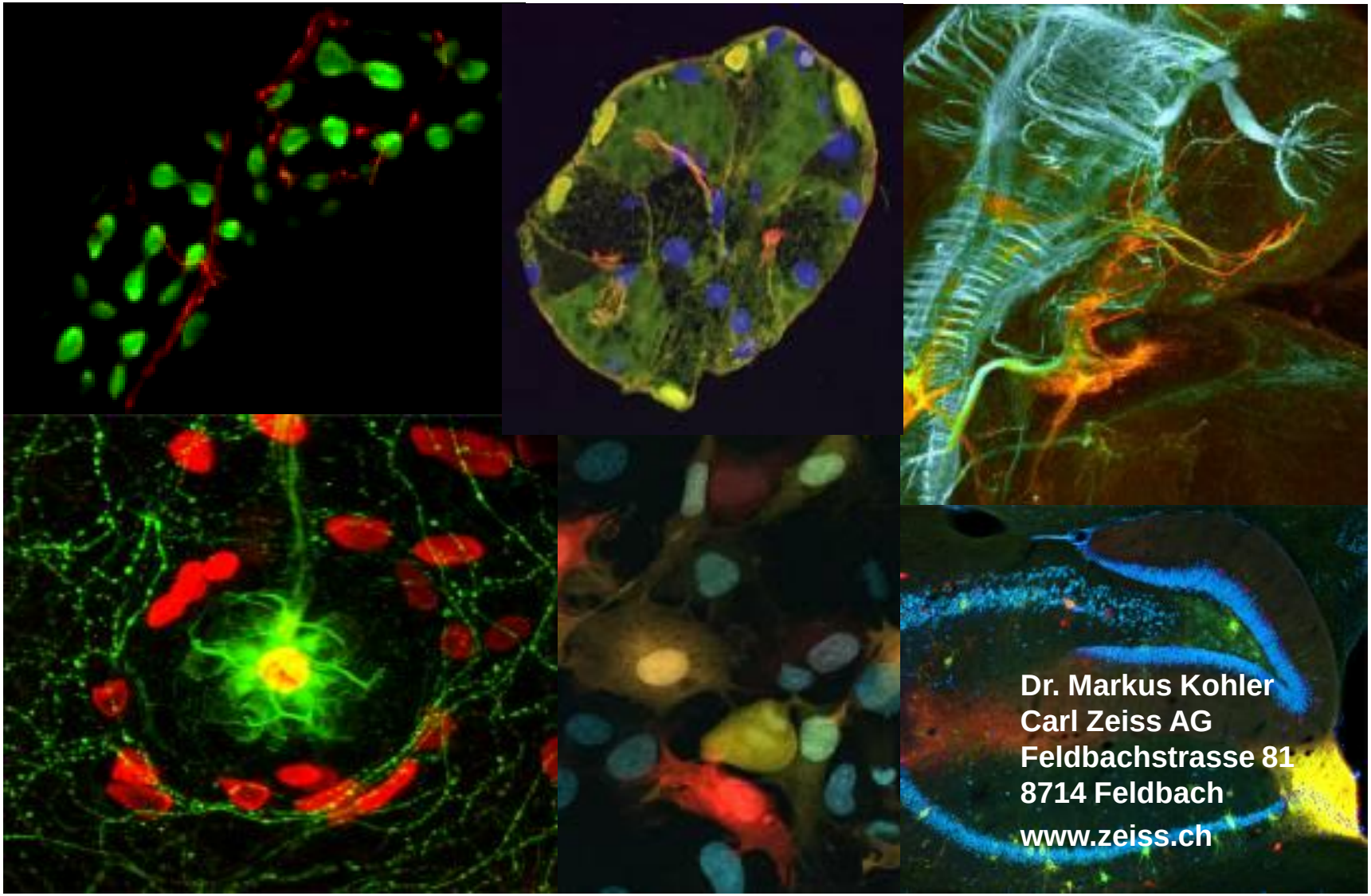
Introduction to Multiphoton Laser Scanning Microscopy



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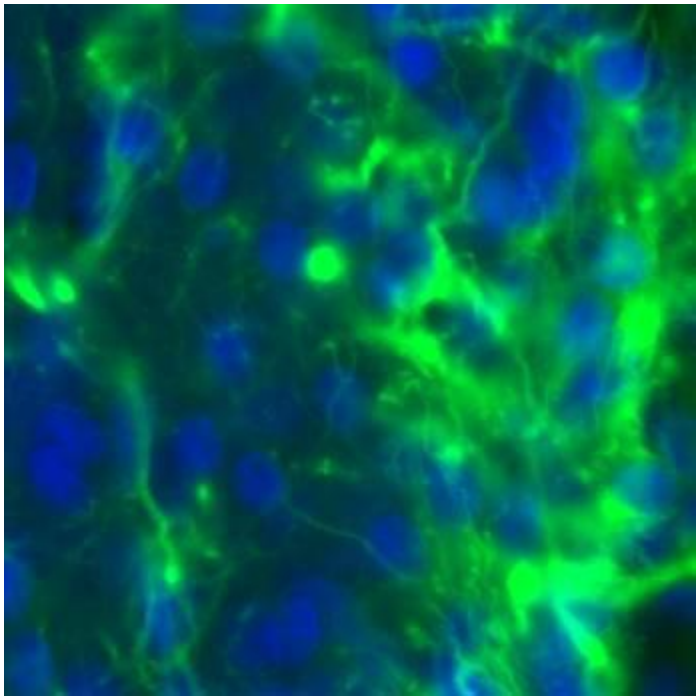
8714 Feldbach
www.zeiss.ch

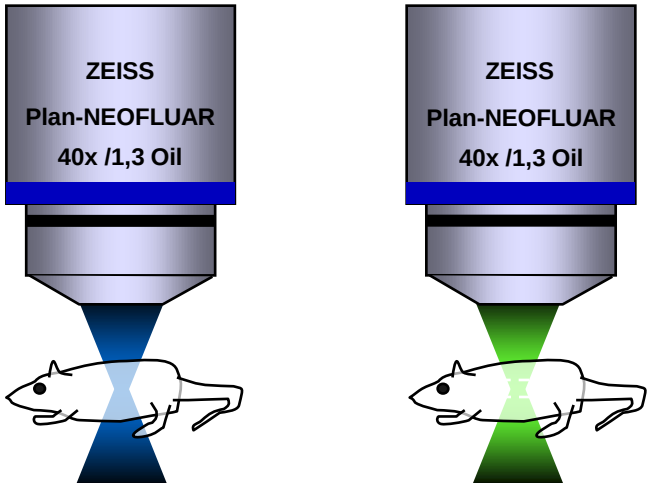
Topics

- Fluorescence Microscopy
- Confocal Laser Scanning Microscopy
- Multiphoton Microscopy

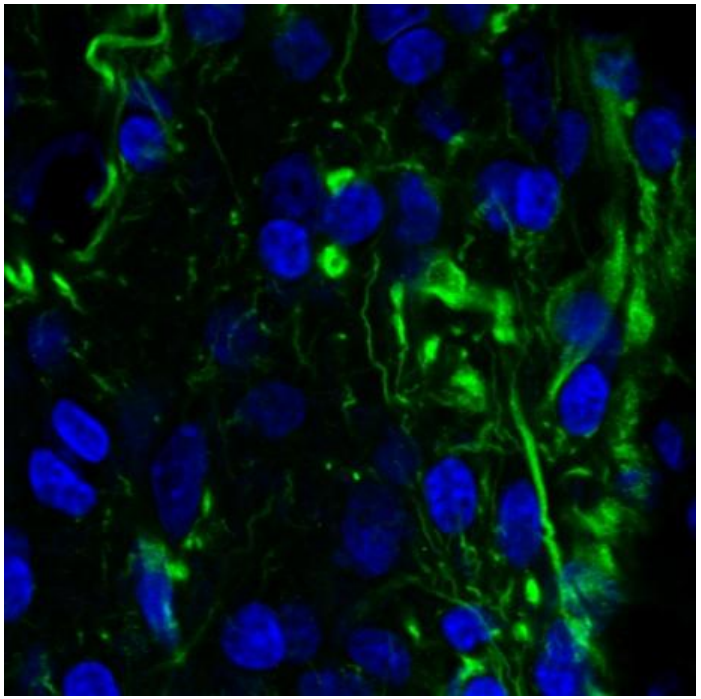
Confocal Laser Scanning Microscopy

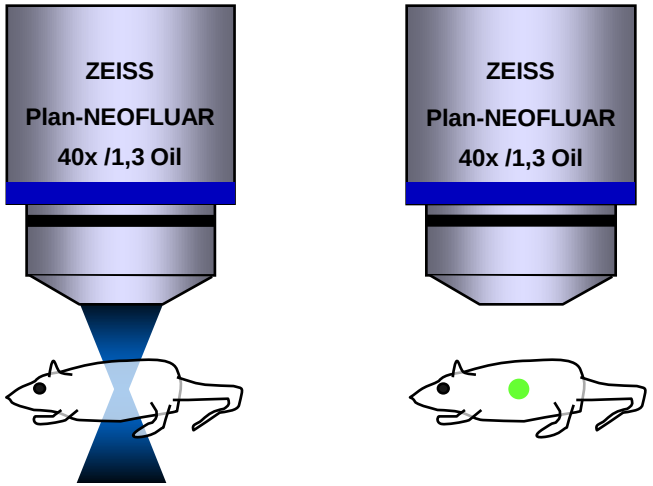
Conventional fluorescence microscopy





Optical sectioning with confocal microscopy





Fluorescence Microscopy

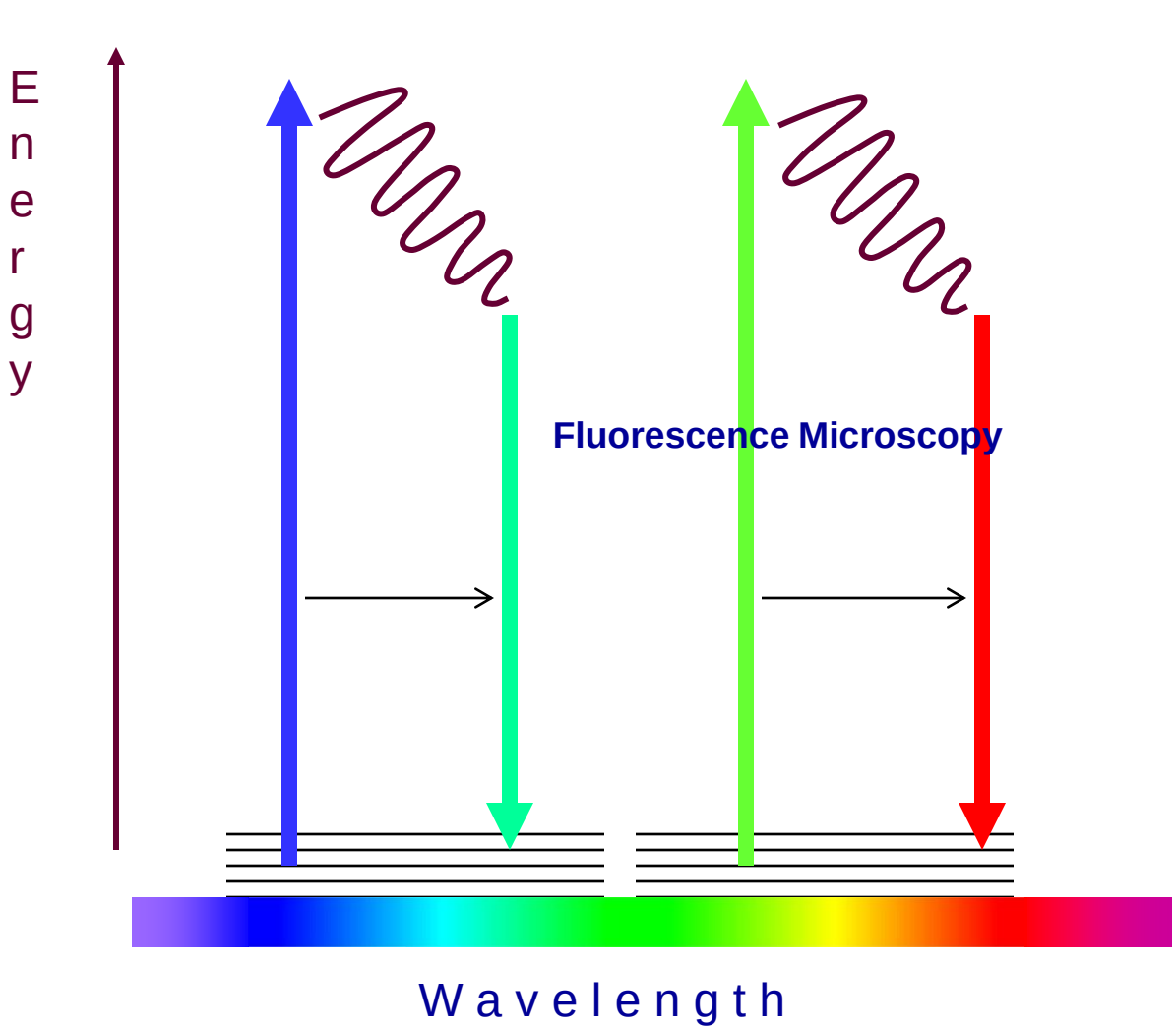
- Absorption of light rises a fluorochrome molecule to an excited state of higher energy content

- The molecule remains in the excited state only for a very short period of time (nsec range)

- The way back to the basic energy level is accompanied by the emission of light (fluorescence)

- Due to internal energy dissipation the emitted light has a longer wavelength (=lower energy) than the exciting light (Stokes shift)

- The quantity of emitted light is very small compared to the quantity of excitation light



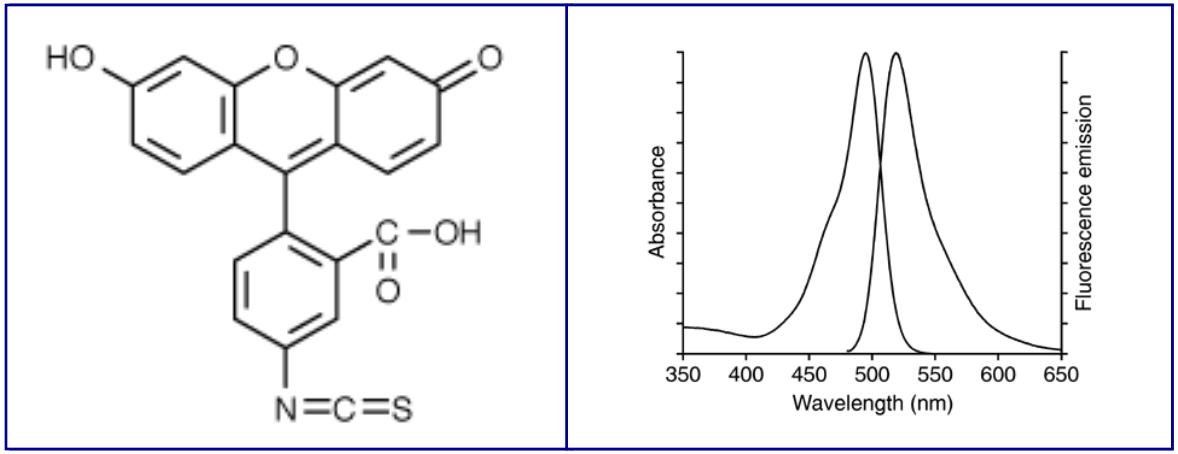
Excitation light

Internal energy dissipation

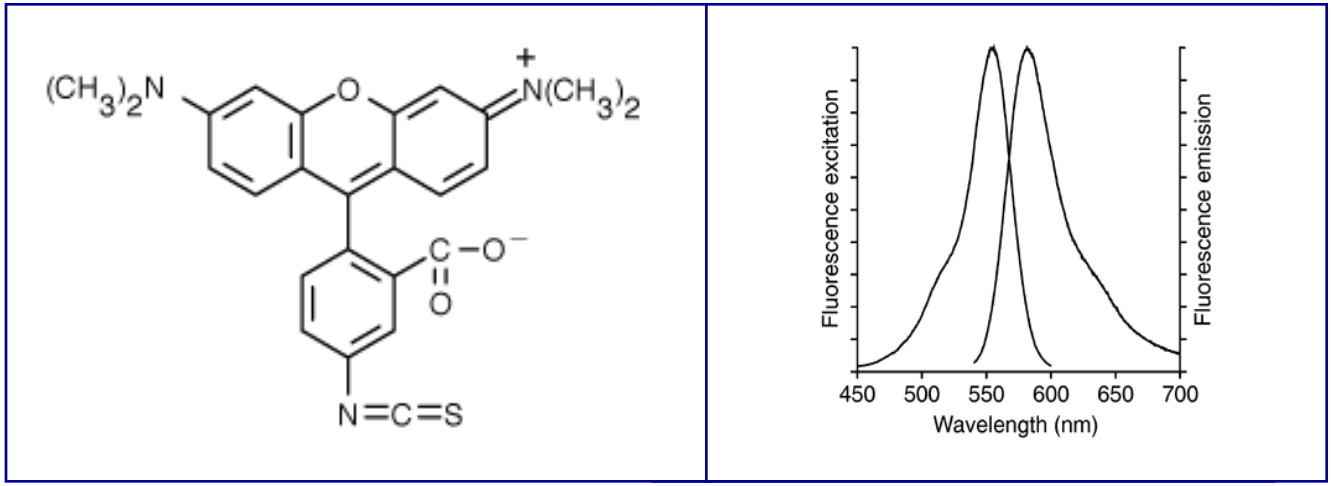
Stokes shift

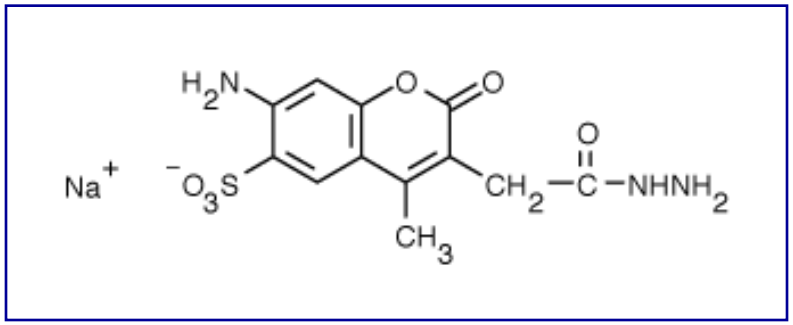
Fluorescence

FITC Fluorescein-5-isothiocyanat (Isomer I)

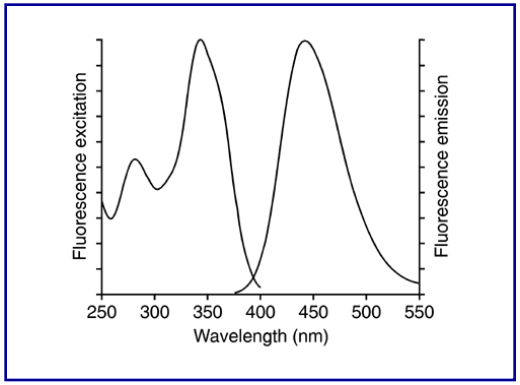


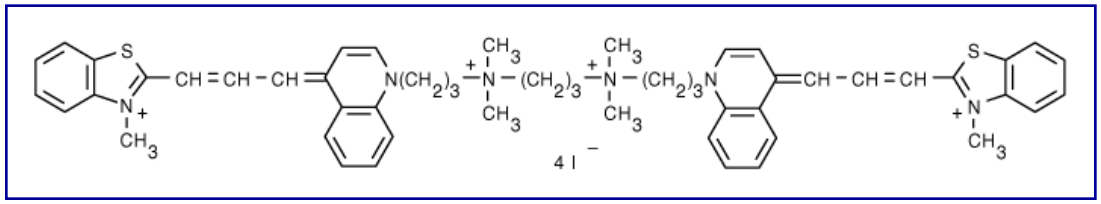
Rhodamine, TRITC Tetramethylrhodamine -5 isothiocyanate(5-TRITC; G isomer)



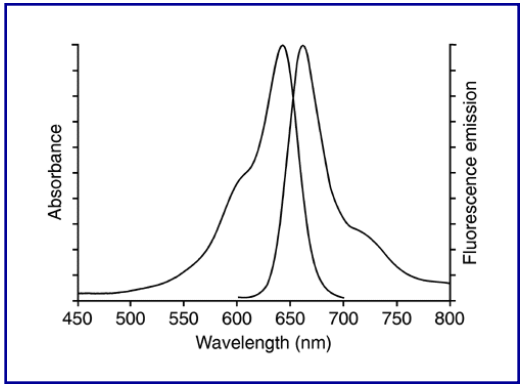


Alexa Fluor™ 350 hydrazide, sodium salt

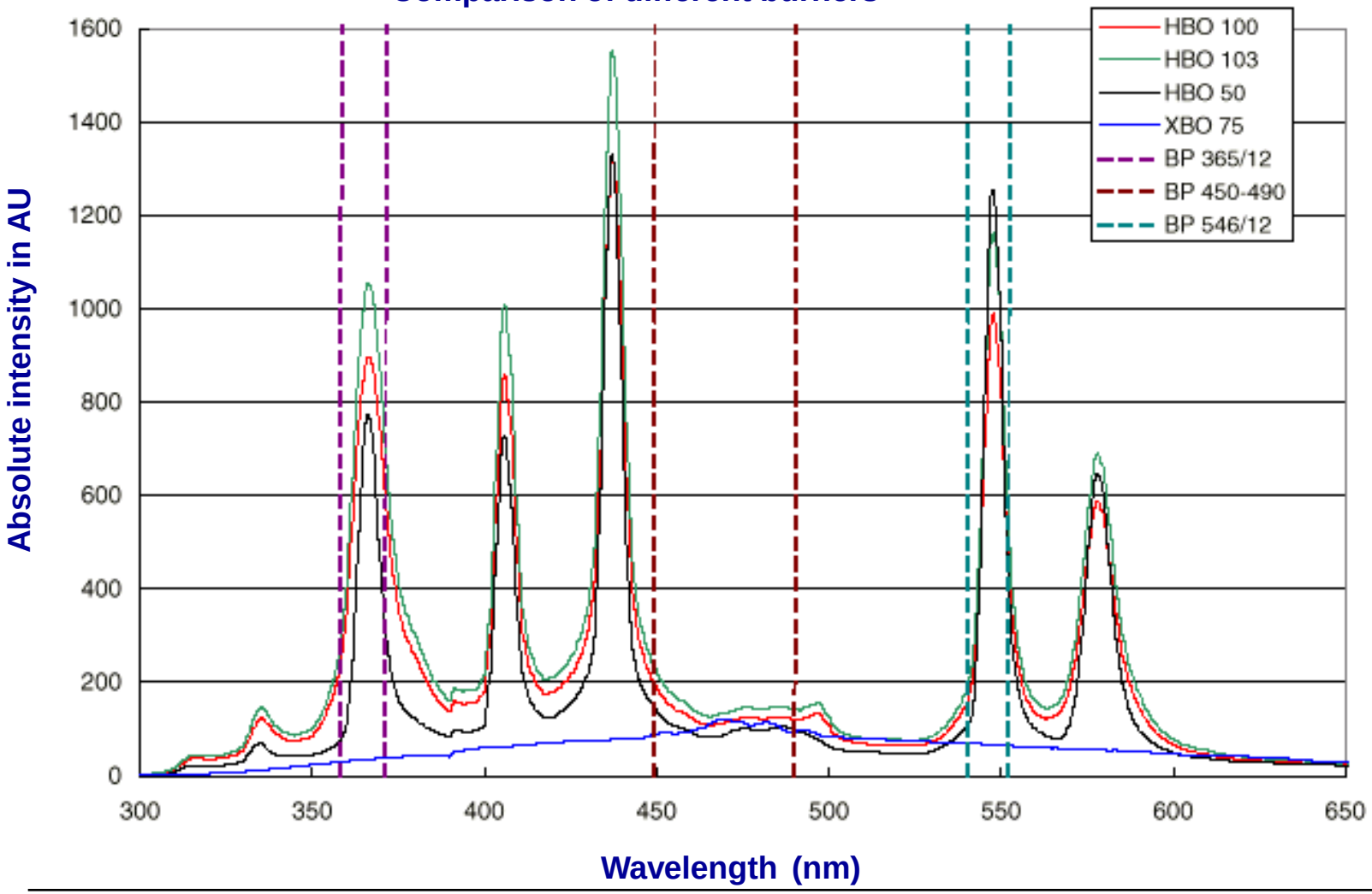


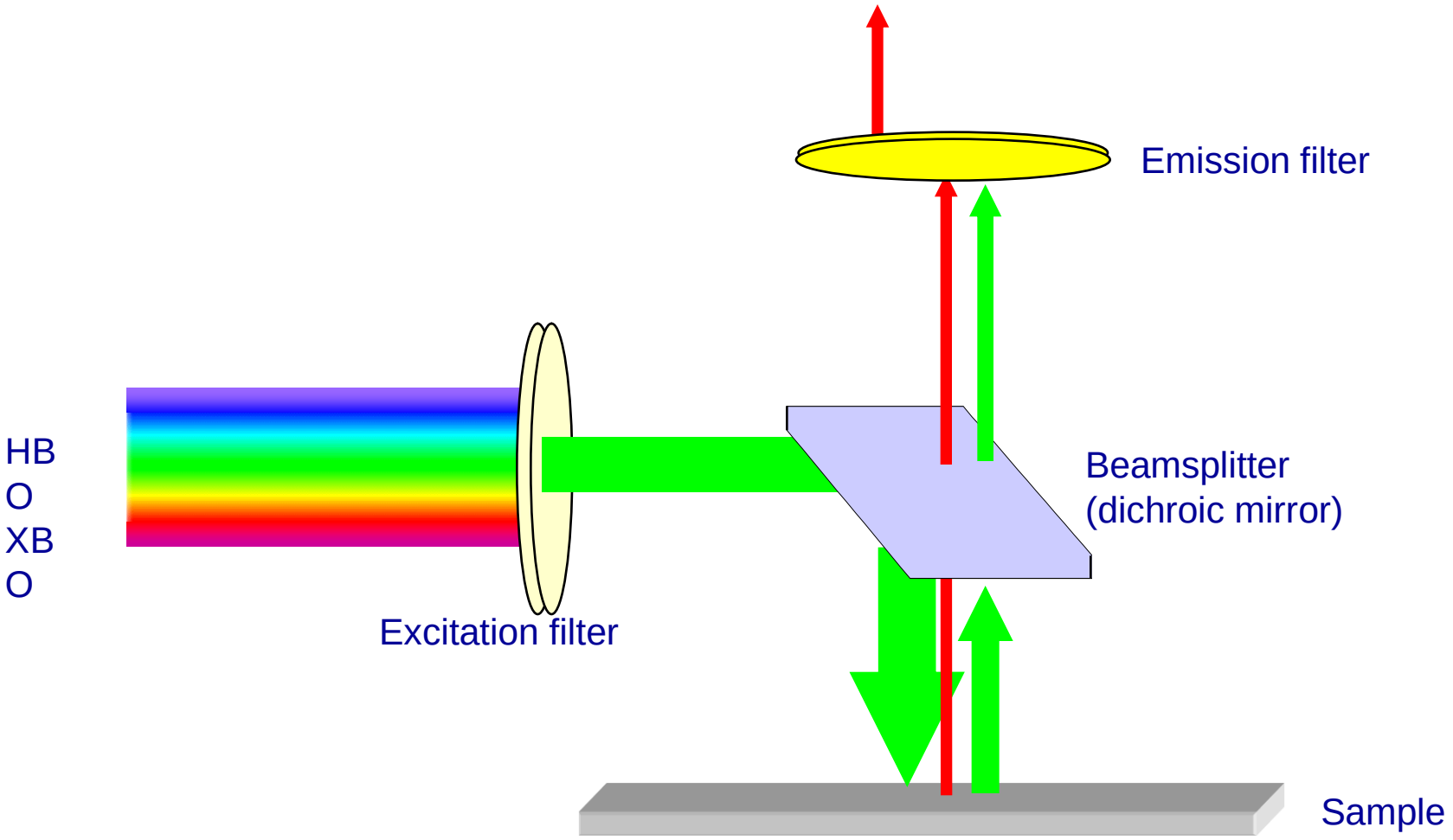


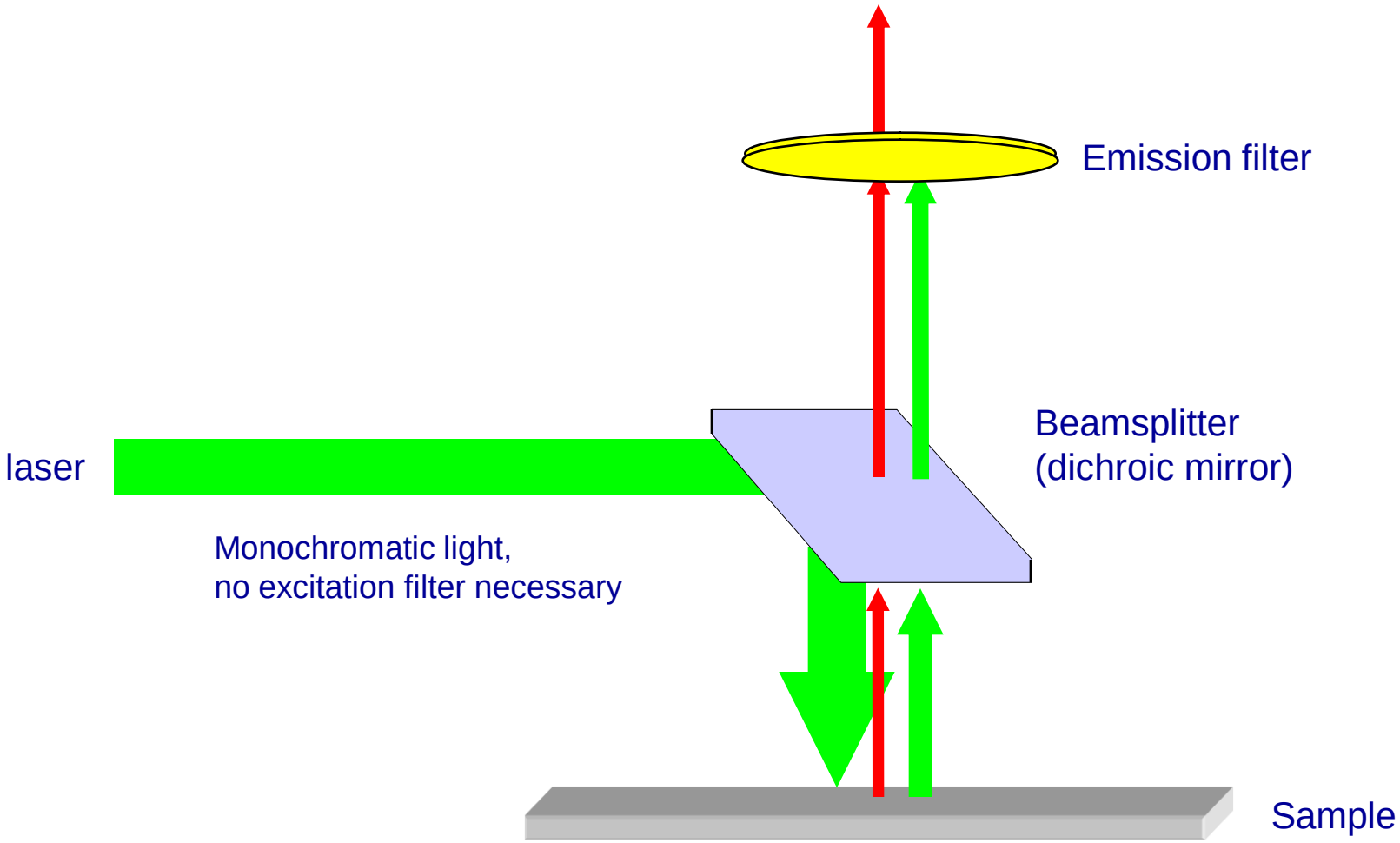
TOTO®-3 iodide (642/660)



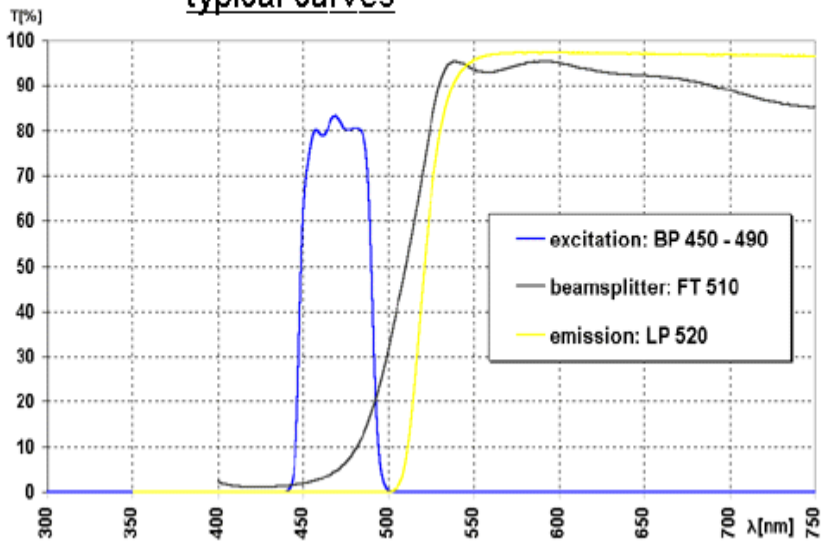
Comparison of different burners



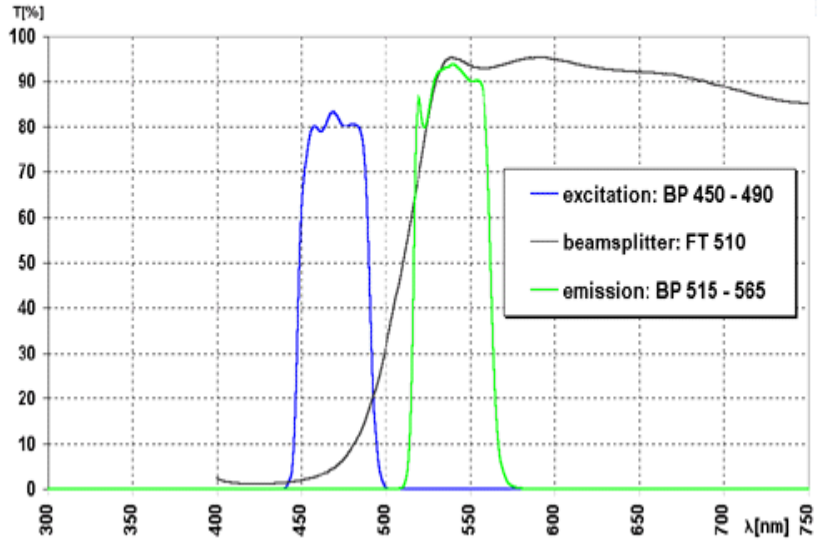

Emission filter
HB
O
XB
O
Beamsplitter
(dichroic mirror)
Excitation filter
Sample

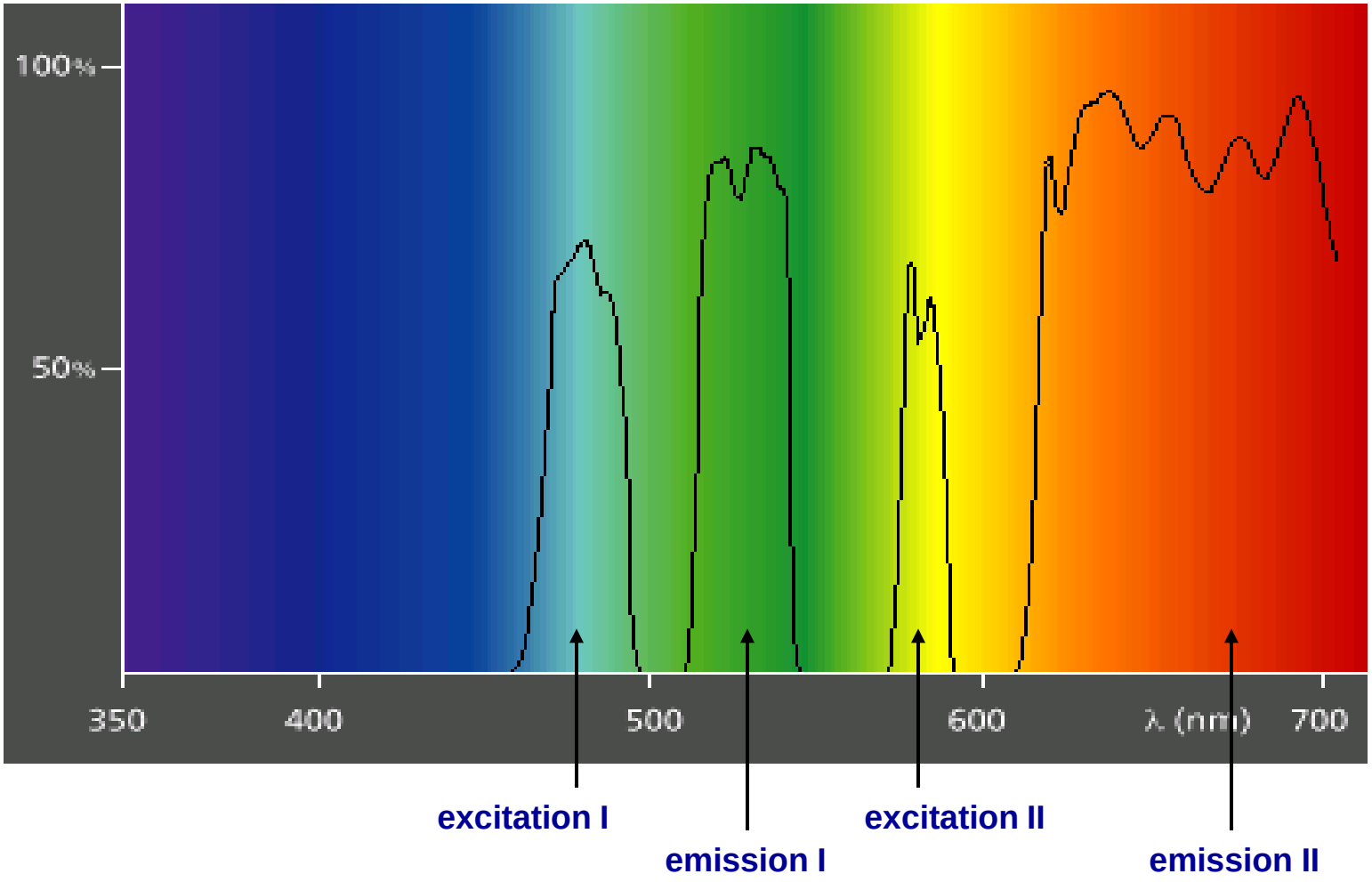

Emission filter
laser
Beamsplitter
(dichroic mirror)
Monochromatic light,
no excitation filter necessary
Sample

Filter set 09 487909 or 488009 typical curves



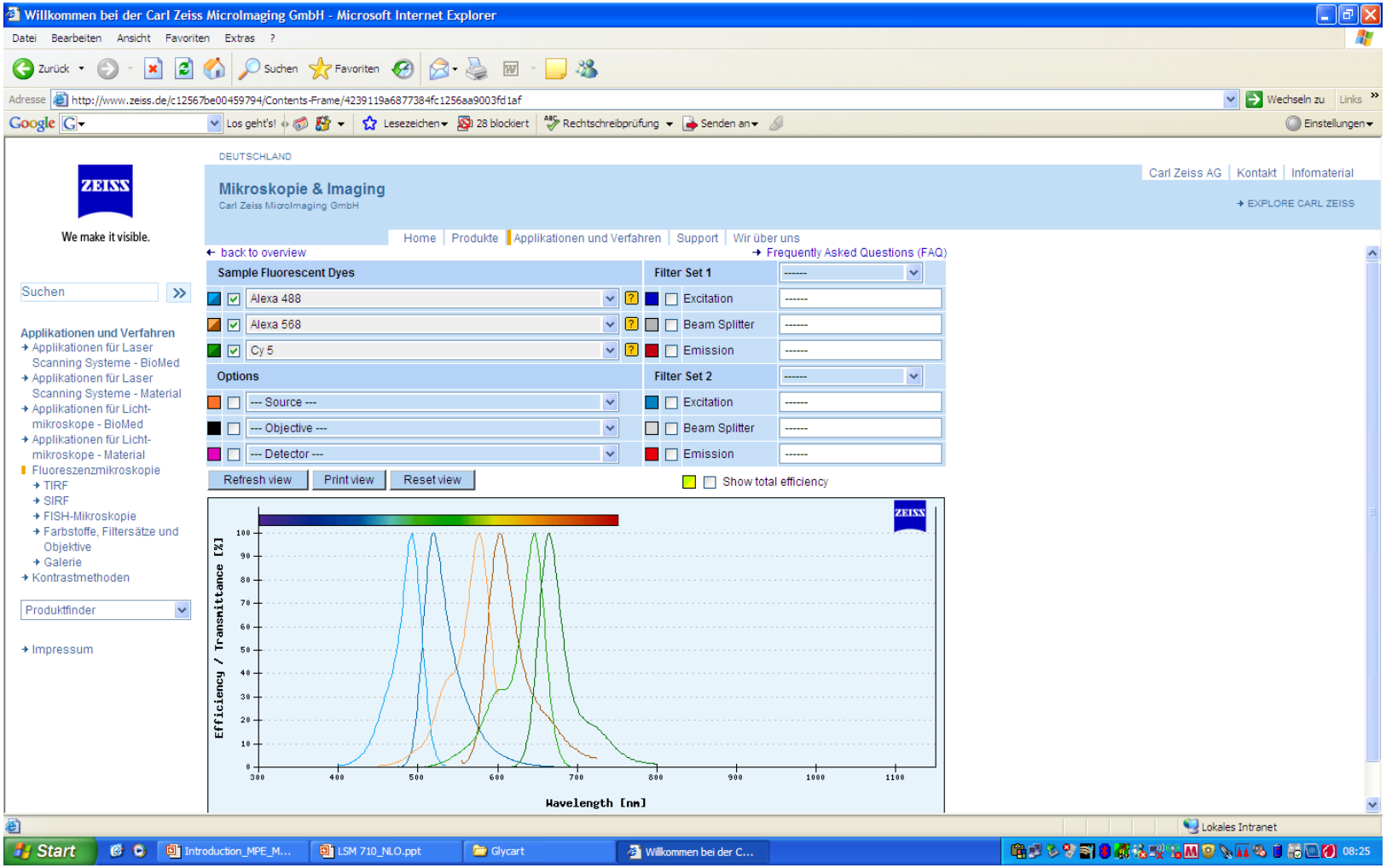
Filter set 10 487910 or 488010 typical curves



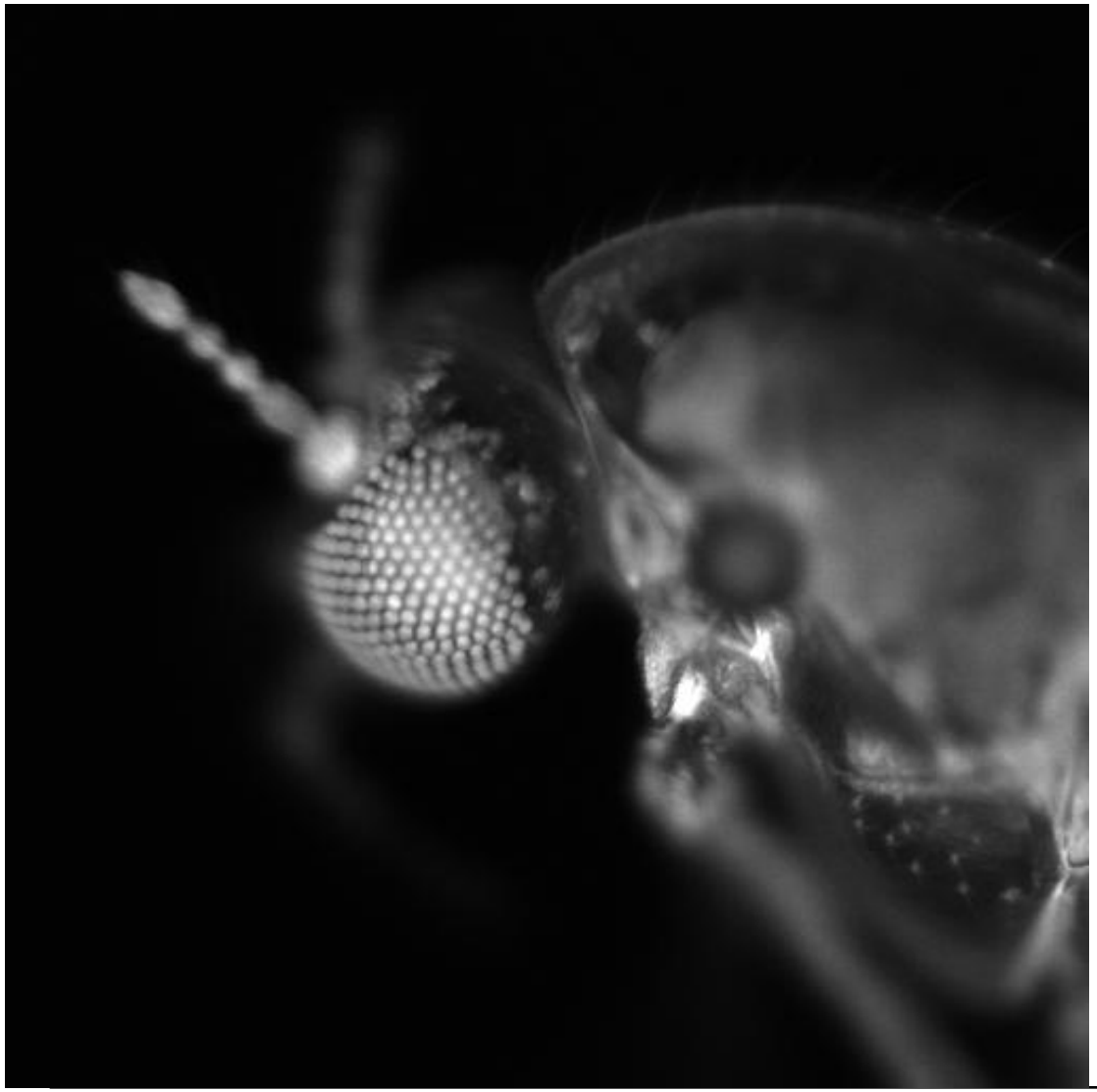

100%
50%
350
400
500
600
λ (nm)
700
excitation I
emission I
excitation II
emission II

Fluorescence Microscopy

www.zeiss.de



Confocal Microscopy



Amber fossil (Chironomide)

thickness app. 300 µm

conventional fluorescence

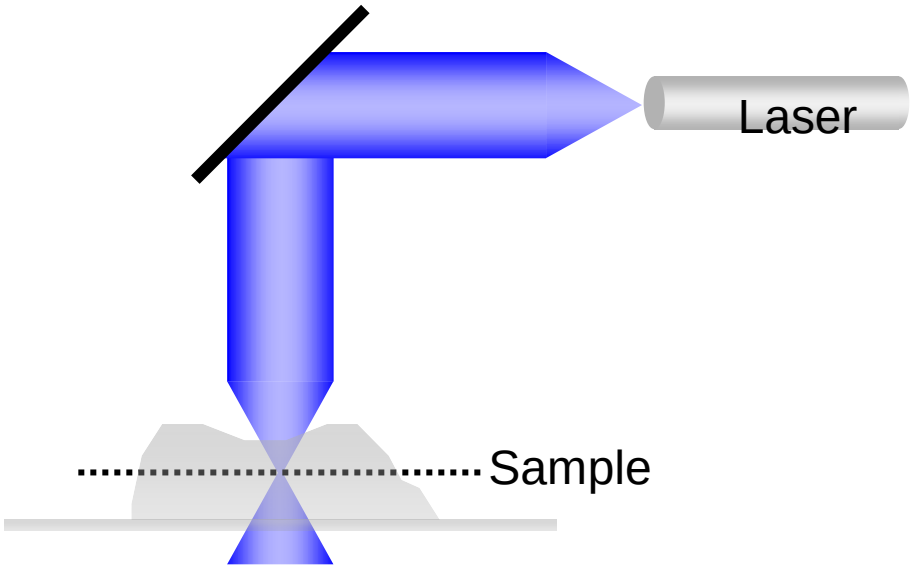
Confocal Microscopy

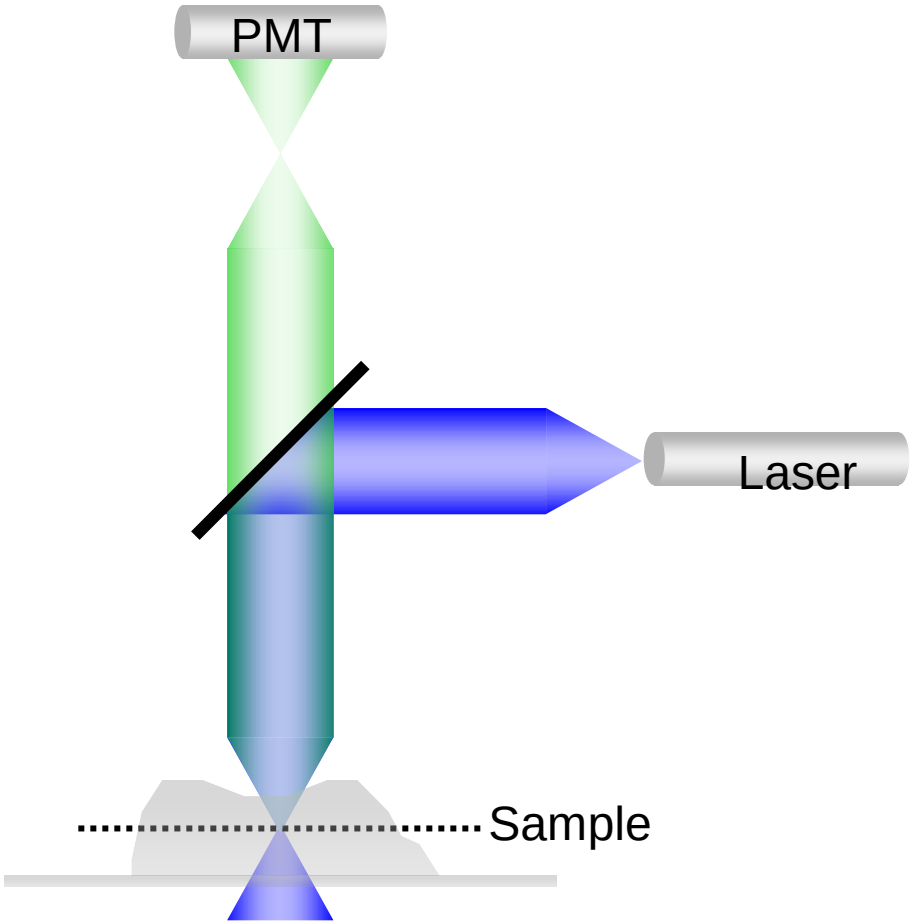
Amber fossil (Chironomide)

thickness app. 300 µm

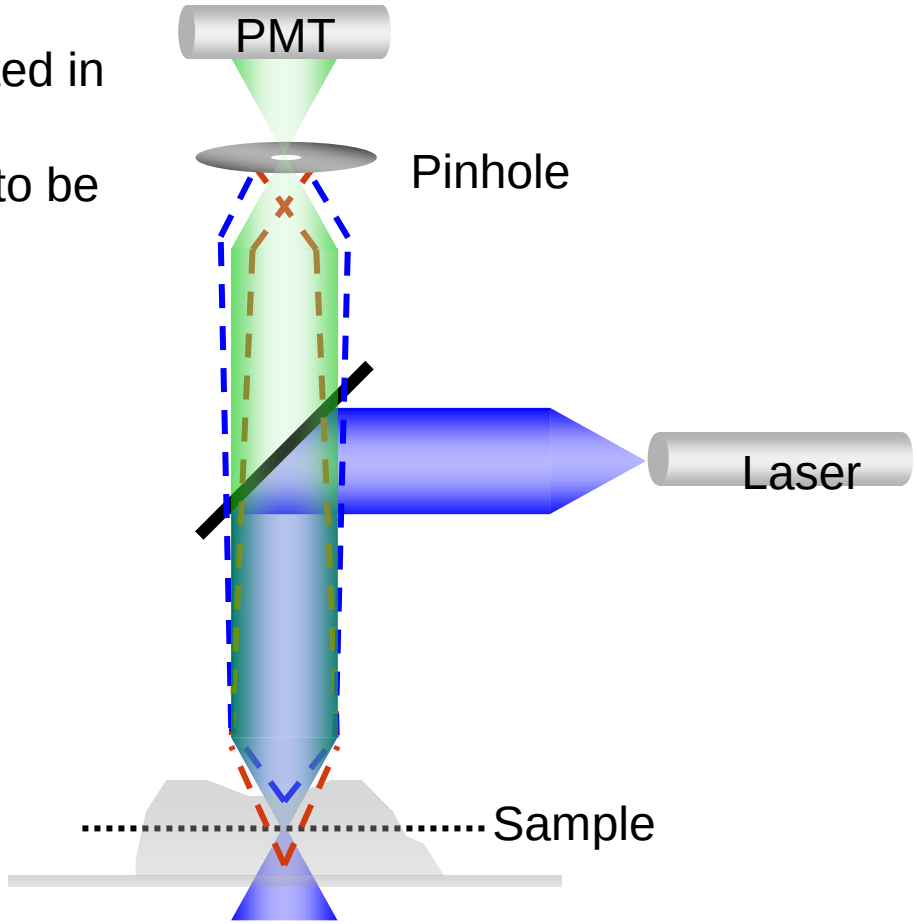
confocal imaging
3D reconstruction

The confocal principle (non multiphoton)

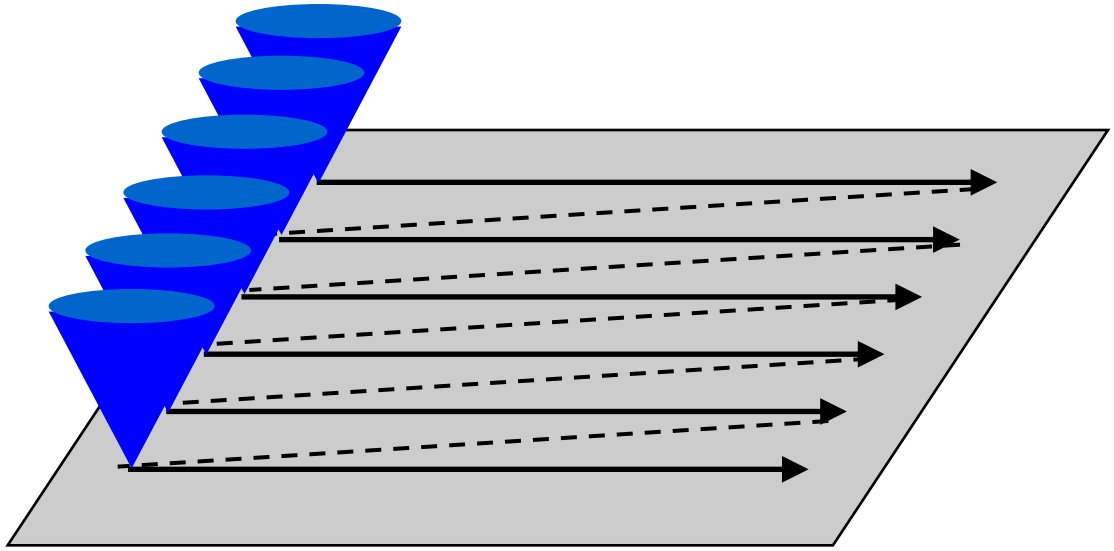



PMT
Laser
Sample

A minute diaphragm, situated in a conjugated focal plane, prevents out of focus light to be detected.

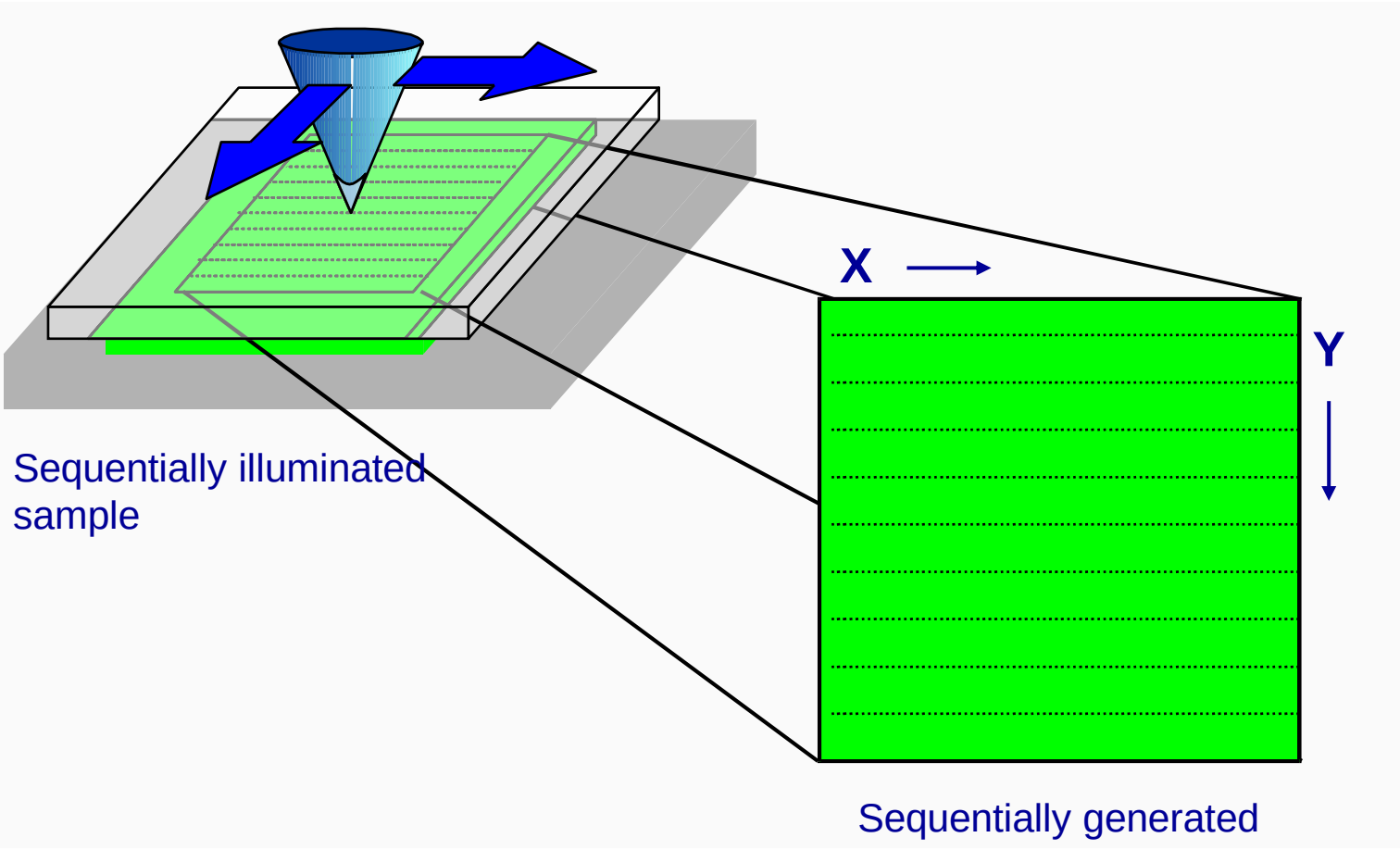


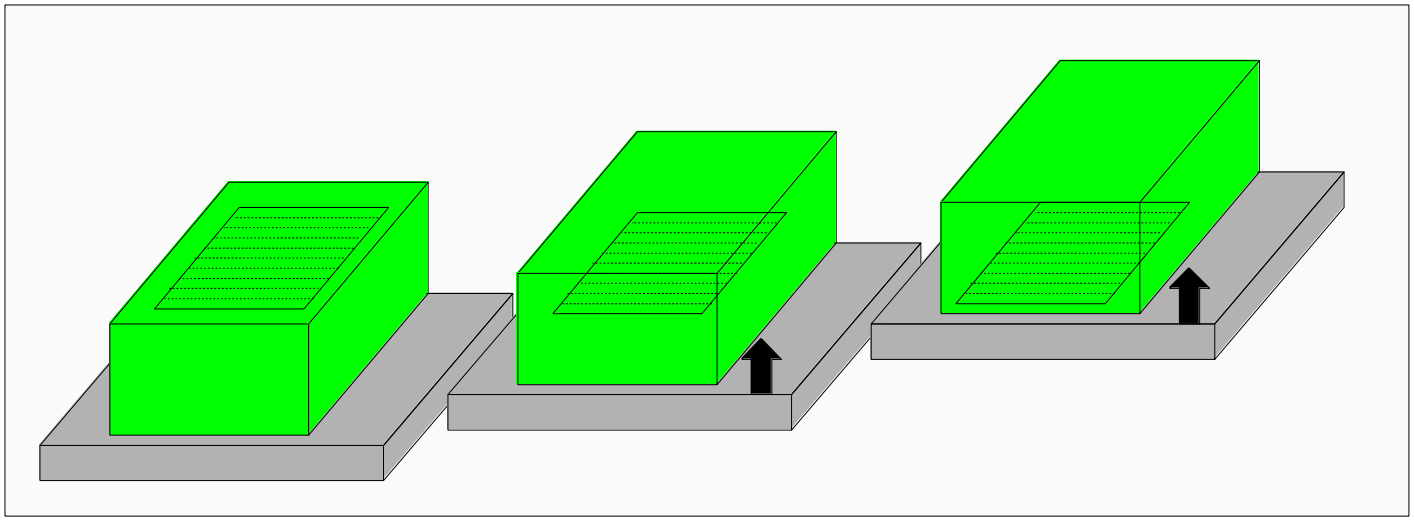
Confocal: Point Scanning



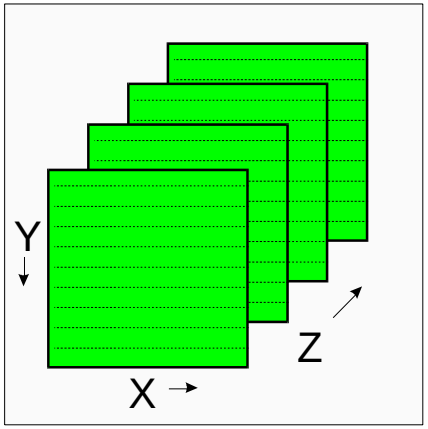
XY scanning

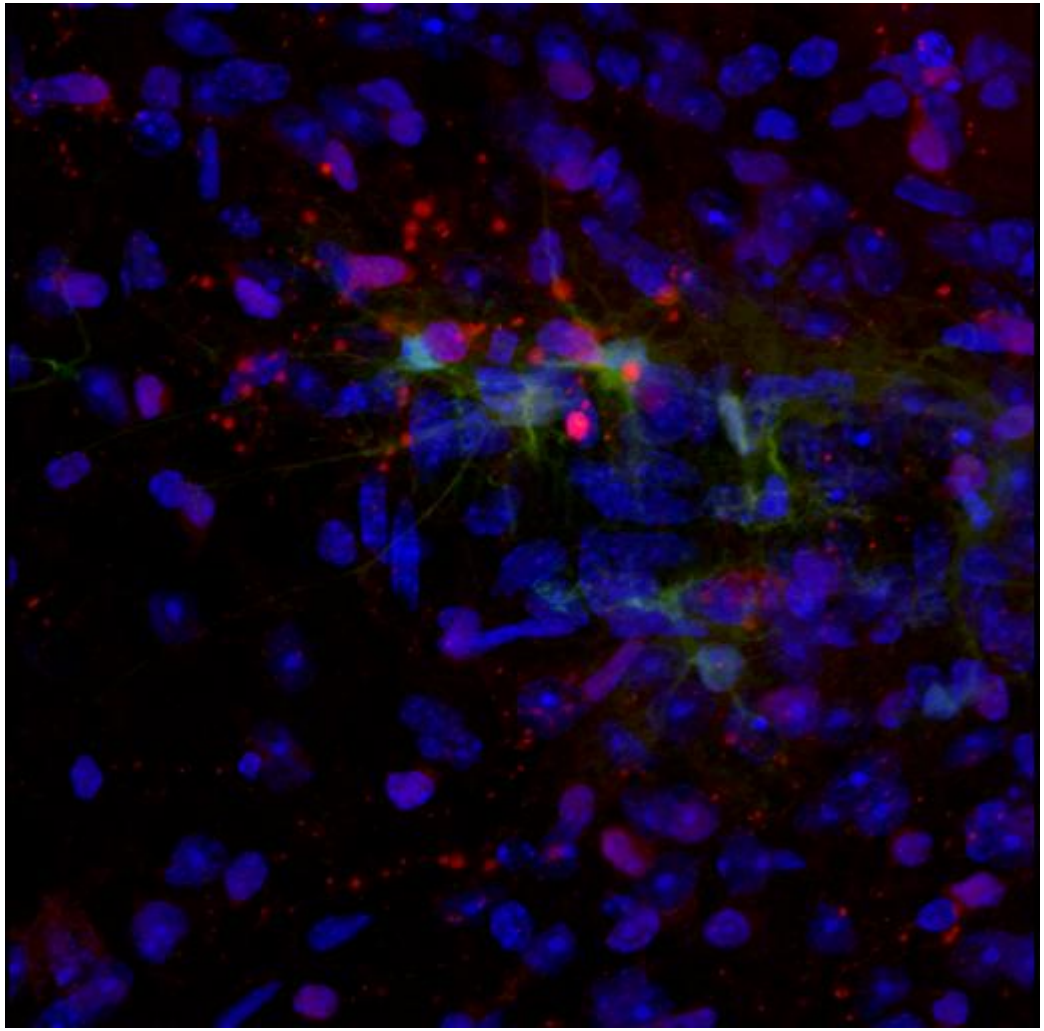
Point scanning confocal systems


X
Y
Sequentially illuminated sample
Sequentially generated image



Optical slicing





Advantages of Confocal Laser Scanning Microscopy

- Efficient excitation by highly focussed laser light
- Adjustable pinhole for the best compromise between resolution and signal detection
- “Optical slices” for sharp three dimensional reconstructions
- Line scans, spline scans, free definable scan fields, changeable resolution…..

Limitations of Confocal Laser Scanning Microscopy

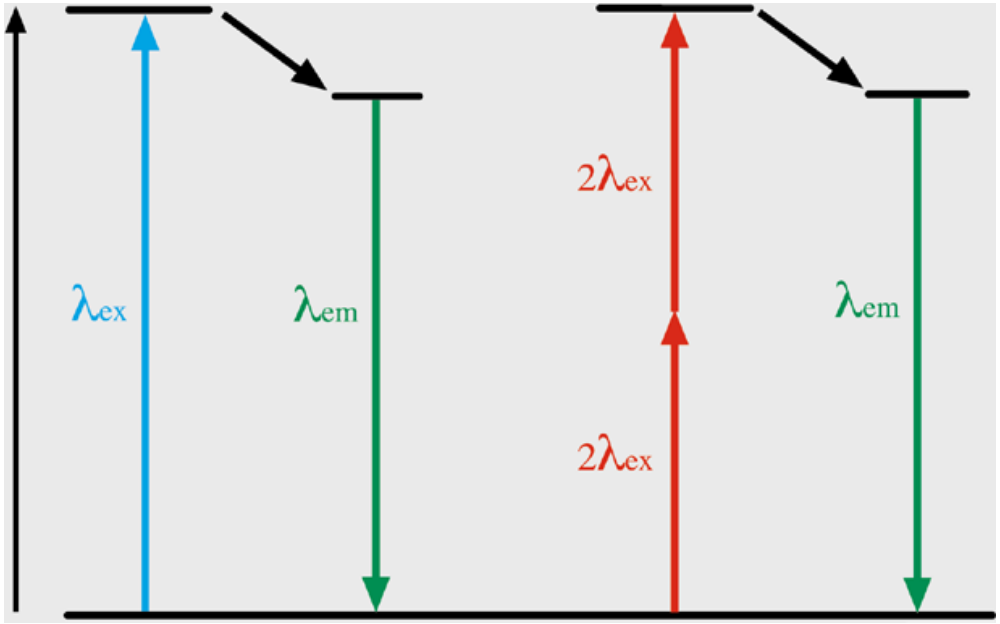
- The excitation wavelengths are limited by the available laser lines
- The sequential scanning is time consuming
- Direct confocal observation of the sample is not possible
- Excitation and bleaching occurs -as in conventional fluorescence- also in out of focus planes

Multiphoton Laser Scanning Microscopy

- Confocal Microscopy **without** pinhole
- The excitation light has a **longer** wavelength than the emitted fluorescence
- Two -or more- near infrared photons have to be absorbed simultaneously by a fluorochrome to emit one visible photon
- Only in the focal spot of the laser the excitation energy is high enough to generate fluorescence
- As the relationship between excitation and emission is no longer linear, multiphoton microscopy belongs to non linear optics (NLO)

Multiphoton processes - the principle

Multiphoton processes described for the first time in 1931 by Maria Goeppert-Mayer (Nobel price in physics 1963)
Über Elementarakte mit zwei Quantensprüngen; Göttinger Dissertation: Ann. Phys. 9: 273-294



Multiphoton processes - the principle

Single Photon Excitation: linear process; proportional to the intensity of the excitation light

$$E_X \propto P_{avg}$$

Multi Photon Excitation: non linear process; proportional to the square of the excitation light intensity per excited area

$$E_X \propto \left(\frac{P_{avg}}{A}\right)^2$$

Multiphoton processes - the principle

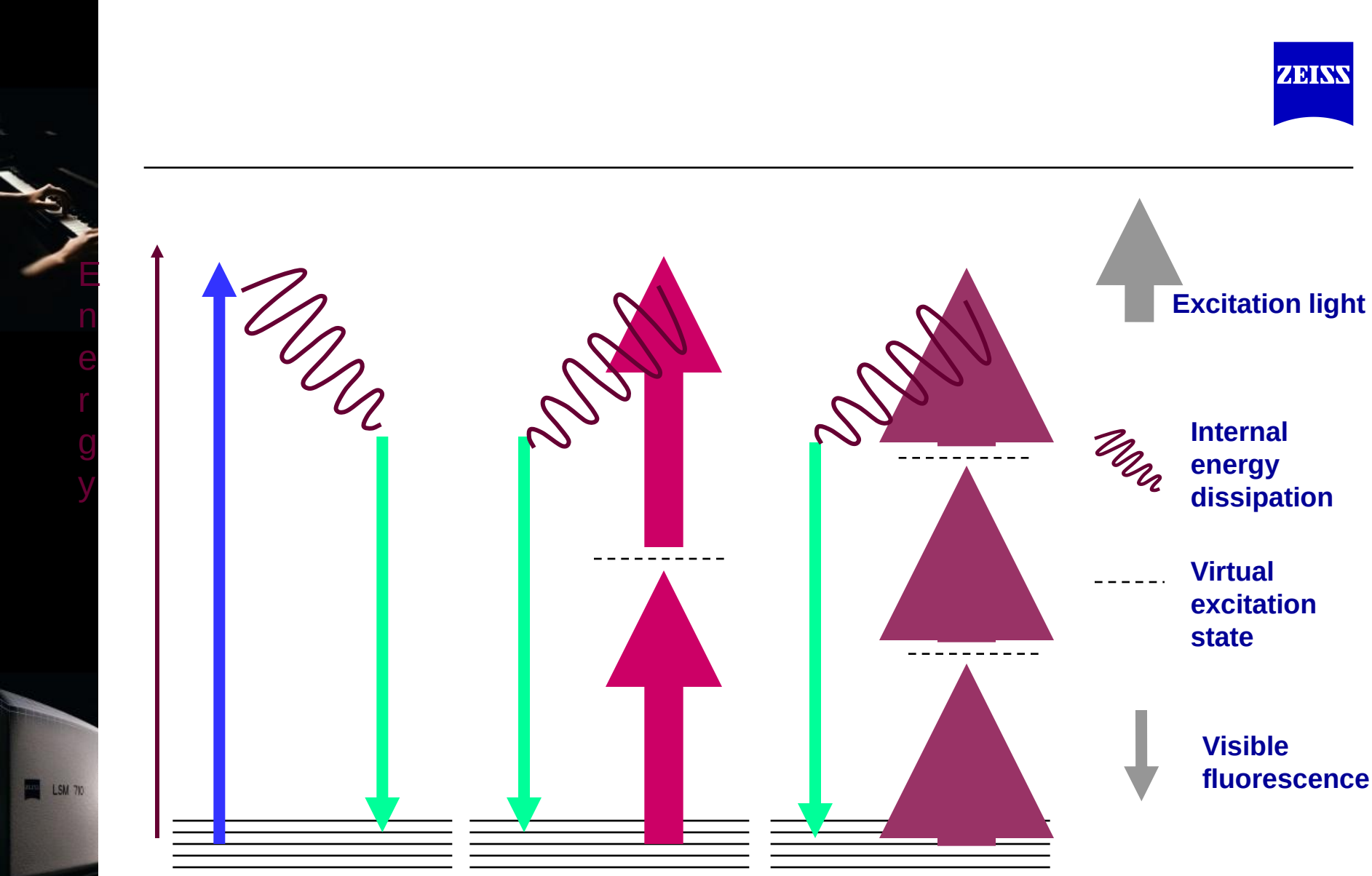
- **Prerequisite for the excitation of fluorochromes with more than one photon of lower energy**
  - very high intensity of the excitation light
  - strong focus of the light
- **-> use of pulsed near infrared lasers with high average power**
- **-> use of objectives with high numerical aperture**

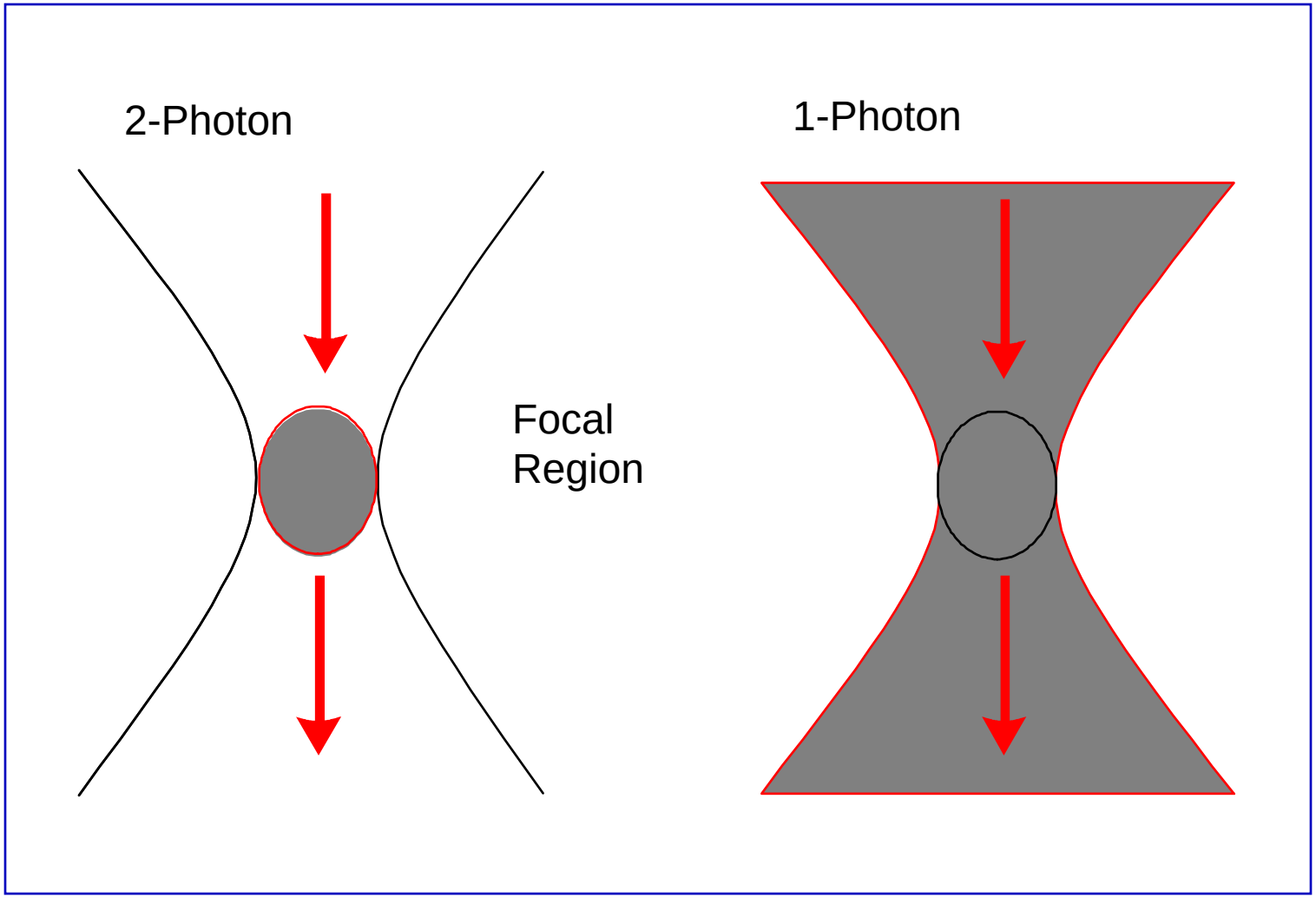
Multiphoton processes - the principle

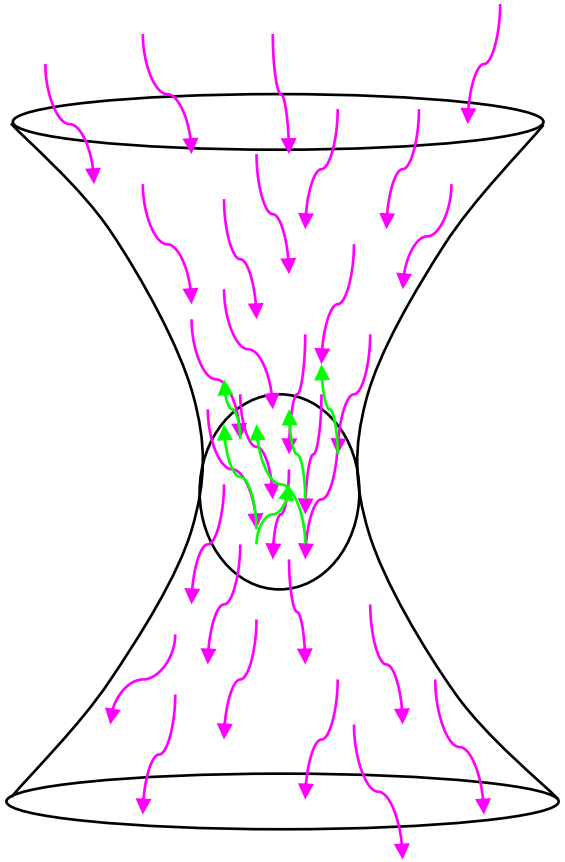
Characteristics of Titan:Sapphire Lasers:

- **Emission Wavelength: < 700 up to >1000 nm**
- **Frequency of pulses: 76-90 MHz (F)**
- **Length of pulses: 100-200 fs (10 $^{-15}$ s)**

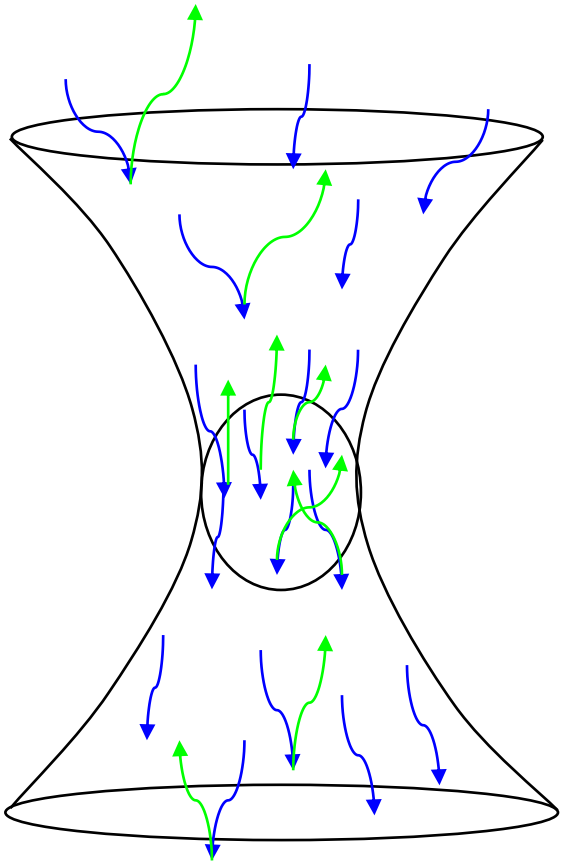
Peak pulse energy $I_{peak} = \frac{P_{ag}}{F_p A_0}$


Energy
Excitation light
Internal energy dissipation
Virtual excitation state
Visible fluorescence

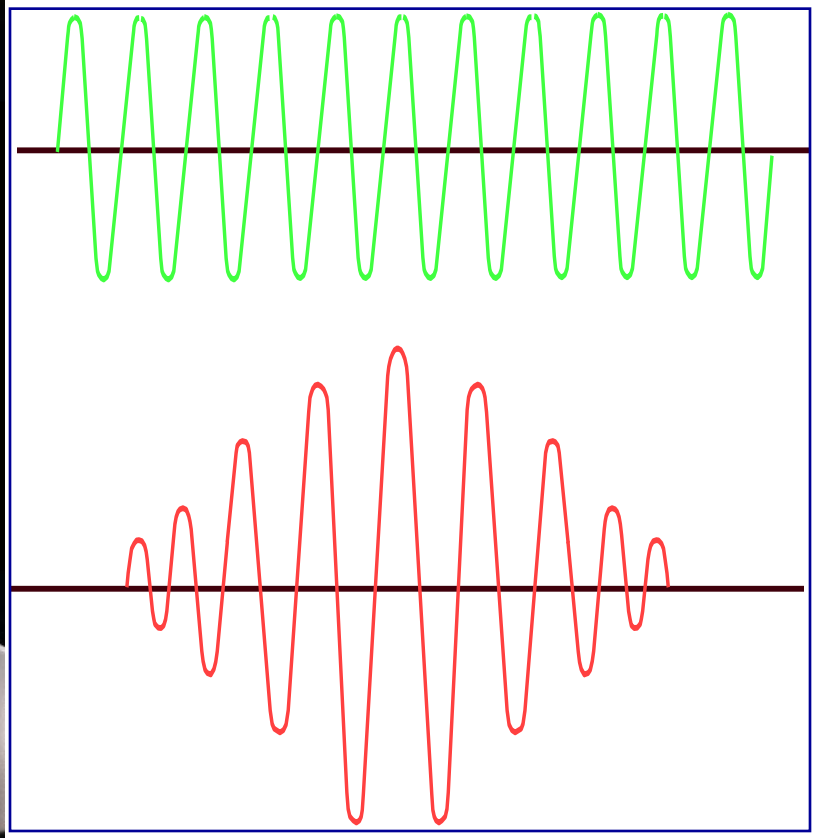

2-Photon
1-Photon
Focal
Region



2 photon

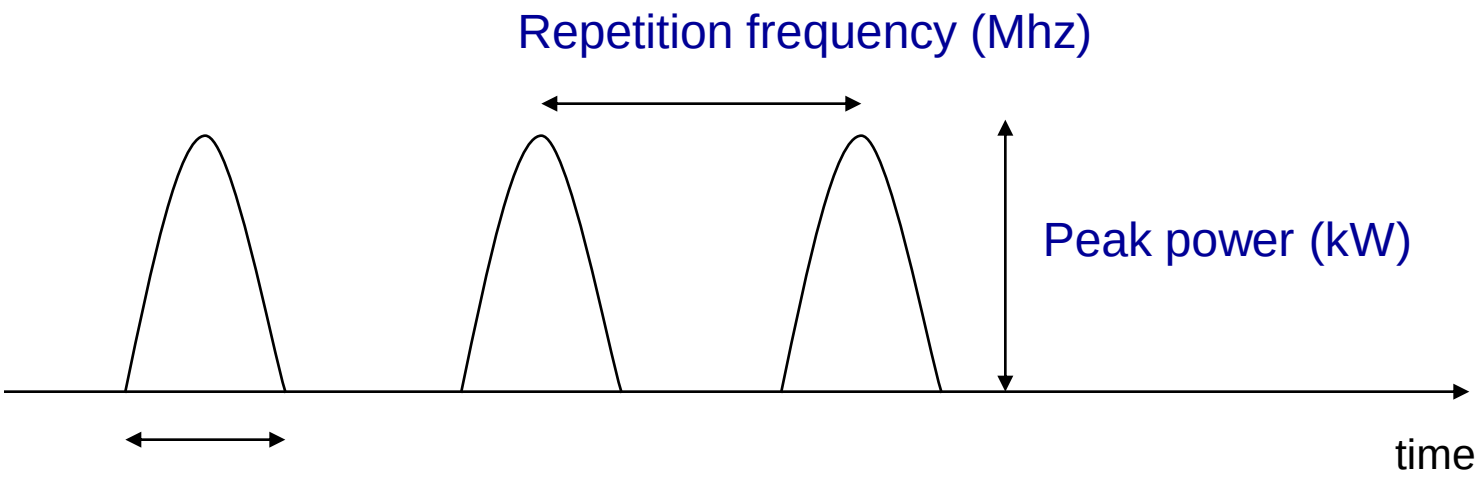


1 photon



CW laser (continuous wave) for microscopy:
Continuous emission with low average power

Pulsed laser:
Very short pulses with a very high peak power
(0,5 W average power with 100 fs pulses correspond to a peak power of 65.8 kW)

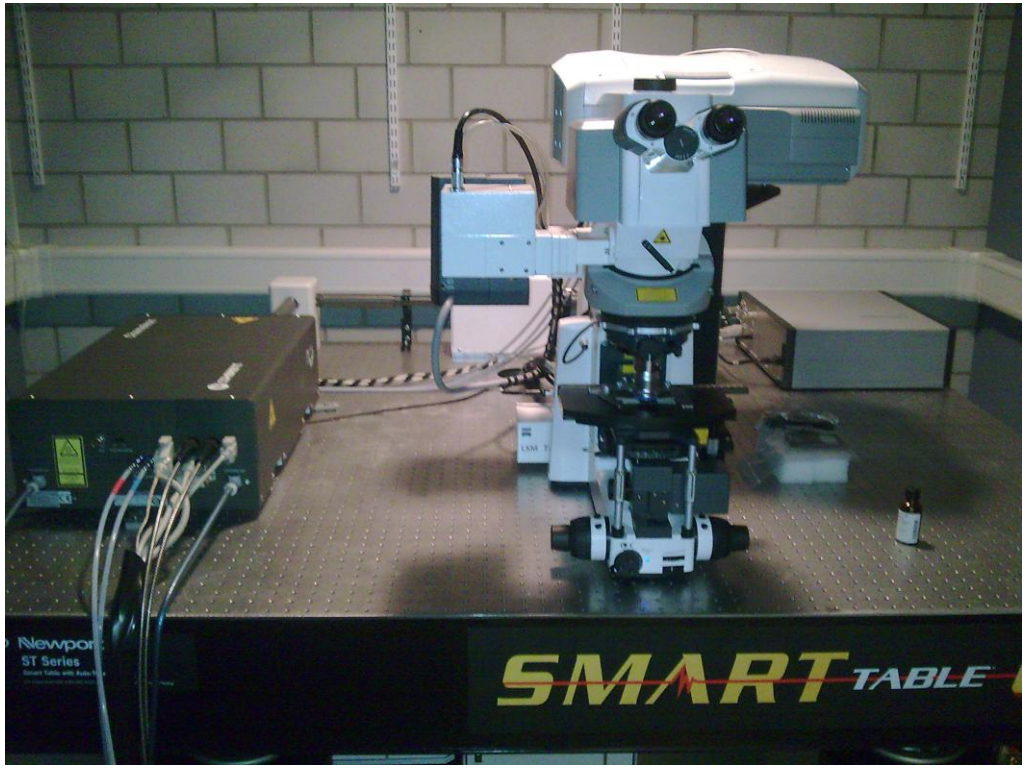

Repetition frequency (Mhz)
Peak power (kW)
time
Pulse length (fs)

Multiphoton Microscopy – practical issues

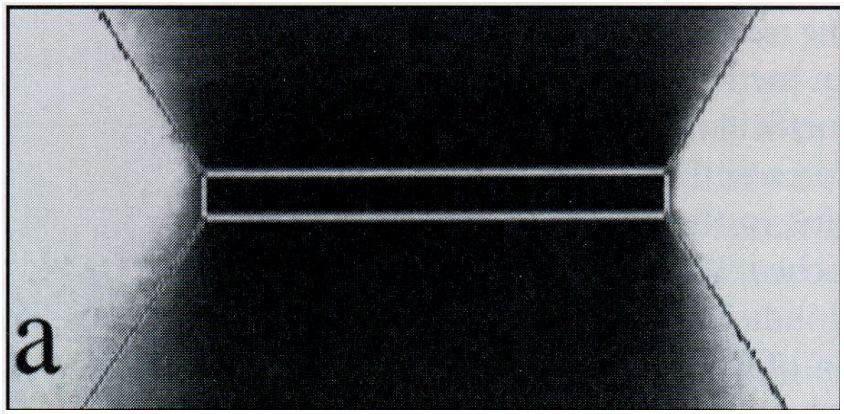
System components of a Multiphoton Microscope

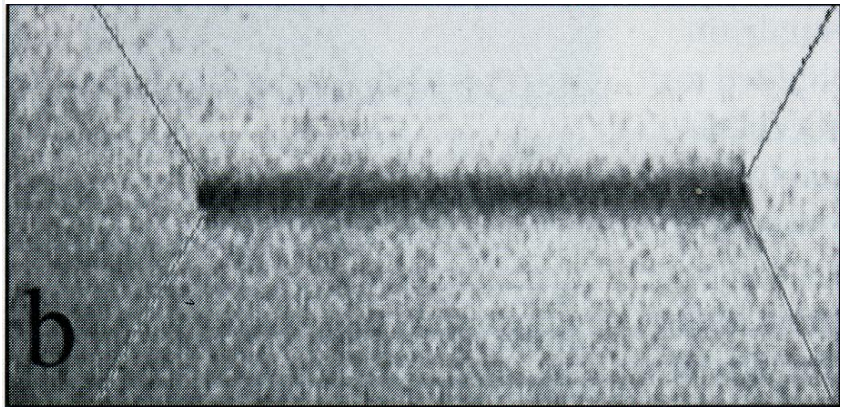
- **Laser Scanning Confocal Microscope**
- **Tunable Ultrafast Laser preferably to be tuned via software**
- **Direct coupling of ultrafast lasers**
- **Integration of laser control into the software of the LSM**

Multiphoton Microscopy – practical issues



LSM 710 NLO attached to the AxioExaminer (fixed stage) Microscope with direct coupled Multiphoton Laser and AOM Box for Laser attenuation





Bleaching of fluorescence gels in Z

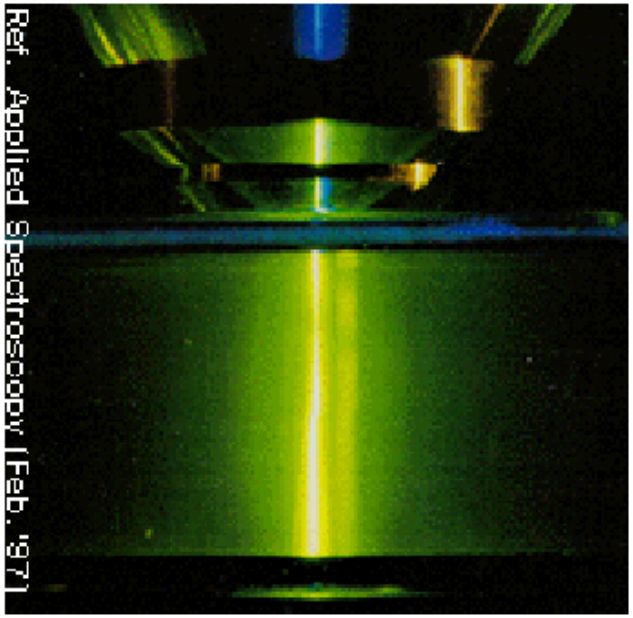
Single photon excitation

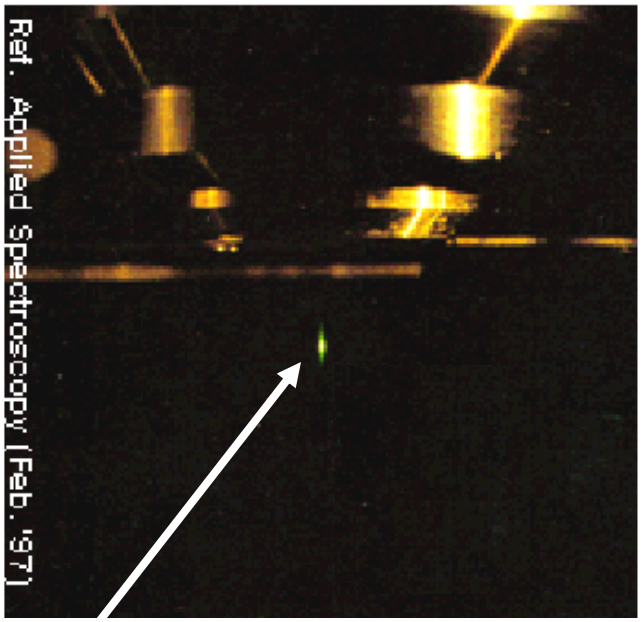
Multi photon excitation

One-Photon – Multiphoton: a comparison

Focal Excitation:

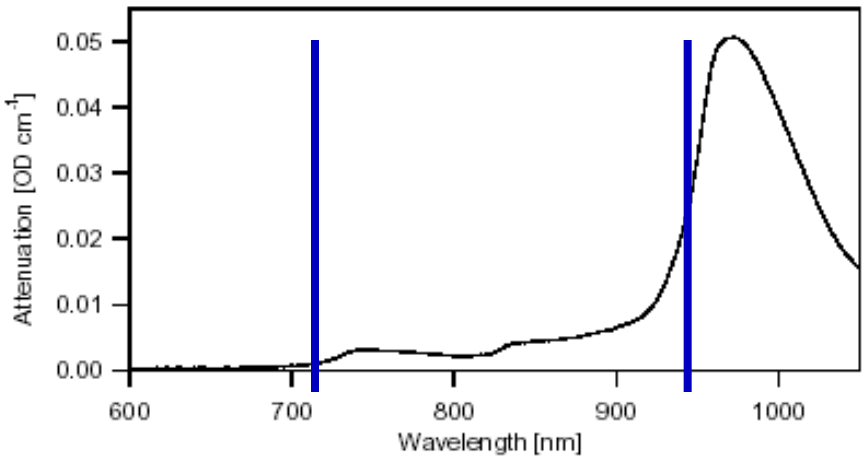
Due to the quadratic dependence on the light intensity only at the focal spot the excitation of the fluorochrome occurs



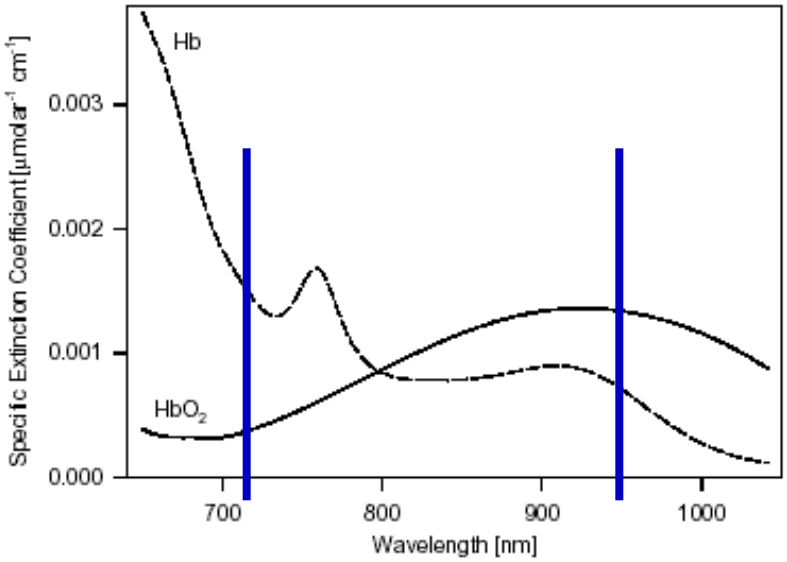


One-Photon – Multiphoton: a comparison

Deep tissue penetration of the excitation light due to less absorption and scattering of NIR light in tissue



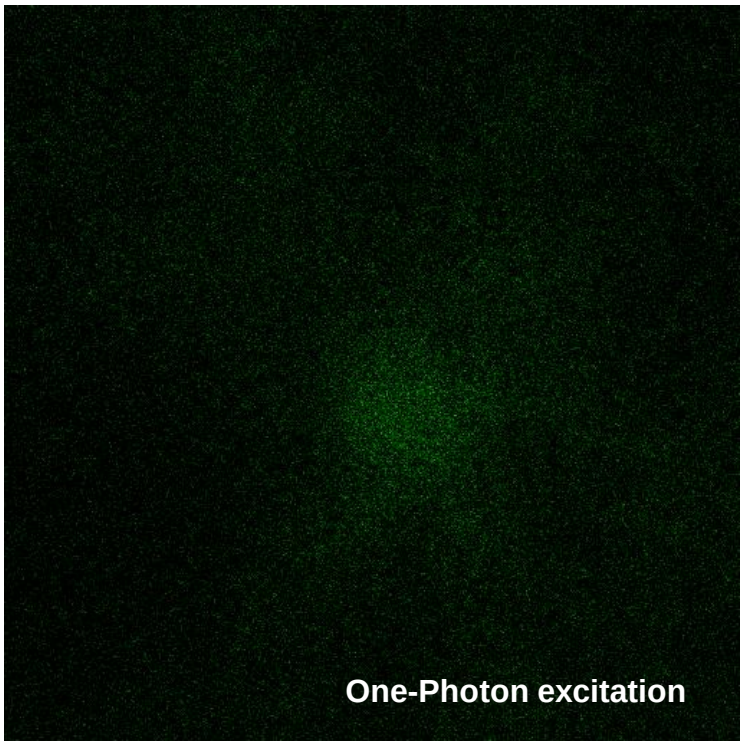
Absorption Spectrum of water

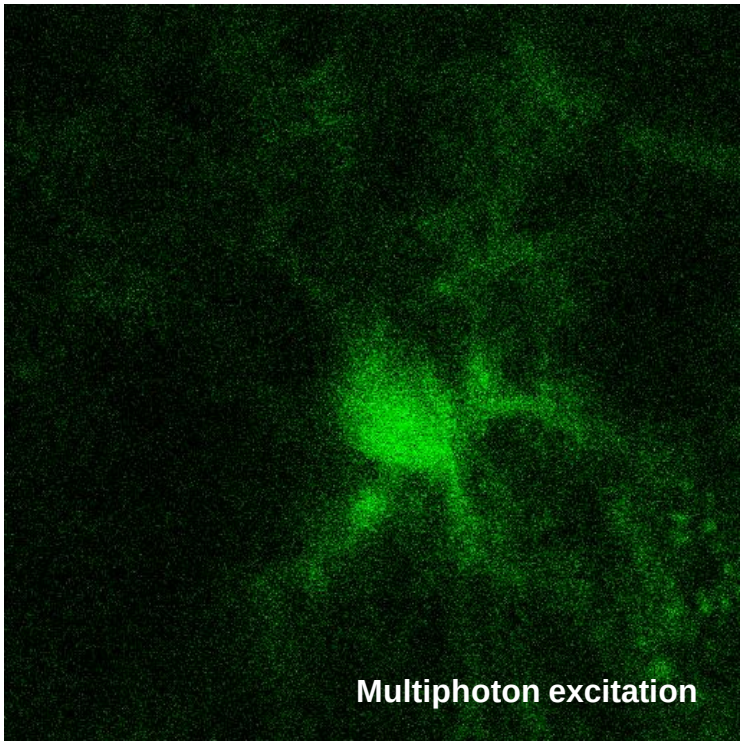


Absorption Spectrum of Oxy- and Deoxyhaemoglobin

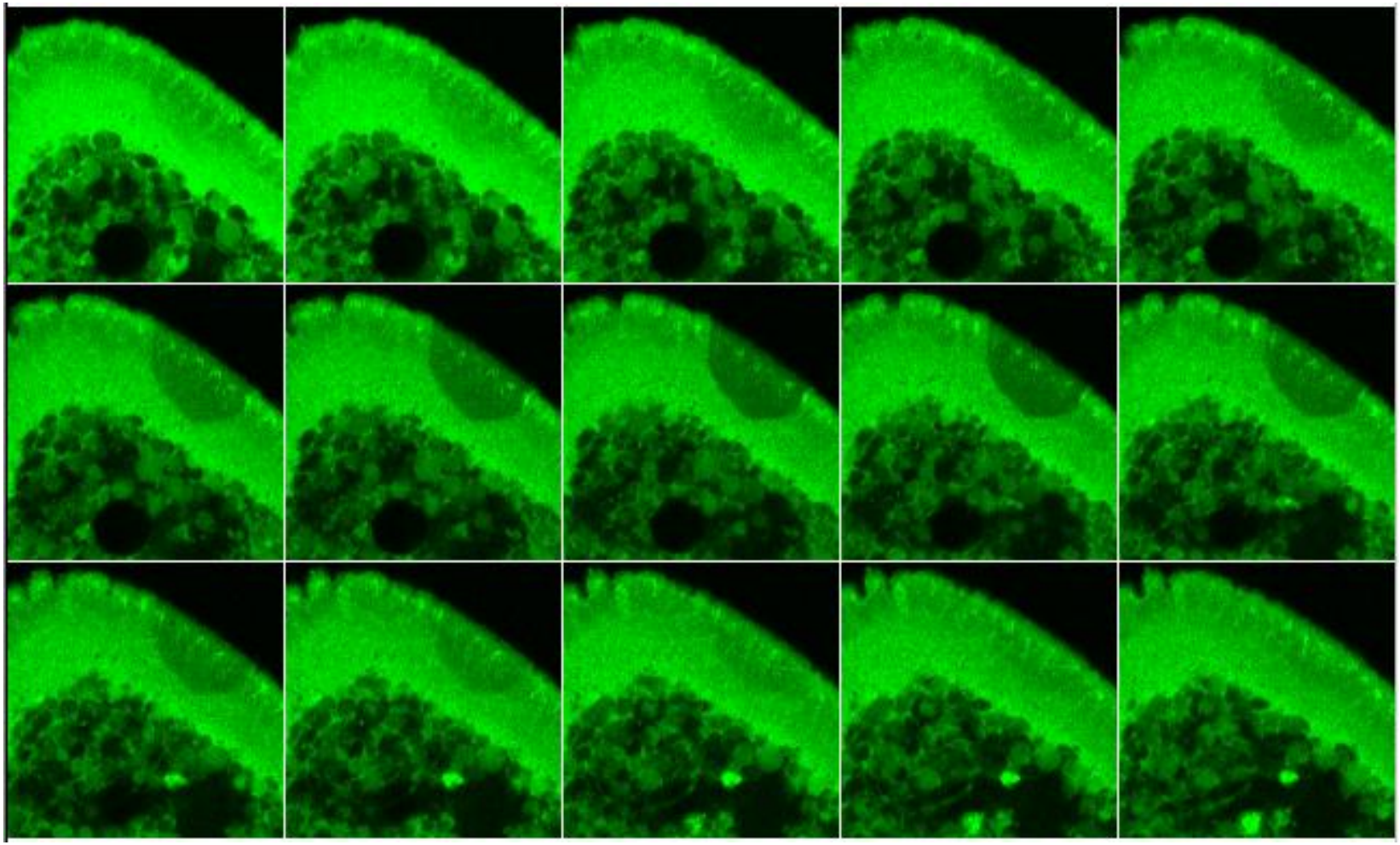
One-Photon – Multiphoton: a comparison

Imaging of fluorescent structures in deep tissue regions





Mouse brain, GFP expressing astrocyte, fixed brain slice, 160 µm deep in the tissue



Bleaching within a Drosophila embryo using multi photon excitation

Examples of Dyes excited with Multiphoton Technique

Fluorochrome	Absorption	Emission
Alexa Fluor 488	720-800	515
Alexa Fluor 568	720-840	596
Alexa Fluor 633	720-900	647
CY2	780-800	506
CY3	780	565, 615
CY5	780-820	670
DAPI, Hoechst	700-820	455, 478
eCFP	800-900	476
eGFP	820-950	509
eYFP	860-950	532
Fluorescein	780 – 820	519
Lucifer Yellow	860-890	533
Mito Tracker red	750-840	600
Propidium Iodide	820-850	617
Rhodamine 123	780-860	550
Sytox Green	740-760 or 880-940	524
TRITC	800-840	572

5 - Uncompromised Multiphoton Imaging

LSM 710 NLO

5 - Uncompromised Multiphoton Imaging
LSM 710 NLO with new Axio Examiner

- Light path and objective to the front
  -> no obstruction of view to the sample
  -> best accessibility to sample
- TFT display as control panel
- All control and focus knobs in the front area of the stand
- Choice of objective holders for 1, 2 or 4 objectives
- Motorized reflector turret
- Focus steps of 25 nm
- Sample space up to 11 cm for whole animal imaging
  -> table and condensor carrier, and transmission NDD port can be mounted and dismounted by user

5 - Uncompromised Multiphoton Imaging
LSM 710 NLO with new Axio Examiner

- Up to 5 NDDs in reflection
- Up to 5 NDDs in transmission
- W Plan Apo 20x 1,0 NA detects 5.6 times more light than IR Achroplan 40x 0.8
  AND
  Higher NA = tighter spot -> less laser power necessary to achieve comparable signal strength

5 - Uncompromised Multiphoton Imaging
LSM 710 NLO with new Axio Examiner

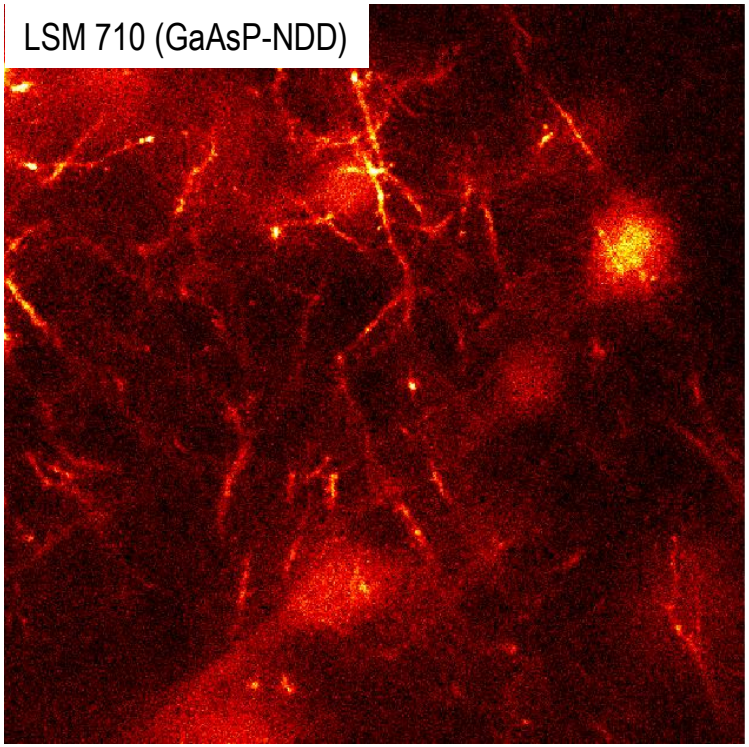
5 - Uncompromised Multiphoton Imaging
LSM 710 NLO with new Axio Examiner

Deep Tissue Imaging

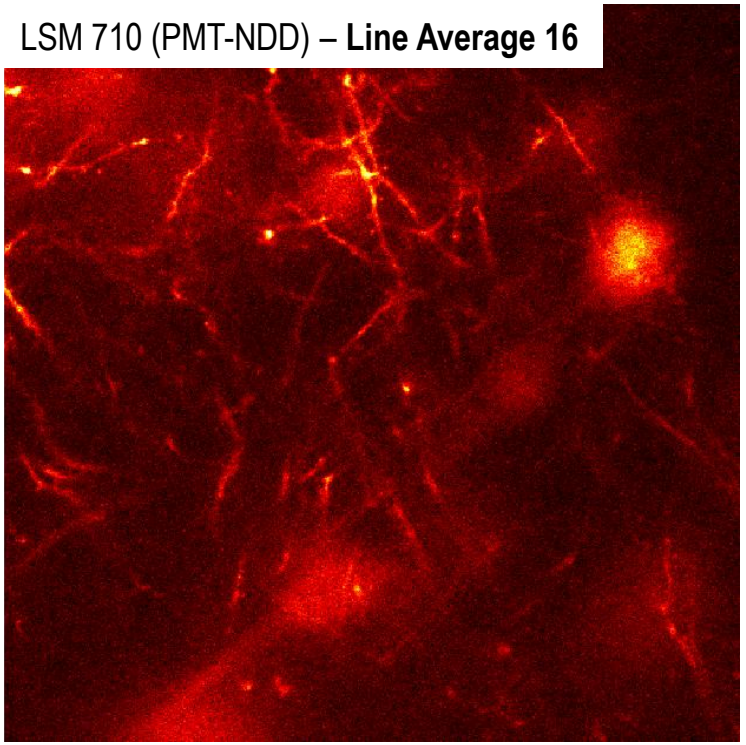
GaAsP increases sensitivity over PMT-NDD by factor ~3

- Sample: Mouse brain (fixed)
- Provided by Steve Turney, Harvard
- Labels: YFP
- Mode: Multiphoton

LSM 710 (GaAsP-NDD)



LSM 710 (PMT-NDD) – **Line Average 16**



Advantages of Multiphoton Laser Scanning Microscopy

- Lower cytotoxicity of infrared light, especially in comparison to UV light
- Deeper penetration depth into living tissue
- No excitation/bleaching of fluorochromes outside of the focal spot
- With LSM 710 NLO combination of cw and pulsed lasers simultaneous
- LSM 7 MP dedicated multiphoton system

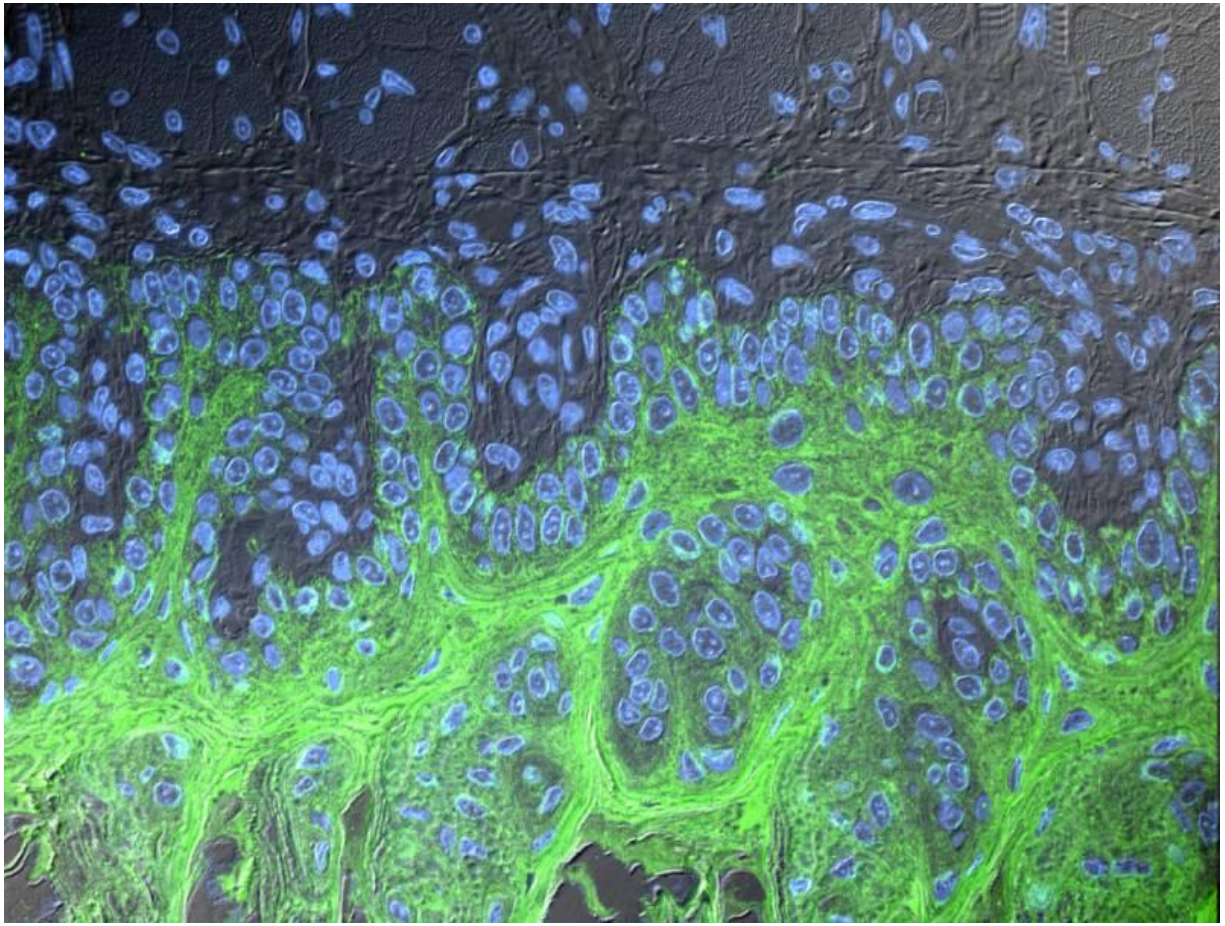
Limitations of Multiphoton Laser Scanning Microscopy

- Pulsed laser is usable just with one tuned wavelength at the time
- Needs some time (seconds) to change the wavelength
- Price for a complete system approx. 800’000 to 1’000’000 Fr

One-Photon – Multiphoton: a comparison

One-Photon Excitation	Multiphoton Excitation
Excitation occurs in the whole light path of the laser beam	**Excitation only at the focal spot of the lens**
Confocal aperture necessary, reduces signal gain	**No confocal aperture required, higher signal gain**
Imaging limited in thick specimens: 70 - 100 µm are feasible	**Visualization of fluorescent dyes up to several 100 µm deep in the tissue sample**
Several lasers to cover a large number of fluorescent dyes	**One laser excites a variety of fluorescent dyes**
Photo toxic events	**Photo toxicity is reduced**
High resolution	**Slightly lower resolution compared to One-Photon excitation**

Thank you very much for your attention!



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