University of Zurich[UZH]

Center for Microscopy and Image Anaylsis

Introduction to light microscopy

Imaging with light / Overview of techniques

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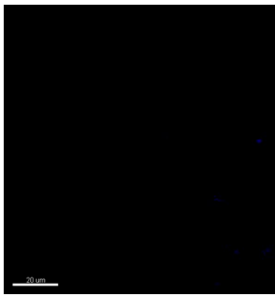
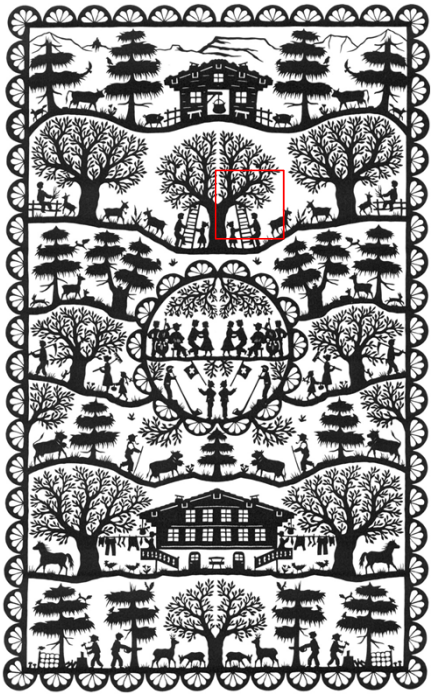


Light interacting with matter

- Absorbtion
- Refraction
- Diffraction
- Scattering



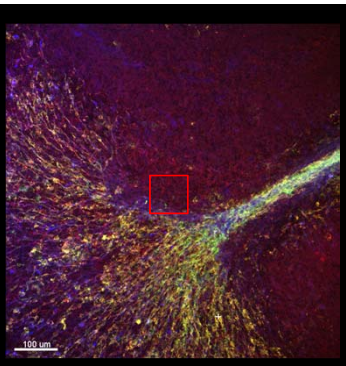
Light interacting with matter

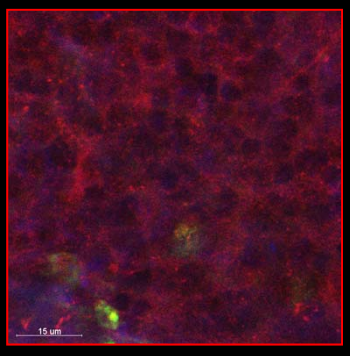
Absorbtion

Refraction

Diffraction

Scattering





Light interacting with matter

Light emitted from fluorochromes

How is an image formed?

Why are there limits in resolution?

Introduction to light microscopy

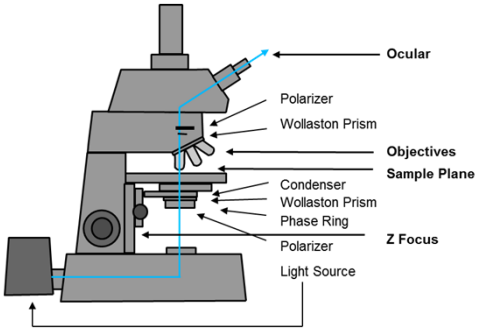
Imaging with light / Overview of techniques

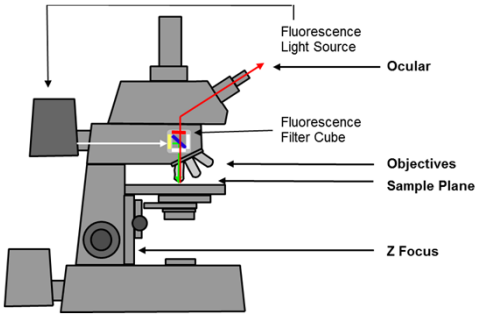
- Image formation in a nutshell
- Resolution limits
- Light emission from molecules and fluorescent imaging

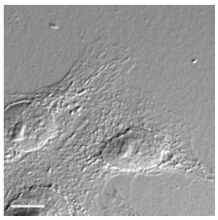
Methods and techniques in microscopy

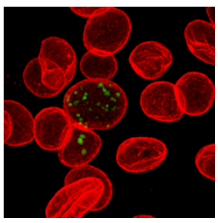
- Widefield microscopy
- Confocal laser scanning microscopy
- Fluorescence energy transfer
- Fluorescence recovery after photobleaching
- In vivo microscopy
- Selective plane illumination microscopy
- Superresolution techniques
- *Correlative techniques – light and electron microscopy*

Fundamental Setup of Light Microscopes

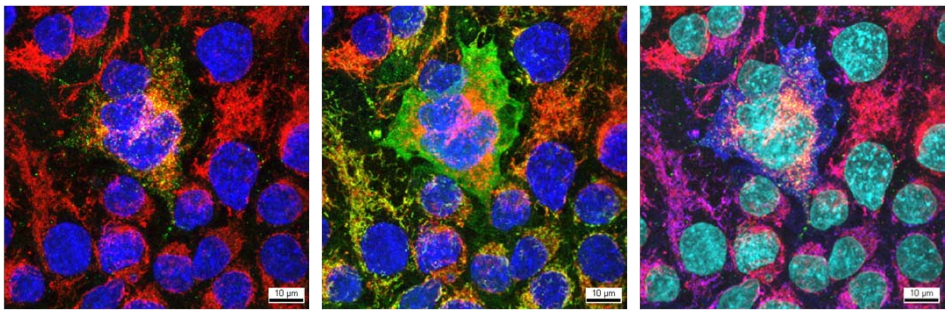






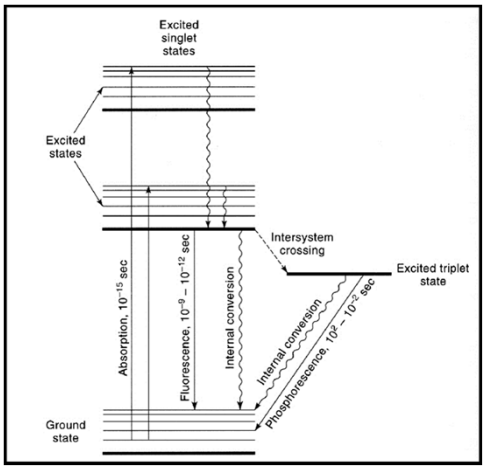


Fluorescence in microscopy



DNA	DNA	DNA
Bax	Bax	Bax
Mitochondria	Mitochondria	Mitochondria
Cytochrome C	Cytochrome C	Cytochrome C

Fluorescence in microscopy



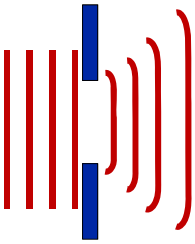
Jablonski scheme

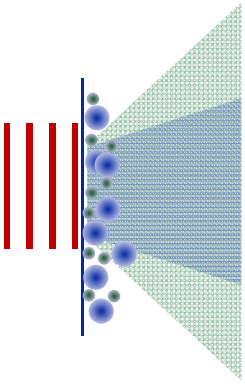
Advantages:

Very high contrast resulting in high sensitivity

Tagging of specific entities possible

Excitation / emission allows for various variants of microscopy techniques

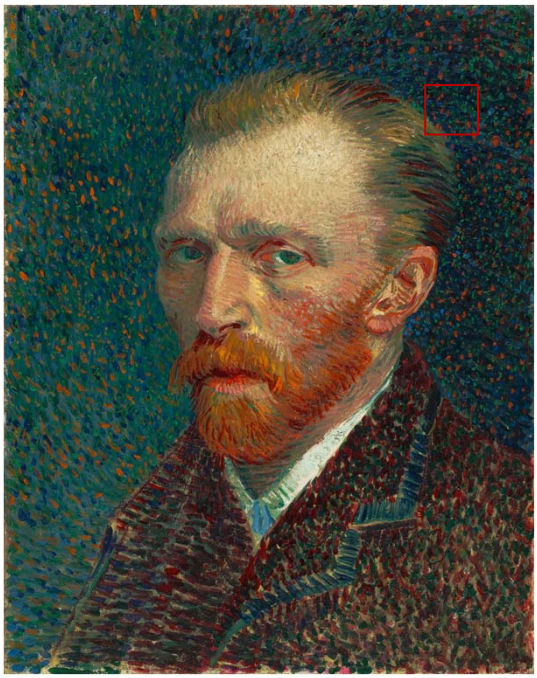




Diffraction at an aperture or substrate

Disturbance of the electric field of a planar wave front by diffraction upon passage through an aperture

A mixture of particles diffracts an incident planar wave front inversely proportional to the size of particles

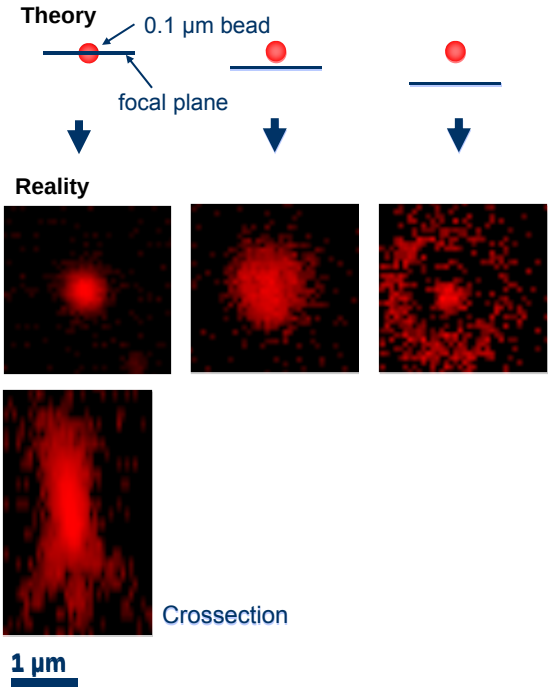


Resolution and aperture angle

Concept:

Object is approximated with self luminous points

Image of each individual point is not influenced by any other points



Spatial resolution in x,y and z

Implications:

Objects smaller than the resolution limit of the chosen objective will always be 1Airy disk

Objects larger than the resolution limit of the chosen objective will always be the size of the object convolved with the optical transfer function

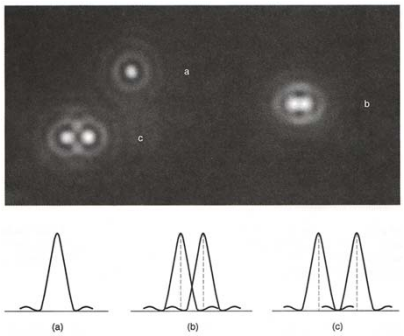
Note: the optical transfer function is a function describing how the imaging is occurring in the microscope

Resolution and size of Airy disk

Concept: an image of an extended object consists of a pattern of overlapping diffraction spots

Resolution: the larger the NA of the objective, the smaller the diffraction spots (airy disks).

Note: this theme of diffraction limited spots and their separation in space and time will again be used and taken up in superresolution microscopy.



a) Single diffraction pattern

b) Two Airy disks with maximum of one overlapping first minimum of the other
objects just resolved

c) Two Airy disks with maximum of one overlapping the second minimum
objects well resolved

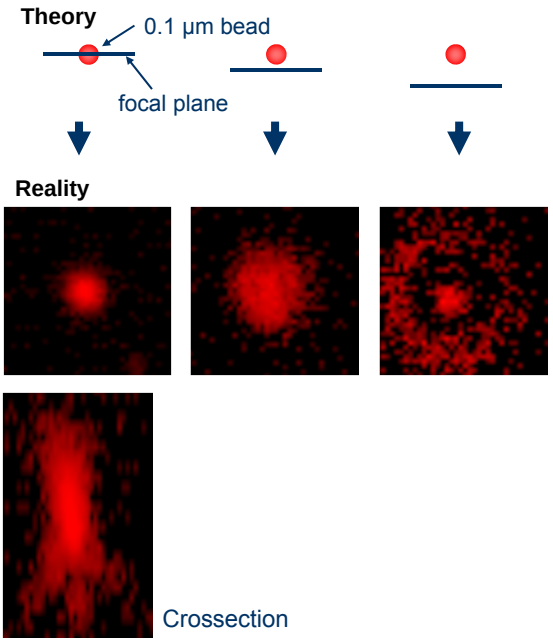
Resolution and Rayleigh criterion

Resolving power of microscope:

$$d = \frac{0.61 \times \lambda}{NA}$$

Concept: an image of an extended object consists of a pattern of overlapping diffraction spots

Resolution: the larger the NA of the objective, the smaller the diffraction spots (airy disks).



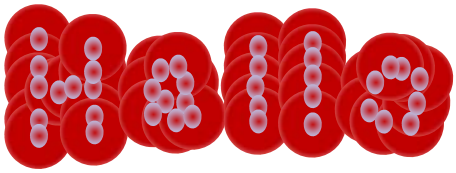
Spatial resolution in x,y and z

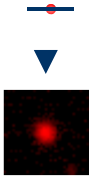
Objects are (always) 3 dimensional

The resulting ‘image’ will also be a 3D ‘image’ in the image space

Again:

an image of an extended object consists of a pattern of overlapping diffraction spots





Resolution and size of Airy disk

Objects are (always) 3 dimensional

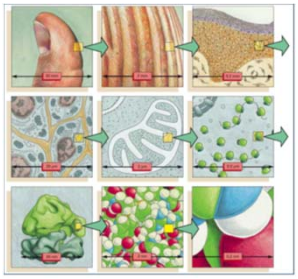
The resulting ‘image’ will also be a 3D ‘image’ in the image space

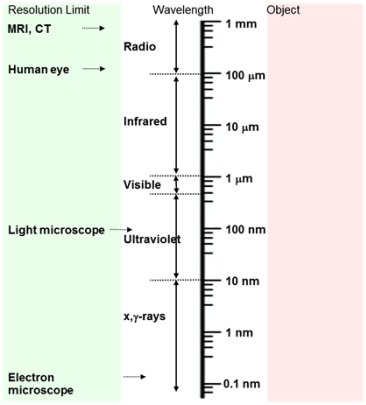
Again:

an image of an extended object consists of a pattern of overlapping diffraction spots

Take home:

In widefield microscopy the out of focus information is increasing the background and results in low contrast images





Resolution limits

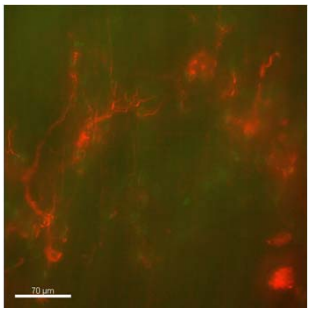
$$d_{xy} = \frac{0.61 \times \lambda}{NA}$$

$$d_z = \frac{n \times \lambda}{NA^2}$$

These formula are used for the calculation of resolution in widefield microscopy.

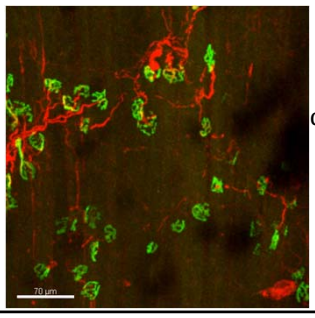
In other techniques like confocal laser scanning, multiphoton microscopy, etc other formula are used.

Comparison of widefield and confocal microscopy



$$d_z = \frac{n \times \lambda}{NA^2}$$

Image acquired with a widefield microscope

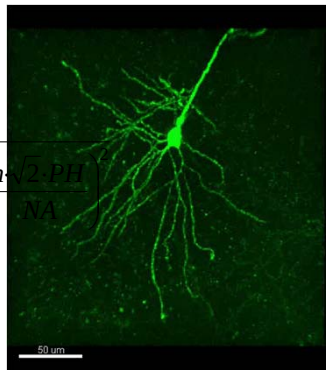


$$dz = \sqrt{\left(\frac{0.88 \cdot \lambda_{em}}{n - \sqrt{n^2 - NA^2}}\right)^2 + \left(\frac{n \cdot \sqrt{2} \cdot PH}{NA}\right)^2}$$

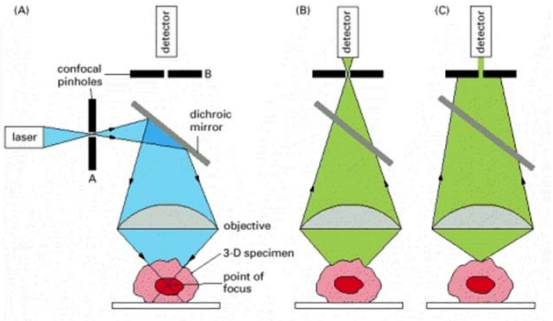
Image acquired with a confocal microscope

Confocal microscopy has a very high signal to noise ratio (prominent in thick samples)

Confocal microscopy allows well resolved 3D imaging (without any image processing)



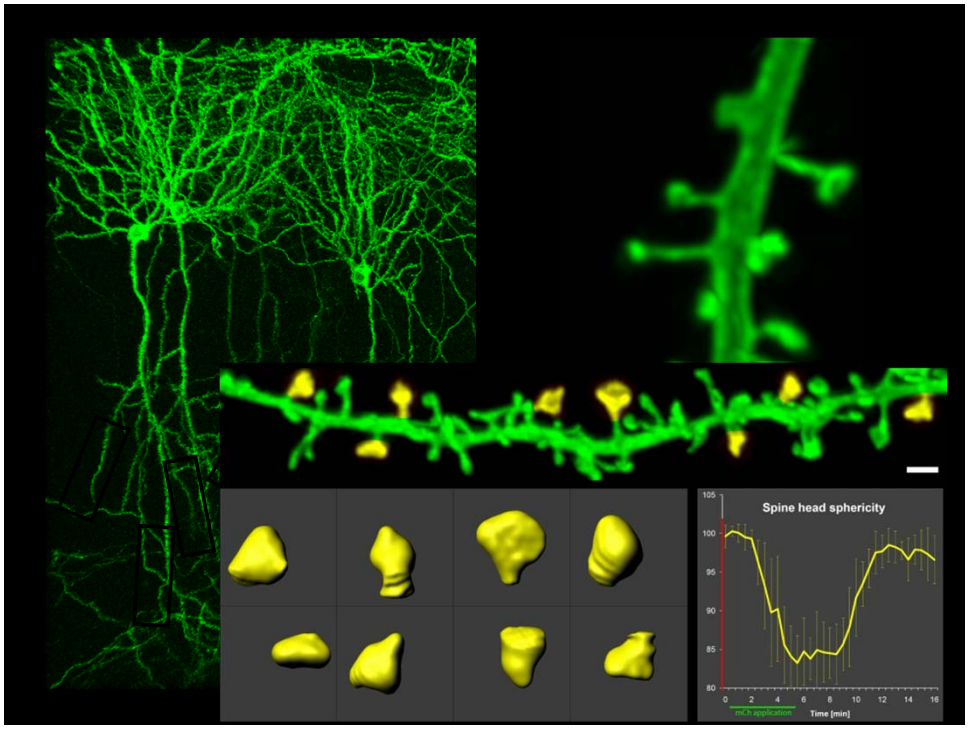
Confocal laser scanning microscopy



Sample is excited by a diffraction limited point of a focused laser spot

Emitted fluorescent light from focus is focused at pinhole and reaches detector

Emitted fluorescent light from out-of-focus is also out-of- focus at pinhole and largely excluded from detector



Spinning disk microscopy

Increase acquisition speed

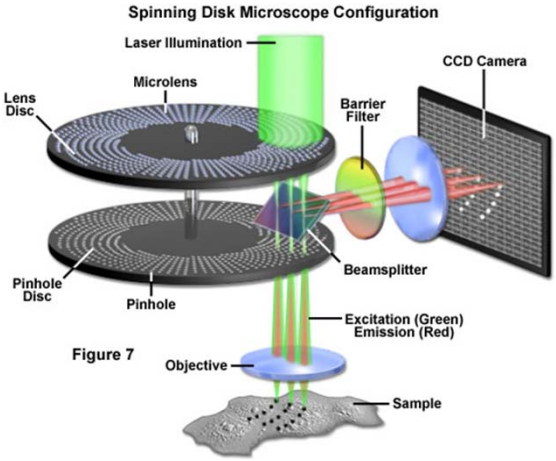
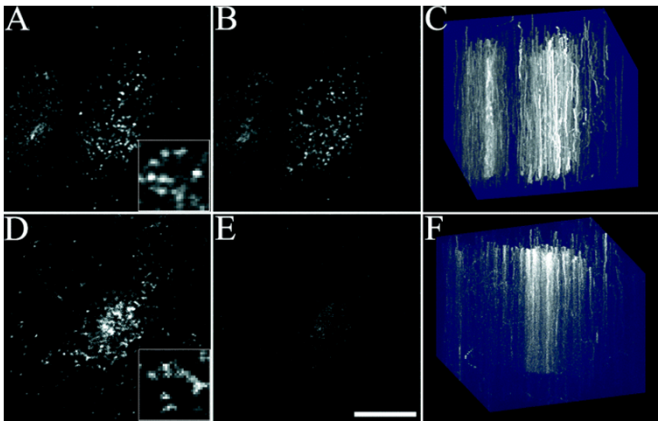
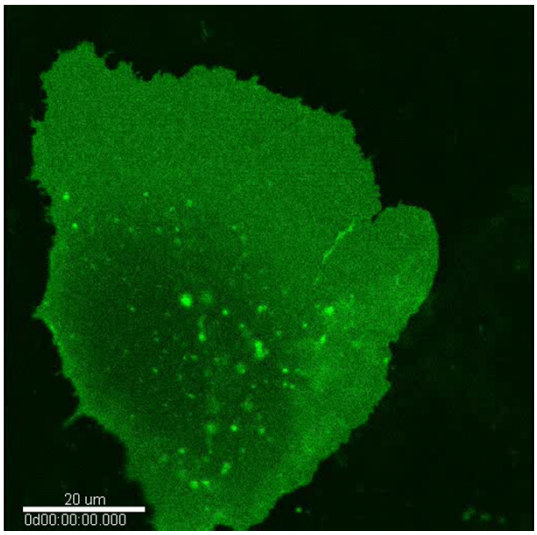


Figure 7

Performance comparison between the high-speed Yokogawa spinning disc confocal system and single-point scanning confocal systems



Journal of Microscopy
Volume 218, Issue 2, pages 148-159, 27 APR 2005 DOI: 10.1111/j.1365-2818.2005.01473.x
http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2818.2005.01473.x/full#f3



Fluorescence recovery after photobleaching

Image sample using widefield microscopy

Bleach defined region using intense illumination

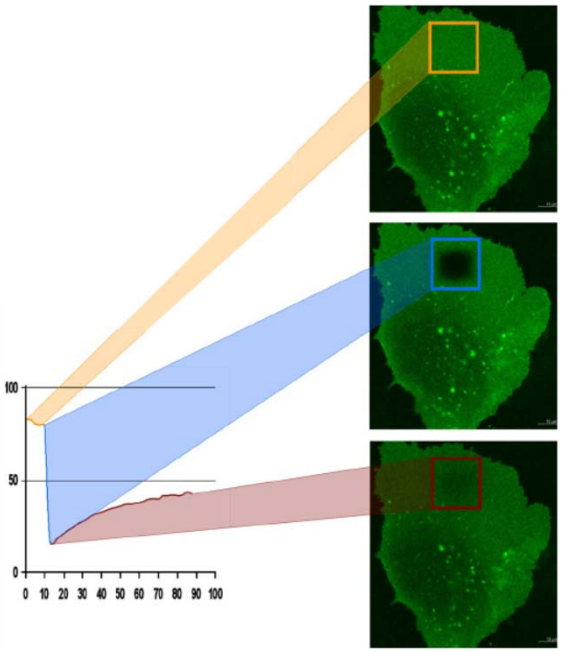
Measure fluorescence intensity over time in the photobleached region

Time for recovery of fluorescence is an indication for:

Diffusion

Mobility

Binding



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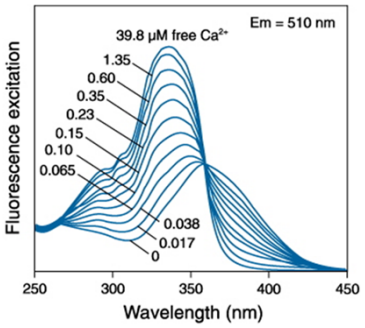
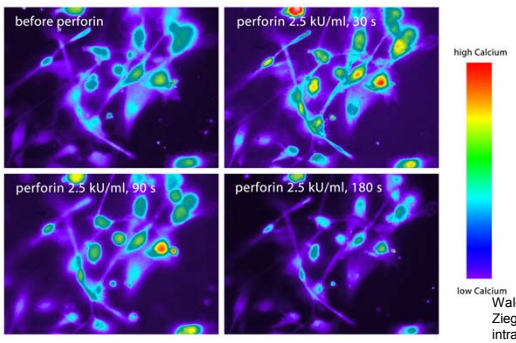




Measuring properties: e.g. Ca^{2+}

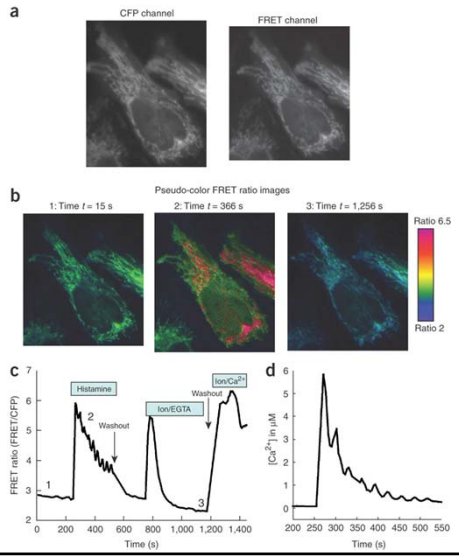
Measurement:
ratio imaging with an excitation of 340 and 380 nm

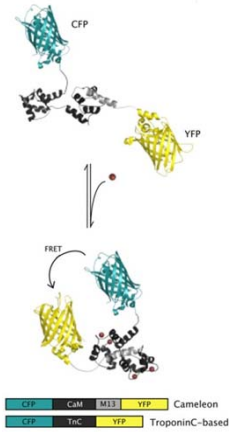
Walch, M., E. Eppler, C. Dumrese, H. Barman, P. Groscurth, and U. Ziegler. 2005. Uptake of granulysin via lipid rafts leads to lysis of intracellular Listeria innocua. *J Immunol*. 174:4220-4227.

Fluorescence resonance energy transfer

Transfer of energy from donor to acceptor without light emission from donor.

Ratio imaging of donor / acceptor or measurment of increase in acceptor emission when exciting the donor.

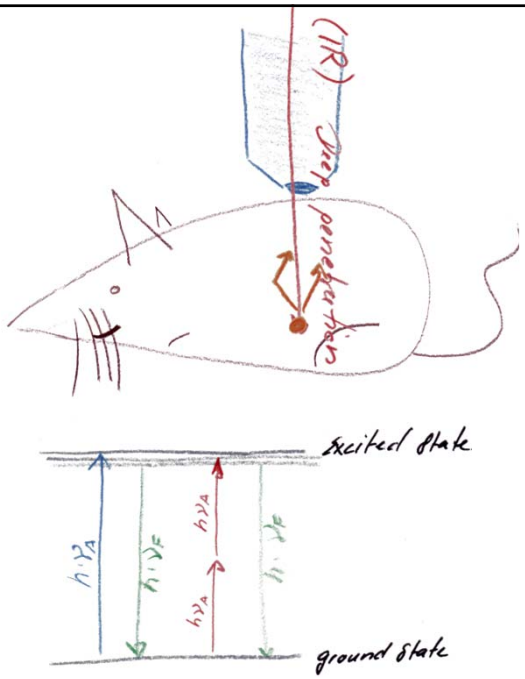




Palmer, A.E., and R.Y. Tsien. 2006. Measuring calcium signaling using genetically targetable fluorescent indicators. *Nature protocols*. 1:1057-65.

McCombs, J.E., and A.E. Palmer. 2008. Measuring calcium dynamics in living cells with genetically encodable calcium indicators. *Methods (San Diego, Calif.)*. 46:152-9.

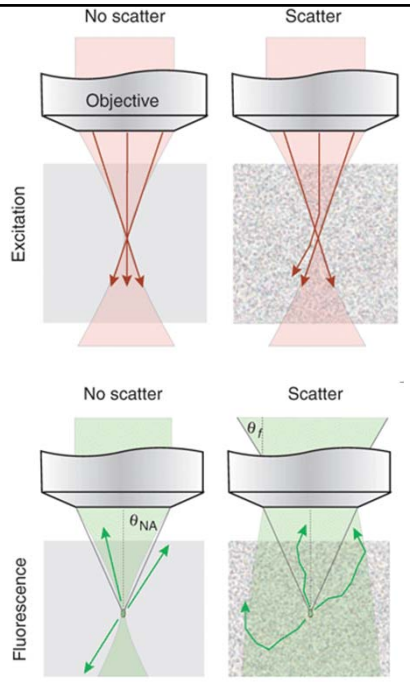
Multiphoton microscopy



Imaging deep into tissue

Pulsed infrared laser (700-1500nm) excites fluorochromes by multiphoton absorbtion

Excitation in a small volume defined by the probability (densitiy of photons high) of a simultaneous multiphoton absorbtion



Helmchen and Denk, Nature Methods 2005

Multiphoton microscopy

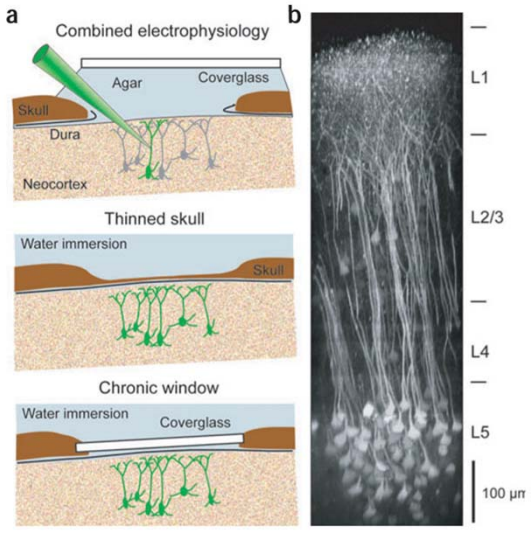
Imaging in scattering tissue and deep into tissue

Pulsed infrared laser (700-1500nm) excites fluorochromes by multiphoton absorbtion

Excitation in a small volume defined by the probability (densitiy of photons high) of a simultaneous multiphoton absorbtion

All fluorescent photons provide useful signals.

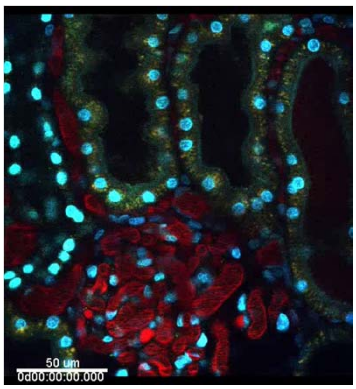
Brain



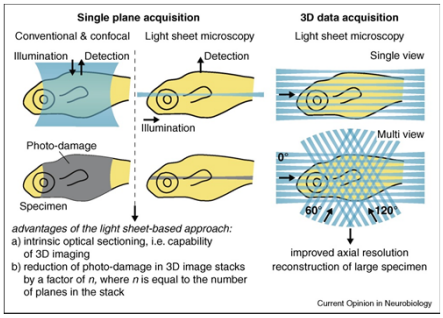
Helmchen, F., and W. Denk. 2005. Deep tissue two-photon microscopy. *Nature methods*. 2:932-40.

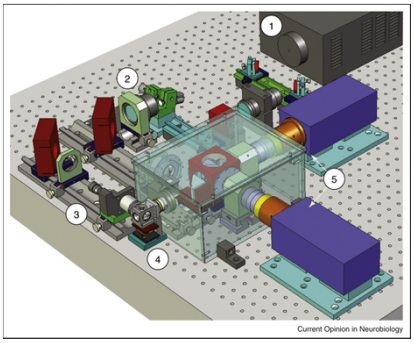
Multiphoton microscopy

Kidney



Living mouse: kidney (Hoechst, 10kD dextran FITC, 150kD dextran Texas Red





Selective Plane Illumination Microscopy

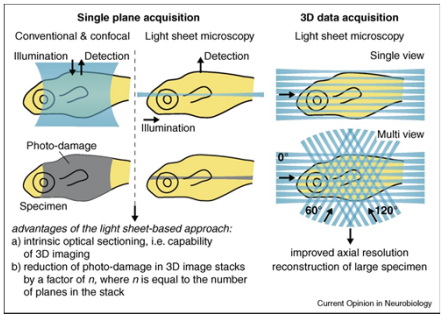
SPIM

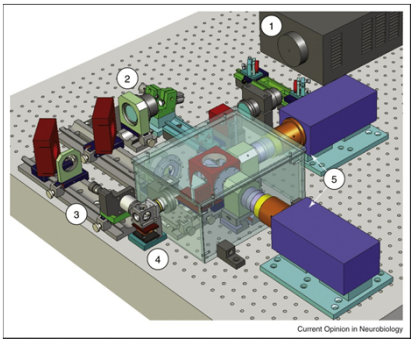
4D imaging

Light-sheet-imaging technique

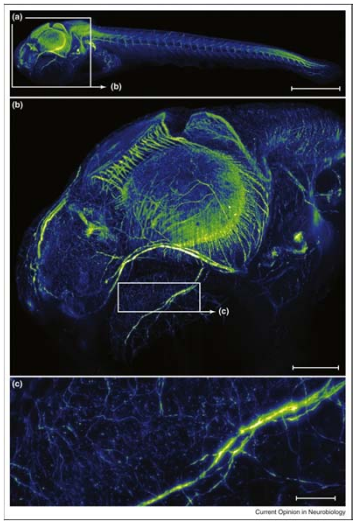
Better signal-to-noise ratio

Low phototoxicity

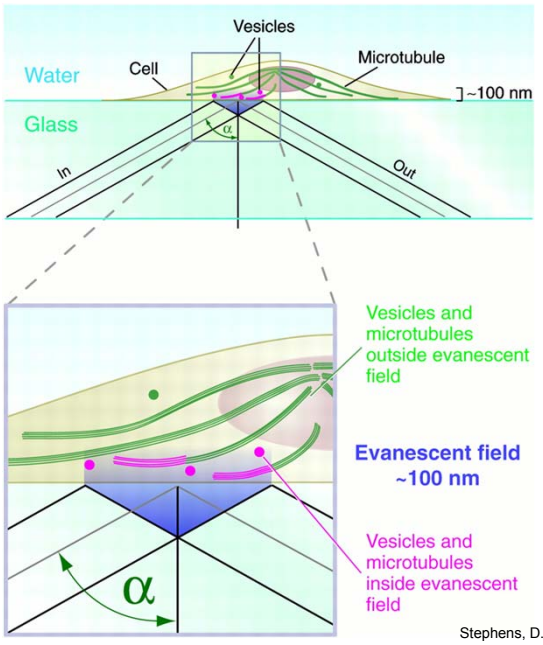




Selective Plane Illumination Microscopy



Keller, P.J., and E.H.K. Stelzer. 2008. Quantitative in vivo imaging of entire embryos with Digital Scanned Laser Light Sheet Fluorescence Microscopy. *Current opinion in neurobiology*. 18:624-32.



Total internal reflection fluorescence microscopy

TIRF

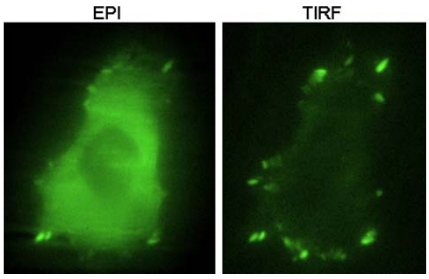
Laser excitation light is directed at a tissue sample through a glass slide at a specific, oblique angle (critical angle)

Most of the light is reflected at the interface between glass and the tissue sample (total internal reflection)

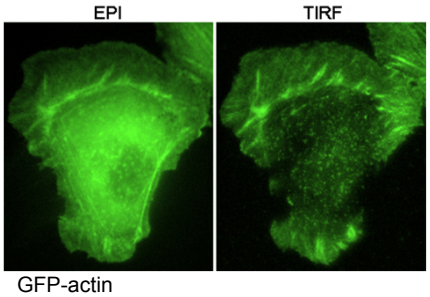
Induction of an evanescent wave parallel to the slide

Decay of the evanescent wave over 200 nm

Stephens, D.J., and V.J. Allan. 2003. Light microscopy techniques for live cell imaging. *Science (New York, N.Y.)*. 300:82-6.



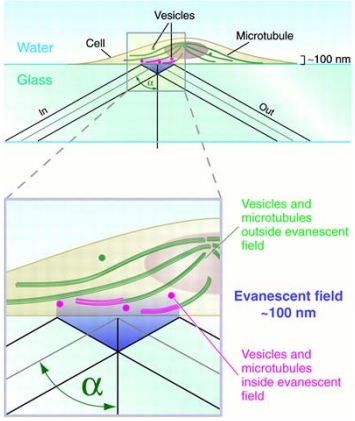
GFP- paxilin

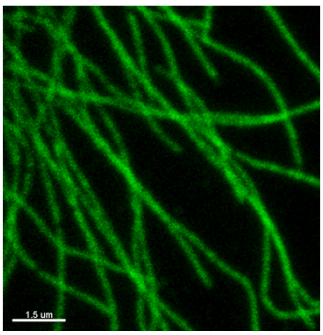


GFP-actin

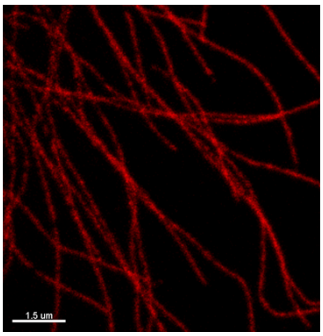
http://www.einstein.yu.edu/aif/instructions/tirf/index.htm

Total internal reflection fluorescence microscopy





Confocal



STED

Superresolution microscopy

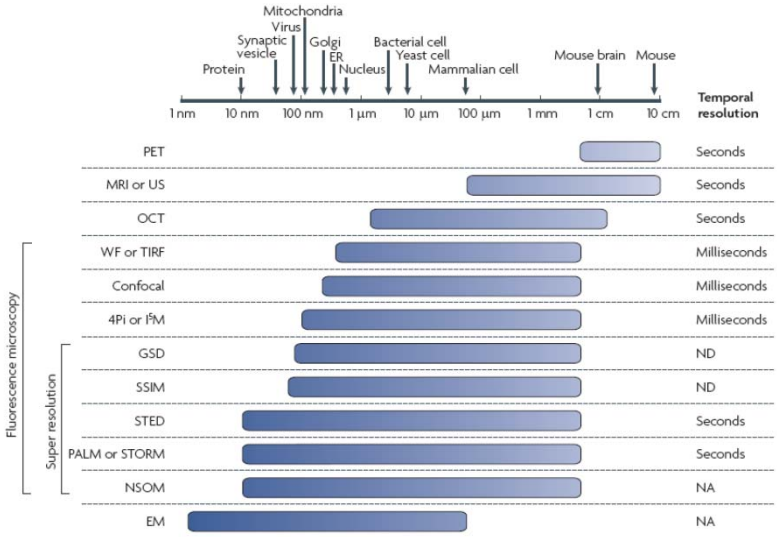
Beyond the diffraction limit

$d = 0.61 \lambda / NA$

Imaging EGFP in living cells has a resolution of approximately 200 (XY) and 500 nanometers (Z)

Sample courtesy Martin Engelke, Urs Greber, Institute of Zoology, University of Zurich

Super resolution microscopy



Super resolution microscopy

Enhanced PSF microscopy

SSIM
Saturated structured illumination microscopy

STED
Stimulated emission depletion

Statistical microscopy

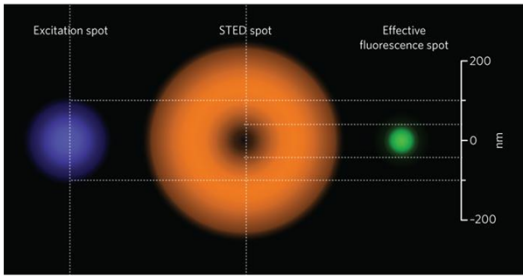
STORM
Stochastic optical reconstruction microscopy

PALM
Photoactivated localization microscopy

GSD
Ground state depletion microscopy

Stimulated emission depletion microscopy

STED



Abbott *Nature* **459**, 638-639 (2009)

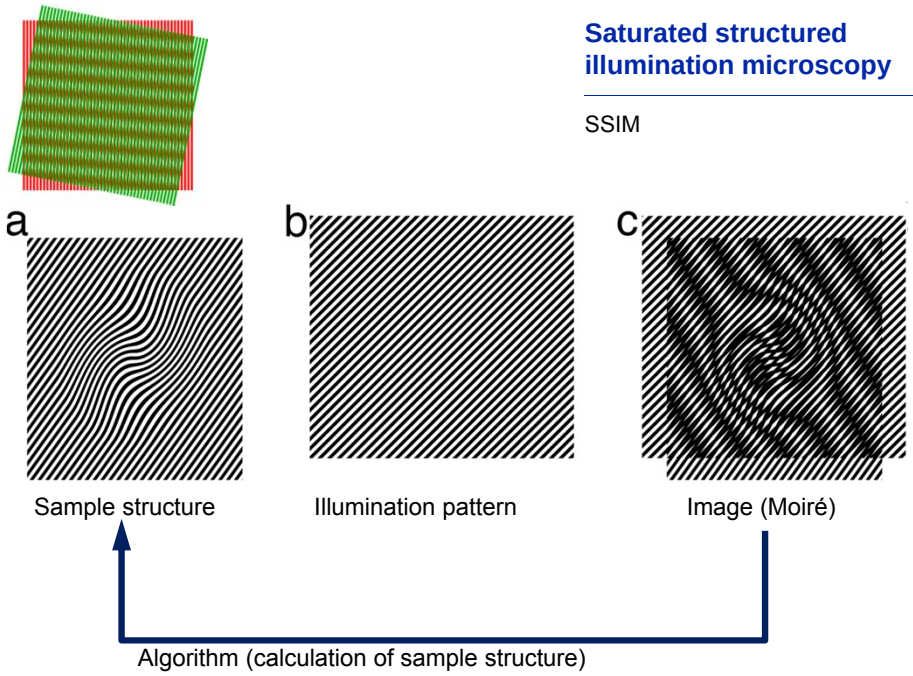
In STED, an initial excitation pulse is focused on a spot.

The spot is narrowed by a second, donut-shaped pulse that prompts all excited fluorophores in the body of the donut to emit (this is the "emission depletion" part of STED).

This leaves only the hole of the donut in an excited state, and only this narrow hole is detected as an emitted fluorescence.

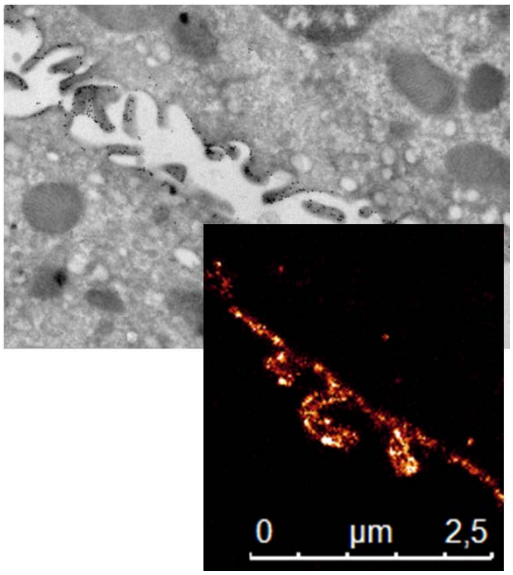
Saturated structured illumination microscopy

SSIM



Statistical microscopy – Imaging single molecules

Position of a single molecule can be localized to 1 nm accuracy or better if enough photons are collected and there are no other similarly emitting molecules within ~200 nm (Heisenberg 1930, Bobroff 1980).



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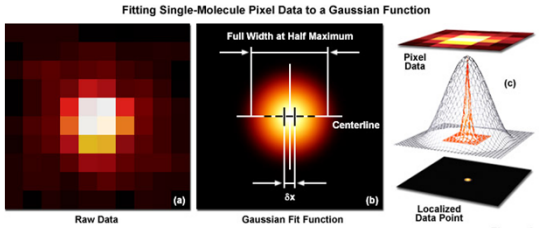

Figure 3

Price and **Davidson**
Florida State University

GSD
Ground state depletion microscopy

PALM
Photoactivated localization microscopy

STORM
Stochastic optical reconstruction microscopy

Literature

Fundamentals of light microscopy and electronic imaging, Douglas B. Murphy; Wiley-Liss, 2001
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Light Microscopy in Biology – A practical approach, A. J. Lacey; Oxford University Press, 2004

Light and Electron Microscopy, E. M. Slayter, H. S. Slayter; Cambridge University Press, 1992

http://microscopy.fsu.edu/primer/index.html

Thank you