



University of Zurich<sup>UZH</sup>

Center for Microscopy and Image Analysis

# Introduction to light microscopy

Imaging with light / Overview of techniques

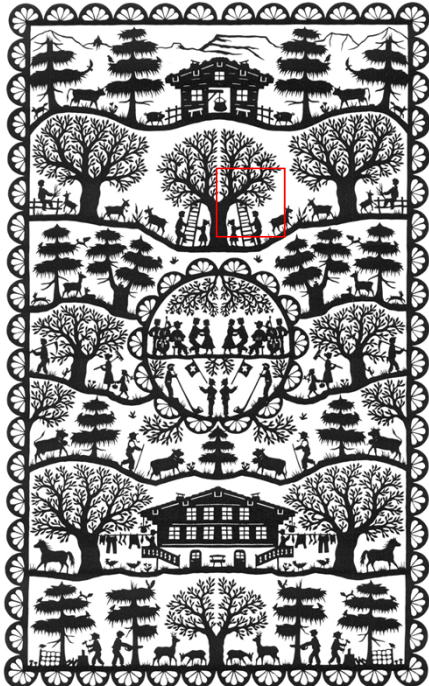
Urs Ziegler

ziegler@zmb.uzh.ch




## Light interacting with matter

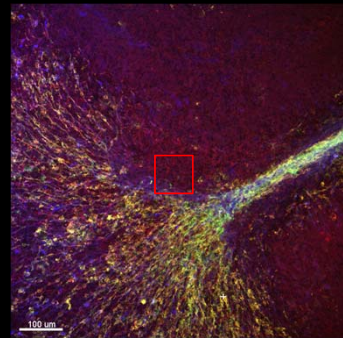
- Absorption
- Refraction
- Diffraction
- Scattering

A detailed woodcut-style illustration of a village scene. The scene is framed by a decorative border of repeating circular motifs. In the center, a large tree stands with a ladder leaning against it. A red box highlights this central tree and ladder. The scene includes various elements like houses, trees, and figures.

### Light interacting with matter

- Absorbtion
- Refraction
- Diffraction
- Scattering

A silhouette illustration of a tree and a ladder leaning against it. This is a simplified version of the central scene highlighted by the red box in the main image. The background is white, and the objects are black.

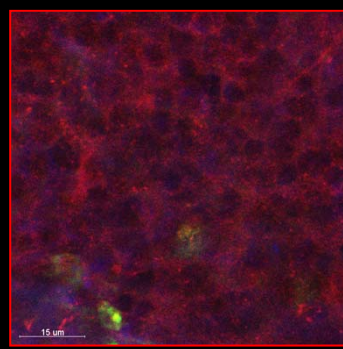
A fluorescence microscopy image showing a biological sample. The image is composed of various colors (purple, blue, green, red) representing different fluorescent components. A red box highlights a specific region in the center. A scale bar in the bottom left corner indicates 100 μm.

### Light interacting with matter

Light emitted from fluorochromes

How is an image formed?

Why are there limits in resolution?

A high-magnification fluorescence microscopy image of the region highlighted by the red box in the top image. The image shows a dense field of red and green fluorescent spots. A scale bar in the bottom left corner indicates 15 μm.

**Imaging with light / Overview of techniques**

**Introduction to light microscopy**

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

**Methods and techniques in microscopy**

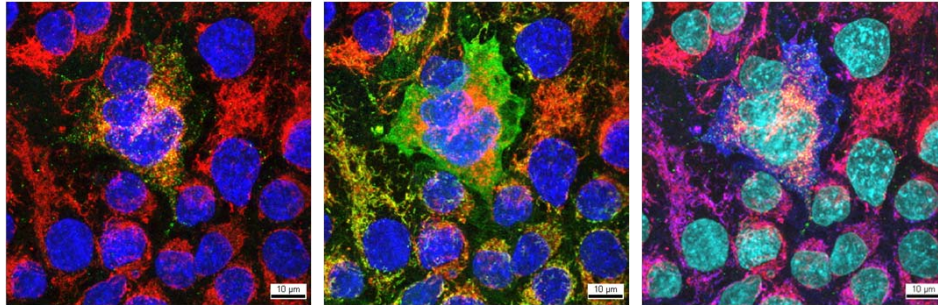
- Widefield microscopy
- Confocal laser scanning microscopy
- Fluorescence energy transfer
- Fluorescence recovery after photobleaching
- In vivo microscopy
- Selective plane illumination microscopy
- Superresolution techniques

*Correlative techniques – light and electron microscopy*

**Fundamental Setup of Light Microscopes**

The diagram illustrates the fundamental setup of a light microscope. It shows the optical path for both brightfield and fluorescence imaging. Key components labeled include the Light Source, Condenser, Wollaston Prism, Phase Ring, Polarizer, Sample Plane, Objectives, Wollaston Prism, Polarizer, and Ocular. The Z Focus is also indicated. A corresponding fluorescence image shows red-stained cells.

### Fluorescence in microscopy

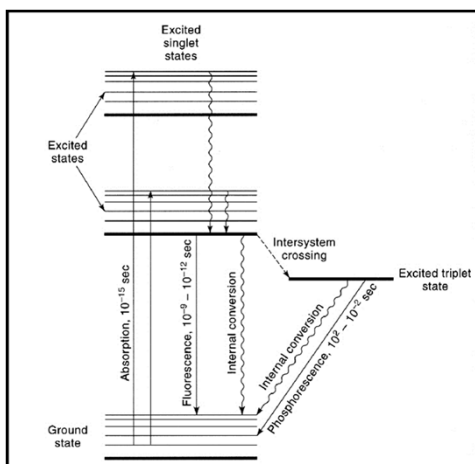


**DNA**  
**Bax**  
**Mitochondria**  
**Cytochrome C**

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

**DNA**  
**Bax**  
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**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



**Theory**

0.1  $\mu\text{m}$  bead  
focal plane

**Reality**

**Crosssection**

1  $\mu\text{m}$

**Spatial resolution in x,y and z**

---

Implications:

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

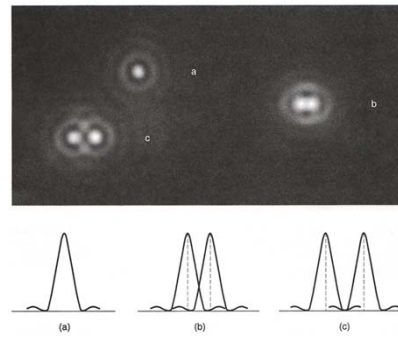
**Resolution and size of Airy disk**

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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

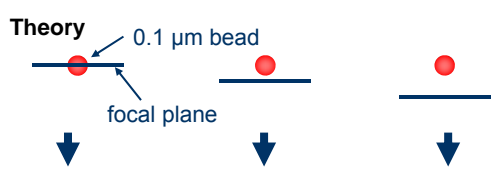
Resolving power of microscope:

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

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).

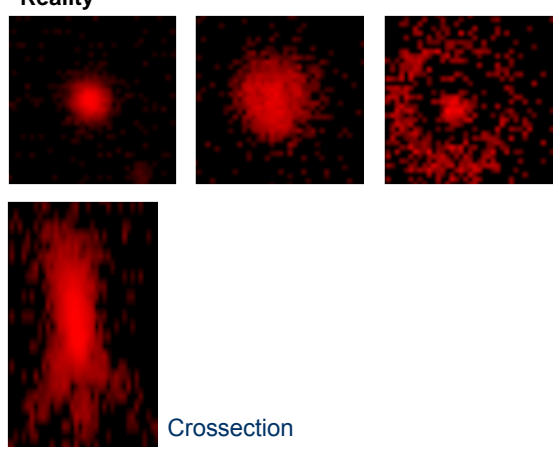
**Theory**



0.1 μm bead

focal plane

**Reality**



Crosssection

1 μm

### Spatial resolution in x,y and z

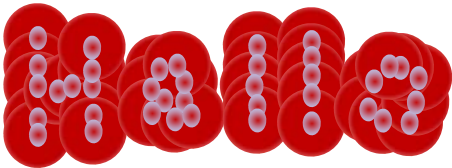
Objects are (always) 3 dimensional

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



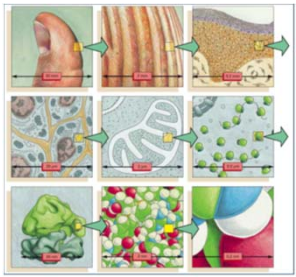
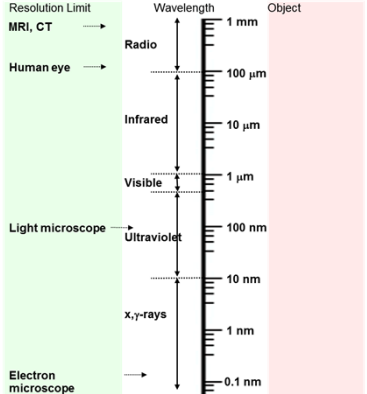
The resulting 'image' will also be a 3D 'image' in the image space

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

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

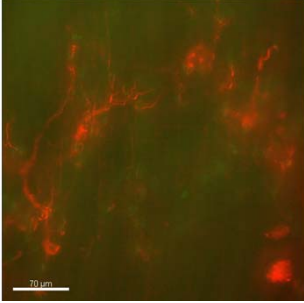
$$d_z = \frac{n \times \lambda}{NA^2}$$

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

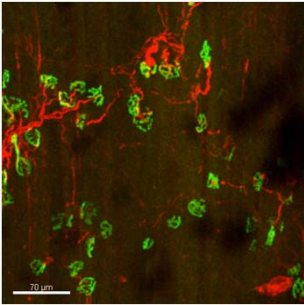


$d_z = \frac{n \times \lambda}{NA^2}$

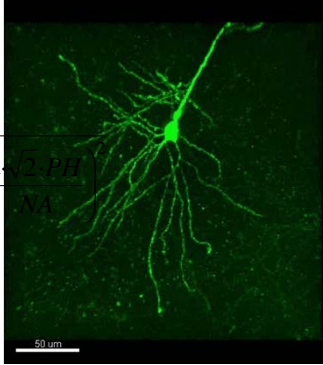
**Image acquired with a widefield microscope**

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)

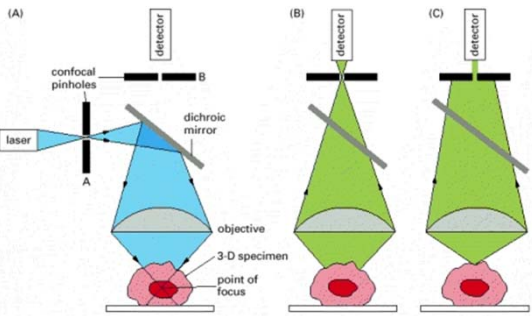


**Image acquired with a confocal microscope**



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

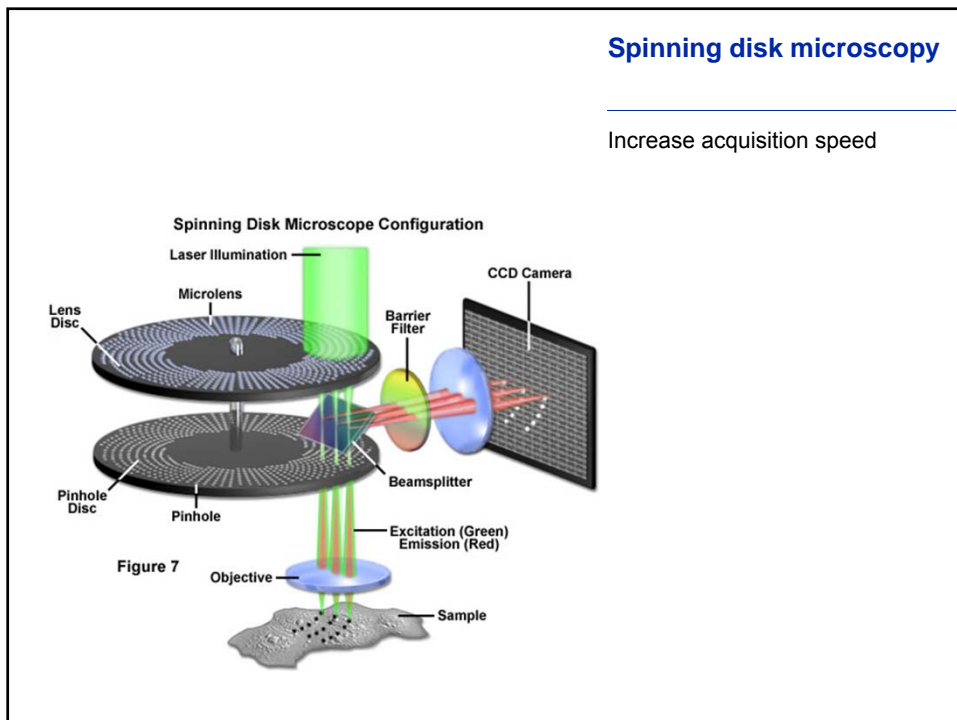
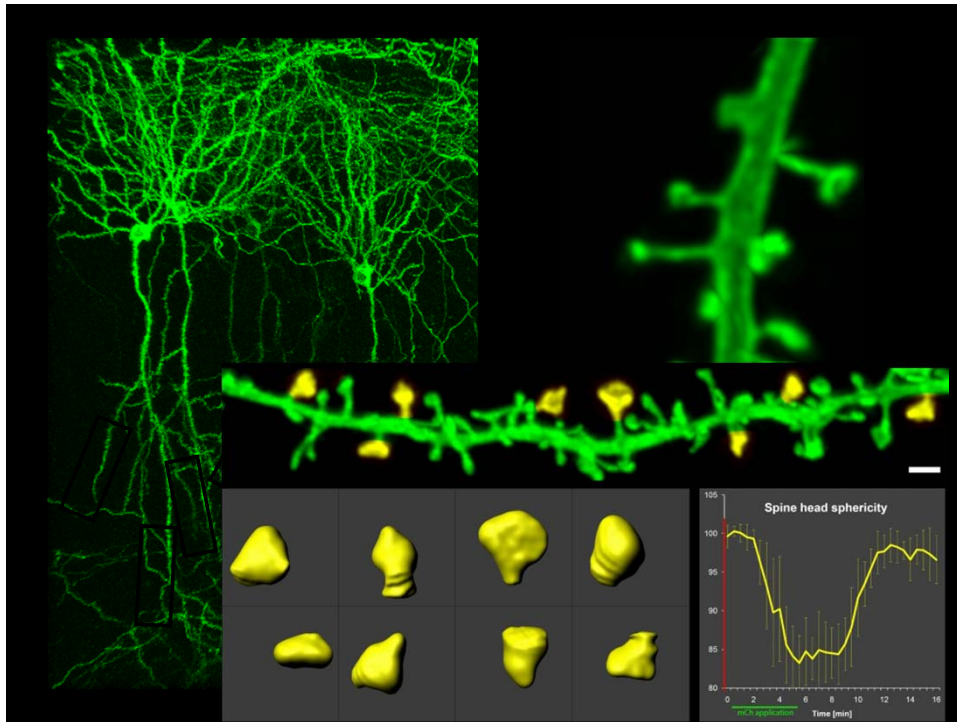
### Confocal laser scanning microscopy



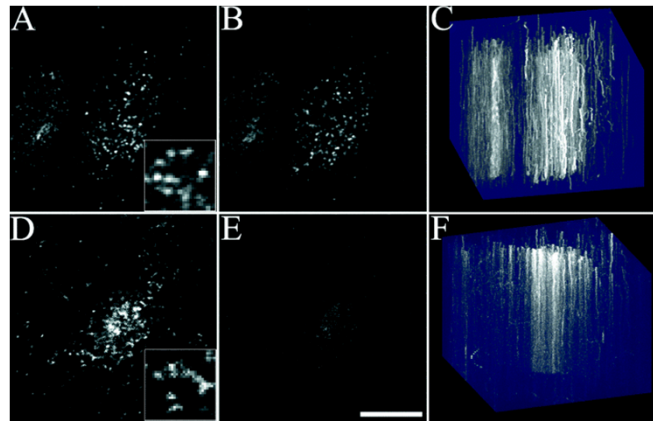
Sample is excited by a **diffraction limited point** of a focused laser spot

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



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

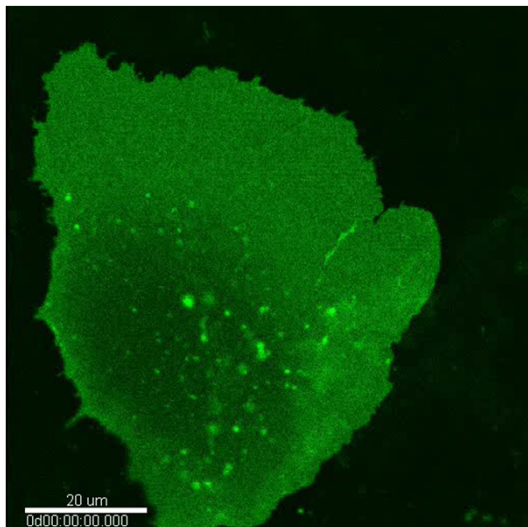


Image sample using widefield microscopy

**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

### Fluorescence recovery after photobleaching

The diagram illustrates the FRAP process. On the left, a graph plots fluorescence intensity (0 to 100) against time (0 to 100). A blue shaded area shows the initial fluorescence, which drops to a lower level (red shaded area) after photobleaching. The recovery of fluorescence is shown as a red curve that gradually returns towards the initial level. On the right, three panels of a green fluorescent sample are shown. The top panel is the initial state with a yellow box indicating the region to be bleached. The middle panel shows the sample after bleaching, with a blue box indicating the region where fluorescence intensity is being monitored. The bottom panel shows the recovery of fluorescence in the bleached region, with a red box indicating the monitored area.

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

### Measuring properties: e.g. Ca<sup>2+</sup>

The graph shows fluorescence excitation versus wavelength (nm) for various free Ca<sup>2+</sup> concentrations. The y-axis is labeled 'Fluorescence excitation' and the x-axis is 'Wavelength (nm)' ranging from 250 to 450. The emission wavelength is noted as Em = 510 nm. The curves represent different Ca<sup>2+</sup> concentrations: 1.35, 0.60, 0.35, 0.23, 0.15, 0.10, 0.065, 0.038, 0.017, and 0. The fluorescence intensity increases with higher Ca<sup>2+</sup> concentrations.

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

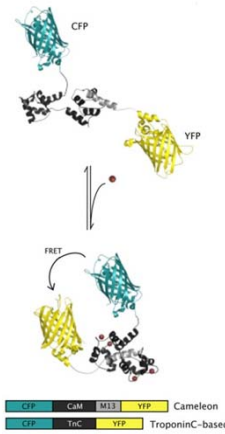
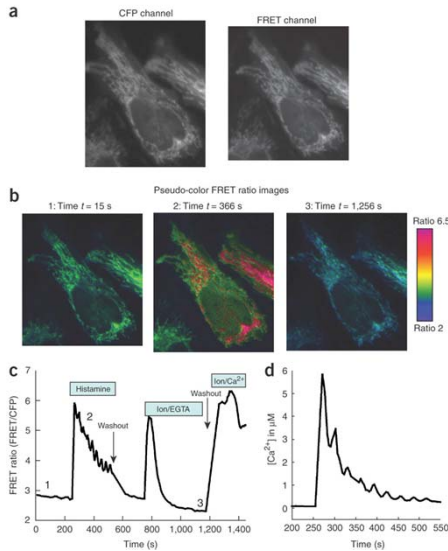
The images show a cell before and after treatment with 2.5 kU/ml of perforin at 30 s, 90 s, and 180 s. A color scale on the right indicates calcium levels, from low (blue) to high (red). The images show an increase in high calcium (red/yellow) areas over time after perforin treatment.

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.

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

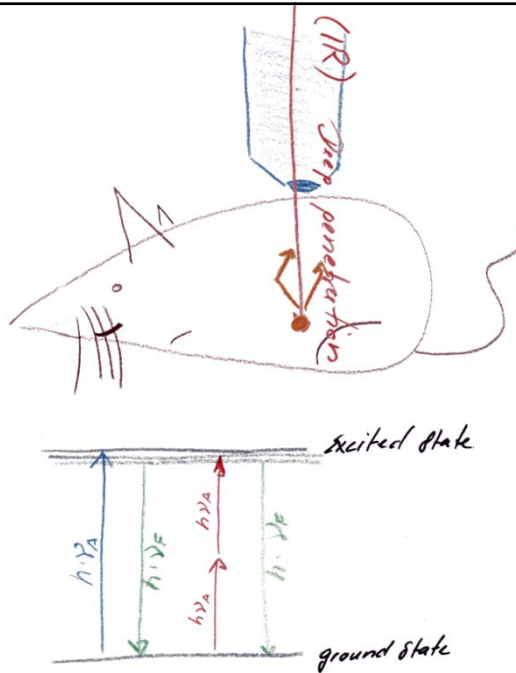
Ratio imaging of donor / acceptor or measurement of increase in acceptor emission when exciting the donor.

### Fluorescence resonance energy transfer



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 absorption

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



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 absorption

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

All fluorescent photons provide useful signals.

### Brain

### Multiphoton microscopy

#### Kidney

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

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

**Single plane acquisition**

Conventional & confocal: Illumination, Detection, Photo-damage, Specimen

Light sheet microscopy: Illumination, Detection

**3D data acquisition**

Light sheet microscopy: Single view, Multi view (60°, 120°), improved axial resolution, reconstruction of large specimen

*advantages of the light sheet-based approach:*  
a) intrinsic optical sectioning, i.e. capability of 3D imaging  
b) reduction of photo-damage in 3D image stacks by a factor of  $n$ , where  $n$  is equal to the number of planes in the stack

Current Opinion in Neurobiology

### Selective Plane Illumination Microscopy

SPIM

4D imaging

Light-sheet-imaging technique

Better signal-to-noise ratio

Low phototoxicity

Current Opinion in Neurobiology

**Single plane acquisition**

Conventional & confocal: Illumination, Detection, Photo-damage, Specimen

Light sheet microscopy: Illumination, Detection

**3D data acquisition**

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Current Opinion in Neurobiology

### Selective Plane Illumination Microscopy

Current Opinion in Neurobiology

Current Opinion in Neurobiology

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.

### Total internal reflection fluorescence microscopy

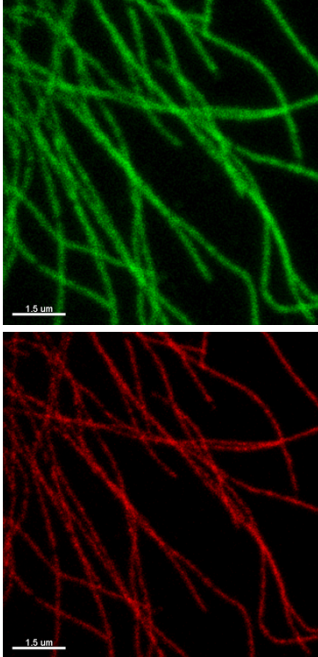
EPI      TIRF

GFP- paxilin

EPI      TIRF

GFP-actin

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



## Superresolution microscopy

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Beyond the diffraction limit

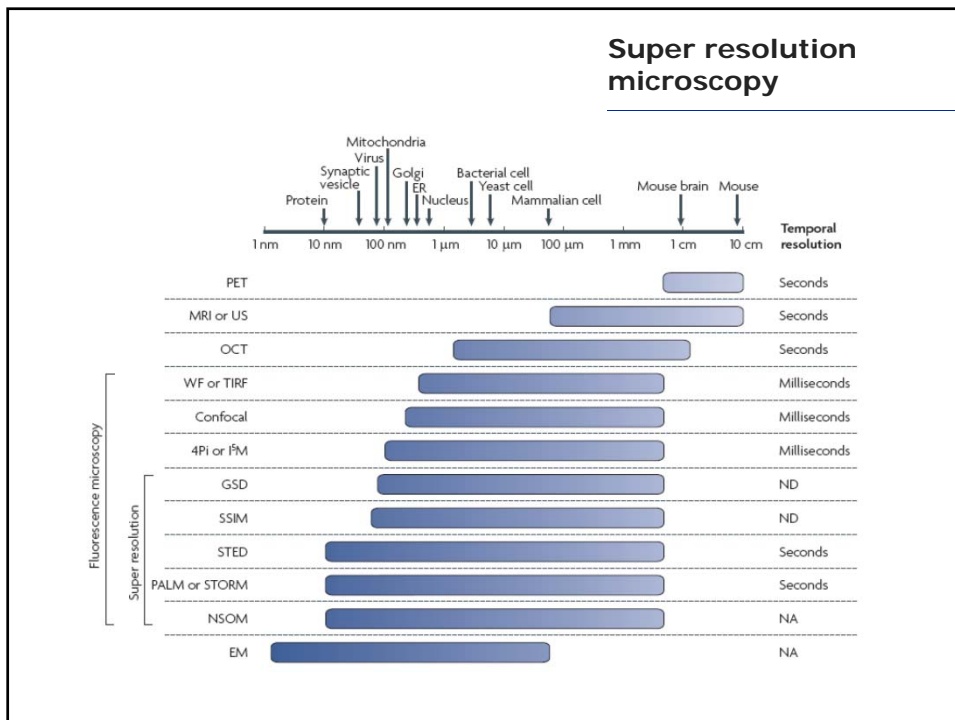
  

$$d = 0.61 \lambda / NA$$
  

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

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### 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

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## Stimulated emission depletion microscopy

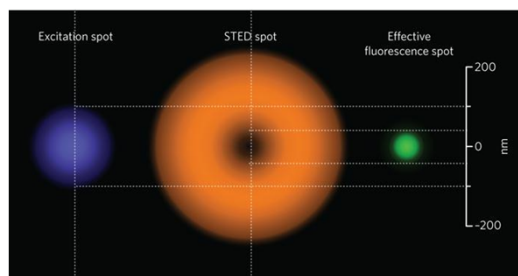
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### STED

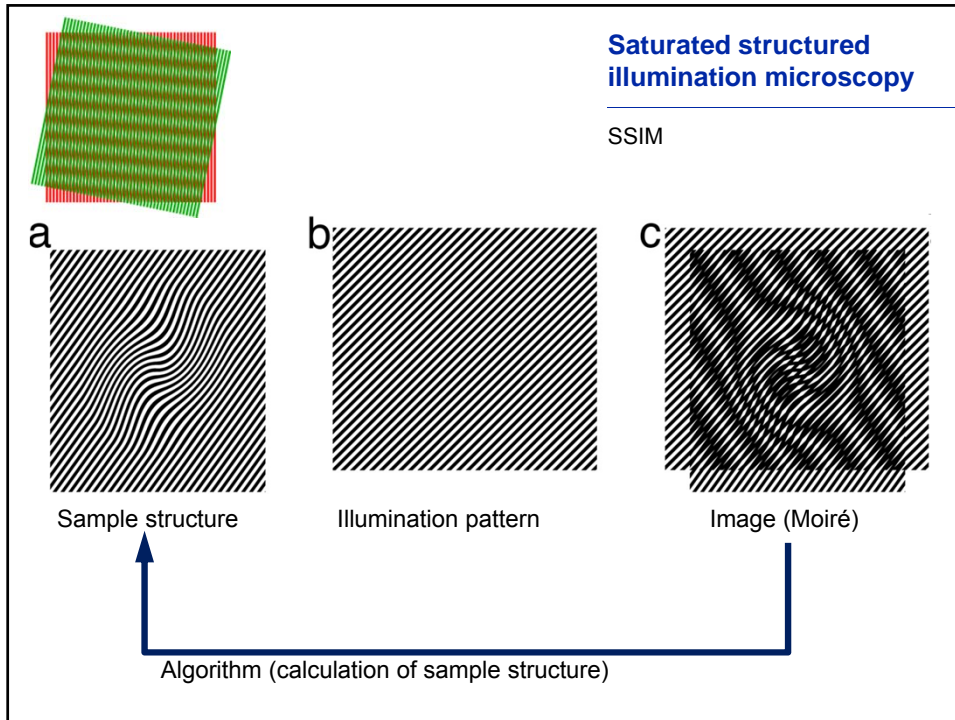
In STED, an initial excitation pulse is focused on a spot.

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.**



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



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).

**Statistical microscopy – Imaging single molecules**

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The image shows a grayscale micrograph of a cell with various organelles. An inset at the bottom right shows a high-resolution image of a specific region, with individual molecules appearing as bright orange spots. A scale bar below the inset is labeled '0 μm 2,5'.

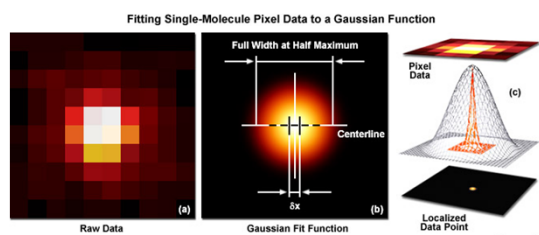
**GSD**  
Ground state depletion microscopy

**PALM**  
Photoactivated localization microscopy

**STORM**  
Stochastic optical reconstruction microscopy

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).

## Statistical microscopy – Imaging single molecules



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  
ISBN 0-471-25391-X

**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

