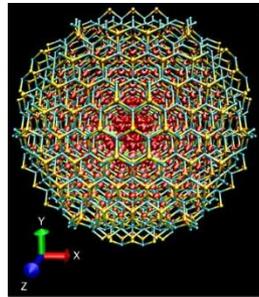


Advanced Microscopy Methods

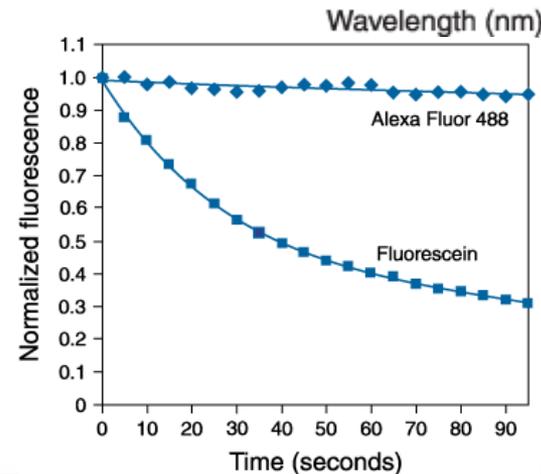
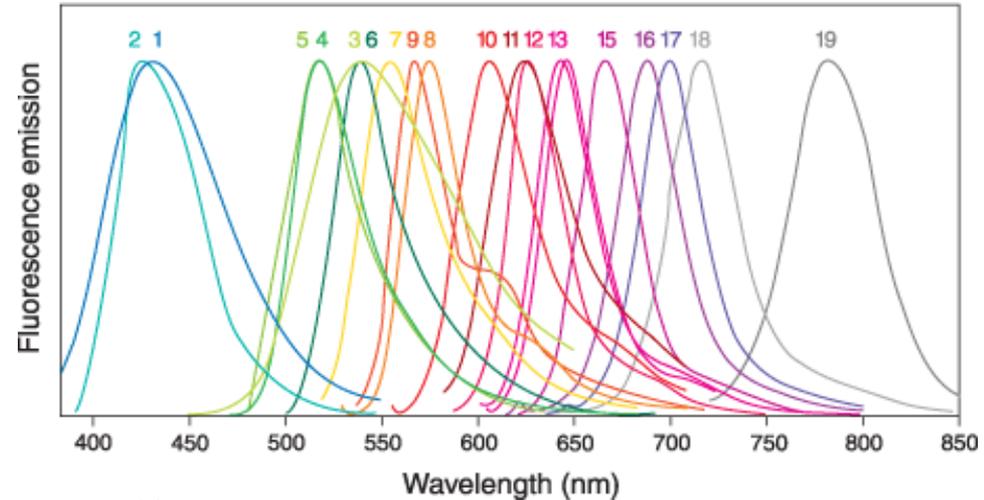
- Novel interesting fluorophores and fluorescent proteins
- Confocal Microscopy in general
- Spectral imaging (Emission Finger printing)
- Spinning disk confocal microscopy
- Multiphoton microscopy
- FRAP (Fluorescence Recovery After Photobleaching)
- FLIP (Fluorescence Loss in Photobleaching)
- FRET
- Superresolution Microscopy

Fluorophore development

- Traditional fluorophores: fluorescein (FITC), rhodamine, DAPI (4',6-diamidino-2-phenylindole) for DNA
- newer dyes: Cy3, Cy5
- further developments: Alexa-Dyes
- quantum dots: „fluorescent“ semiconductors; emission wavelengths depends on the size; one excitation wavelength for several emission wavelengths



- | | | | |
|--------------------|---------------------|---------------------|---------------------|
| 1. Alexa Fluor 350 | 6. Alexa Fluor 514 | 11. Alexa Fluor 594 | 16. Alexa Fluor 660 |
| 2. Alexa Fluor 405 | 7. Alexa Fluor 532 | 12. Alexa Fluor 610 | 17. Alexa Fluor 680 |
| 3. Alexa Fluor 430 | 8. Alexa Fluor 546 | 13. Alexa Fluor 633 | 18. Alexa Fluor 700 |
| 4. Alexa Fluor 488 | 9. Alexa Fluor 555 | 14. Alexa Fluor 635 | 19. Alexa Fluor 750 |
| 5. Alexa Fluor 500 | 10. Alexa Fluor 568 | 15. Alexa Fluor 647 | |



bleaching

Interesting fluorescent proteins

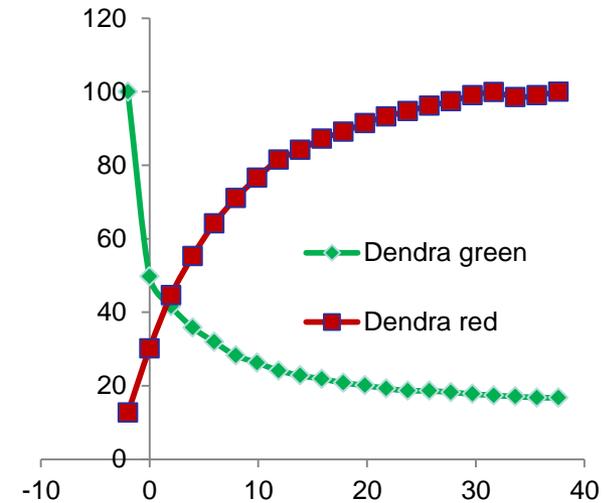
<i>fluor. protein</i>	<i>Ex-Peak (nm)</i>	<i>Em-Peak (nm)</i>	<i>quantum yield</i>	<i>comment</i>
<i>EBFP</i>	380	440	0.18	<i>Clontech</i>
<i>ECFP</i>	433 (453)	475 (501)	0.4	<i>Clontech</i>
<i>EGFP</i>	488	507	0.6	<i>Clontech</i>
<i>wildtype GFP</i>	397 (475)	509	0.77	<i>Aequorea victoria</i>
<i>EYFP</i>	513	527	0.61	<i>Clontech</i>
<i>Citrine</i>	516	529	0.76	<i>Griesbeck et al. 2001</i>
<i>DsRed</i>	558	583	0.29	<i>Clontech, tetramer</i>
<i>DsRed2</i>	563	582	0.55	<i>tetramer,</i>
<i>HcRed1</i>	588	618	0.02	<i>Clontech, dimer</i>
<i>PA-GFP</i> (Patterson 2002)	400 before act. 504 (397) after	515 before act. 517 after act.	0.13 0.79	<i>photoactivatable GFP, T203H mutant of mammalian codon-optimized wildtype GFP</i>
<i>PS-CFP</i>	400 before act. 490 after act.	468 before act. 511 after act.	0.2 0.23	<i>photoswitchable CFP, turns from cyan to green after intense illum. at 405 nm</i>
<i>mOrange</i>	548	562	0.69	<i>Shaner et al., 2004</i>
<i>mStrawberry</i>	574	596	0.29	<i>Shaner et al., 2004</i>
<i>mCherry</i>	587	610	0.22	<i>Shaner et al., 2004</i>
<i>dTomato</i>	554	581	0.69	<i>Shaner et al., 2004, dimeric</i>

New developments: photo-convertible fluorescent proteins

Dendra 2

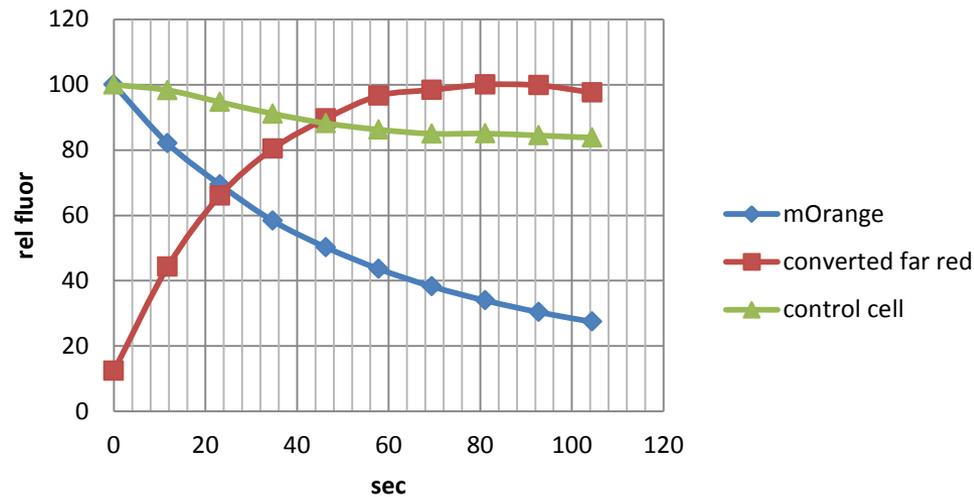
pre-UV exposure

after 30 sec UV exposure
(350 nm, DAPI excitation)
50% intensity

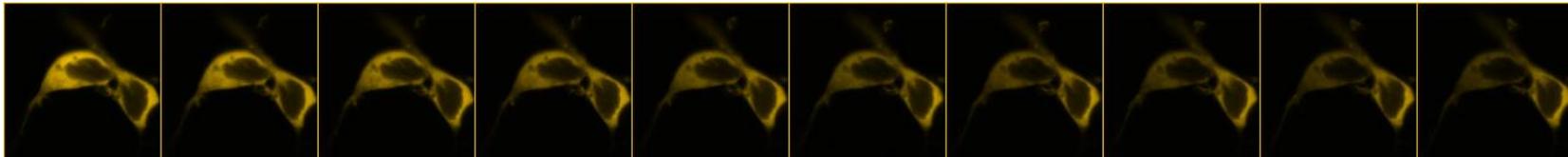


Photoconvertible fluorescent proteins

mOrange conversion to far-red
(2x bleaching with 100% 488 nm in between)



mOrange



Far-Red

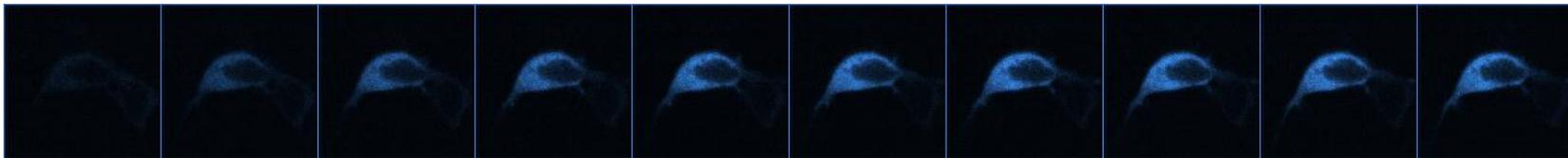
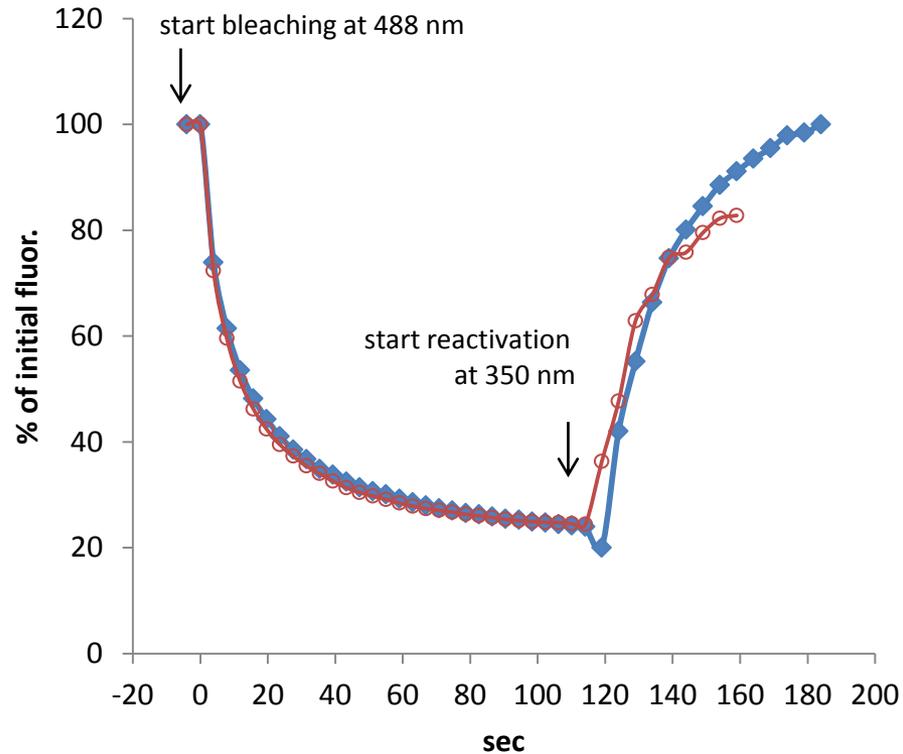


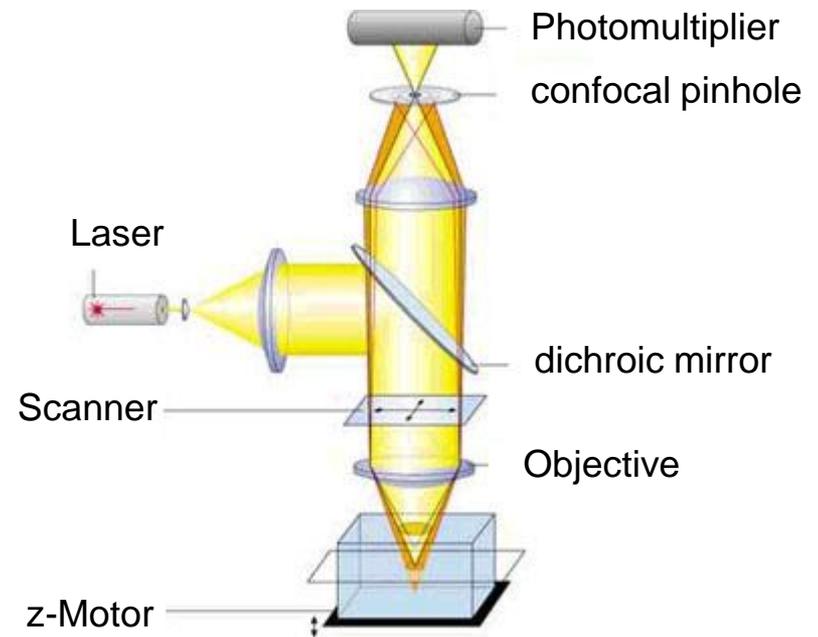
Photo-switchable fluor. protein Dronpa



Confocal Laser Scanning Microscopy



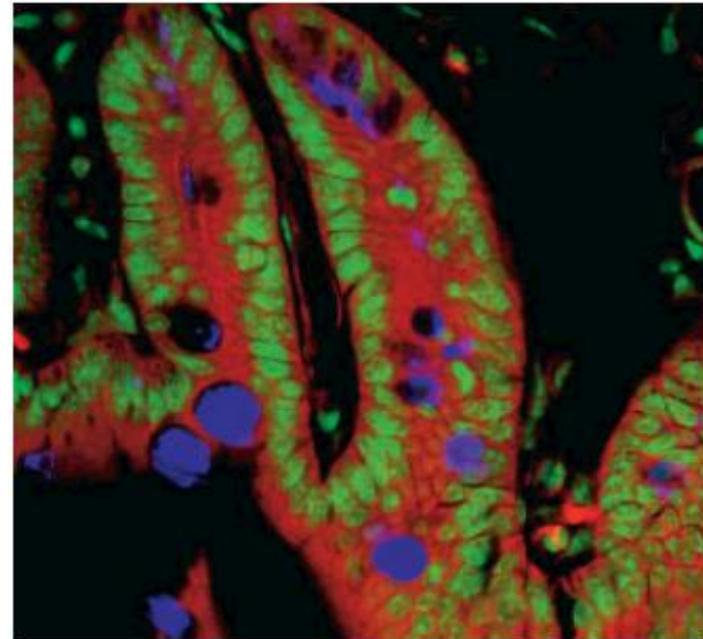
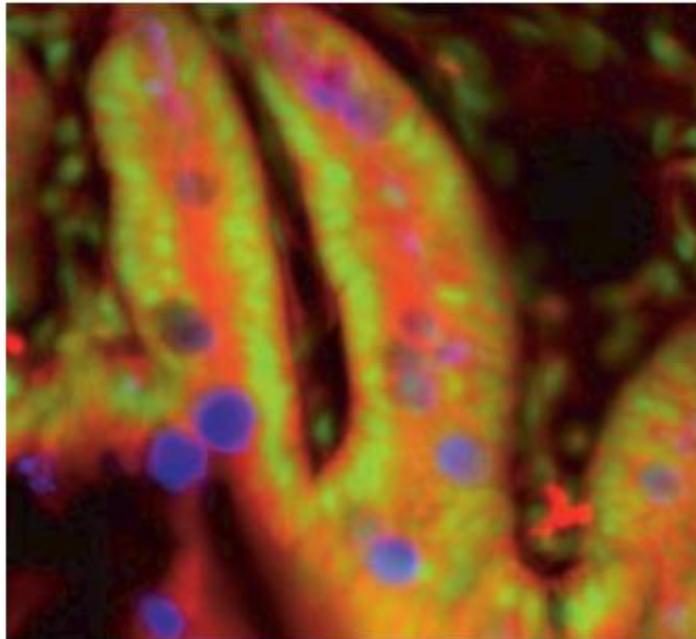
Detector
Laser (Light fiber to the microscope)



Modern Laser Microscopes



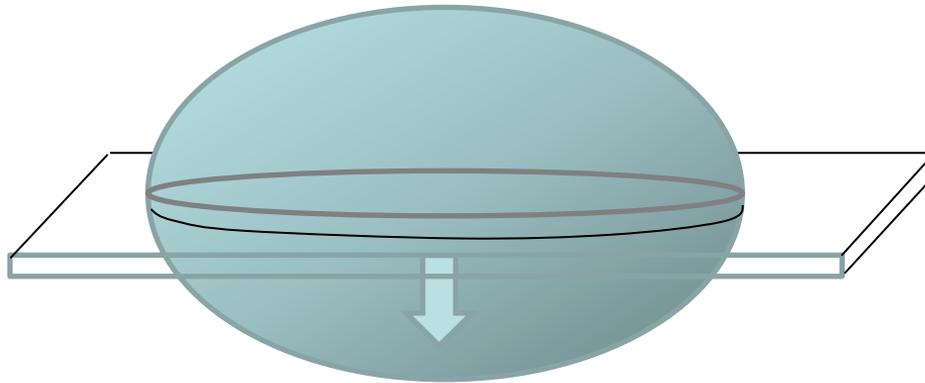
Confocal microscopy removes the blur from thicker objects



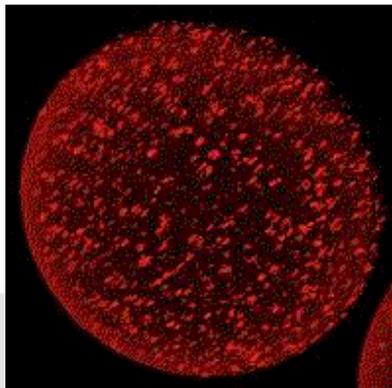
<http://zeiss-campus.magnet.fsu.edu/tutorials/opticalsectioning/confocalwidefield/index.html>

Optical sectioning and 3D-projections

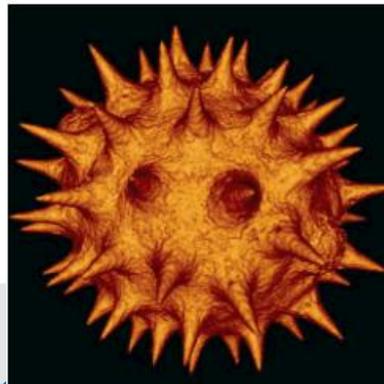
Acquisition of a „z-stack“ (image slices along the z-axis) allows reconstruction of a 3D-projection, which can be shown as animation



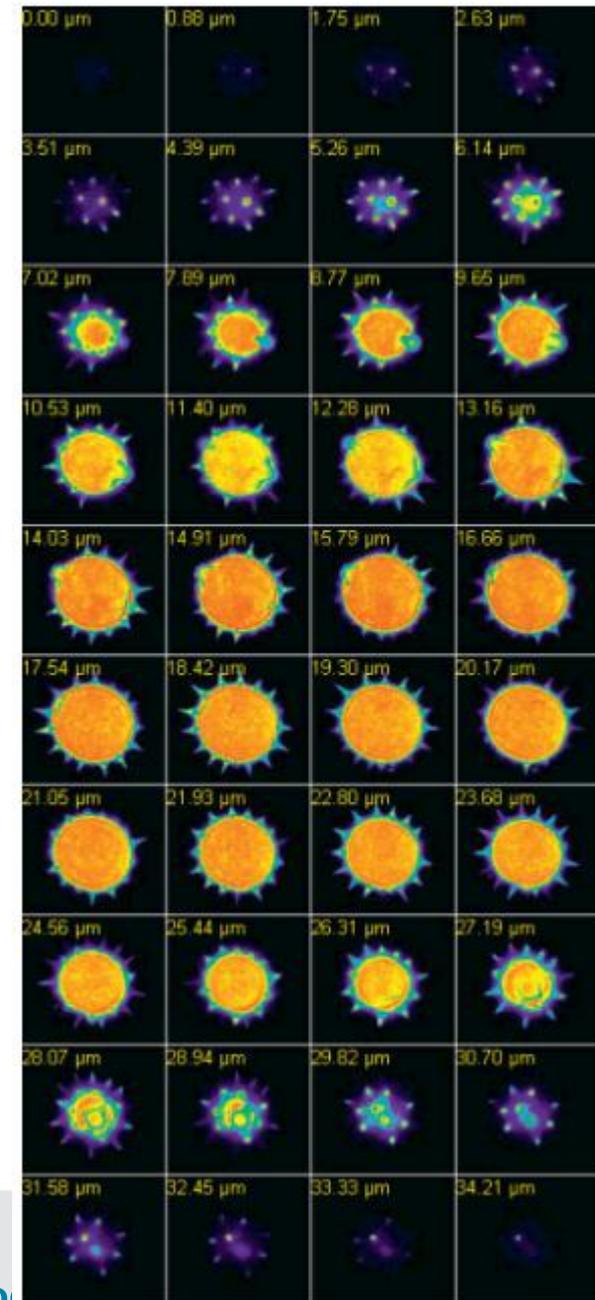
projection



3D rendering



z-stack

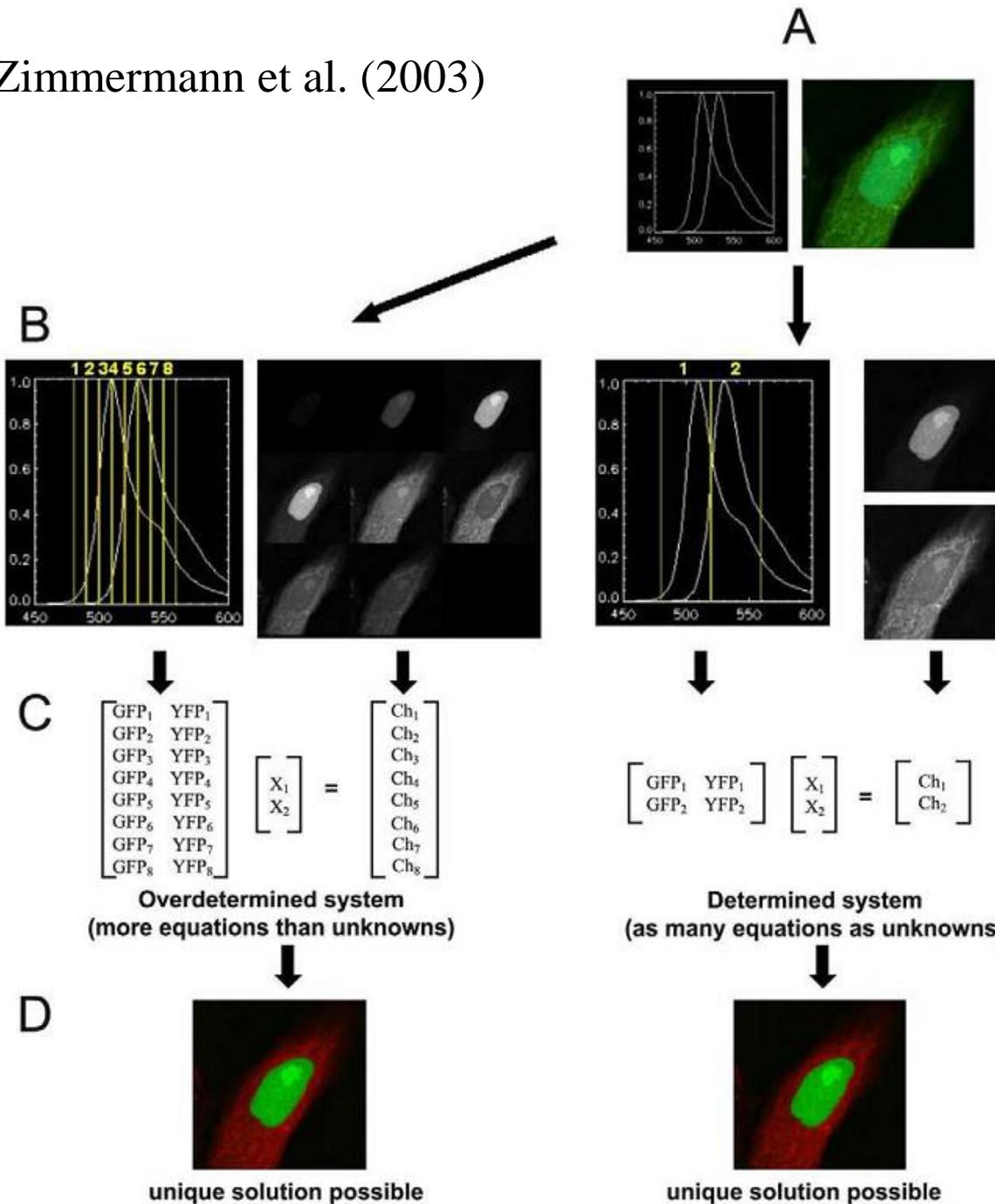


Spectral imaging

Resolving spectral information on a pixel-by-pixel basis

- „Emission finger printing“: emission scan of a microscopy sample („lambda stack“ of images) at a given excitation wavelength (e.g. with Zeiss LSM META systems or with Leica confocal microscopes...)
- Excitation scan (at a constant emission wavelength; e.g. using a monochromator light source)
- Appropriate filter sets with different excitation and emission (e.g using filter wheels)
- Increases the number of markers to be measured in parallel
- Can be used to discriminate fluorophores with overlapping spectra
- Can be used to discriminate specific fluorescence from autofluorescence

Zimmermann et al. (2003)



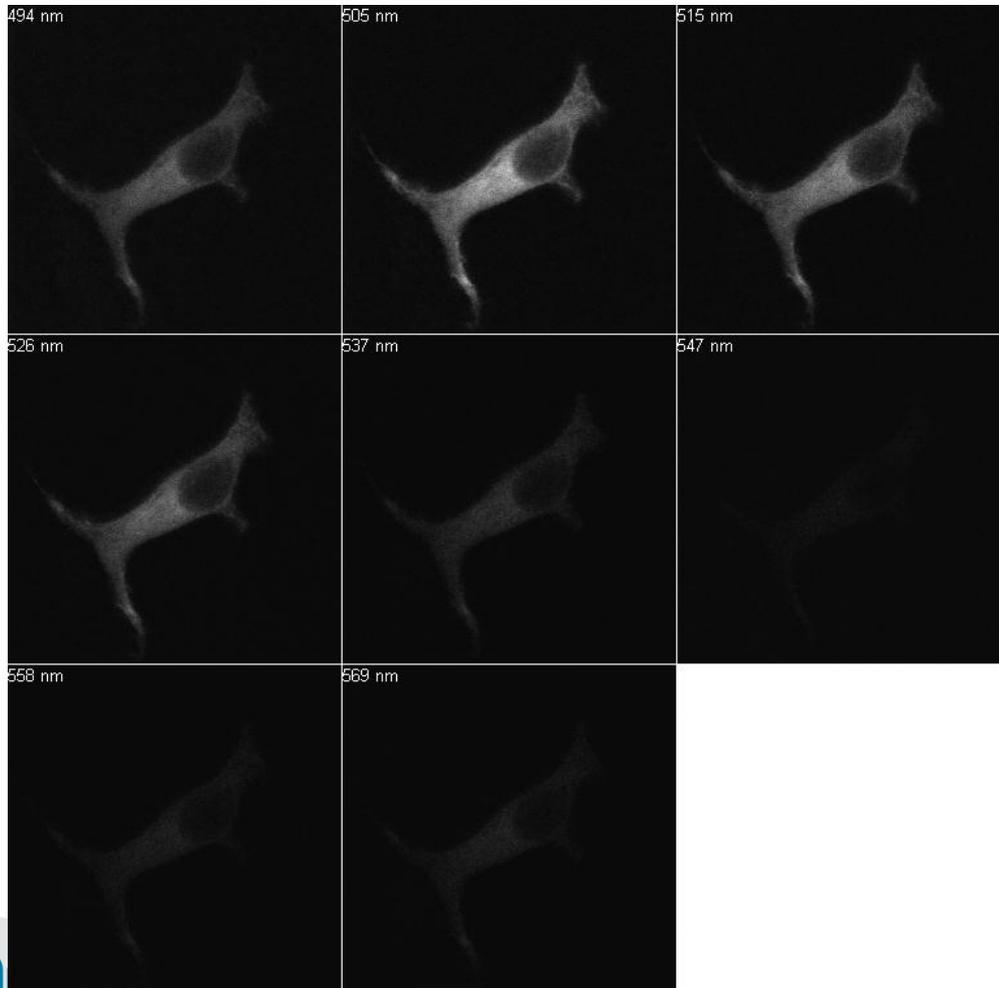
Sample with overlapping fluorophores

Emission curves separated into 8 channels (left) or 2 channels (right)

Equation matrix for the channel signals based on reference intensities in the channels (GFP_n and YFP_n) and the unknown contributions of the fluorophores

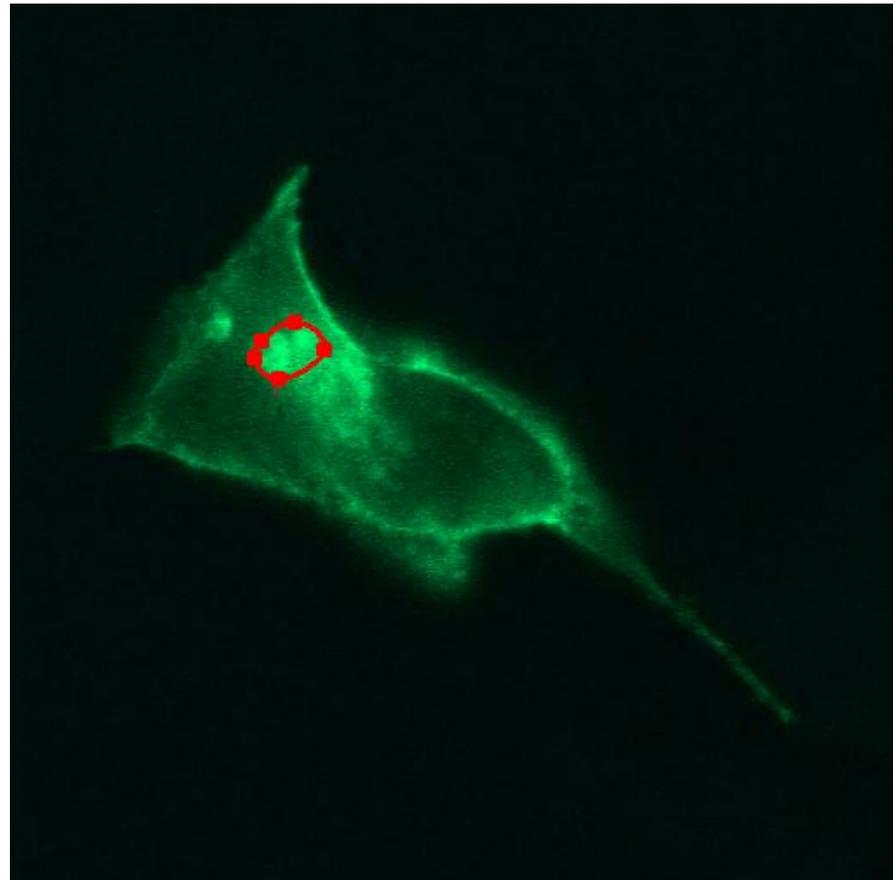
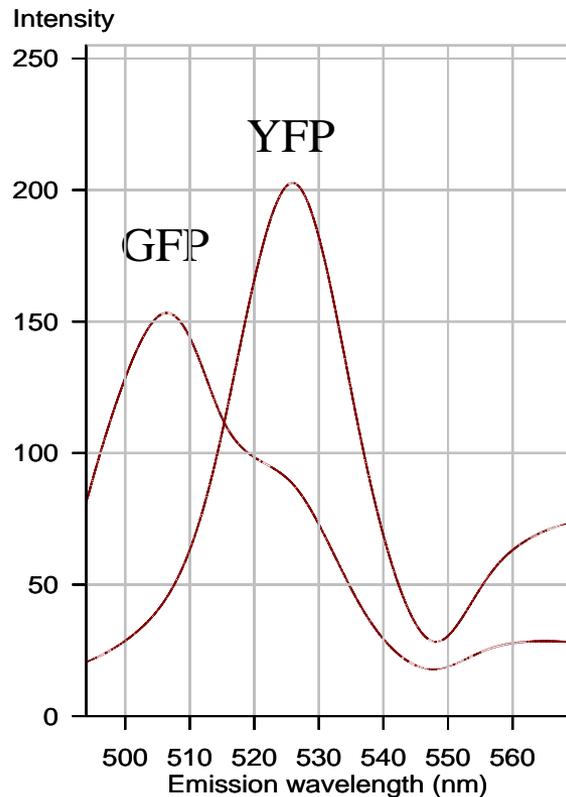
Unmixed fluorescence (pseudo-coloured)

Example for Emission Fingerprinting on a Zeiss LSM510 META: Separation of GFP and YFP

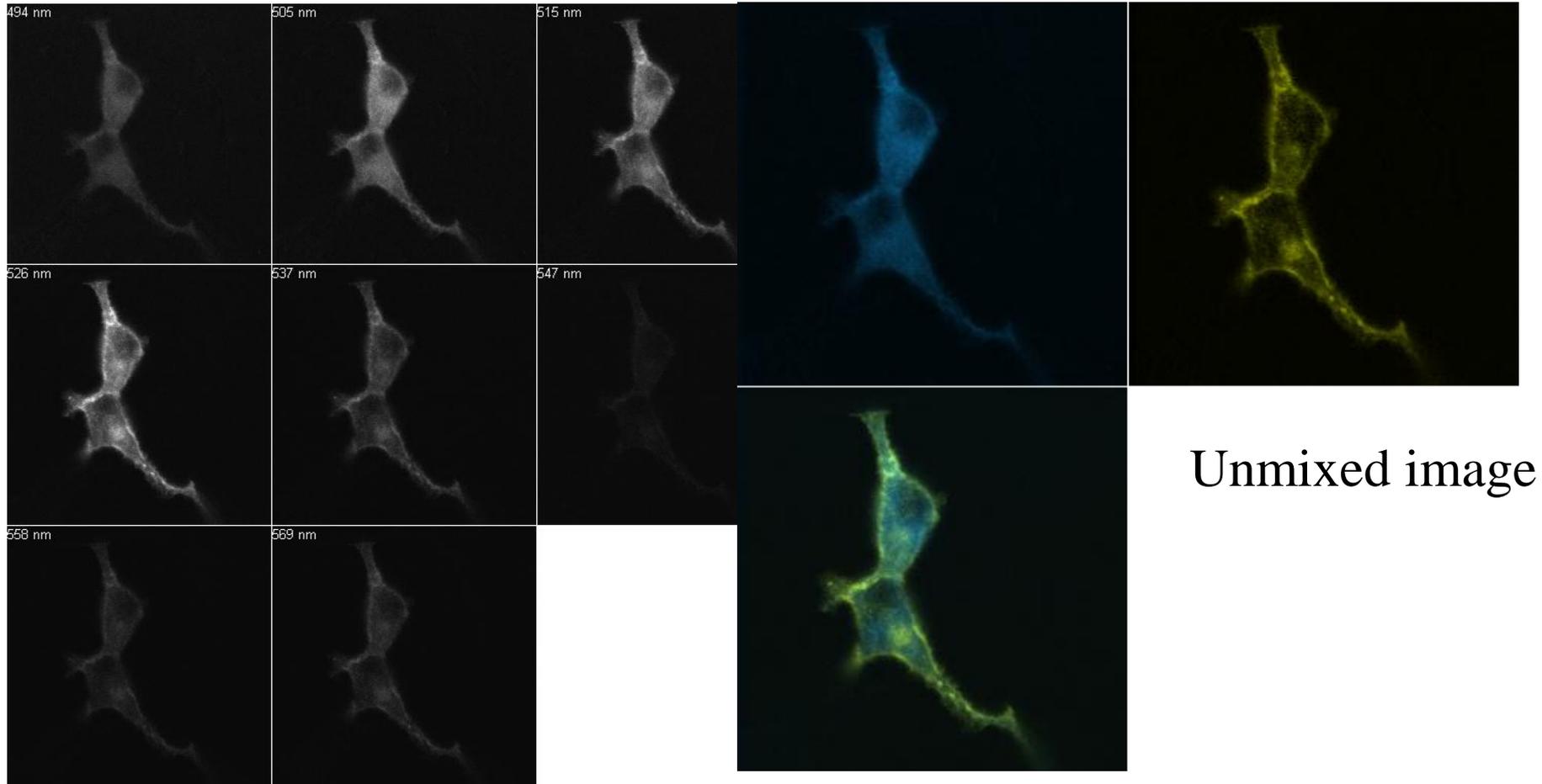


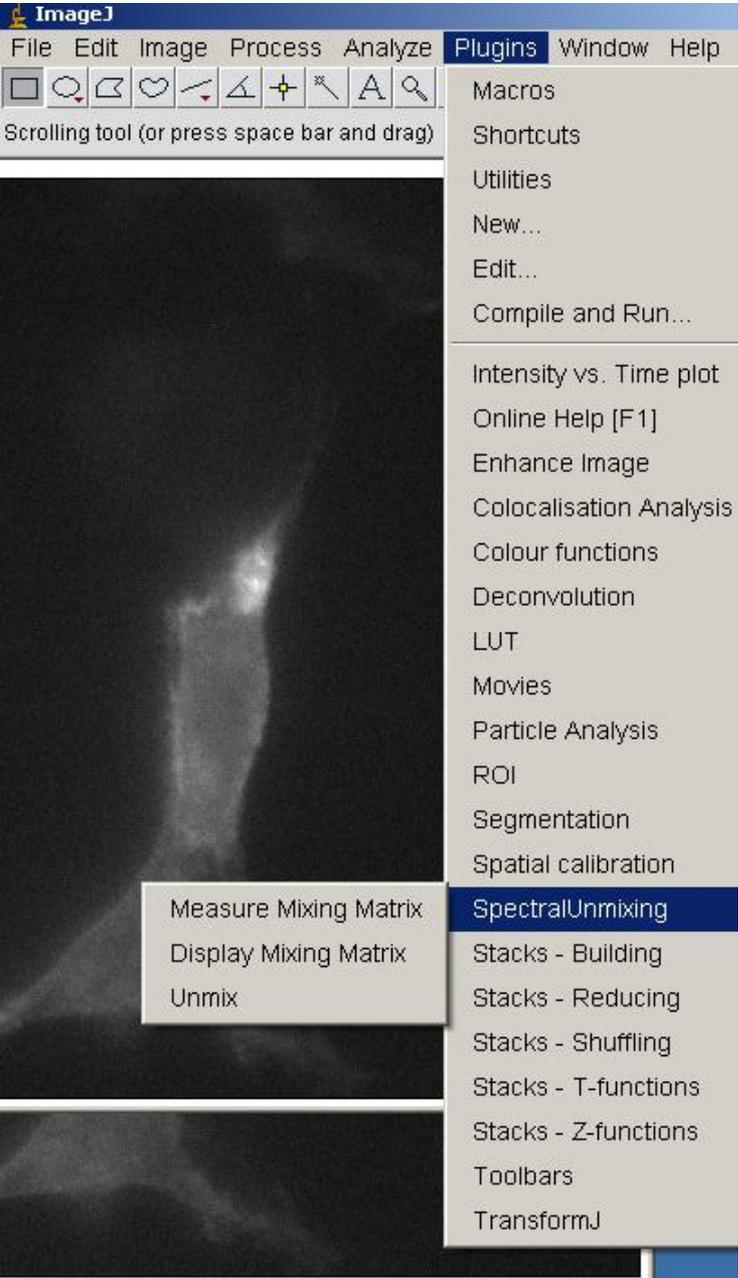
Acquisition of a reference lambda stack for the first fluorophore (GFP)

Obtain the spectral emission curve for the first fluorophore and repeat the procedure for the second fluorophore



Unmixing of a mixed sample (GFP-Actin and YFP-membranes)

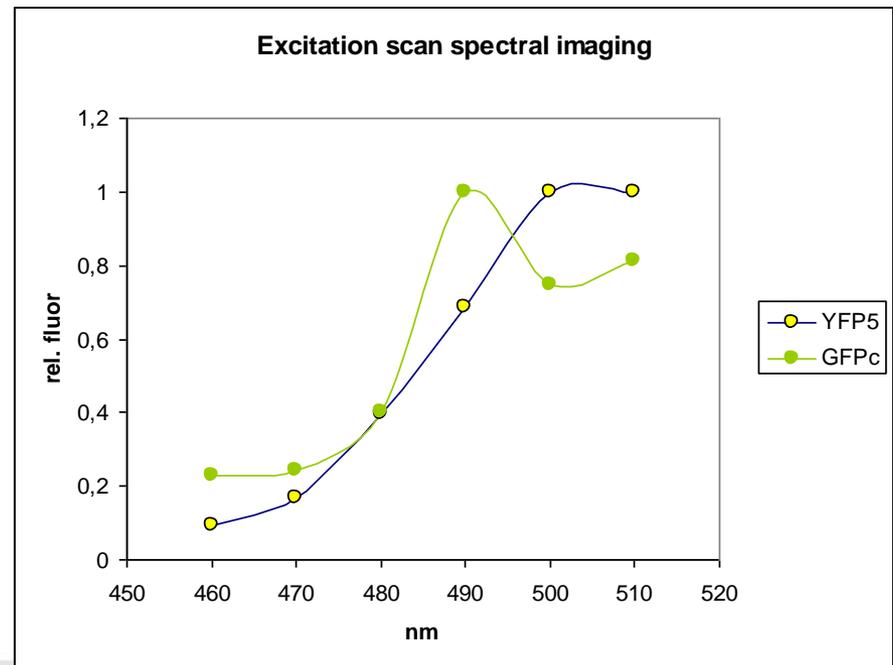




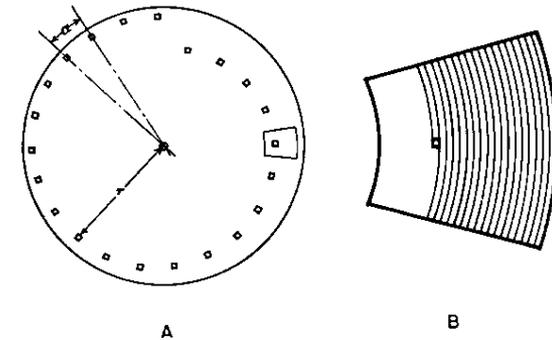
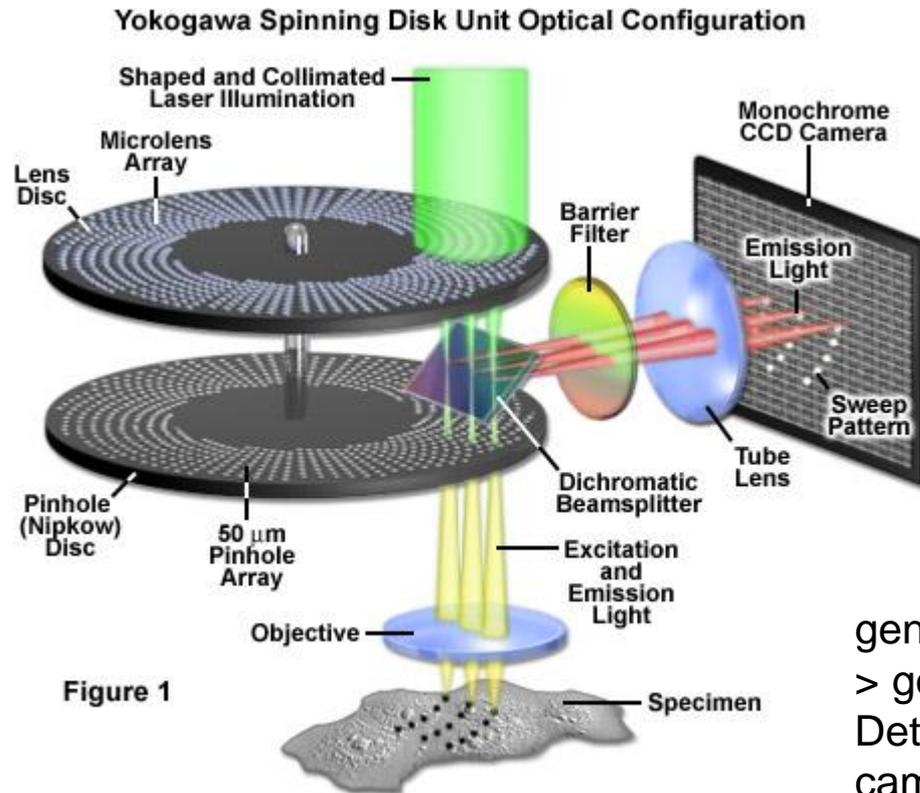
Example of excitation scanning spectral imaging

(using a Polychrome IV monochromator
for excitation)

> Analysis-Plugin available for *ImageJ*



„Realtime“ confocal microscopy, Spinning disk confocal microscopy (with Nipkow-disks)



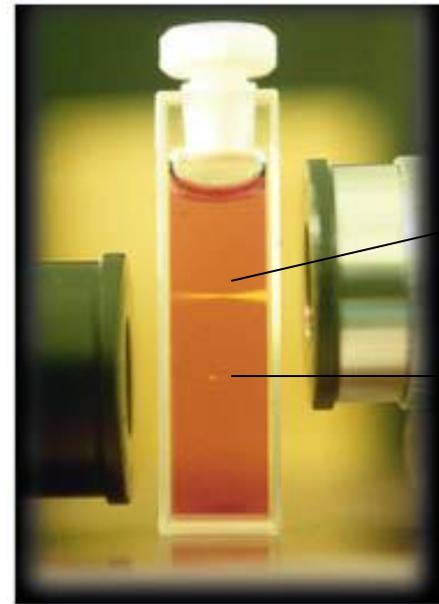
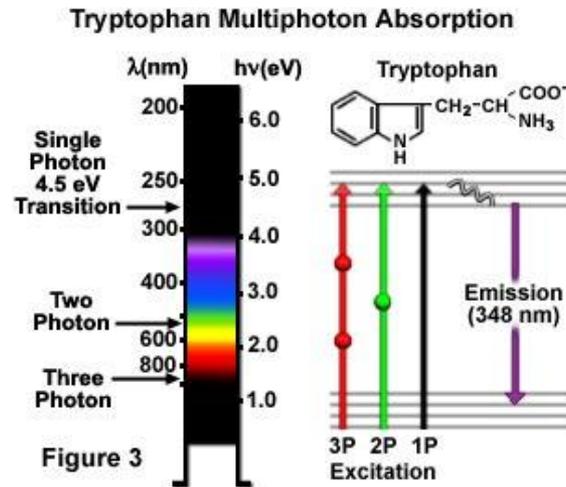
gentle scanning (less bleaching
> good for sensitive life cells
Detection of the signal with a CCD-camera

<http://zeiss-campus.magnet.fsu.edu/tutorials/spinningdisk/yokogawa/index.html>

Multiphoton Laser Scanning-Microscopy

A quantum physical phenomenon is used: at very high light densities (using pulsed lasers, about 900 nm infrared light) packages of 2 or more photons occur (just in the focal plane !). These have the same energy as single photons of higher energy (shorter wavelength, e.g. 450 nm). Thus these photon packages can excite a fluorophore, which emits then at for instance 520 nm (emitted wavelength is shorter than the excitation light wavelength !). An important advantage is that the 900 nm light has a much deeper penetration into tissue (approx. 1 mm), while conventional excitation can image just down to 0.25 mm. Another advantage is a reduced overall bleaching effect, as excitation photon packages occur just in the focal plane.

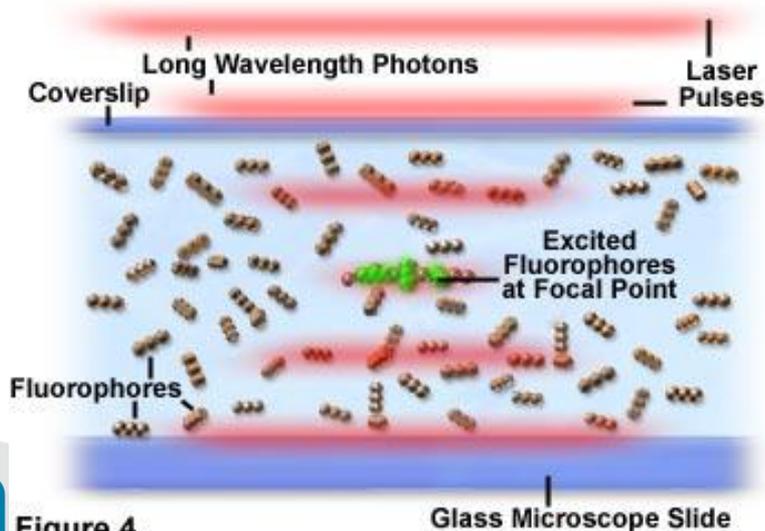
Multiphoton Laser Scanning-Mikroskopie II



conventional excitation
(1-Photon > cone of
excitation light)
2-Photon excitation:
only a spot of
excitation

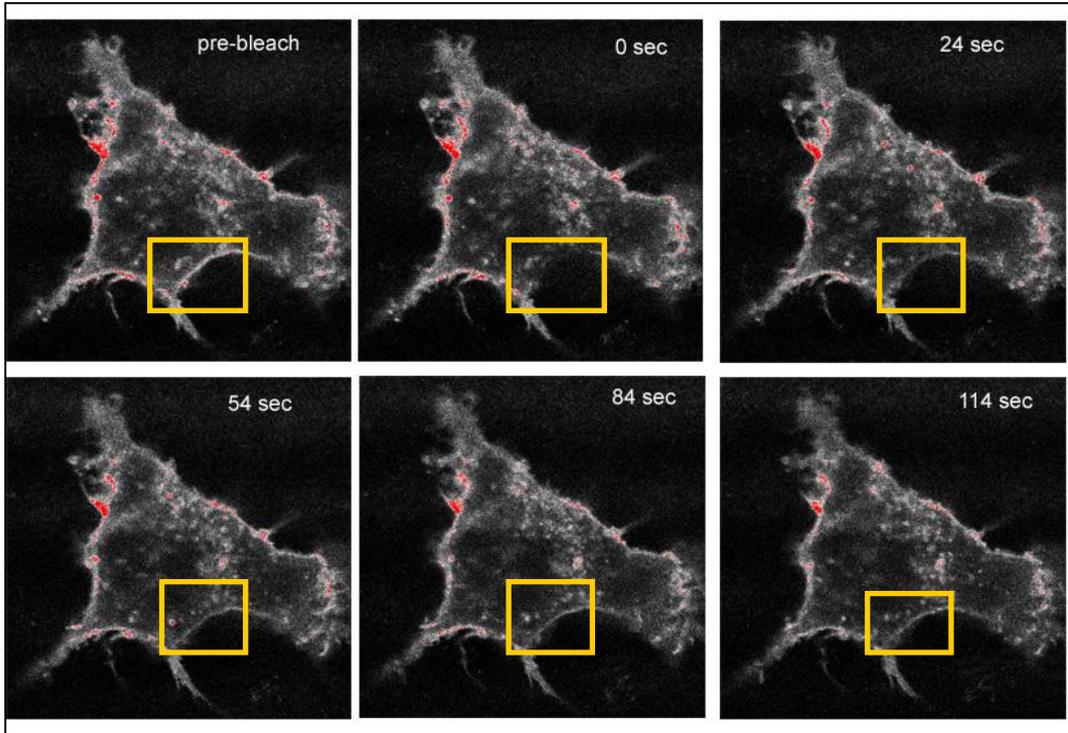
1-photon excitation (top) and
two-photon excitation (bottom)
demonstrated in a cuvette of
fluorescent dye

Fluorophore Excitation in Multiphoton Microscopy

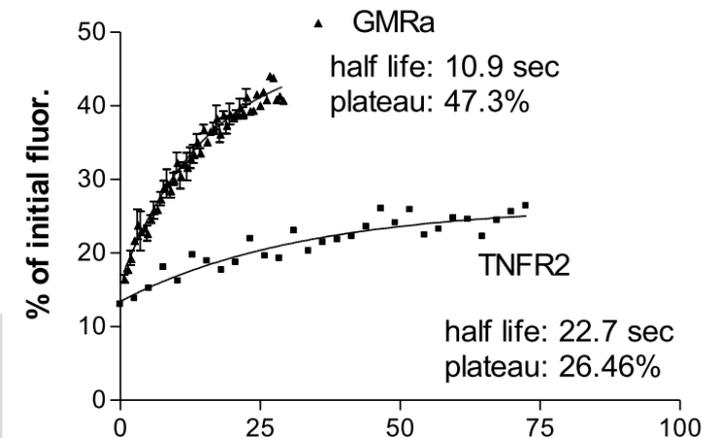
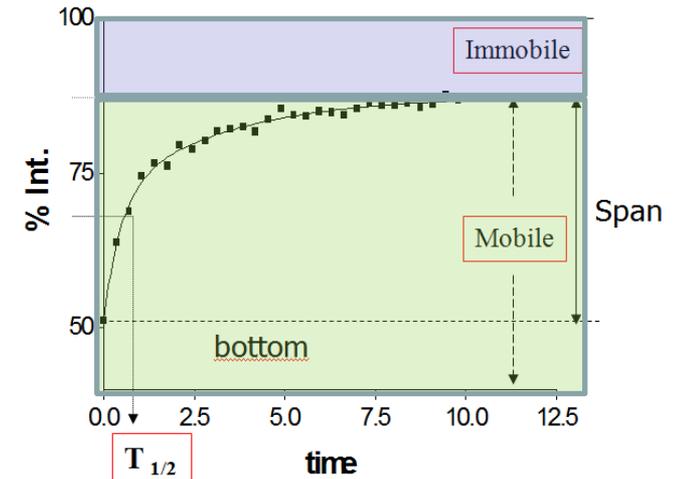


FRAP: Fluorescence Recovery After Photobleaching

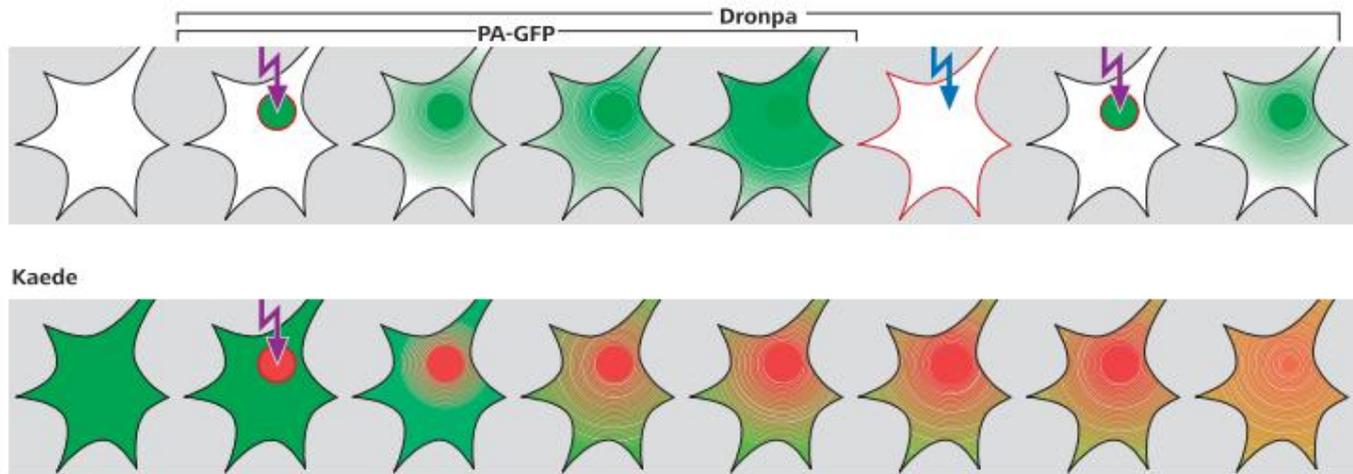
to determine diffusion and mobility of molecules



$$y = \text{span} (1 - e^{-kx}) + \text{bottom}$$



inverse FRAP with novel fluorescent proteins



Protocol: FRAP analysis on Zeiss LSM510

- Capture an image of the whole cell before bleaching
- Define a bleaching / scan region (and maybe in addition another scan region that is not bleached)
- Perform a time series with 1 scan prebleach, about 70 iterations of bleaching with 100% laser power and then 50-100 scans of the bleach region (and also the non-bleached control region if you specified one)- a good time resolution can only be obtained if just the small bleach region (and maybe the control region) is scanned - and not the whole cell; averaging of 2 or 4 scans reduces the electronic noise and leads to better quantifications.
- Capture an image of the whole cell after the FRAP time series (with the same conditions as the prebleach image – for calculating the total loss of fluorescence.
- If you want to save disk space: extract the FRAP region and save just this region instead of the whole image
- It is recommended to use the [WCIF version of ImageJ](#) for analysis: You can open the LSM-files with the built-in feature (which also allows opening the time values of the image series). Measure the mean fluorescence in a control region or for the whole cell for both the prebleach and the postbleach images and calculate the loss of overall fluorescence due to the bleaching in the region of interest (this is necessary for obtaining correct recovery values for the bleach region).
- Import the FRAP-image sequence, define a measurement region and apply the „intensity versus time plot“ plug-in – this will draw a graph of the FRAP curve; clicking the list button, shows a list of the numerical values (the first 4 parameters are dimension and position of the region, the rest are the fluorescence intensity values).
- Copy the fluorescence raw data from the list to the corresponding column of an Excel template

- Calculate the difference of mean fluorescence from the background and normalize the fluorescence values to 100% for the initial fluorescence.
- Divide the percent values by the correction factor calculated from the total loss of fluorescence (e.g. if total fluorescence decreased from 1 to 0.9 then divide the mean fluorescence of the FRAP regions for each time value by 0.9 to compensate for the loss in total fluorescence). A similar compensation can be obtained by normalizing the FRAP fluorescence values to the control scan region that was not bleached. This method also compensates more exactly for the bleaching effect in the course of scanning of the time series (this scanning-dependent bleaching effect is opposed to the recovery of fluorescence in the bleach region due to diffusion of non-bleached molecules in the bleach region). This “dynamic correction” gives a somewhat better estimation of the curve (and the kinetics of the recovery) – but leads in principle to results that are very similar to the curve obtained with the “constant correction factor” (by calculating the total loss in fluorescence based on the intensities of the images that were captured before and after the FRAP-time series)
- For non-linear regression analysis (curve fit of the data to a single exponential association algorithm): Copy the data to a fitting program (such as GraphPad Prism) and perform the fitting with a “bottom to span” algorithm:

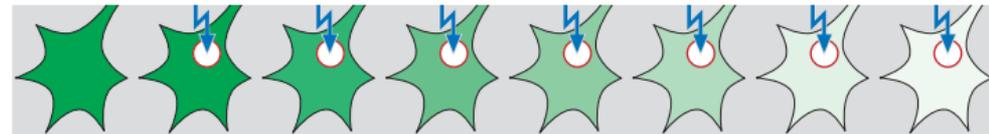
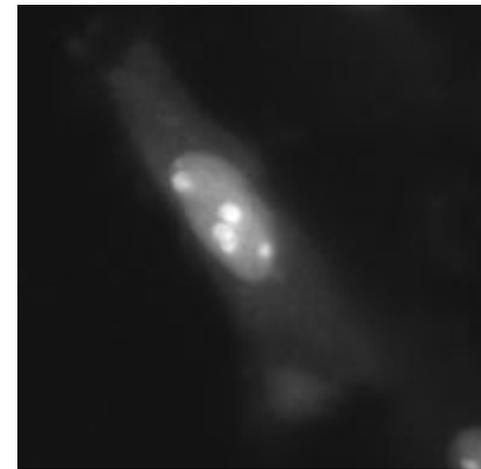
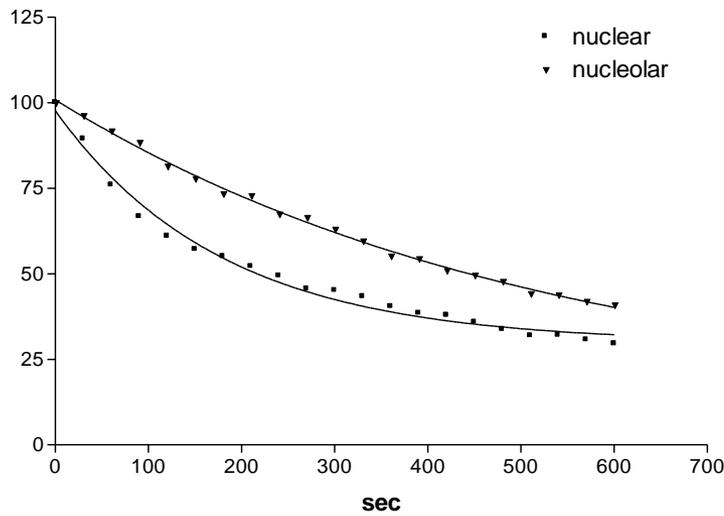
$$y = span \times (1 - e^{-kx}) + bottom$$

FLIP: Fluorescence Loss in Photobleaching

... to determine the dynamic shuttling of molecules between different compartments of the cell

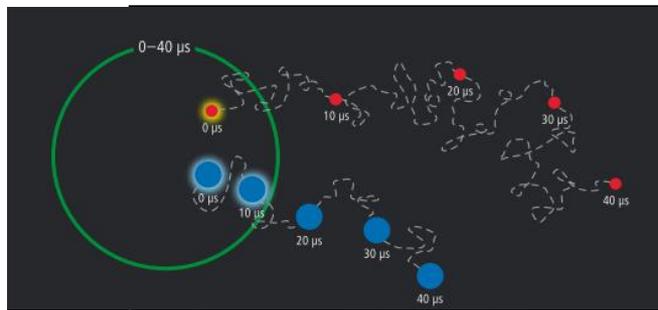
A certain compartment A is repetitively bleached by the laser – and the fluorescence decrease in a different compartment B is monitored. Molecules that shuttle from B to A are bleached in A > thus the compartment B gets dimmer when there is a dynamic distribution of molecules between A and B.

nuclear FLIP
(bleach in nucleus outside nucleoli)



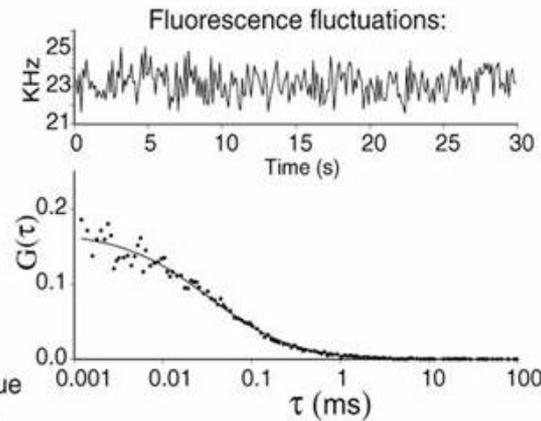
FCS: Fluorescence Correlation Spectroscopy

... to determine diffusion coefficients and interactions between molecules. The sample is illuminated by the laser at a very small spot, the movements of molecules in this spot (in and out) cause fluorescence fluctuations, which are analyzed by correlation functions



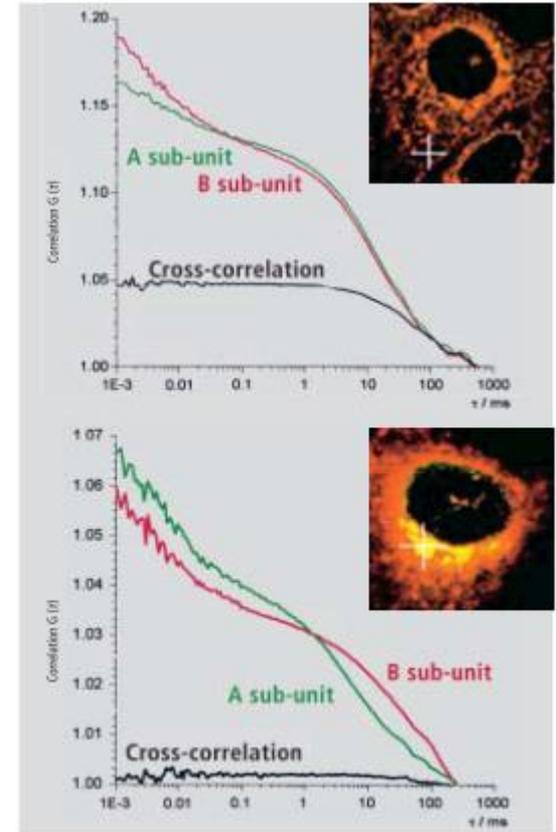
Fluctuations in fluorophore concentrations due to diffusion in and out of the focal volume provide information on the mobility of the labeled probe from which a diffusion coefficient can be obtained.

Concentration range:
? ← nM → ~1 μM



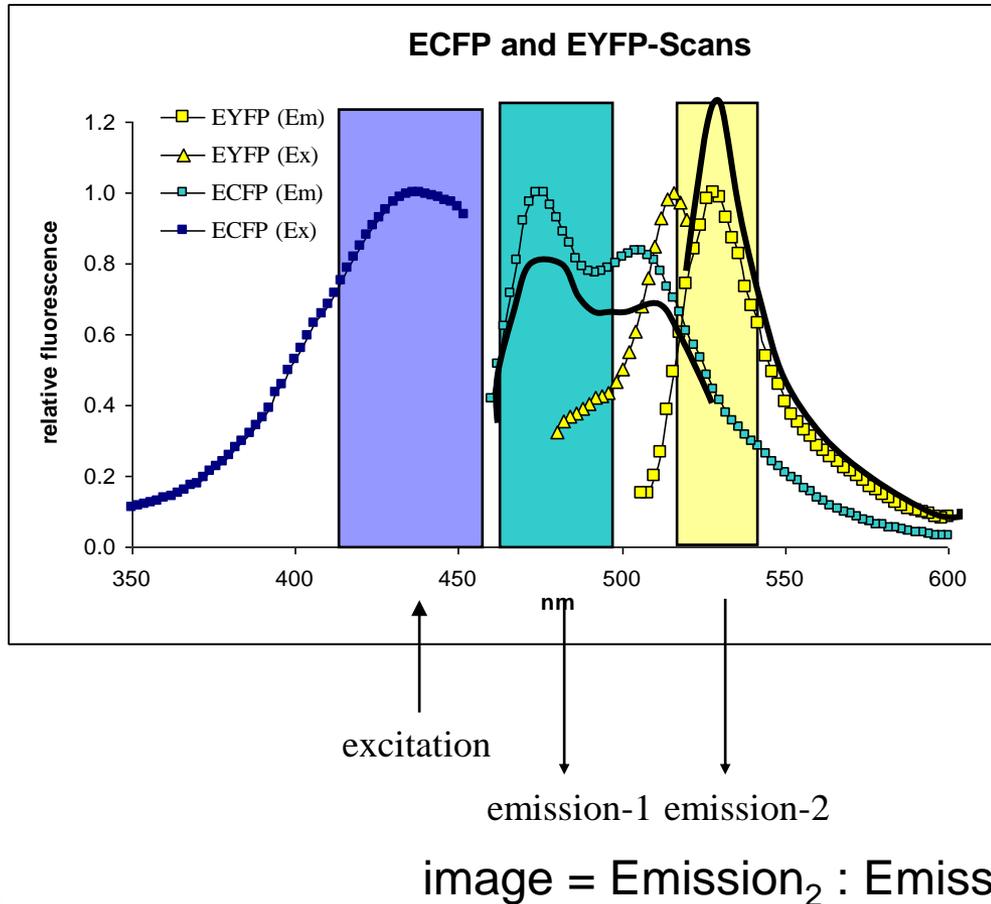
$$G(\tau) = \frac{\langle \delta I(t) \delta I(t+\tau) \rangle}{\langle I(t) \rangle^2}$$

$$= \frac{C}{V_{eff}} \frac{1}{1 + \frac{4D\tau}{\omega_{xy}^2}} = \frac{1}{\sqrt{1 + \frac{4D\tau}{\omega_z^2}}}$$



2 Filter-FRET Microscopy (Ratio Imaging)

Ratio of donor emission and acceptor emission at the excitation wavelength of the donor

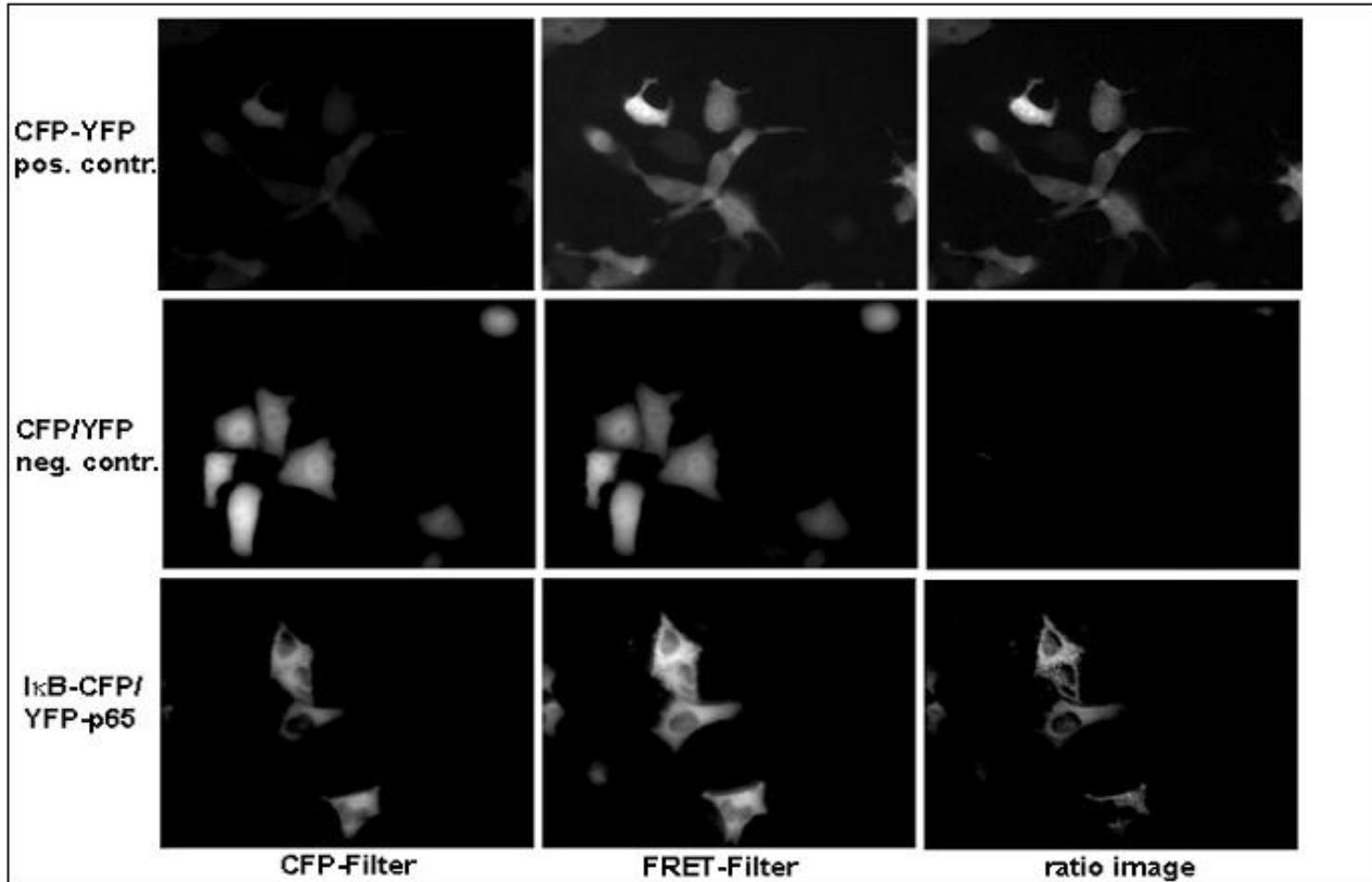


Limitations:

- concentration dependent
- controls are difficult
- donor and acceptor have to colocalize completely (100%)

> just useful for FRET-biosensors with covalent linkage between donor and acceptor

Example for Ratio Imaging FRET-Microscopy



3-Filter FRET Microscopy

3 Images are taken (under constant camera settings):

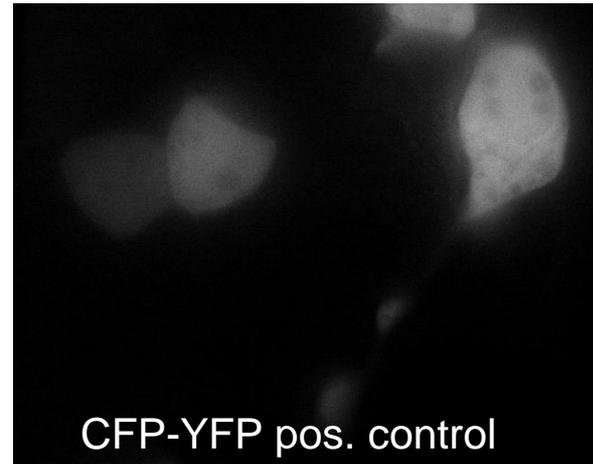
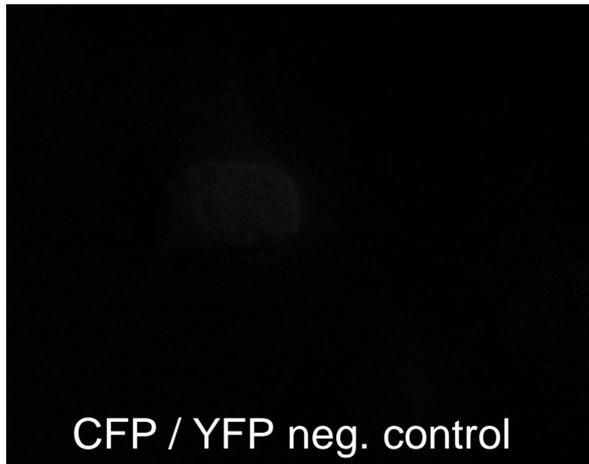
1. CFP (CFP-excitation and emission),
2. YFP (YFP-excitation and emission – this signal is not affected by FRET)
3. FRET-Filter (CFP-excitation and YFP-emission).

A normalized FRET signal (image) can be calculated by using correction factors obtained with single stained samples:

$$\text{FRET}_{\text{N}} = I_{\text{FRET}} - \text{corr}_{\text{CFP}} \times I_{\text{CFP}} - \text{corr}_{\text{YFP}} \times I_{\text{YFP}}$$

corr_{CFP} : ca. 0.59

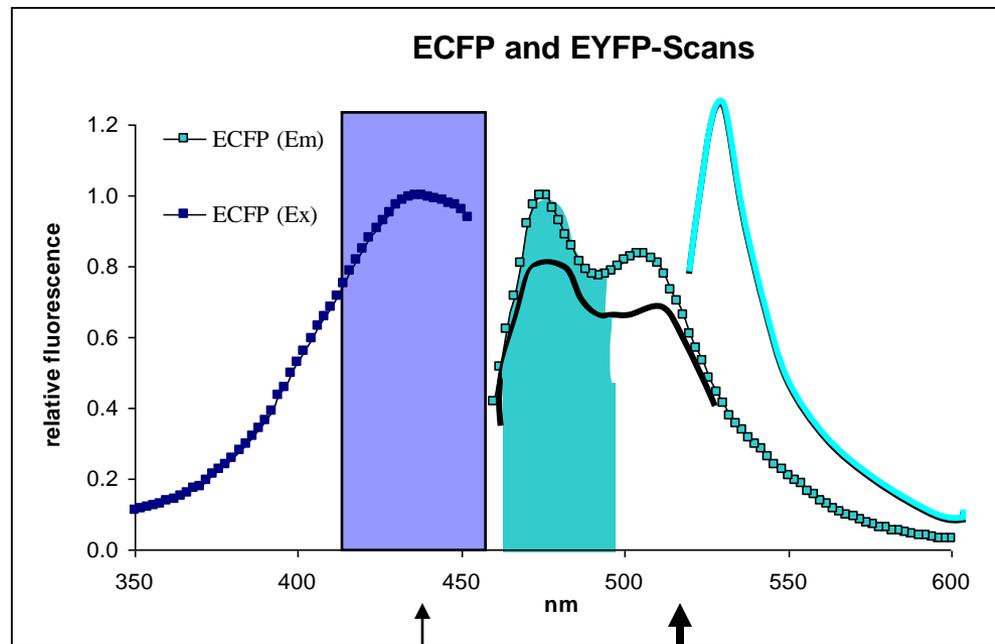
corr_{YFP} : ca. 0.18



FRET Microscopy by acceptor bleaching and monitoring donor recovery

(do not use for CFP / YFP !)

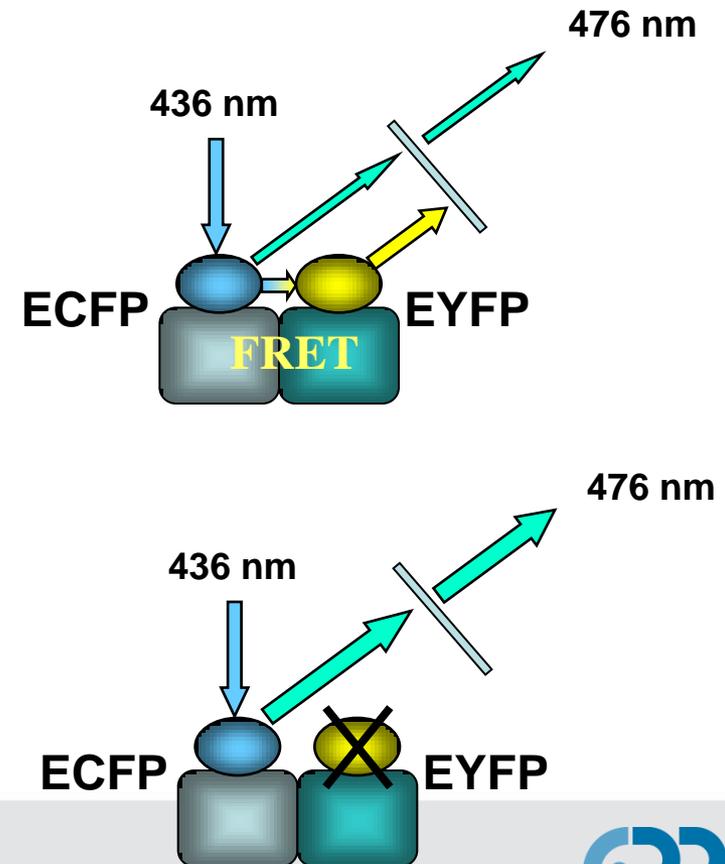
Donor recovery after acceptor bleaching: An image of the donor in the presence of the acceptor is taken, then the acceptor is bleached (partially), followed by acquisition of a second donor image



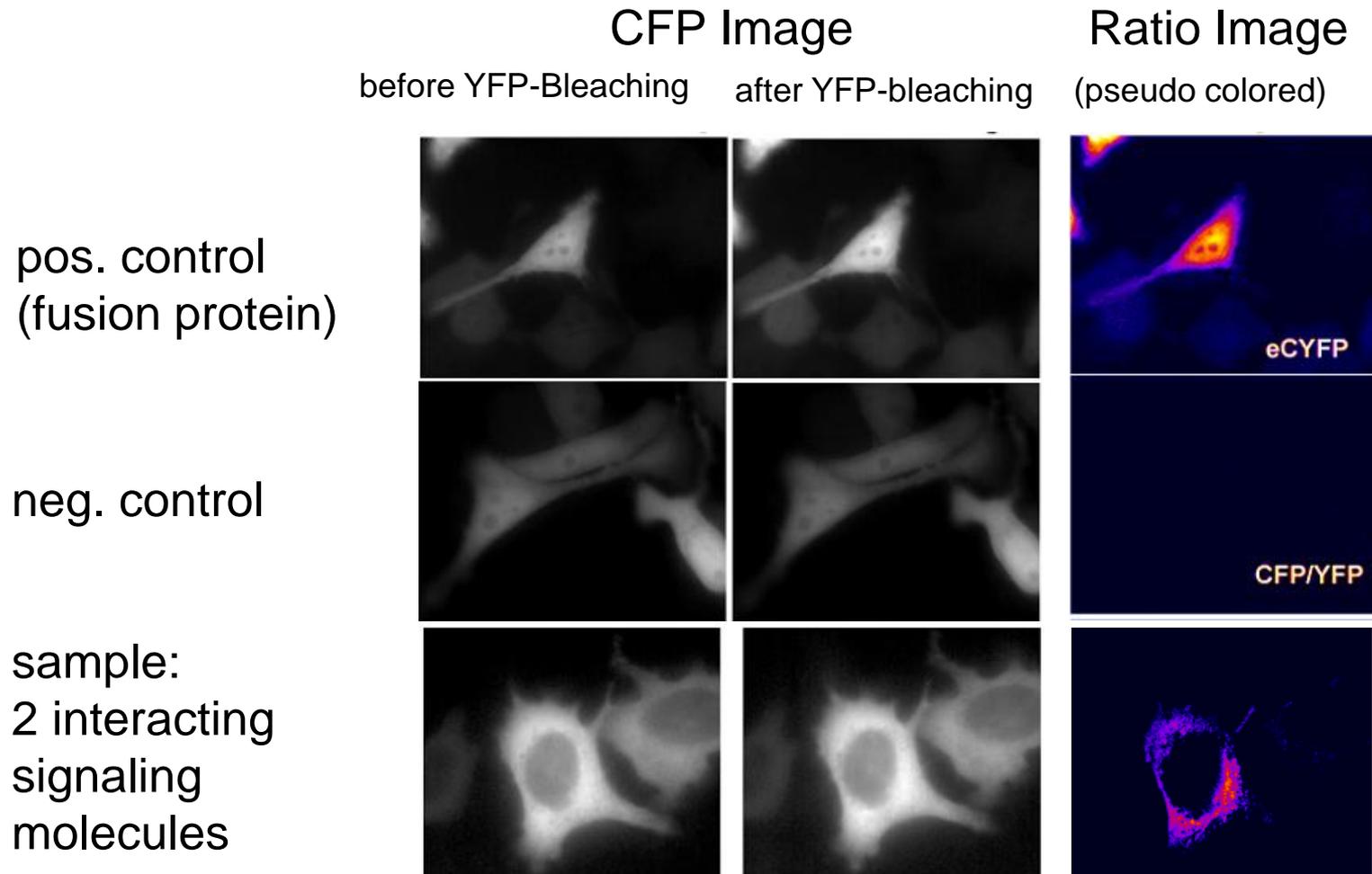
excitation

bleaching of acceptor

Donor Image 1
Donor Image 2



Example for a „Donor Recovery“ FRET-Microscopy

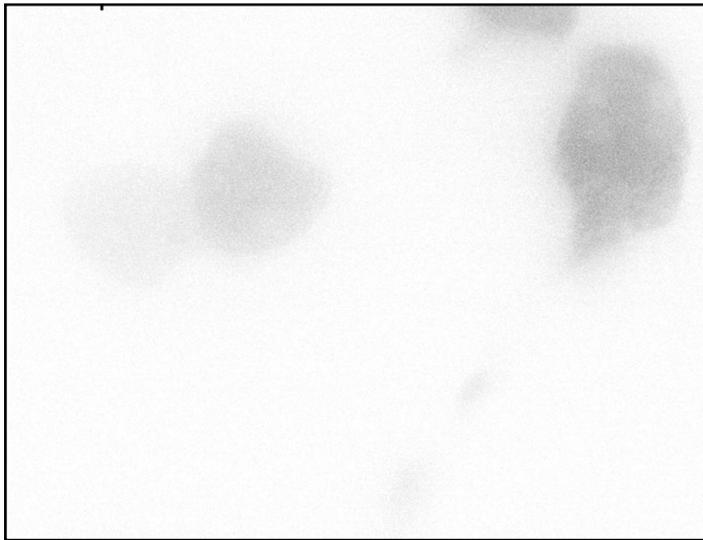


Advantages:

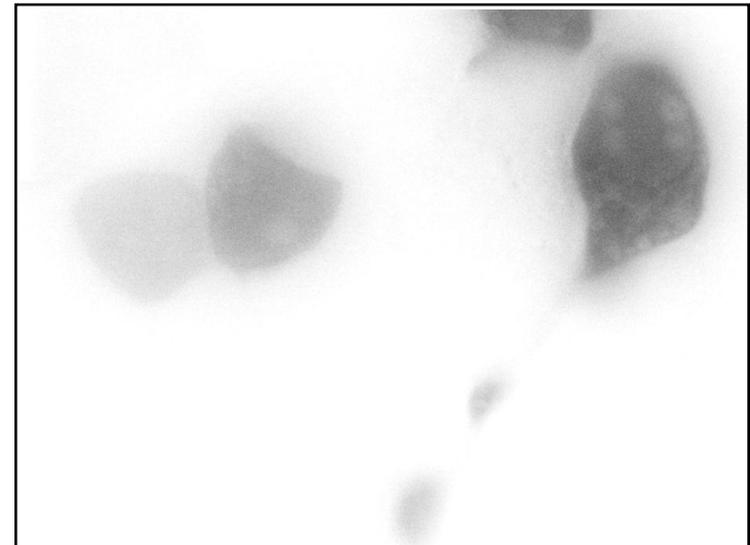
concentration independent; donor and acceptor do not have to colocalize; internal control; Ratio-image (after Acceptor-bleaching/before Acceptor-Bleaching) = FRET-image

Comparison between the 3-filter method and the donor recovery method

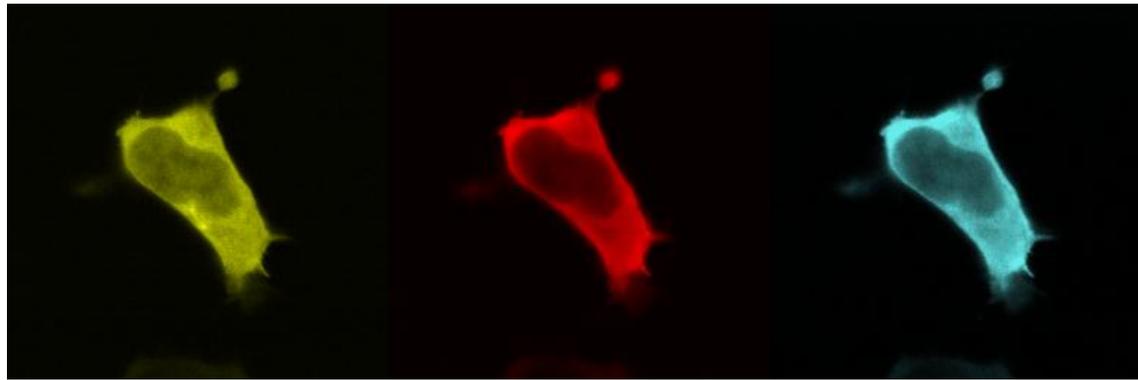
DRAP
(Donor Recovery After
Acceptor Photobleaching)



3-Filter corrected FRET image
(according to Youvan)



Normalization of FRET signals for concentrations

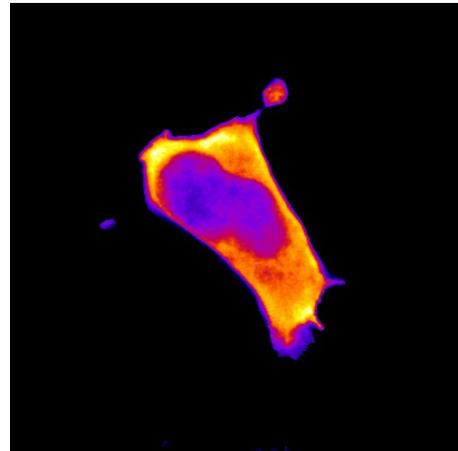


YJab1

HA-IKK2

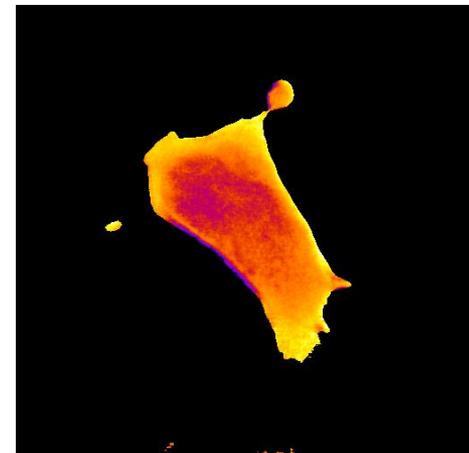
FRET channel raw data

$$\text{FRETc} = (\text{FF} - d \times \text{DF} - a \times \text{AF})$$



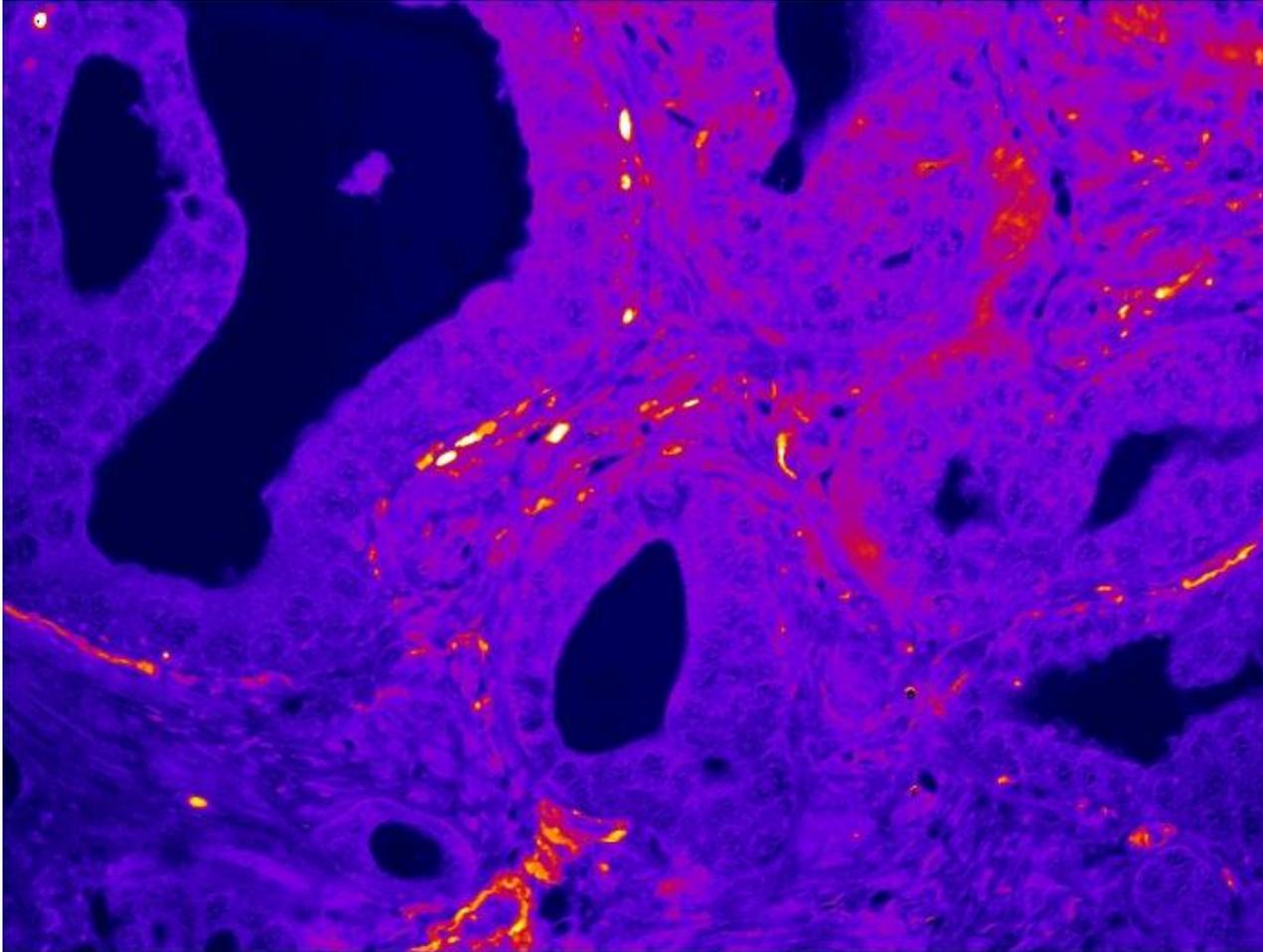
corrected FRET image
(not normalized for conc.)

$$N_{\text{FRET}} = (\text{FF} - d \times \text{DF} - a \times \text{AF}) / (\text{DF} \times \text{AF})^{1/2}$$



FRET efficiency image
(normalized for conc.)

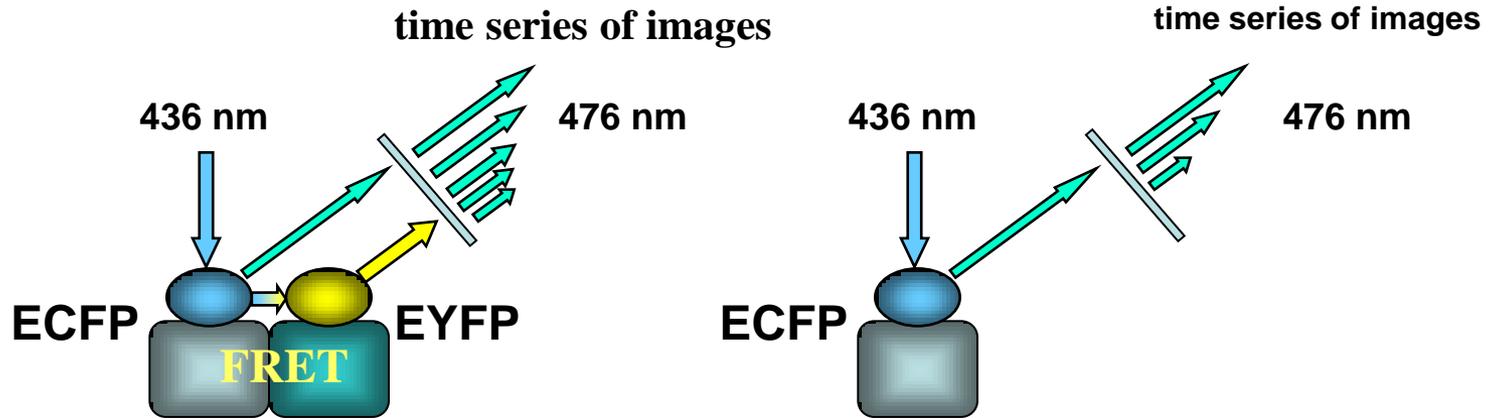
FRET analysis of protein interactions in patient material



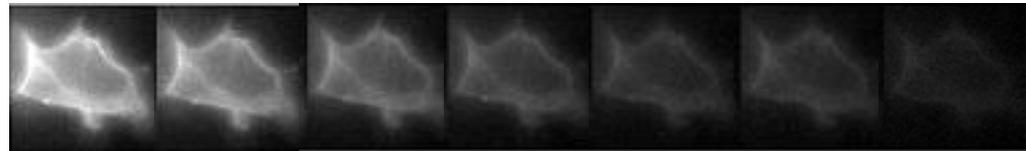
> Protein interactions of signaling molecules can be quantified and visualized in tissue sections

FRET Microscopy by analyzing the kinetics of donor bleaching

... this is slowed down in presence of a FRET acceptor



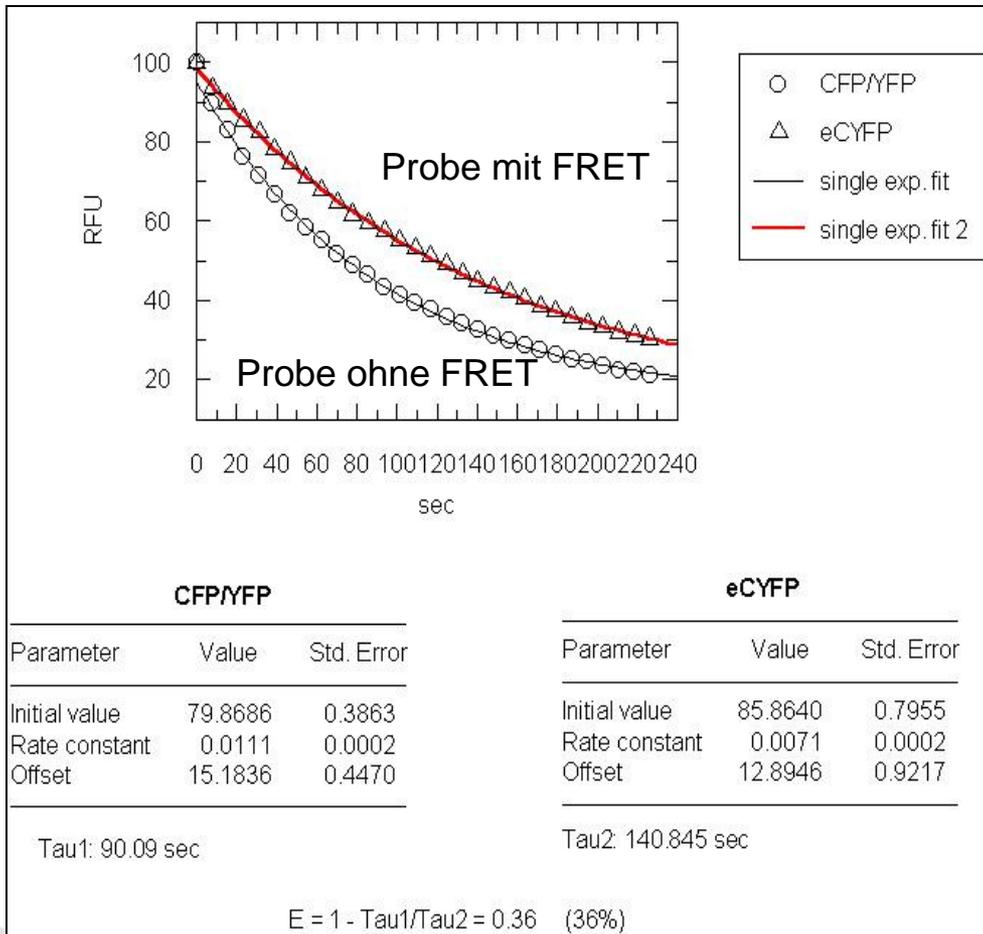
CFP-Protein alone



CFP- and YFP-Protein



Donor-bleaching kinetics



advantages:

concentration independent
donor and acceptor don't have to
colocalize completely

Limitation: requires external control,
difficult to obtain a FRET-image

single exponential decay

$$y = A_0 \cdot e^{-kt} + \text{offset}$$

y... Fluor. Signal

A_0 ... starting signal

k... decay constant

t... time

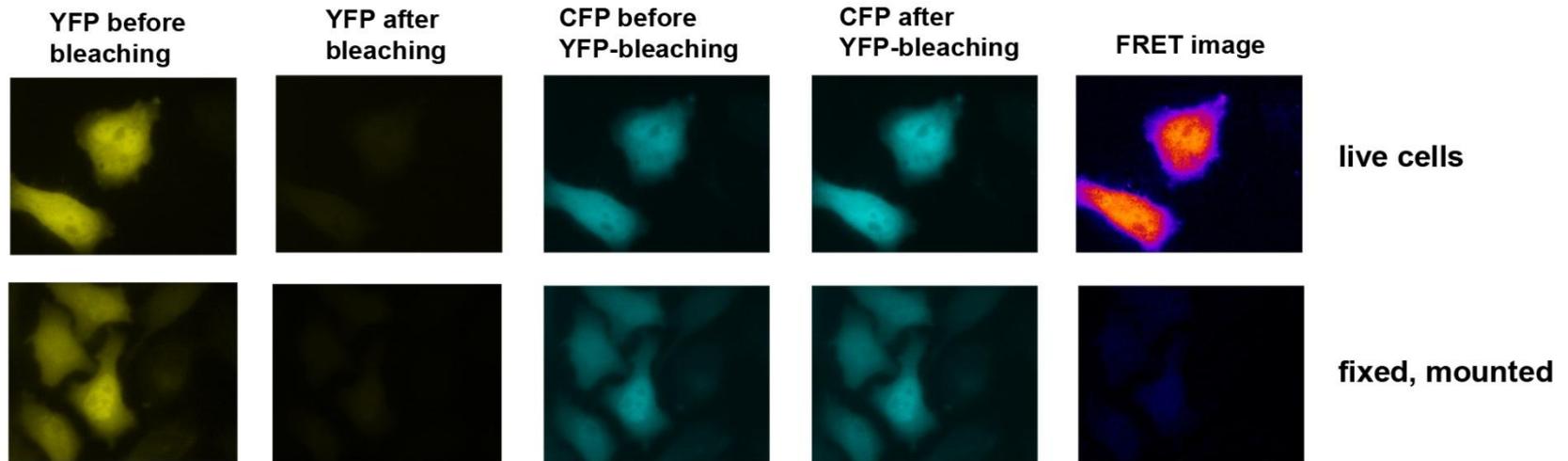
offset... final value

$$\text{Fluorescence half time Tau: } \tau = 0.69/k$$

$$\text{FRET}_{\text{eff}} \cdot E = 1 - (\tau_{\text{without}} / \tau_{\text{with Akzeptor}})$$

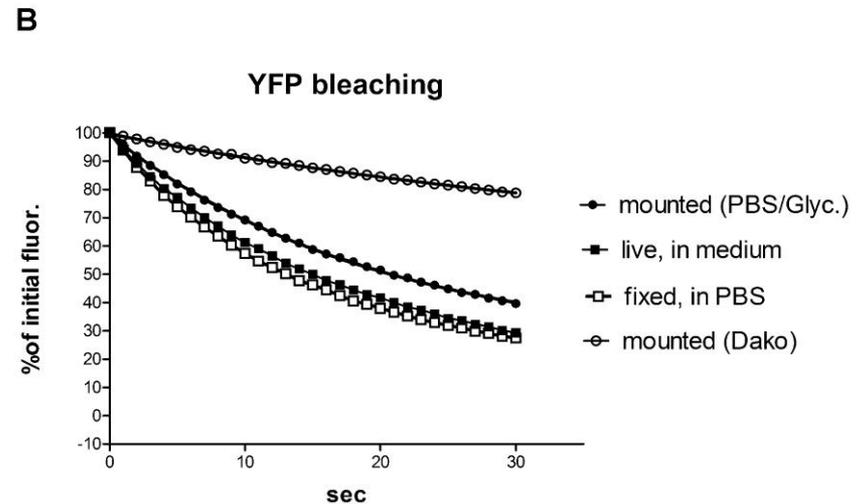
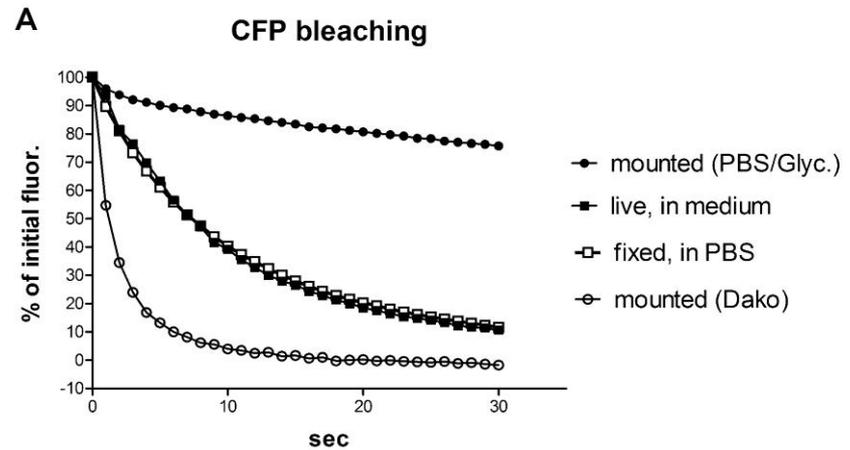
The Secret Nature of Some Fluorescent Proteins

Markedly Faster Bleaching in Commercial Mounting Fluids and Photoactivation at YFP-Excitation

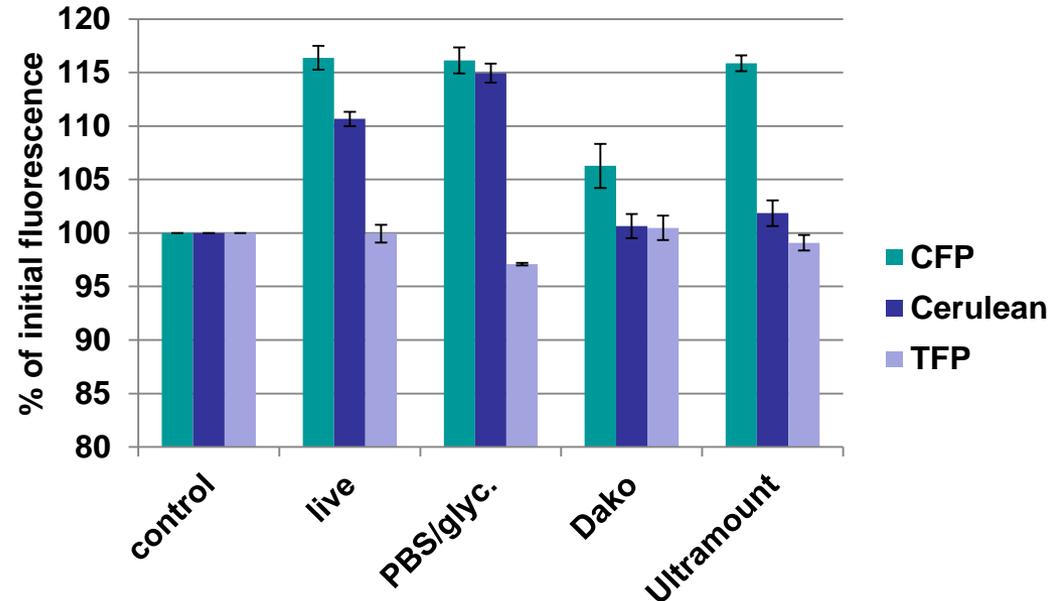
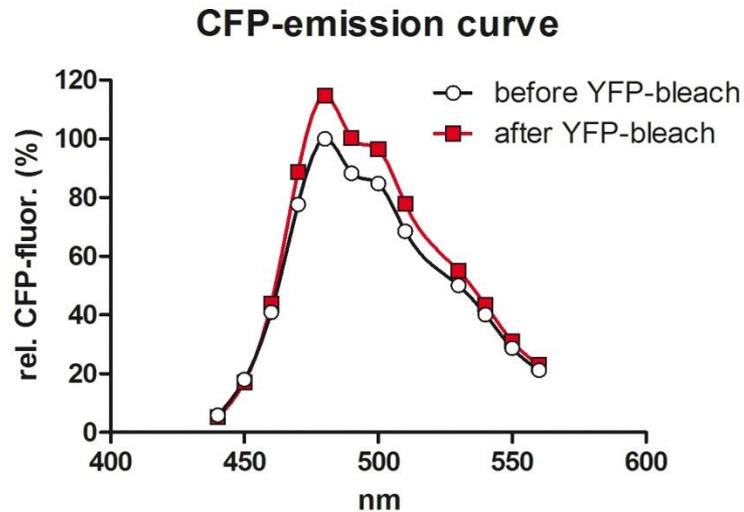


Attempts to perform acceptor-bleaching FRET microscopy fail in fixed, mounted samples

Differences in bleaching kinetics in different mounting fluids



Photoactivation of cyan fluor. Proteins upon YFP-excitation (Ex500-514)



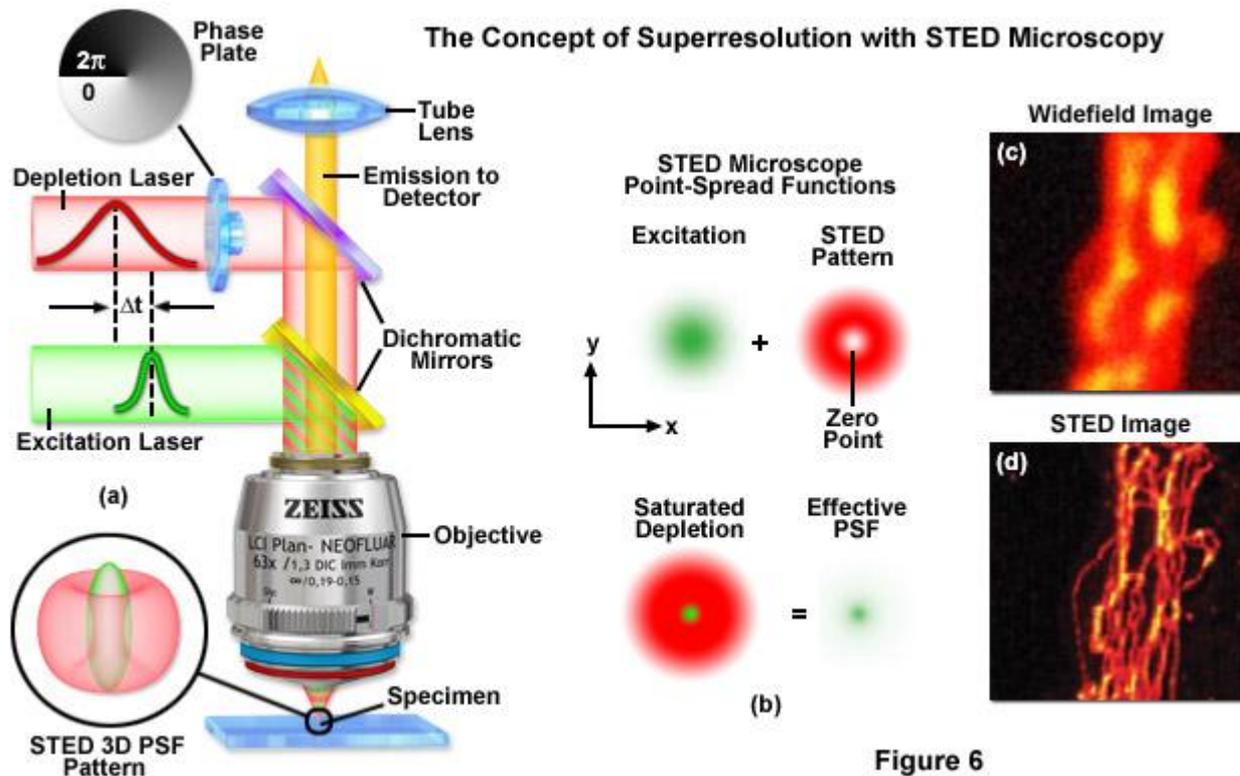
Not only CFP, but also Cerulean shows this phenomenon

FRET microscopy by bleaching of acceptor and monitoring the donor increase can lead to artefacts

Superresolution Microscopy I

STED: Stimulated Emission Depletion

A second laser (depletion laser) „trims“ the excitation spot (point-spread function, PSF) to a smaller size. Resolution appr. 80 nm.



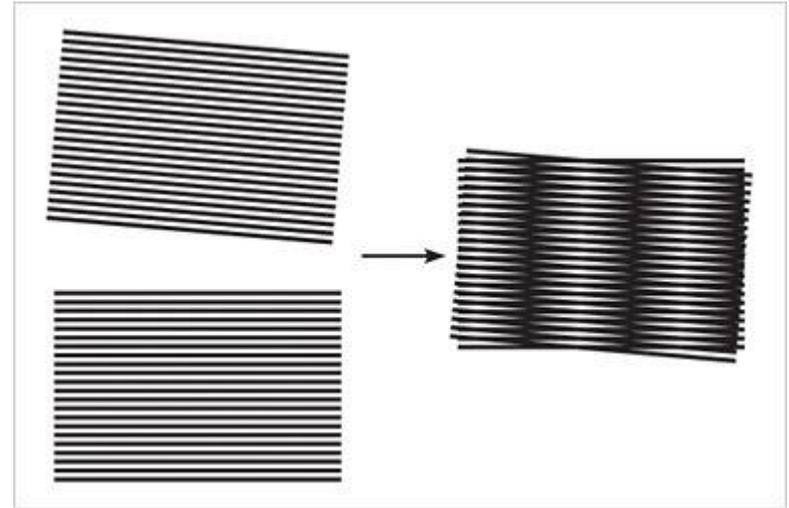
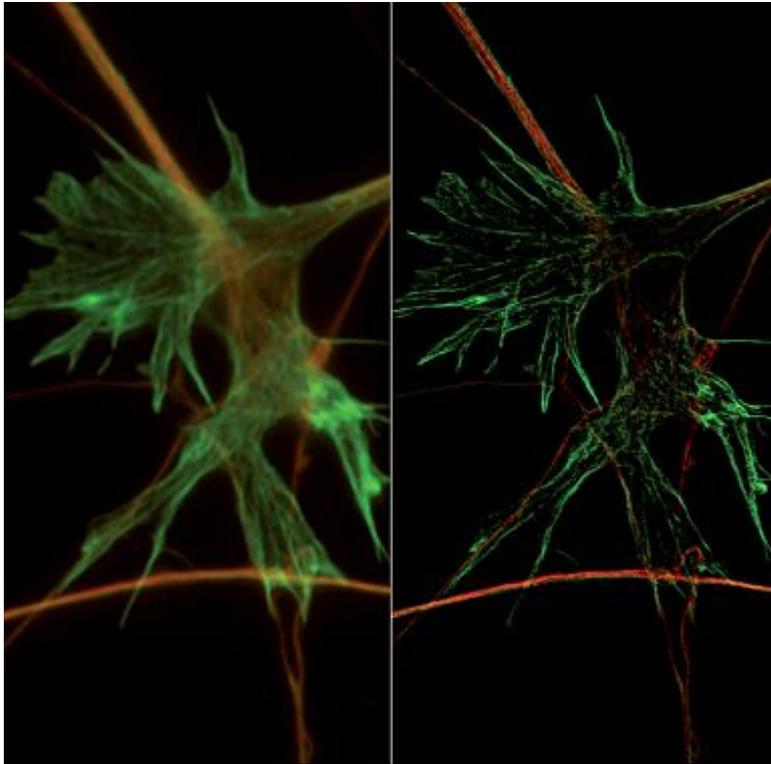
<http://zeiss-campus.magnet.fsu.edu/articles/superresolution/introduction.html>

Superresolution Microscopy II

Structured Illumination Microscopy (SIM)

normal image

SIM image



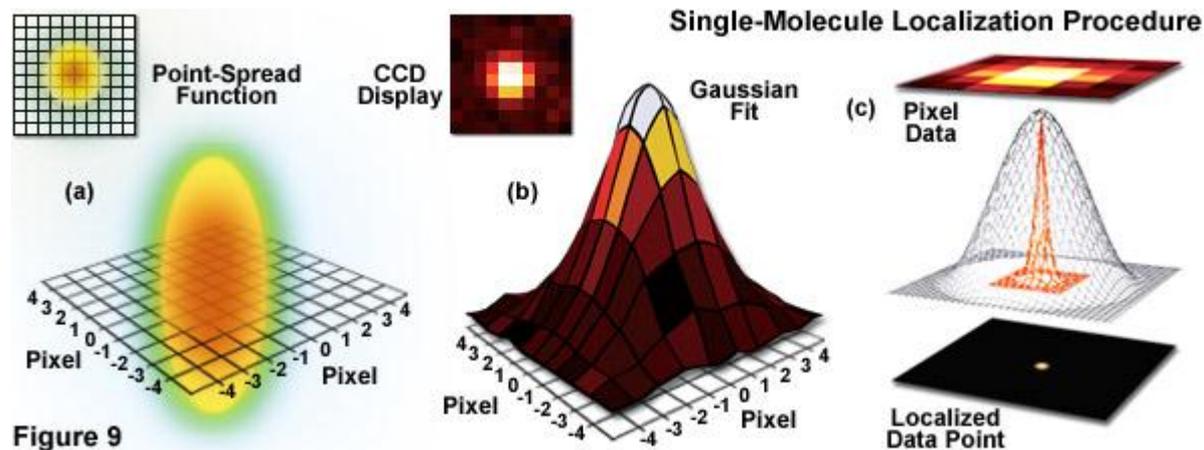
A known pattern is projected into the image plane at different angles and interferes with sample structures, creating Moiré pattern. Superresolution information can now be captured by the microscope from these structures by mathematical algorithms.
(from www.zeiss.de)

Superresolution Microscopy - by single molecule detection

STORM: Stochastic Optical Reconstruction Microscopy using single fluorescent molecules

PALM: Photoactivated Localization Microscopy

Resolution: appr. 30 nm, based on statistical calculation of the center of a Gaussian Fit of a single molecule. Requires a sensitive camera (e.g. EMCCD: Electron-multiplying charge-coupled device cameras) – and some software, but no specific hardware



<http://zeiss-campus.magnet.fsu.edu/articles/superresolution/introduction.html>