Fluorescence Microscopy

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7h Annual Course of Principles of Fluorescence Techniques
Genova, Italy
June 29 – July 2, 2009

The Microscope

Investigation of Lake water:

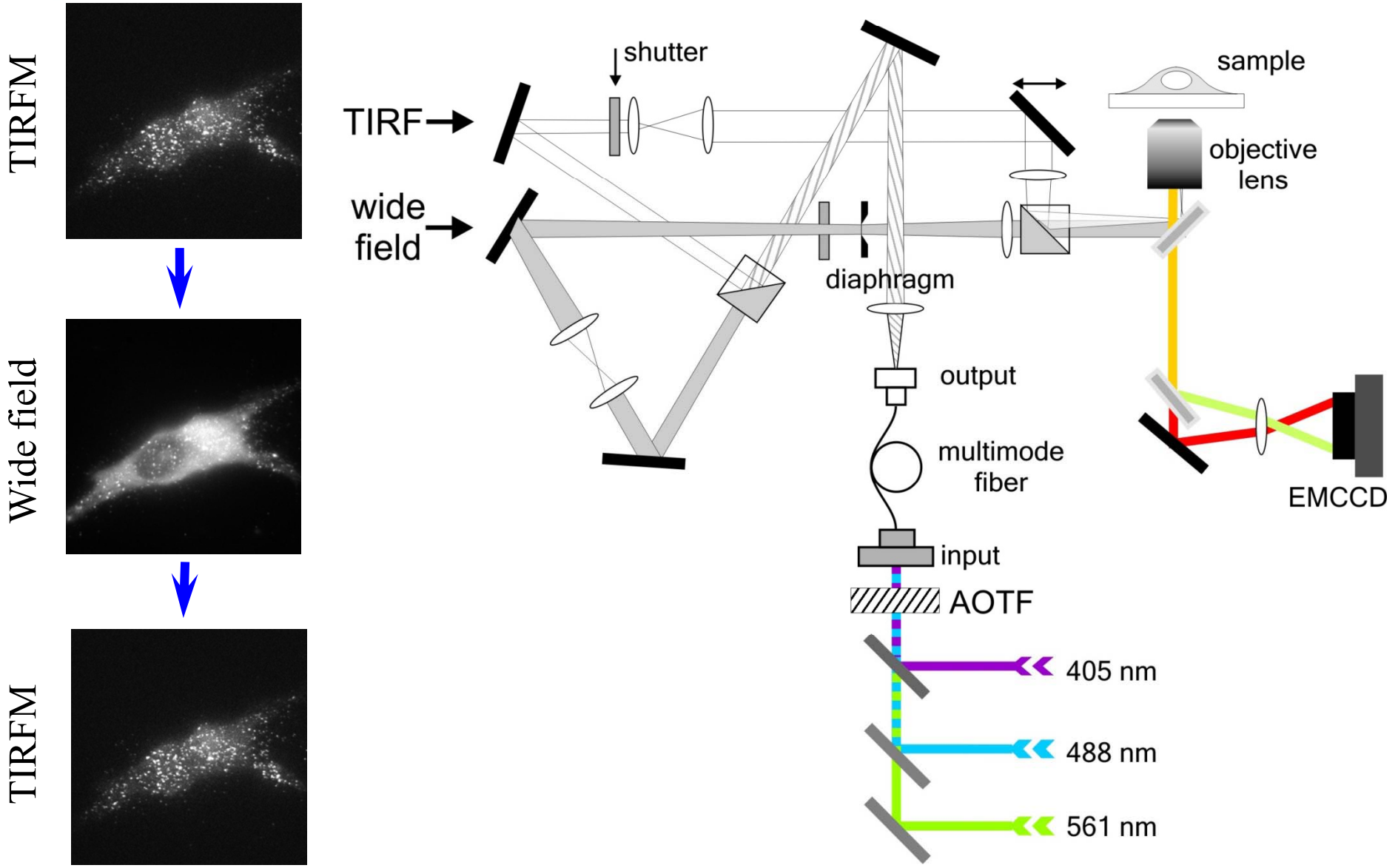
"I found floating therein divers earthy particles, and some green streaks, spirally wound serpent-wise, and orderly arranged, after the manner of the copper or tin worms, which distillers use to cool their liquors as they distil over. …all consisted of very small green globules joined together: and there were very many small green globules as well.

Investigation of the plaque of an old man who never brushed his teeth:

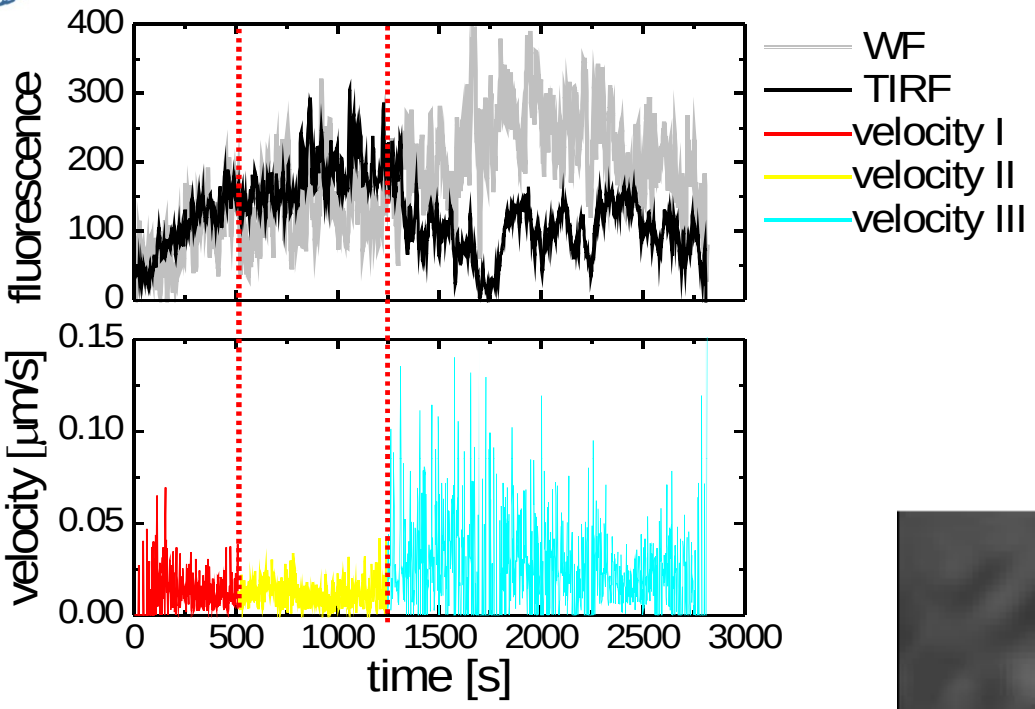
He found "…an unbelievably great company of living animalcules, a-swimming more nimbly than any I had ever seen up to this time. … Moreover, the other animalcules were in such enormous numbers, that all the water…seemed to be alive.

- Von Leeuwenhoek

Wide-Field / TIRF Fluorescence Microscopy

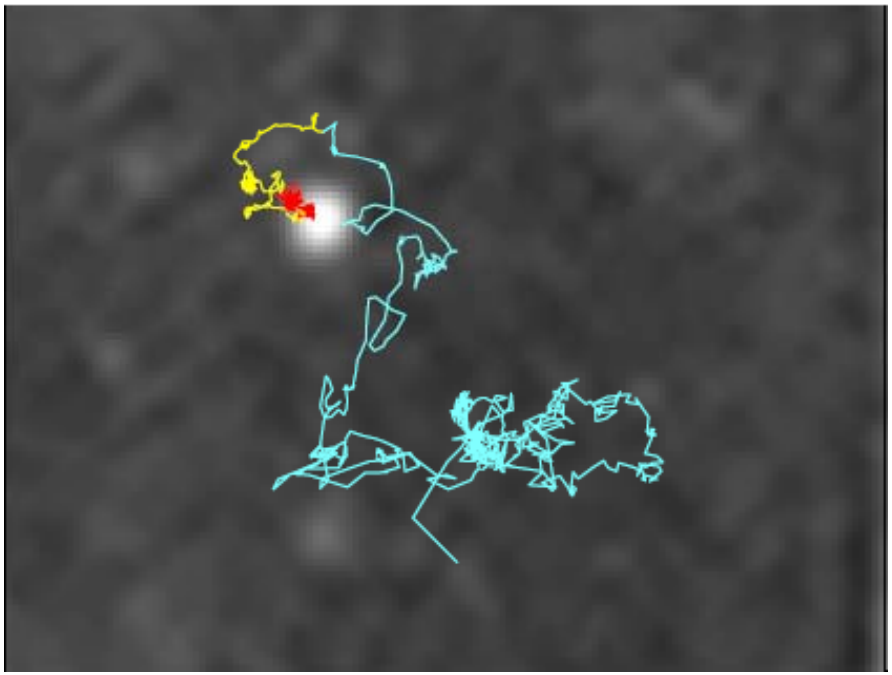


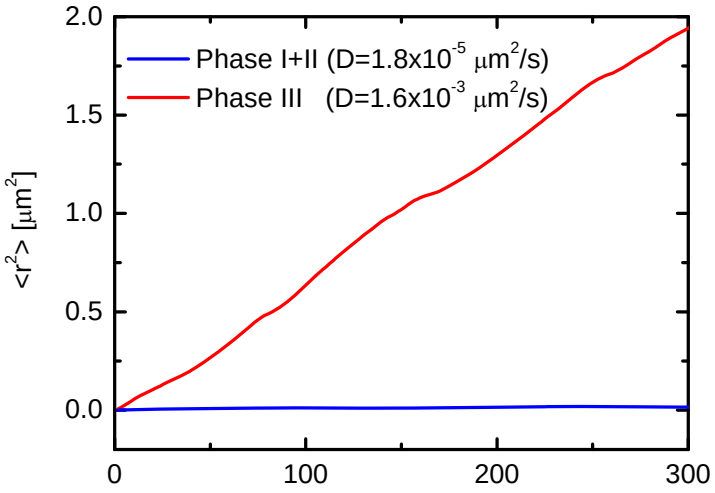
Release of HIV Particles



N= 18 Events

$\tau = 1500 \pm 700$ s





Green Fluorescent Protein

The Nobel Prize in Chemistry 2008

"for the discovery and development of the green fluorescent protein, GFP"

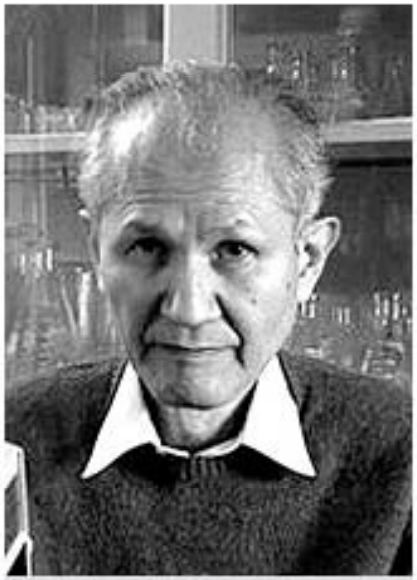


Photo: J. Henriksson/SCANPIX

Osamu Shimomura

1/3 of the prize

Photo: J. Henriksson/SCANPIX

Martin Chalfie

1/3 of the prize

Photo: UCSD

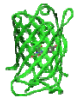
Roger Y. Tsien

1/3 of the prize

http://nobelprize.org/nobel_prizes/chemistry/laureates/2008/

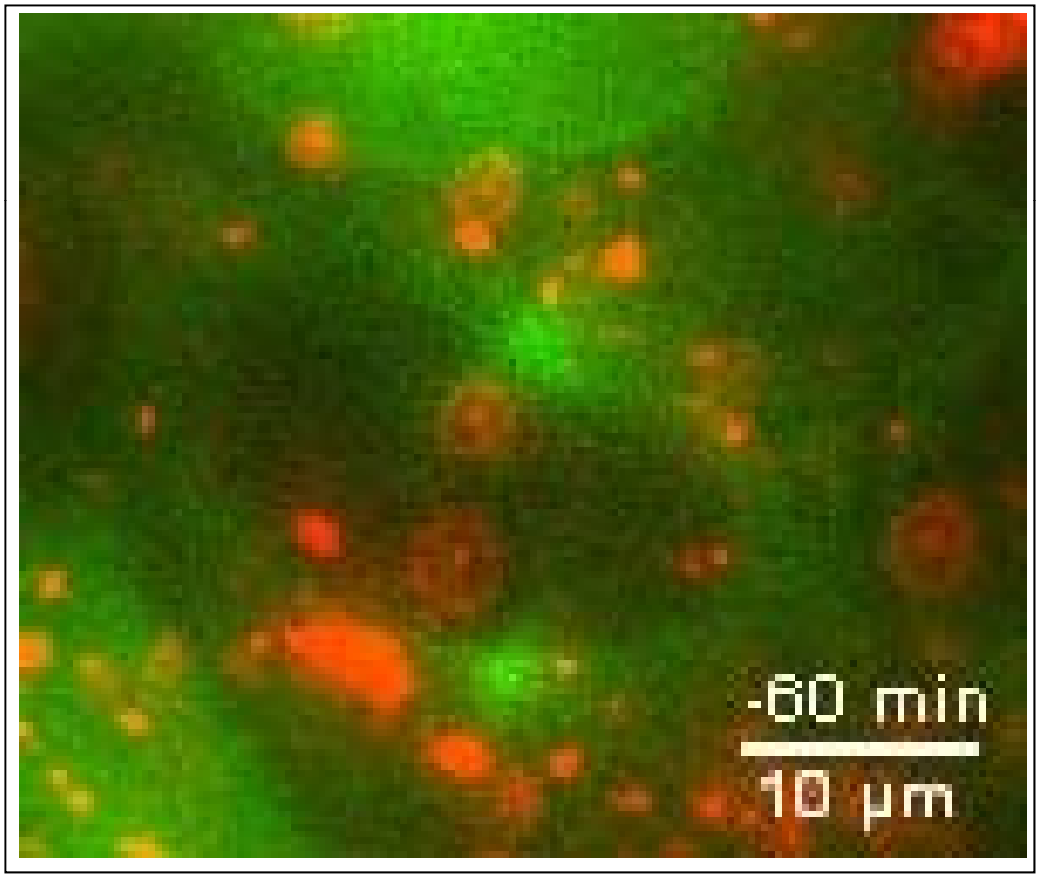
Fluorescence Video Microscopy


eGFP-labeled microtubules


eGFP – End product of infection with nuclear localization signal

Duration: 4.5 hr
Resolution: 500 ms every 12 s
Cell Type: HUH7


Cy5-labeled DNA/PEI particles



- t ~ -60 min
  Nuclear envelope breaks down and spindle apparatus forms
- t ~ 0 min
  Daughter cells form, nuclear membrane builds up again
- t ~ 120 min
  GFP accumulation detectable inside the nucleus of both daughter cells.

Ralf Bausinger

Bausinger et al., Angew. Chem. Int. Ed. 45 (2006) 1568

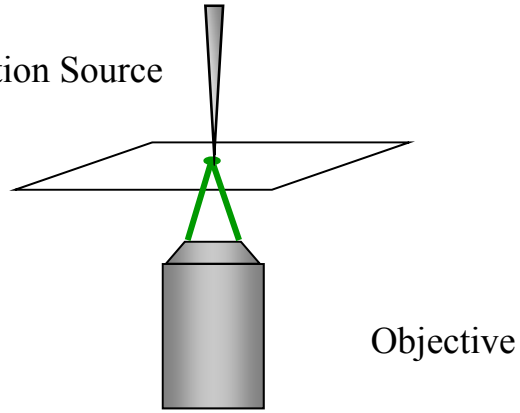
Other Fluorescent Methods

Near-Field Optical Microscopy

Excitation through a fiber

Exit aperture < 100 nm

• Resolution higher than the diffraction limit

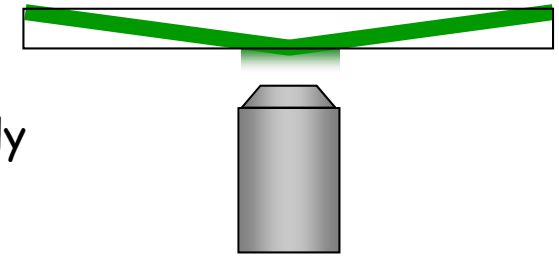


Total-Internal-Reflection

Excitation is from the evanescent wave of the total-internal-reflected light.

Depth of excitation is only 100 nm (compared to 1.5 μm)

• Entire field of view can be measured simultaneously

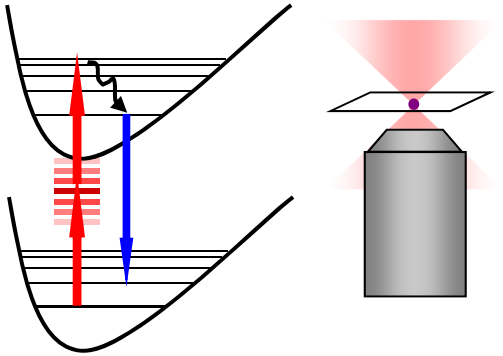


Two-Photon Excitation

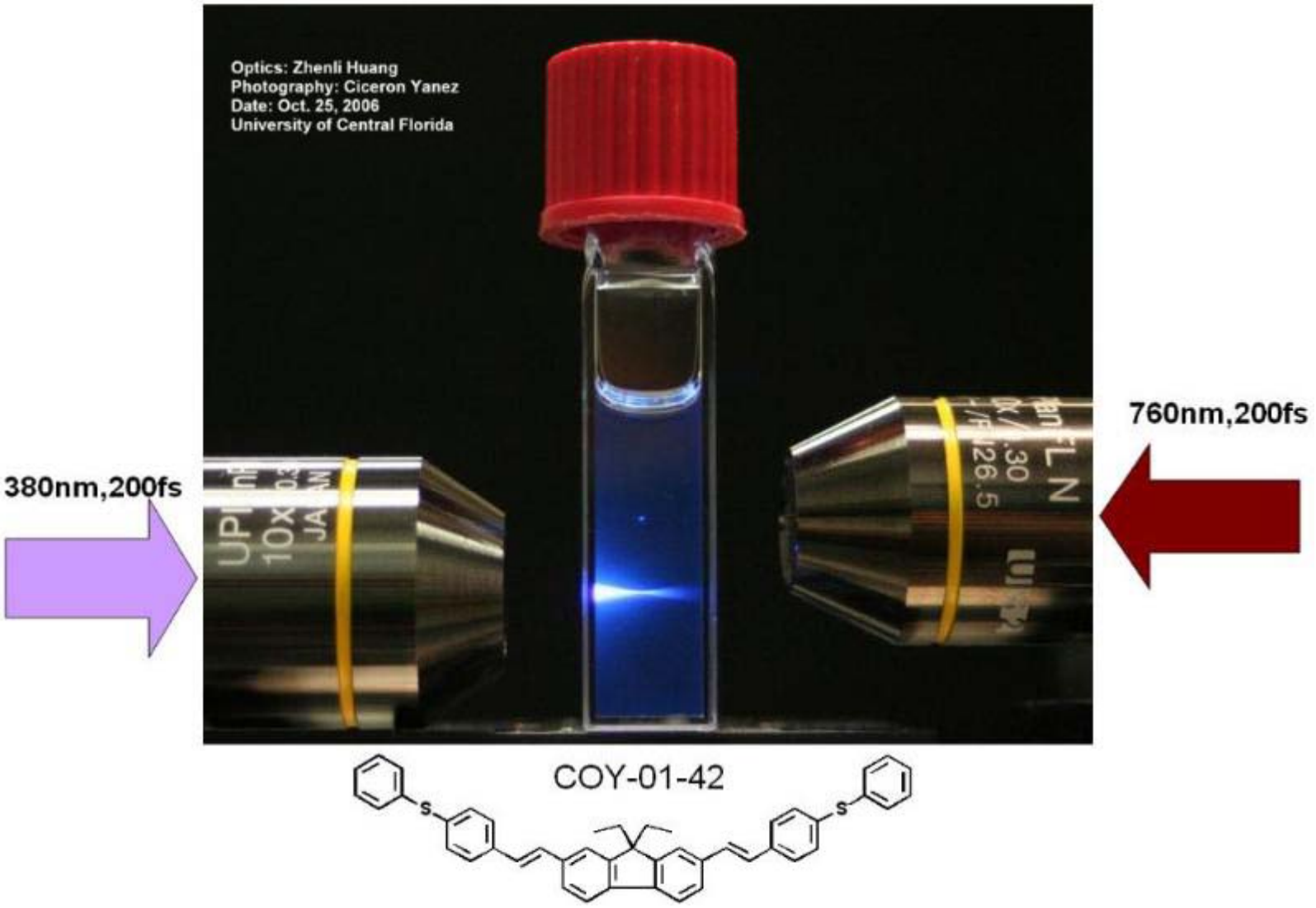
Two photons of half the energy are absorbed simultaneously

Excitation occurs only at the focus of the objective

• Confocal images are obtained without a pinhole.

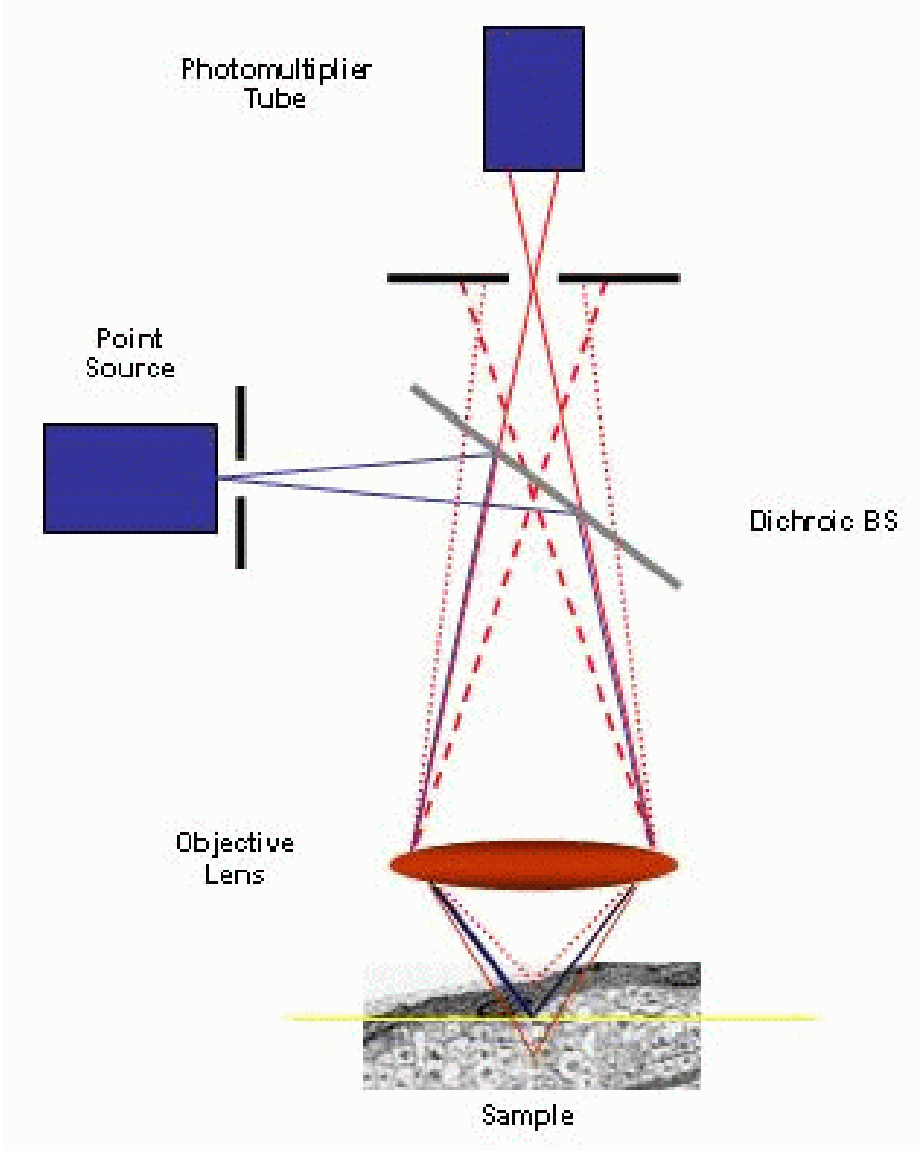


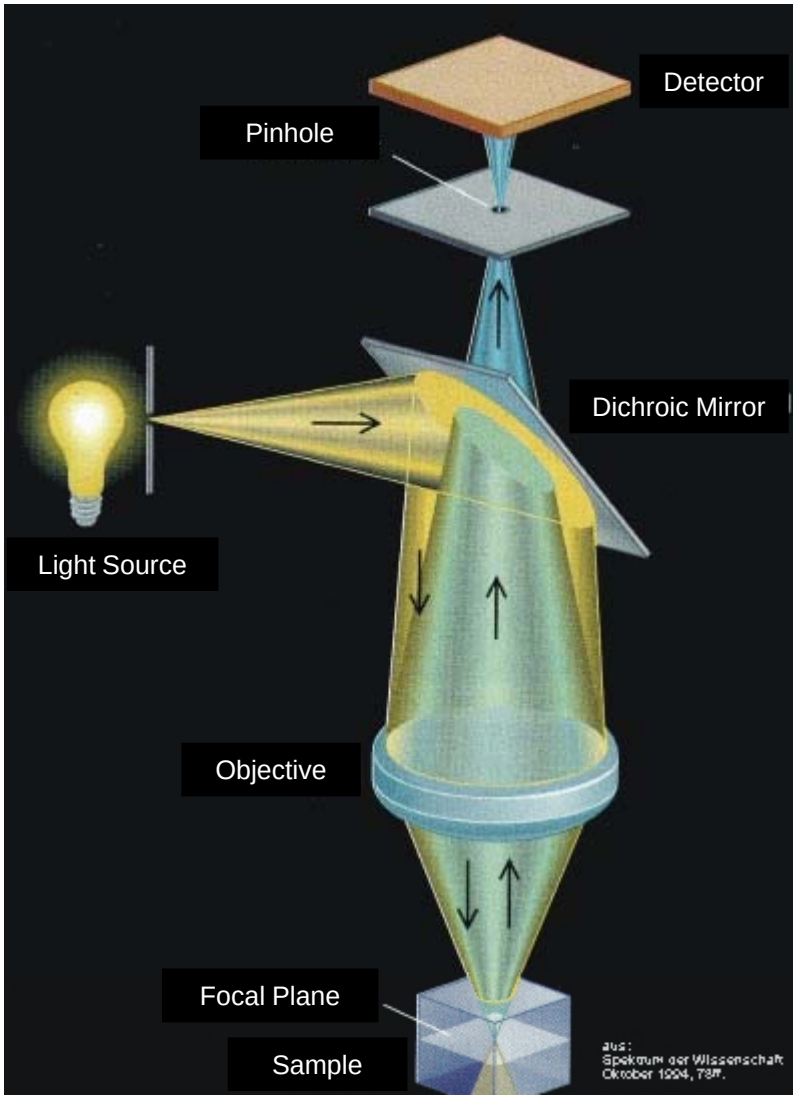
Two-Photon Excitation



http://belfield.cos.ucf.edu/one%20vs%20two-photon%20excitation.html

Confocal Microscopy

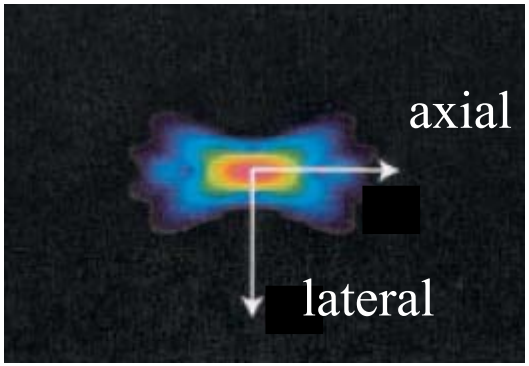




Confocal Microscopy

3-D Resolution

Point Spread Function (PSF)

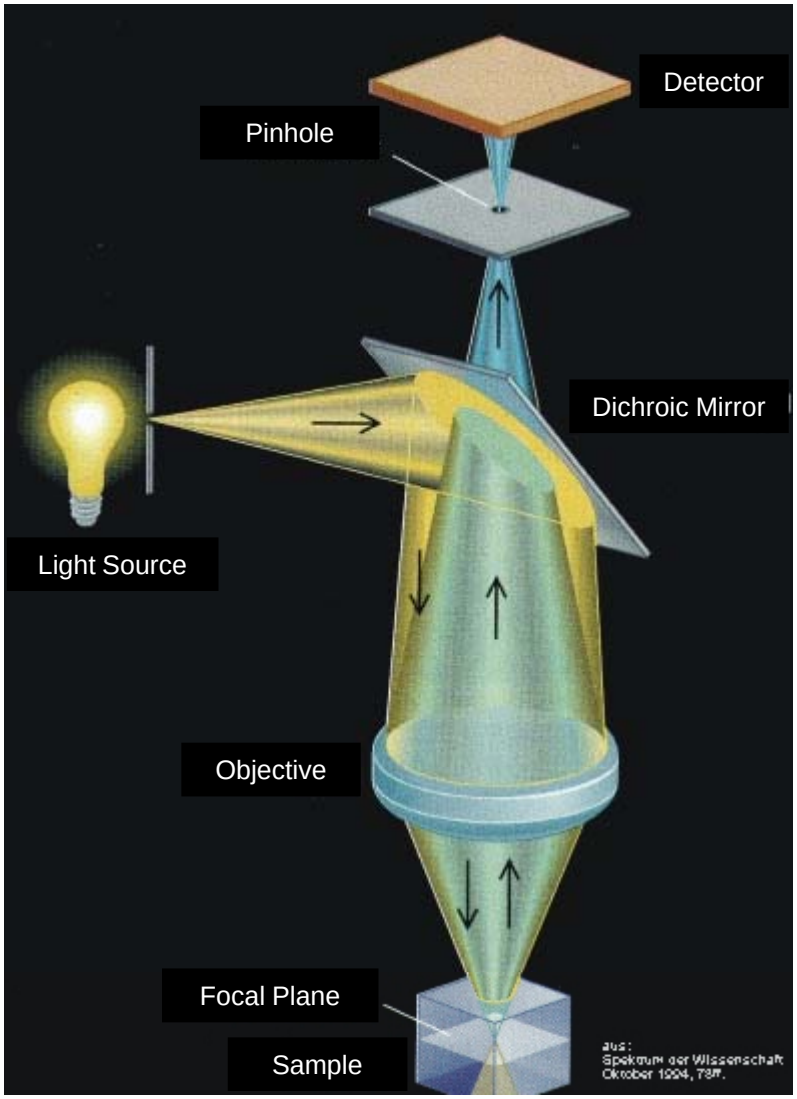


Hess & Webb (2002) *Biophys J* **83**:2300

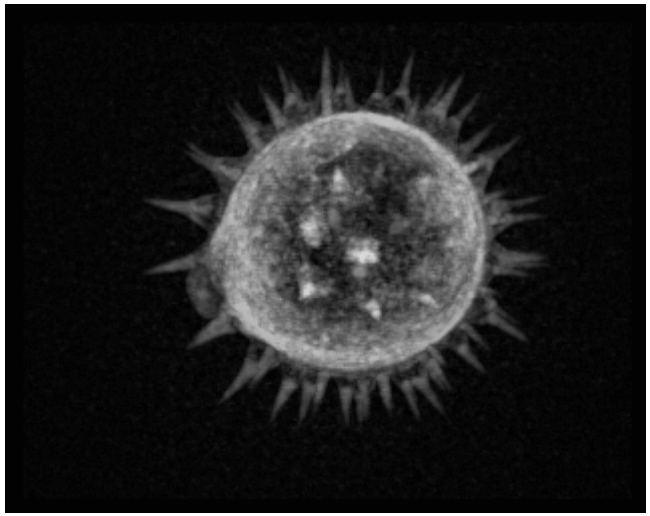
$$PSF = I_0 \exp\left[-\frac{2\left(x^2 + y^2\right)}{w_r^2} - \frac{2z^2}{w_z^2}\right]$$

w_r = ~ 0.25 μm

w_z = ~ 0.8 - 1.0 μm

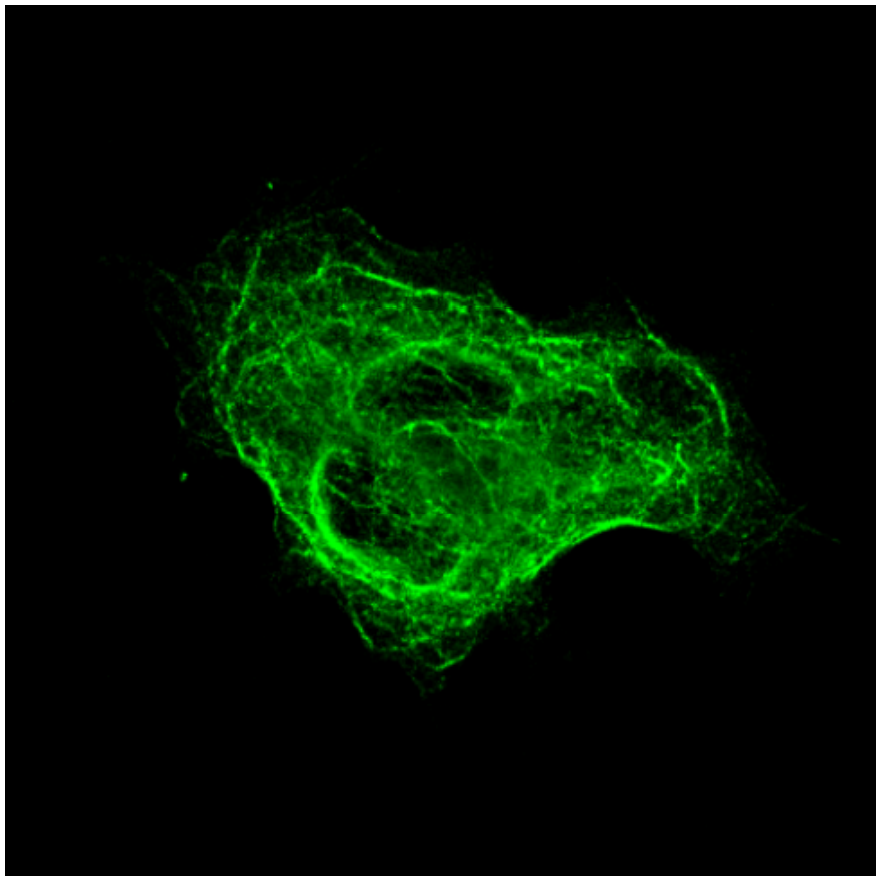


3D Confocal Images



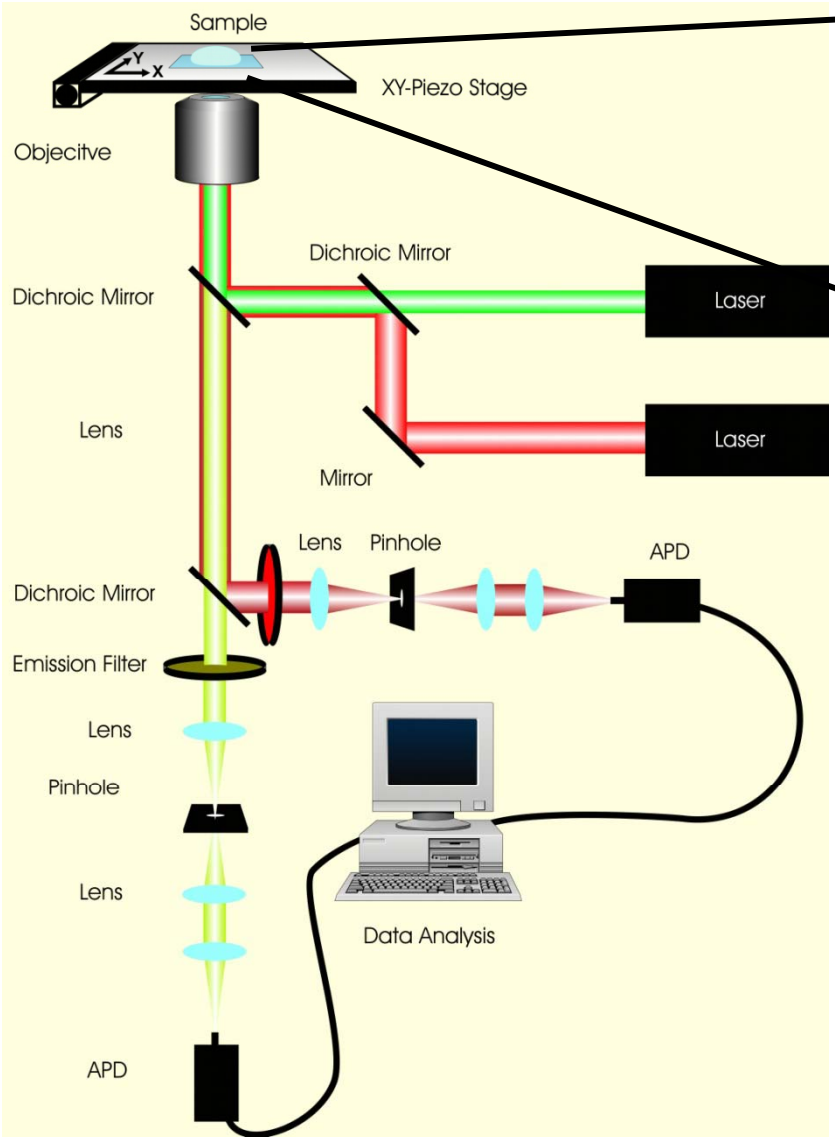
- 3D Reconstruction of Pollen

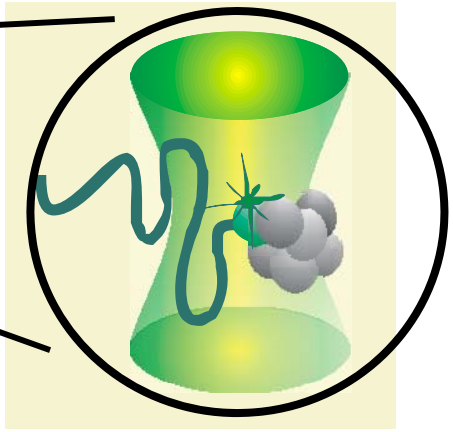
from http://www.meyerinst.com/html/openlab/samples/dcipolxx-3d-lg.gif



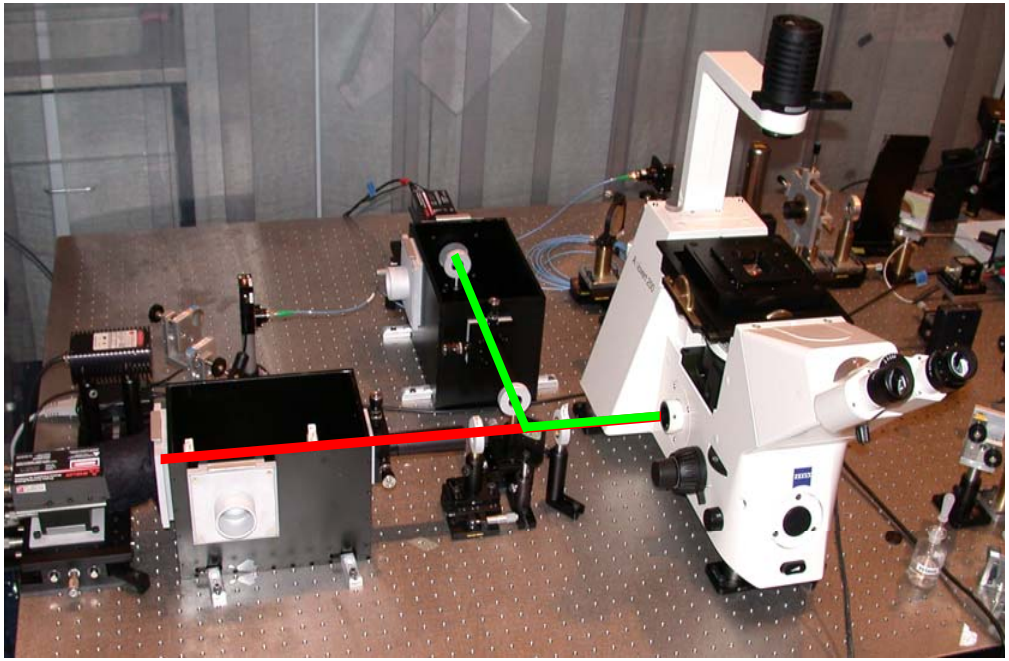
- A HeLa cell with GFP labeled Microtubules (courtesy of Ralf Bausinger)

Experimental Setup

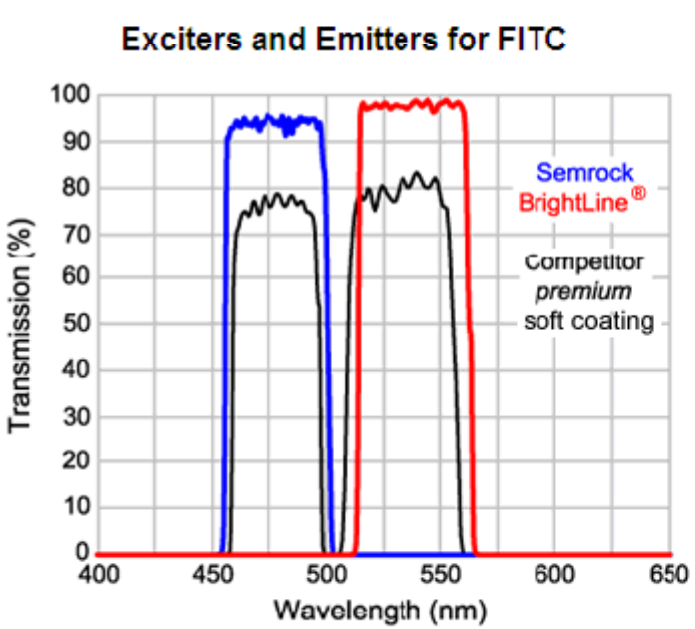


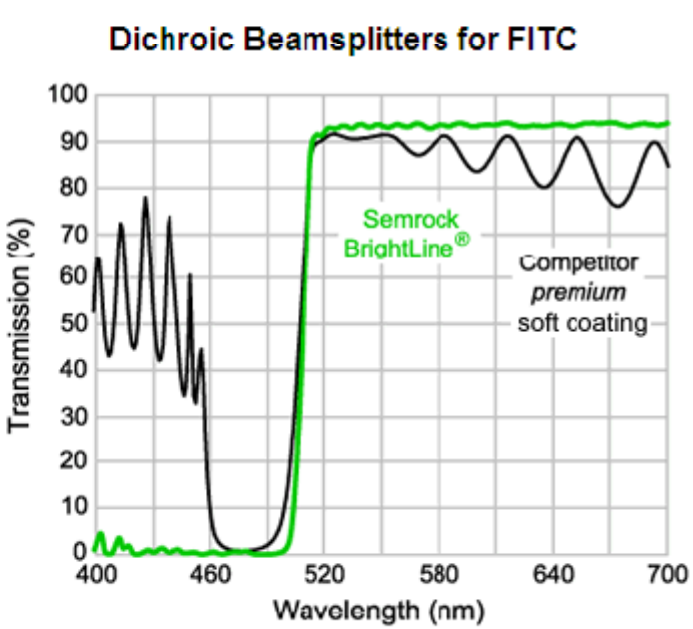


Two Channel Confocal Microscope



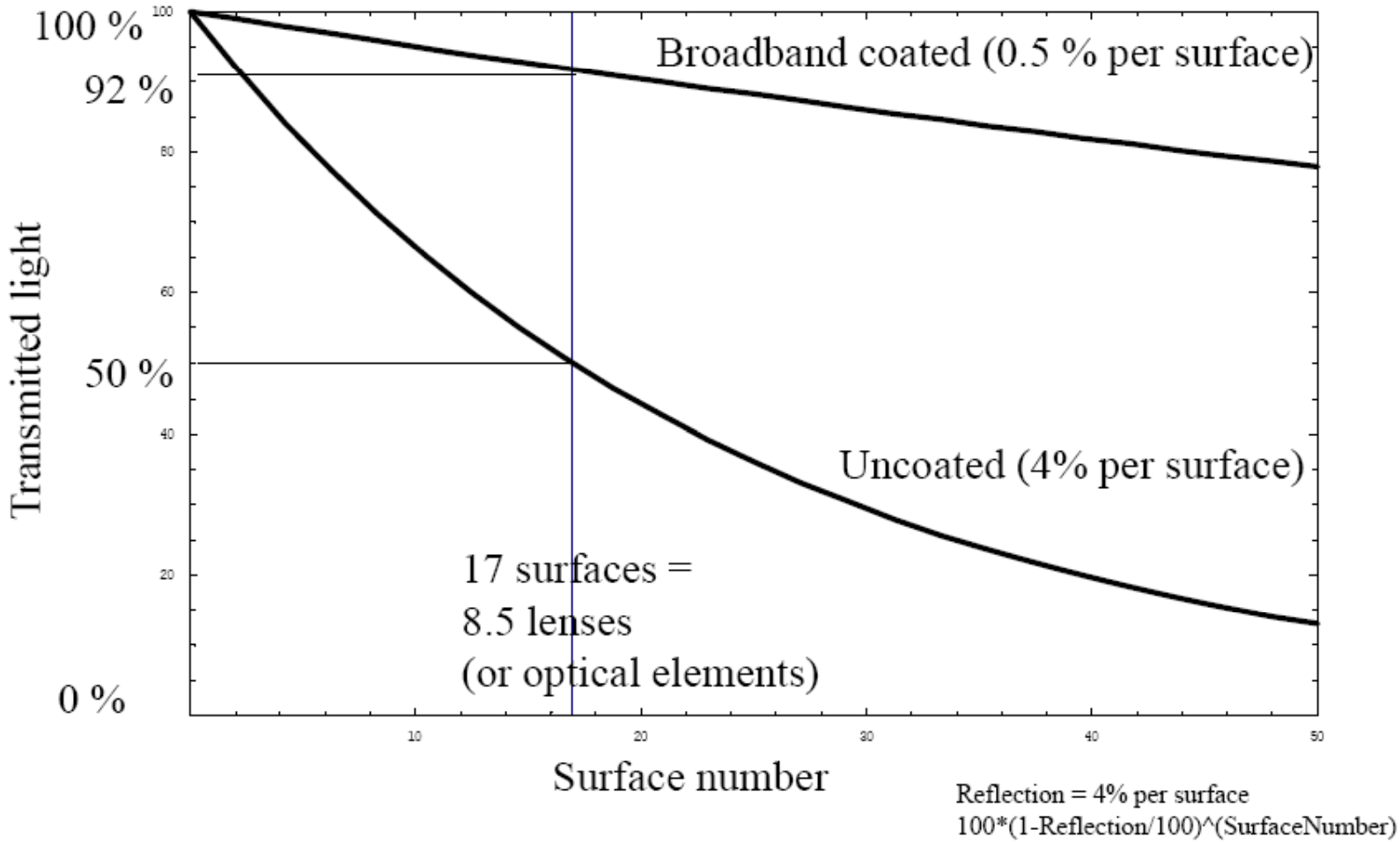
Filters / Dichroics





http://www.semrock.com/Catalog/SpectralComparison.htm

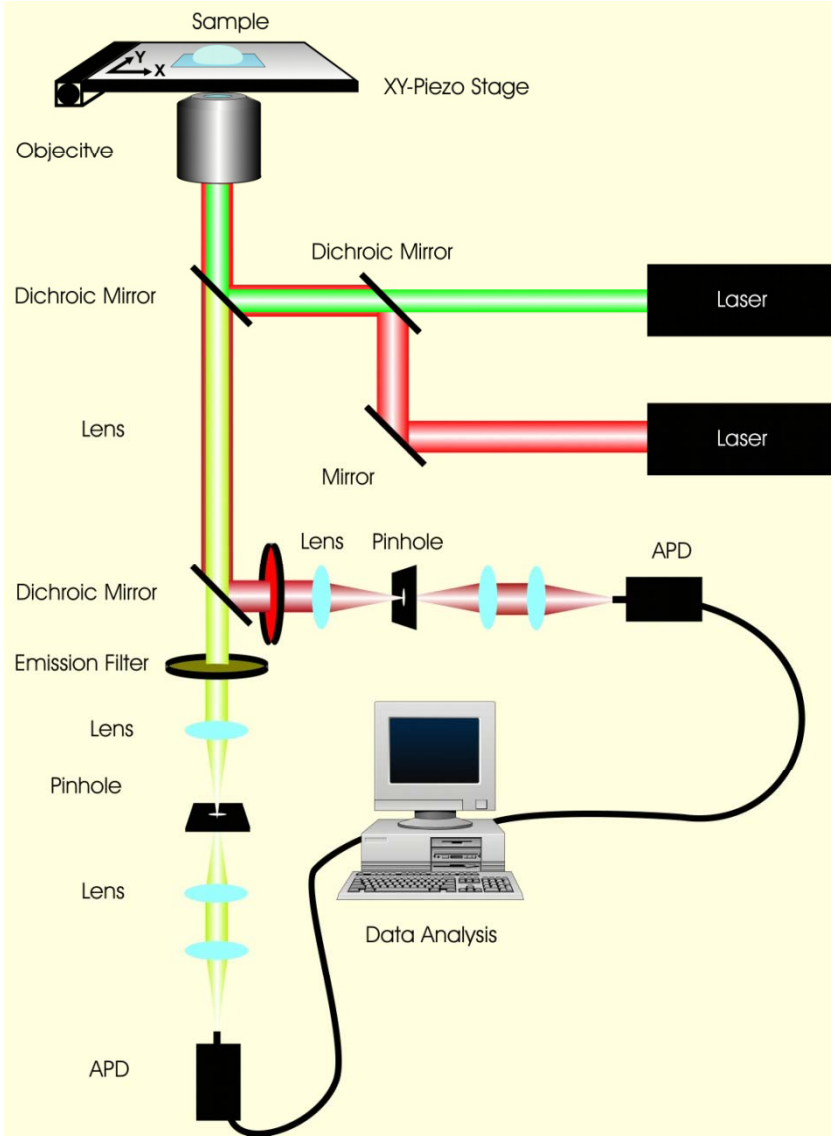
Anti-reflection coating



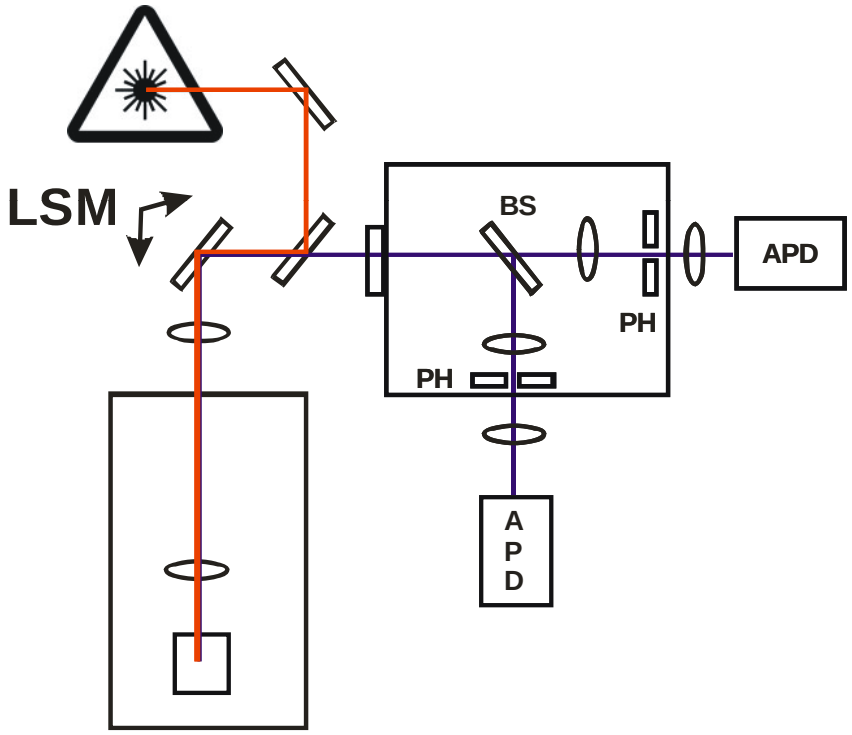
Reflection = 4% per surface
100*(1-Reflection/100)^(SurfaceNumber)

Scanning / D-Scanning

Sample Scanning



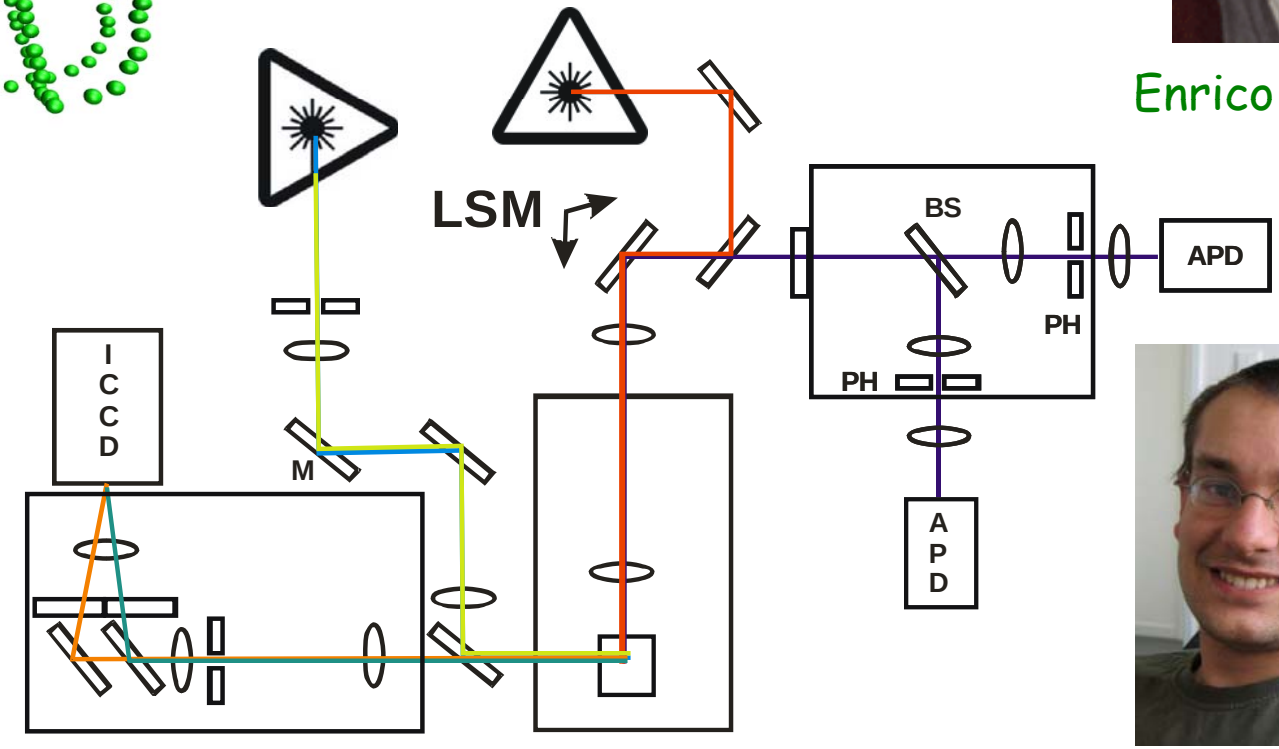
Laser Scanning



3-D Orbital Particle Tracking

with simultaneous bright field imaging

Enrico Gratton



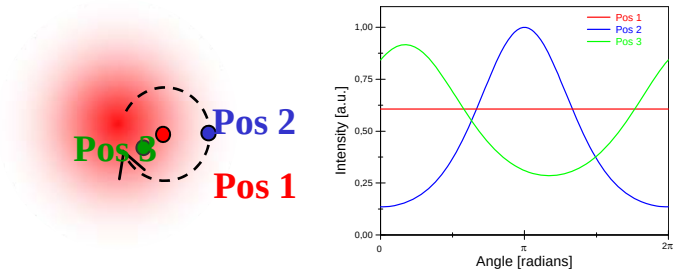
Iko Katayama

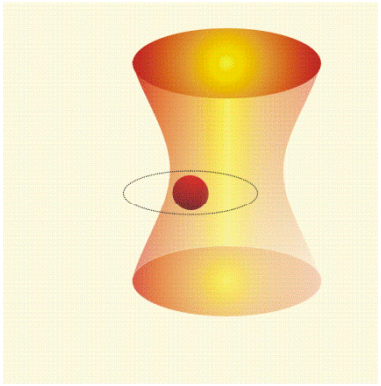
Bright Field

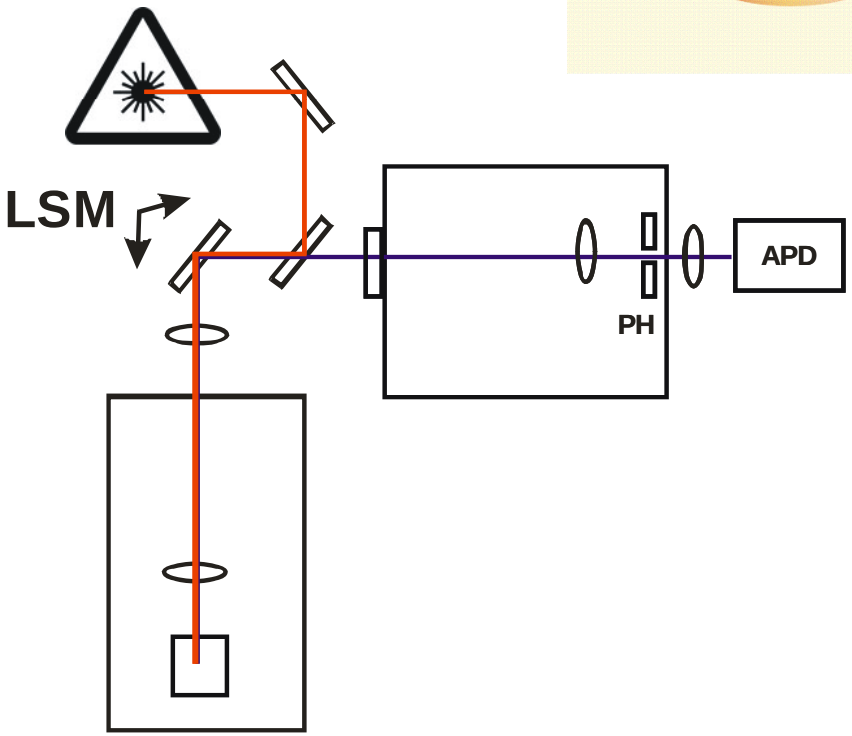
Dr. Ondrej Burkacky

3-D Orbital Particle Tracking

3D orbital tracking microscope (x,y)





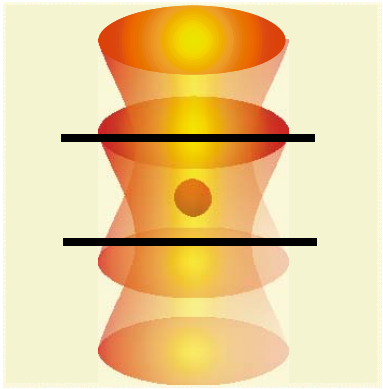


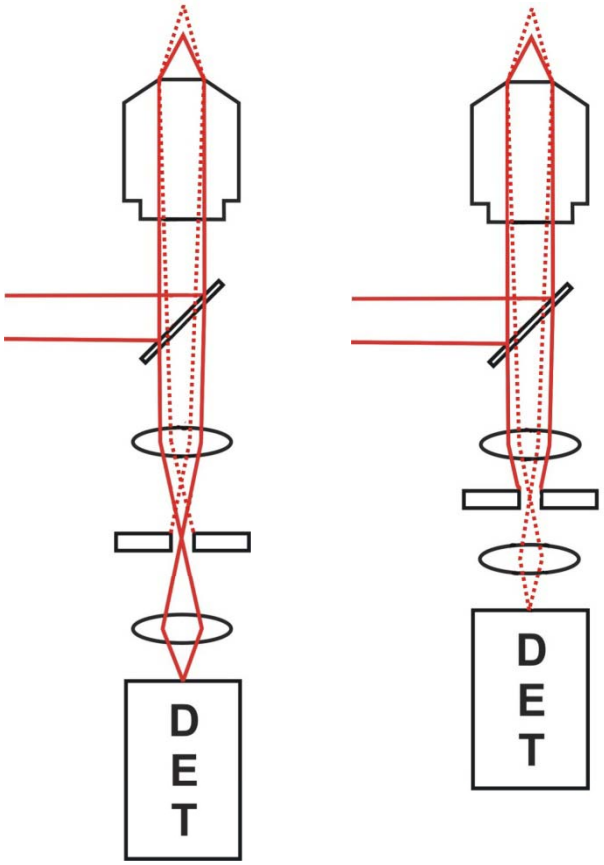
3-D Orbital Particle Tracking

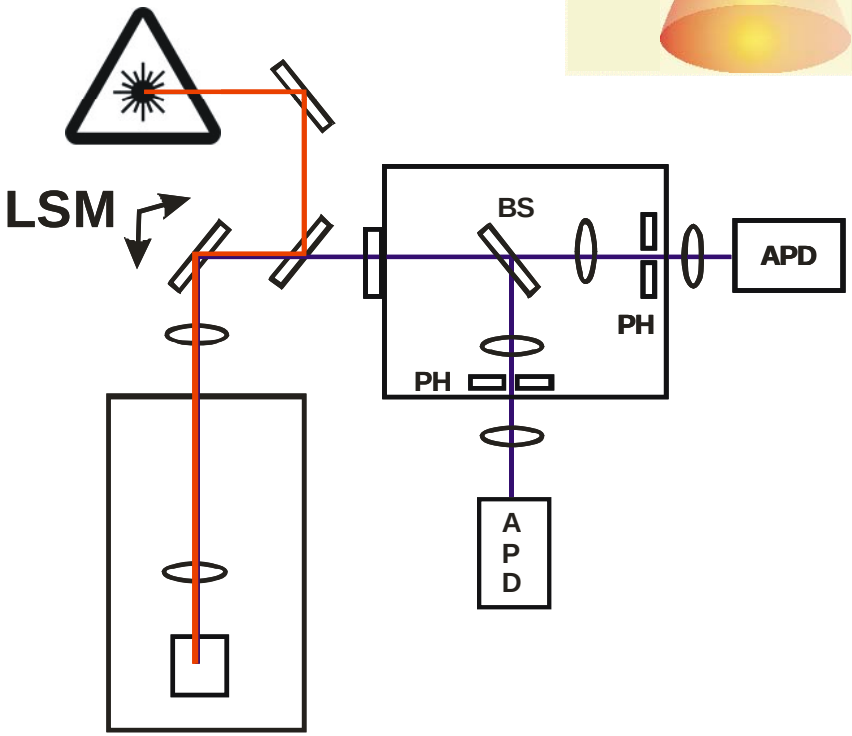
3D orbital tracking microscope (z)

Focus, Det 1

Focus, Det 2







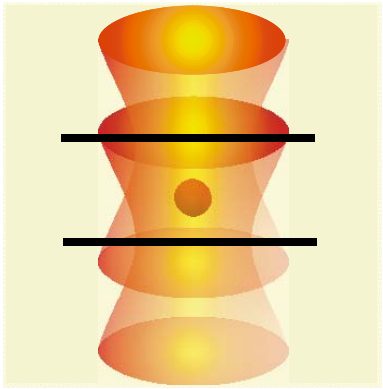
3-D Orbital Particle Tracking

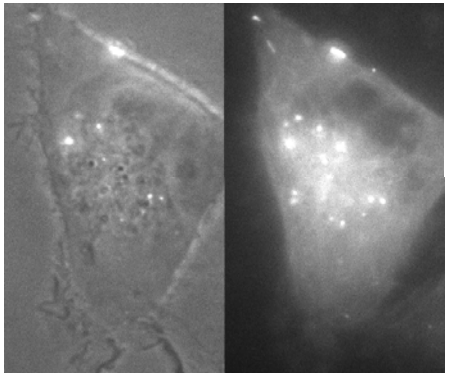
3D orbital tracking microscope (z)

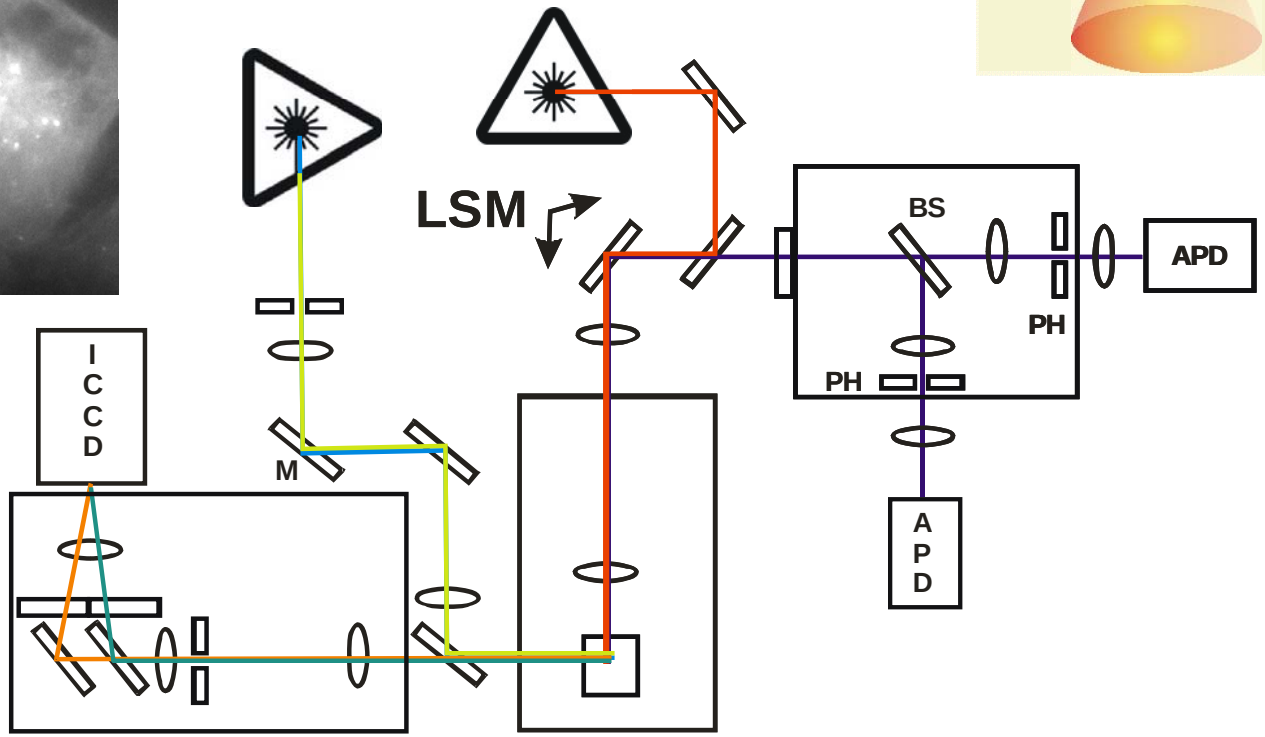
with simultaneous bright field imaging

Focus, Det 1

Focus, Det 2



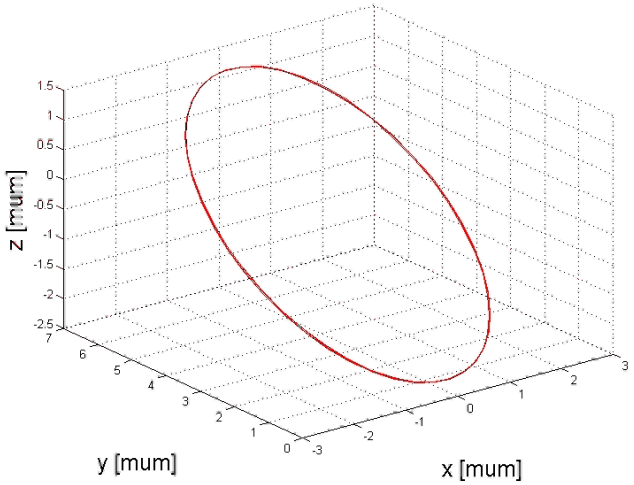




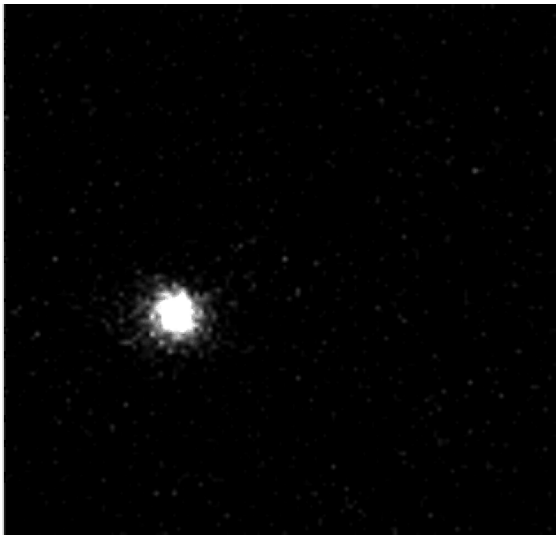
Bright Field

Simultaneous Wide-field Imaging

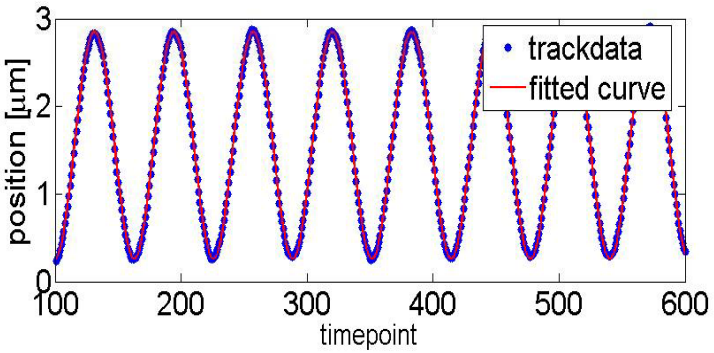
Wide-field without Tracking

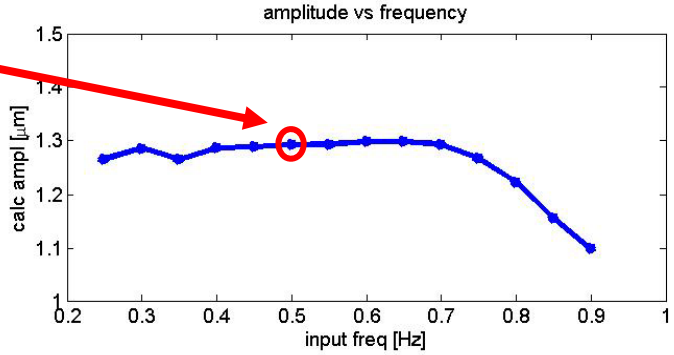


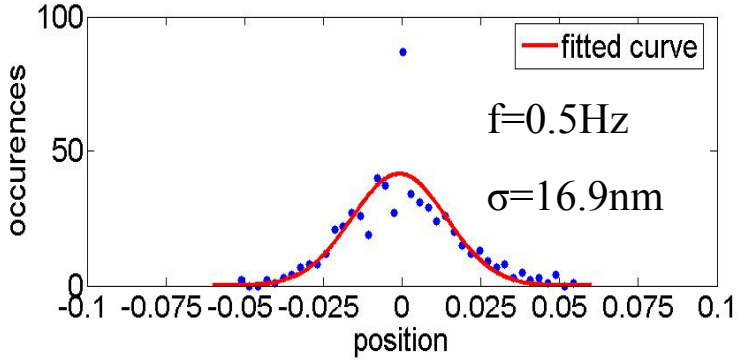
Wide-field with tracking

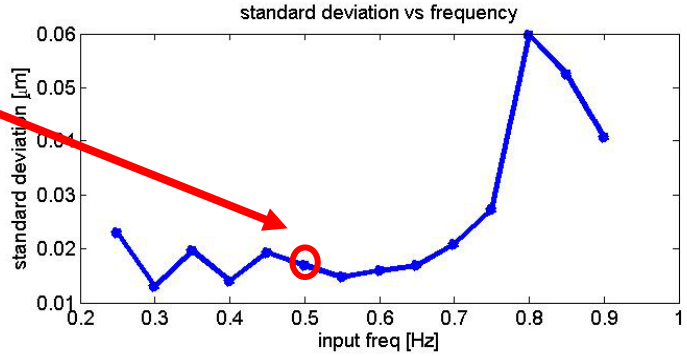


Tracking Accuracy vs Motion





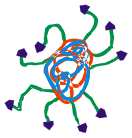




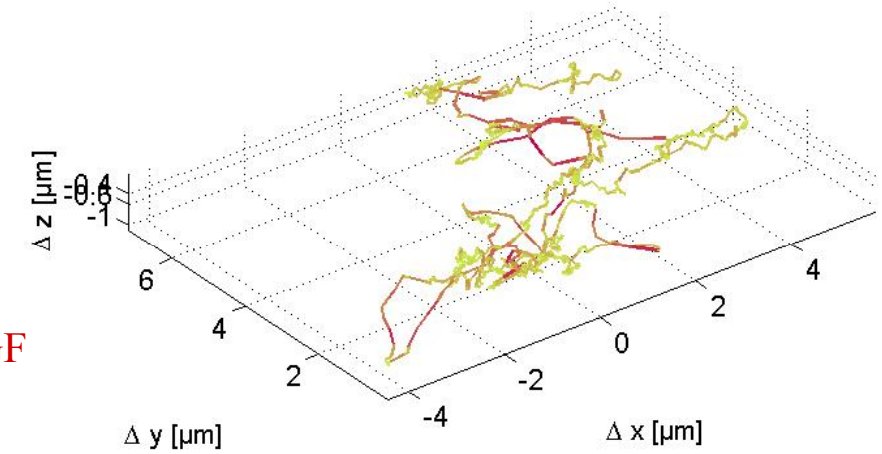
Tracking Single Particles in Real Time in 3D

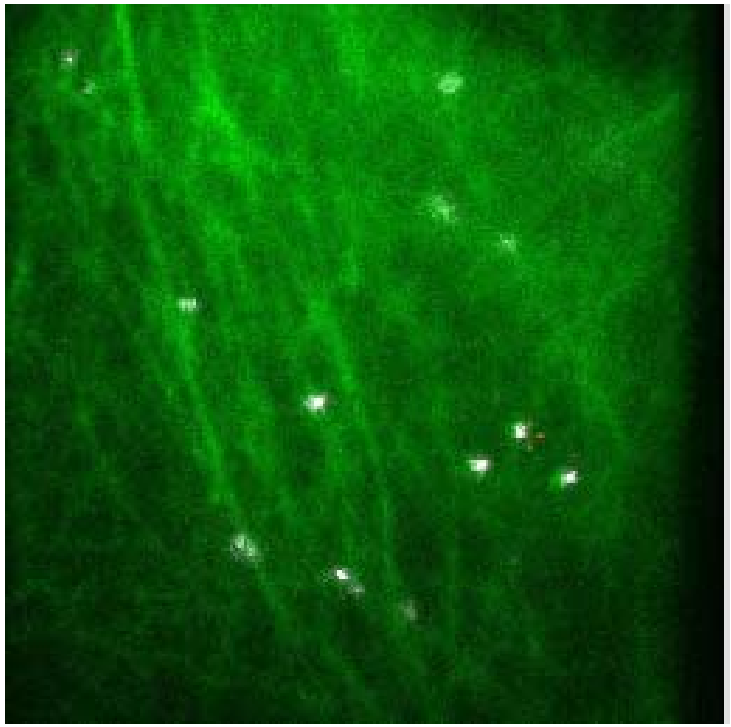
Post injection: < 10 min
Duration: ~ 5 min
Resolution: 500ms / 32ms
Cell Type: Huh7

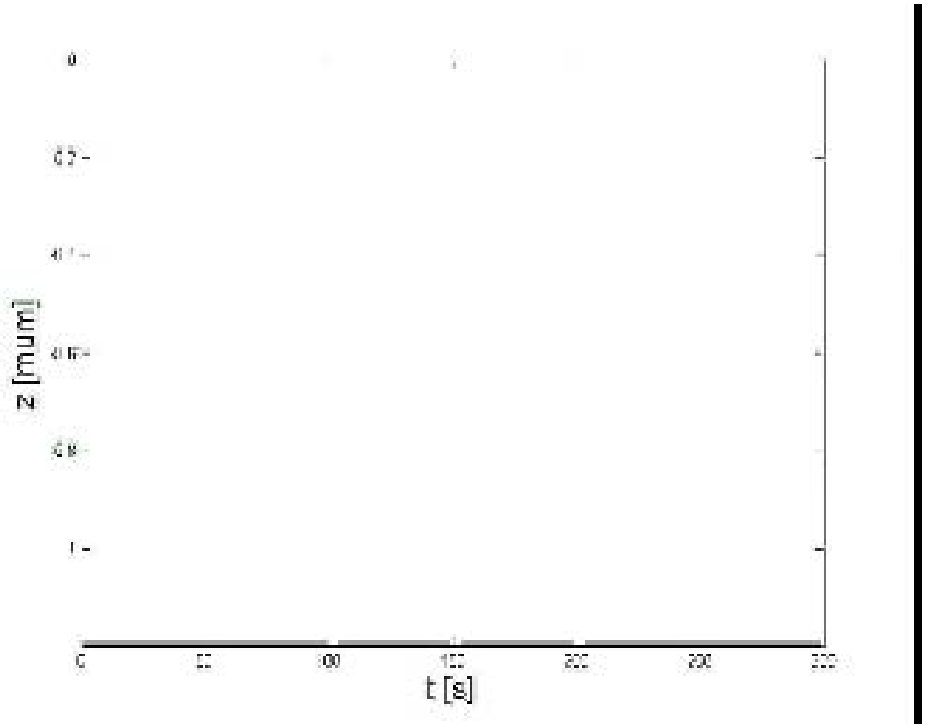
GFP labeled microtubulin



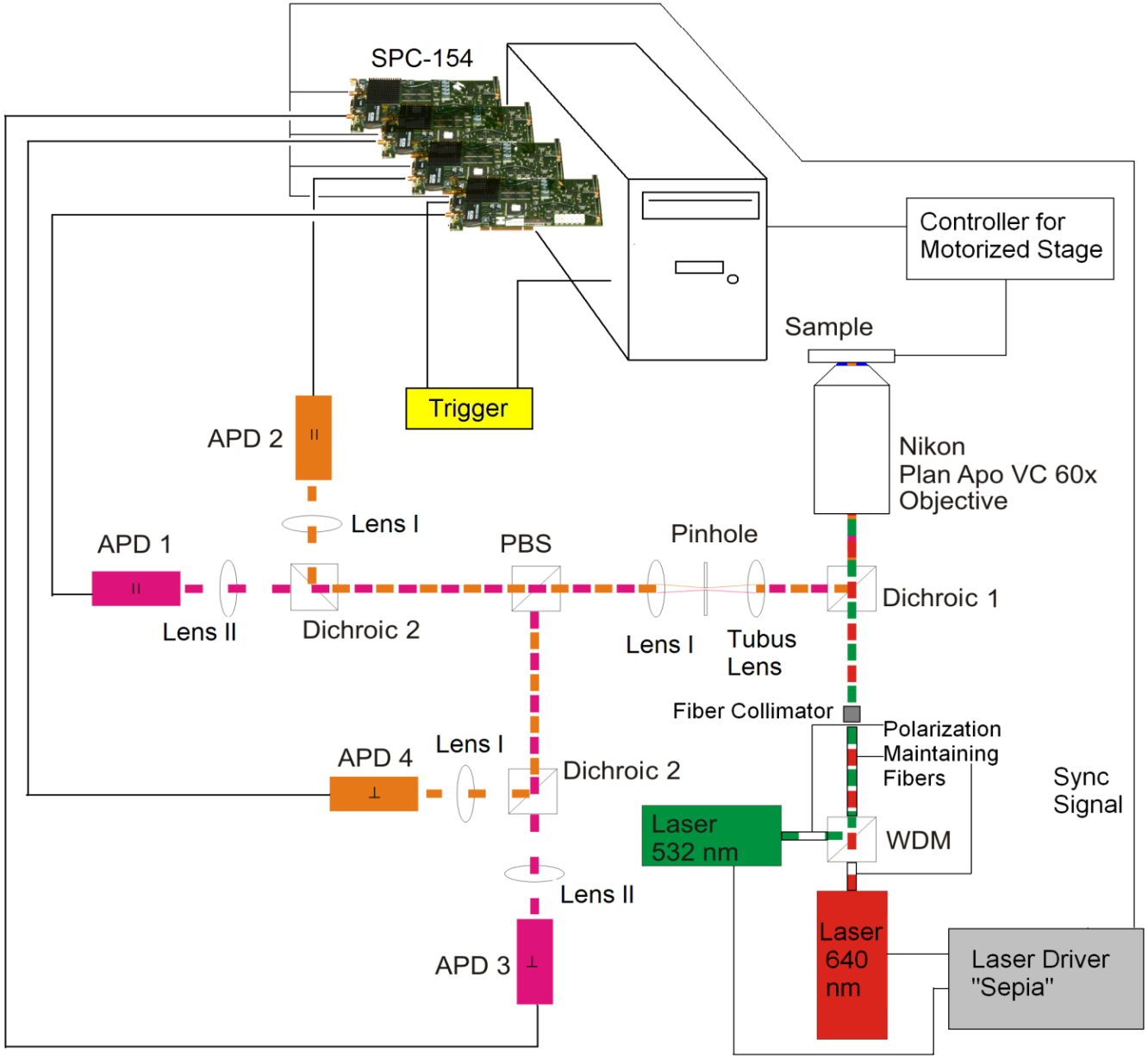
Cy3/Cy5 labeled DNA/PEI/PEG/EGF complexes







Multiparameter Fluorescence Detection Setup



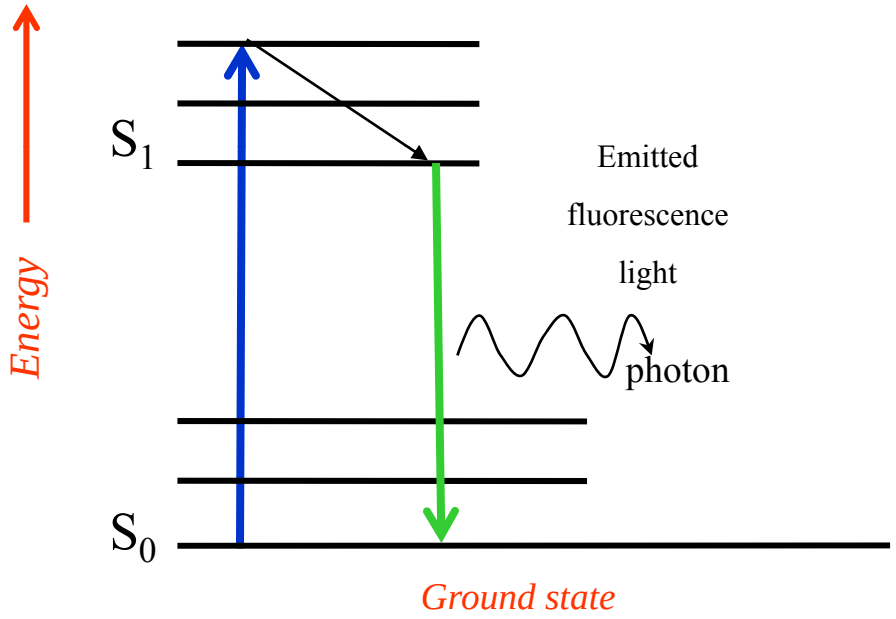
Fluorescence Lifetime Imaging Microscopy (FLIM)

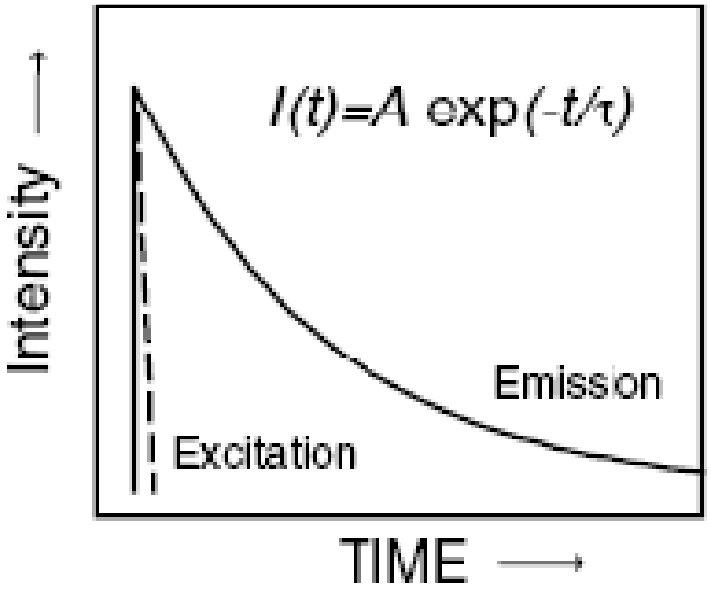
Don C. Lamb

Laboratory for **F**luorescence
Applications in **B**iological Systems
Institute of Physical Chemistry
Munich, Germany

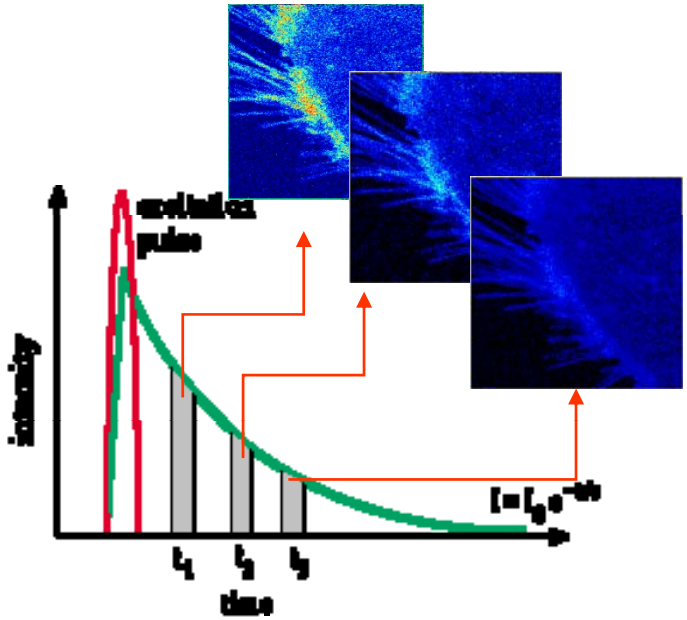
7h Annual Course of Principles of Fluorescence Techniques
Genova, Italy
June 29 – July 2, 2009

Lifetime: Background



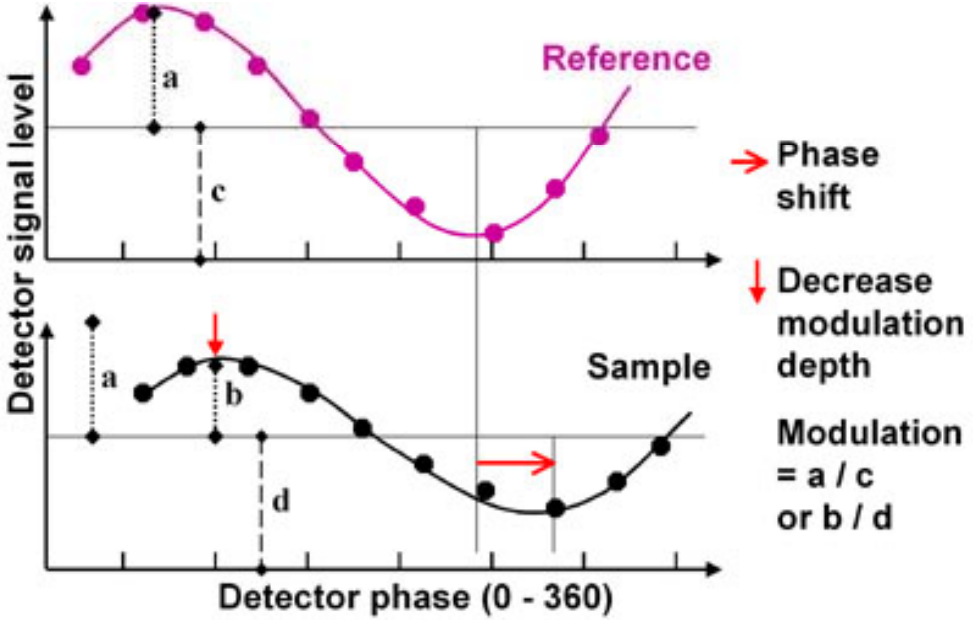


Time Domain and Frequency Domain FLIM



A sample is flashed many times by a short duration laser source

The histogram of the time intervals between the excitation flash, and 1st emitted photon is measured



http://lambert-instruments.asweb.nl/beeldbank/frequency-reference-sample.jpg

A sample is excited by a modulated light source

The fluorescence emission has the same frequency but is modulated and phase-shifted from the excitation source

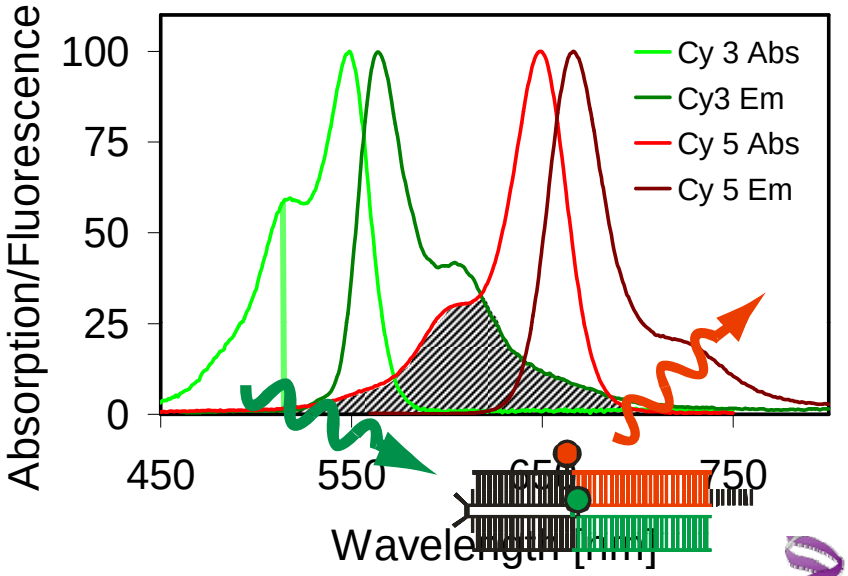
Why FLIM?

FLIM is used for :

- **FRET**
- **Intracellular mapping of Ion concentration and pH imaging**
- **Biochemical reactions (oxidation/reduction) processes**
  - **NAD and NADH**
- **Long lifetime imaging (phosphorescence).**
  - **For example O_2 concentration in the cell or in tissues**

Förster Resonance Energy Transfer (FRET

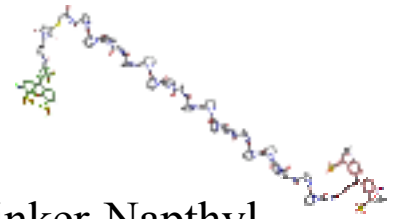
- spectral properties of D and A



$$k_T = \frac{9000(\ln 10)\,\phi_D\,\kappa^2 J}{128\pi^5 N_A n^4 \tau_D R^6}$$

$$J = \frac{\int d\lambda\, F_D(\lambda)\varepsilon_A(\lambda)\lambda^4}{\int d\lambda\, F_D(\lambda)}$$

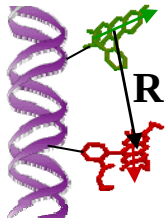
- D-A separation

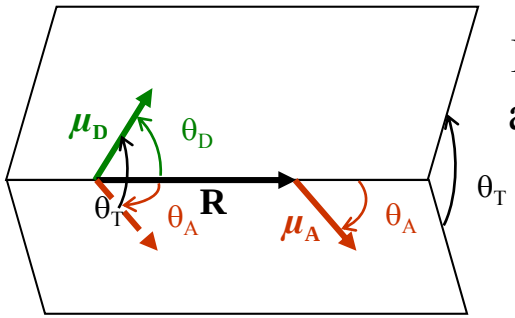


Dansyl-(Pro)$_n$-Linker-Napthyl

- relative orientation of D and A

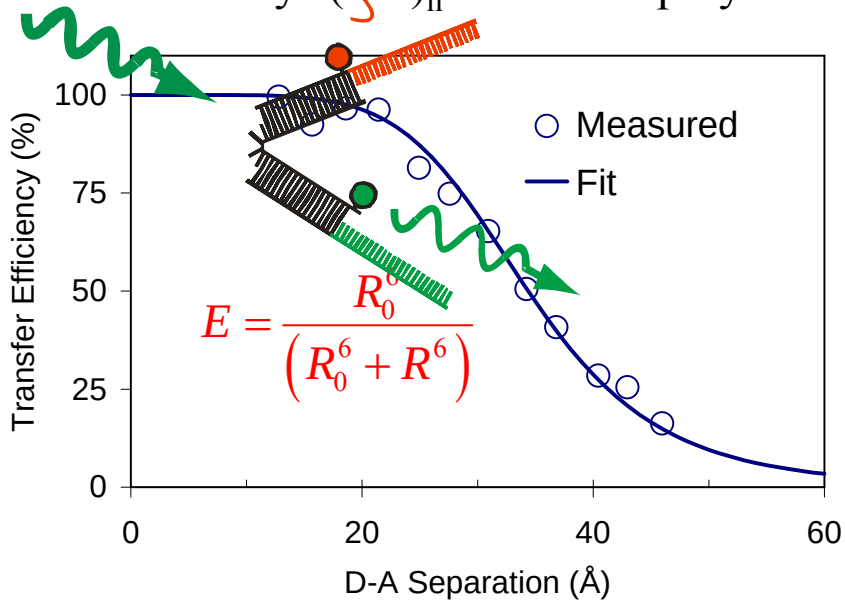
$$\kappa^2 = \left(\cos\theta_T - 3\cos\theta_D \cos\theta_A\right)^2$$





For flexible dyes averaged over all orientations

$\kappa^2 = 2/3$



$$E = \frac{R_0^6}{\left(R_0^6 + R^6\right)}$$

Data taken from: Stryer and Haugland (1967) *PNAS* **98**:719

Energy Transfer Efficiency

Energy Transfer Efficiency

$$E = \frac{k_T}{k_T + \sum_{i \neq T} k_i} = \frac{1}{1 + \left(R/R_0\right)^6}$$

$$= 1 - \frac{\tau_{DA}}{\tau_D}$$

Donor Lifetime

$$= \left(1 - \frac{F_{DA}}{F_D}\right)\frac{1}{f_A}$$

Donor Intensity

$$= \frac{\varepsilon_A(\lambda_{ex})}{\varepsilon_D(\lambda_{ex})}\left(\frac{F_{AD}}{F_A} - 1\right)\left(\frac{1}{f_D}\right)$$

Acceptor Intensity

$$= \frac{F_A}{F_A + \gamma F_D}$$

Donor and Acceptor Intensity

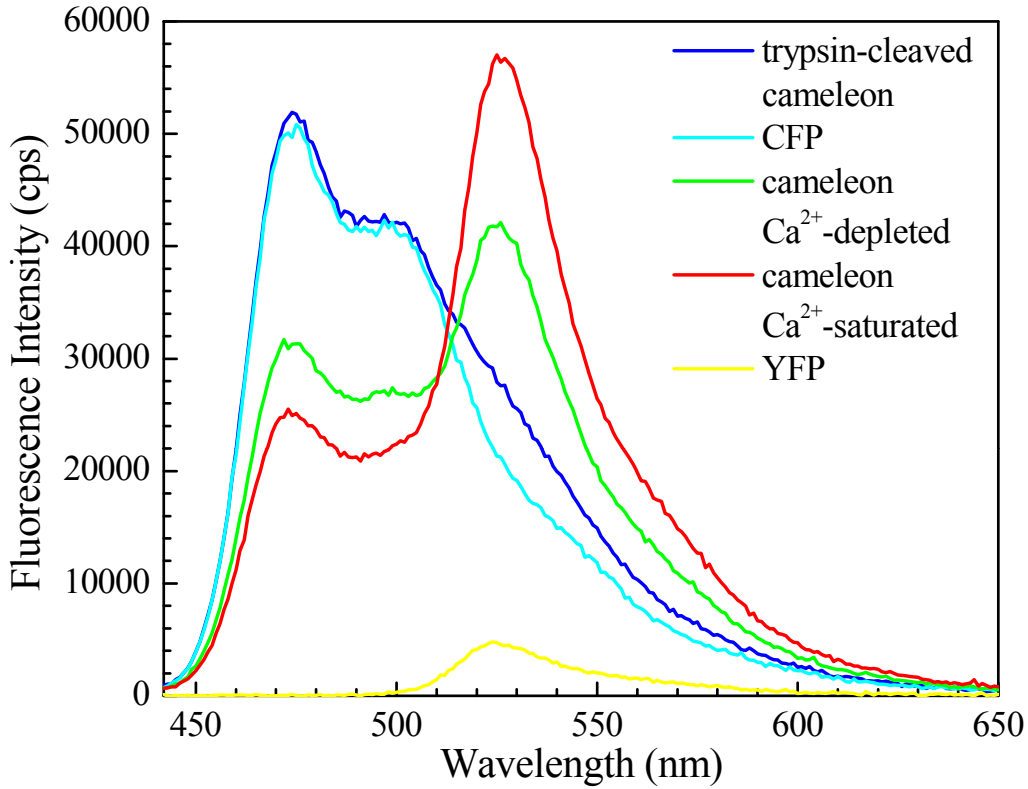
D-A Separation

$$R = R_0\left(\frac{1}{E} - 1\right)^{\frac{1}{6}}$$

$$= R_0\left(\frac{\tau_{DA}}{\tau_D - \tau_{DA}}\right)^{\frac{1}{6}}$$

$$= R_0\left(\frac{\gamma F_D}{F_A}\right)^{\frac{1}{6}}$$

FRET Detection



Summary of the FRET detection

- Quenching of the donor (intensity and lifetime)
- Increase of the acceptor fluorescence
- Decrease of the steady-state polarization

The Challenges of FLIM

FAB

- At every pixel there are contributions of several fluorescent species, each one could be multi-exponential.

- To make things worse, we can only collect light for a limited amount of time (100-200 microseconds per pixel) which result in about 500-1000 photons per pixel.

- This is barely enough to distinguish a double exponential from a single exponential decay.

- Resolving the decay at each pixel in multiple components involves fitting to a function, and is traditionally a complex computational task “for experts only”.

A major problem is **data analysis and interpretation**

The Major Issues with FLIM

FAB

- Rather difficult technique
- Fitting is slow
- Results depend on initial conditions
- Interpretation requires expertise

Can we avoid all these problems?

- No expertise necessary
- Instantaneous results
- Independent of initial choices
- Quantitative results
- Intuitive simple interface

The Laboratory for Fluorescence Dynamics (Enrico Gratton) at the University of California, Irvine proposed a change in paradigm:

Use a different representation of the decay where each molecular species has its own unique representation and where each process (FRET, ion concentration changes) can be easily identified.

We need to go to a new “space”

Calculating the S and G Components of the Phasor

FAB

$$g_i(\omega) = \int_0^\infty I(t)\cos(\omega t)dt \,/\, \int_0^\infty I(t)dt$$

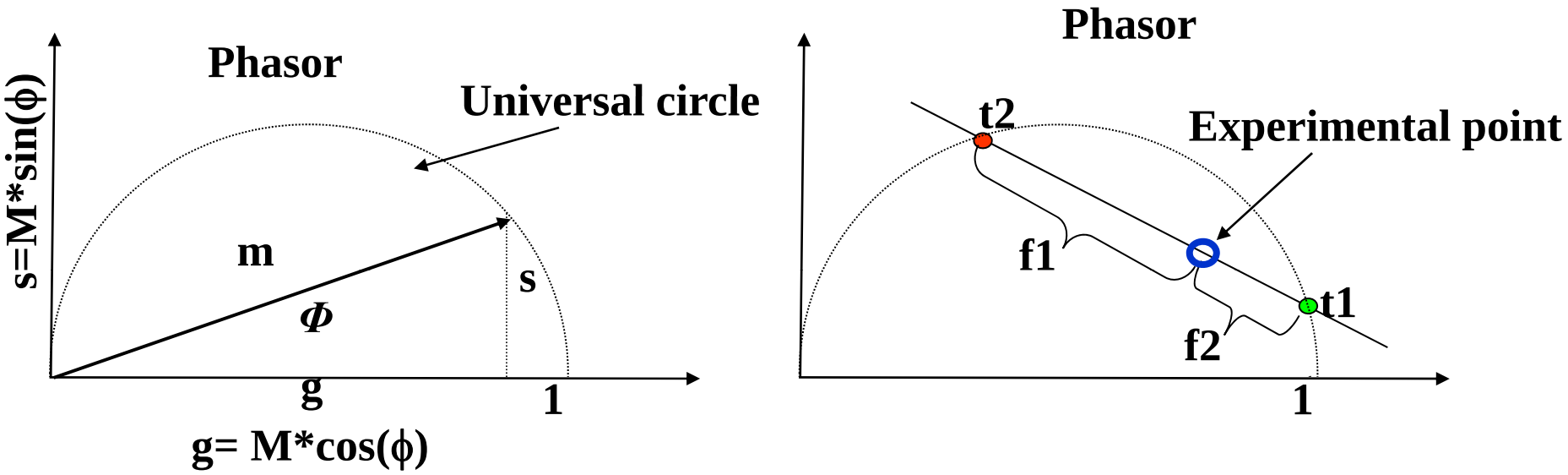
$$s_i(\omega) = \int_0^\infty I(t)\sin(\omega t)dt \,/\, \int_0^\infty I(t)dt$$

Time-domain

components of a phasor. I(t)
is measured

Note that I(t) can contain raw multiexponential data!!

Phasor Algebra

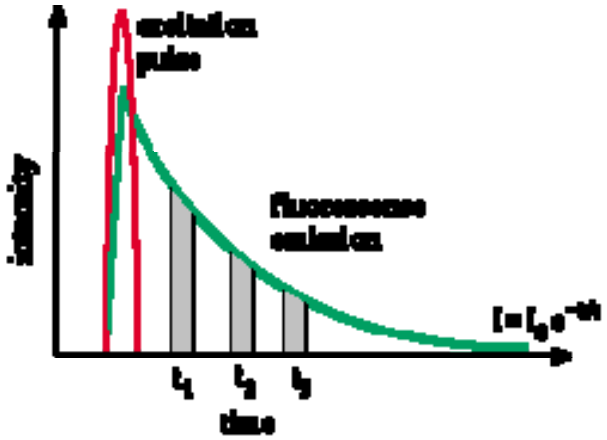


Simple rules to the Phasor plot:

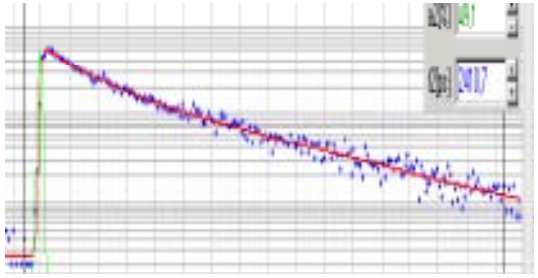
1) All single exponential lifetimes lie on the “universal circle”
2) Multi-exponential lifetimes are a linear combination of their components
3) The ratio of the linear combination determines the fraction of the components

Different Approaches, Same Information

From decay data

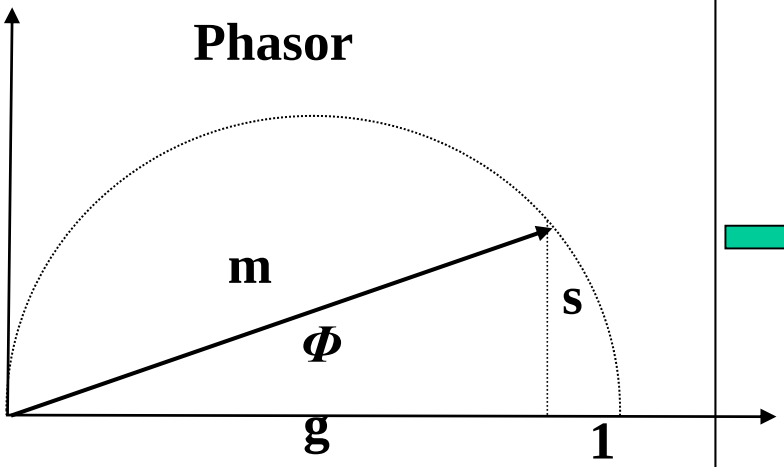


Fitting of exponentials

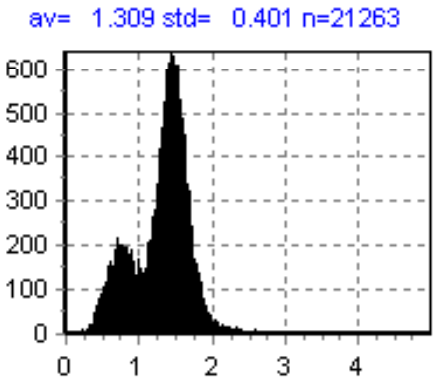


Multiexponential analysis

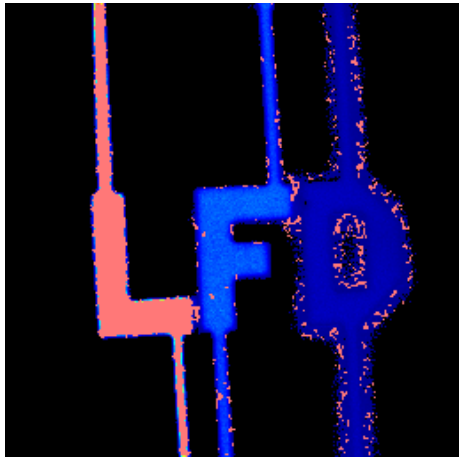
Phasor

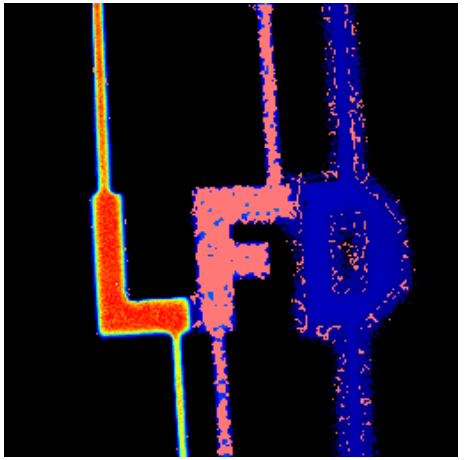


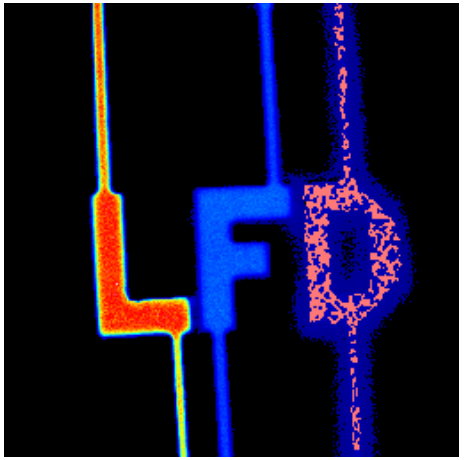
Lifetime distribution

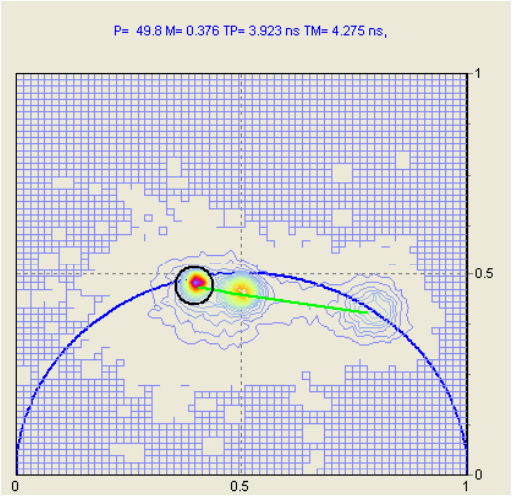


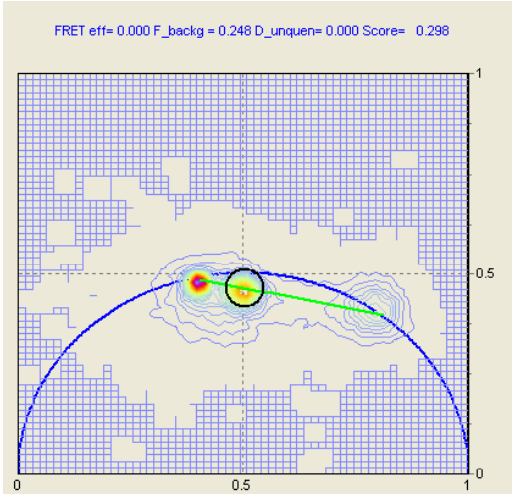
Separating Different Lifetime Components

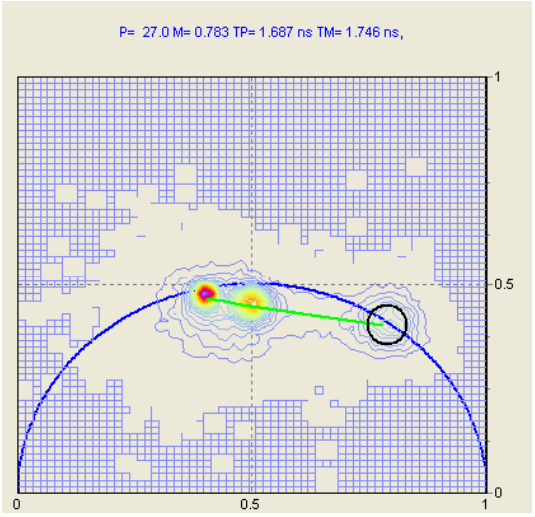






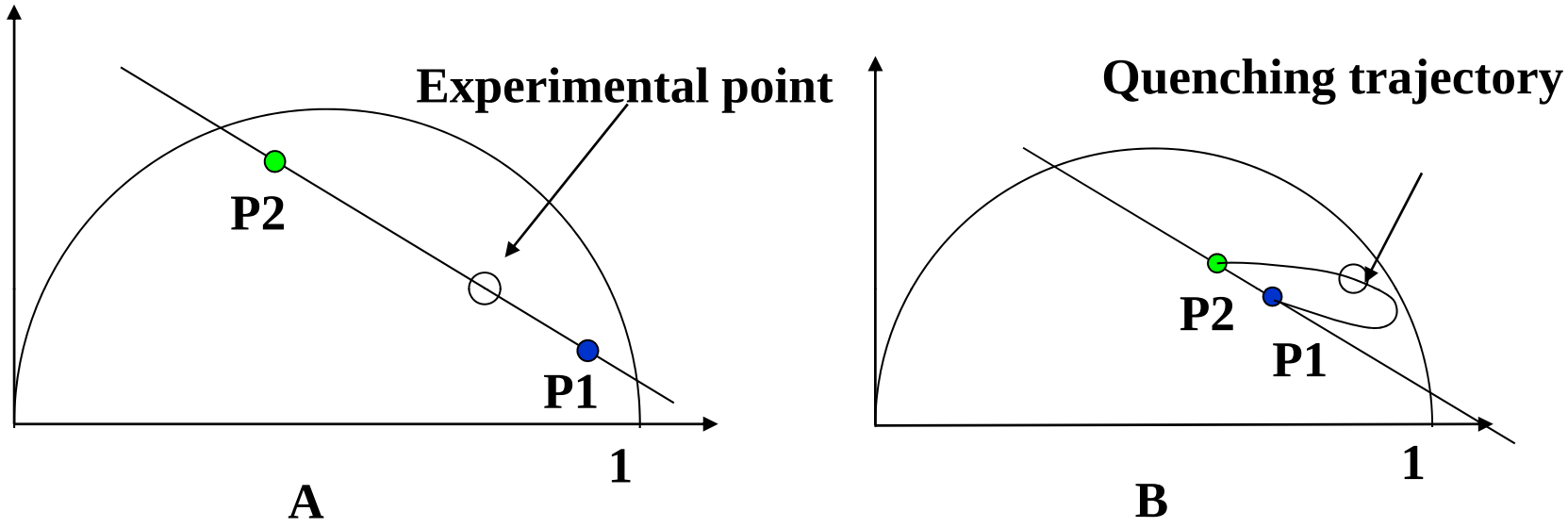






Fluorescein **Mixture** **Rhodamine B1**

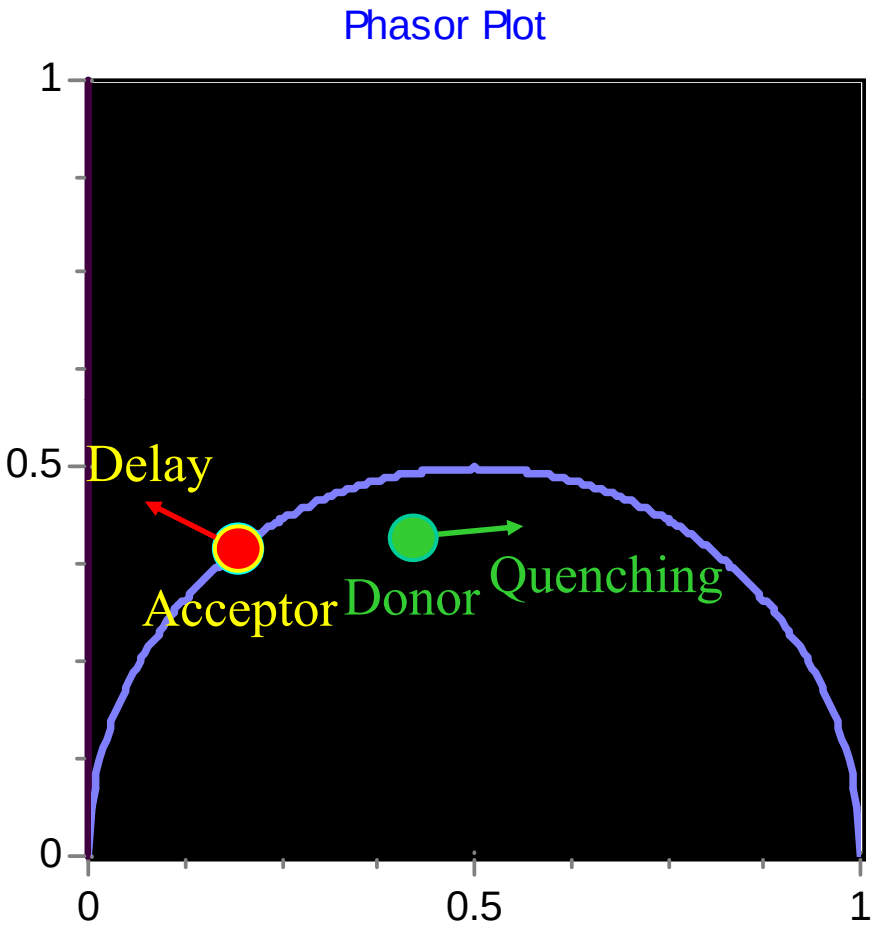
Distinguishing multi-exponential Components from FRET



Simple Rules for FRET:

1) If the experimental point lies on a straight line then it is **NOT** FRET
2) FRET efficiencies follow a “quenching trajectory”
3) Quantitative FRET efficiencies can be obtained from the position on the quenching trajectory

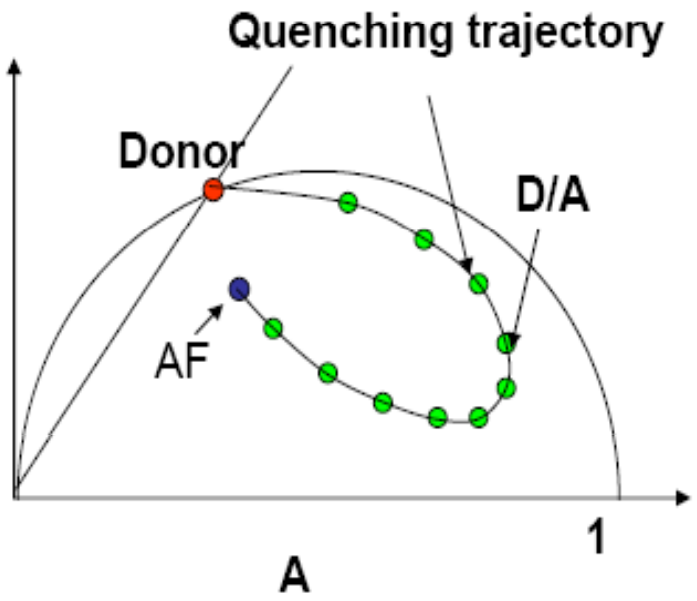
Distinguishing multi-exponential Components from FRET

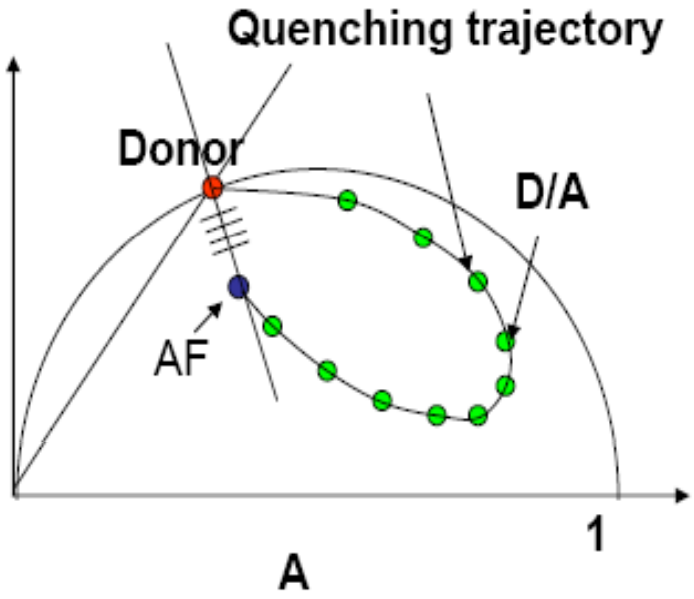


Delay of the excitation of the acceptor due to FRET moves the acceptor phasor to the left (yellow arrow). If the delay is sufficiently long, the phasor could fall outside the semicircle. The donor phasor moves to the right (red arrow) due to quenching (shorter lifetime).

Understanding Phasor Plots

After quenching all the Donor fluorescence, what is left?
Cellular Autofluorescence





As the lifetime of the Donor is quenched, the phasor of the quenched Donor is added to the phasor of the autofluorescence

If there is a fraction of Donor that cannot be quenched, the final point will be along the line joining the Donor with the autofluorescence phasor

Features of the Phasor Approach

Many of the obstacles in FLIM data analysis can be removed.

The accuracy of lifetime determination is improved .

The speed of data analysis is reduced to almost instantaneous for an entire image or several images.

The analysis is “global” over the image and across images.

The interpretation of the FLIM experiment is straightforward. Minimal prior spectroscopy knowledge is needed.

The Phasor analysis method can be applied to all modes of data acquisition (frequency-domain and time-domain).

Quantitative Analysis can be performed.

Conclusions

Fluorescence microscopy provides a powerful means of investigating processes in living cells.

Multiple fluorescence microscopy methods exists with various advantages and disadvantages.

FLIM can provide information over FRET, quenching

The phasor approach reduces the problem of fitting exponential components to the exploration of regions of the phasor plot by representing “molecular species” rather than a sum of exponential decays,

The analysis of the trajectories in the phasor plot provides a quantitative resolution of “processes” such as linear combination of two (or more species) and the calculation of FRET efficiency to a simple arithmetic.

You don’t need to be an expert spectroscopist

A global analyze of many cells (or experiments) can be performed simultaneously