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Fluorescence Lifetime Imaging (FLIM)

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Exton, PA

What is FLIM?

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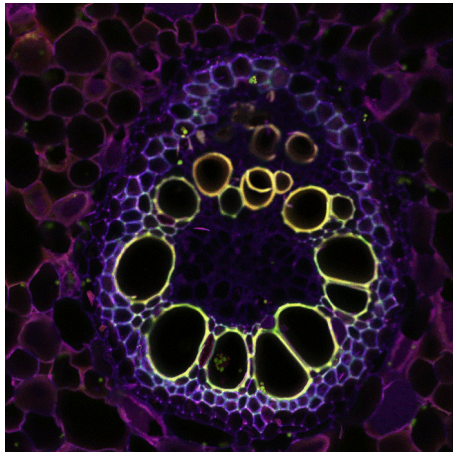
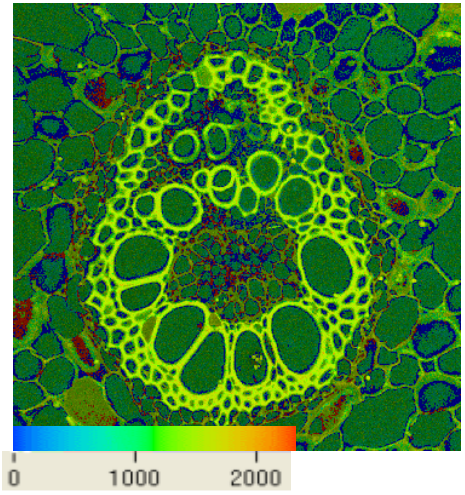
Fluorescence **L**ifetime **I**maging **M**icroscopy – **FLIM**

- Fluorescence based method
- Analysis of the lifetime of the excited state of fluorescent molecules
- Combination of this analysis with imaging
  - ⇒ Spatially resolved distribution of fluorescent lifetimes
  - ⇒ Additional information



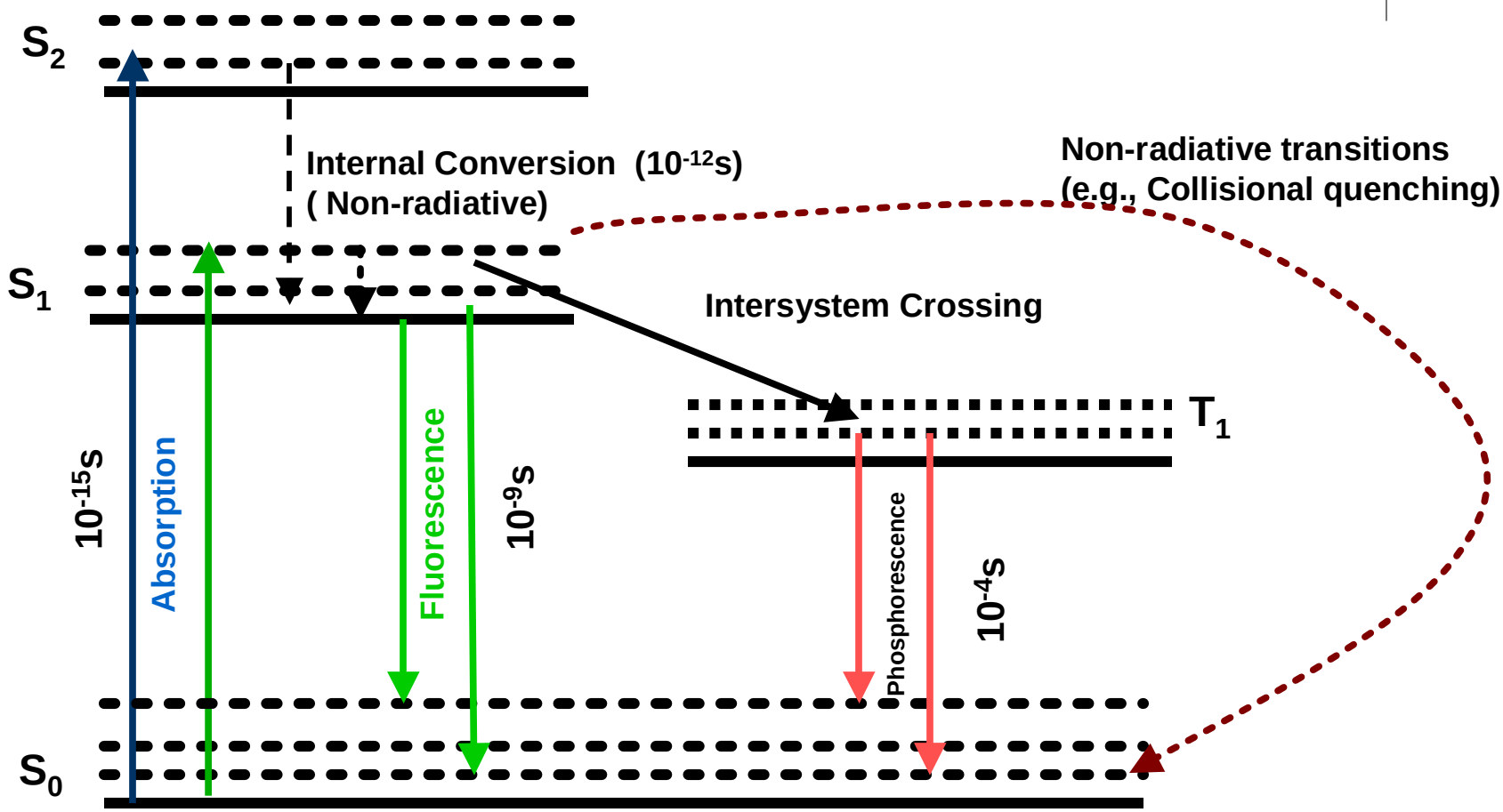
Conventional confocal intensity image



Fluorescence lifetime image /ps

Sample: Prionium, stained with Safranin and Fast green

Excitation-emission cycle and Fluorescence Lifetime



Jablonski Diagram

Excitation-emission cycle and Fluorescence Lifetime

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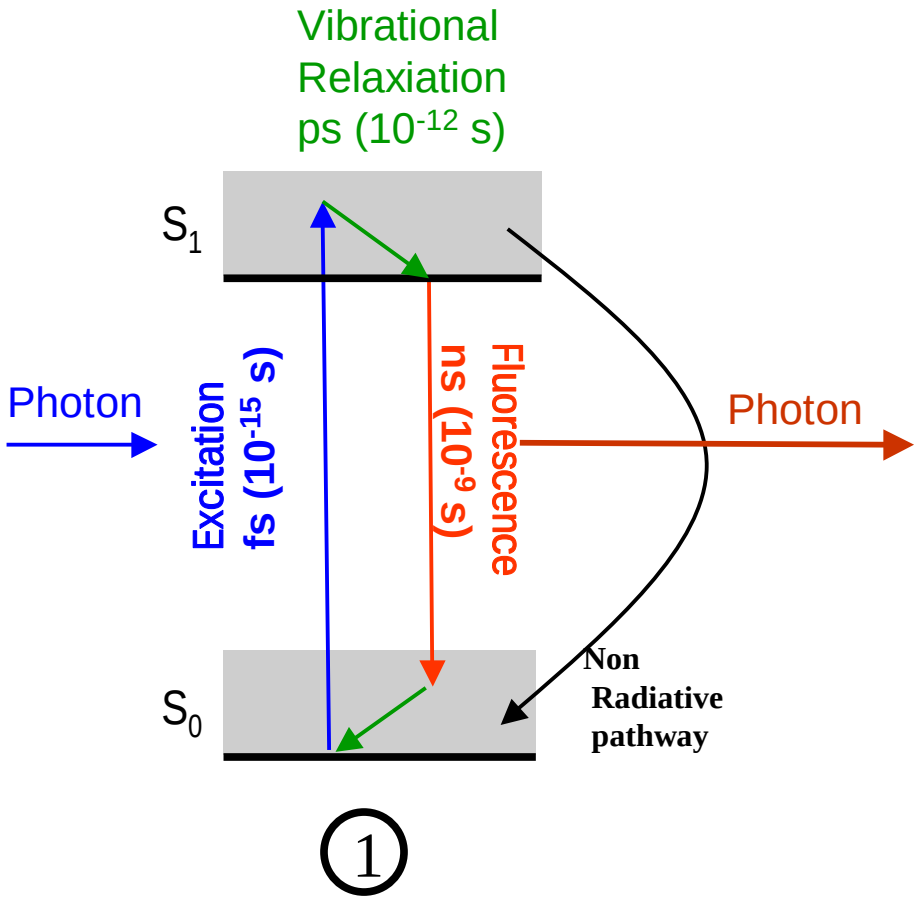

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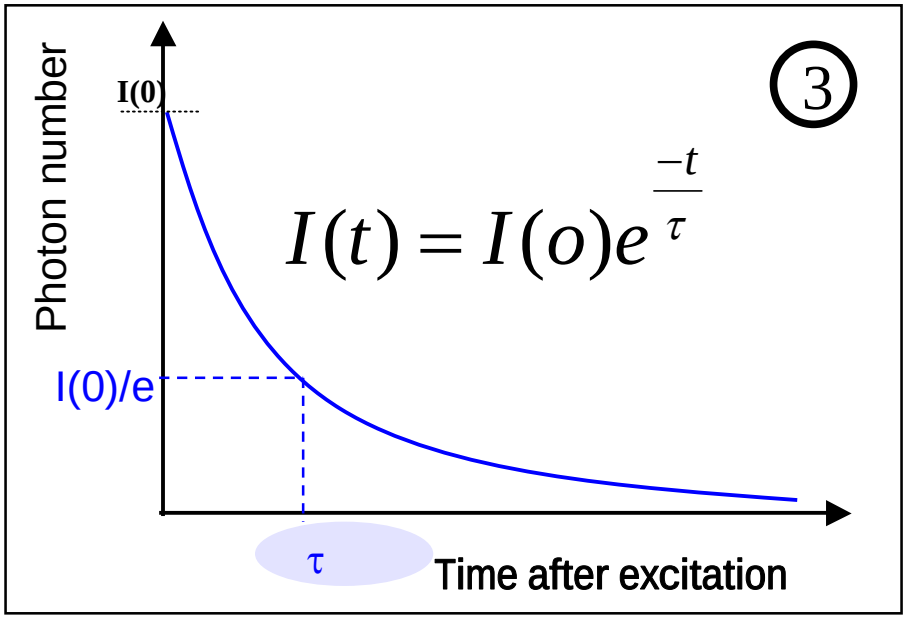

There is a distribution of times associated with the return of an electron to the ground state and the emission of a photon.

Fluorescence Lifetime: Time Domain Measurements

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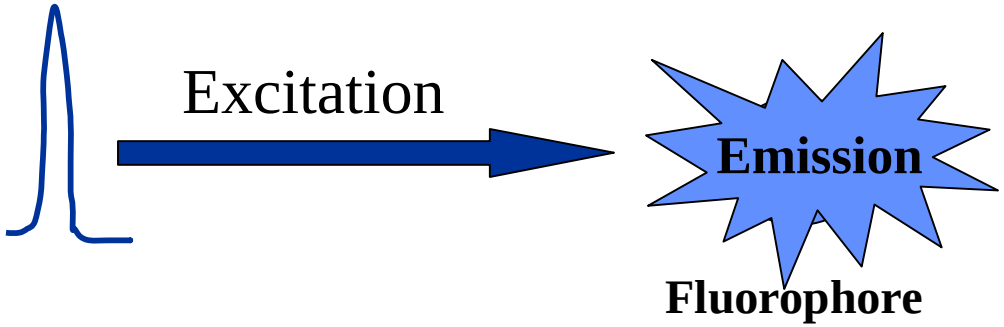
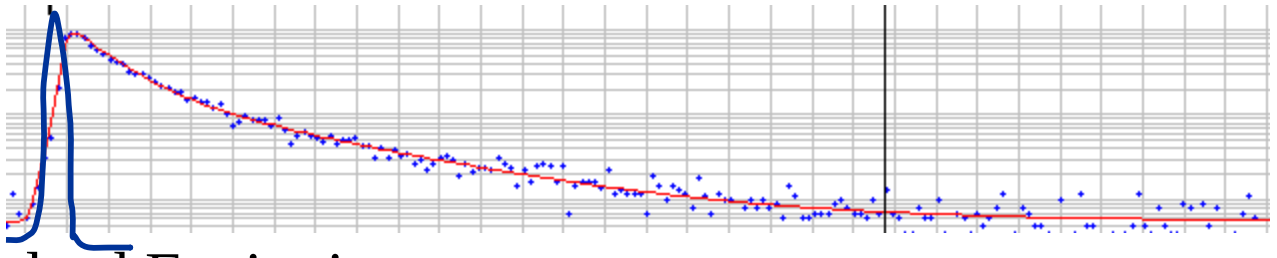


Excitation pulse width should be shorter than fluorescent lifetime. Typical pulse widths < 10 ps



Pulsed Excitation

Examples for time domain measurement techniques

- Time Correlated Single Photon Counting (TCSPC)
- Streak Camera Measurements

Fluorescence Lifetime: Frequency Domain Measurements

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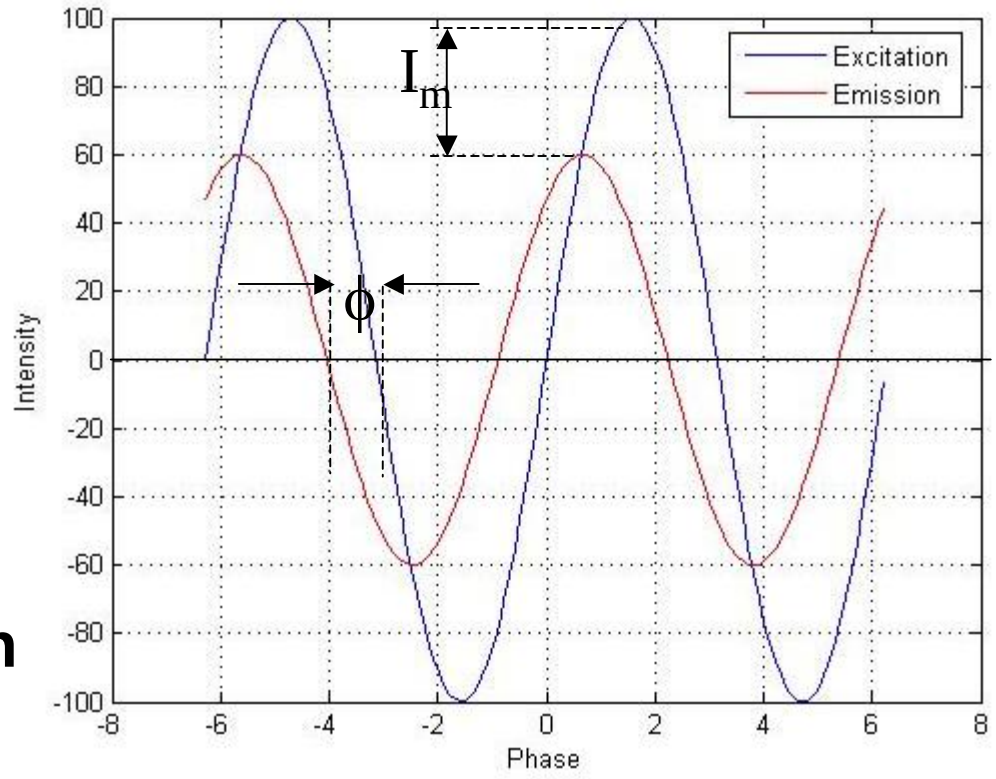
- **Sample excited with intensity modulated light**
- **Intensity of light is varied at high frequency.**
- **Emission delayed relative to the excitation – measured in phase shift (ϕ).**



Commonly used with wide-field imaging techniques

Fluorescence Lifetime: TCSPC Measurements

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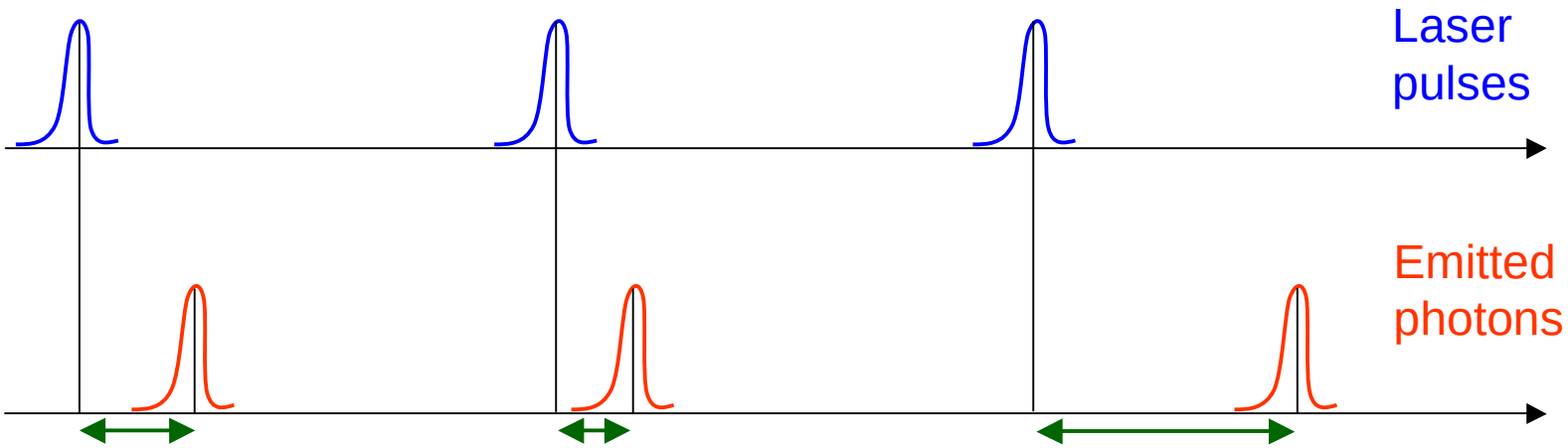
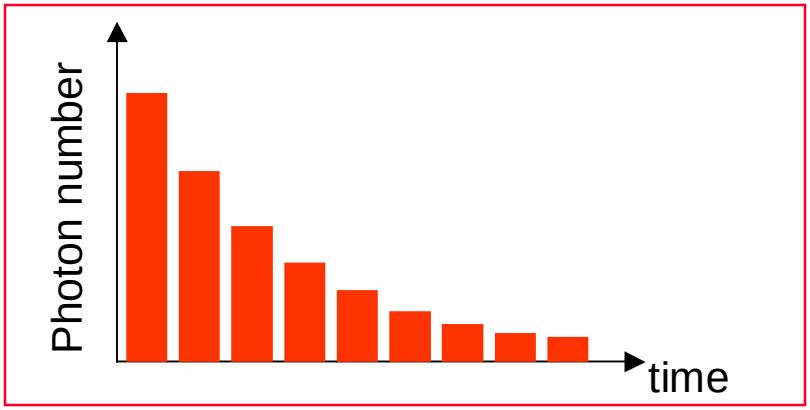
- excitation with a pulsed laser



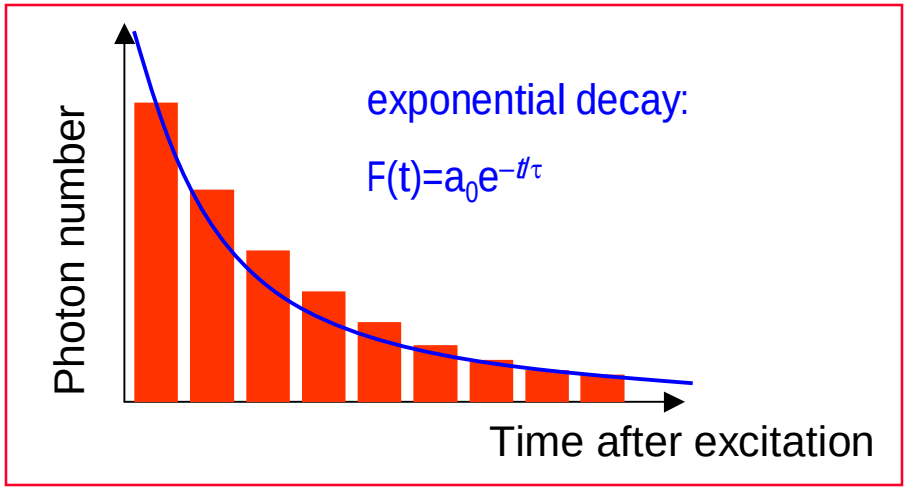
- measuring the time between laser pulse and fluorescence photon
- calculation of a histogram of numbers of photons over time after laser pulse (lifetime decay curve)

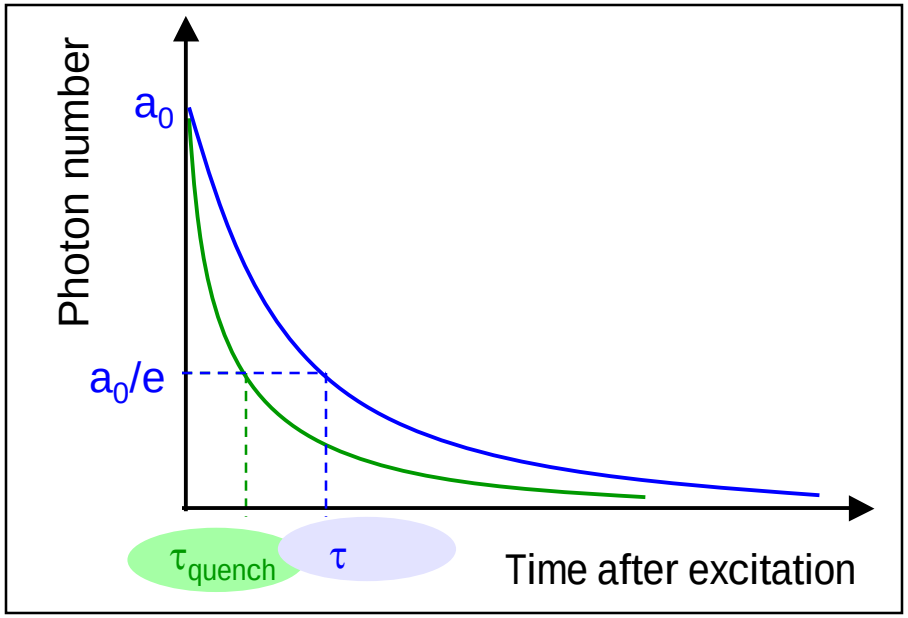


+ good time resolution!
+ high sensitivity!

Lifetime measurement: TCSPC

Time **C**orrelated **S**ingle **P**hoton **C**ounting





Data analysis

- Fit of an exponenetial curve to the histogram
- fit parameter of the curve: amplitude (number of photons at t=0) and time constant τ (fluorescence lifetime)

τ - fluorescence lifetime:

- time at which amplitude a_0 of the fit curve decays to a_0/e ($e \cong 2.3$)
- average time between excitation and emission
- characteristic property of dyes, usually in ns range
- depends on environment (ions, pH, O_2 …)

Fluorescence Lifetime : Dye Mixture

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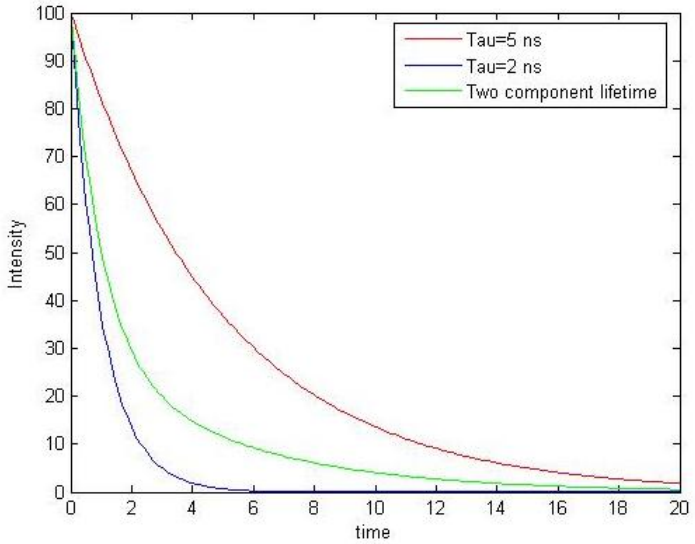
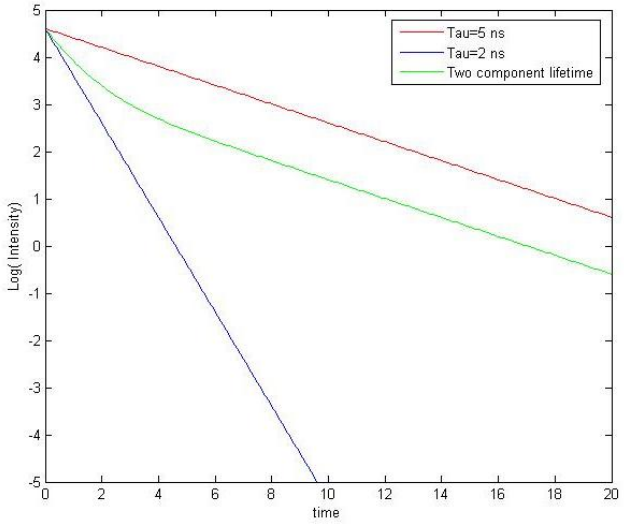
Dye A: Lifetime τ_1 =5ns

$$I1 = I0.\exp\left(\frac{-t}{\tau_1}\right)$$

Dye B: Lifetime τ_2 =2ns

$$I2 = I0.\exp\left(\frac{-t}{\tau_2}\right)$$





30% Dye A + 70% Dye B

$$I_{mix} = 0.3\exp\left(\frac{-t}{\tau_1}\right) + 0.7.\exp\left(\frac{-t}{\tau_2}\right)$$

FLIM – Fluorescence Lifetime Imaging

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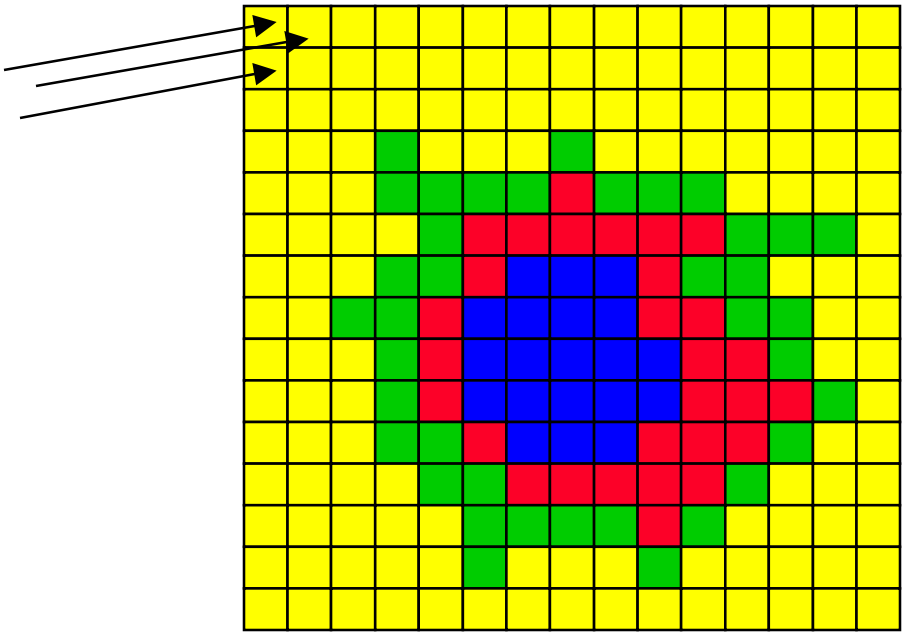
FLIM: Lifetimes are measured at each pixel and displayed as color contrast.

It combines information about spatial distribution of a fluorescent molecule together with information about its microenvironment (pH, etc).

In this way an extra dimension of information is obtained.

Imaging modes: wide-field, confocal, multiphoton



Fluorescence lifetime image

Wavelength -color lock-up table

τ_1 τ_2 τ_3 τ_4

Steps in Fluorescence lifetime imaging (FLIM)

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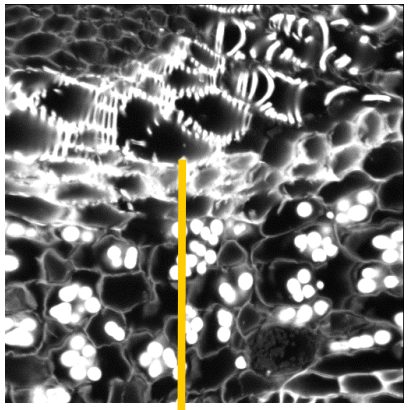
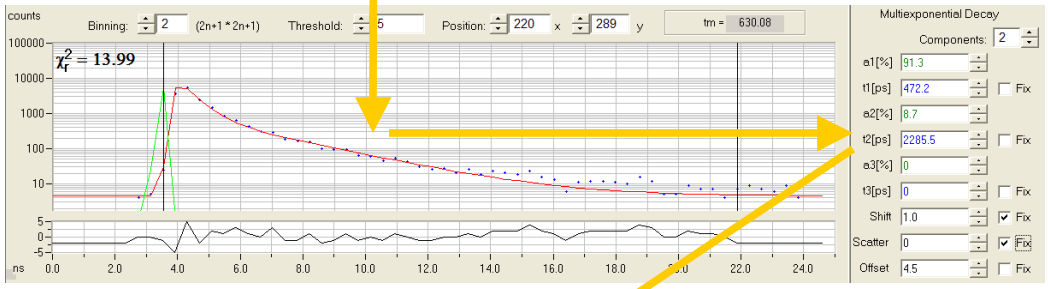
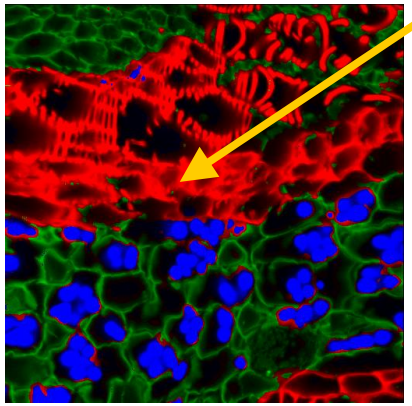
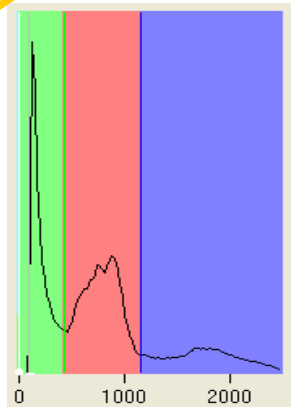


Intensity image





Lifetime image



Lifetime distribution/ ps

1) Data aquisition: measurement of lifetime decay curves with spatial resolution

2) exponential fit of decay curves in each pixel, calculate fluorescence lifetime in each pixel

3) transformation of fluorescence lifetimes in color code

Sample: Prionium, stained with Fast green, Safranin orange, and autofluorescence

Spatial resolution of FLIM images

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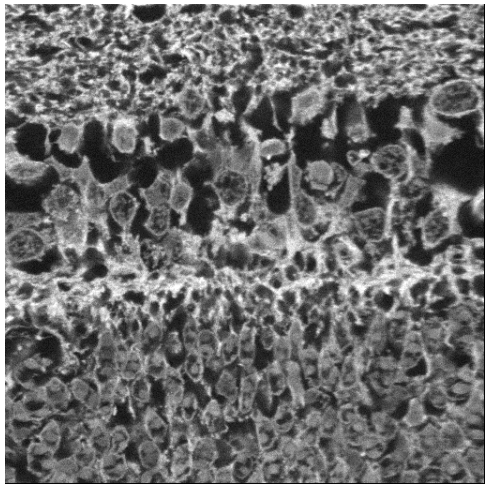
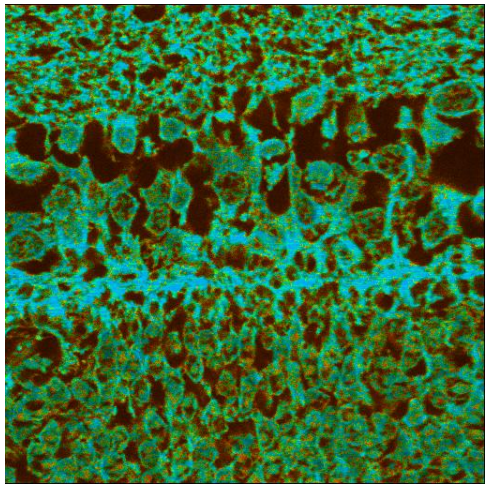
Resolution of FLIM image acquired in TCSPC Mode using PMTs (Leica SP2 D FLIM) equals conventional confocal intensity imaging.

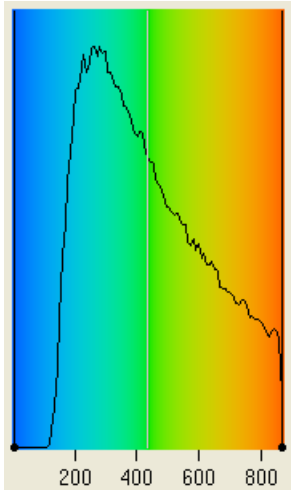
Resolution using image intensifiers+CCD camera is much lower (not shown here).



Intensity image



Lifetime image

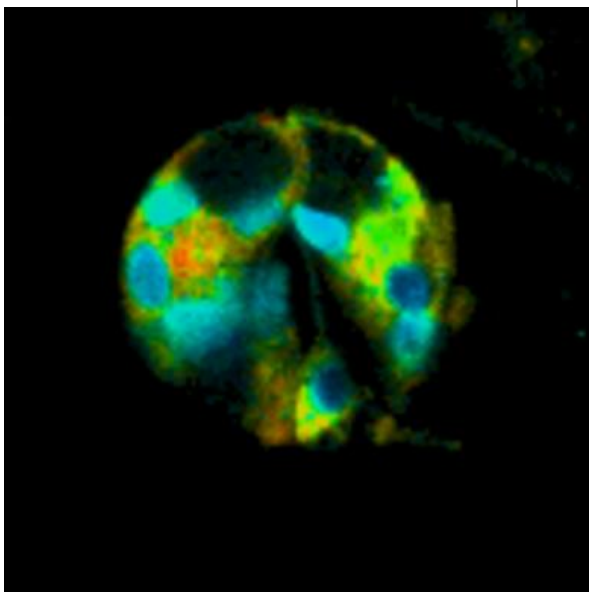


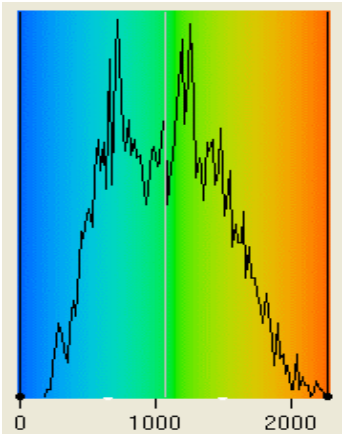
Lifetime distribution /ps

Sample: Cat retina, Azan-staining

Types of fluorescent markers

- auto fluorescence: NADH, Flavins, chlorophyll
- fluorescent proteins (CFP, GFP, YFP, ..)
- Fluorescent markers bound to antibodies (FITC, ..)
- Ion indicators e.g. Calcium, Sodium, pH (Fluo-3, Na-green, Oregon Green, DM-NERF, CI-NERF ..)





Lifetime distribution/ ps

Lifetime image of guard cell:

Expression of yellow chameleon (ECFP, Calmodulin, M13 and EYFP) and autofluorescence (chloroplasts in blue)
Excitation @ 405 nm

Courtesy:
Xiaodong Xie, Dept. of Biol. Sciences, Lancaster University

Leica FLIM: Main components

FLIM Components

- Pulsed Laser
- Detector working in single photon counting mode with high time resolution, good quantum efficiency and low noise
- In Computer: High performance counting card for data collection and processing

Leica setup

- pulsed IR laser **(MP FLIM)** and/or pulsed PQ laser diode 405 nm **(D FLIM)**
- Up to 2 spectral (internal) FLIM detectors from Hamamatsu
- SP C 830 from B&H in separate PC

Leica MP FLIM – Available lasers for MP excitation:

- Coherent: Chameleon (repetition rate 90 MHz=pulse distance of 11.1 ns), Mira
- Spectra Physics: Mai Tai (repetition rate 80 MHz=pulse distance of 12.5 ns), Tsunami

Leica FLIM 2 using Spectral FLIM detector, SPC-830 countercard and software

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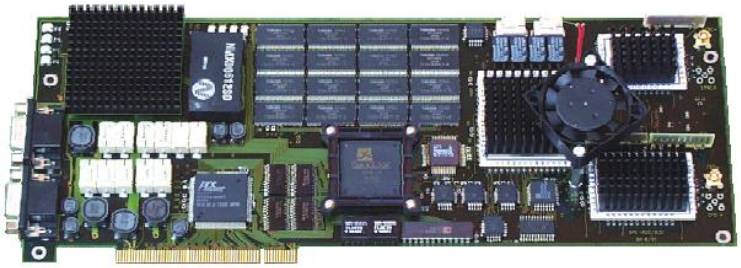
•Internal FLIM detector : Hand selected Hamamatsu R7400-U01 PMTs

•Counterbord SPC 830:

- compact PCI board with <10 ps resolution
  including software for online data acquisition, processing, and evaluation
- SPC 730: 8 MB histogram memory
  (@ 256 x 256 pixels: 64 time channels for each pixel)
- SPC 830: 32 MB histogram memory
  (@ 512 x 512 pixels: 64 time channels for each pixel)
- count rates up to 4 MHz



Leica FLIM: lasers

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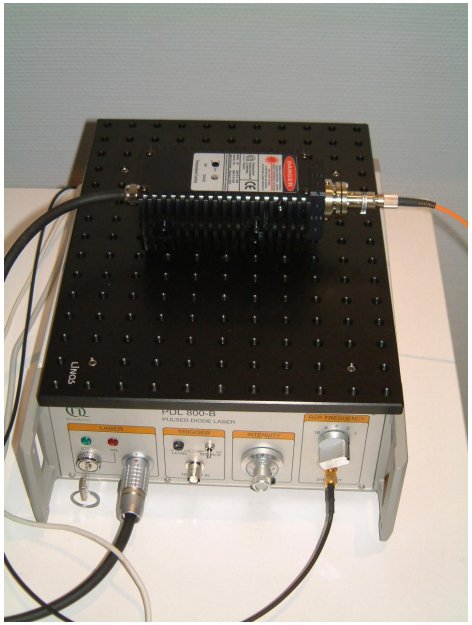
- **MP FLIM – multiphoton excitation using tunable IR lasers**
  - Coherent:
    Chameleon (repetition rate 90 MHz=pulse distance of 11.1 ns), Mira
  - Spectra Physics:
    Mai Tai (repetition rate 80 MHz=pulse distance of 12.5 ns), Tsunami

- **D FLIM – pulsed diode excitation:**
  - pulsed 405 nm laser diode from PicoQuant
  - pulse length: < 70 ps @ 1 mW average power
  - maximum average power: > 3 mW
  - peak power: a few 100 mW
  - repetition rate: 40, 20, 10, 5, 2.5 MHz
    = pulse distance: 25, 50, 100, 200, 400 ns



Leica FLIM: comparison of advantages of MP and D FLIM

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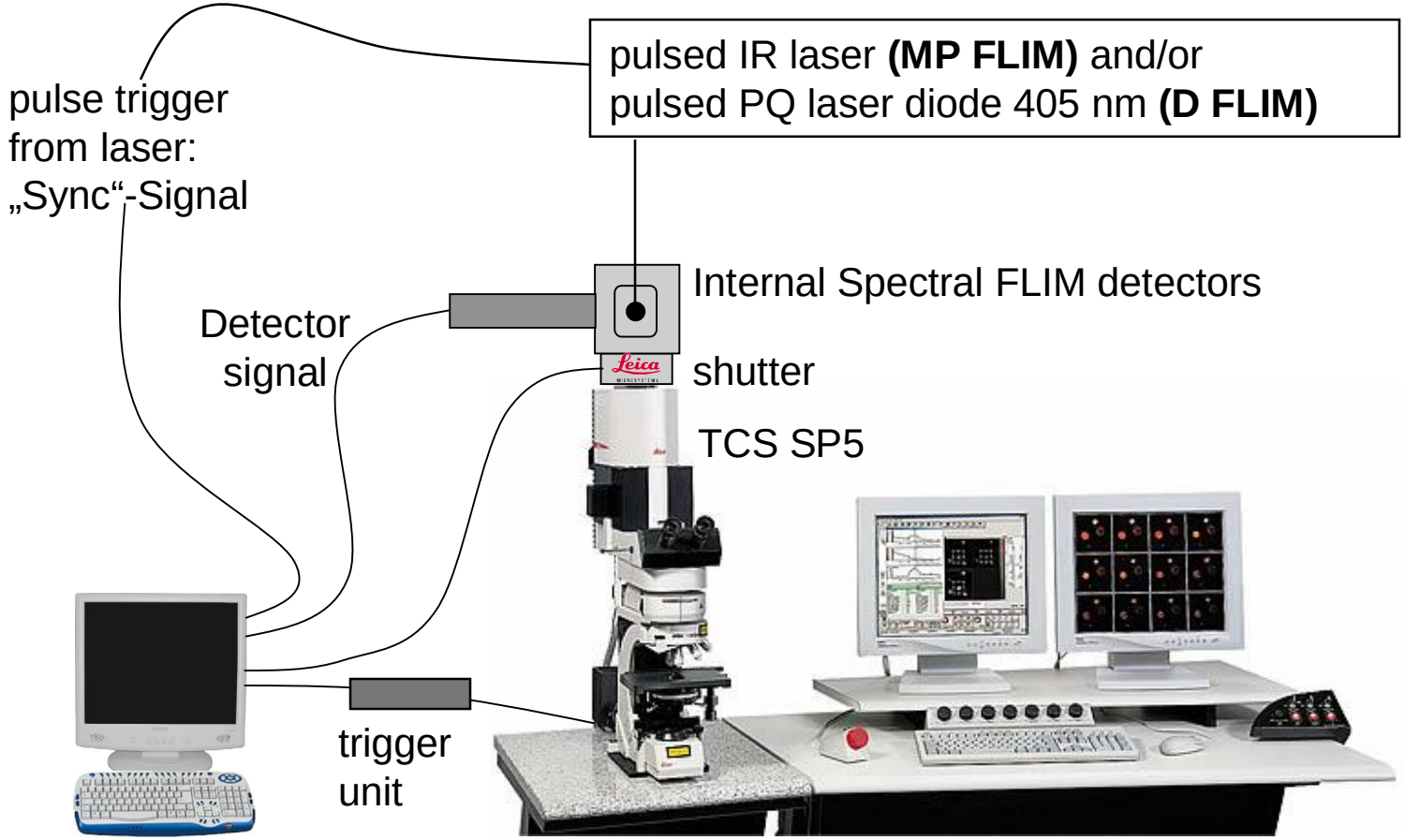
- **MP FLIM – multiphoton excitation using tunable IR lasers**
  - tunable excitation wavelength => variety of dyes observable
  - deep tissue penetration
  - no out of focus bleaching
  - laser can be used for regular MP intensity imaging
- **D FLIM – pulsed diode excitation:**
  - Different repetition rates available (2.5 to 40 MHz) => also longer lifetimes can be observed without “pile-up” effect => adaptation to different dyes
  - no 3 photon excitation => no excitation of DNA, NADH, … => less photo damage in living cells (important in long term experiments)
  - dyes can be observed that are not excitable with MP (like cy-dyes)
  - laser more affordable, can be used for regular intensity imaging and photoactivation, ROI and beampark funcktion available

Both, MP and D-FLIM can be attached to one system!

TCSPC FLIM: Hardware

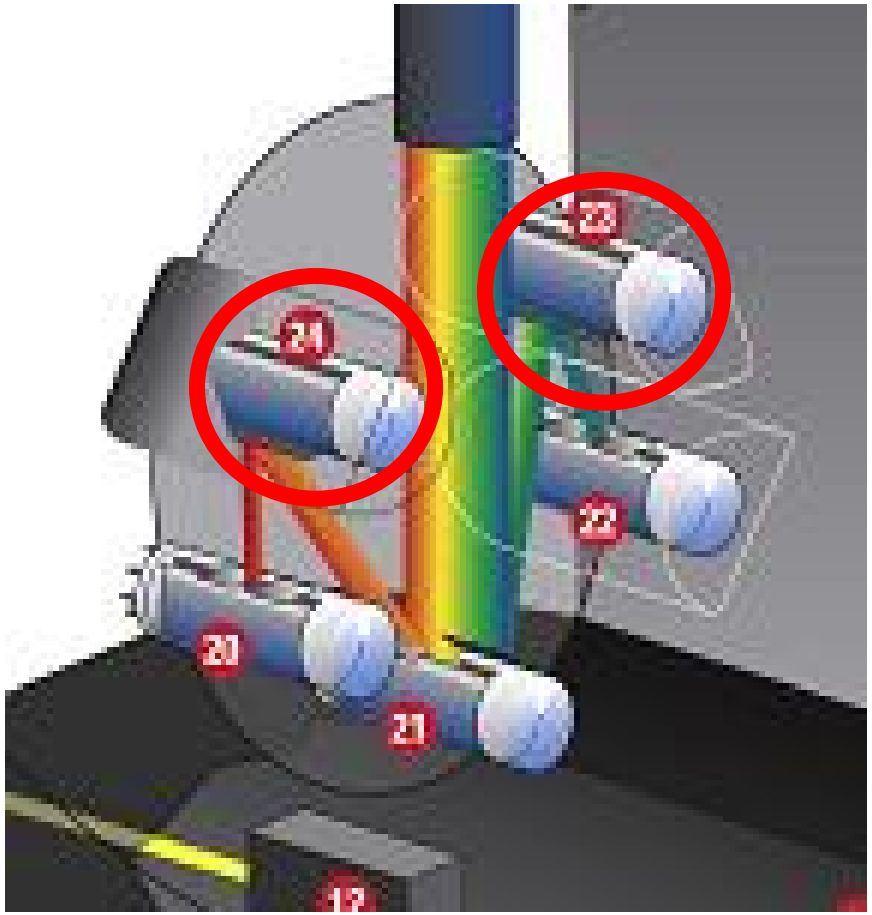


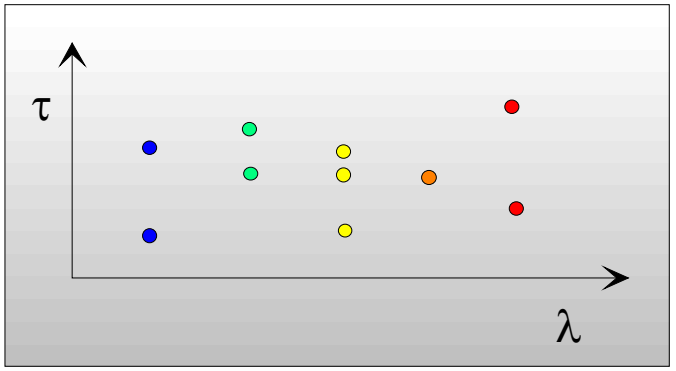
B&H FLIM hardware and
software on a separate PC

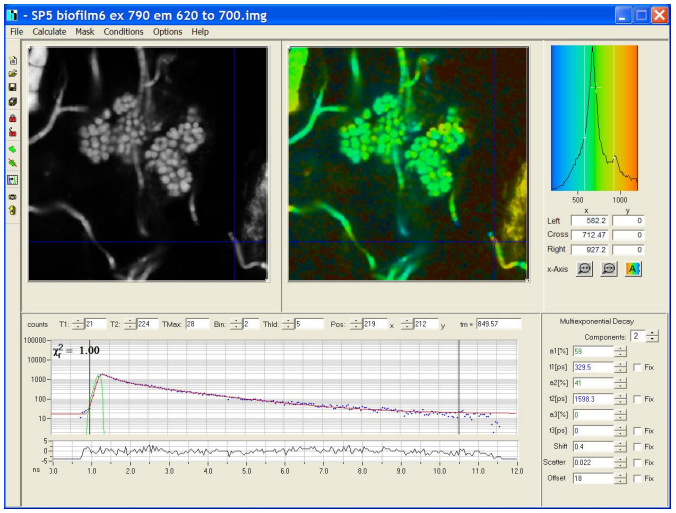
from SP5 scanhead to SPC830:
trigger output (frame, line & pixel clock)

Spectral (internal) FLIM with one or two channels

Fast Detectors for Spectral FLIM: Lifetime-Wavelength Recording







Leica FLIM: Advantages of spectral (internal) FLIM

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- Freedom and flexibility in choice of spectral range for FLIM (filter-free FLIM). Optimal adjustment to experimental conditions, removal of autofluorescence by selection of spectral detection range.
- Allows to measure lifetime over wavelength and thus to separate populations with same lifetime by spectrum or with same spectrum by lifetime.
- Increase in sensitivity in FRET analysis by simultaneous observation of donor and acceptor. Increase in sensitivity in FRET analysis by simultaneous observation of donor and acceptor.
- The FLIM detectors can be used for regular intensity imaging as well => cost efficiency. Quantum efficiency of internal FLIM detectors is slightly lower compared to “normal” detectors.

Leica FLIM: Becker & Hickl SPC-830 acquistion package: SPCM

- On separate PC, independent from Leica TCS SP2
- 2 program packages:
  1) SPCM: Image acquisition, Set parameters of SPC 730/830
  2) SPCImage: Data analysis
     - Fluorescence decay curve for each single pixel
     - Single or multiple exponential fit to each point
     - Intensity and lifetime image

Leica FLIM: Becker & Hickl SPC-830 acquistion package: SPCM

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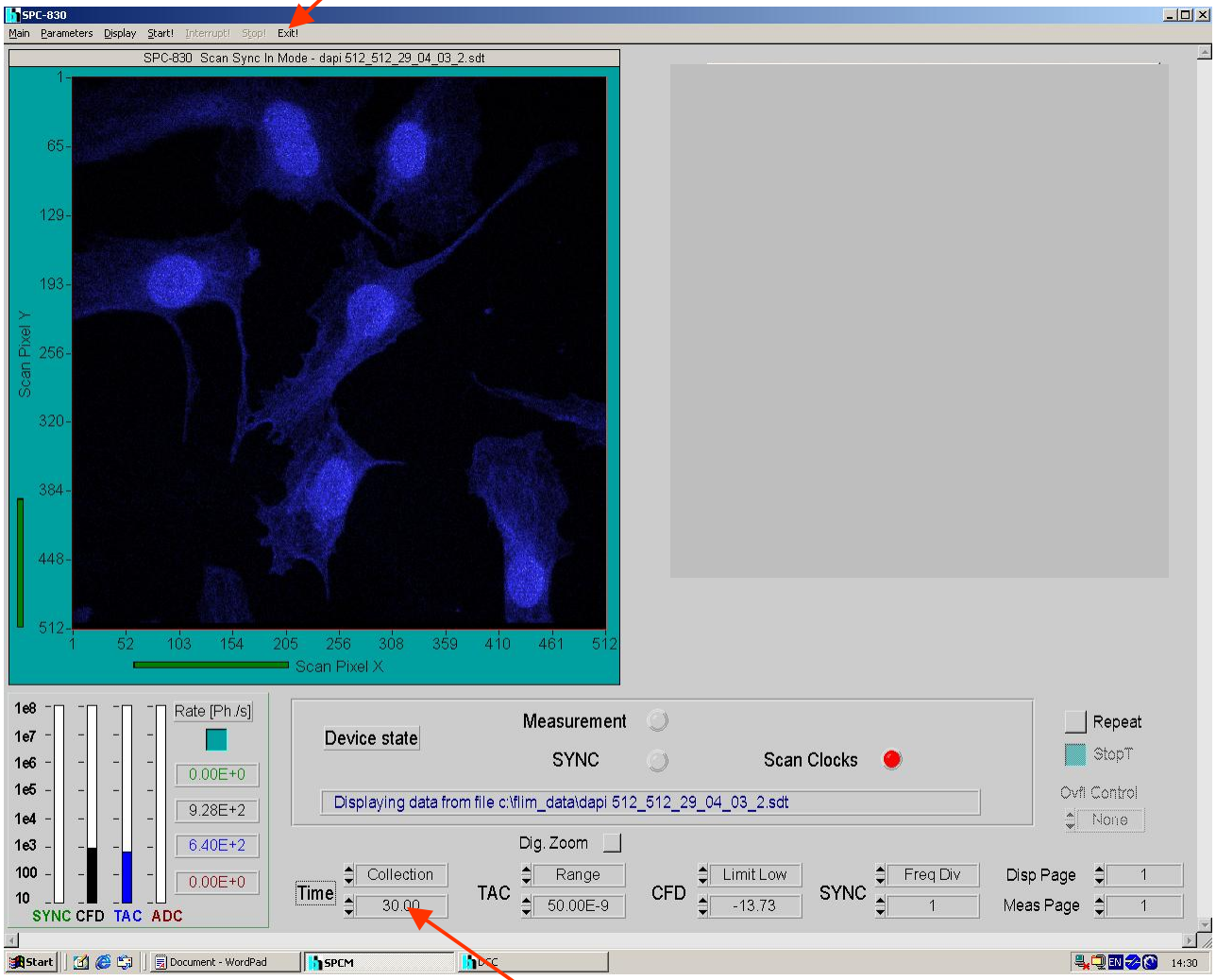
Start measurement



Measurement time

Haridas Pudavar, PhD, Application and Technology Support, Leica Microsystems Inc. 10/25/09

Leica FLIM: Becker & Hickl SPC-830 acquistion package: SPCM

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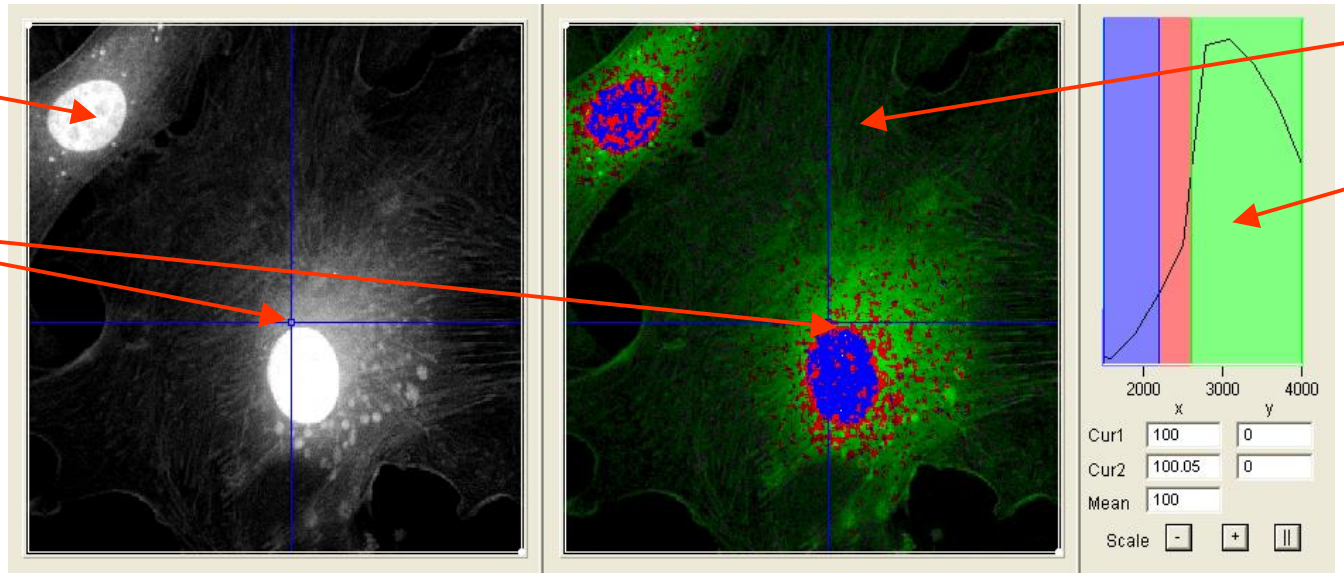

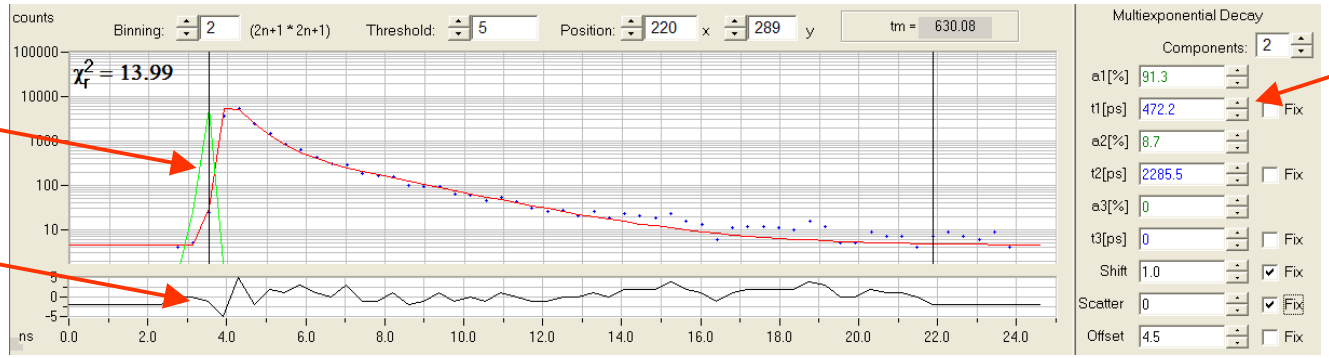

Leica FLIM: Becker & Hickl SPC-830 acquistion package: SPCM

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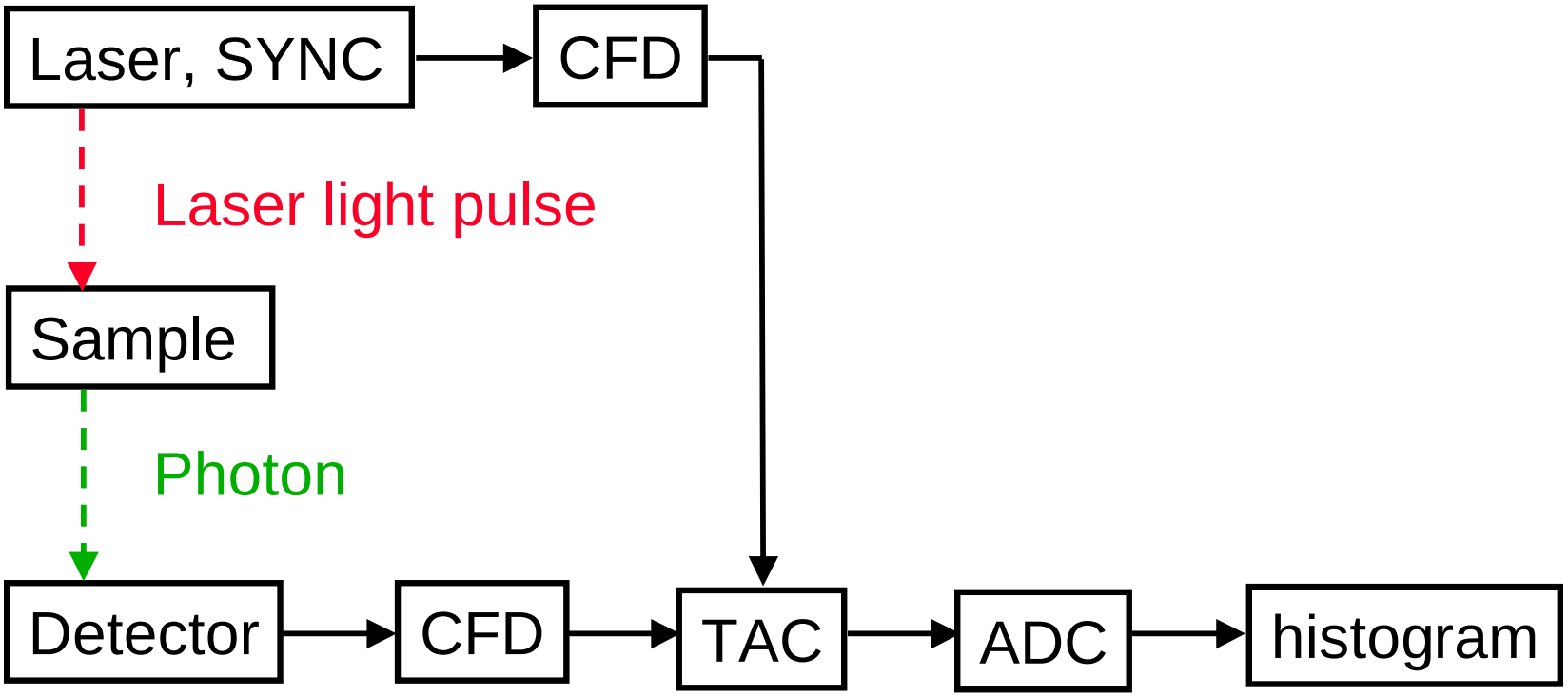
What means SYNC, CFD, TAC, and ADC in the B&H software SPCM?



Leica FLIM: Becker & Hickl SPC-830 acquistion package: SPCM

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What means SYNC, CFD, TAC, and ADC in the B&H software SPCM?

	Full Name	Function	Meaning of bar display
SYNC	**Sync**hronization signal	Signal from the laser when a laser pulse is generated	Shows average pulse rate during scanning, due to blanking number is smaller than real laser repetition rate
CFD	**C**onstant **F**raction **D**iscriminator	Discriminates electronic background noise from signal of photons and removes the noise, removes temporal jitter of detector pulses	Shows number of all events recognized by the cards as being photons
TAC	**T**ime to **A**mplitude **C**onverter	Measures the time between photon and laser pulse and converts it into a voltage amplitude	Shows number of all photons that can be related to a laser pulse
ADC	**A**nalogue to **D**igital **C**onverter	Converts the analogues voltage signal into a digital signal (needed to build up the histogram)	Shows number of all photons that can be also addressed to an xy position in the image

Leica FLIM: Becker & Hickl SPC-830 acquistion package: SPCM

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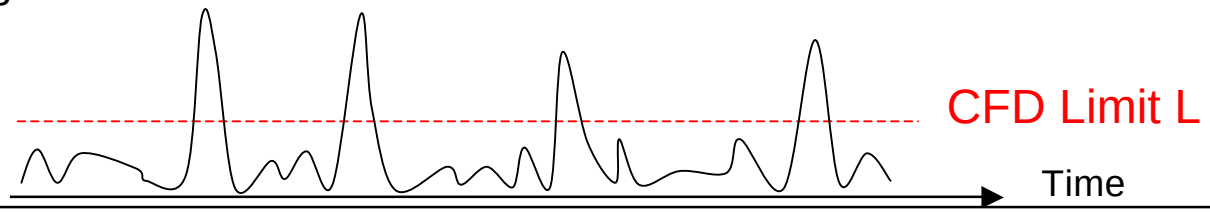
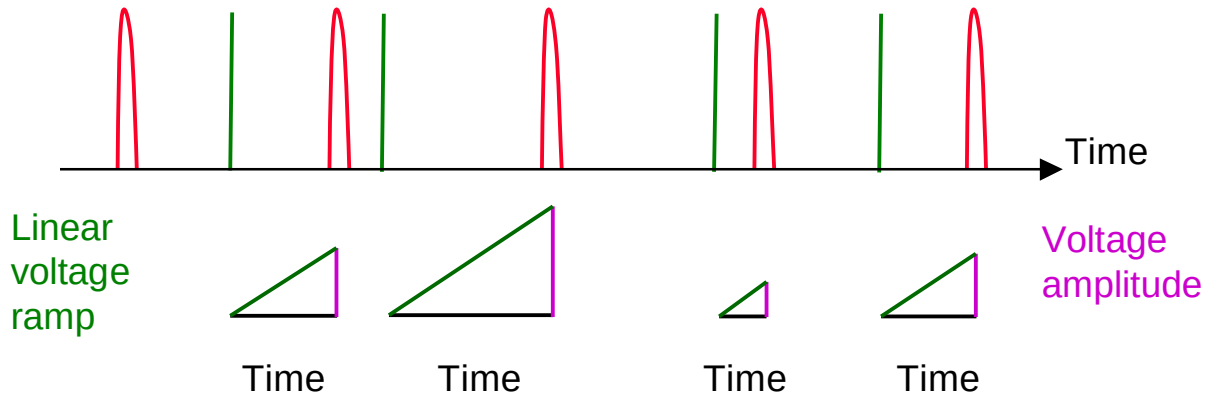
What do CFD, TAC, and ADC do?

CFD	Signal from Detector 	CFD Limit L discriminates between photon and noise
TAC	Blue – laser pulse, Green – photon 	Time measured in **reversed start-stop mode**: time between photon and next laser pulse => higher pulse rate possible, TAC Offset adds a fixed time to measured time
ADC	Amplified TAC Signal → ADC → Address of histogram memory => photon is related to a certain time bin	

FLIM applications

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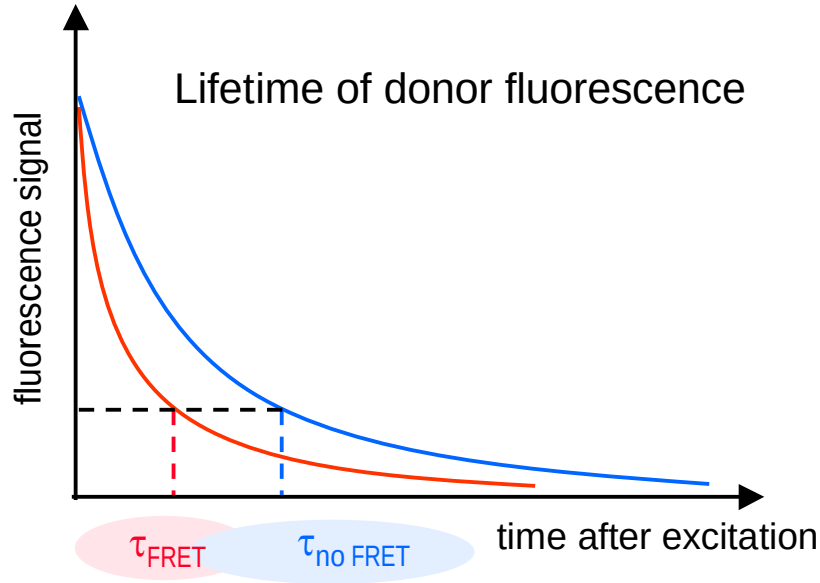
- Dye separation
- Energy transfer (FRET)
  for distance measuremens
- Concentration measurements of ions
  (Ca^{2+}, Na^{+}, pH, ...), small ligands, oxygen
- Environmental studies (viscosity,
  refractive index, membran potential)
- Protein studies (Proteomics)
- Intracellular signal transduction

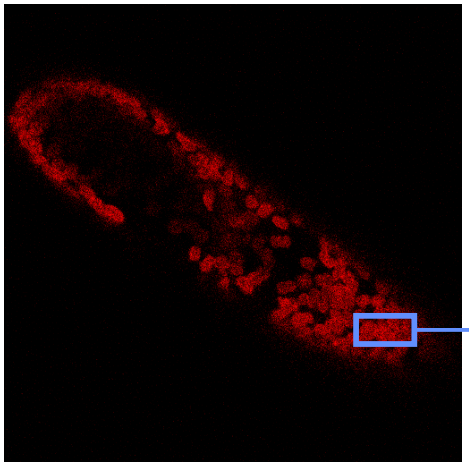
Example: FRET

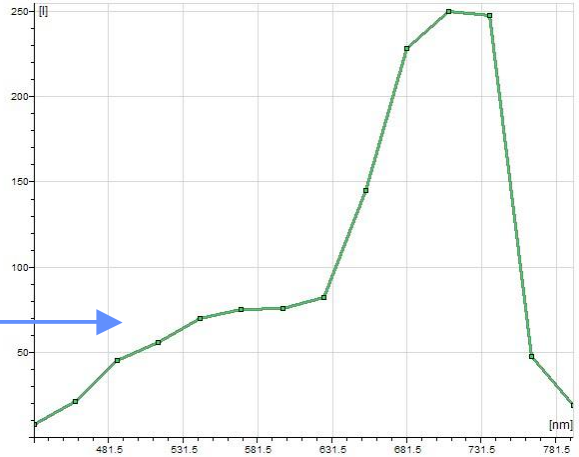


$$FRET_{eff} = \frac{\tau_{no\ FRET} - \tau_{FRET}}{\tau_{no\ FRET}}$$

Measurement of autofluorescence – Lifetime of chlorophyll

Leica MICROSYSTEMS

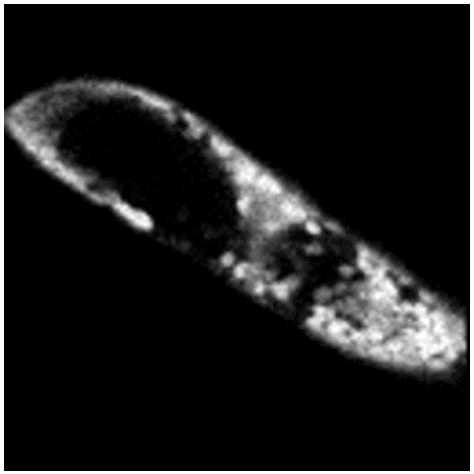




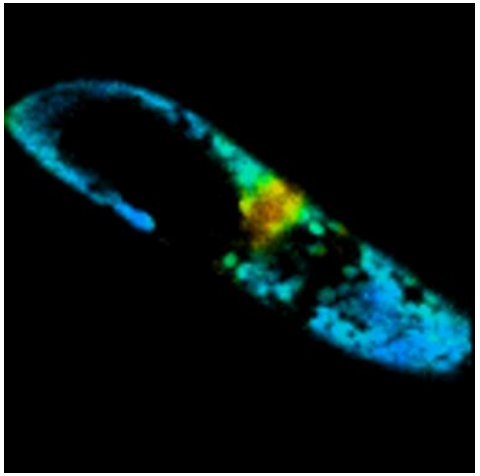
Sample: Living Diatomee
excitation @ 405 nm

Conventional confocal image showing chlorophyll fluorescence

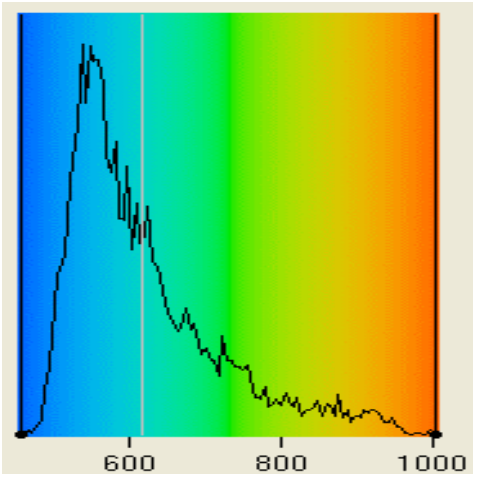
lambda-scan showing Chlorophyll emission spectrum



Intensity image



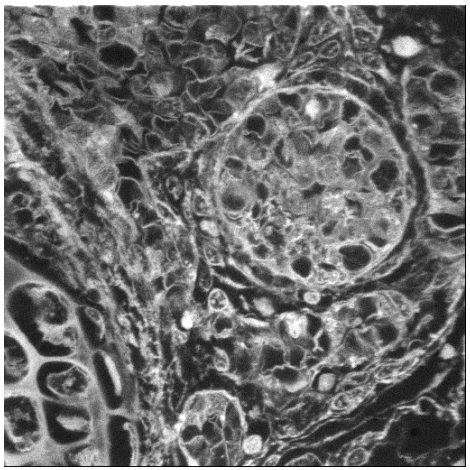
Fluorescence lifetime image

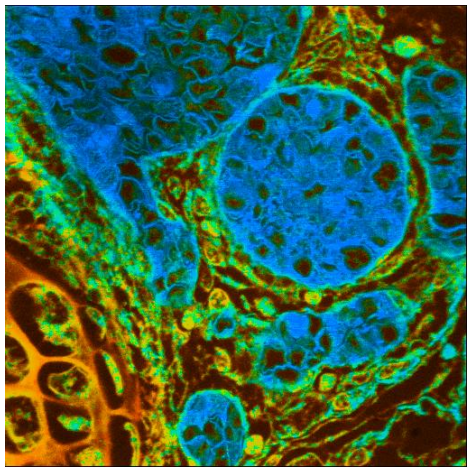


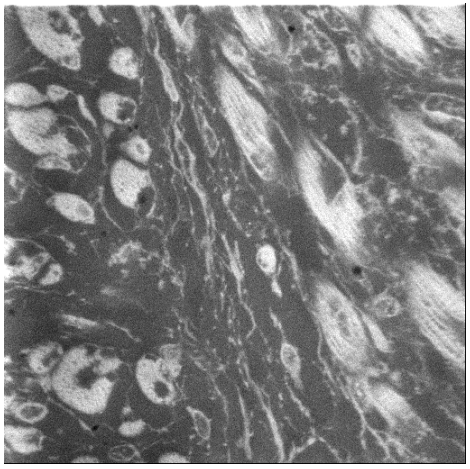
Lifetime distribution/ ps

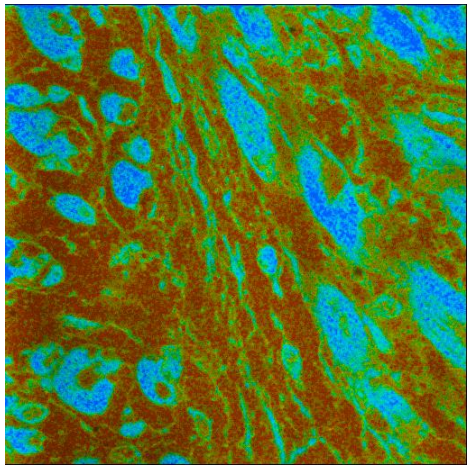
Measurement of autofluorescence – Differentiate different cell types by lifetime

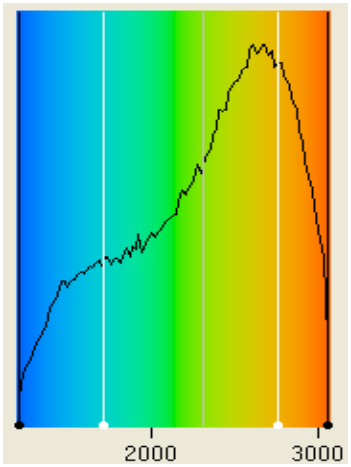
Leica MICROSYSTEMS











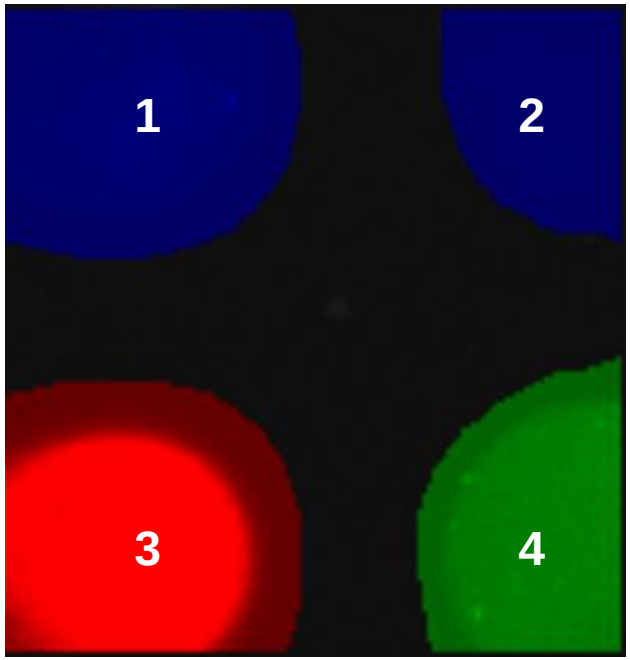
Intensity image

Lifetime image

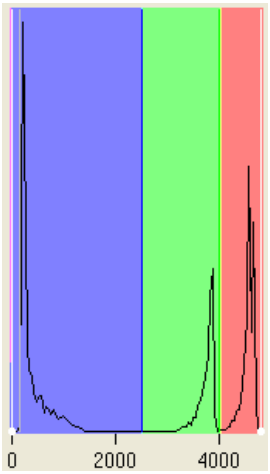
Lifetime distribution/ ps

Sample: Mouse tissue sections, unstained, excitation @ 405 nm

Lifetime as additional contrast: Distinguish 4 yellow dyes by their life time



Fluorescence lifetime image



Lifetime distribution/ ps

well	dye	buffer	lifetime
1	DASPI	Methanol	0.217 ns
2	Stilben	Methanol	0.282 ns
3	Coumarin	Methanol	4.65 ns
4	Fluorescein	NaOH buffer	3.85 ns

Sample: 4 different yellow, fluorescent dyes (see table) dissolved in methanol or buffer, in multi-well plate

Excitation @ 405 nm

Lifetime as additional contrast: Distinguish 2 green dyes by their life time

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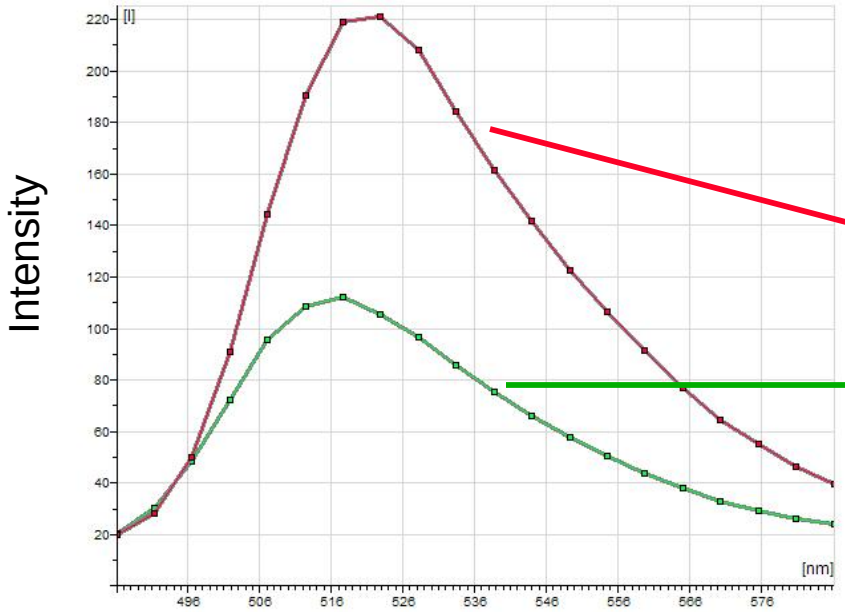
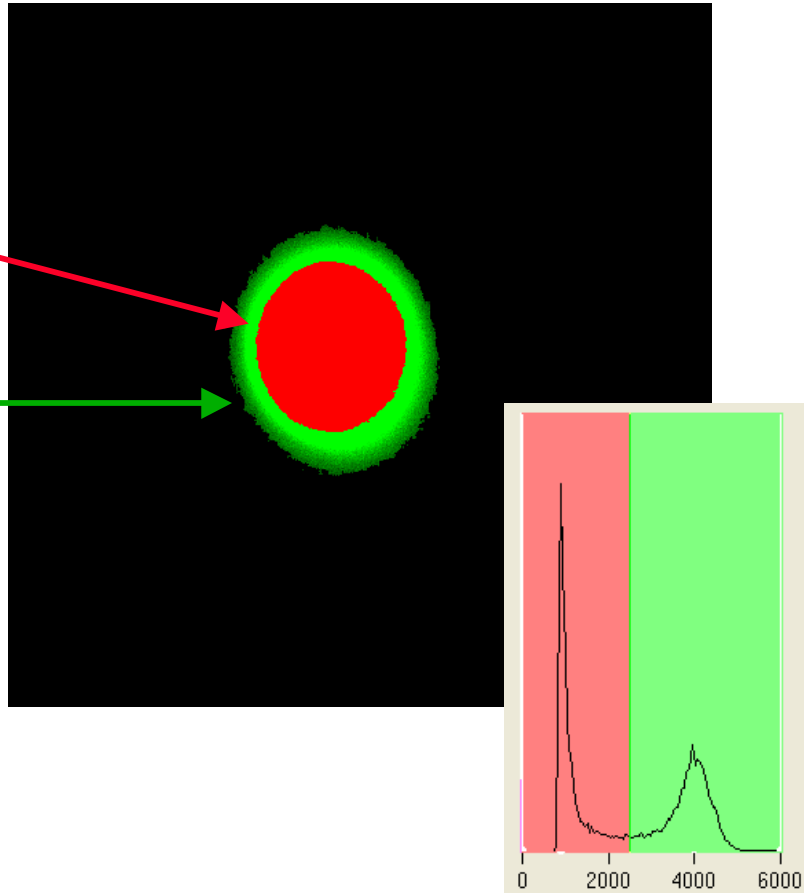
Fluorescence emission spectrum
(acquired with Leica SP2 AOBS)

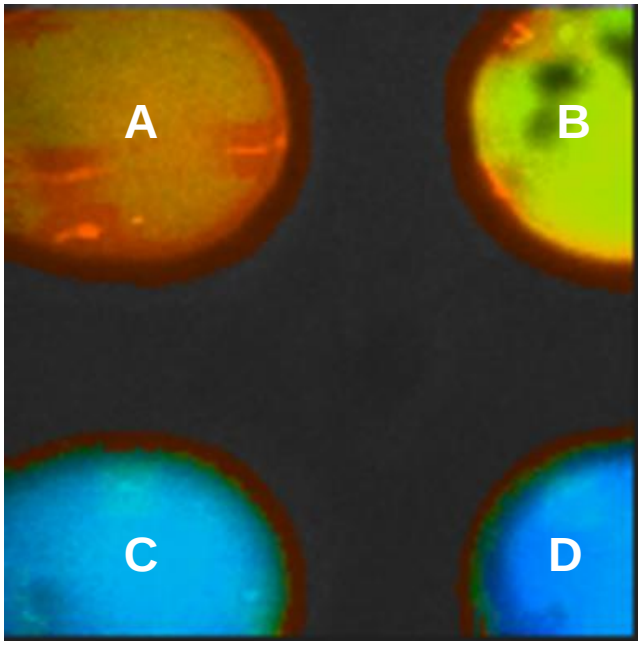


Fluorescence Lifetime Image

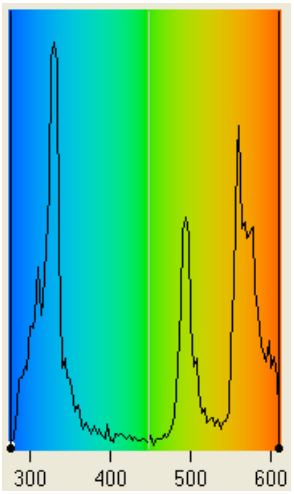


Sample: Bead - Focal Check DoubleGreen,
Molecular Probes, F-36905, Excitation @ 405 nm

Environmental studies: Effect of local surrounding of the dye on its lifetime



Fluorescence lifetime image



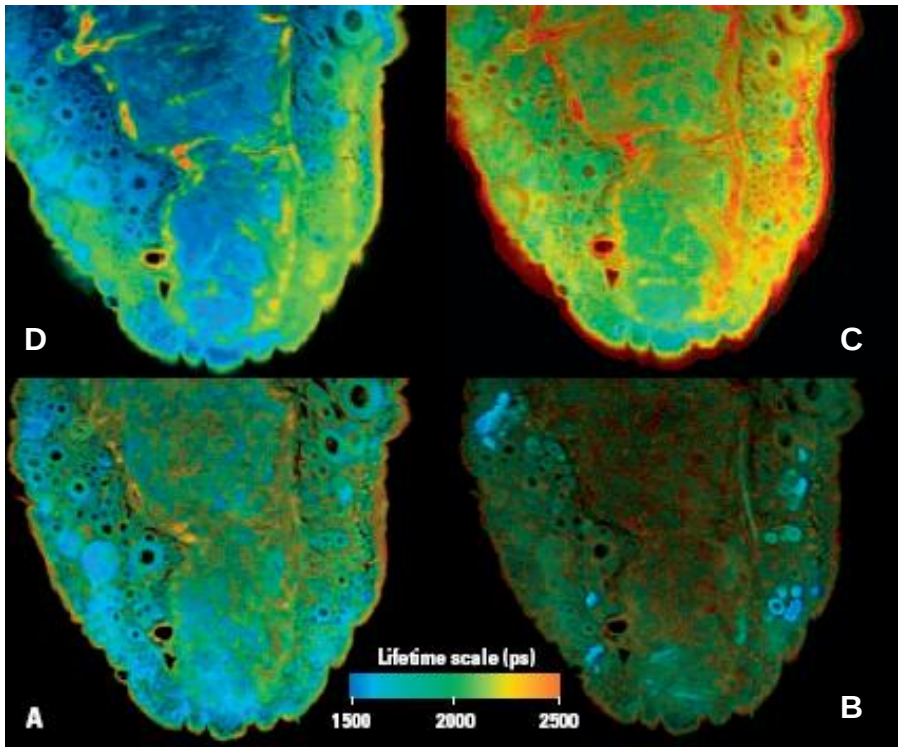
Lifetime distribution/ ps

Sample:
DASPI dissolved in methanol/glycerol mixture,
glycerol concentration: **A>B>C>D**,
in multi-wellplate
Excitation @ 405 nm

Result: The higher the viscosity the longer the lifetime.

well	lifetime
A	567.5 ps
B	500.7 ps
C	331.7 ps
D	308.5 ps

Spectral FLIM: a new dimension



Schistocera gregaria (nervous system)

A – Ex. @ 780 nm, Em. @ 500-550 nm
B – Ex. @ 780 nm, Em. @ 574-647 nm
C – Ex. @ 405 nm, Em. @ 500-550 nm
D – Ex. @ 405 nm, Em. @ 435-485 nm

- Spectral FLIM:
  - Simultaneous data acquisition in two FLIM channels
  - Any emission band
  - High efficiency and transparency
  - Can be combined with MP and D FLIM
- New Dimension:
  - Lifetime-Wavelength Recording

Lifetime as contrast: Distinguish 3 dyes by their life time in fixed cells (FluoCells, BPAE)

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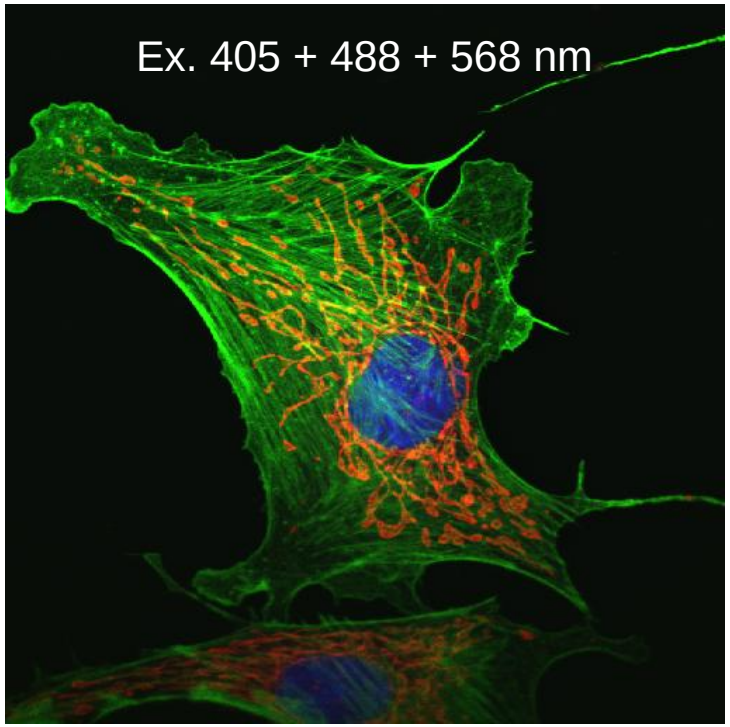
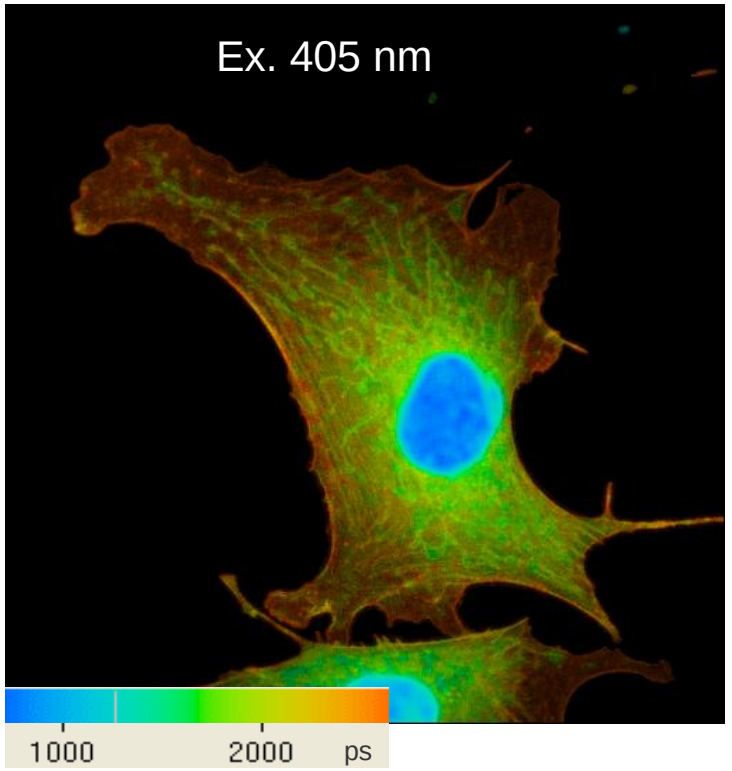




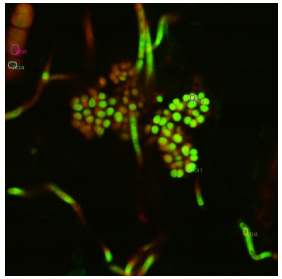
Intensity image
Blue: DAPI (nucleus)
Red: Mitotracker Red (mitochondria)
Green: BODIPY FL phallacidin (actin)

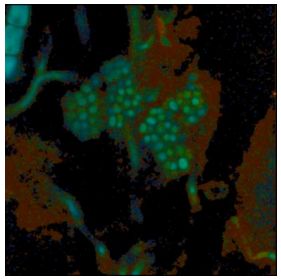
Lifetime image
Blue: DAPI (nucleus)
Green: Mitotracker Red (mitochondria)
Red: BODIPY FL phallacidin (actin)

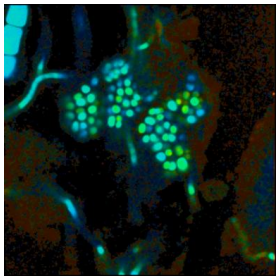
Molecular Probes, F-14780

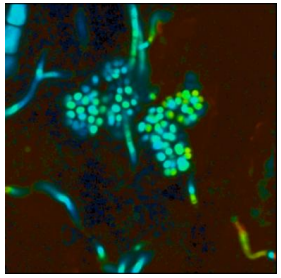
Spectral FLIM on cyanobacteria

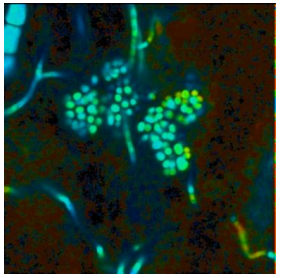
Excitation @ 790 nm, 80 MHz

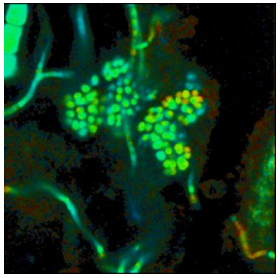

Intensity image

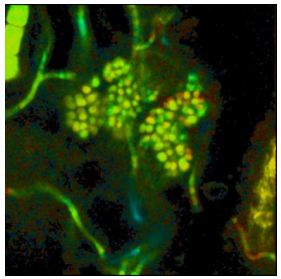

540 to 560 nm

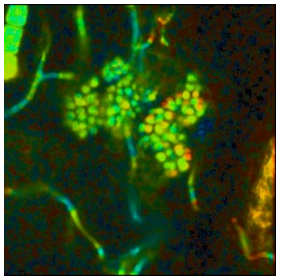

560 to 580 nm

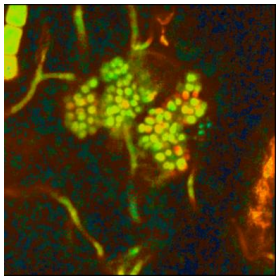

580 to 600 nm


600 to 620 nm


620 to 640 nm

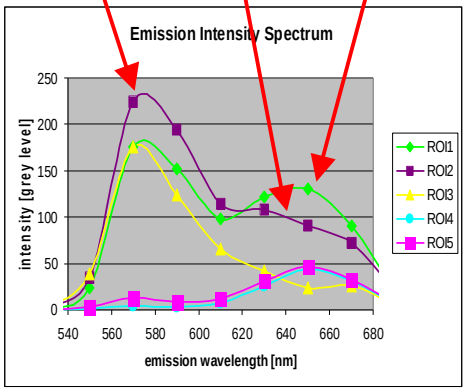

620 to 640 nm

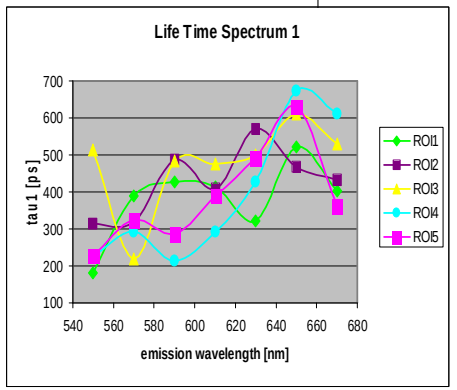

640 to 660 nm

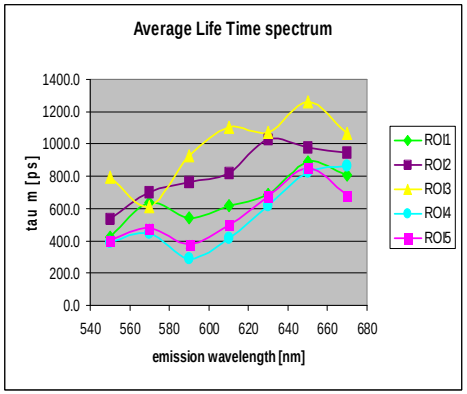

660 to 680 nm

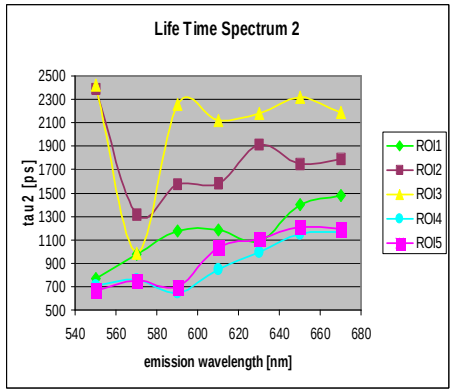
Phycocyanin?

Phycoerythrin? Allophycocyanin?









LUT τ_m [ps]:

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FLIM-FRET

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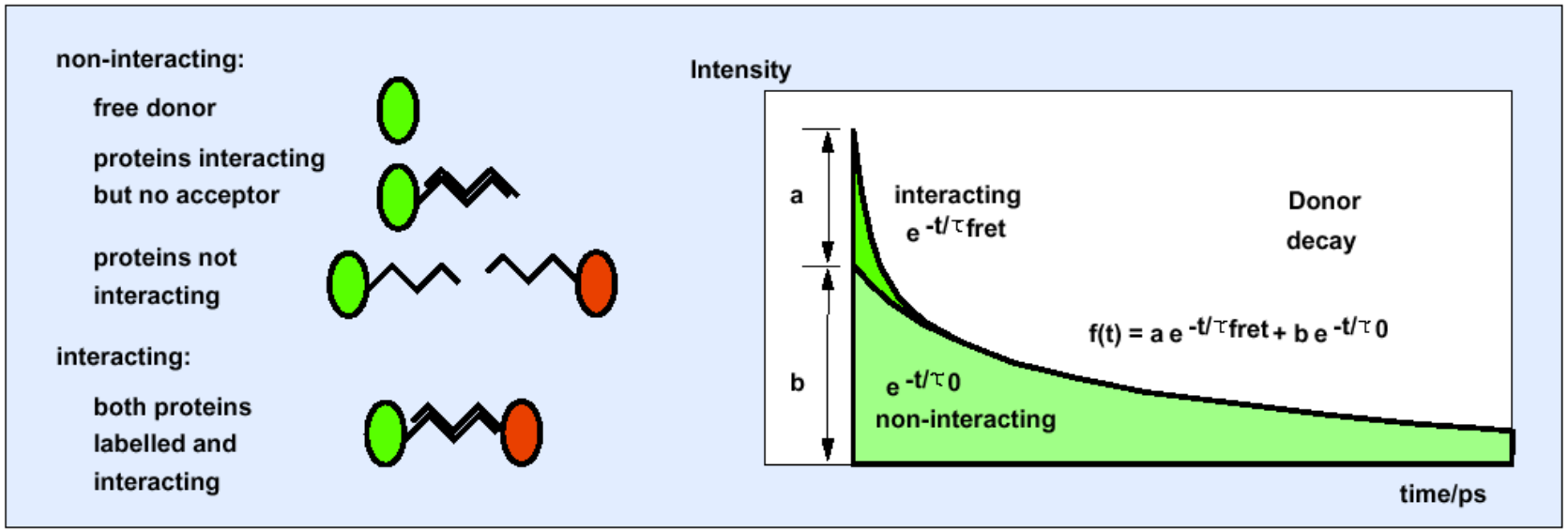
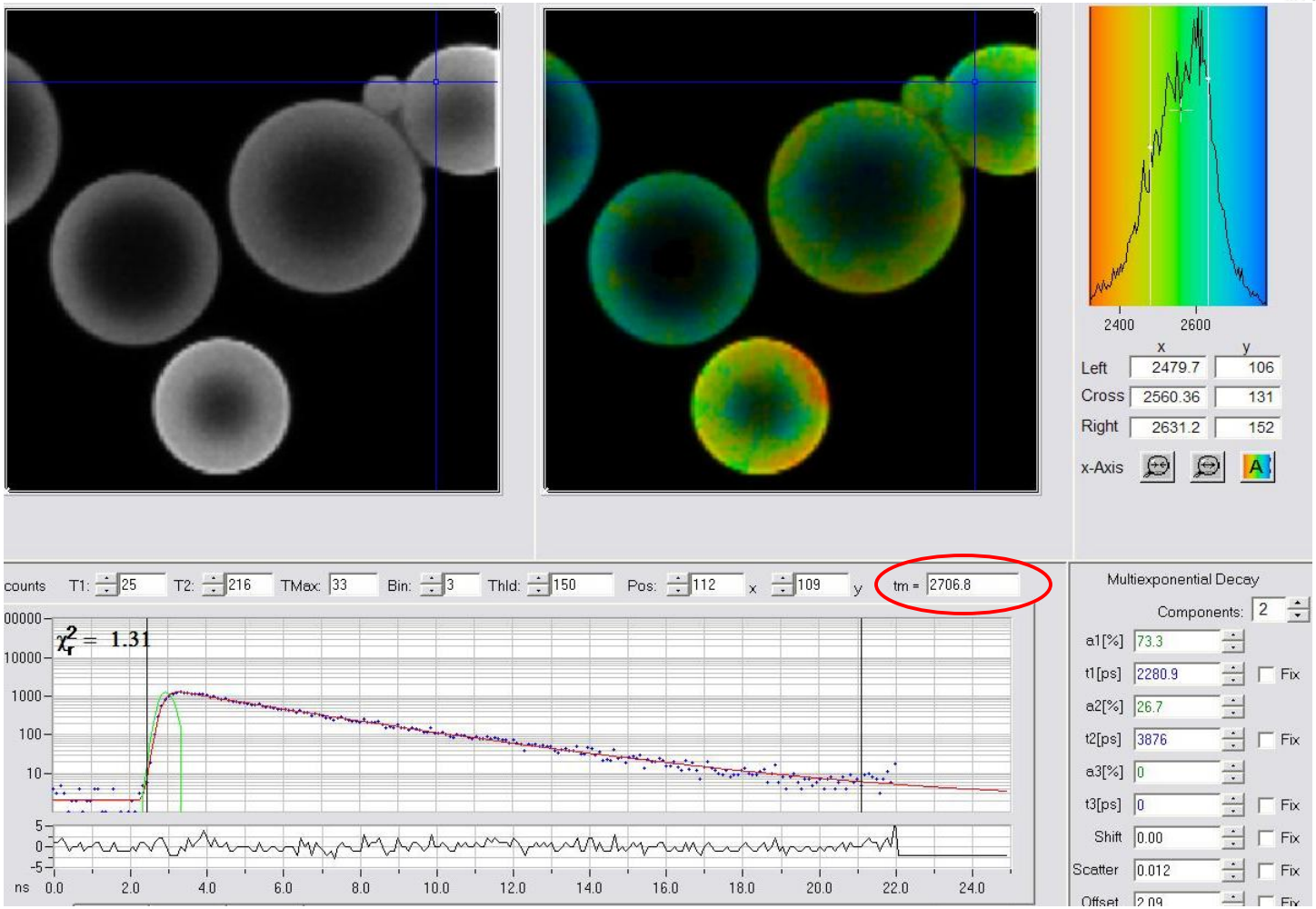


$$E_{fret} = 1 - \tau_{fret} / \tau_0$$

$$(r / r_0)^6 = \tau_{fret} / (\tau_0 - \tau_{fret}) \quad \text{or} \quad (r / r_0)^6 = \frac{1}{E_{fret}} - 1$$

$$N_{fret} / N_0 = a / b$$

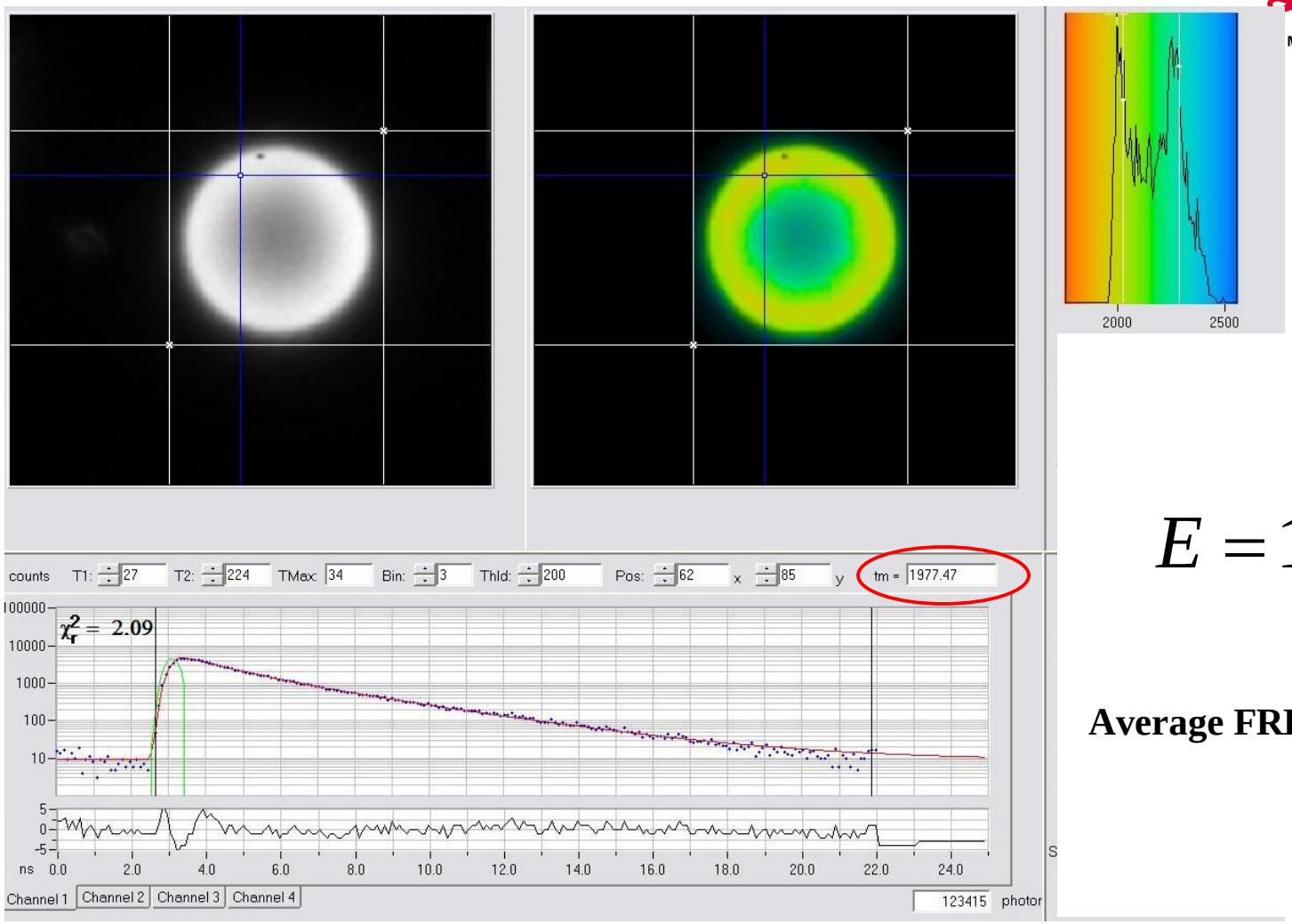
Beads tagged with eCFP



Ex: 405nm Em : 465-505nm

Samples provided by Prof. Eicke Latz, UMASS Medical School

Beads tagged with eCFP-eYFP



$$E = 1 - \frac{\tau_{FRET}}{\tau_D}$$

Average FRET Efficiency=0.27

Ex: 405nm Em : 465-505nm

Samples provided by Prof. Eicke Latz, UMASS Medical School

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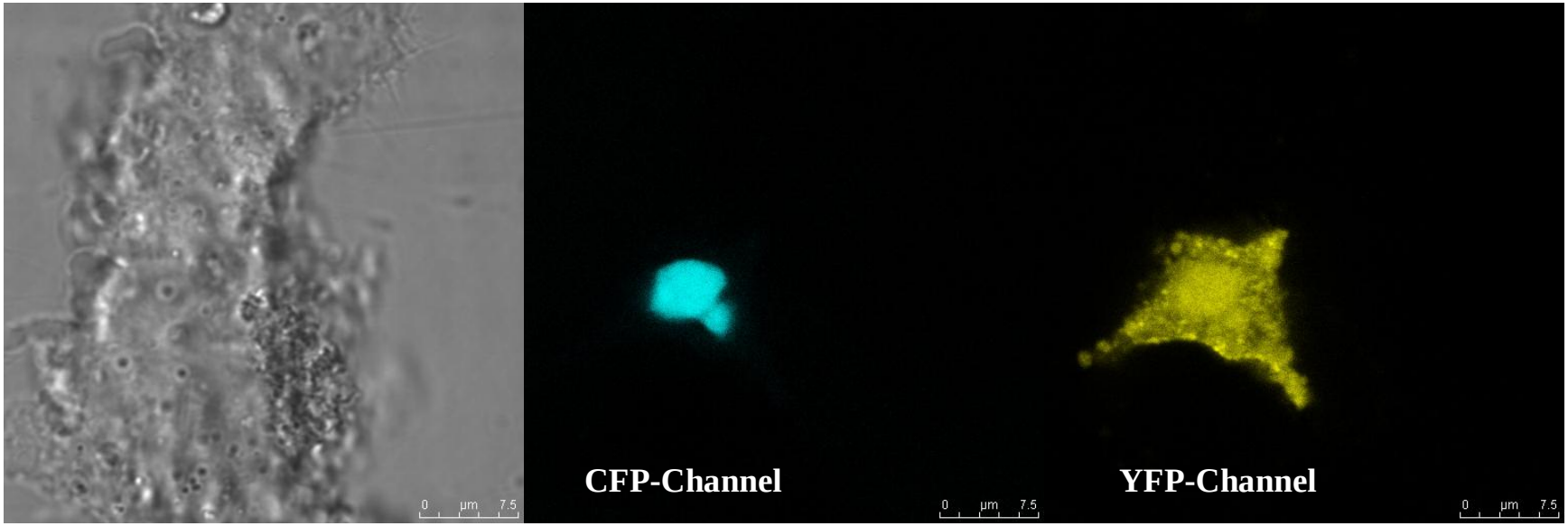
eCFP-NIPPP1 - eYFP-pcDNA



Ex: 458nm Em : 465-505nm Em: 525-600nm

Constructs obtained from Prof. Swedlow/ Prof. Mycek (AQLM-2008)

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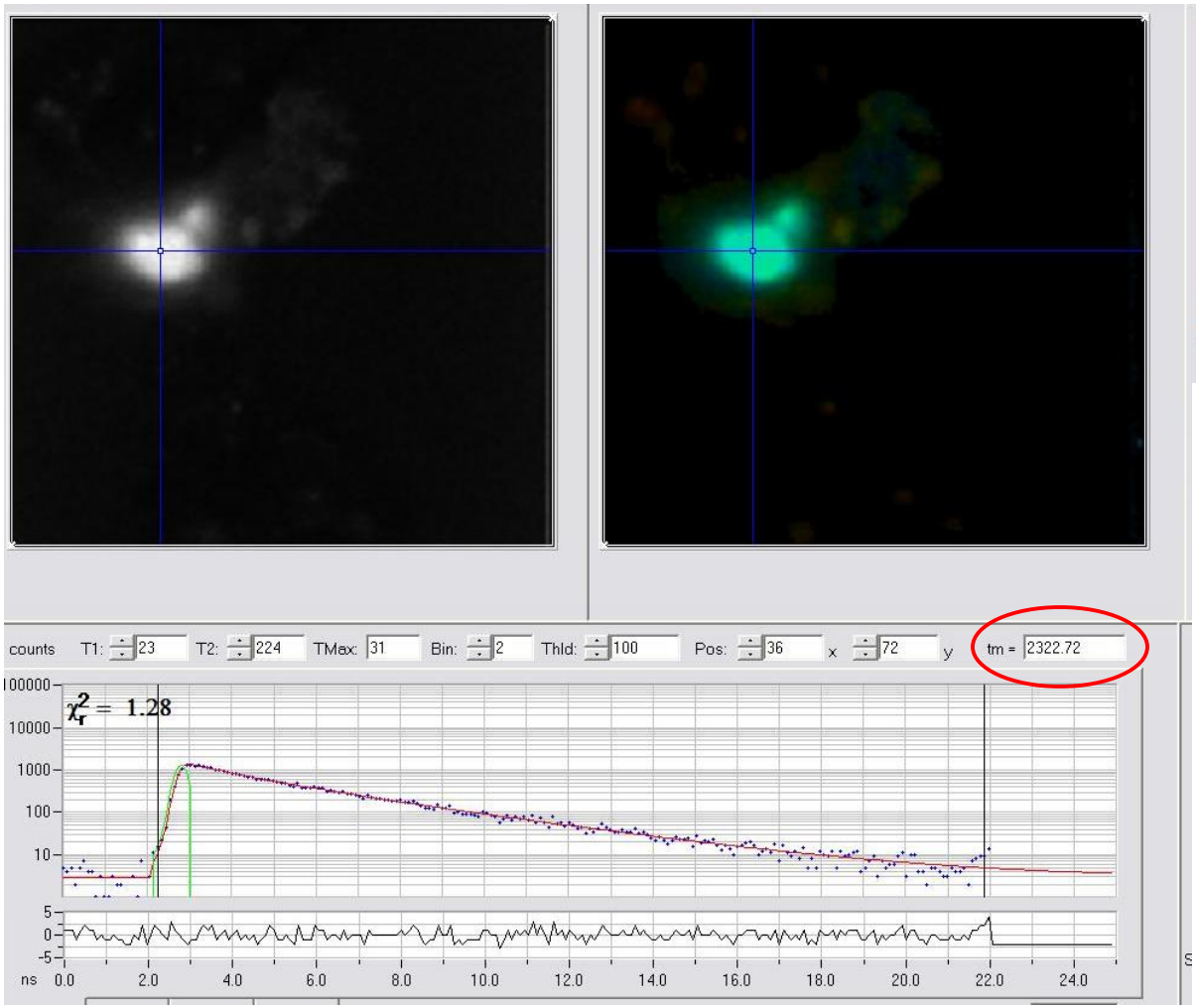
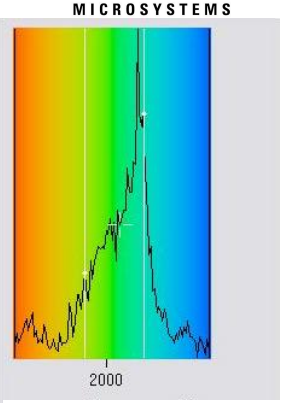
eCFP-NIPPP1-mutant-eYFP-PP1





Ex: 405nm

Em : 465-505nm

Constructs obtained from Prof. Swedlow/ Prof. Mycek (AQLM-2008)

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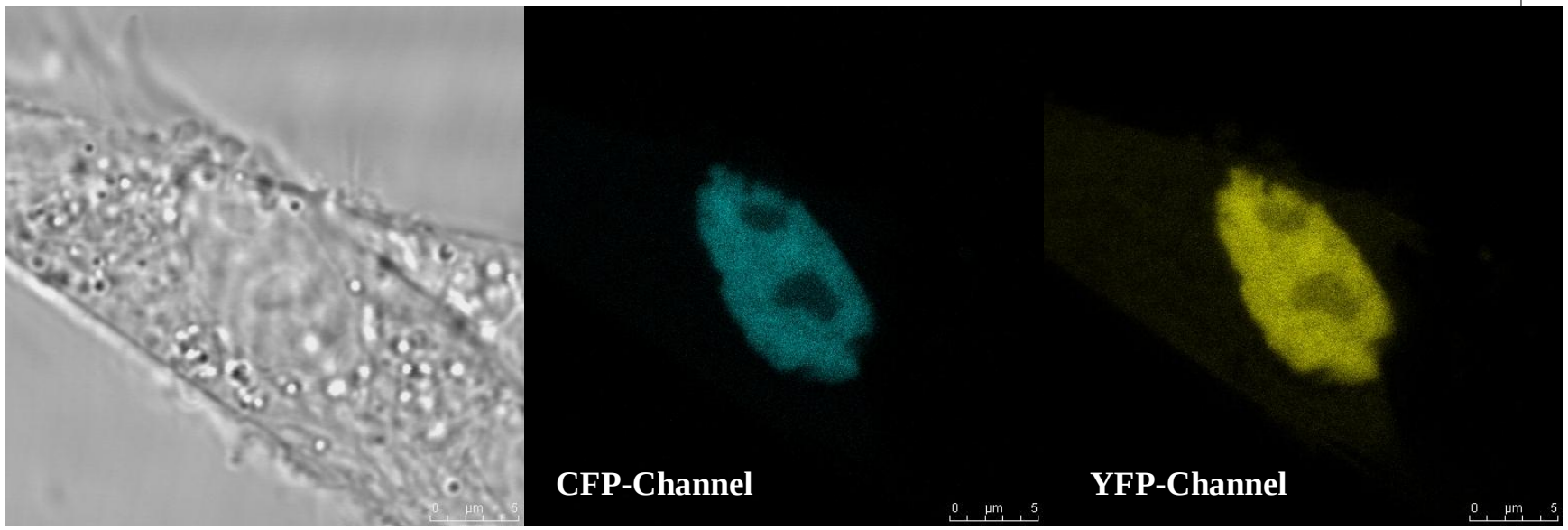
eCFP-NIPPP1 - eYFP-PP1



Ex: 458nm Em : 465-505nm Em: 525-600nm

Constructs obtained from Prof. Swedlow/ Prof. Mycek (AQLM-2008)

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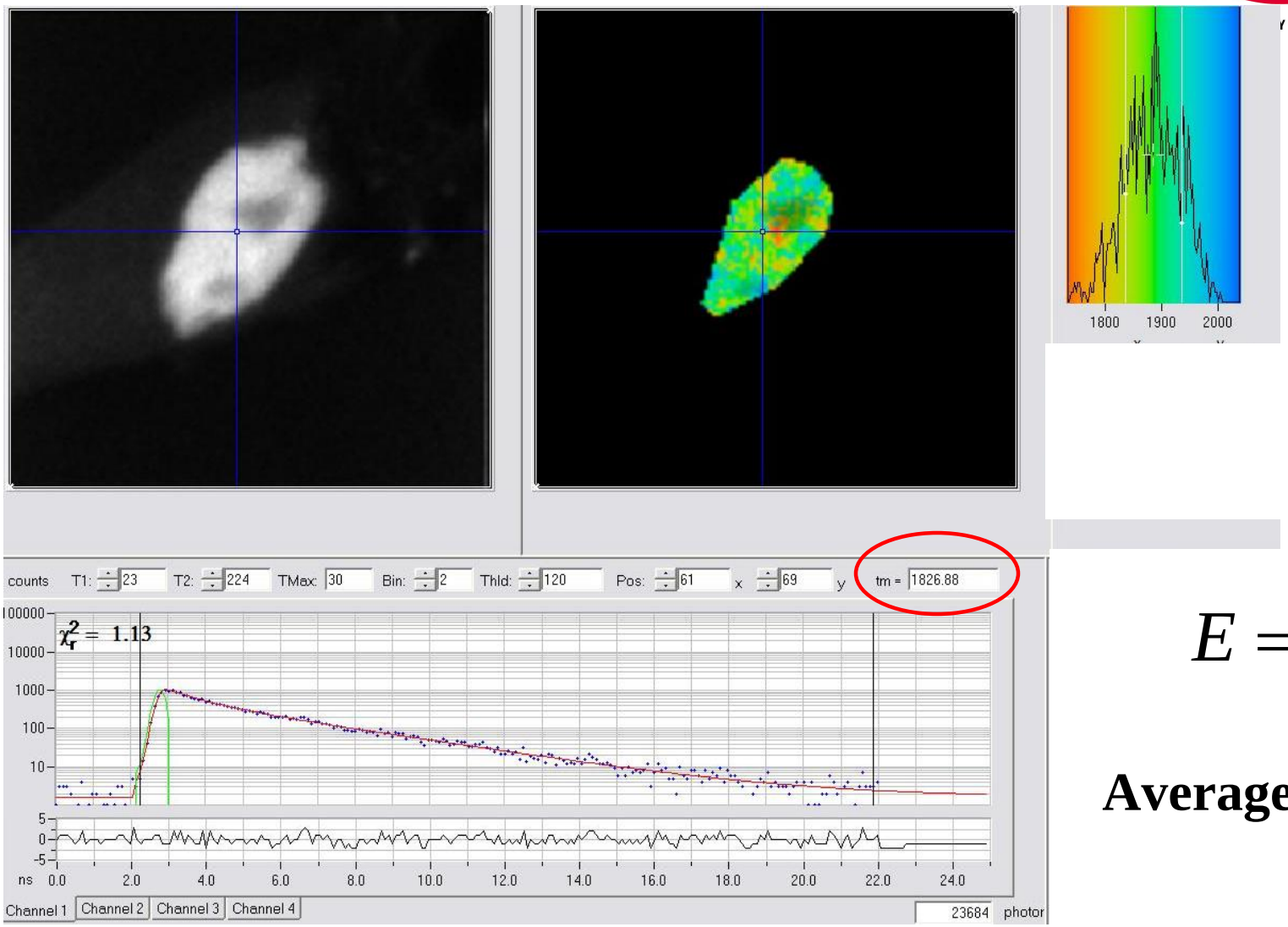
eCFP-NIPPP1 - eYFP-PP1



$$E = 1 - \frac{\tau_{FRET}}{\tau_D}$$

Average E=0.21

Ex: 405nm Em : 465-505nm

Constructs obtained from Prof. Swedlow/ Prof. Mycek (AQLM-2008)

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Fluorophores and lifetimes

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Probe:	$\lambda_{exc}/\lambda_{em}$	τ_a (ns)	τ_b (ns)
BCECF	490/520	3.0 (acid)	3.8 (base)
Fluo-3	490/520	2.44 (no Ca^{2+})	0.79 (Ca^{2+})
Lucifer Yellow		3.3	
Sodium Green		1.1 (low Na^{+})	2.4 (high Na^{+})
Hoechst		2.2 (no acc., 7-AAD)	1.4 (acceptor,7-AAD)
FITC	490/520	4.0 (pH > 7)	3.0 (pH < 3)
TRITC	543/	2.0	
Rhodamine 700	659/669	1.6 (pH 9)	1.55 (pH 6)
Rhodamine 700		1.55 (H_2O)	2.99 (Ethanol)
Cy3	550/570	0.27	0.5 (antibody conjug.)
Cy5	633/	1.0	
GFP free (S65T)	488/507	2.68	
CFP		1.3	
YFP		3.7	

FLIM standards

Leica MICROSYSTEMS

Compound	Conditions	Emission Wavelength Range (nm)	τ (ns)
Phenol	5 mm acetate, pH 6	285-320	3.16
PPD	Ethanol	315-390	1.24
BBO	Toluene	380-470	1.03
DCS	Toluene	430-510	0.12
DCS	MeOH	500-540	0.46
Erythrosin B	H_2O	535-580	0.08
Rhodamine B	H_2O	575-620	1.58
DCM	MeOH	590-680	1.22
Pyridine 2	MeOH	680-800	0.30
Rhodamine 800	H_2O	700-750	0.74

Tips and tricks:
How to do proper FLIM acquisition and analysis

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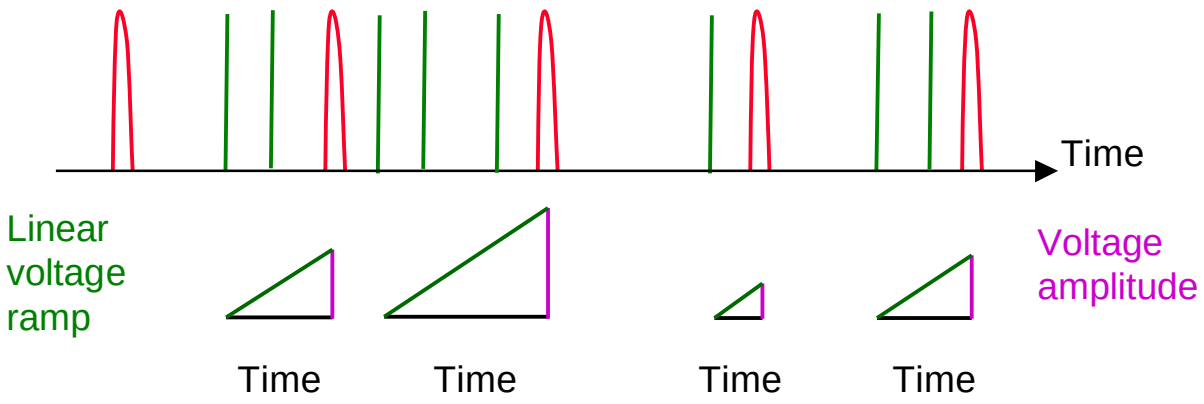
Why is the maximum count rate limited and what is the maximum one could use?

If the count rate is in the same range as the laser pulse rate the probability is high, that two or more photons arrive within one cycle (between 2 laser pulses). However, only the first photon is registered. This leads to a distortion of the photon histogram and calculated live times are too short. This is called the “pile-up” effect.

Blue – laser pulse, Green – photon



Tips and tricks:
How to do proper FLIM acquisition and analysis

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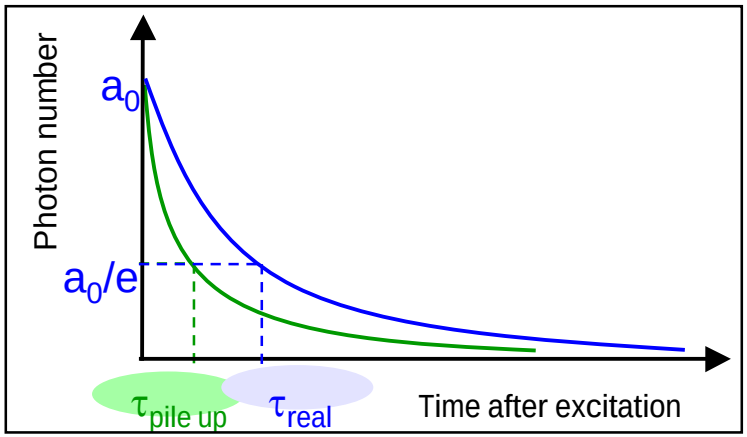
Why is the maximum count rate limited and what is the maximum one could use?

"pile-up" effect:

⇒ Reduce laser intensity until
At least: photon count rate
< 5% of laser repetition rate
Better: photon count rate
< 1% of laser repetition rate



Laser repetition rate	Photon count rate < 5%	Photon count rate < 1%
80 MHz	$< 4*10^{+6}$	$< 8*10^{+5}$
40 MHz	$< 2*10^{+6}$	$< 4*10^{+5}$
20 MHz	$< 1*10^{+6}$	$< 2*10^{+5}$
10 MHz	$< 5*10^{+5}$	$< 1*10^{+5}$
5 MHz	$< 2.5*10^{+5}$	$< 5*10^{+4}$
2.5 MHz	$< 1.25*10^{+5}$	$< 2.5*10^{+4}$

Tips and tricks:
How to do proper FLIM acquisition and analysis

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How can I measure FRET using FLIM?

1) Do not use fixed samples
2) Select spectral detection range to detect donor only
3) Measure lifetime of donor without acceptor being present: τ_D
4) Measure lifetime of donor in the presence of acceptor: $\tau_{D=>A}$
   - Take 2 component fit
   - Fix τ_D to the value obtained in 2)
   - Get $\tau_{D=>A}$ from the fit
   - Get corresponding amplitudes a_D and $a_{D=>A}$
5) Calculate FRET efficiency according to following formula:

$$\mathbf{FRET_{eff} = (\tau_D - \tau_{D=>A})/\tau_D}$$

5) Calculate the population undergoing FRET according to following formula:

$$\mathbf{FRET_{pop} = a_{D=>A}/(a_D + a_{D=>A})}$$

Formulas according to B. Vojnovic, Plymoth, Optical Workshop, April 2004

Tips and tricks:
How to do proper FLIM acquisition and analysis

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How many photons do I need for a proper analysis of lifetimes?

The number of photons depends on the expected number of components and how close are the corresponding lifetimes together. As a rule of thumb one could say:

components	Number of photons in the maximum	condition
1	> 100	
2	> 1.000	$\tau_2 > 2\tau_1$
3	> 10.000	$\tau_2 > 2\tau_1$, $\tau_3 > 2\tau_2$

If lifetimes are closer together they still can be separated. However in this case more photons have to be collected. The limits could be tested in a reference system (for instance mixture of dyes of known lifetimes)

Tips and tricks:
How to do proper FLIM acquisition and analysis

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What is “Incomplete decay” and when do I use it?

If the lifetime is too long for a given laser pulse rate the photon histogram does not decay within on laser cycle. The photons will appear within the next cycle and effect its shape.

If:

$A * \exp(- T / \tau) > \mathrm{SQRT}(A)$ (A – amplitude)

one should choose the option “incomplete decay” in B&H software.

Limitation:

Either: any stray light should be avoided carefully and “Offset” fixed to zero.

Or: the offset must be measured in control experiment and afterwards fixed to this value within the real experiment.

A better way to treat this problem is to reduce the pulse rate of the laser (easily possible in D FLIM)

Tips and tricks:
How to do proper FLIM acquisition and analysis

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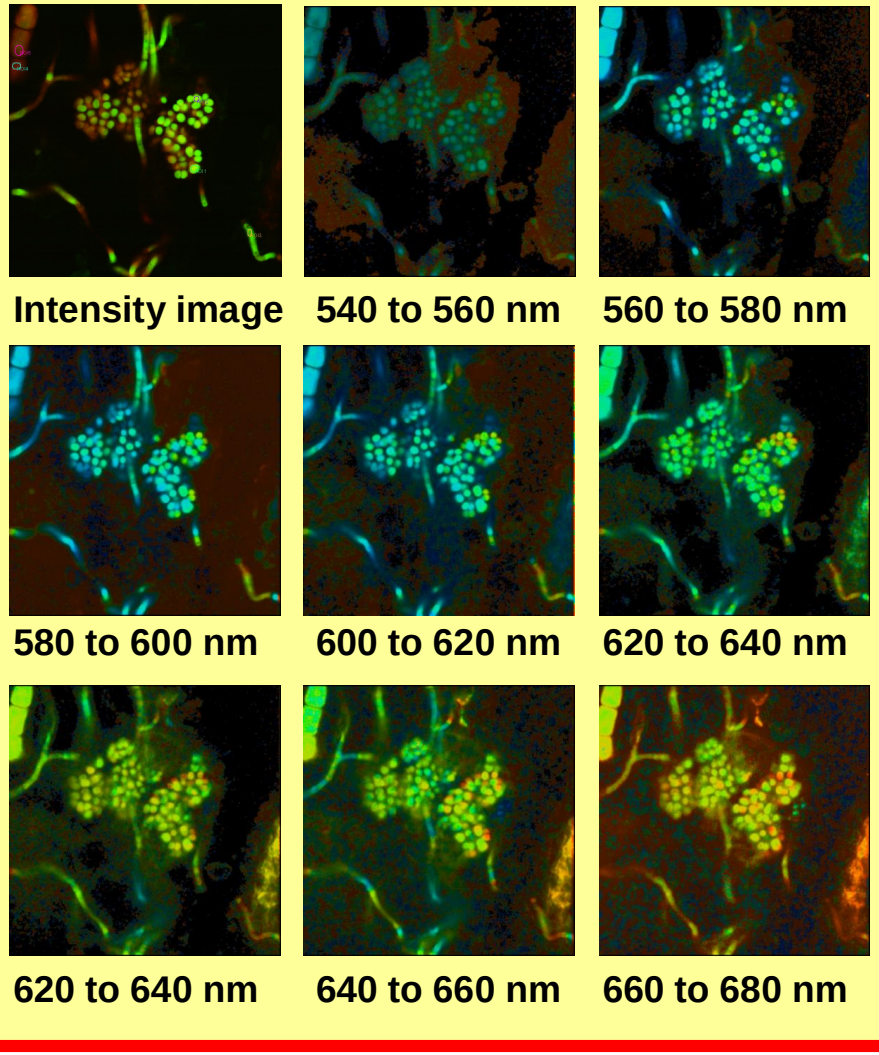
The live time at regions of high fluorophore concentration is higher than that of regions of lower concentrations. What is the reason and what should I do?

This might be due to too high photon counting rate at the regions of high intensity. Reduce further laser intensity.

Thank you !

Excitation @ 790 nm, 80 MHz



Questions ????????

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Nonlinear Optical Effects

$$P_i(\mathbf{E}) = P_{0i} + \chi_{ij}^{(1)}\mathbf{E}^j + \chi_{ijk}^{(2)}\mathbf{E}^j\mathbf{E}^k + \chi_{ijkl}^{(3)}\mathbf{E}^j\mathbf{E}^k\mathbf{E}^l + \ldots$$

Permanent Dipole Moment

Linear Polarization

Second-Order Nonlinear Term
- *Electro-Optic Pockels Effect*
- *Second-Harmonic Generation*

Third-Order Nonlinear Term
- *Optical Kerr Effect*
- *Electric Field-Induced Second Harmonmic Generation*
- *Degenerate Four-Wave Mixing*
- *Third Harmonic Generation*
- ***Two-Photon Absorption***

$$TP_{abs} \alpha I^2$$

Where **I** is the incident light intensity

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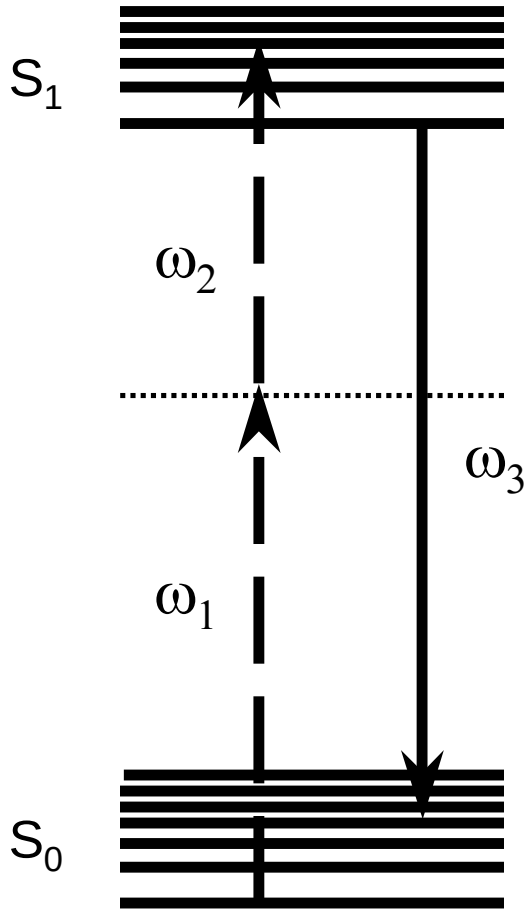
Two-Photon Induced Upconverted Fluorescence in Dyes

Energy level diagram showing two-photon induced fluorescence or lasing

$$TP_{abs} \alpha I^2$$

Where **I** is the incident light intensity



Pulsed Lasers

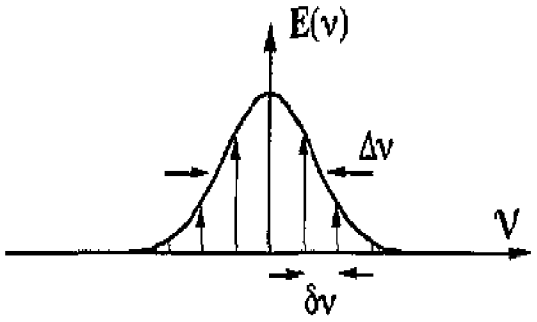
Power= Energy / time

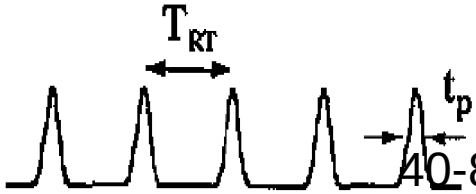
For 10 mW average power of pulsed laser
Output (1ps pulse width with 80 MHz rep rate)

Peak Power =

$$10^{-3} \times \frac{1}{80 \times 10^{6} \times 10^{-12}} = 0.125 \times 10^{3} W$$

10-20ns

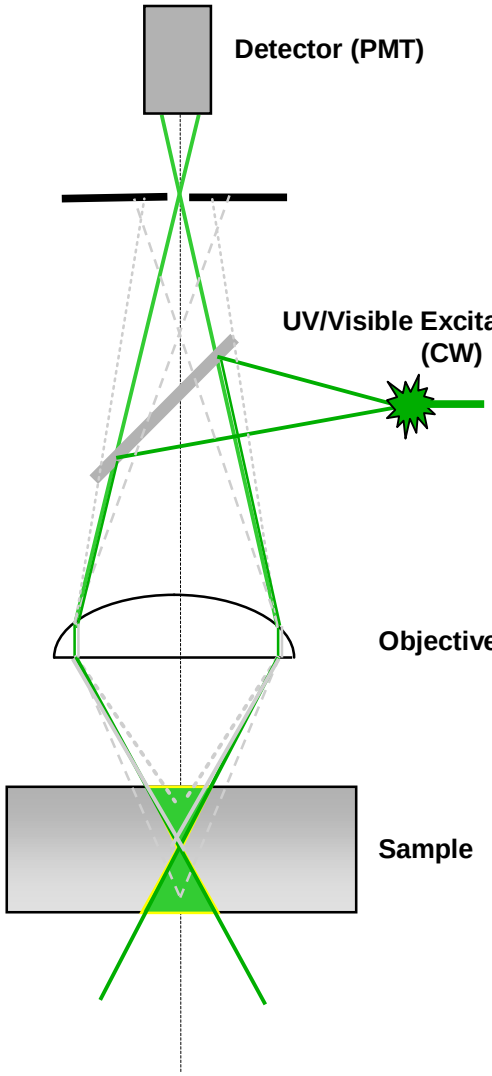




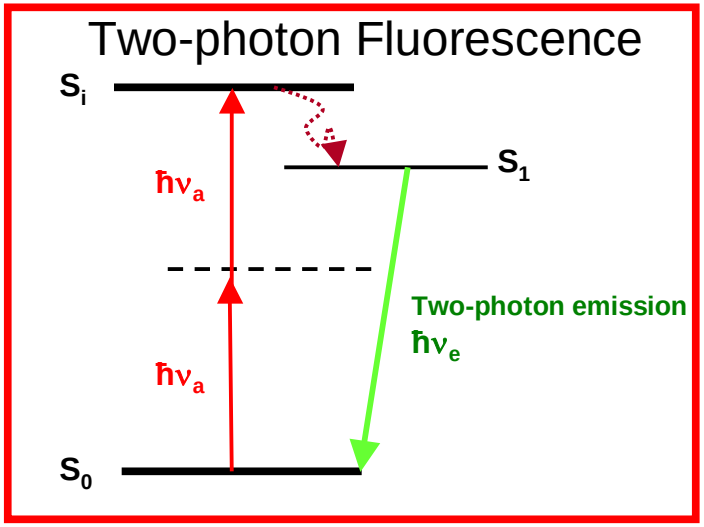
40-80 MHz

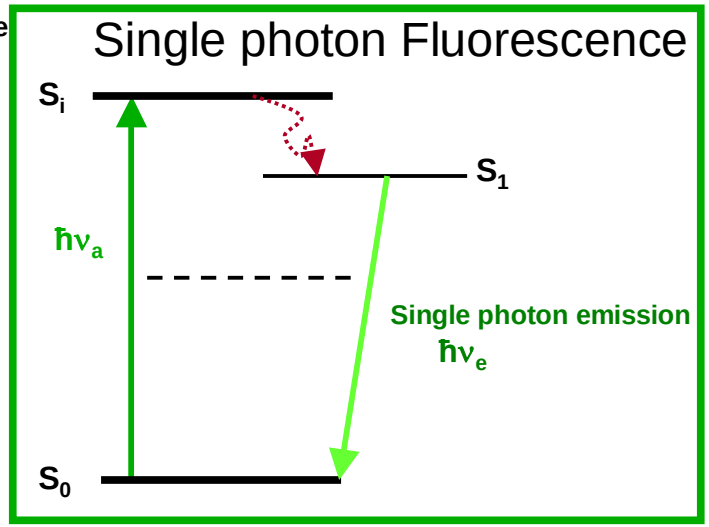
λ

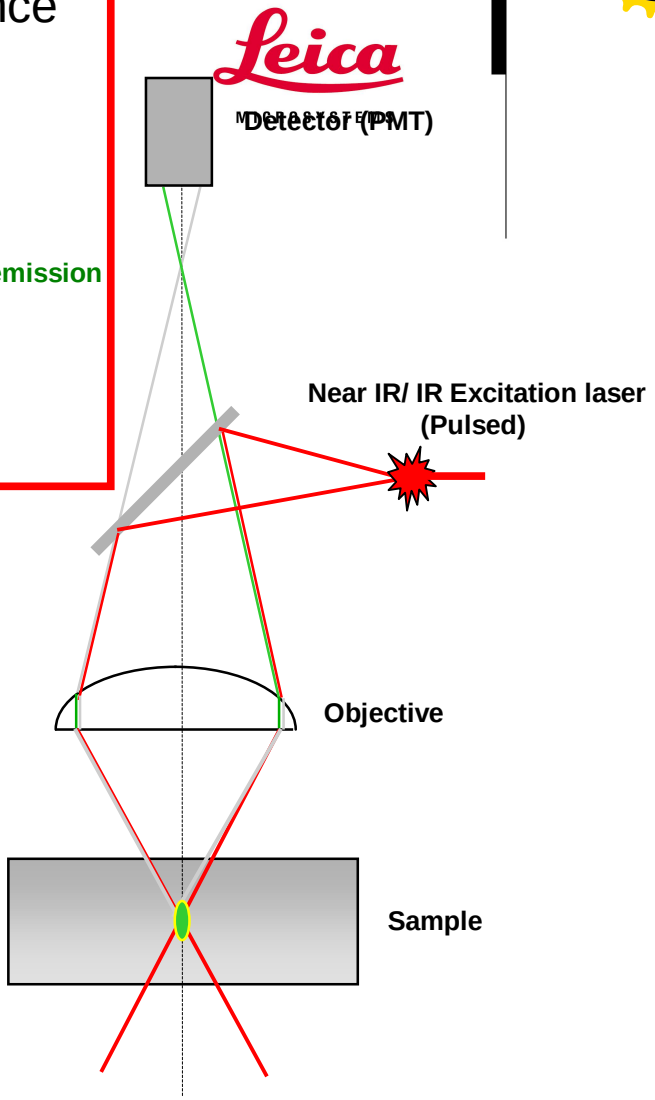
5-15nm



Confocal (Single Photon)







Multi-Photon (e.g., Two-photon)

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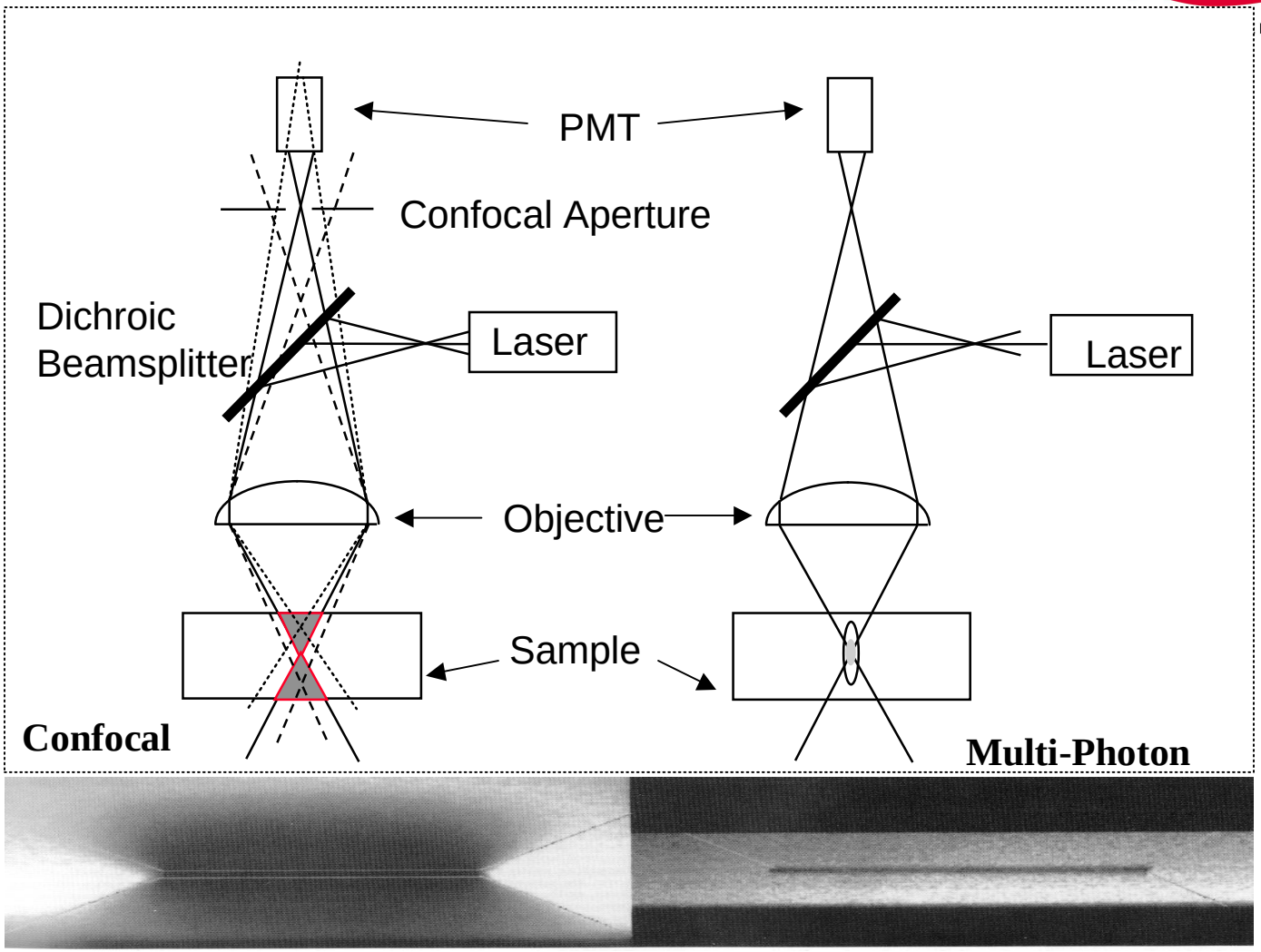
Two-photon Imaging



Technology of TCS SP2- MP

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Basic Principle

$$Fl \cong \frac{\langle P \rangle^2}{T \cdot f}$$

Fl= no. fluorescence photons/sec
P = average laser power
T = pulse length
f = laser repetition rate

Technology of TCS SP2- MP

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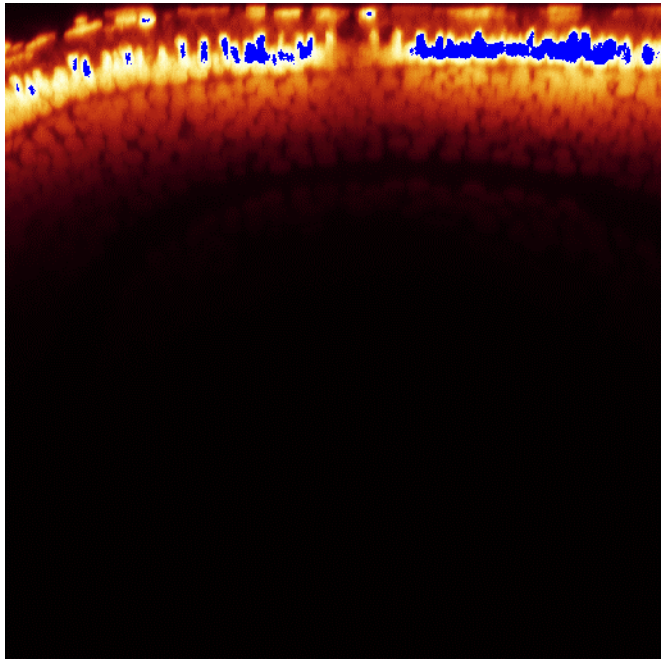
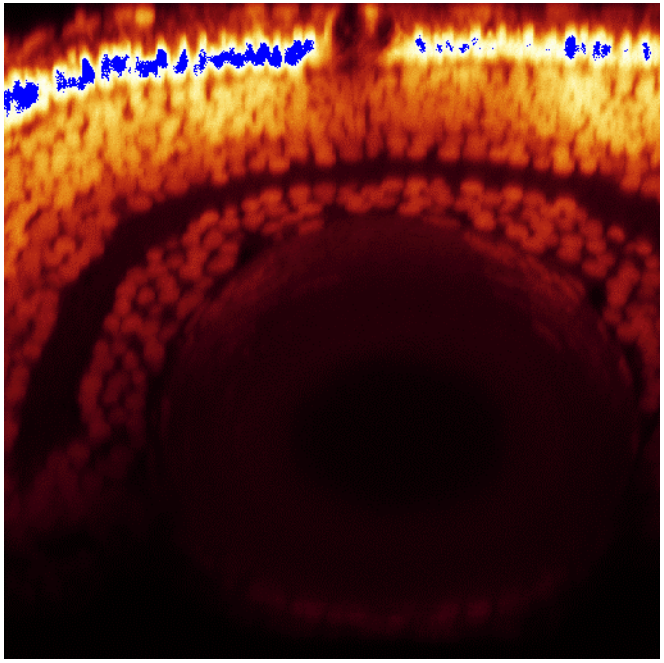
Advantages

Eye of zebrafish larvae (stained with DAPI)

Image size (xz): 125 µm x 125 µm - Objective: 63x 1.2 Water - Detection range: 400nm – 500nm



Ex: UV / 365 nm
PMT: 360V



Ex: IR / 780 nm
PMT: 360V

Two-photon Excitation wavelengths for some common dyes

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Blue/Cyan Dyes

Dye	Excitation
Alexa	350 780-800 nm
Hoechst	780-1000 nm
DAPI	780-1000 nm
CFP	800-900 nm

Green Dyes

Dye	Excitation
Oregon Green	800-860 nm
Alexa	488 800-830 nm
eGFP	920-990 nm
BODIPY	900-950 nm
FITC	750-800 nm
DiO	780-830 nm

Yellow/Orange Dyes

Dye	Excitation
YFP	890-950 nm
DiA	800-860 nm

Red Dyes

Dye	Excitation
DiI	830-920 nm
Rhodamine B	800-860 nm
Alexa 568	780-840 nm

http://research.stowers-institute.org/wiw/external/Technology/NLO/index.htm#TwoPhotonFluorescence

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Two-photon Excitation wavelengths for some common dyes

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Laser Options – TPE of fluorochromes

	Dye	1P Ex/Em (nm)	2P Ex (nm)
Cell Wall Stain	Calcofluor White	440/500-520	780>820
Nucleic Acid Stains	DAPI, Hoechst	350/470 350/460	780>820
	Feulgen	480/560	780>820
Cell Viability	Fluorescein Di Acetate	495/520	780>820
Calcium	Calcium Green/Texas Red (770)	488/530, 596/620	780
	Calcium Green	488/530	780>820
	Yellow Cameleon	464/527	780>820
Protein Conjugates	AMCA	431/498	780
	FITC	490/525	780>820
	CY2 (760 nm)	489/506	780>800
	CY3 (760 nm)	550/570	780
	CY5 (760 nm)	649/670	780<820
	TRITC	541/572	800-840
	Texas Red	596/620	780

	Dye	1P Ex/Em (nm)	2P Ex (nm)
Gene Expression	BFP	395/509	780>820
	CFP	434/477	780>840
	GFP	488/507	860<960
	YFP	514/527	860<960
	Yellow Chameleon	434/477-527	780>840
	DsRed	543/580	900<1064
Mito Tracers	Rhodamin 123	507/529	780-860
Neuronal Tracer	DID (760-780)		780
Neurotrans mitter Release	FM 1-43	510/626	830