TIGP, Fall 2007

TIME-RESOLVED FLUORESCENCE MICROSCOPY

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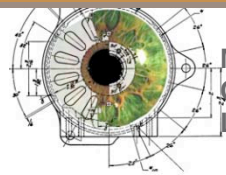


MODERN
OPTICS
LABORATORY

NATIONAL
YANG-MING
UNIVERSITY

Salvador Dali

Outline

1. Basics of Fluorescence Lifetime
   - types of luminescence
   - types of fluorescence
   - basic properties of fluorescence
   - definition of the fluorescence lifetime
   - The probability nature
   - Ways of fluorescent lifetime measurements
   - **Summary**
2. The Time-Correlated Single Photon Counting
   - Basic principles
   - Scheme of working
   - Instrumentation
   - **Summary**
3. Data analysis
4. The dynamics of fluorescence lifetime
   - The experimental window
   - Quenching and sensitivity to microenvironment
   - Resonance energy transfer
   - **Summary**
5. Applications
   - RET: Virus diagnostics
   - FLIM: Autofluorescence sensing
   - **Summary**

BASICS OF FLUORESCENCE LIFETIME

Luminescence

(from Latin *lumen* – light) emission of ultraviolet, visible or infrared photons from electronically excited specimen.
Eilhardt Wiedemann, 1888: "all those phenomena of light which are not solely conditioned by the rise in temperature".

1. **ORGANIC COMPOUNDS**
   - AROMATIC HYDROCARBONS
   - FLUORESCEIN
   - RHODAMINES
   - COUMARINES
   - AMINOACIDS
   - …
2. **ORGANOMETALLIC COMPOUNDS**
   - RUTHENIUM COMPLEXES
   - COMPLEXES WITH LANTHANIDES
   - …
3. **INORGANIC COMPOUNDS**
   - URANYL ION (UO_2^+)
   - LANTHANIDE IONS (EU^{3+}, TB^{3+}, ETC.)
   - DOPED GLASSES (ND, MN, CE, ETC.)
   - CRYSTALS (ZNS, CDS, ZNSE, ETC.)

Photoluminescence
- fluorescence
- phosphorescence

Radioluminescence

Cathodoluminescence

Electroluminescence

Thermoluminescence

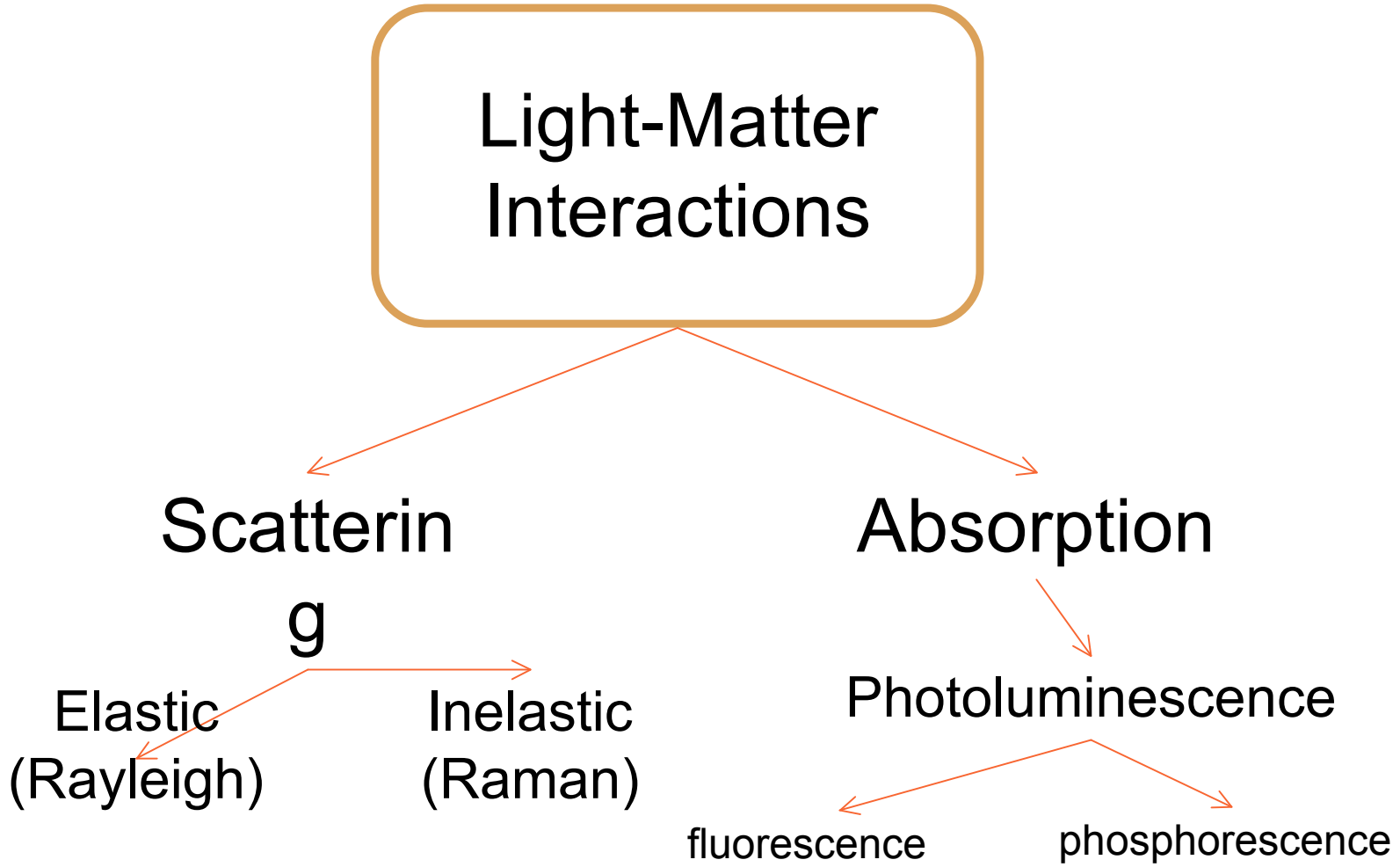
Chemiluminescence

Bioluminescence

Triboluminescence

Sonoluminescence

Light-Matter Interactions



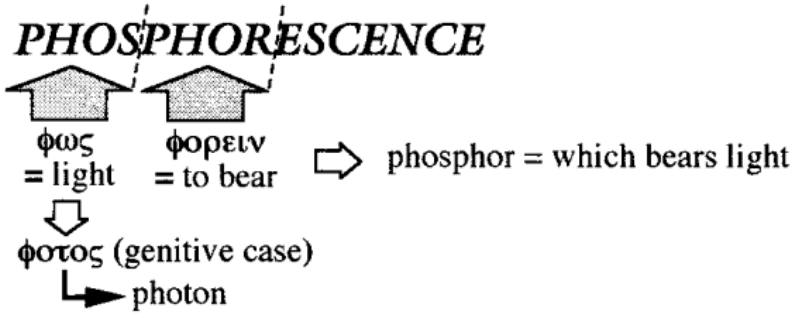
Ethymology

First observed in 1565 by Nicholas Monardes in the infusion of *Lignum Nephriticum.* Repeated by Boyle and Newton.

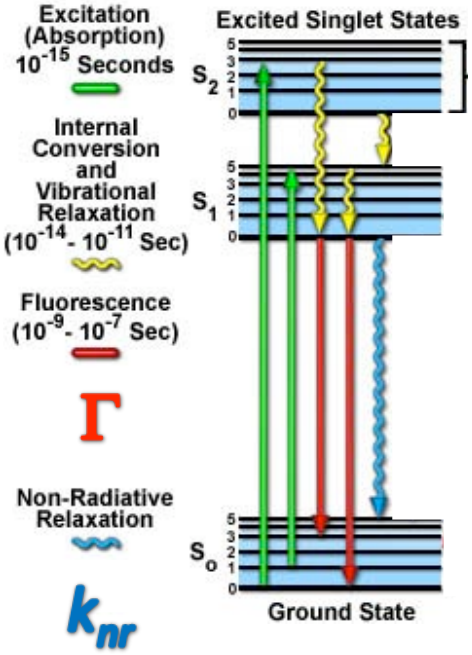
The term introduced by Sir George Gabriel Stokes in 1842. Blue light observed from quinine similar to that seen from fluorspar.

Spars – in the 18th century transparent stones, easily melted.
Fluor – (fluere – liquid in Latin).
Fluorspars are not fluorescent! Presence of Europium ions.

First observed in 1608 in the calcinated Bolognian Phosphor.



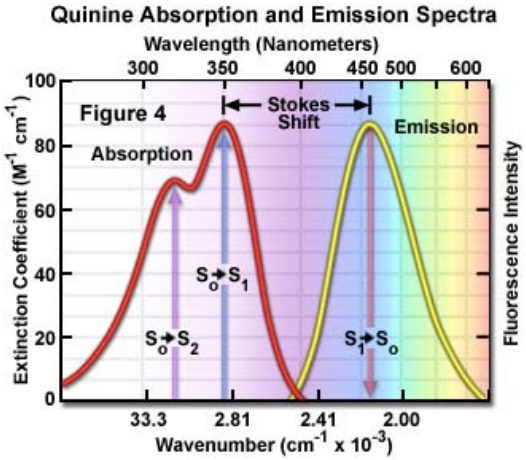
Fluorescence properties



Spectra

Stoke's shift

Mirror image rule



Fluorescence brightness

Quantum yield – number of emitted photons relative to the numbers of absorbed photons

$$Q = \frac{\Gamma}{\Gamma + k_{nr}}$$

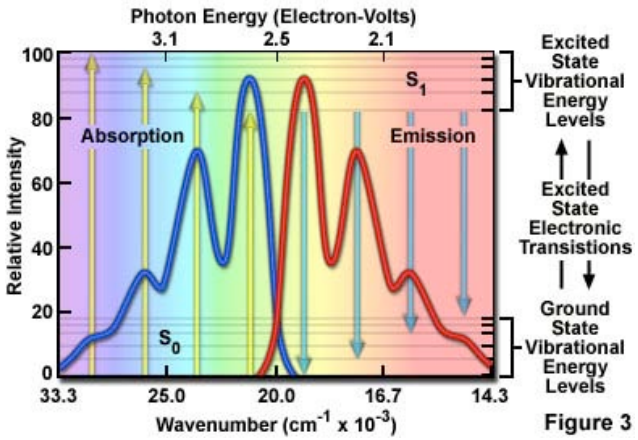
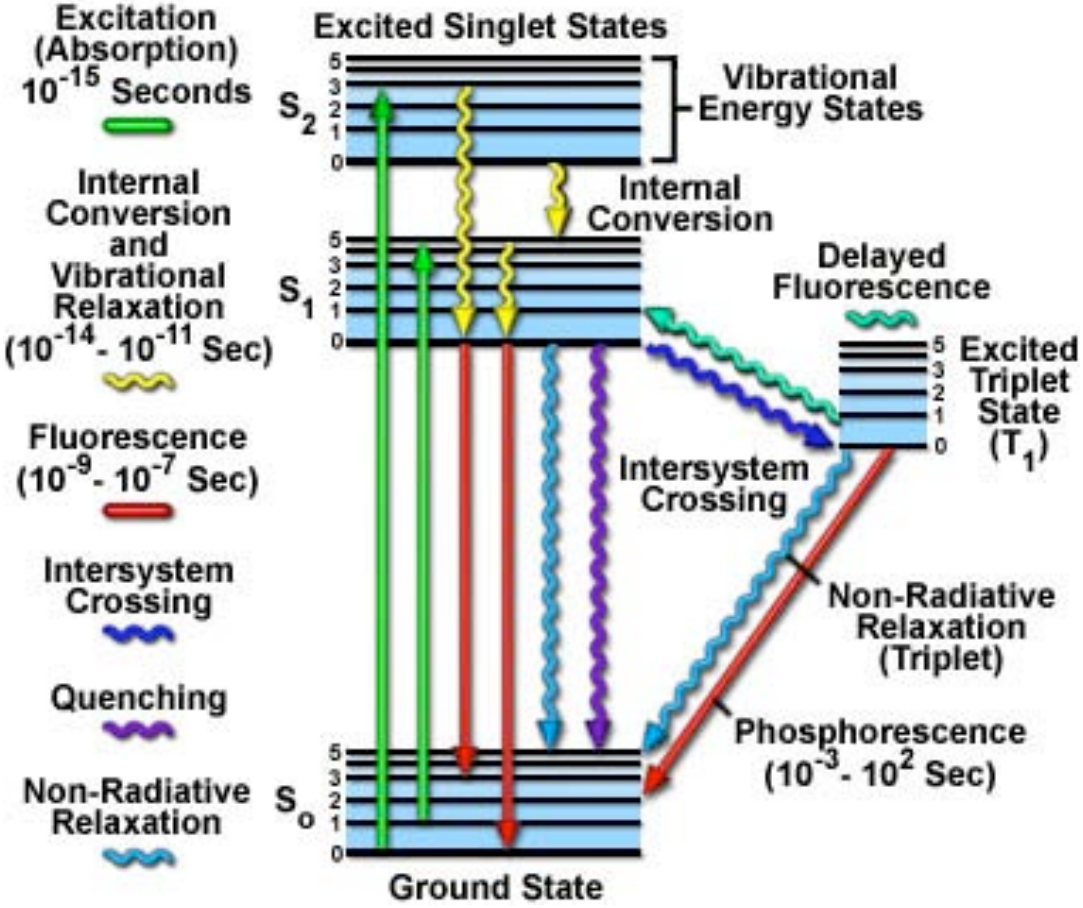
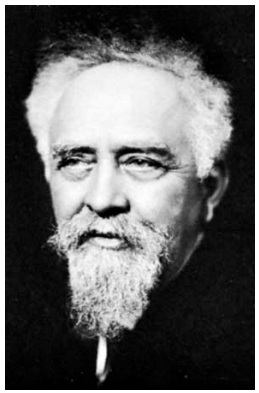


Figure 3

Perrin-Jablonski Diagram



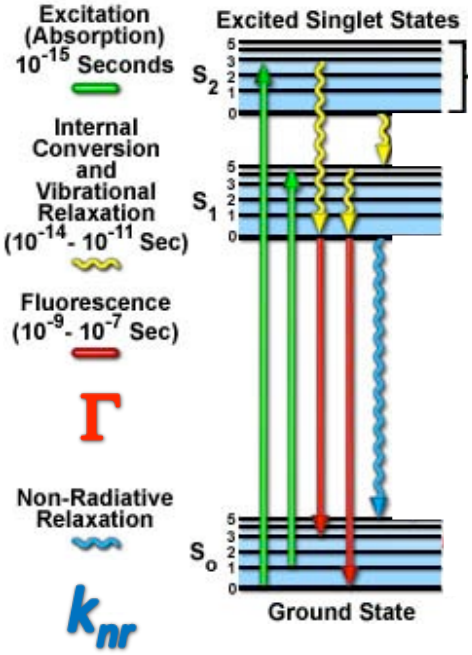
Alexander Jablonski
(1898-1980)



Jean Perrin
(1870-1942)

The diagram design is a (C) by Michael W. Davidson and The Florida State

Fluorescence properties



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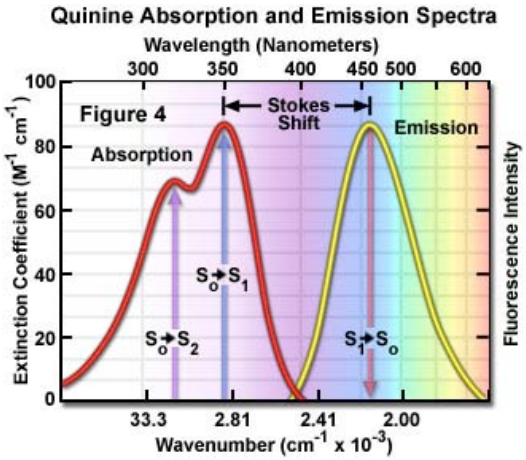

Fluorescence brightness

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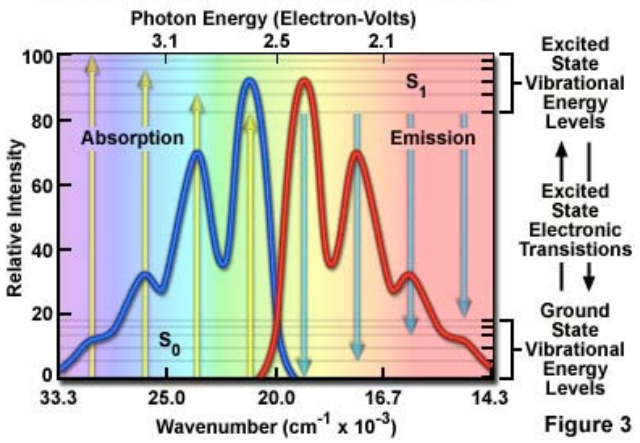
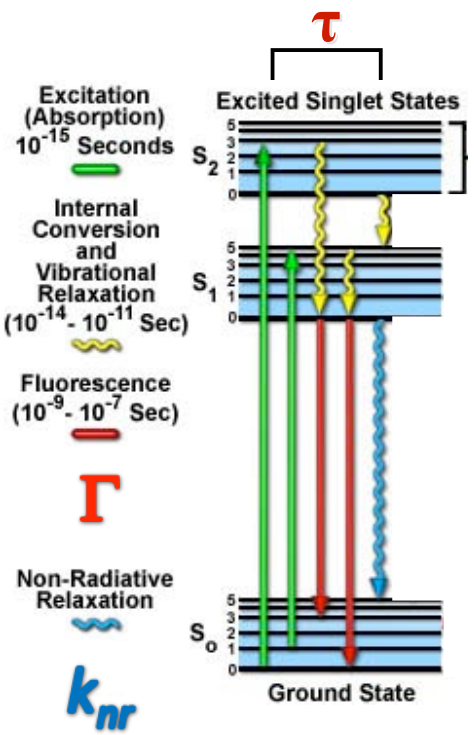


Figure 3

Fluorescence lifetime

Fluorescence Lifetime Definition



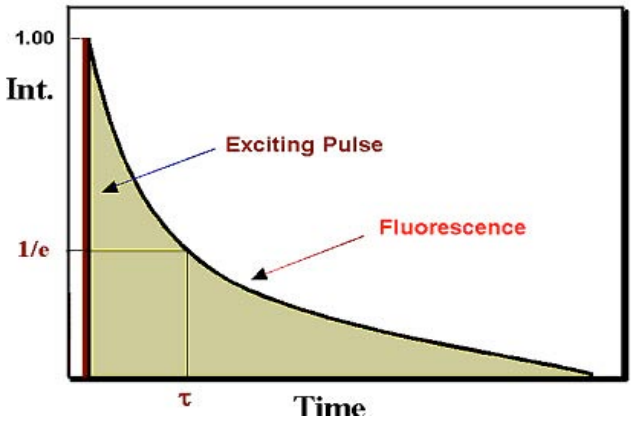
τ

The fluorescence lifetime [lifetime of the excited state] is defined by the average time the molecule spend in the excited state prior to return to the ground state.

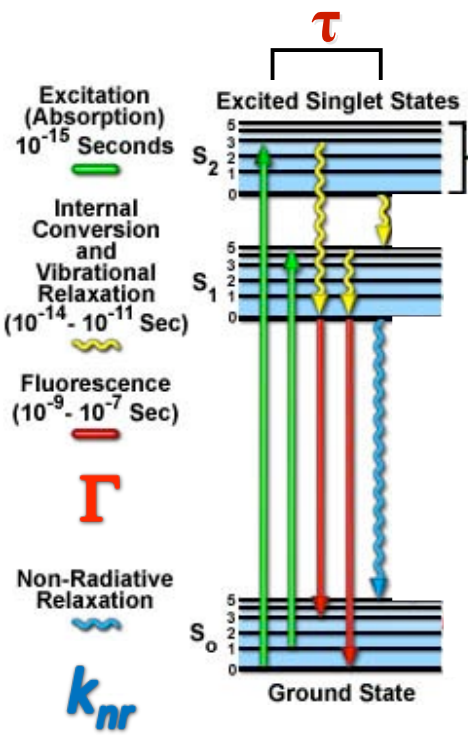
1. $\tau = \dfrac{1}{\Gamma + k_{nr}}$

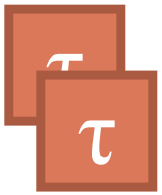
2. $n(t) = n_0 \exp(-t/\tau)$

3. $I(t) = I_0 \exp(-t/\tau)$



Multi-exponential decays





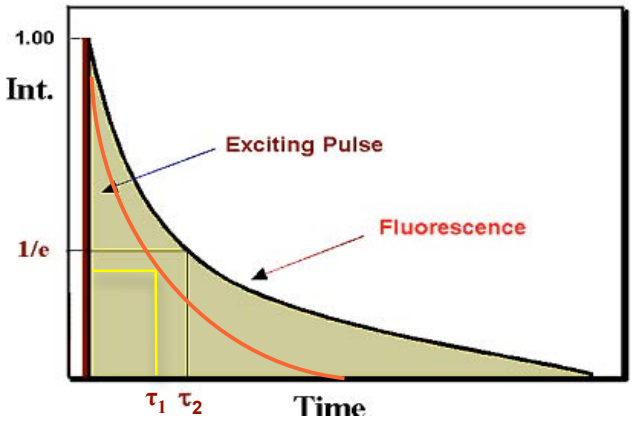
Multimple components

In case of two- and more components with different lifetime are present, the decay will become **multiexponential**.

1. $I(t) = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}$

α – pre-exponential factor

2. $I(t) = \sum_i \alpha_i e^{-t/\tau_i}$



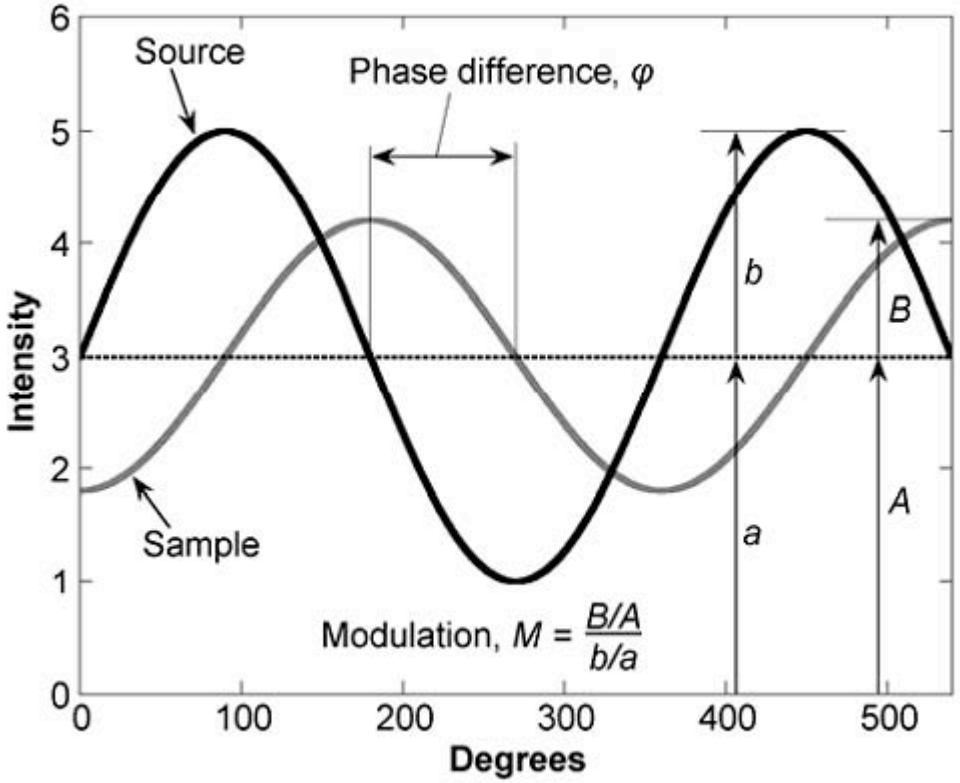
Measurement

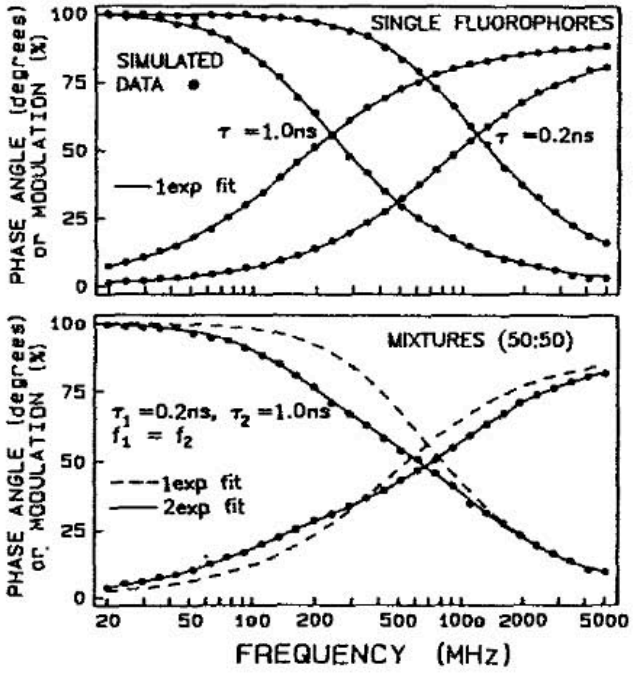
Following the excitation, the decay time is calculated from the slope of a plot of **log I(t)** versus **t**.

Time- and Frequency Domains

Frequency domain measurements

With the modulated light serving as a source for excitation, the emission is delayed in time.





$$\tan\varphi = \omega\tau_{\varphi}$$

Time-domain measurements

Analog
vs
Counting

The 3 steps:
Collection
Waveform restoration
Calculation

Summary

1. The **fluorescence** is a type of **luminescence** – property of compounds to emit light, when excited with different energy carriers.

2. The fluorescence has a number of properties, such as **spectra**, **quantum yield** and **lifetime**, whereas the fluorescence itself is not the only way of releasing the excess of energy (Perrin-Jablonski diagram).

3. Lifetime is a probability process, whereas excited molecules have the same probability of emitting a photon over some period of time – a decay law.

4. The lifetime is well described by exponential decay.

5. The lifetime is measured currently mostly with frequency and time-domain measurement techniques.

TIME-CORRELATED SINGLE PHOTON COUNTING

History

1926 — First experiment of lifetime measurement by Gaviola. Phase fluorometry applied.

1984 — D. O'Connor and D. Phillips. First monography on TCSPC

TCSPC is used primarily to record fluorescence decay of dye solutions in cuvettes.

+ Amazingly sensitive and accurate technique with excellent time-resolution
- Slow acquisition, single dimension.

The Becker&Hickl Era.

1983 — SPC-100, the first model of TCSPC board

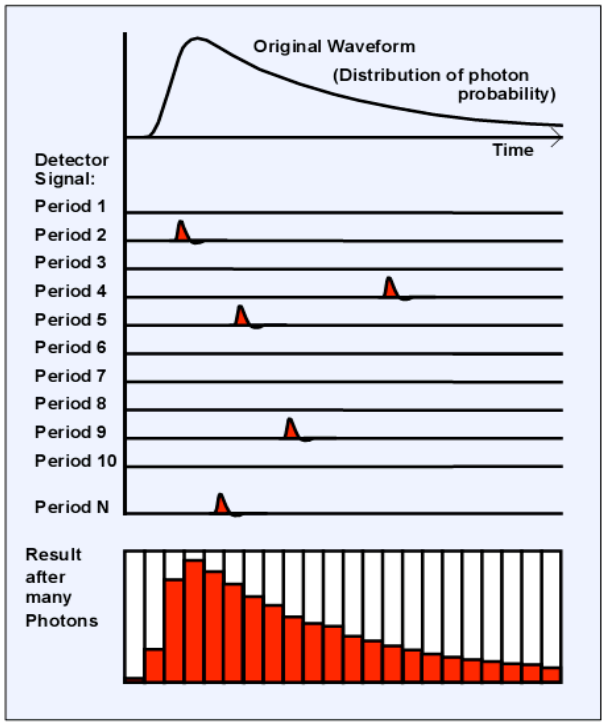
1992 — FPGA-based board introduced: the TCSPC on the rise.

1992 — Introduction of the first multidimensional system. 100 times faster than before!

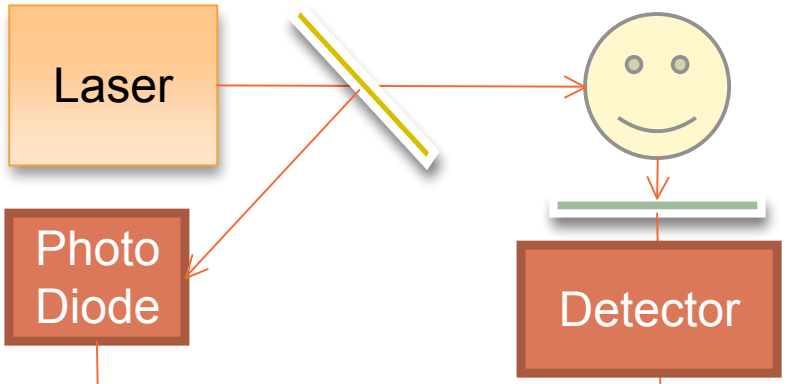
The principle

The task:
to reconstruct the fluorescence decay waveform.

Solution:
repetitive excitation and memory channels.



Building from the scratch



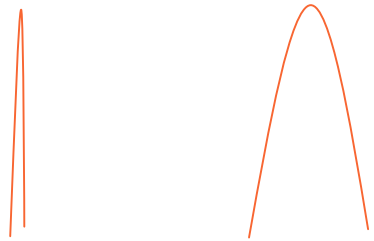
- Detect the time of photon arrival in a high-precision manner.
- Maintain low level of deviation.
- Assign each photon to the corresponding memory channel

TCSPC

The instrumentation: Light sources

The light source:

The δ-function consideration



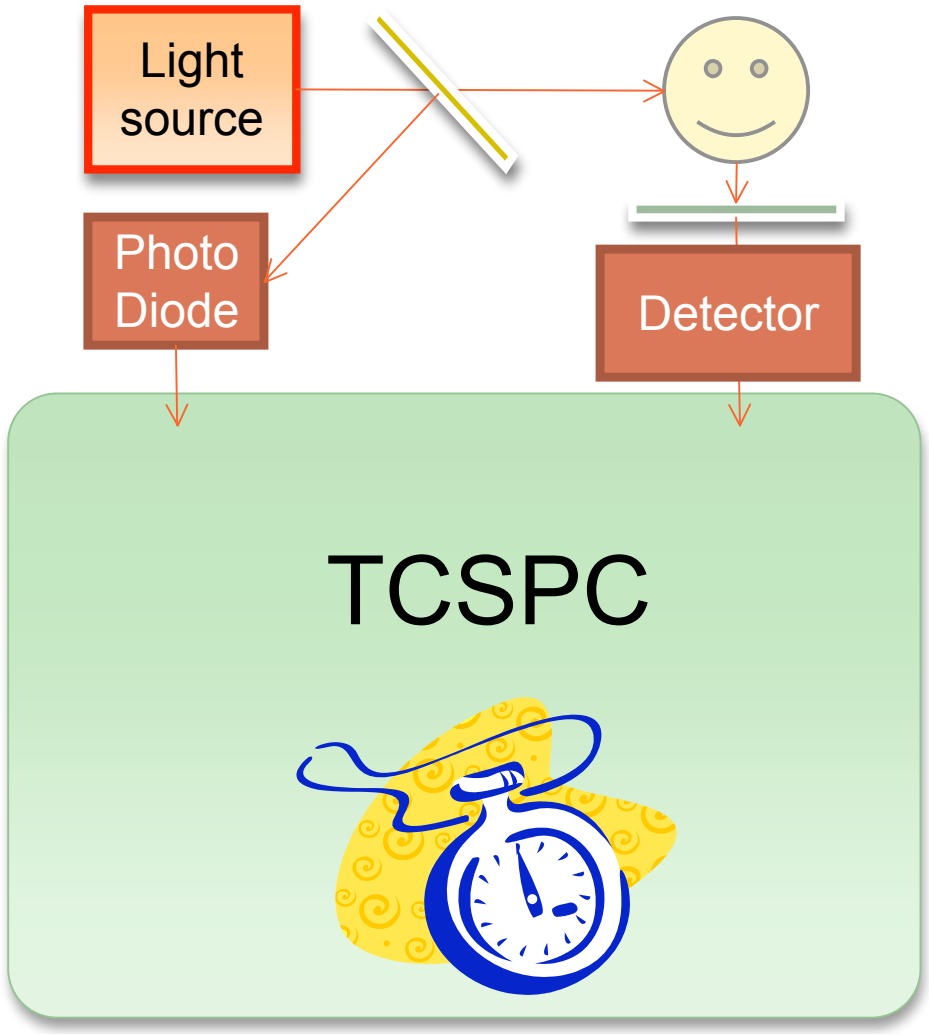
We want

We get

The evolution

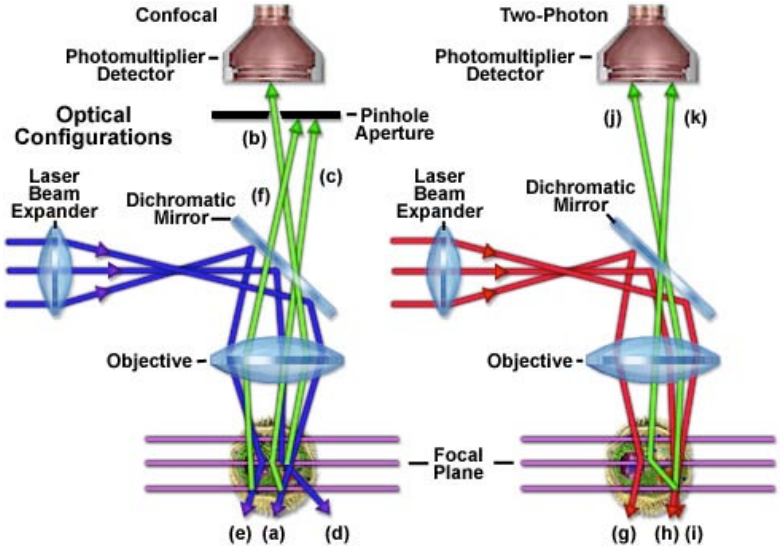
Nanosecond flashlamp	2ns, 50KHz
Cavity pumped dye lasers	5ns, 80MHz
The Ti: Sapphire: the One	100fs, 80MHz

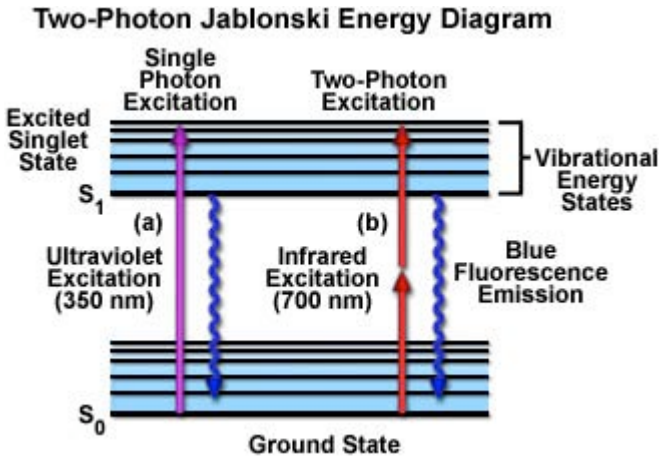
Building from the scratch

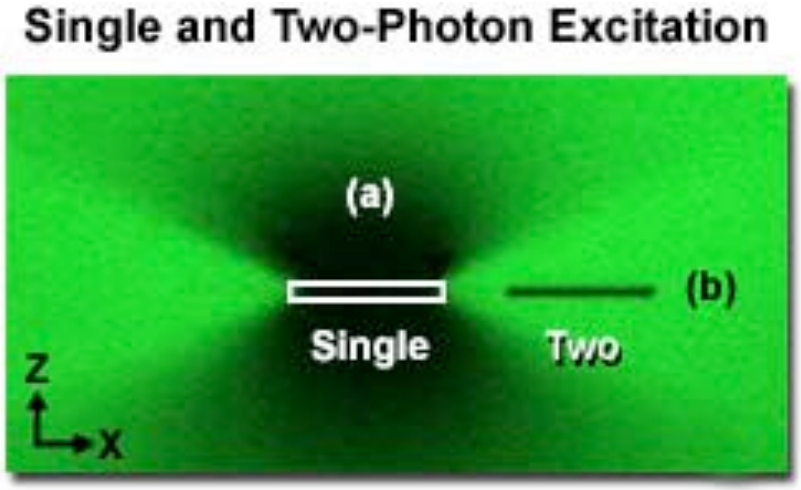


The instrumentation: The modality

The excitation:

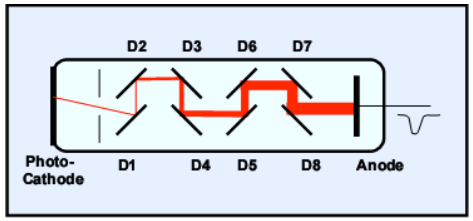




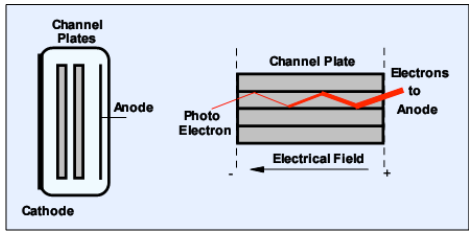


The instrumentation: Detectors

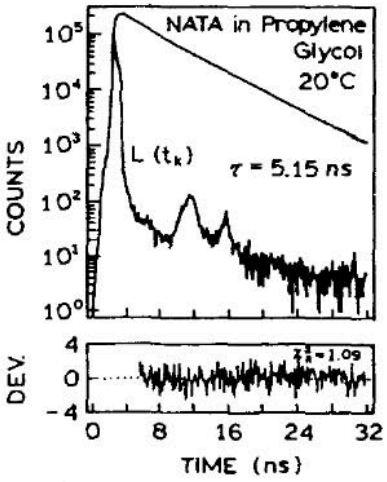
The Detector:

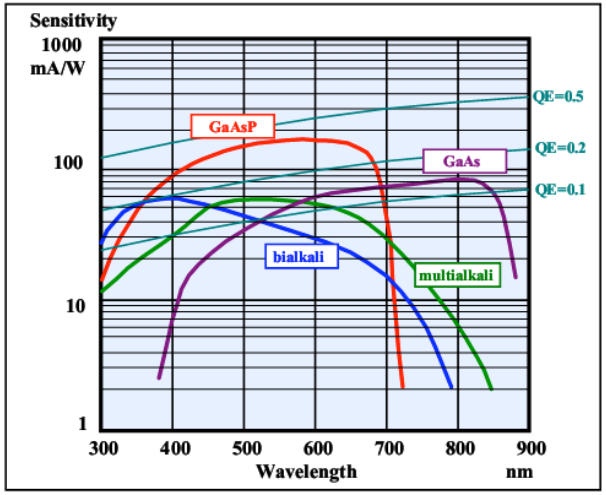


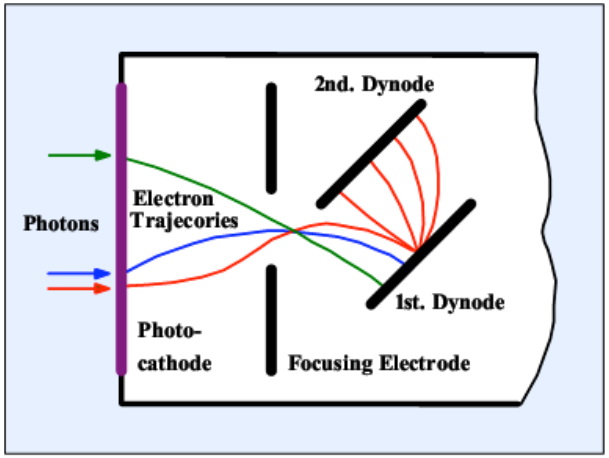
PMT
Photo
Multiplier
Tube



MCP
Multi
Channel
Plate







Parameters & limitations

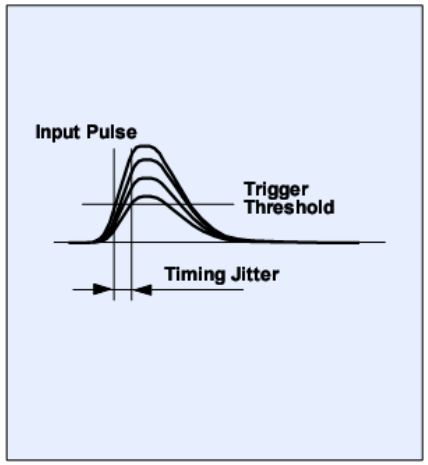
- Transit-time spread (!)
- Height distribution (jitter) (!)
- Spectral response
- Dark count rate
- Afterpulsing
- Quantum efficiency

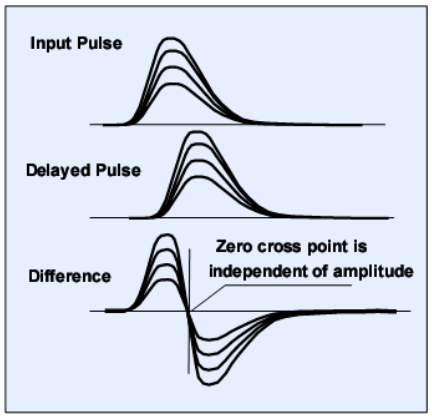
TCSPC

The instrumentation: CFDs

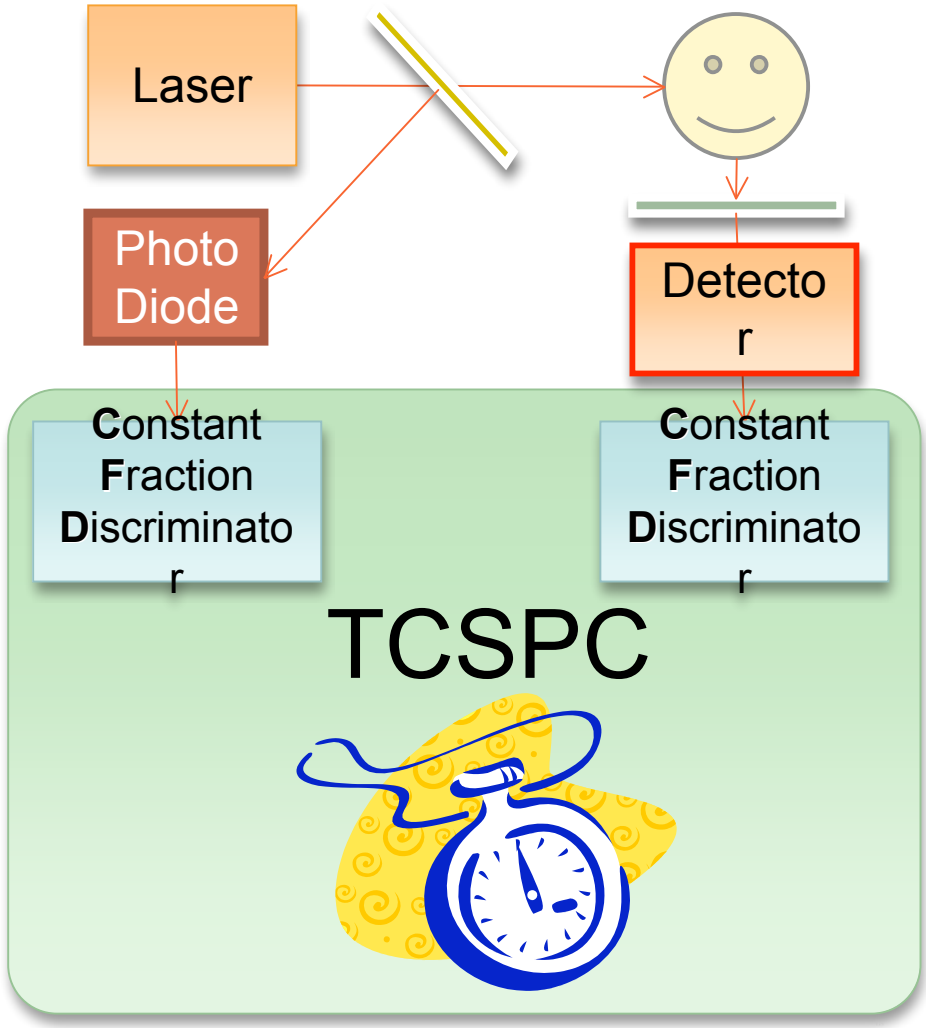
The Constant Fraction Discriminator

- Height distribution (jitter) (!)
- Dark Count





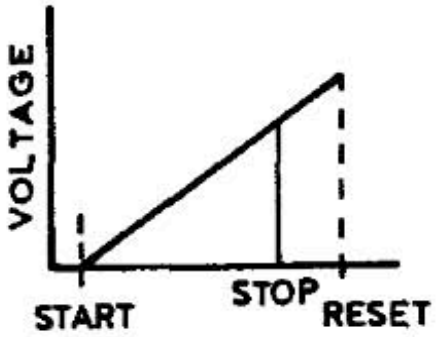
Building from the scratch

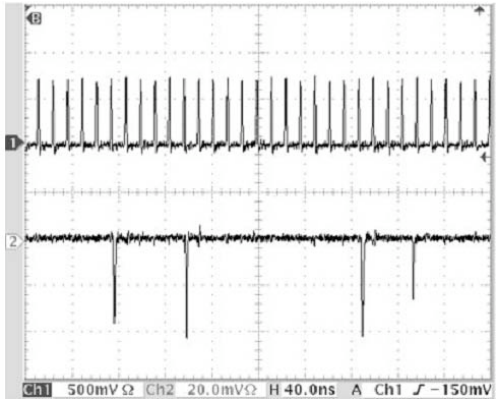


The instrumentation: TACs

The Time to Amplitude Converter

The TAC Principle





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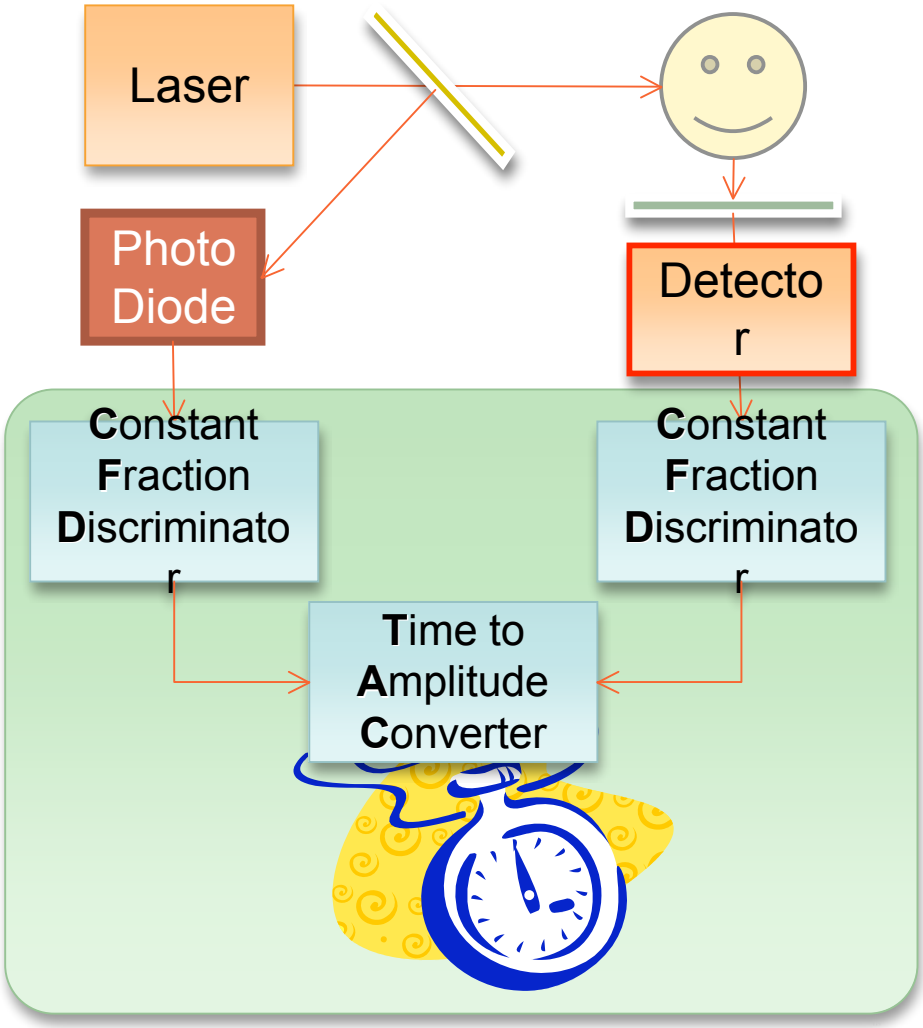
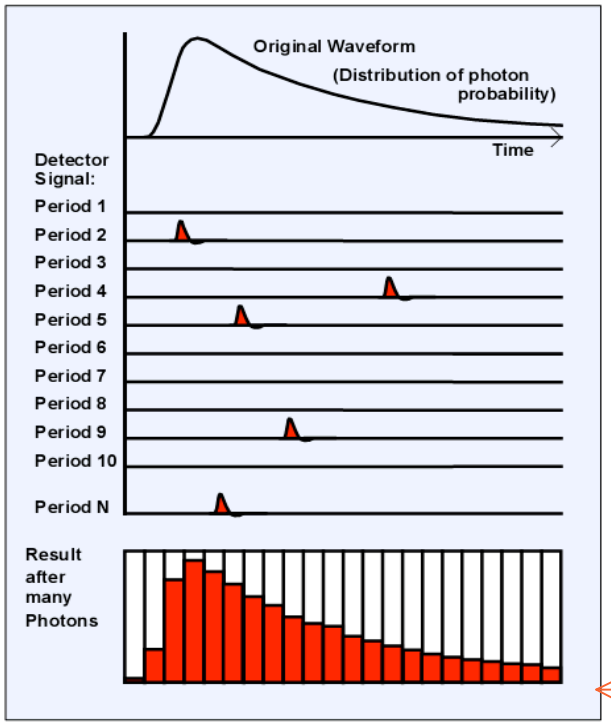


TCSPC

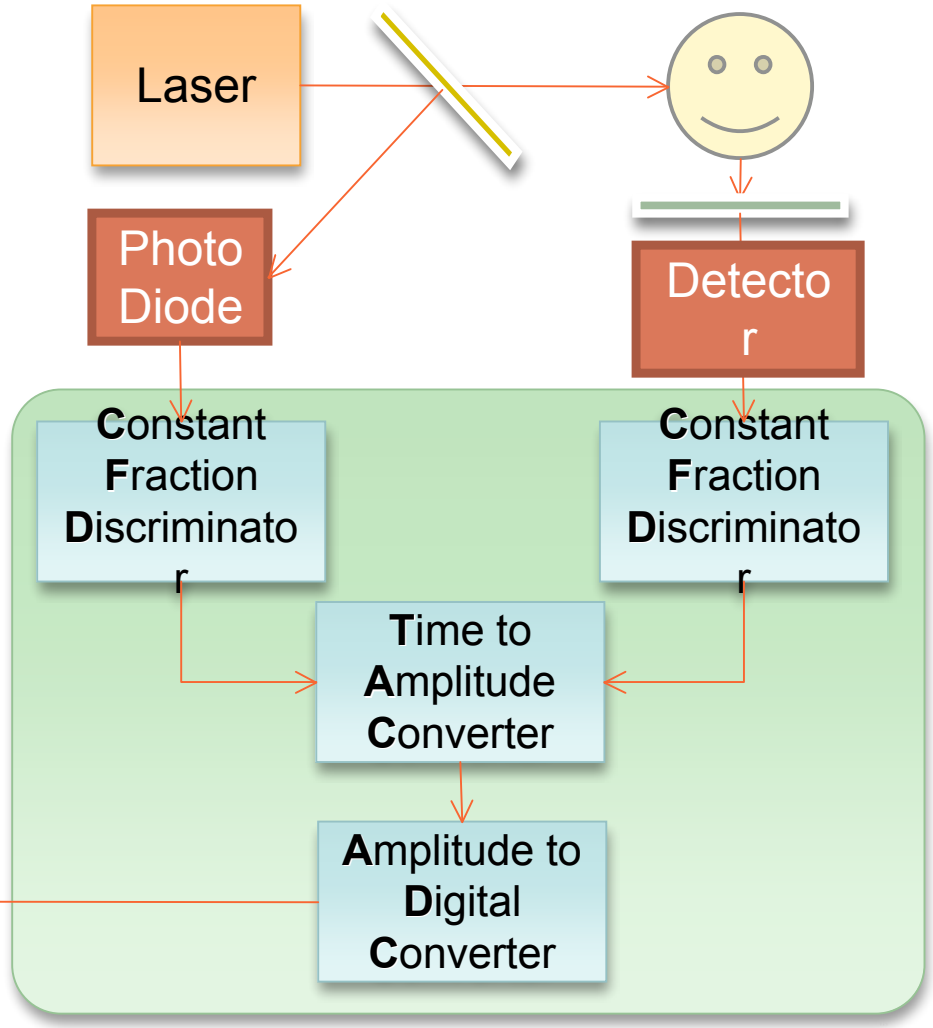
The instrumentation: ADCs

The task:
to reconstruct the fluorescence decay waveform.

Solution:
repetitive excitation and memory channels.

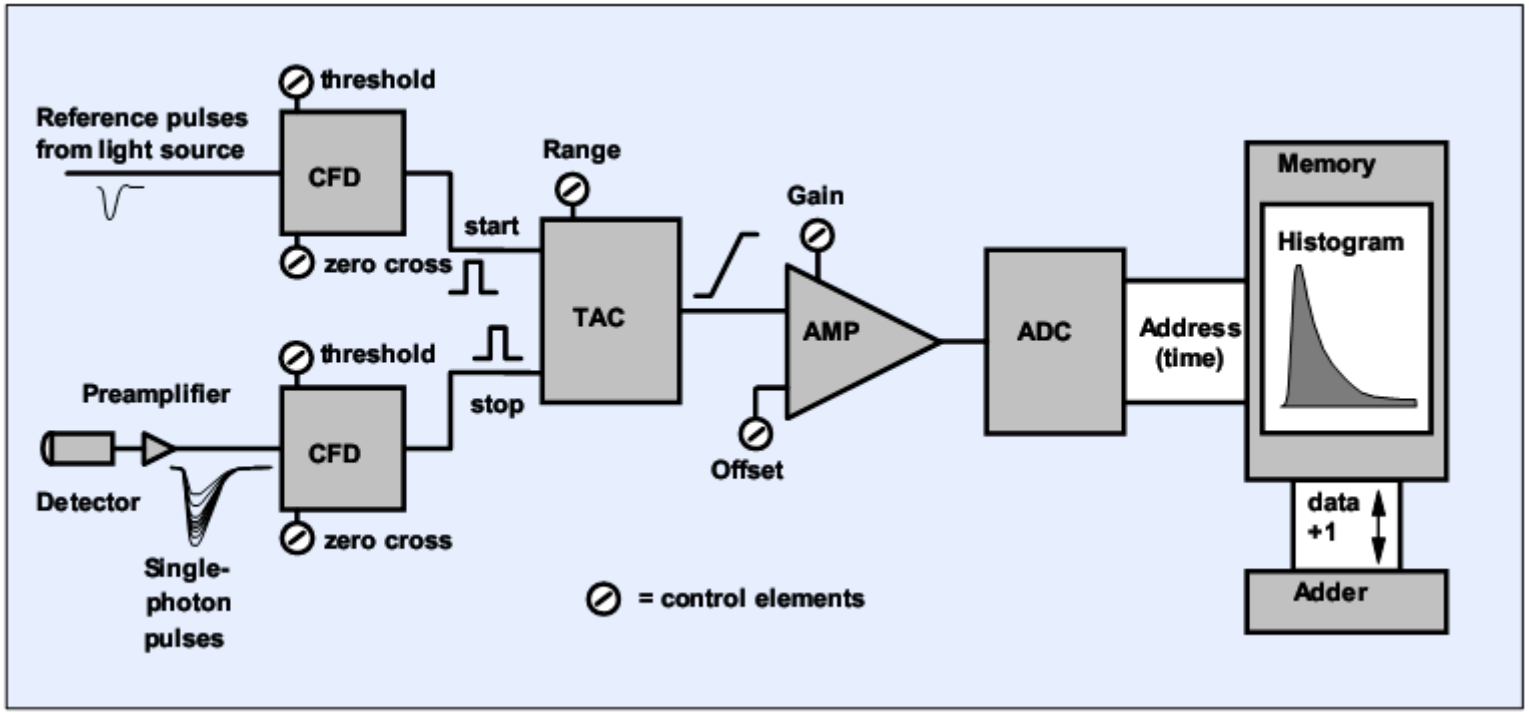


The stopwatch concept:
The measurement units: laser pulses
Arrival of the photon – start/stop



TCSPC

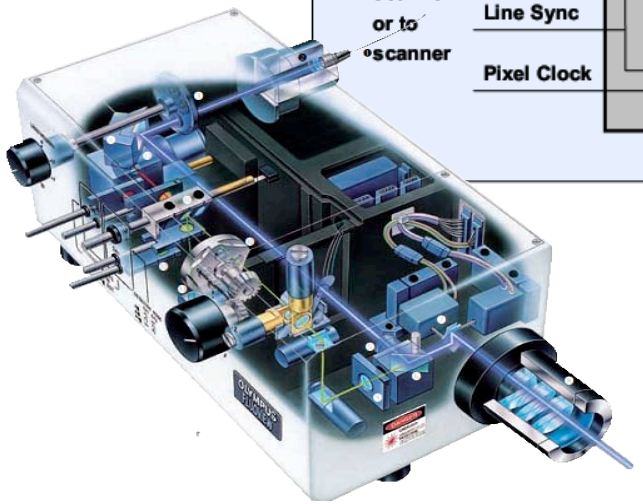
The classical scheme

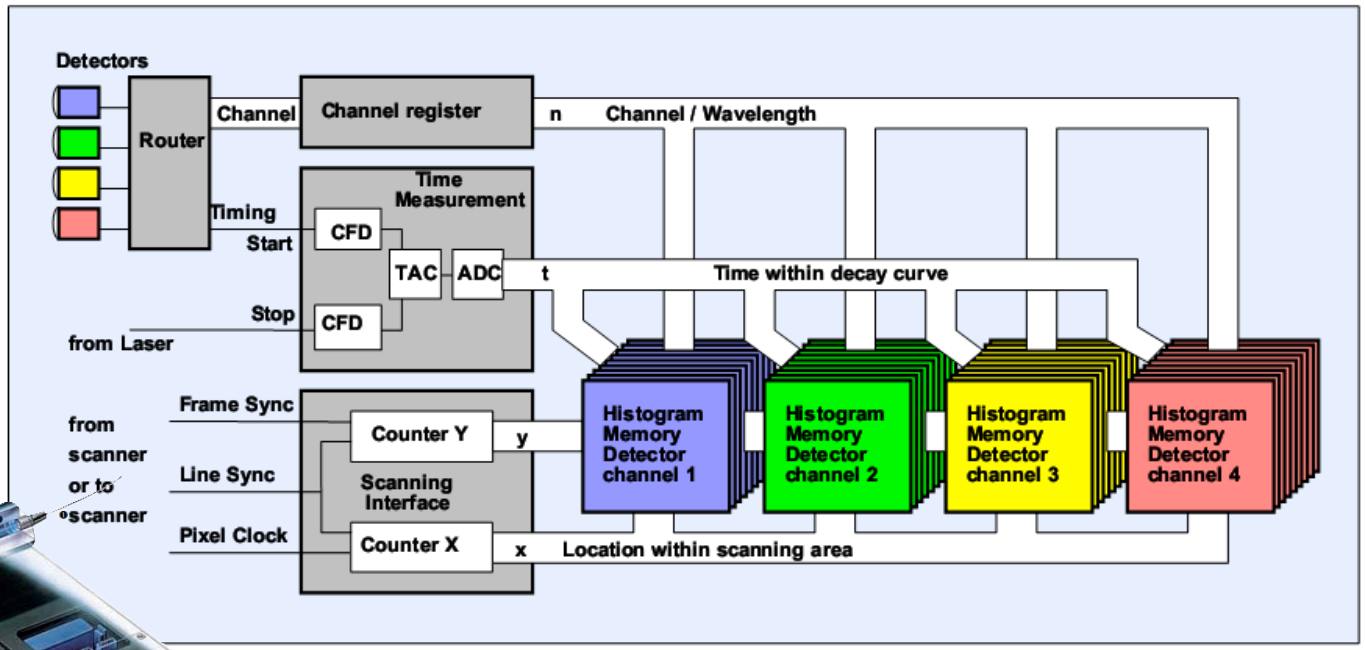


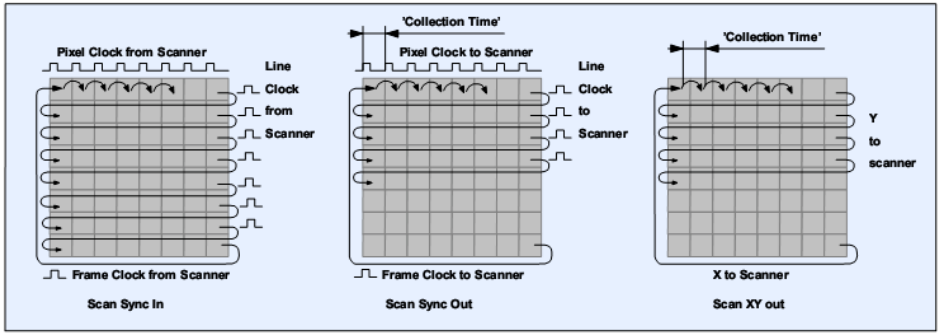
Start-stop problem and reversed mode

Introducing new dimensions: spatial

Multidimensional TCSPC:

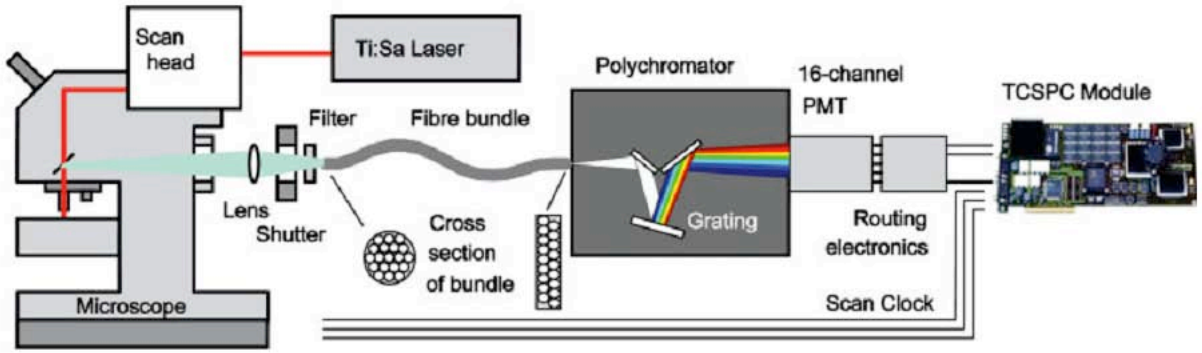


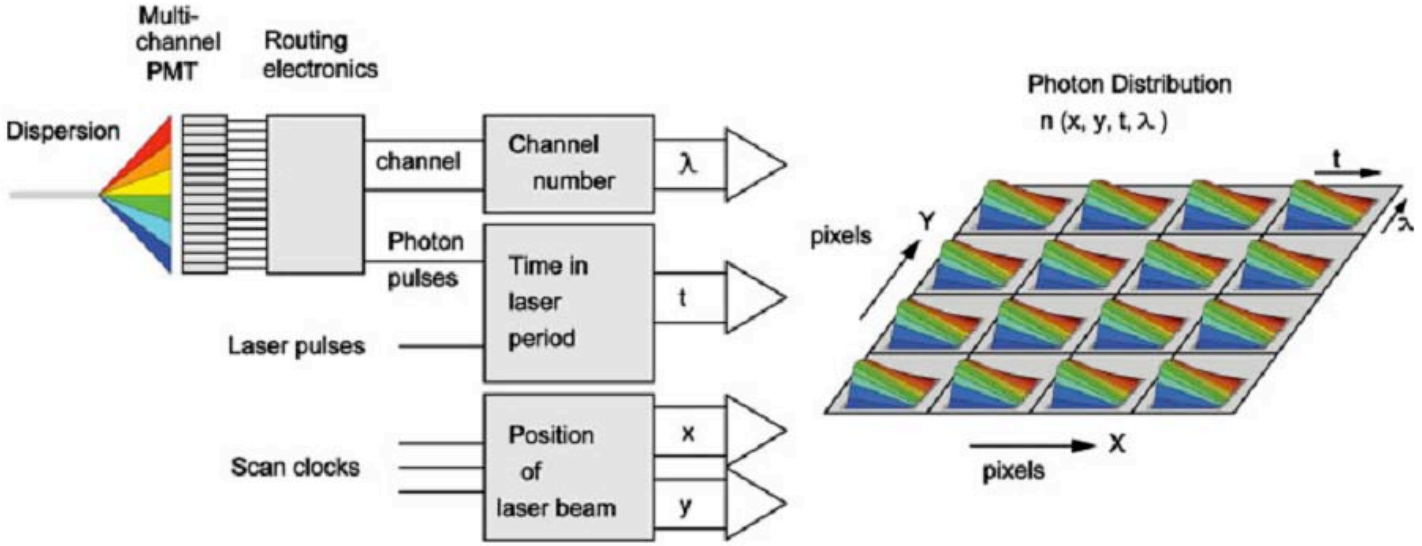




Introducing new dimensions: spectral

Multidimensional TCSPC:





Introducing new dimensions: spectral

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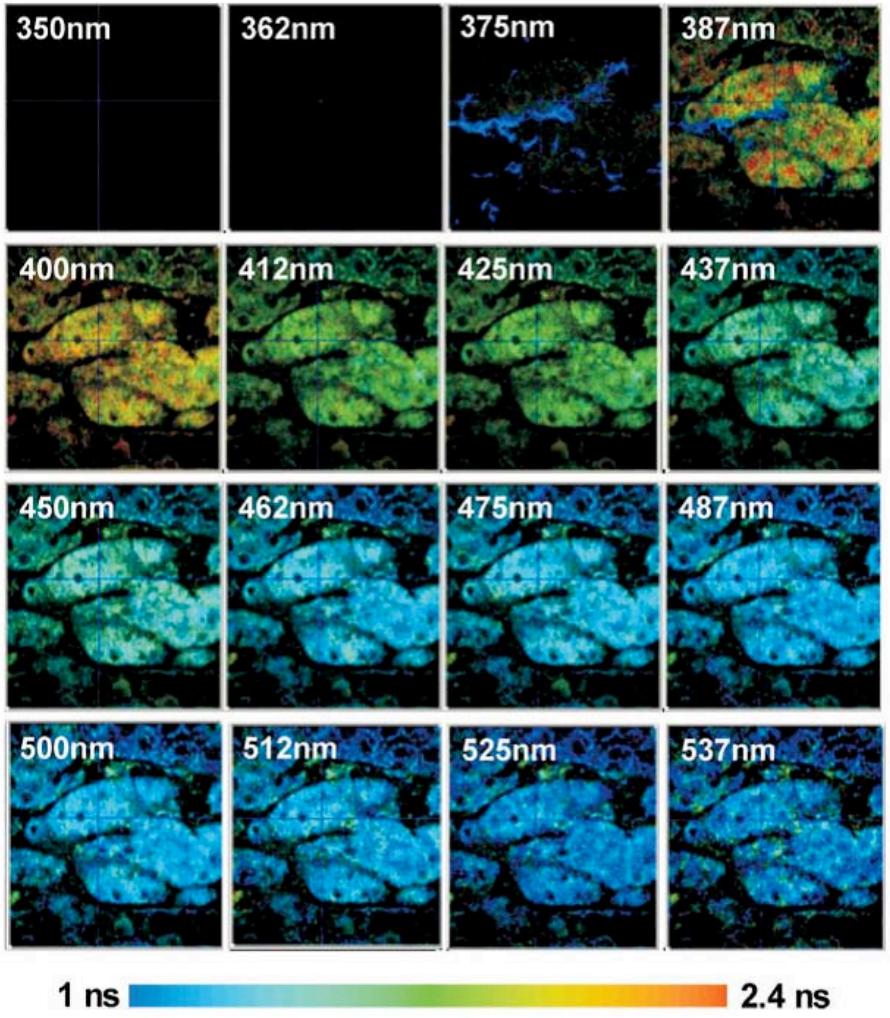


Introducing new dimensions: spectral

Multidimensional TCSPC:

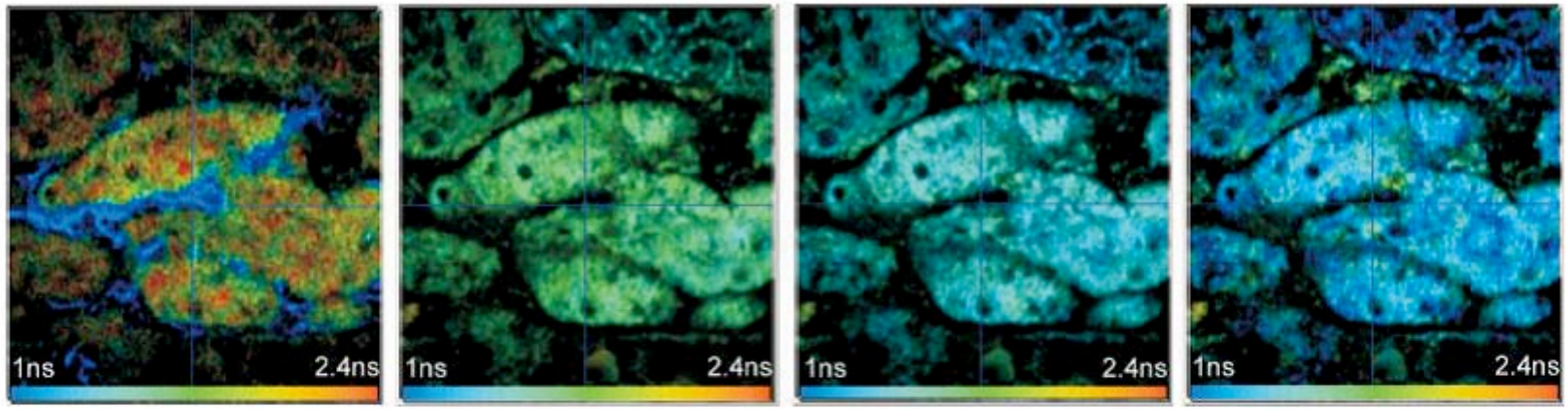
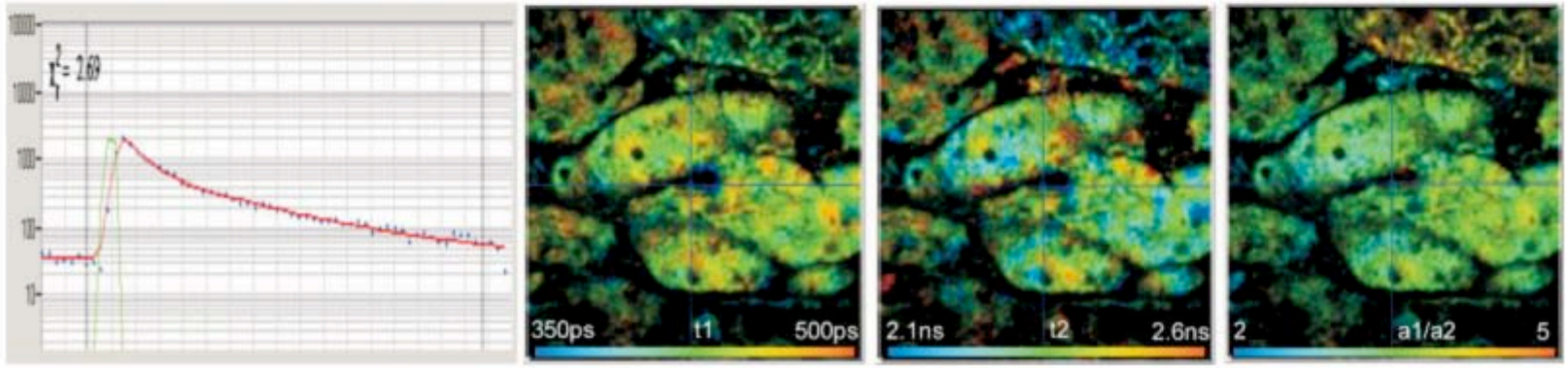
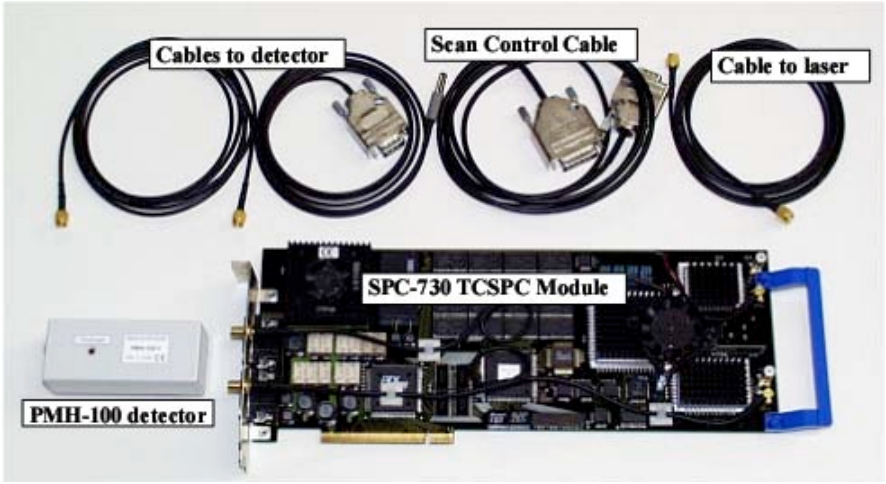


Fig. 5. Single-exponential lifetime images in the wavelength intervals from 350 to 387 nm, 400 to 437 nm, 450 to 487 nm, and 500 to 537 nm.



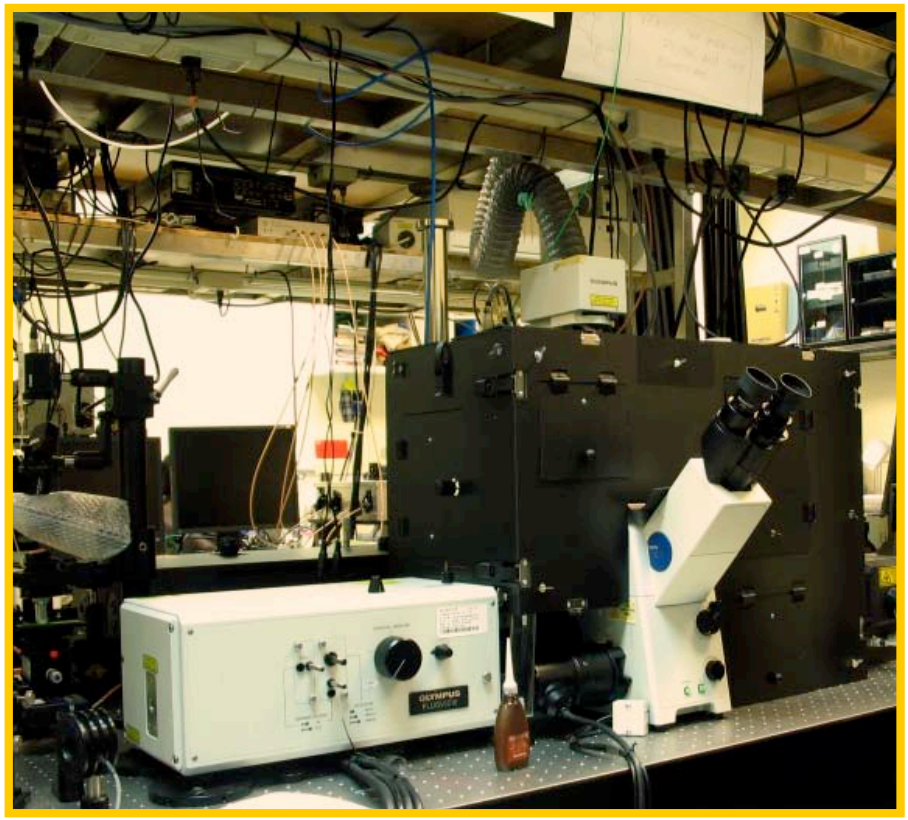
The instruments in reality

The Board



Detectors

The real lab



The TCSPC characteristics

Time resolution:

Not limited by the standard Single Electron Response (SER), which limits the resolution of the analog techniques. The TCSPC is rather dependent on the Transit Time Spread (TTR), which varies in the range of 15-400 ps depending on the detector. The resolution of the board is fs per channel.

Counting efficiency:

$$SNR = \sqrt{N}$$

Near-ideal efficiency

Sensitivity:

$$S = \frac{(Rd * N/T)^{1/2}}{Q}$$

Rd – dark count rate (300 s^{-1}),
N- number of time channels (256)
T – measurement time (100s)
Q – quantum efficiency (0.1)

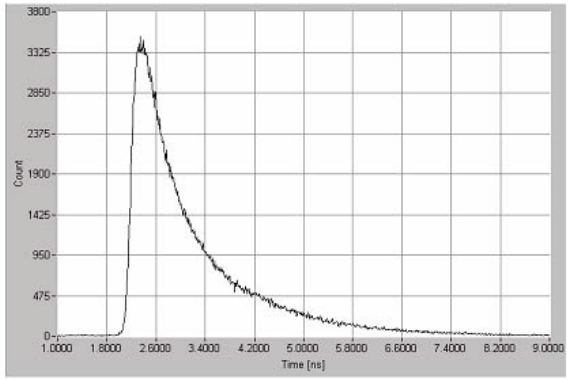
S=280 photons/s

Count rate:

Pile-up problem and insignificance for the TCSPC. Possible rates: $5x10^6$/s

Acquisition time:

More than 300.000 in 100 ms.



Summary

1. The **TCSPC** systems were sensitive and precise from the beginning, however, limitation in the light source repetition rate and slow electronics made the acquisition times enormously big.

2. The basic principle of the TCSPC is based on the repetitive excitation, collection of the photons, placing them in the corresponding memory channel and further building of the decay curve.

3. Upon the introduction with the high-repetition rate lasers with ultra-short pulses (Ti:Sapphire) the main limitation factor of the system is the detector with the transit time spread around 300ps, and time uncertainity introduced by afterpulses, dark noise, amplitude jitter and color sensitivity.

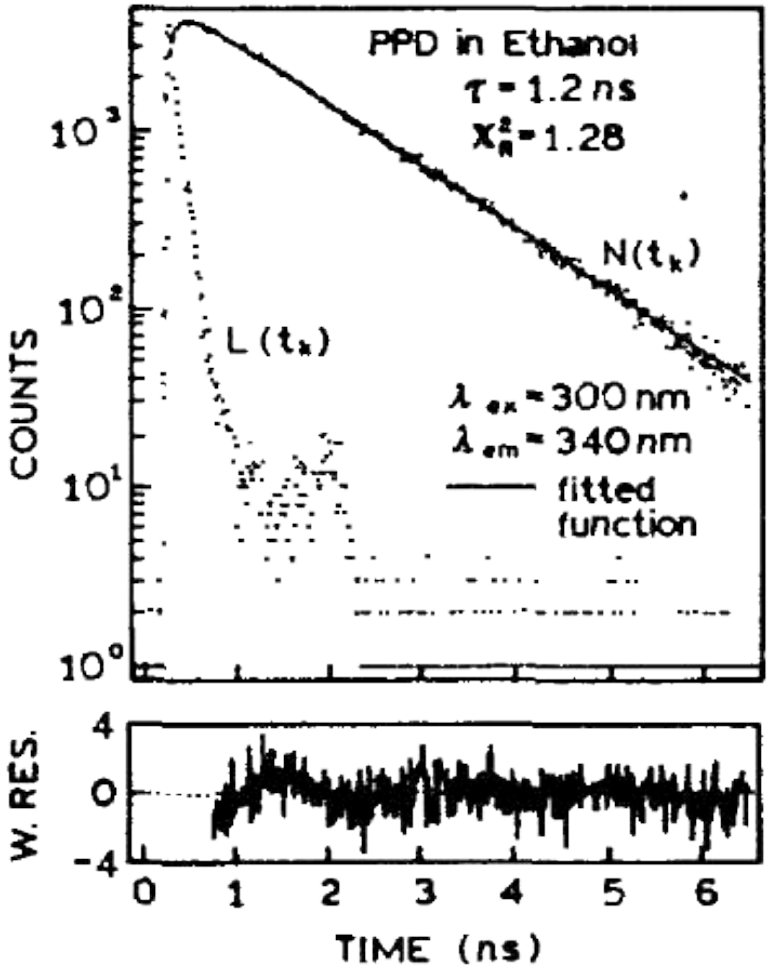
4. The limitations of the PMTs are addressed by the electronics of TCSPC with the noise and jitter filltered and corrected by the CFDs, high-precision working TAC and ADCs.

5. Currently additional modalities are introduced to the market: multidimensional TCSPC allows to correlate lifetime with other parameters, such as spatial coordinates (scanning) and spectrum-resolved.

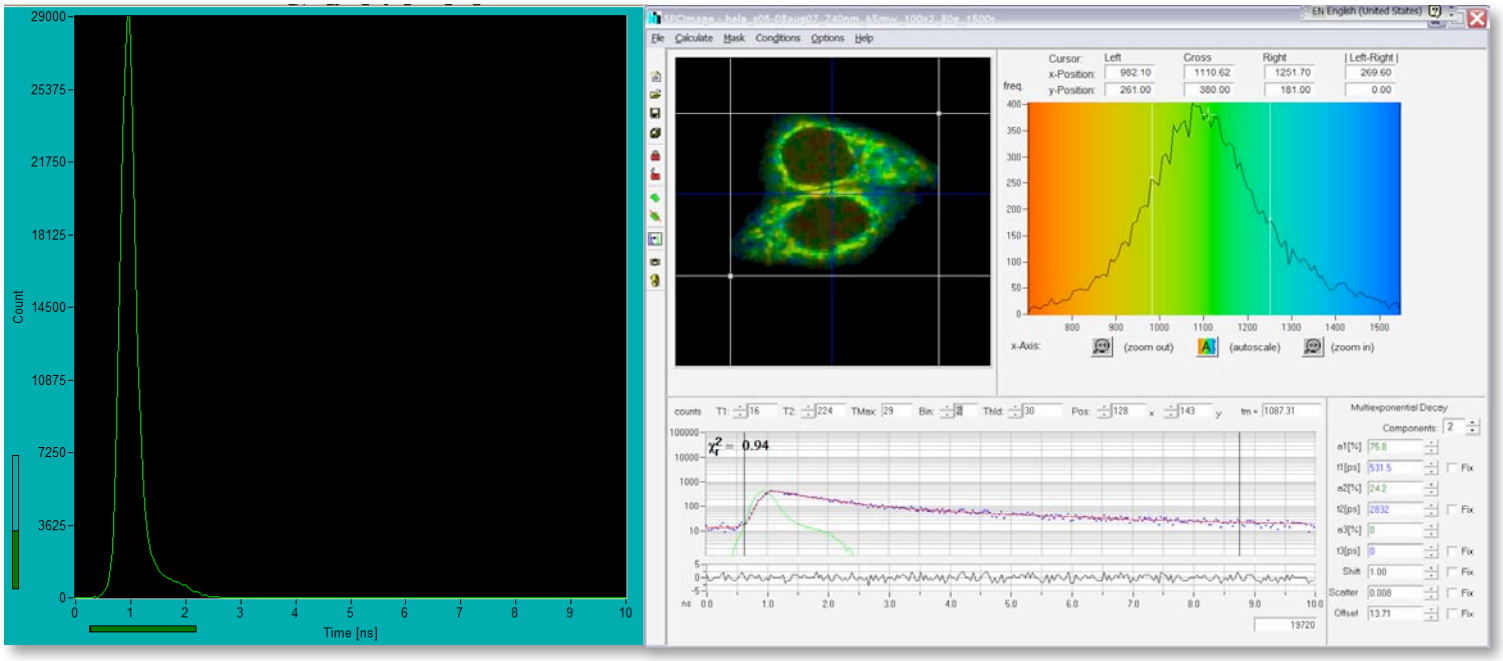
DATA ANALYSIS

Got the data. What next?

- Development of the decay model.
- Calculation of the Instrument Response Function.
- Convolution of the model with IRF
- Fitting of the resulting model with the data.



The Instrument Response Function

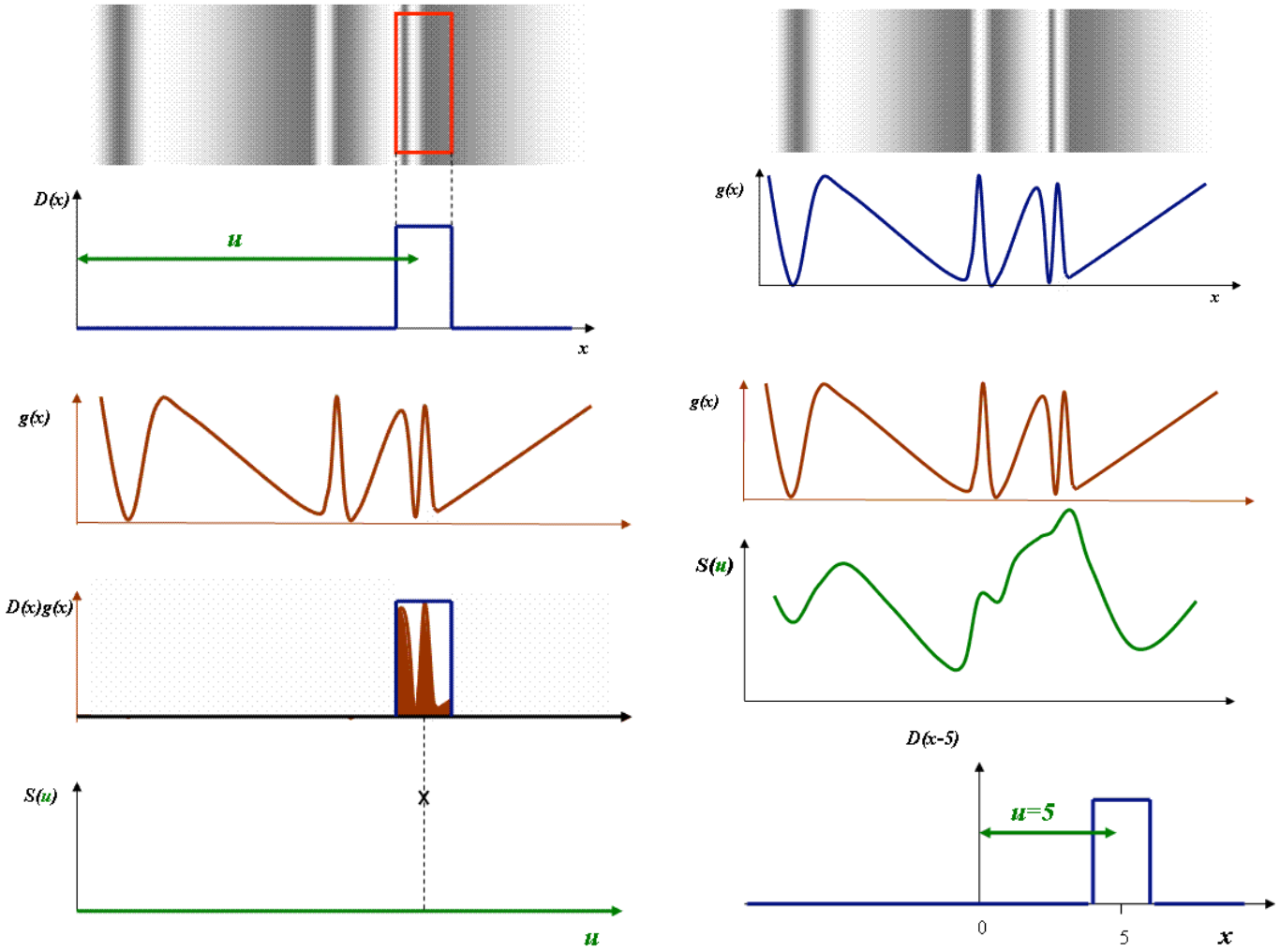


Measurement:

-Scattering for single-photon excitation

- Second Harmonic Generation for 2-photon excitation.

The Instrument Response Function



$$f(x) = g(x) \oplus h(x)$$

$$S(u) = \int g(x)D(x-u)dx$$

Deriving the model function

$$I_0 + \sum_{i=1}^{n} a_i \left(\exp\left(-t/\tau_i\right)\right)$$

Data set for analysis

Instrument response (IRF)

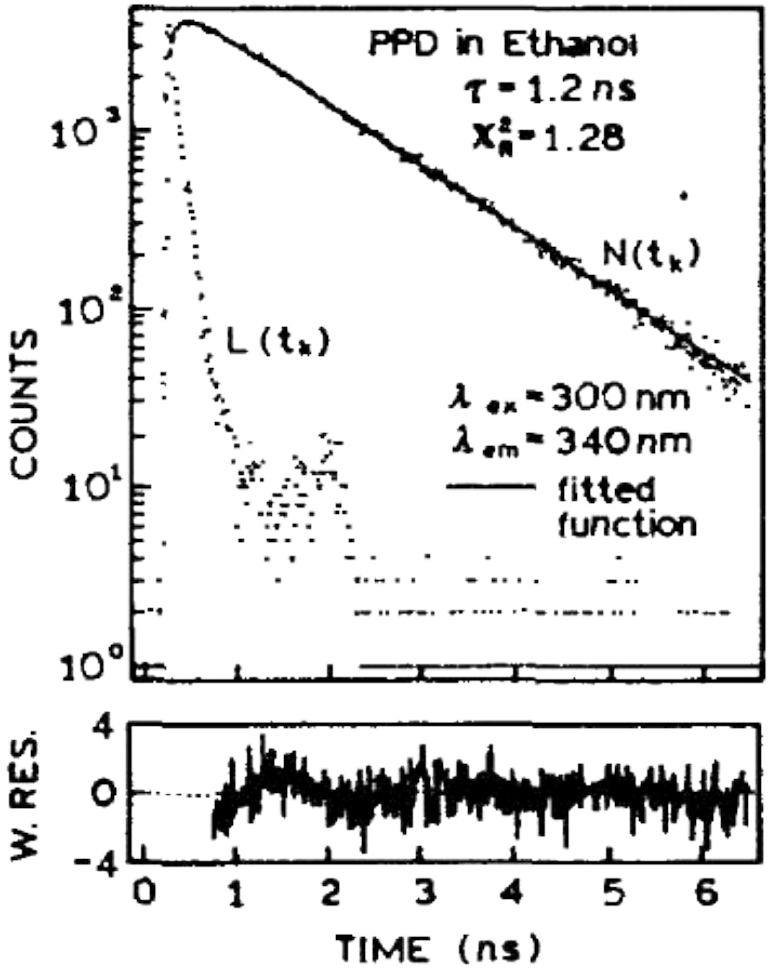
+ N(t_k) Measured photons

distribution over **t**

+ Nc(t_k) Calculated decay

+ Residuals

$$I(t) = \int_{-\infty}^{\infty} L(t_k)\left\{I_0 + \sum_{i=1}^{n} a_i \left(\exp\left(-t/\tau_i\right)\right)\right\} dt$$



Convolution improves the temporal resolution

Fitting the data

Standard deviation

$$\sigma_k = [N(t_k)]^{1/2}$$

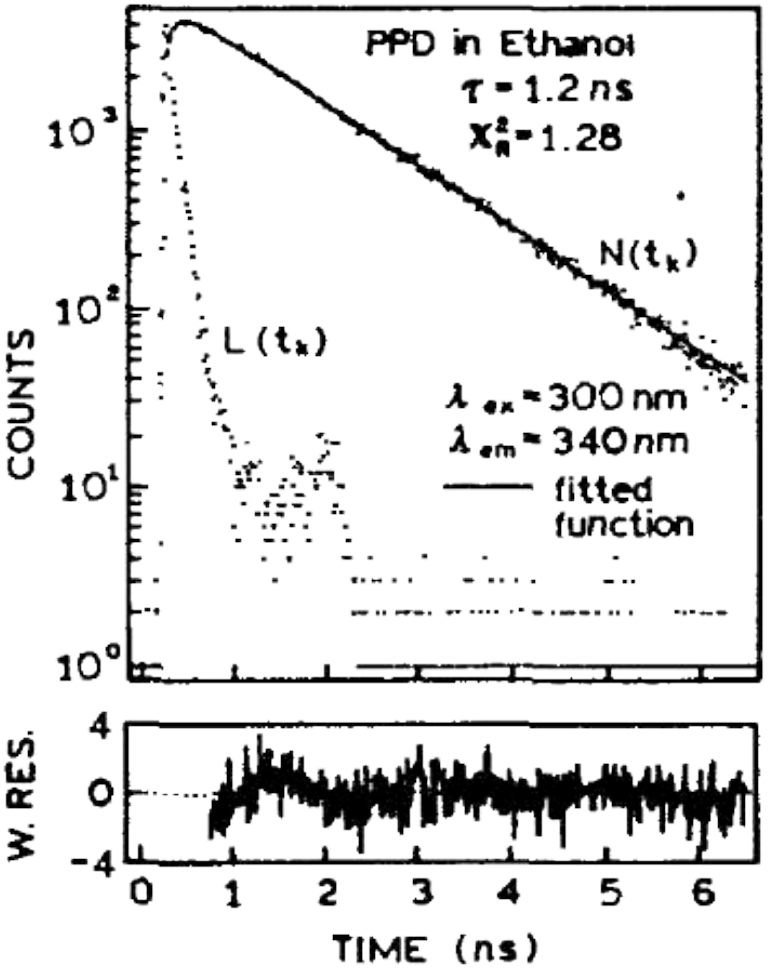
Goodness-of-fit parameter calculation

$$\chi^2 = \sum_{k=1}^{n} \frac{[N(t_k) - N_c(t_k)]^2}{N(t_k)}$$

Reduced goodness-of-fit

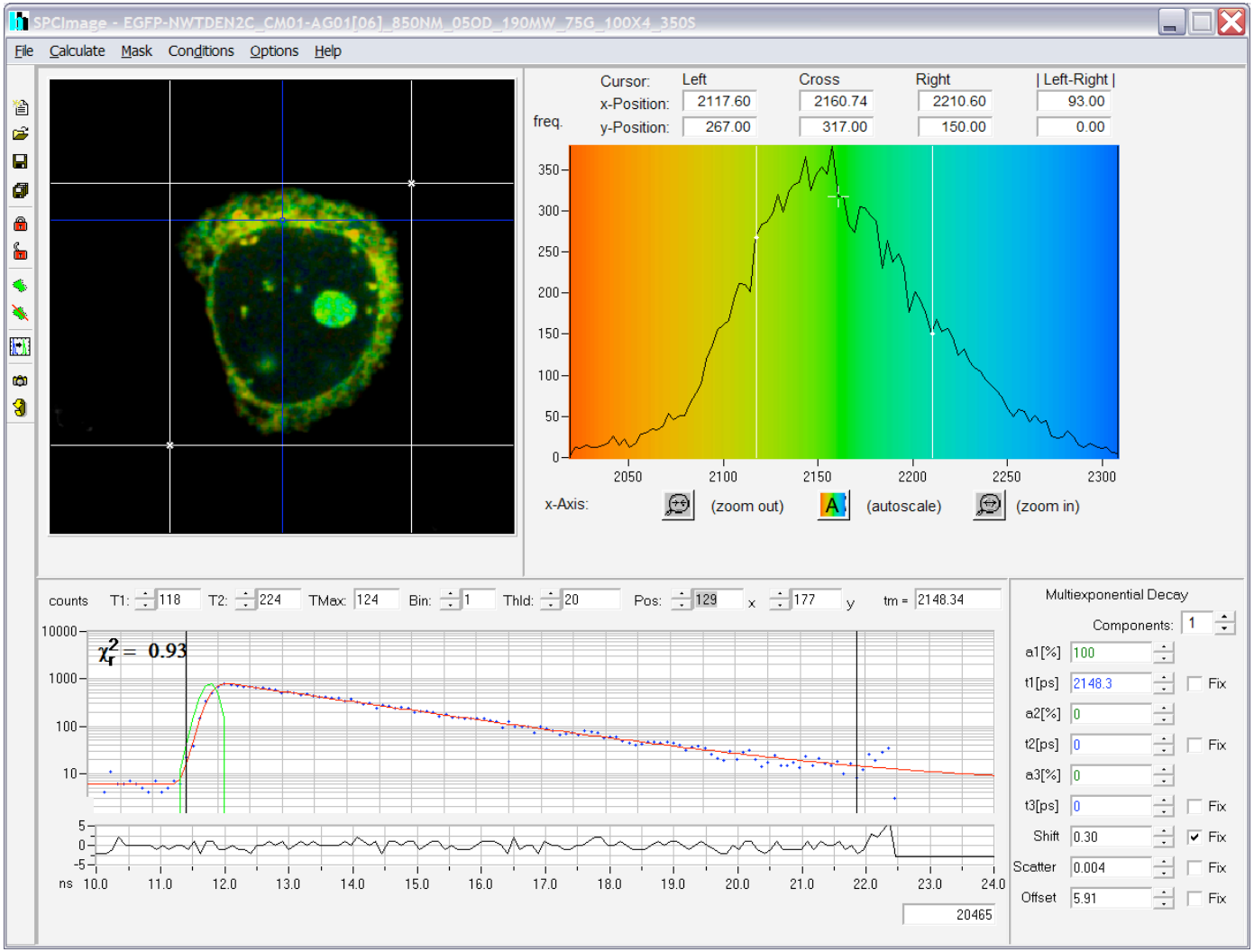
$$\chi_R^2 = \frac{\chi^2}{n - p}$$

Where
n- number of time channels,
p – number of parameters



Levenberg-Marquardt Algorithm.

Color-Coding



Summary

1. The data obtained is convolved by the Instrument Response Function. To restore the original values and increase the temporal resolution a separately measured Instrument Response Function is convolved with the model function.

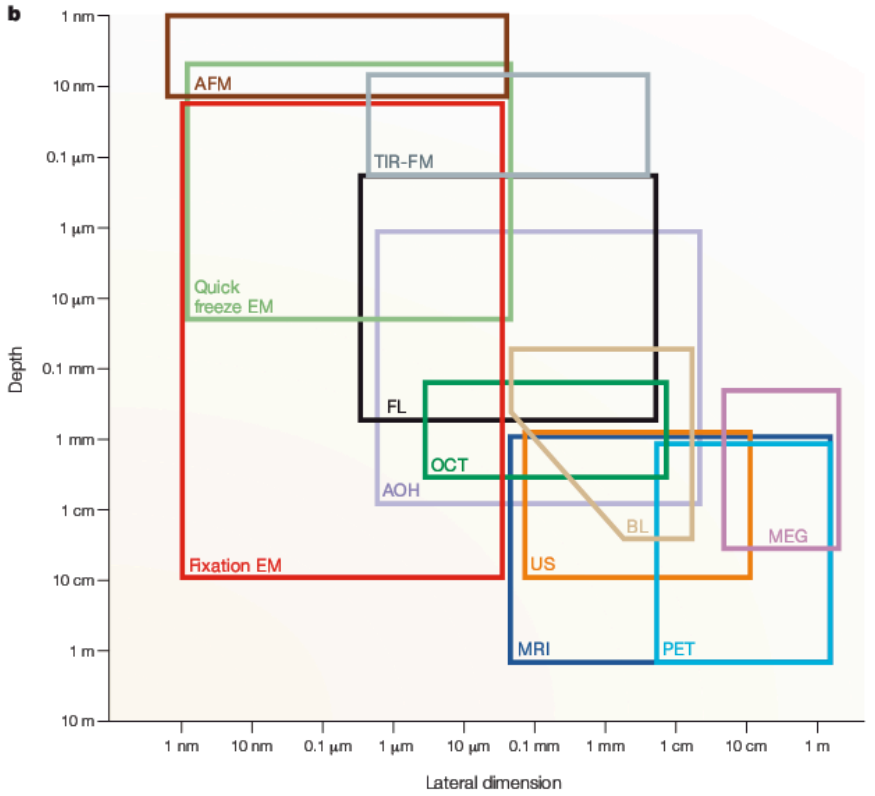
2. Upon convolution the temporal resolution increases almost x10.

3. After the convolution the fitting procedure is applied to let the model function follow the measured decay.

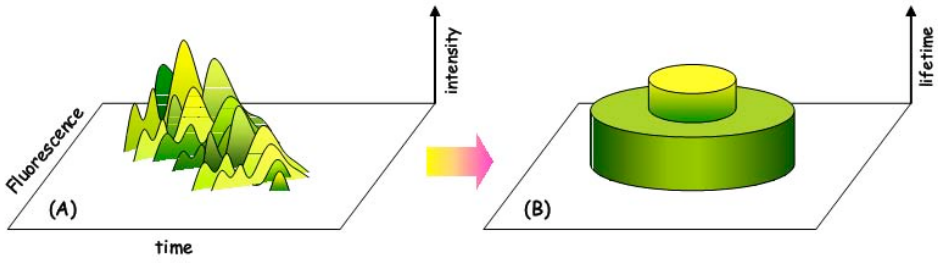
THE APPLICABILITY OF FLIM

FLIM and the others

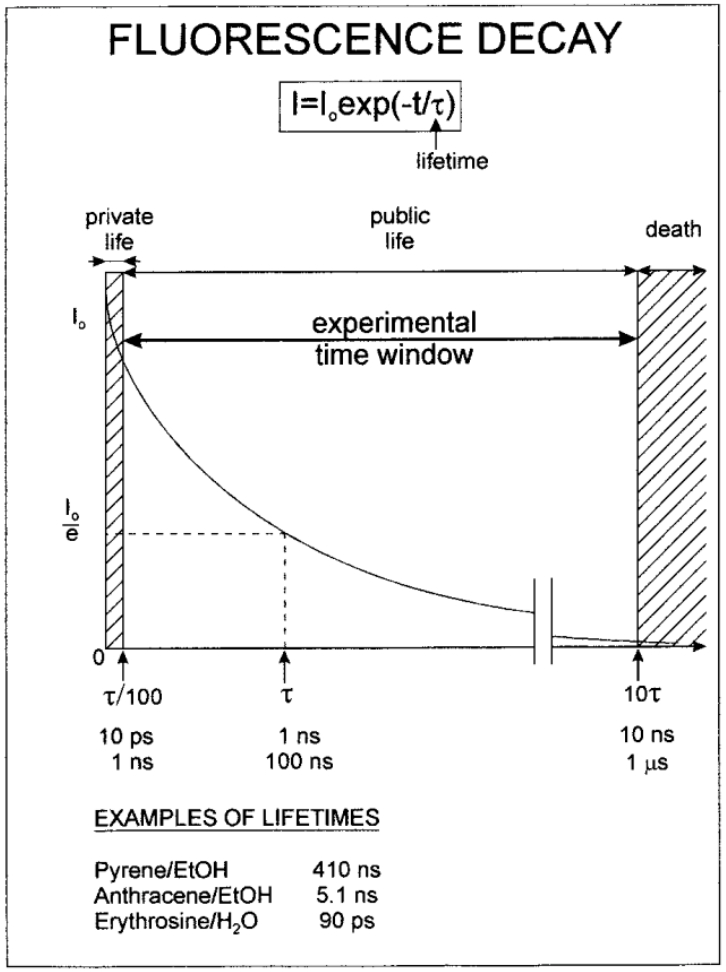
- Atomic Force Microscopy
  - Limited penetration depth
  -Ability to perform the live objects imaging
- Electron Microscopy
  -Good penetration depth
  - Pre-processing required
- Fluorescence Microscopy
  -Dependence on the excitation and emission wavelengths
  - Dependence on the intensity of the excitation
  - Dependence on the sample concentration



R. Tsien, “Imagining Imaging Future”, Nature 2003.



Experimental time window



The 150fs resolution by the current generation lasers

Picoseconds resolution by the detectors

Confocal/2-photon microscopy: excitation volume of 0.1fL

Spatial resolution: 200-300 nm.

FLIM probing / Quenching

a. Close proximity: Static
In the close distance. Intramolecular reactions.

c. Collisional: Dynamic quenching
In solutions. Intermolecular reactions.

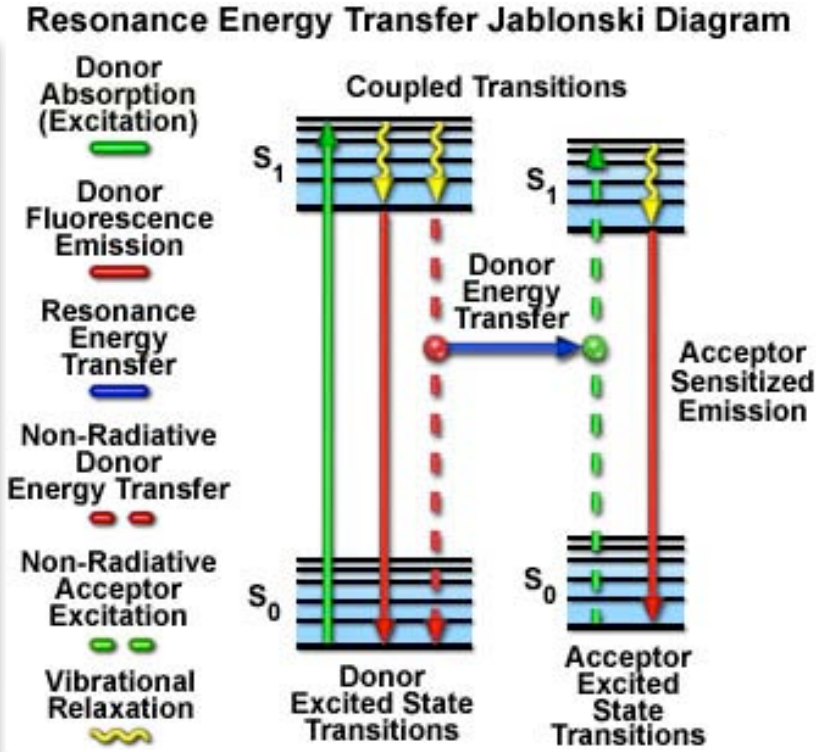
b. Distant: Non-radiate transfer of energy At the distance of 2-10 nm. Non-radiative energy transfer. Intermolecular

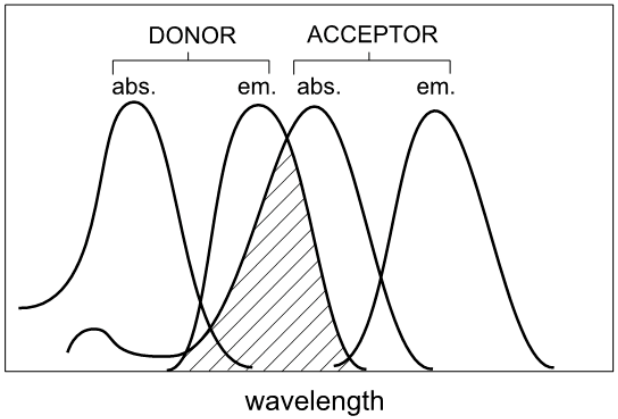
Conditions for FRET to happen

1.Spectral overlap between donor emission and acceptor excitation.

2.Relative orientation of the donor and acceptor transition dipoles.

3.Necessary level of the donor quantum yield.





FLIM probing / Quenching

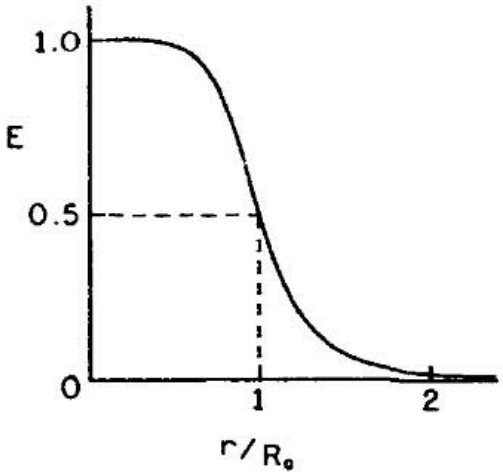
Rate calculation

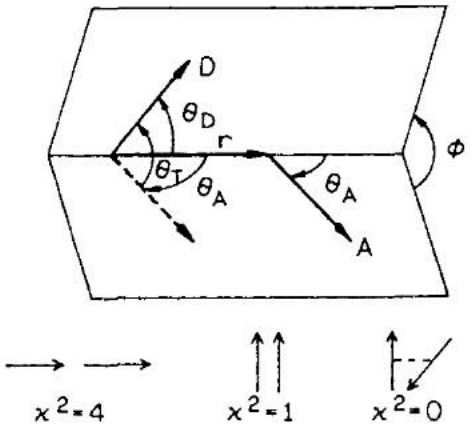
$$k_T = \frac{Q_D \kappa^2}{\tau_D r^6}\left(\frac{9000 \ln 10}{128 \pi N n^4}\right)\int_0^\infty F_D(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda$$

τ_D – is the lifetime of the **D** w/o **A**
Q_D - quantum efficiency of the donor,
κ – orientation factor, r – distance between molecules, F_D – normalized fluorescence intensity, ε - extinction coefficient

$$k_T = \frac{1}{\tau}\left(\frac{R_0}{r}\right)^6$$ **R_0 - Forster distance**

$$E = \frac{R_0^6}{R_0^6 + r^6} = \frac{1}{1 + (r/R_0)^6}$$



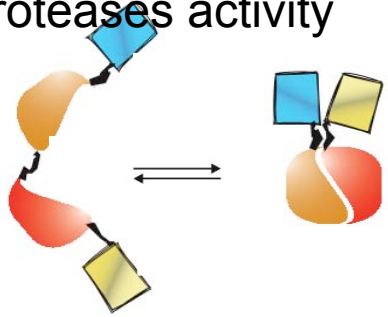


The FLIM-FRET saga

Interactions

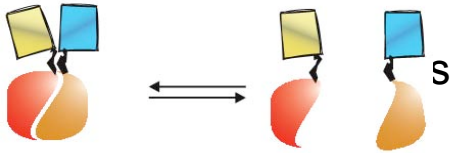
- FRET efficiency of fluorophores pairs
- Caspases activity
- Detection of apoptosis
- Proteases activity

Conformation



Protein-protein interactions
Dimers formations
Contraction of cells
Detection of clustered distribution
Genotyping (via PCR)
Location-specific activity of molecules
DNA-protein interactions
Molecules
Relative o s

Disruption



Summary

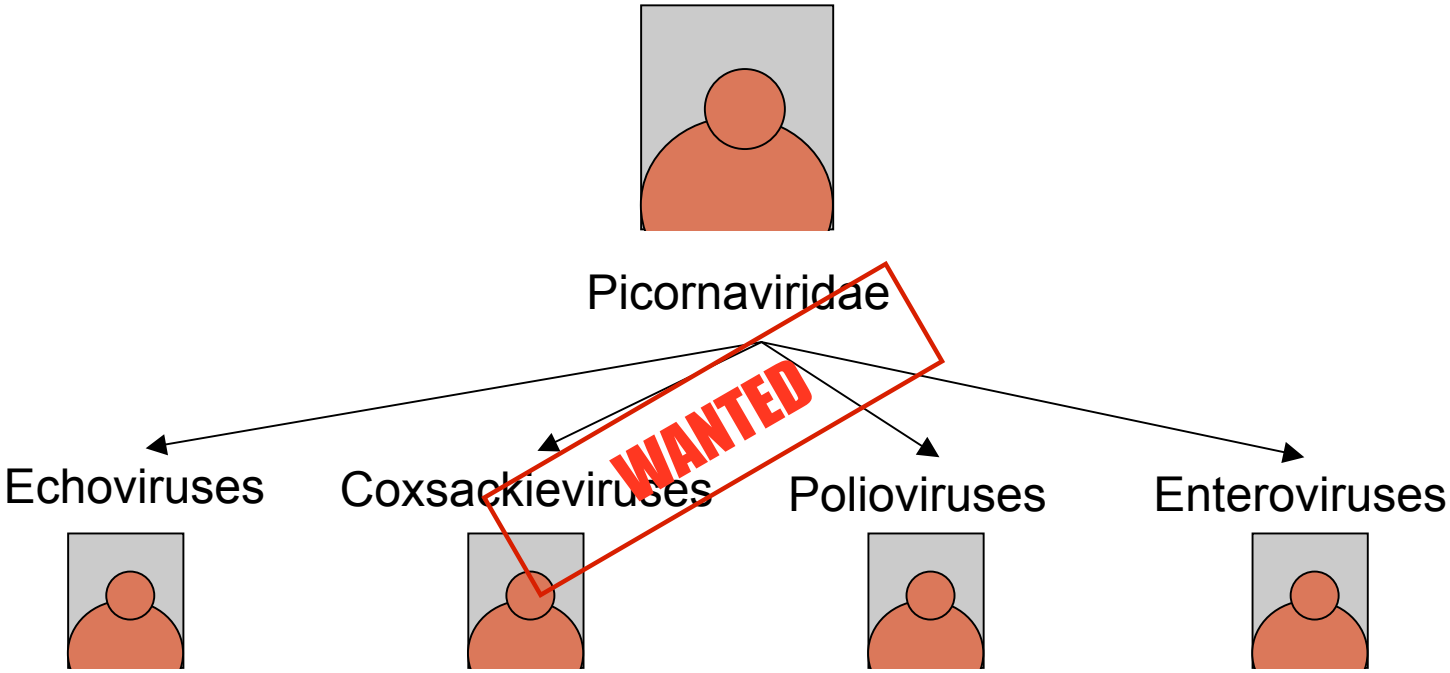
1. The applicability of FLIM is explained with the time scale of the average lifetime comparable with the dynamical processes in the environment.

2. Combination of FLIM with RET provides with the indirect “Spectroscopic Ruler”.

3. The FLIM/RET combination provides with a rich toolkit, whereas the imagination is the limit.

THE APPLICATIONS :: VIRAL DIAGNOSTICS BY MEANS OF FLIM/FRET

Enterovirus



• Hand-foot-and-mouth disease •
• Encephalitis •
• Aseptic meningitis •
• Pulmonary oedema •
• Poliomyelitis-like paralysis •

Enterovirus

PATHOLOGY

Associated with:

• Hand-foot-and-mouth disease •
• Encephalitis •
• Aseptic meningitis •
• Pulmonary oedema •
• Poliomyelitis-like paralysis •

RECENT OUTBREAKS

Taiwan, 1998
the biggest outbreak,
129106 infected
78 dead

Singapore, 2000
thousands infected
4 dead

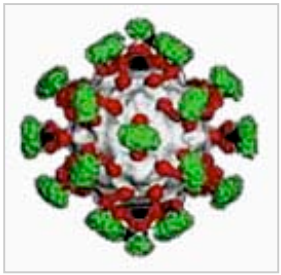
- Australia
- Hungary
- the United States
- Brazil
- Bulgaria
- Malaysia

Diagnostics

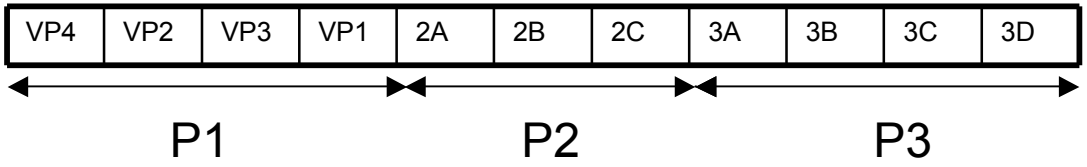
Asymptomatic

Serious neurological outcomes.
Enterovirus 71 – the most common non-polio enterovirus associated with poliomyelitis-like paralysis.

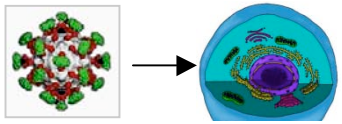
The Infection

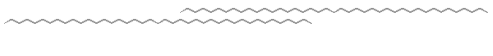


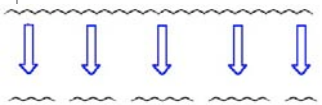
Single-stranded positive charged RNA

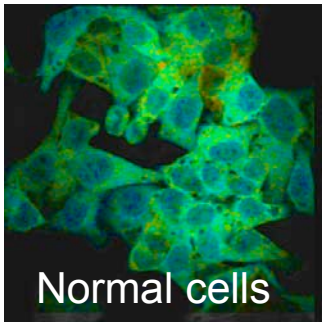


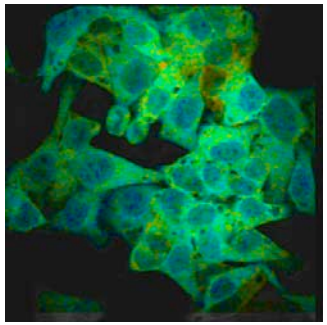
Viral infection → Translation into a single polyprotein → Maturation cleavage with 2A protease

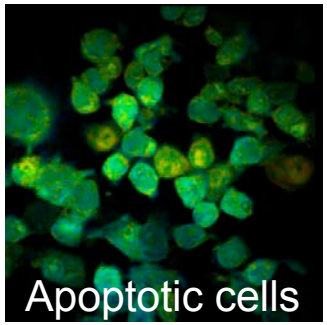




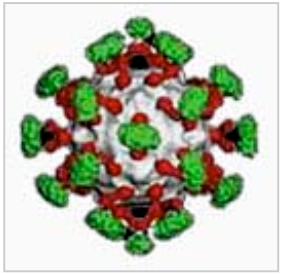




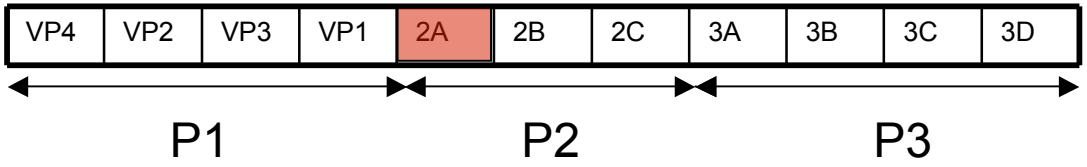




The Infection



Single-stranded positive charged RNA



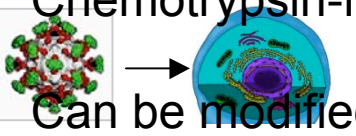
The 2A protease ($2A^{pro}$)
Viral infection

Translation into a single polyprotein

2A

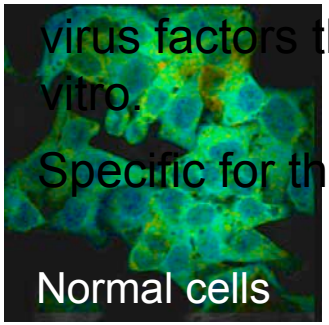
Maturation cleavage

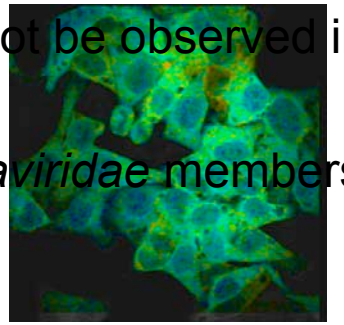
Chemotrypsin-like cysteine protease

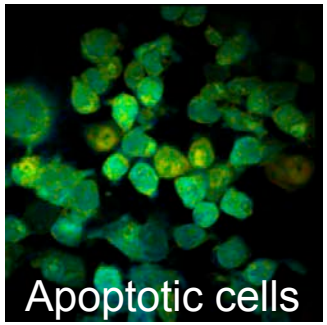


Can be modified by a number of host and virus factors that can not be observed in vitro.

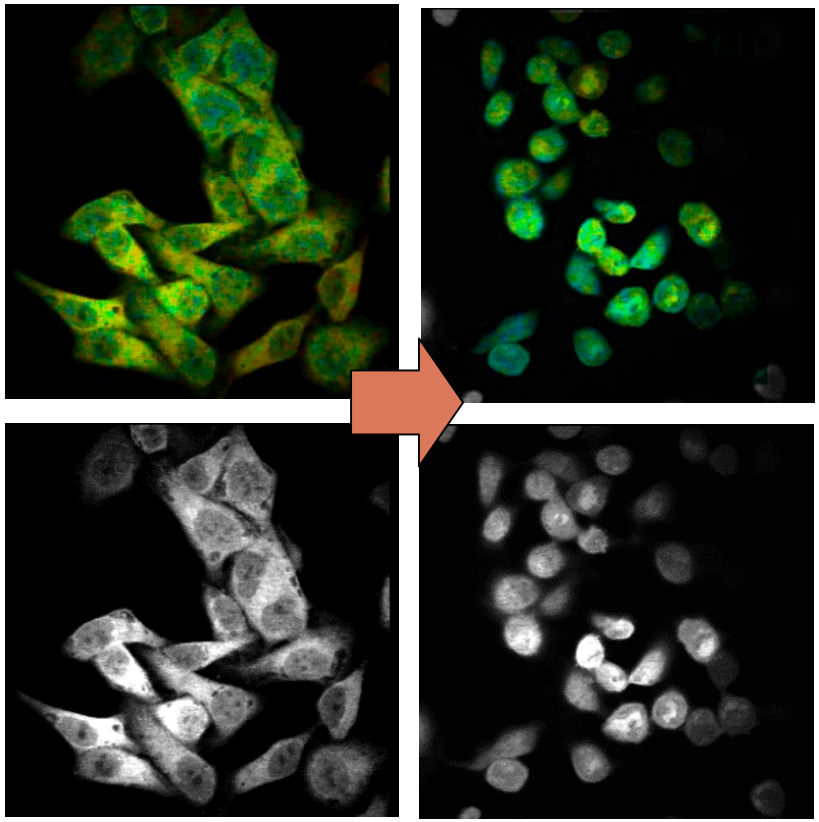
Specific for the *Picornaviridae* members

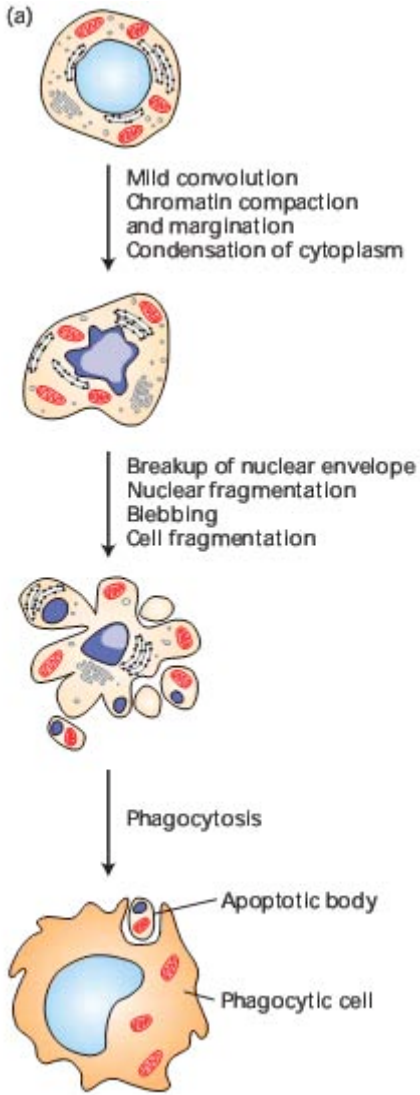

Normal cells




Apoptotic cells

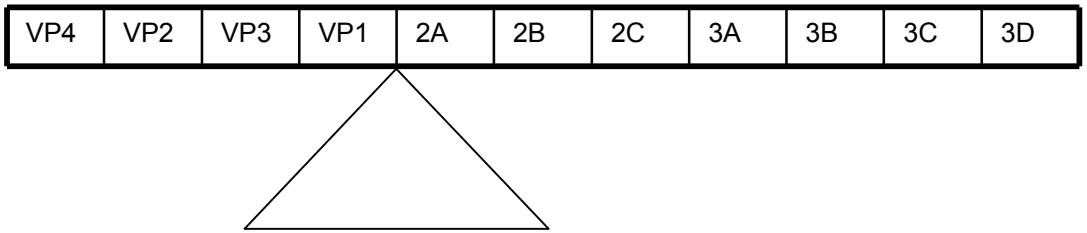
The Infection



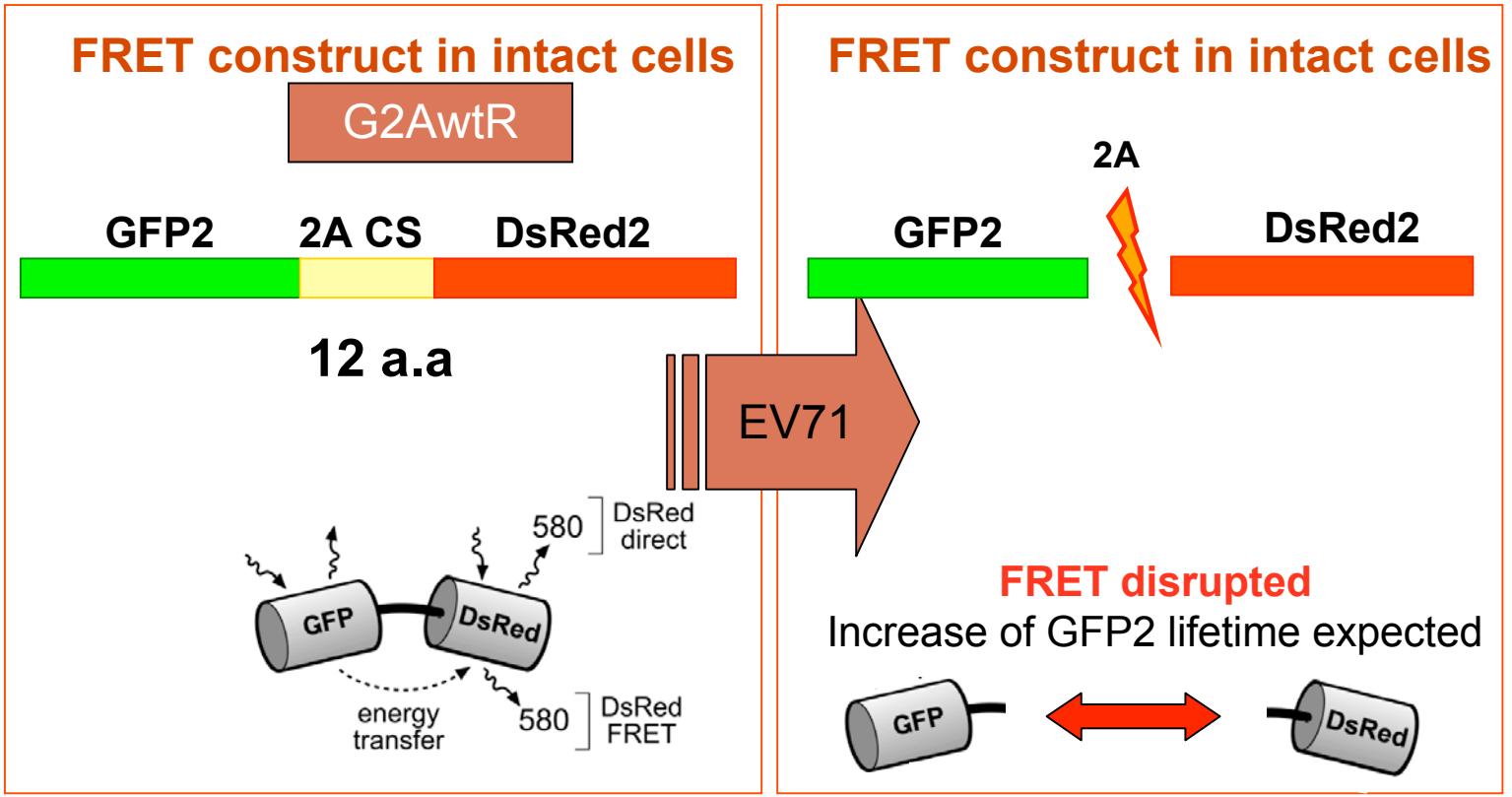


The key event: activation of the caspases – death executioners.

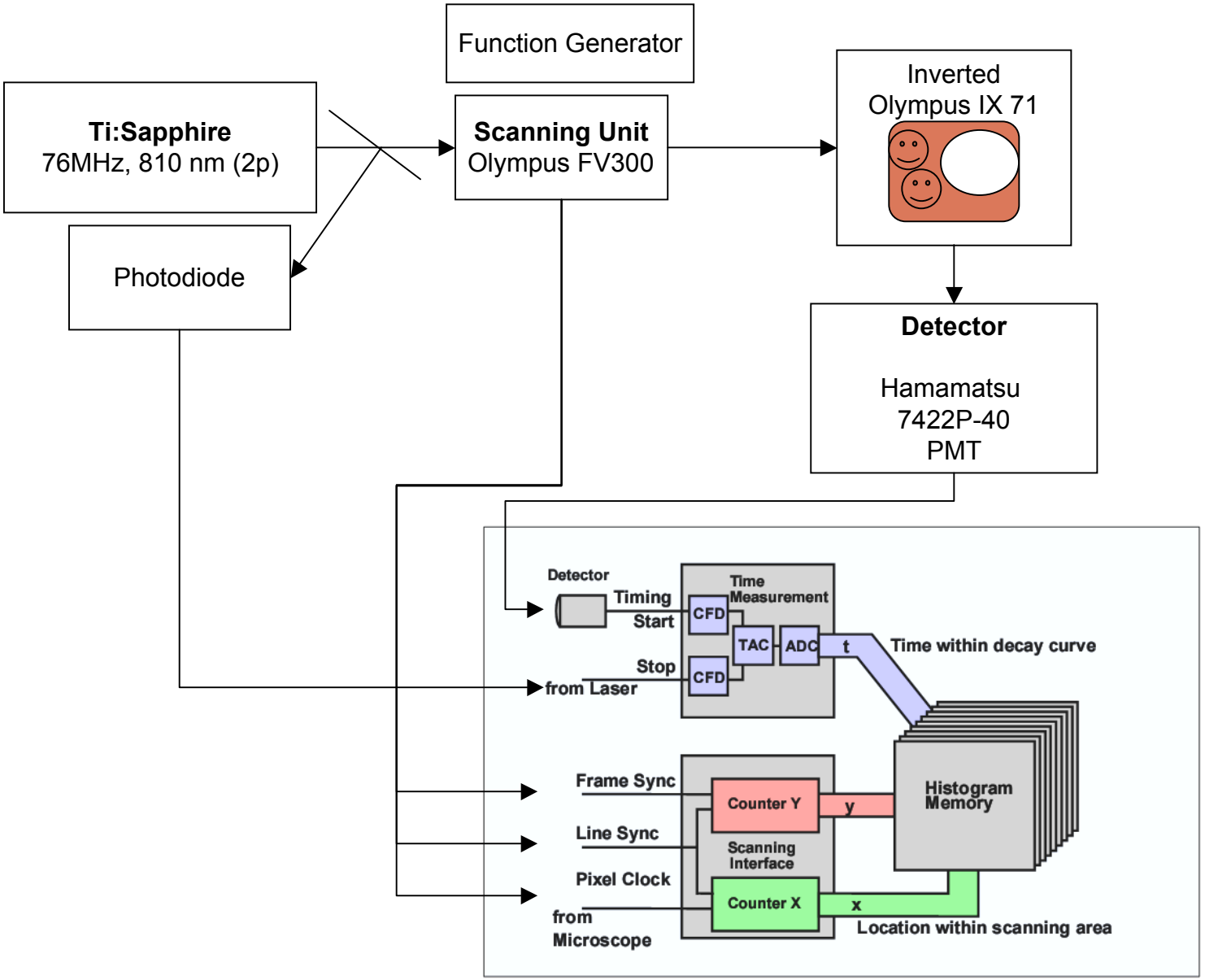
The Assay Construct



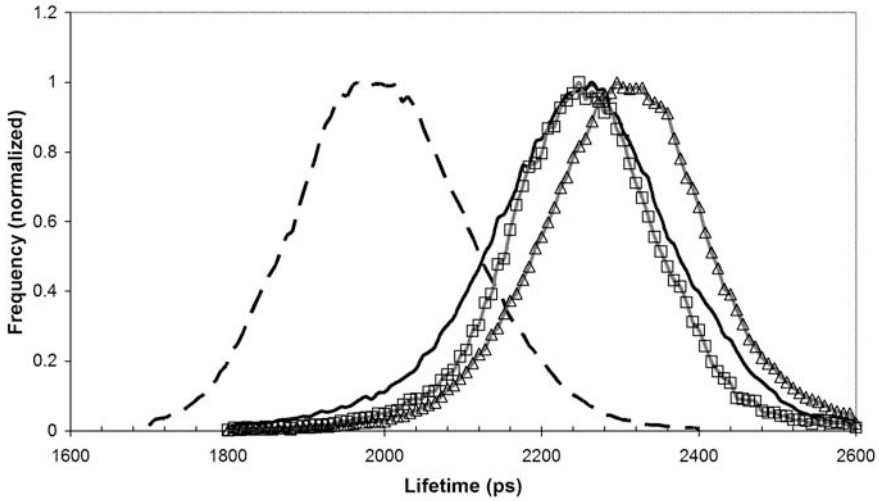
Cleavage recognition site

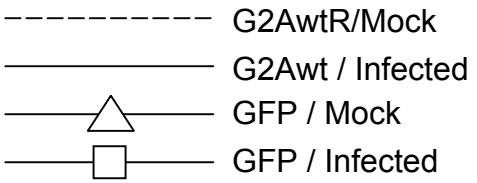


System Setup

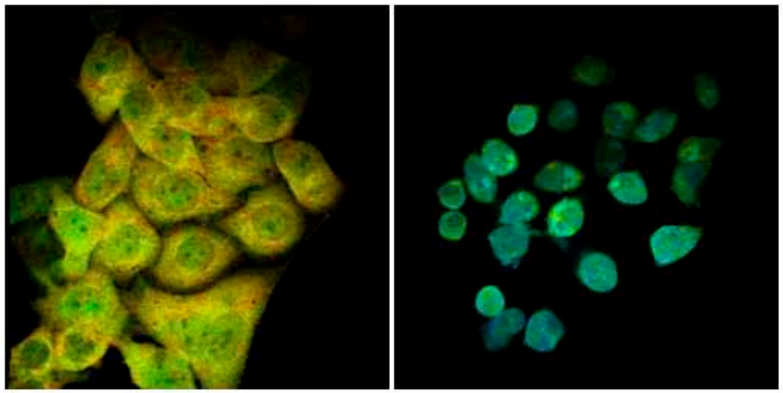


Results ::



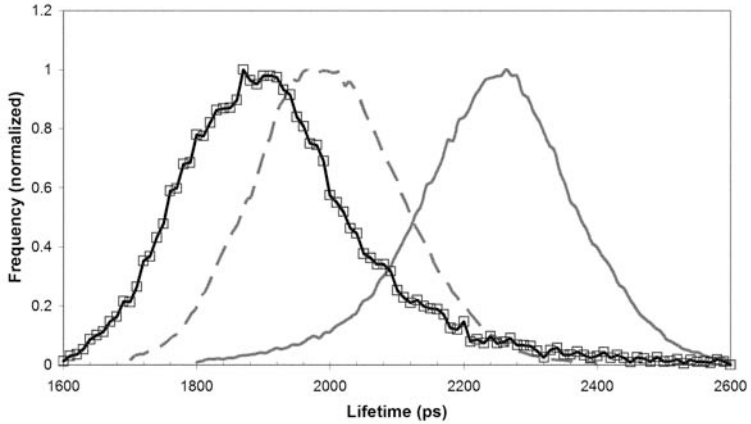


The lifetime observed in infected samples follow the histogram of GFP alone.

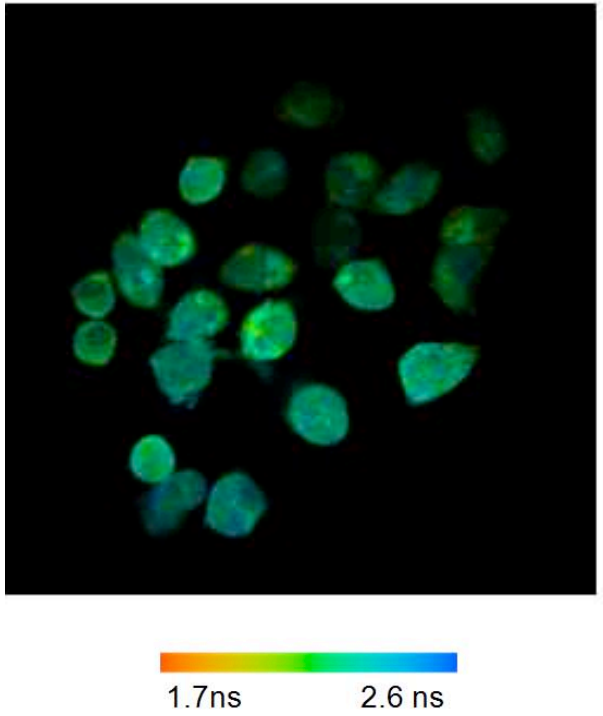


Controls

2A causes apoptosis:



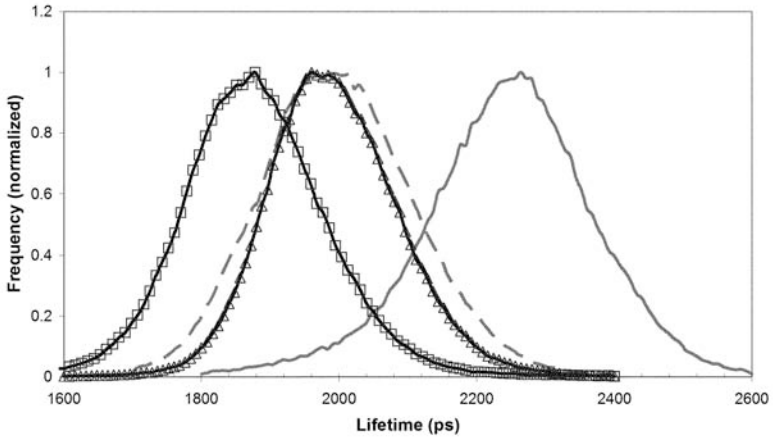
G2AwtR lifetime histogram measured upon the initiation of apoptosis in cells by cisplatin



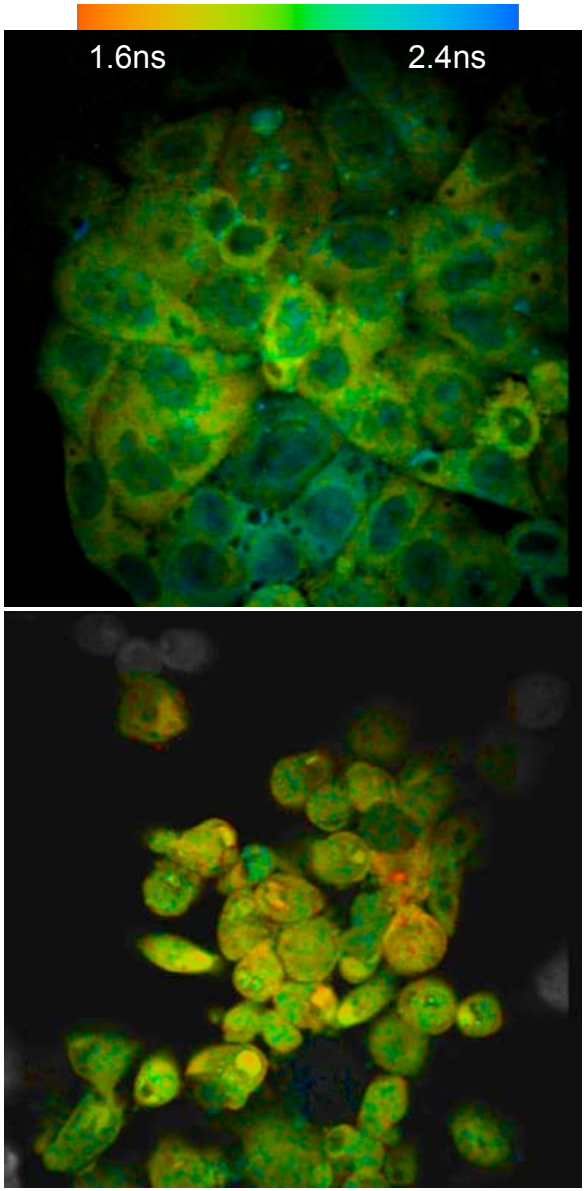
Controls

How specific is the disruption?

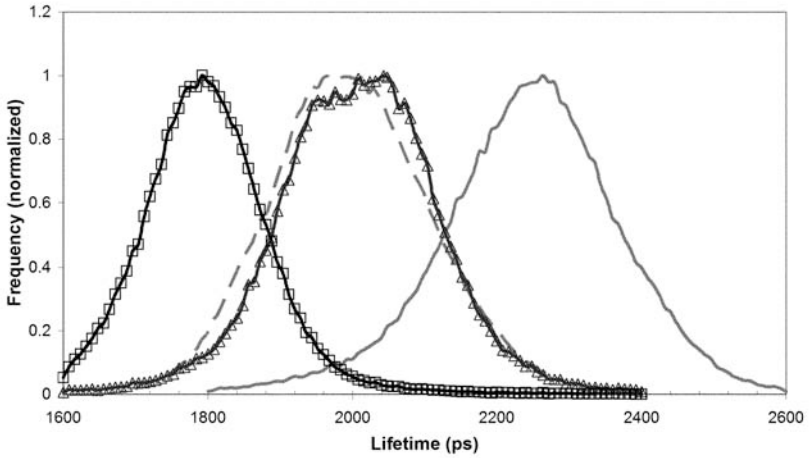
Mutant controls



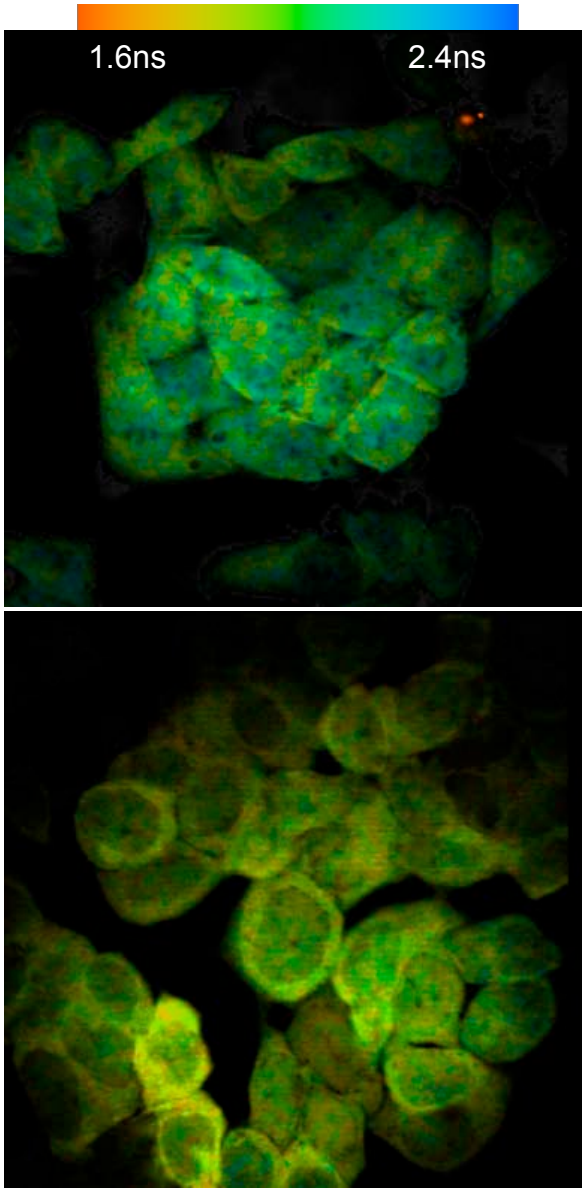
Averaged lifetime histograms of the mutant construct G2AmutR in mock and EV71 infected HeLa cells.



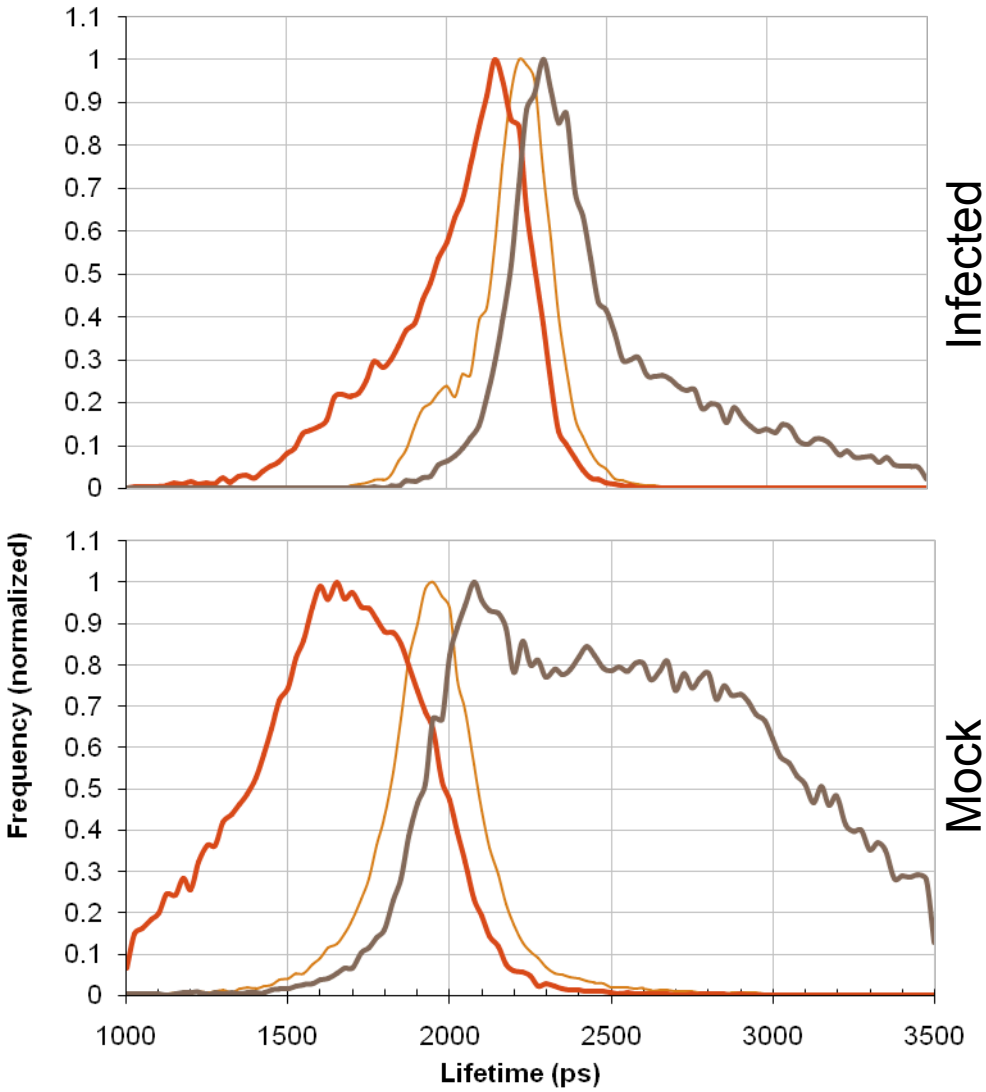
Controls



Averaged lifetime histograms for G2AwtR in mock and HSV-infected HeLa cells.

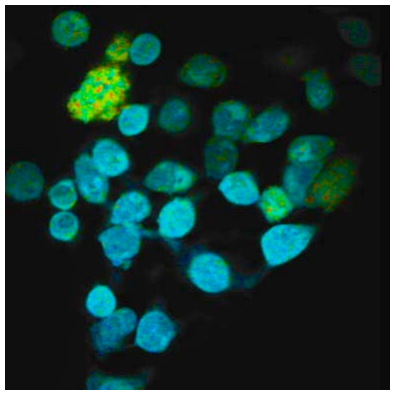


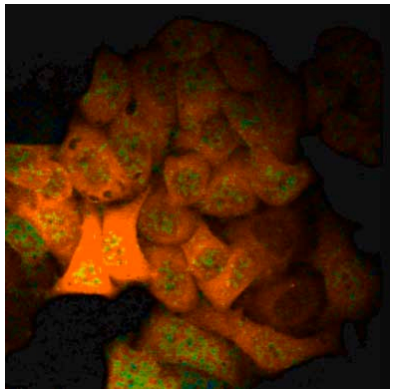
Results



Infected

Mock

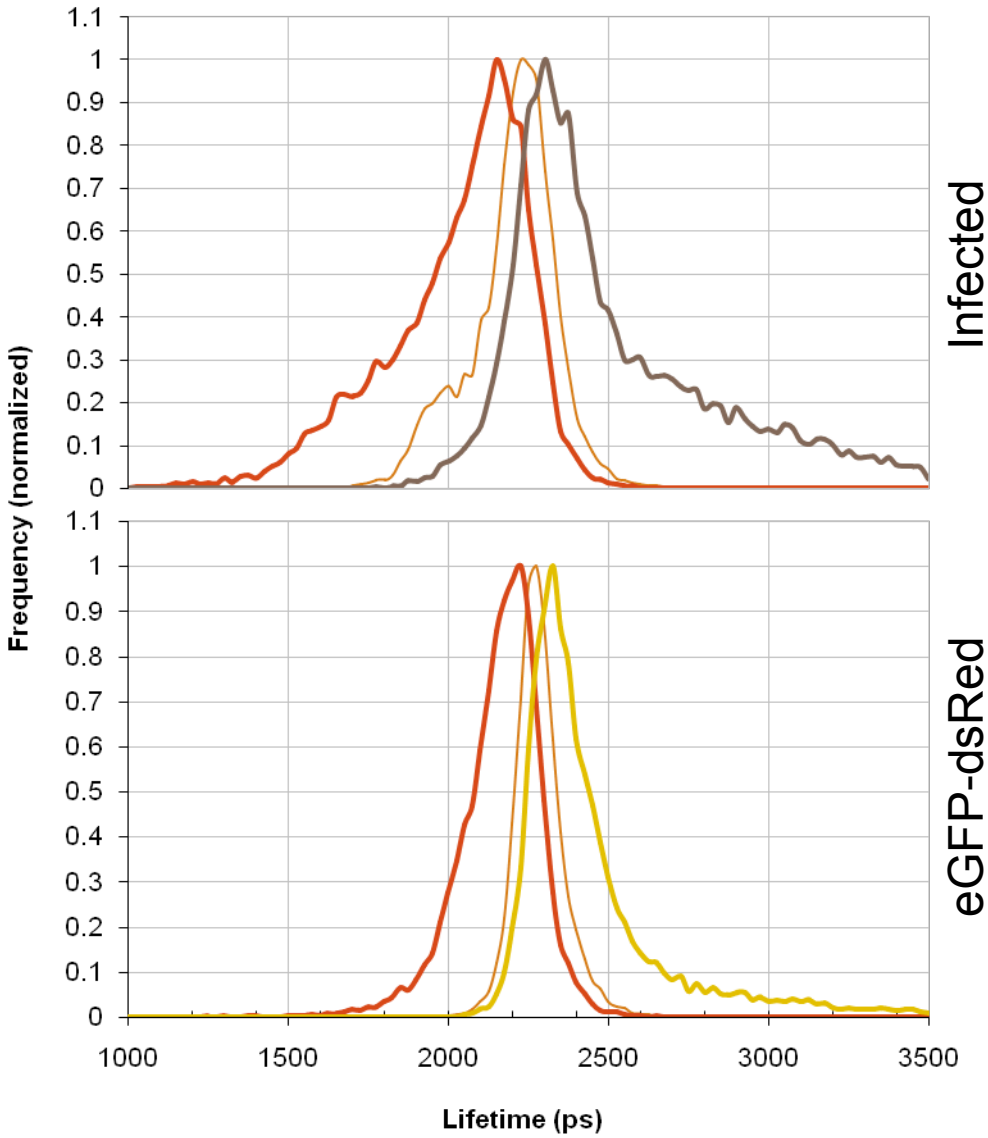




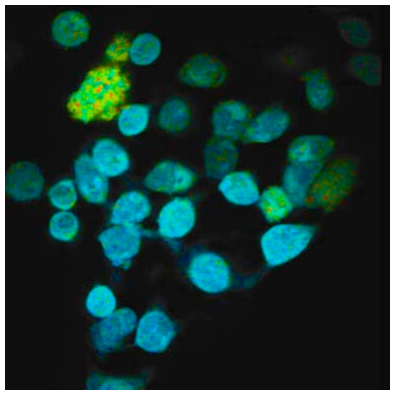
2.5 ns

2 ns

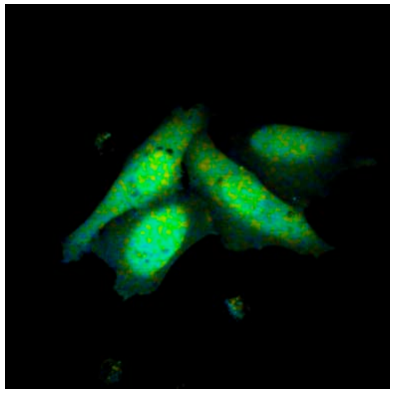
Results



Infected

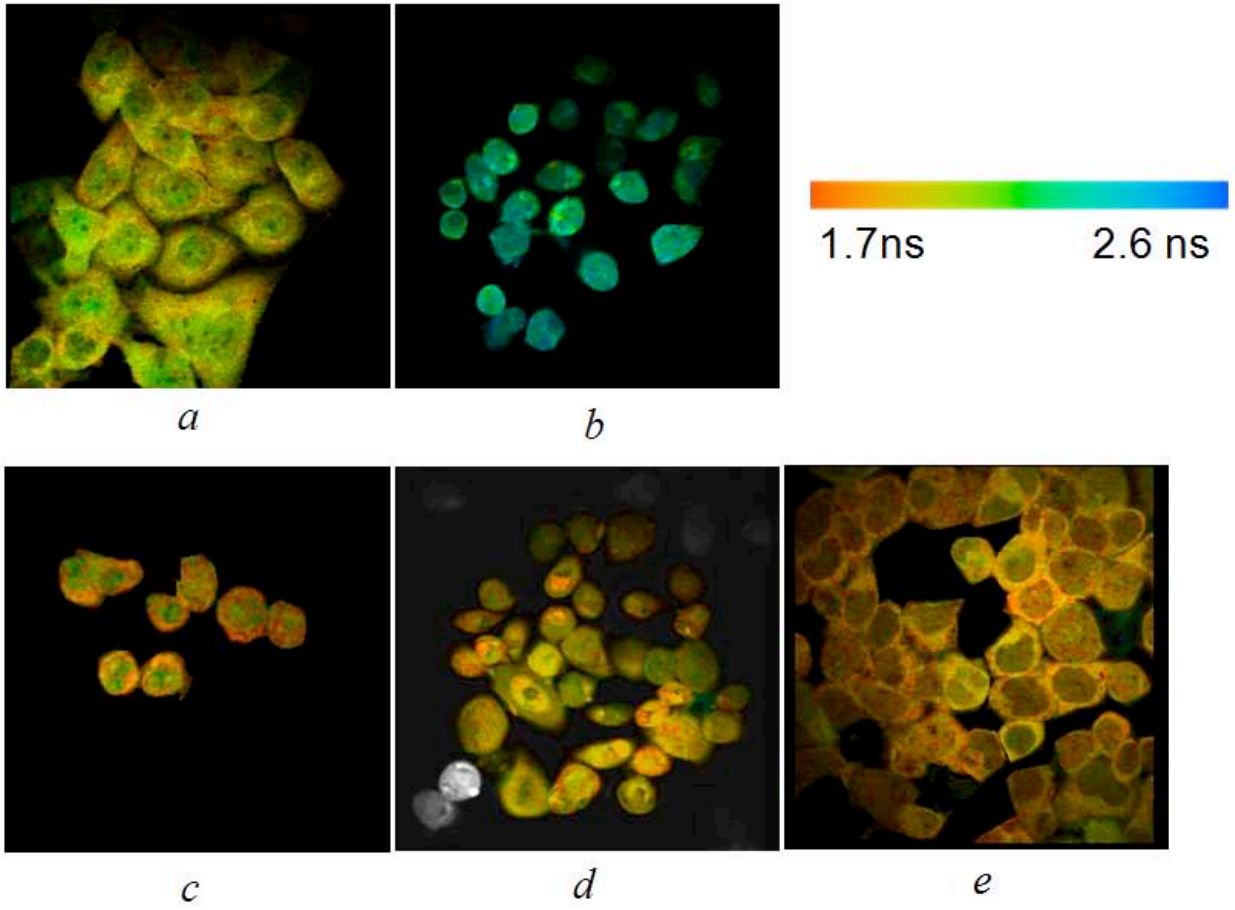


eGFP-dsRed

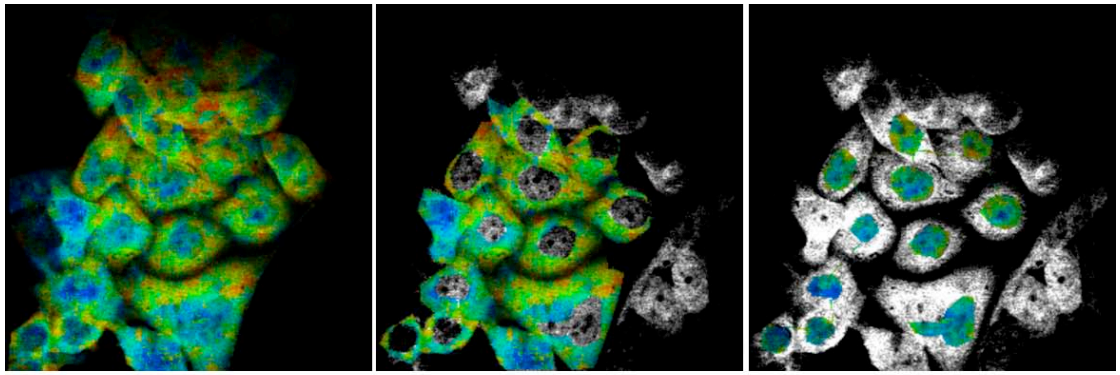


Results ::

Lifetime color coded images (lifetime measured in the GFP channel

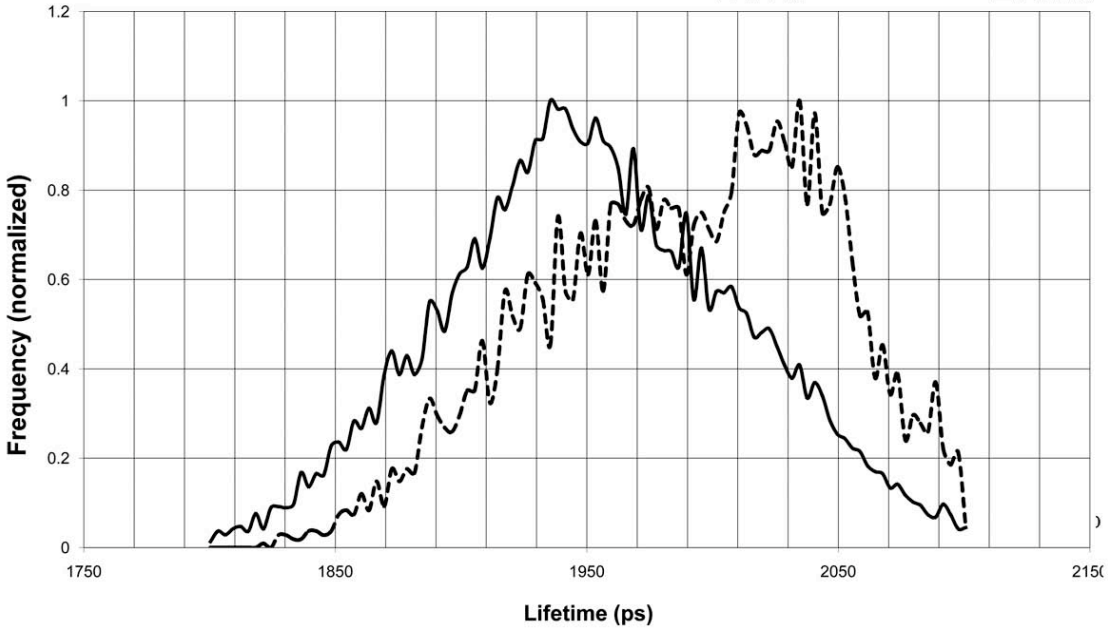


Results



a b c


1.8ns
2.1ns


Frequency (normalized)
1.2
1
0.8
0.6
0.4
0.2
0
1750
1850
1950
2050
Lifetime (ps)

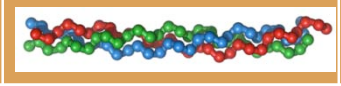
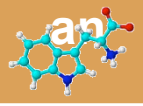
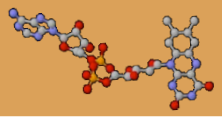
Potential applications

- The 2Apro microscopy based assay has a high efficiency in detecting the enteroviral infection. High specificity.
- Infection cycle of many viruses depend on the maturation cleavage. The proposed assay may help to trace the activity of these viruses during infection.
- The $2A^{pro}$ is specific for all *Picornaviridiae*. Thus detection of these viruses is possible with the given assay. The medical treatment of the patients with these infections doesn't differ in case of different species, thus the method has a potential for clinical diagnostics.
- Specificity of the proteases is observed for some other viruses, such as HIV, which makes the assay a potential method for this virus also.

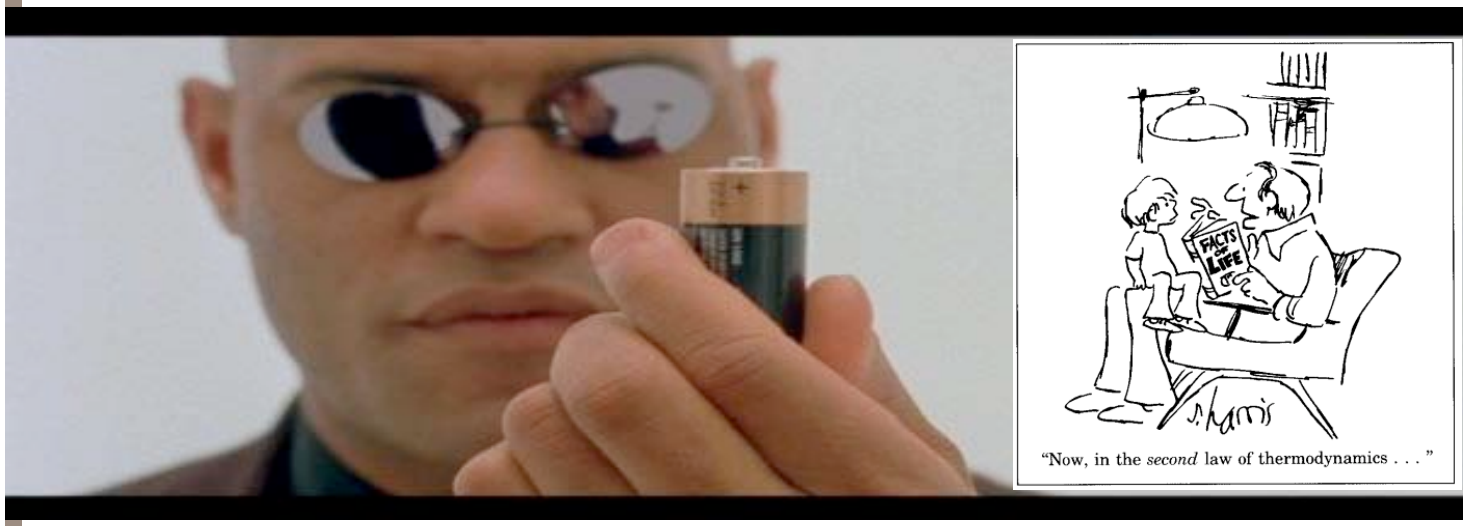
THE APPLICATIONS :: AUTOFLUORESCENCE IMAGING

Autofluorescence

The main sources of light in cells:

	Collagen 	Tryptophan 	Flavins 	NADH 
Location	Connective tissues Extracellular matrix Inside certain cells Main component : •Cartilage •Fascia •Ligaments	Basic component of cells	Basic component of cells	Basic component of cells
Observation	• Second Harmonic Generation • Fluorescence	Fluorescence	Fluorescence	• Fluorescence • Fluorescence Lifetime

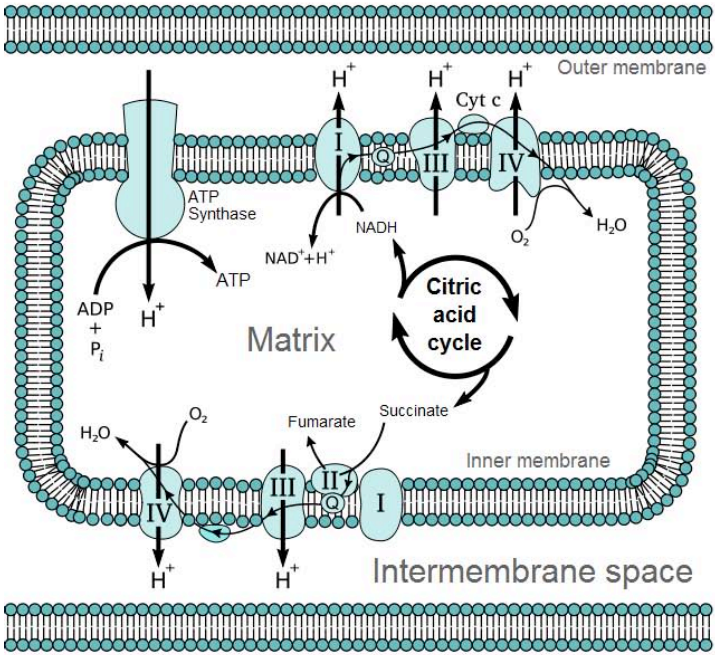
NADH – The battery of life



ATP (Adenosine triphosphate) is the main energy source in cells, enabling all the synthesis and other endergonic reactions, as well as increasing the rate of exergonic.

NADH (Reduced nicotinamide adenine diphosphate) is a part of the oxidative phosphorylation pathway, which is the most efficient way to produce ATP in aerobic cells.

NADH – The 2 “forms of existence”



The NADH, involved in the first step of the oxidative phosphorilation, serves as an electron donor, creating the intermembrane potential.

The potential is used by ATP synthase to phosphorylate ADP and AMP.

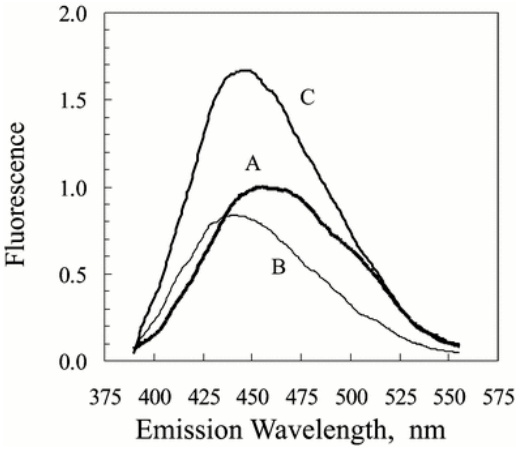
2

When involved in the ATP production, the molecules bind to enzymes (bound pool, mainly located in mitochondria).

Otherwise, distributed as single molecules in cytosole (free pool).

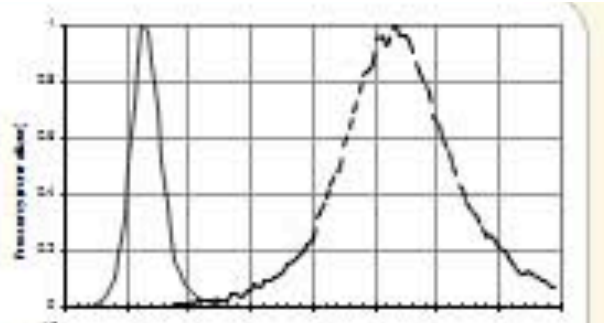
THE RATIO BETWEEN TWO POOLS ILLUSTRATE THE METABOLICAL ACTIVITY OF THE CELLS.

NADH – Detecting and distinguishing



The spectra:
Difference between the peaks for free (A) and bound forms (B, C) are just 10-20 nm, whereas the spectrum width ~150 nm wide.

Low Sensitivity



The lifetime:
Difference between the peaks for free and bound forms are well spearated: 400 ps vs ~2800 ps.

Excellent Sensitivity

The MOL autofluorescence profile

The dynamics of the NADH fluorescence in response to apoptosis inducer Staurosporine STS.

HWWang[1], V. Ghukasyan[1], CTChen[2], HWGuo[1], JSYu[1], YHWei[2], and FJKao[1]
Institute of Biophotonics Engineering, Institute of Biochemistry and Molecular Biology,
National Yang-Ming University

Completed

The influence of aging on the NADH fluorescence lifetime

V. Ghukasyan[1], CTChen[2], YHWei[2], and FJKao[1]
Institute of Biophotonics Engineering,
National Yang-Ming University

In progress

The yeast Prometheus: the increasing autofluo-rescence phenomena; correlations with lifetime.

V. Ghukasyan[1] and FJKao[1]
Institute of Biophotonics Engineering,
National Yang-Ming University

In progress

The NADH lifetime characterization

V. Ghukasyan[1] and FJKao[1]
Institute of Biophotonics Engineering,
National Yang-Ming University

The MOL autofluorescence profile

The dynamics of the NADH fluorescence in response to apoptosis inducer Staurosporine STS.

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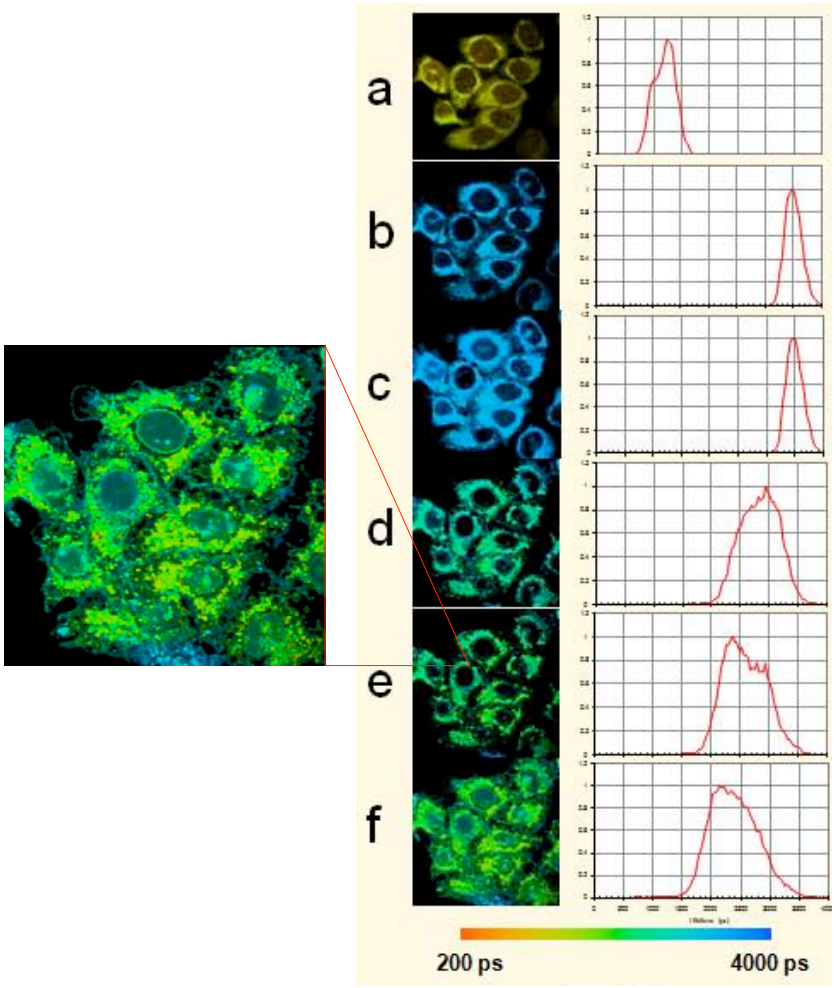

Lifetime observation (τ_m) in HeLa cells at 15 (a), 30(b), 45 (c), 60(d), 75 (e) minutes and 10 hours (f) after treatment with apoptosis inducer staurosporine (STS). Magnified image shows characteristic to apoptosis morphological changes.

The MOL autofluorescence profile

The dynamics of the NADH fluorescence in response to apoptosis inducer Staurosporine STS.

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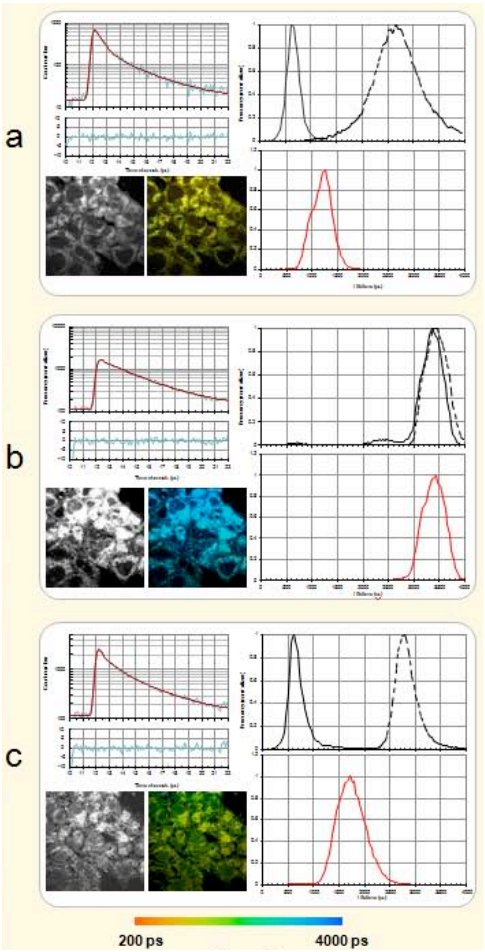
Completed



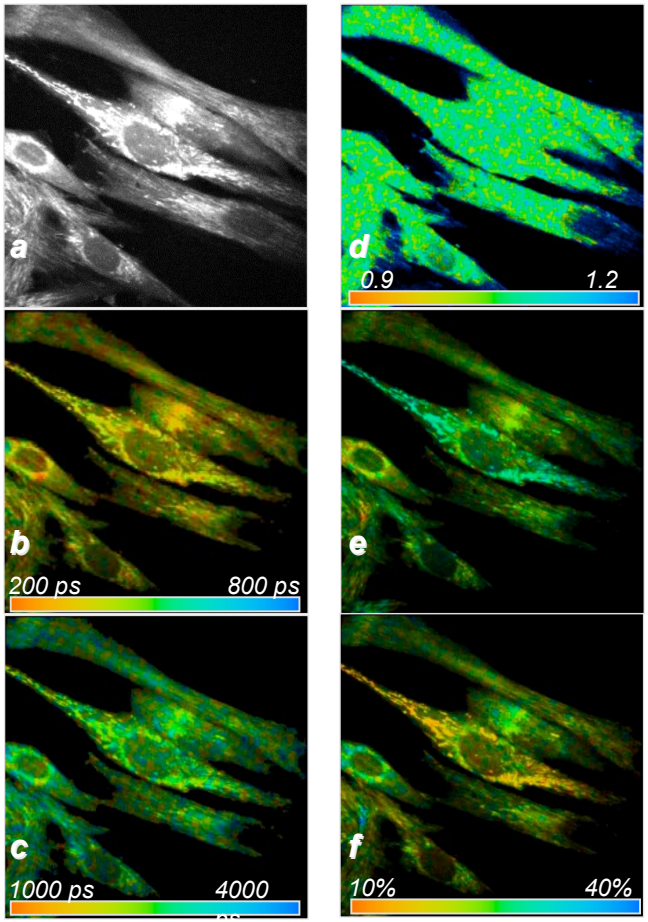
2-exponential lifetime observation in 143B cells at 15 (a), 30 (b) and 45 (c) minutes upon STS treatment. τ_1 and τ_2 (black) as well as $\tau_{av.}$ (red) are shown along with the fluorescence decays.

The MOL autofluorescence profile

The influence of aging on the NADH fluorescence lifetime

V. Ghukasyan[1], CTChen[2], YHWei[2], and FJKao[1]
Institute of Biophotonics Engineering,
National Yang-Ming University

In progress



Goal
Study dependence of fibroblast donor's age and NADH lifetime. Expected difference should reflect changes in metabolism observed earlier.

Representative FLIM data for fibroblasts. Images: *a).* fluorescence intensity image, *b).* color-coded image of the τ1 lifetime distribution, *c).* color-coded image of the τ2 lifetime distribution, *d).* χ2 distribution, *e.* distribution of the pre-exponential factor a1, *f).* distribution of the pre-exponential factor a2.

The MOL autofluorescence profile

The influence of aging on the NADH fluorescence lifetime

V. Ghukasyan[1], CTChen[2], YHWei[2], and FJKao[1]
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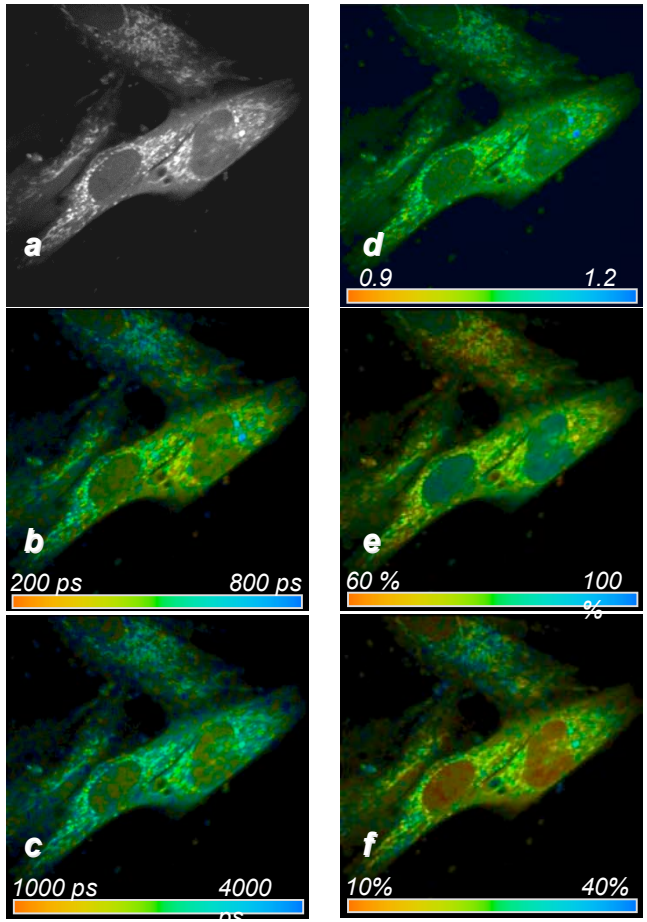
In progress



Representative FLIM data for fibroblasts from donor C (13 yrs old).
Images: ***a).*** fluorescence intensity image, ***b).*** color-coded image of the τ1 lifetime distribution, ***c).*** color-coded image of the τ2 lifetime distribution, ***d).*** χ2 distribution, ***e.*** distribution of the pre-exponential factor a1, ***f).*** distribution of the pre-exponential factor a2.

The MOL autofluorescence profile

The influence of aging on the NADH fluorescence lifetime

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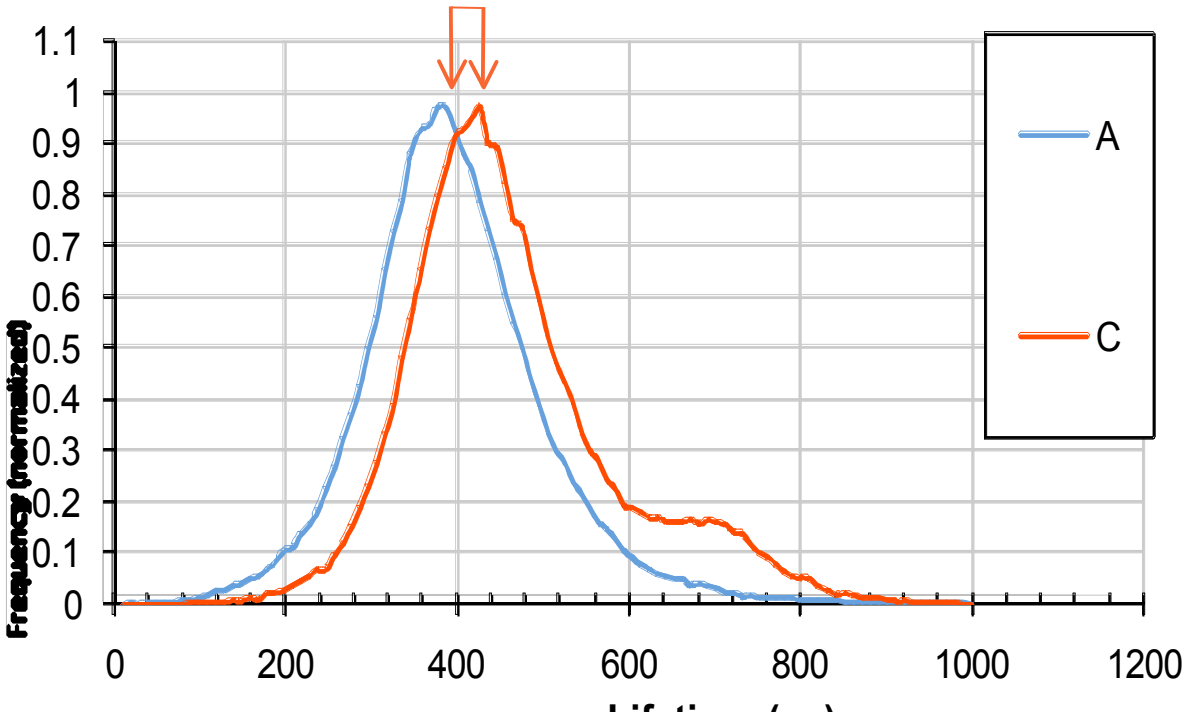
Institute of Biophotonics Engineering,
National Yang-Ming University

In progress

Averaged data from 5 samples from each donor.



The experimental conditions

Measurements taken from HeLa, grown on cover slides, coated with FBS.

Initial concentration: $1x10^4$ /ml. First measurement taken after 12 hours upon placing on the cover slide with further measurements taken every 24 hours.

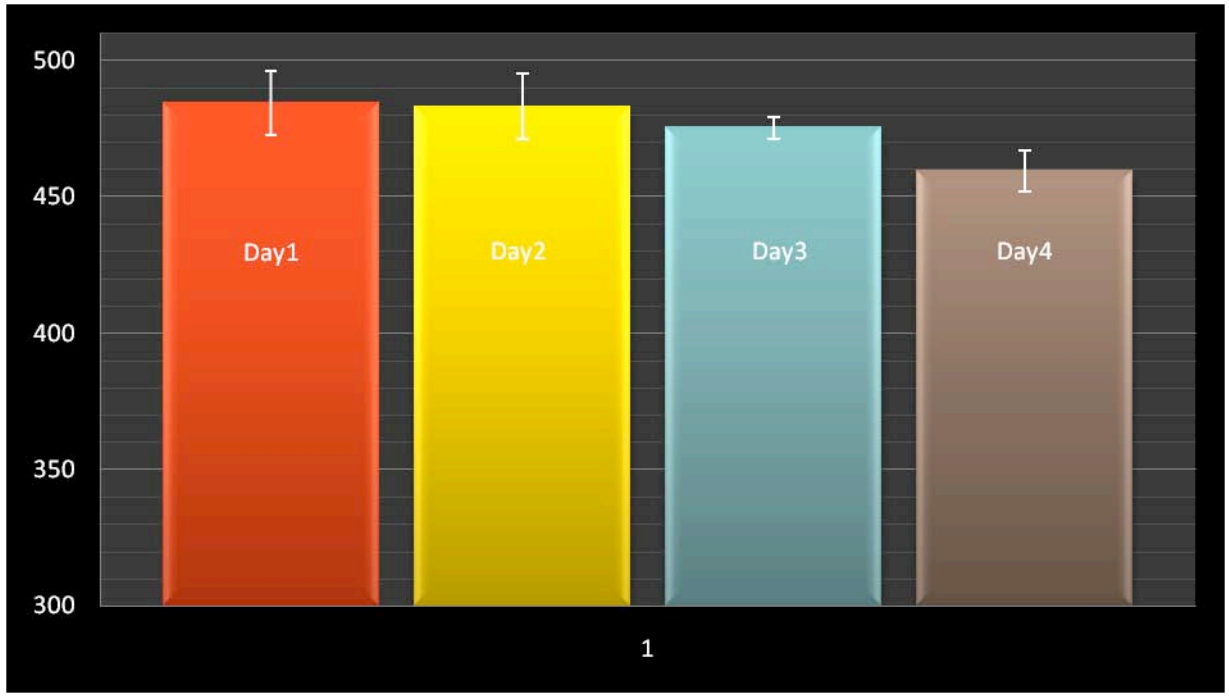
1 hour before the measurement the cover slides were placed in a chamber and 1 ml of fresh DMEM (5% FBS) added and incubated.

Objective: 100x, NA=1.4, Plan ApoChromat
Laser operated at 76MHz at 740 nm (2-photon)
Laser power measured at the focal plane: 4-5 mW
Image acquisition: 1500 s.

This measurement is a part of overall studies aimed to characterize the NADH lifetime dynamics.

Results

The first lifetime component is gradually decreasing as the cell culture density is increasing.



The τ_1 dynamics

Precautions/controls

The first lifetime component is gradually decreasing as the cell culture density is increasing.

An Artifact?

Possible causes/controls:

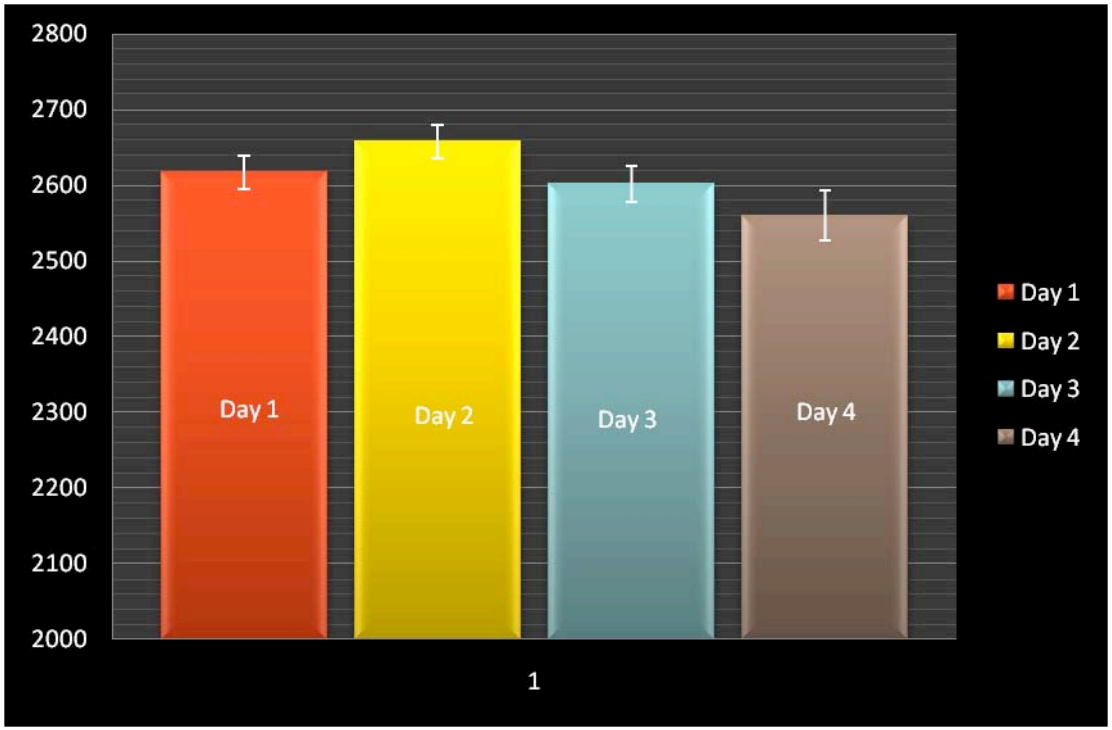
a.Limited oxygen supply

- Should result in changed pre-exponential factors ratio. (see next slides)

b. Limitation of nutrition elements:

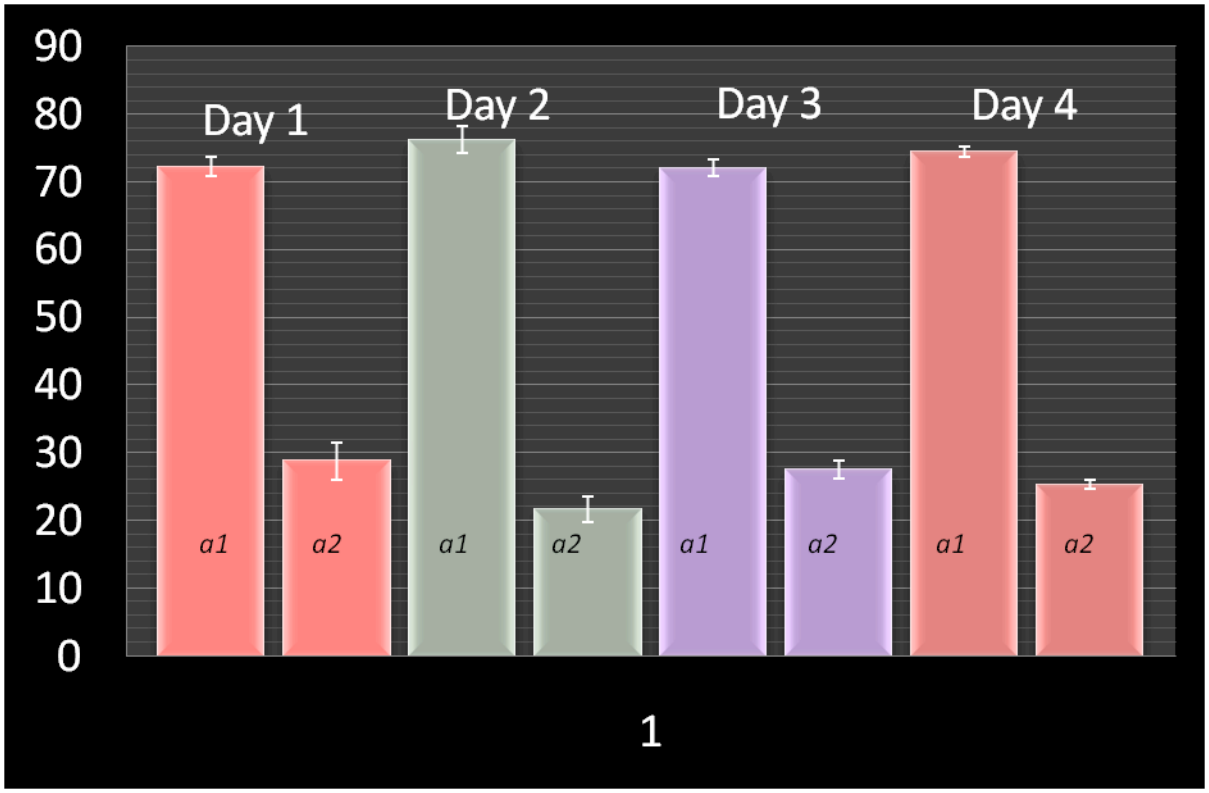
- For the purpose of the experiments the cells, grown on cover slides, were replaced to the chamber and covered with the fresh media with further incubation for 10 minutes.

Results



The long lifetime component τ_2 doesn't exhibit any distinguishable dynamics.

Results



No distinguishable trends in pre-exponential factors.

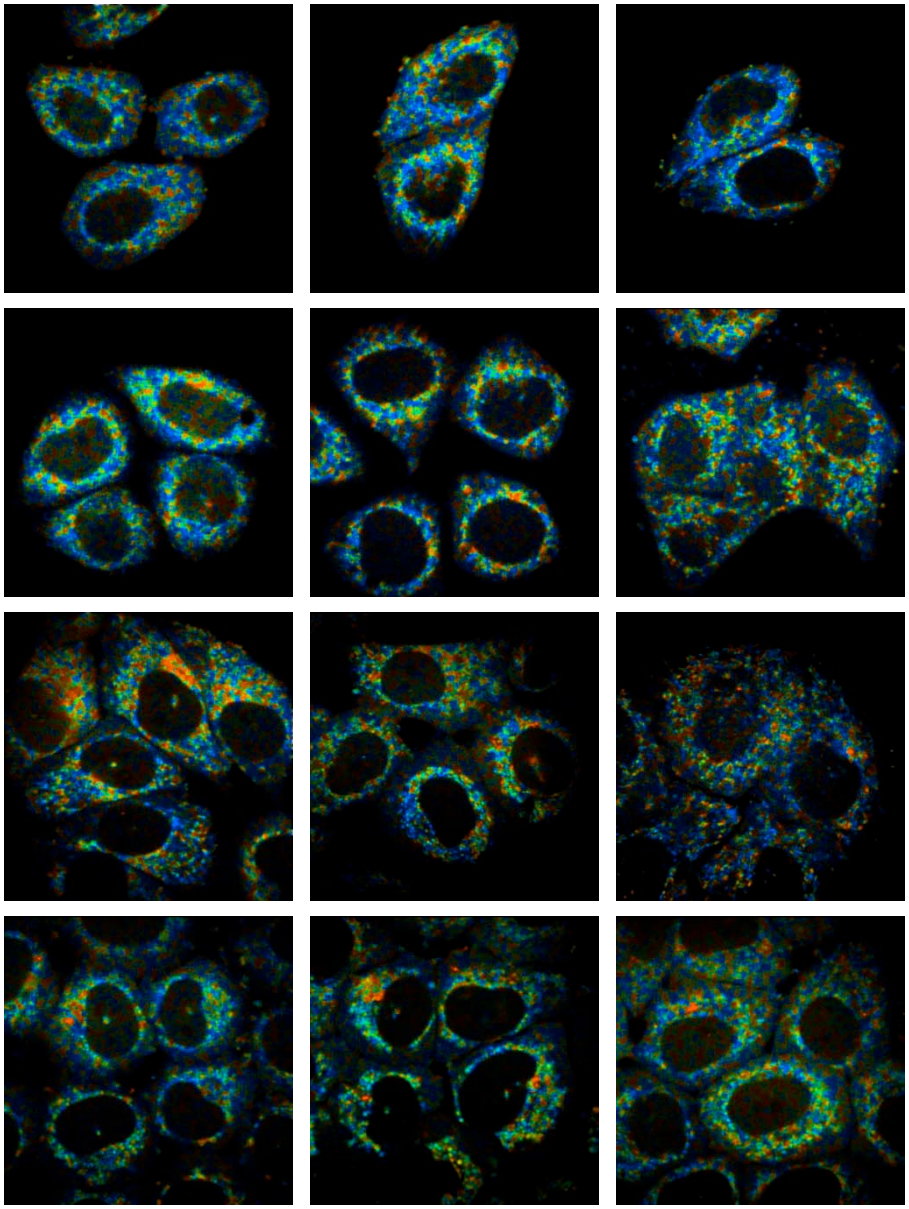
Results

Day 1

Day 2

Day 3

Day 4



Resume

Among the currently available systems, FLIM, especially in combination with RET, provides with unsurpassed possibilities at high temporal and spatial resolution. This is especially true in case of live cells observation.

See you there…

Thank you!

MODERN
OPTICS
LABORATORY