FLIM, FRET

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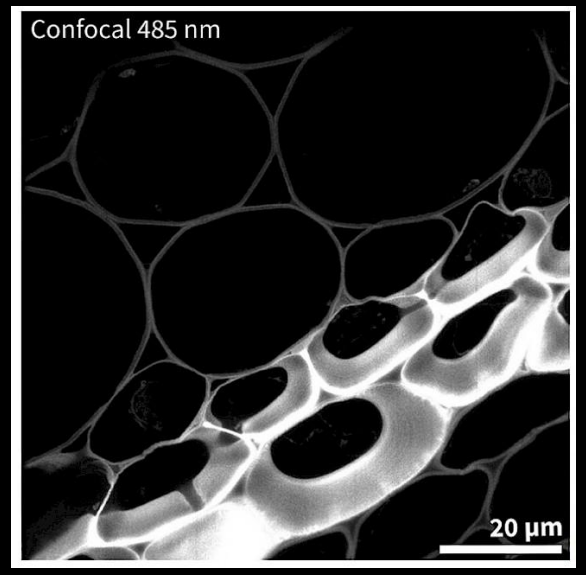
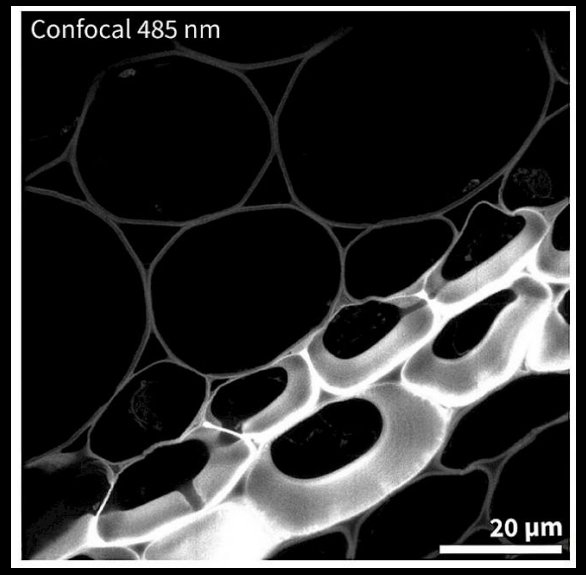


- Conventional fluorescence microscopy
  - Identification is based on differences in fluorescence spectral characteristics between dyes
  - Several dyes can be used simultaneously

https://www.abberior-instruments.com/products/expert-line/flim/



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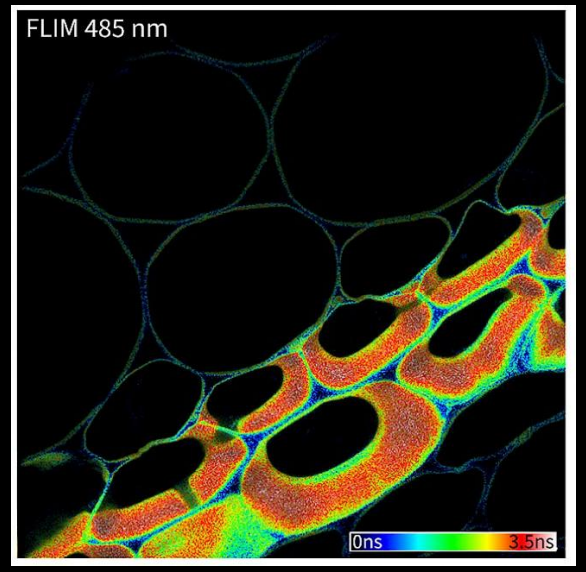


- Each fluorescent dye has its own **lifetime** in the excited state
  - Possible to distinguish even dyes having the same fluorescent color as well as to identify autofluorescence

https://www.abberior-instruments.com/products/expert-line/flim/

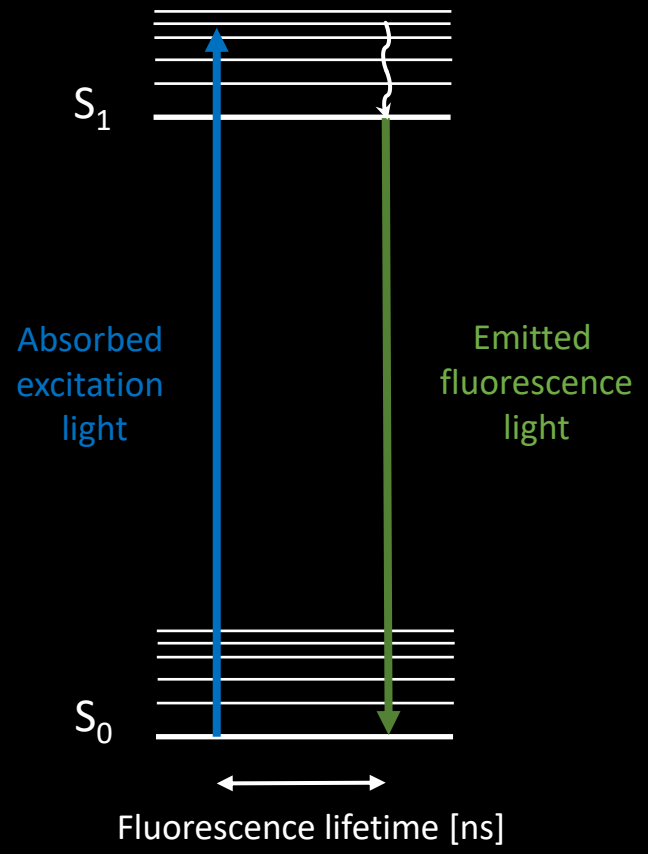
Outline:

- FLIM (fluorescence lifetime imaging microscopy)
  - Time-domain FLIM
  - Phasor approach
  - Applications
- FRET (fluorescence resonance energy transfer)
  - Sensitized emission
  - Acceptor photobleaching
  - FLIM-FRET
  - Phasor approach
  - Applications
- Conclusion

FLIM

What is FLIM

- ***Fluorescence lifetime imaging microscopy***
- Fluorescence lifetime - average time that a molecule remains in an excited state prior to returning to the ground state by emitting a photon

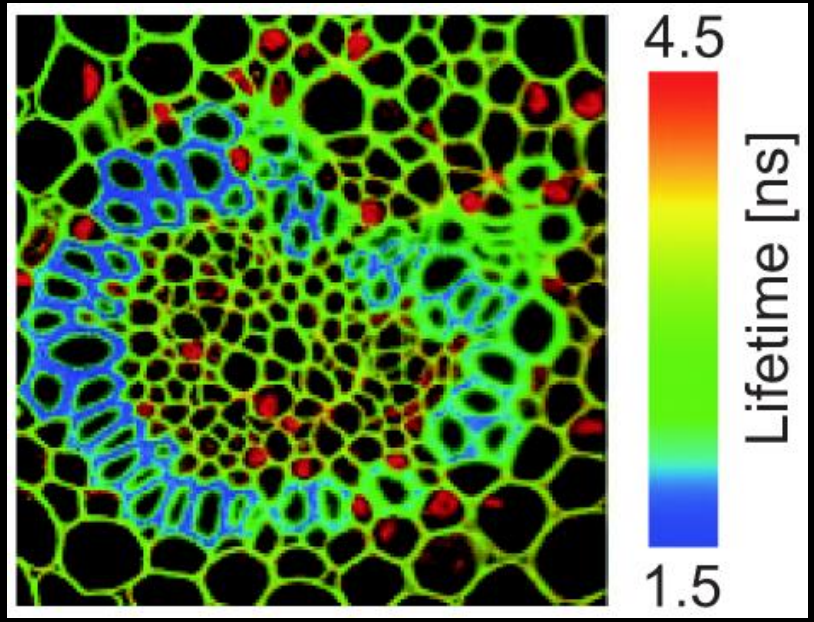


FLIM

- Fluorescence lifetime *(τ)* of a fluorophore
  - depends on **its molecular environment**
  - does NOT depend on its concentration, absorption by the sample, sample thickness, photo-bleaching and/or excitation intensity

-> visualize factors that affect the fluorescence lifetime properties of the dye molecule - state of the environment around the molecule

-> provides contrast in images (fluorophores with similar excitation and emission spectra but with different lifetimes)



https://www.picoquant.com/applications/category/life-science/fluorescence-lifetime-imaging-flim

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- *τ* changes when the molecules undergo **de-excitation through other processes than fluorescence**
  - *dynamic quenching* through molecular collisions with small soluble molecules like ions
  - *energy transfer* to a nearby molecule

-> fluorescent lifetime mirrors any process in the micro-environment that quenches the fluorophores

FLIM

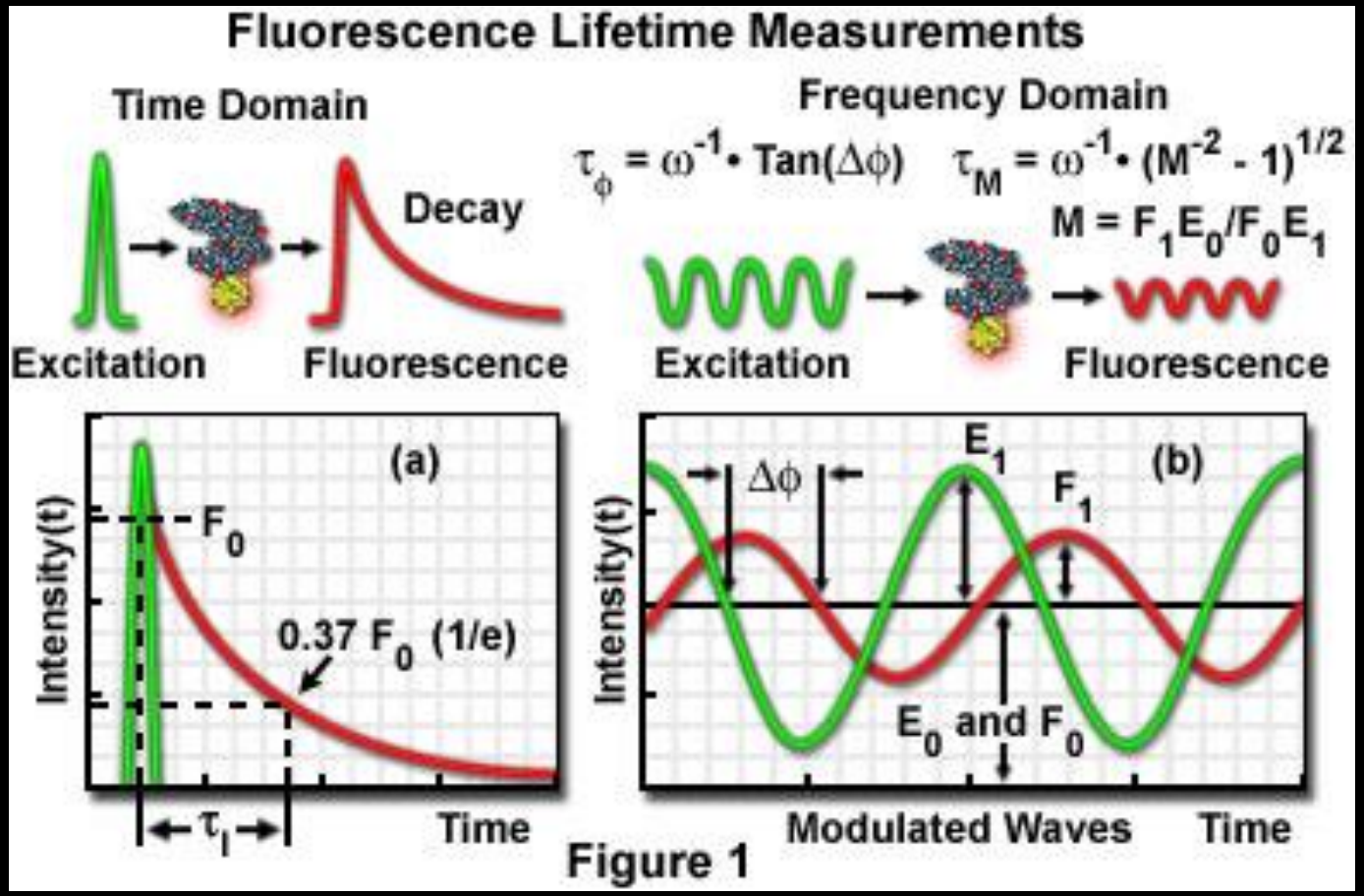
Factors affecting the fluorescence lifetime:

- **Environment** - ion intensity, hydrophobic properties, oxygen concentration
- **Molecular binding**
- **Molecular interaction** by energy transfer when two proteins approach each other

FLIM measurements

- Time-domain FLIM
- Frequency-domain FLIM
- Phasor approach

FLIM measurements

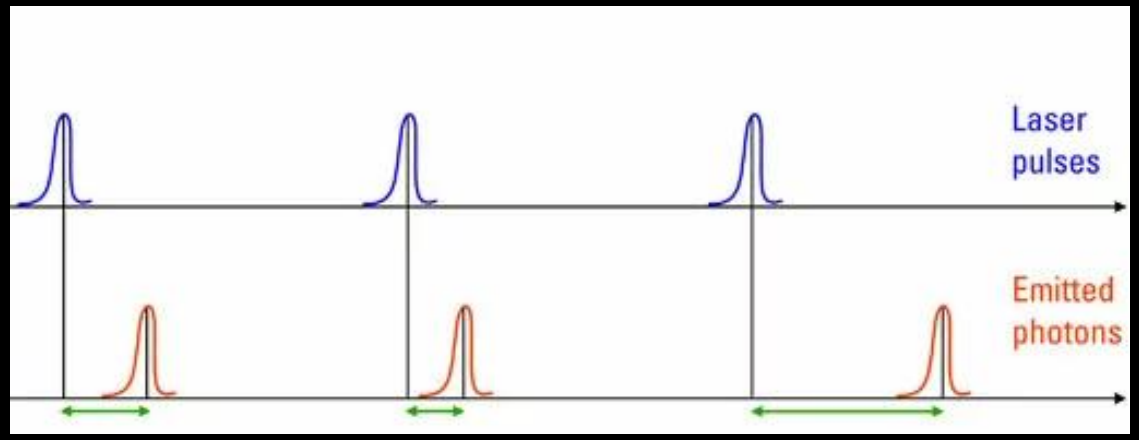


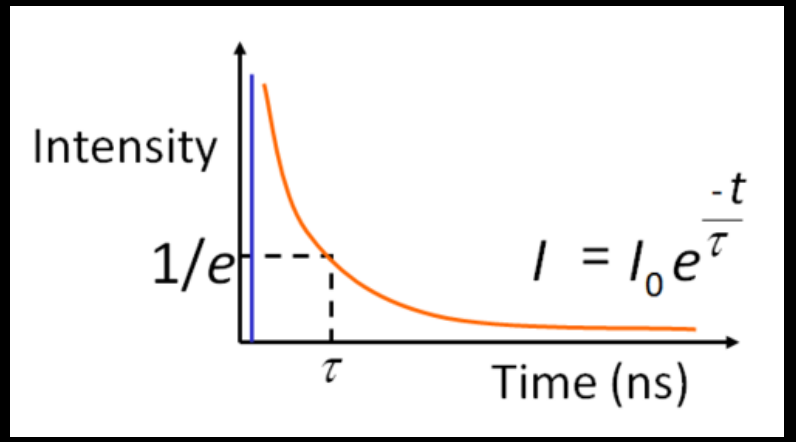
https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/confocal/applications/flimintro/

Time-domain FLIM

Time-correlated single photon counting (TCSPC)

- Typically a confocal microscope with pulsed laser and PMT detector
- Detector records a histogram of photon arrival times at each spatial location
- Lifetimes are derived from exponential fits to the decay data





$$I = I_0 \sum_i \propto_i e^{\frac{-t}{\tau_i}}$$

Multi-exponential decay

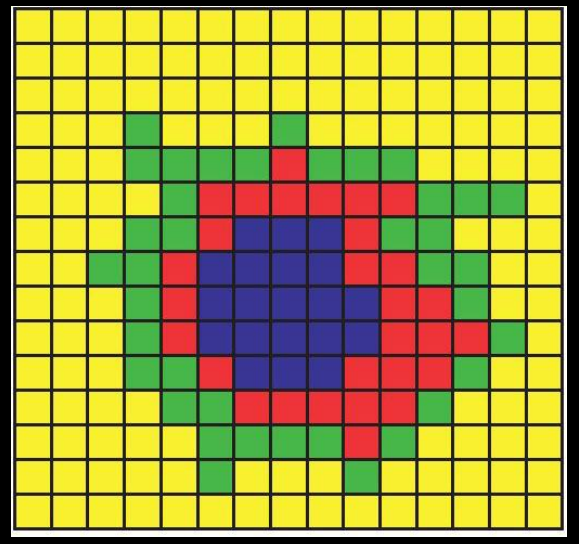
https://www.leica-microsystems.com/science-lab/what-is-flim-fluorescence-lifetime-imaging/

Time-domain FLIM

Time-correlated single photon counting (TCSPC)

- Typically a confocal microscope with pulsed laser and PMT detector
- Detector records a histogram of photon arrival times at each spatial location
- Lifetimes are derived from exponential fits to the decay data

- FLIM image of the sample - one pixel at a time

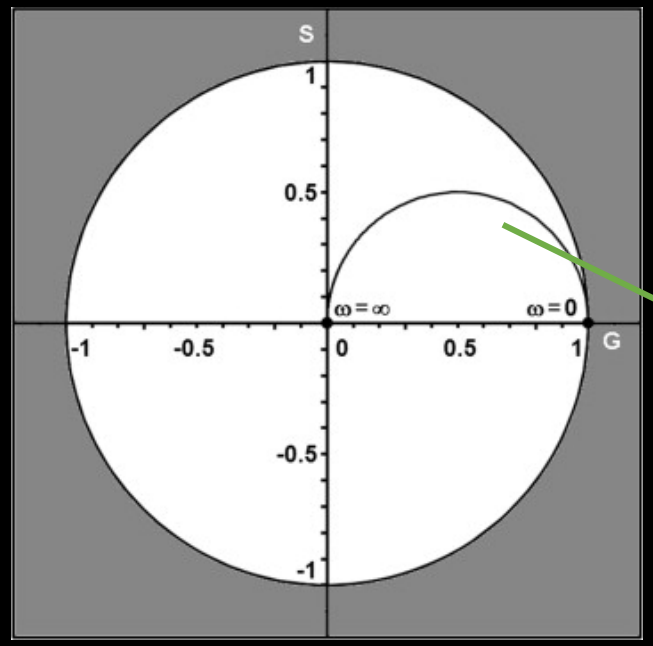


Camera-based detection on widefield, multi-beam confocal and TIRF microscopes operate both in time domain and frequency domain

https://www.leica-microsystems.com/science-lab/what-is-flim-fluorescence-lifetime-imaging/

Phasor approach

- Cosine and sine Fourier transforms of *I(t)*



$$G(\omega) = \frac{\int_0^\infty I(t)\cos(\omega t)\,dt}{\int_0^\infty I(t)dt}$$

ω - angular frequency

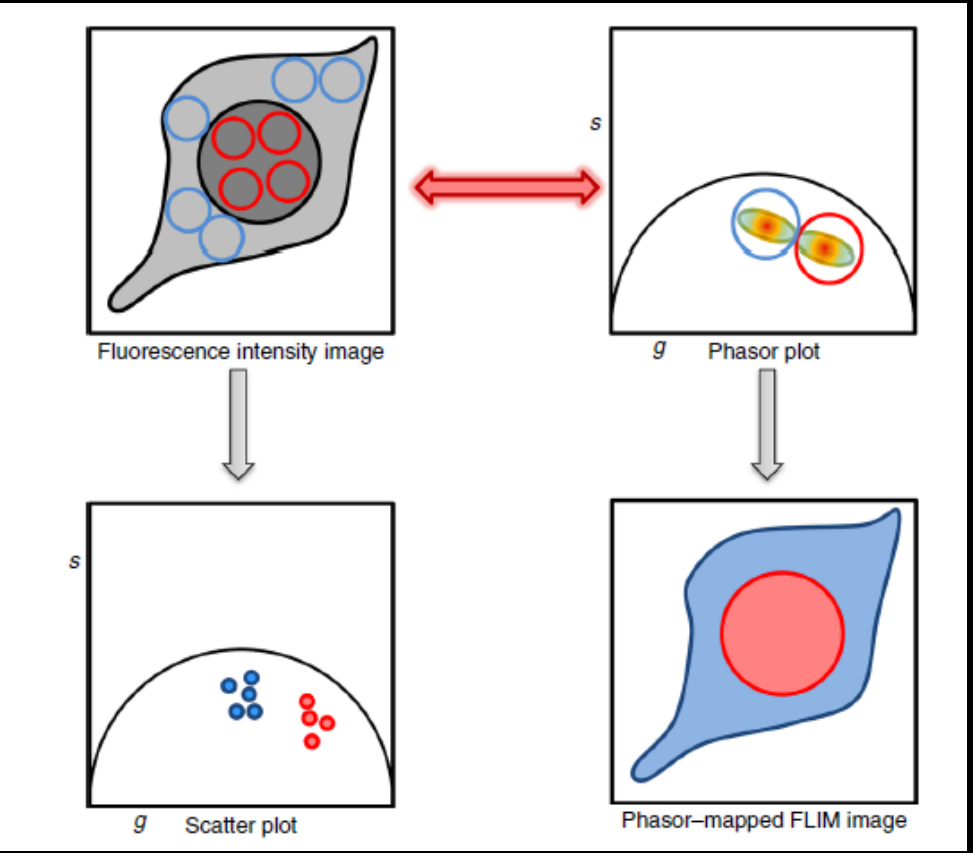
$$S(\omega) = \frac{\int_0^\infty I(t)\sin(\omega t)\,dt}{\int_0^\infty I(t)dt}$$

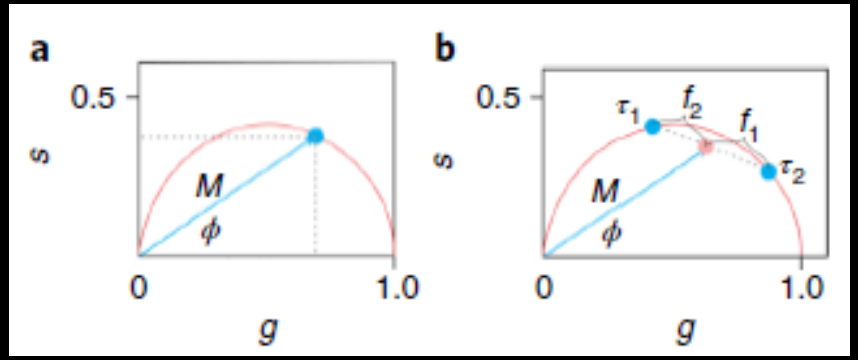
"Universal" semicircle – for all exponential decays

Phasor space – for all possible decay functions

Phasor plots of luminescence decay functions, M. N. Berberan-Santos, Chemical Physics, Vol. 449, p. 23-33, 2015

Phasor approach





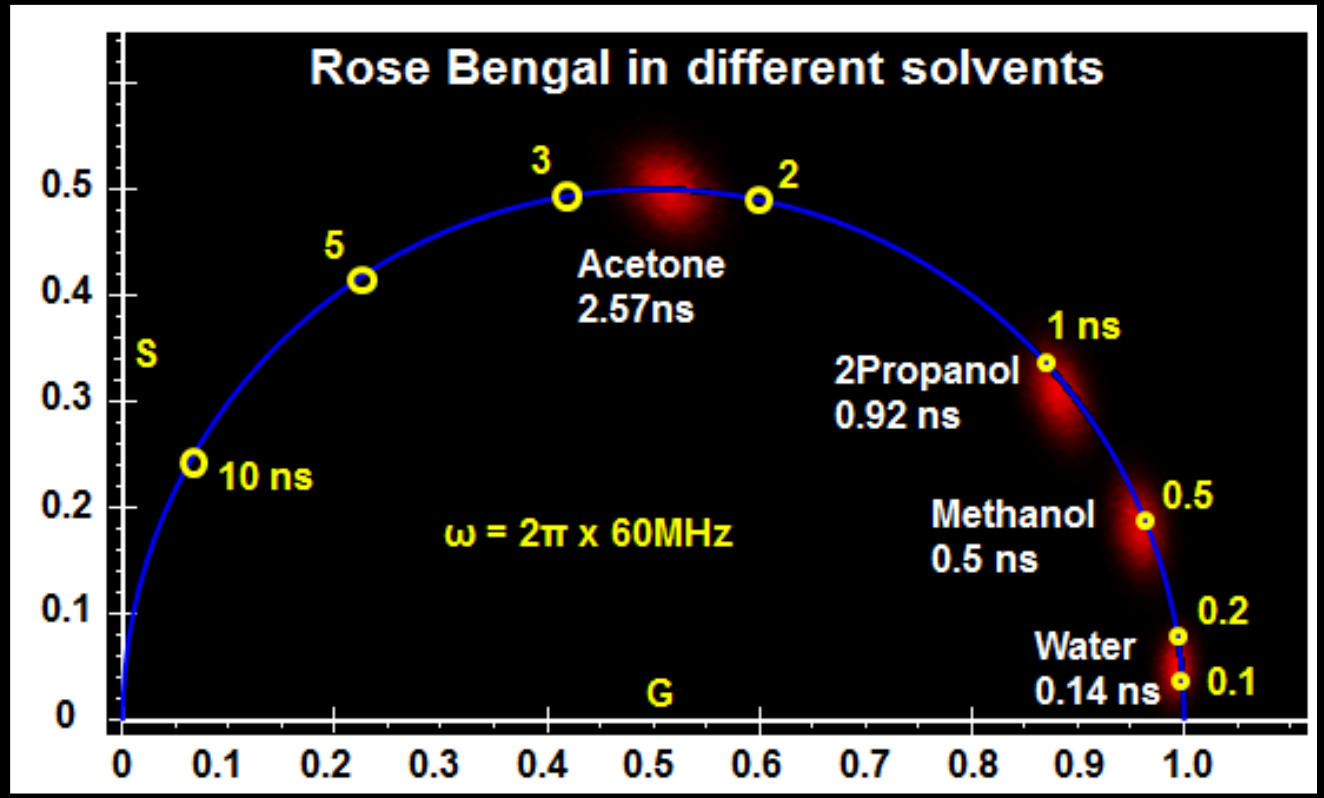
Mono-exponential decay

Multi-exponential decay

Phasor = point in the phasor space (corresponding to fluorescence decay from a pixel)

Fit-free analysis of fluorescence lifetime imaging data using the phasor approach, Ranjit S. et al., Nature Protocols, Vol. 13, p. 1979-2004, 2018

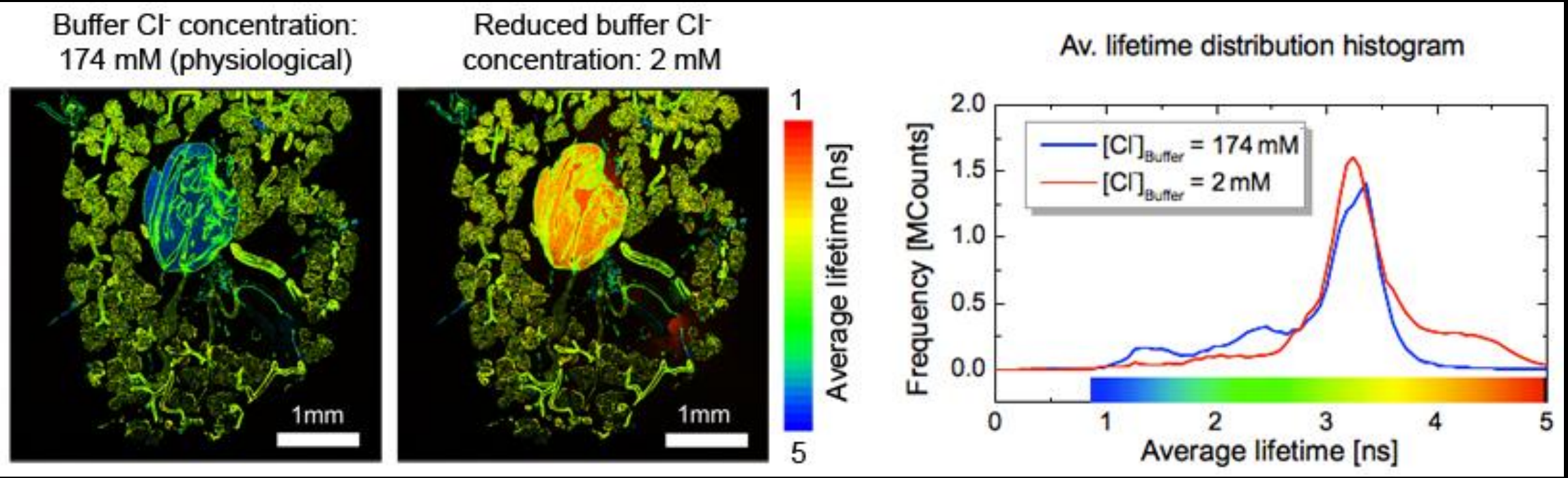
Phasor approach



https://iss.com/resources/pdf/technotes/FLIM_Using_Phasor_Plots.pdf

FLIM applications

- Fluorescence lifetime is sensitive to hydrogen ion concentration (**pH**), oxygen, and calcium ion concentrations



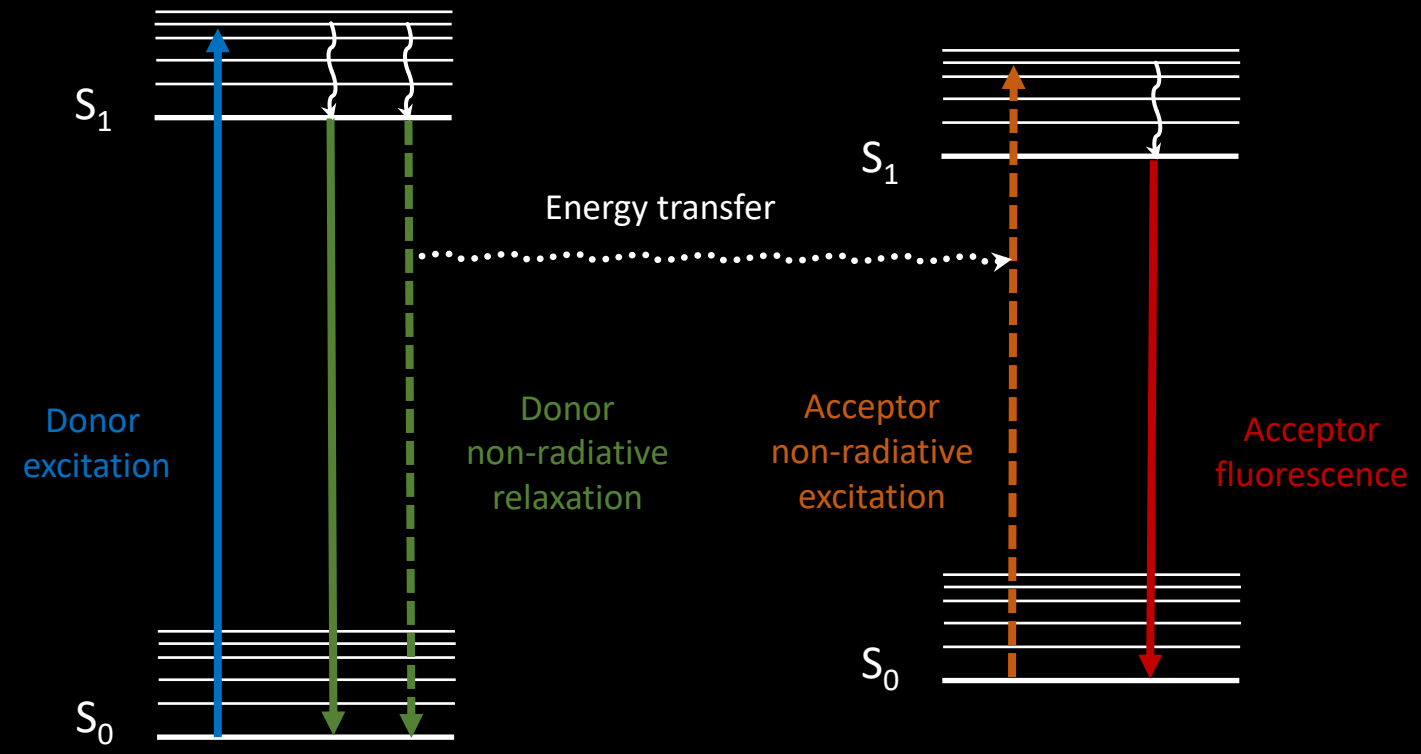
- Monitoring of metabolism on the basis of free and enzyme-bound NADH FLIM
- FRET

https://www.picoquant.com/applications/category/life-science/fluorescence-lifetime-imaging-flim

FRET

What is FRET

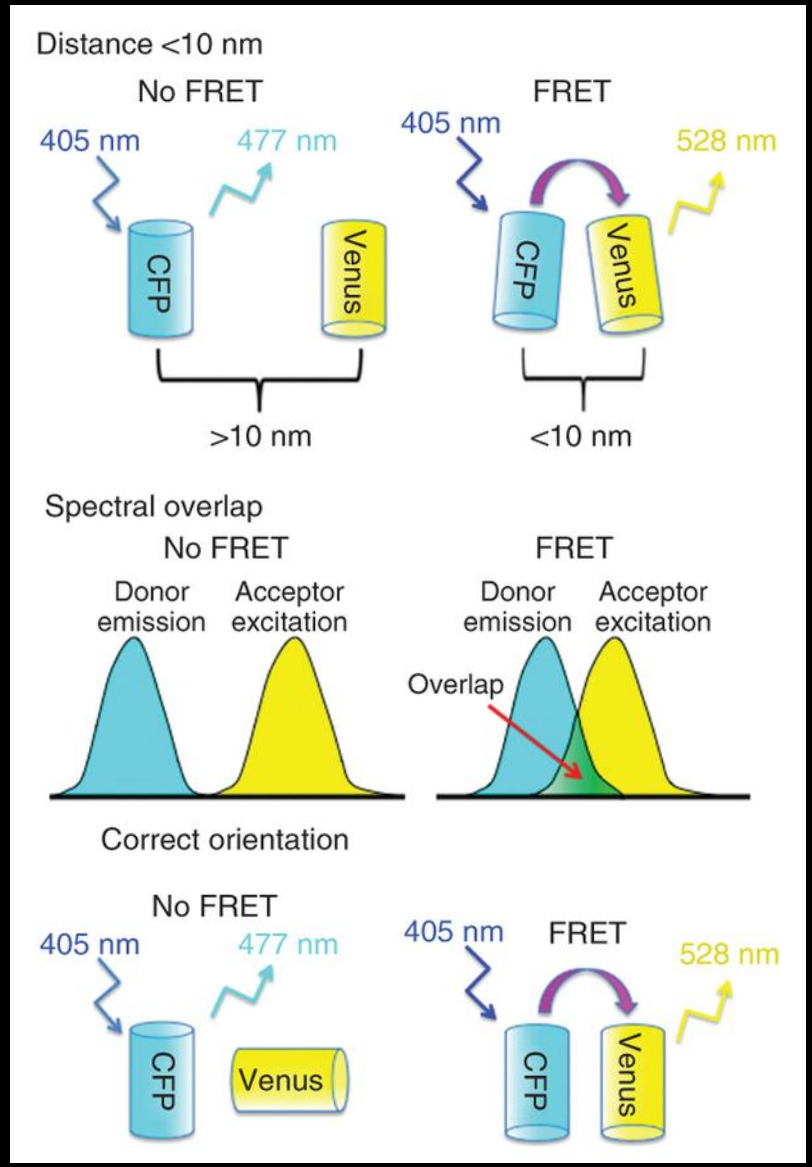
- **“Fluorescence Resonance Energy Transfer” (Förster Resonance Energy Transfer)**
- Energy transfer between 2 chromophores
- Nonradiative dipole-dipole coupling



FRET

FRET efficiency depends on:

- **Distance** between the *donor* and the *acceptor* (typically in the range of 1-10 nm)
- **Spectral overlap** of the *donor* emission spectrum and the *acceptor* absorption spectrum
- **Relative orientation** of the *donor* emission dipole moment and the *acceptor* absorption dipole moment
- **High quantum yield** of the *donor* molecule



Joshua A. Broussard et al., Nature Protocols 8, 265–281 (2013)

FRET

FRET efficiency

- Fraction of energy transfer event occurring per donor excitation event
- Depends on separation distance between *donor* and *acceptor*

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

Donor-acceptor separation distance

Förster distance - at which the energy transfer efficiency is 50%

$$E = \frac{k_{ET}}{k_f + k_{ET} + \sum k_i}$$

Rate of energy transfer

Radiative decay rate

Rate constants of other de-excitation pathways

FRET

What can be determined by FRET:

- Quantify **protein-protein interactions**
- Protein-DNA interactions
- Interactions between complexes
- Protein **conformational changes**
- Monitor **complex formation**

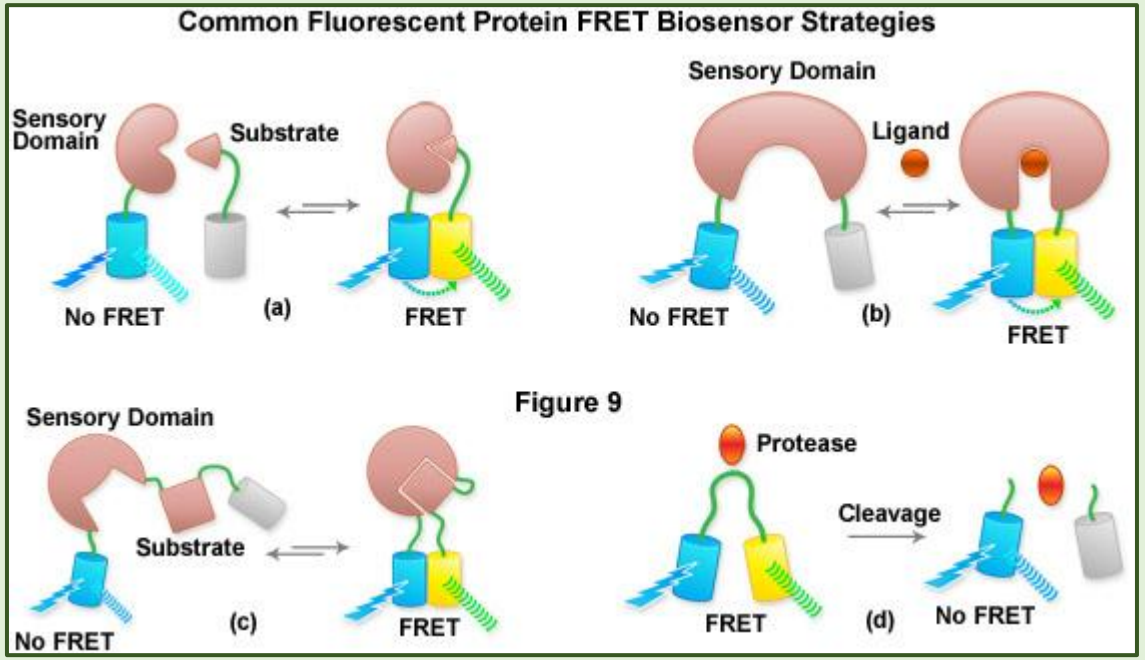


Figure 9

FRET

What can be determined by FRET:

- Quantify **protein-protein interactions**
- Protein-DNA interactions
- Interactions between complexes
- Protein **conformational changes**
- Monitor **complex formation**

FRET measurements

- Sensitized emission (direct FRAP) *(in vivo)*
- Acceptor photobleaching *(fixed samples)*
- FLIM-FRET (fluorescence lifetime shortening)
- Phasor approach

Sensitized emission

Measure

- Fluorescence of donor D (D_{ex}/D_{em})
- Fluorescence of acceptor A (A_{ex}/A_{em})
- FRET (D_{ex}/A_{em})

For cells expressing both donor and acceptor

$$E = \frac{FRET_{corr}}{A}$$

Donor cross-talk correction

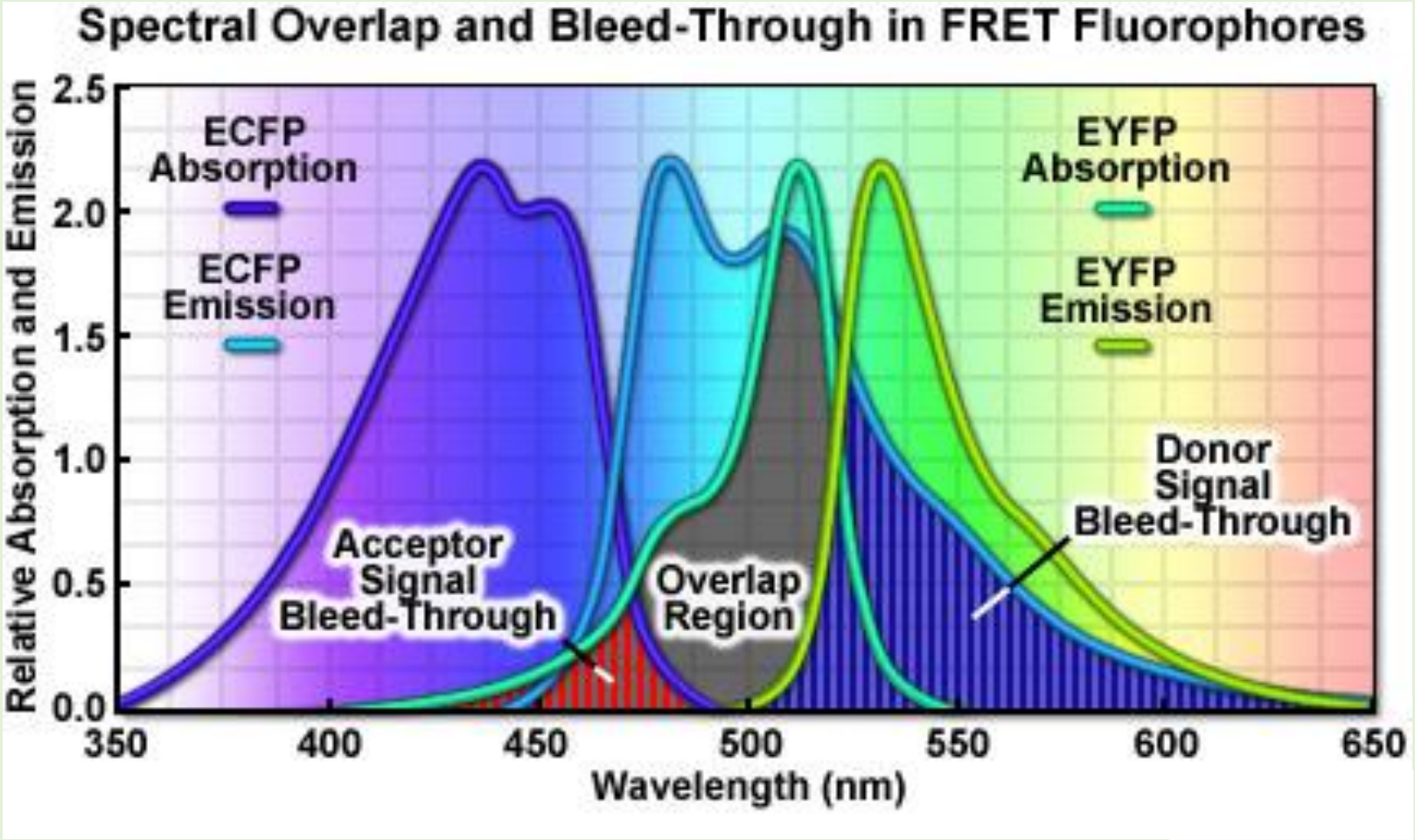
$$FRET_{corr} = FRET - R_A * A - R_D * D$$

Acceptor cross-excitation correction

Controls:

- *Background* (without labeled molecules)
- *Positive control* – sample expressing both donor and acceptor that are known to interact
- *Negative control* – sample expressing both donor and acceptor with elimination of their direct interaction

Sensitized emission



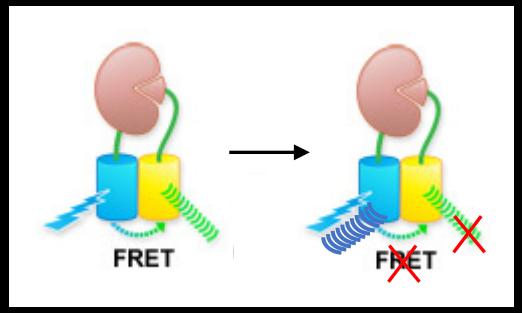
→ **artificially high FRET values**

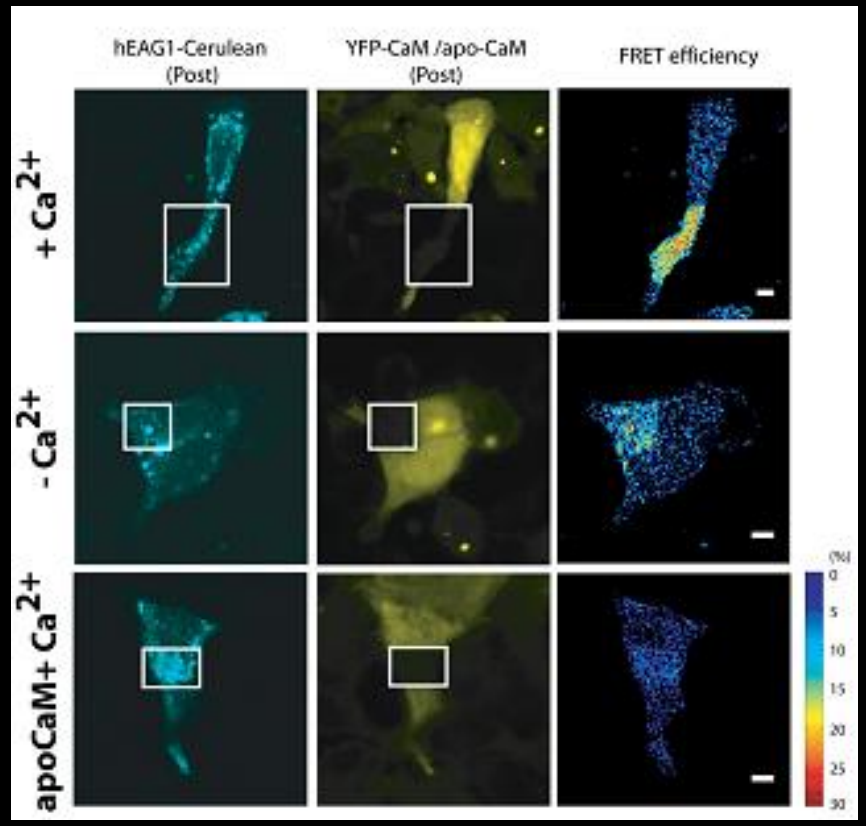
http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/spectralfret.html

Acceptor photobleaching

Measure

- Fluorescence of donor D (D_{ex} /D_{em})
- Fluorescence of acceptor A (A_{ex} /A_{em})





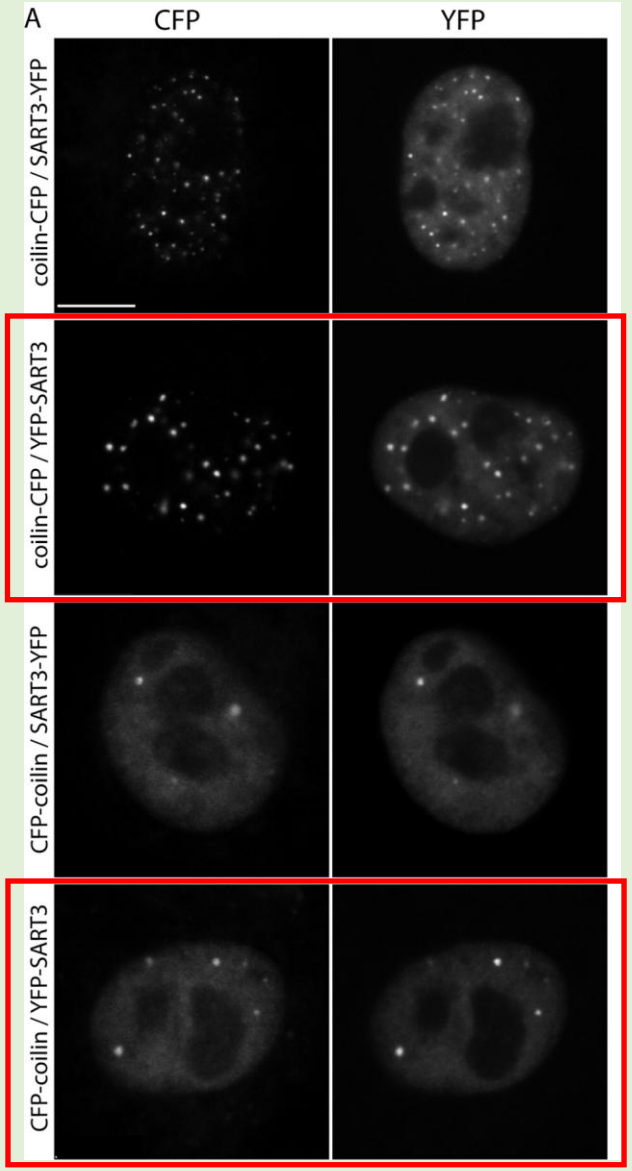
The acceptor molecule is bleached, resulting in brightening (unquenching) of the donor fluorerscence

$$D_{measured} = D_{total} - FRET \longrightarrow D_{measured} = D_{total}$$

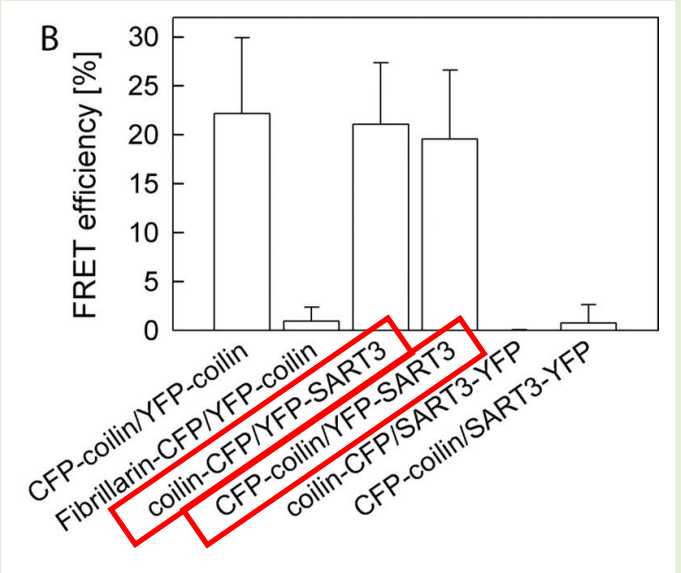
➢ *Positive and negative control*

doi:10.1371/journal.pone.0010873.g001

Example of FRET



In vivo kinetics of U4/U6·U5 tri-snRNP formation in Cajal bodies



FRET analysis of SART–coilin interaction

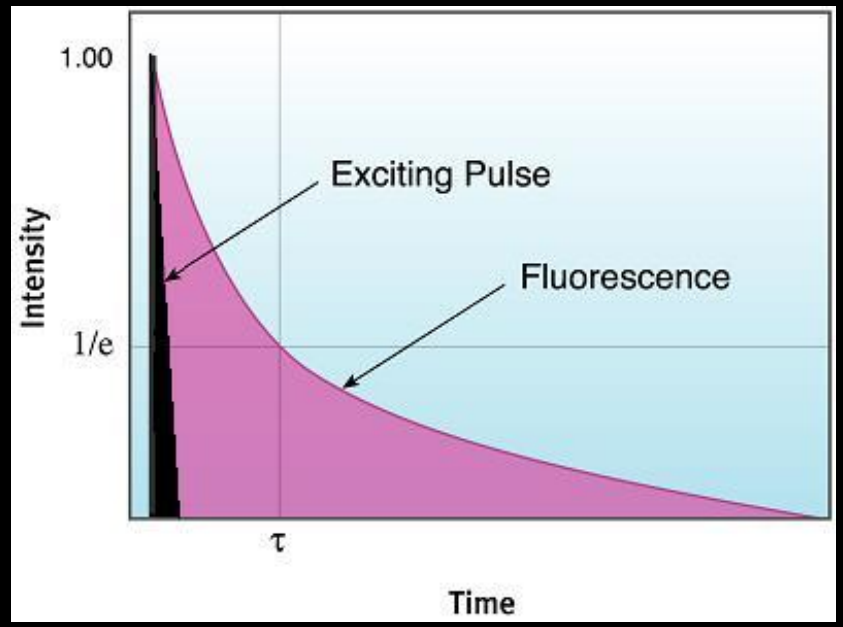
- FRET was measured in CBs by acceptor photobleaching
- Positive FRET signal between coilin and SART3 was detected when SART3 was tagged at the N terminus but not at the C terminus
- CFP-coilin/YFP-coilin served as a positive control
- YFP-coilin/fibrillarin-CFP as a negative control

Novotny et al., Molecular Biology of the Cell 22(4):513-23 (2011)

FLIM-FRET

Fluorescence lifetime imaging microscopy

- The most rigorous method

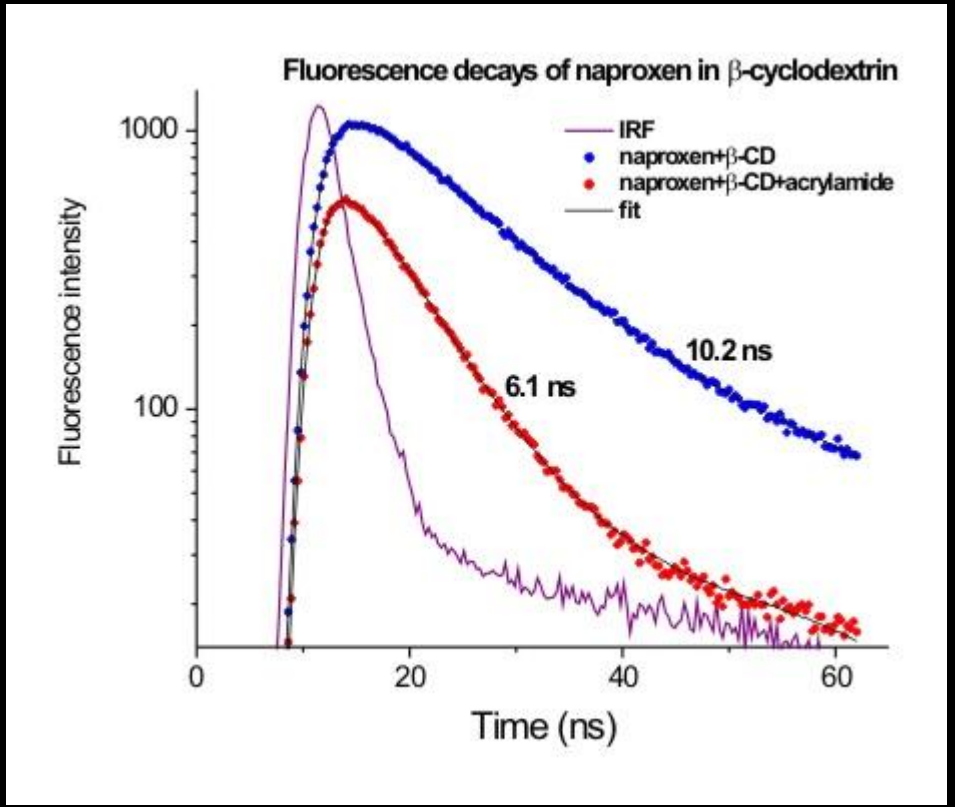


- **The donor fluorescence is quenched by the FRET interaction**, and the amount of quenching can be determined by measuring the decrease in fluorescence decay time of the donor in the presence of FRET

FLIM-FRET

- FLIM-FRET measures the change in the decay function of the FRET donor on interaction with an acceptor

-> **shortening of donor lifetime** is observed



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

http://microscopy.berkeley.edu/courses/tlm/fluor_techniques/FLIM.html

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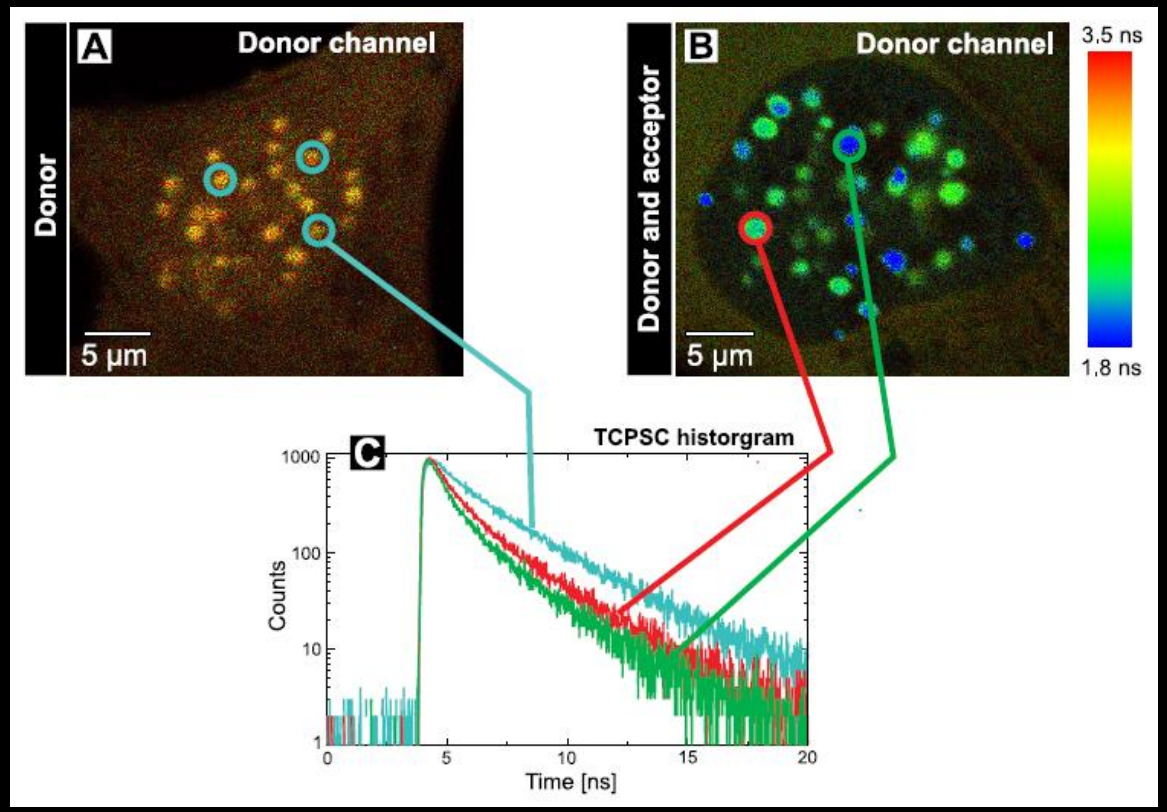


FLIM-FRET measurements of the human kinetochore proteins CENP-B-Cerulean (donor) and EYFP-CENP-A (acceptor)

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

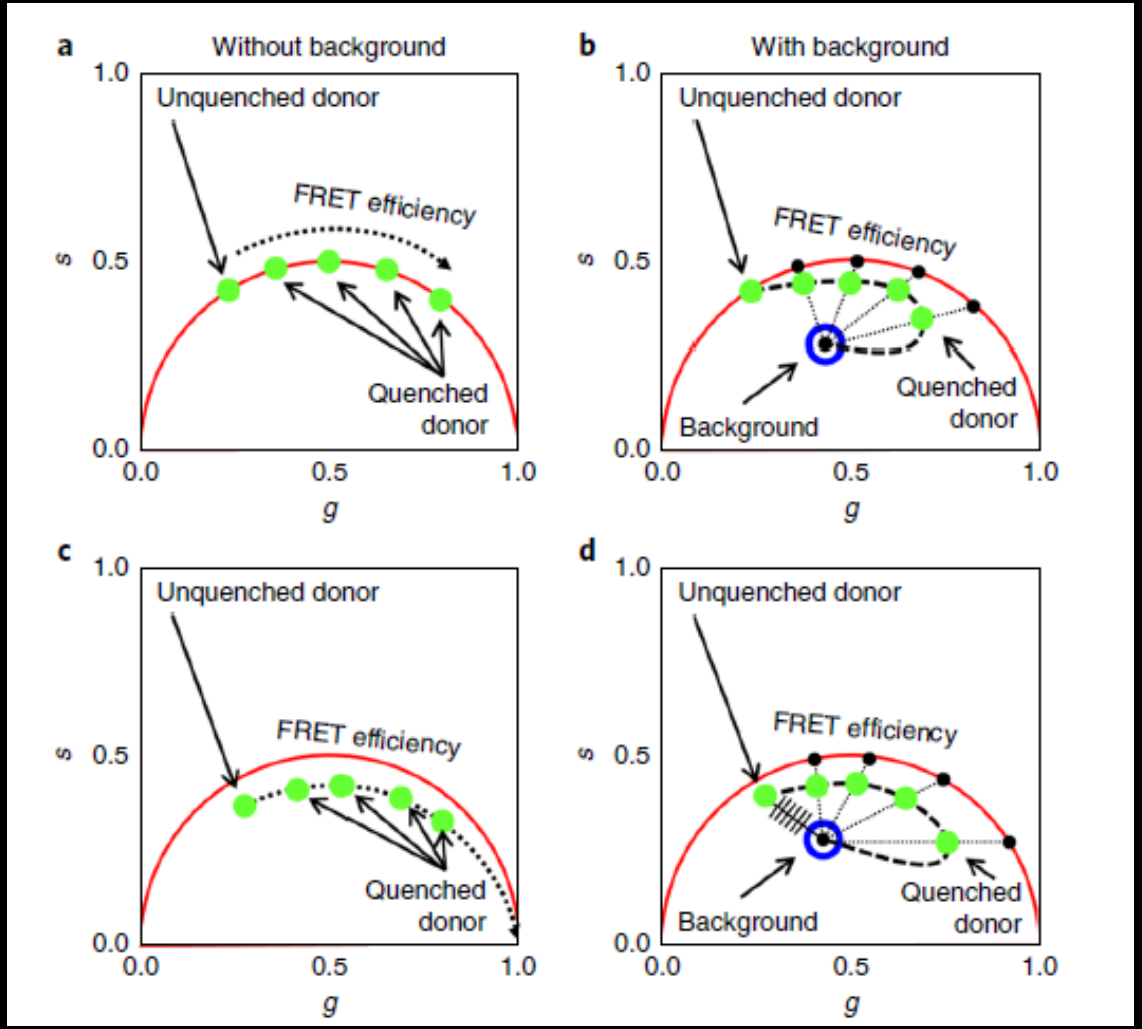
- FLIM-based FRET measurement does not have to cope with problems like donor bleedthrough or directly excited acceptor fluorescence

http://www.picoquant.com/images/uploads/page/files/7350/appnote_flim_overview.pdf

Phasor approach

The trajectories for a donor with mono-exponential lifetime

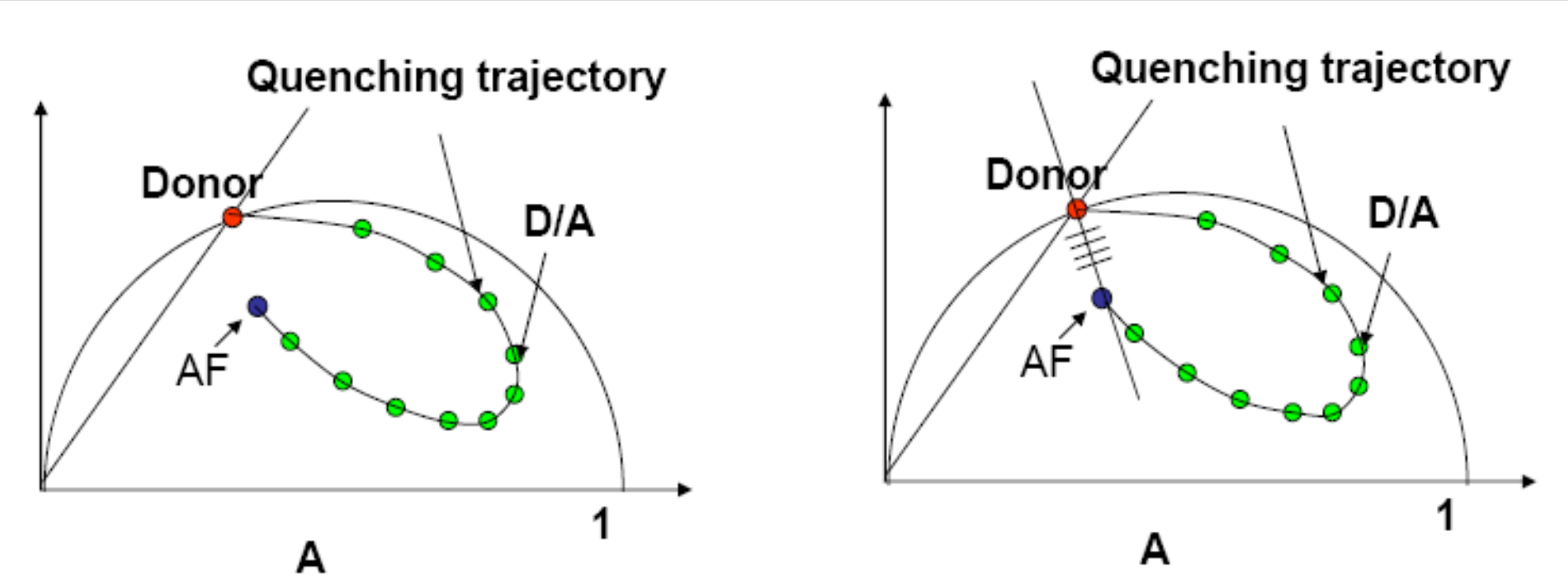
The trajectories for a donor with multi-exponential lifetime



Fit-free analysis of fluorescence lifetime imaging data using the phasor approach, Ranjit S. et al., Nature Protocols, Vol. 13, p. 1979-2004, 2018

Phasor approach

- FRET trajectory approach (E. Gratton)



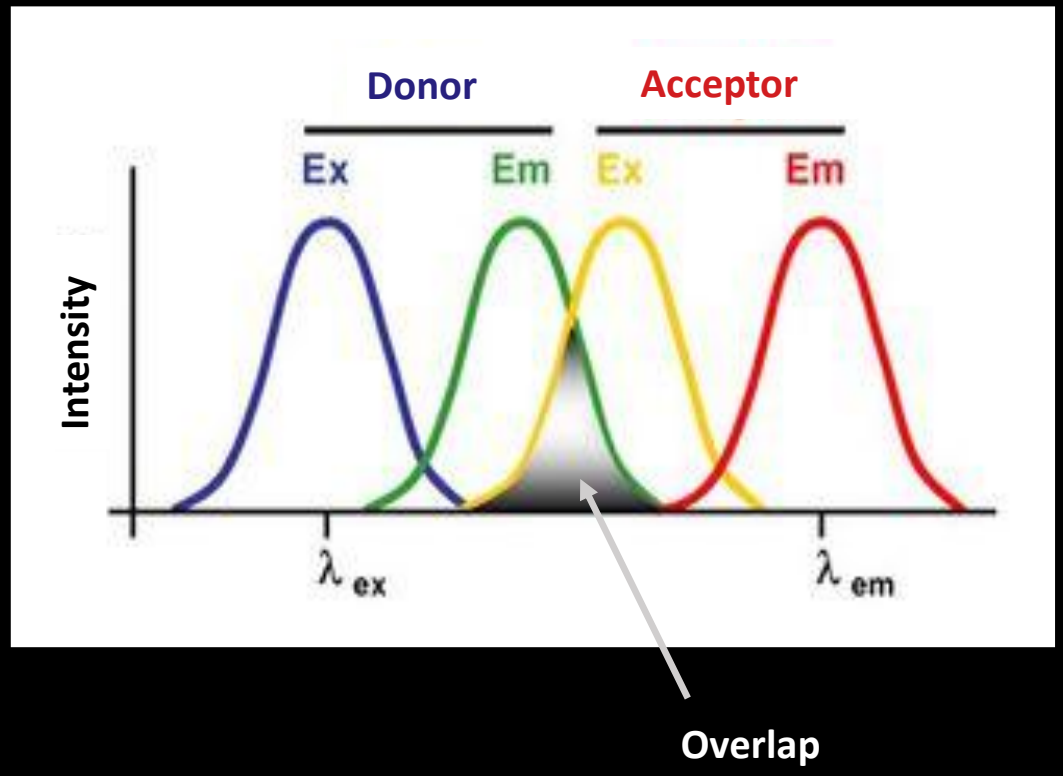
Phasor of the quenched Donor is added to the phasor of the autofluorescence

The final point will be along the line joining Donor with autofluorescence phasor

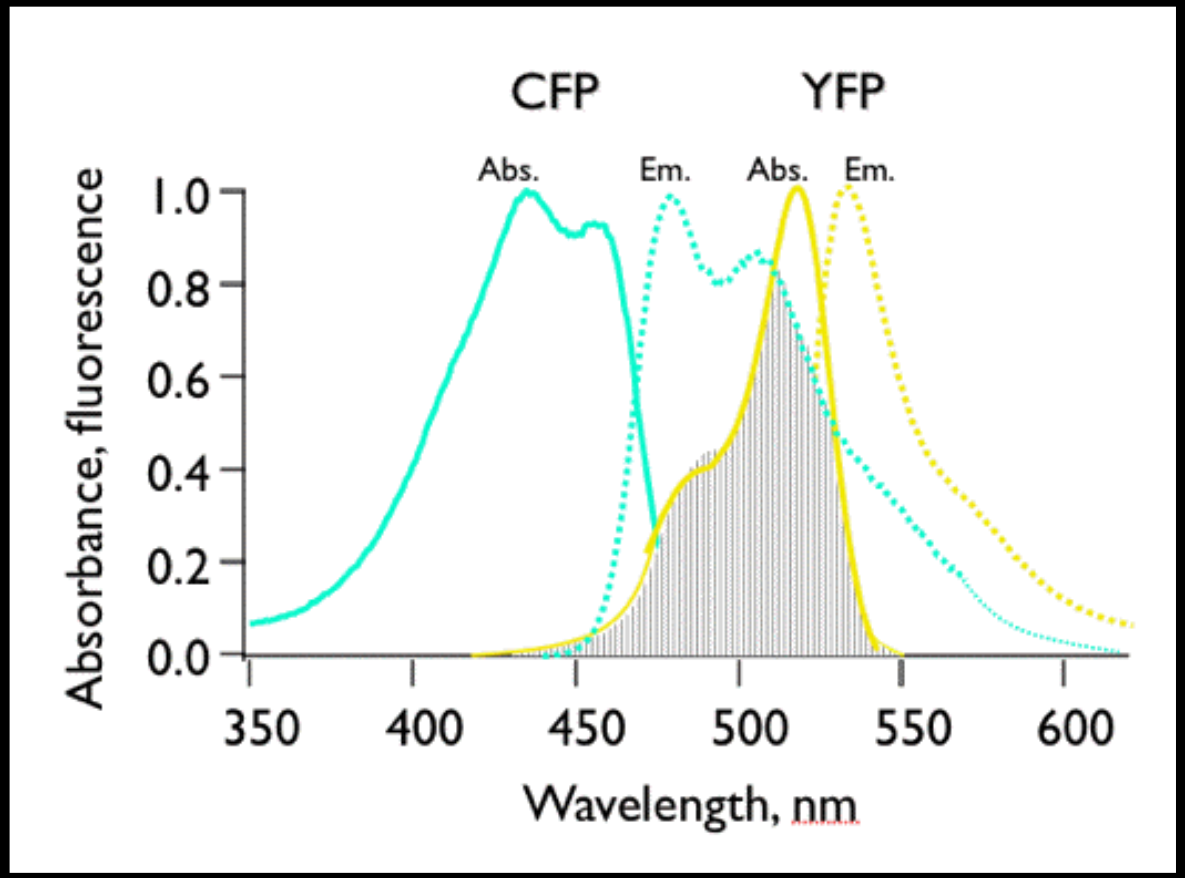
https://iss.com/resources/pdf/technotes/FLIM_Using_Phasor_Plots.pdf

Fluorophores used for FRET

- **Maximal overlap** of donor emission and acceptor excitation
- **Minimal direct excitation** of the acceptor at the excitation maximum of the donor



Fluorophores used for FRET



- CFP/YFP - a common example of FRET pair

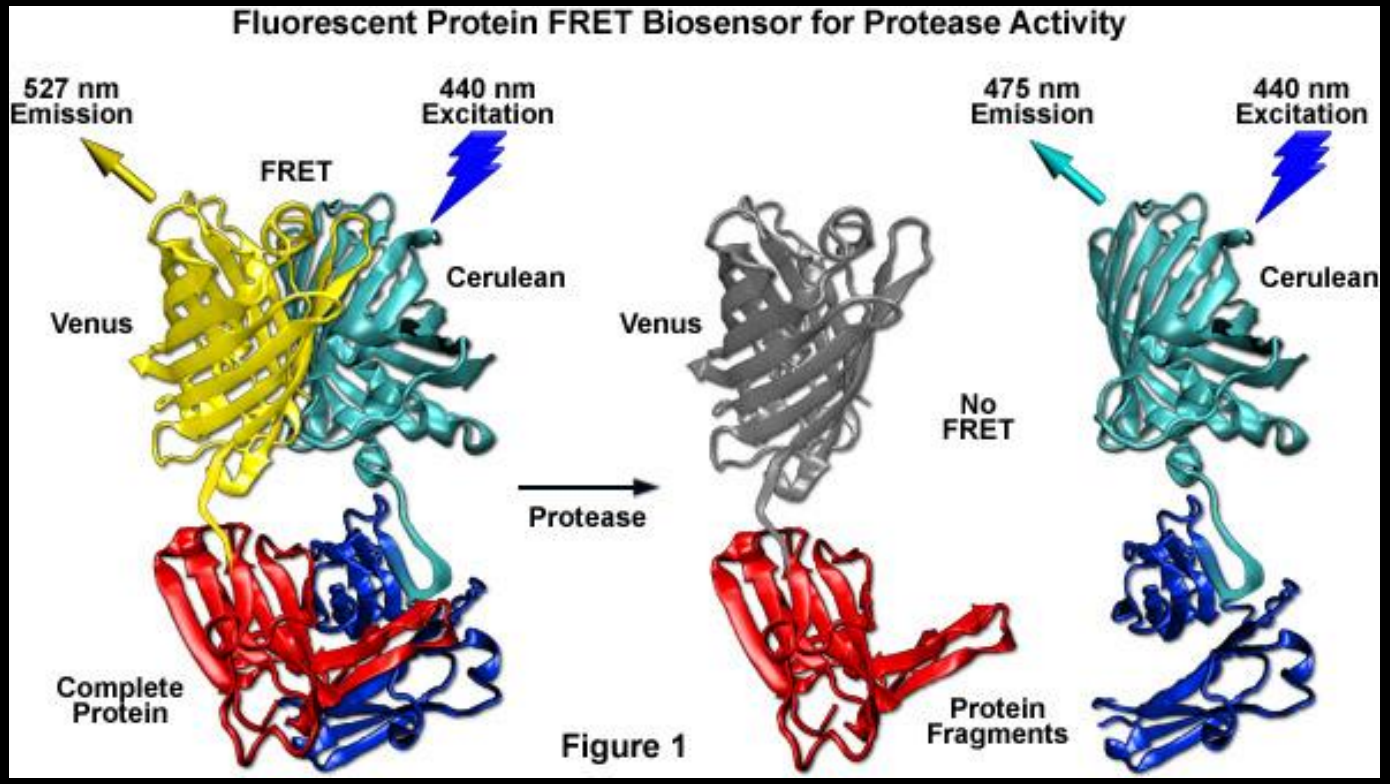
Fluorophores used for FRET

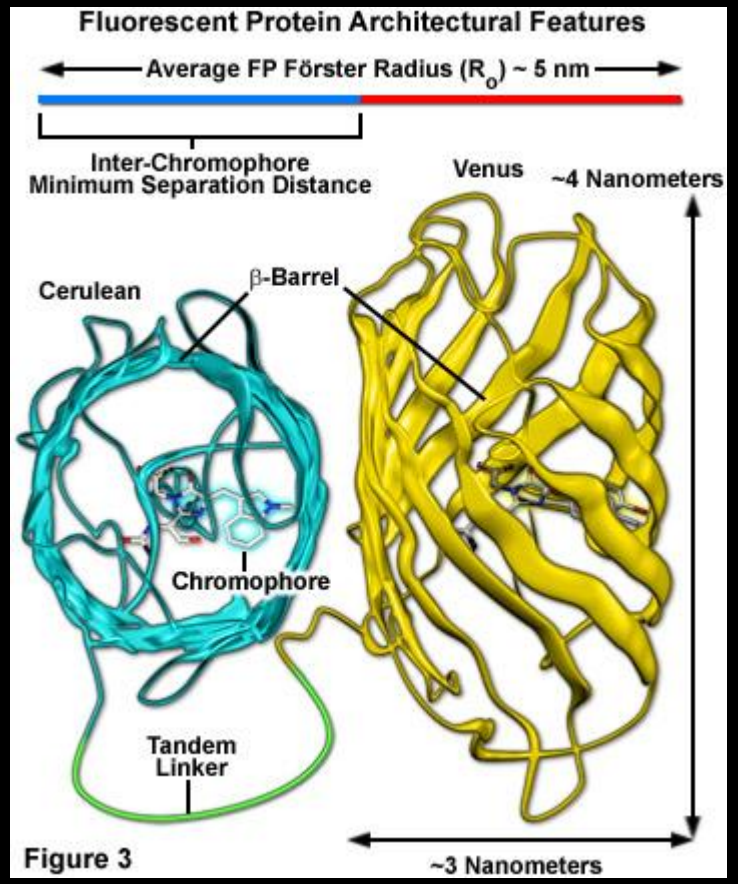
Protein Pair	Donor Quantum Yield	Acceptor Molar Extinction Coefficient ($mM^{-2}cm^{-1}$)	Förster Distance (nm)
ECFP-EYFP	0.4	83	4.9
mTurquoise2-sEYFP	0.93	101	5.9
mTurquoise2-mVenus	0.93	92	5.8
EGFP-mCherry	0.6	72	5.4
Clover-mRuby2	0.76	113	6.3
mClover3-mRuby3	0.78	128	6.5
mNeonGreen-mRuby3	0.8	128	6.5
eqFP650-iRFP	0.24	105	5.8
mAmetrine-tdTomato [d]	0.58	138	6.6
LSSmOrange-mKate2 [d]	0.45	63	7.0
EGFP-sREACh	0.6	115	5.8
EGFP-ShadowG	0.6	89	4.7
EGFP-activated PA-GFP	0.6	17	4.4
EGFP-Phanta	0.6	98	5.8
mTagBFP-sfGFP	0.63	83	4.6
mVenus-mKOκ	0.57	105	6.3
CyOFP1-mCardinal [d]	0.76	87	6.9

A Guide to Fluorescent Protein FRET Pairs, Bajar BT et al., 2016, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5038762/

FRET biosensors

- Molecular probes
- Processes such as calcium wave induction, cyclic nucleotide messenger effects, pH changes, membrane potential fluctuations, phosphorylation, and intracellular protease action





http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/spectralfret.html

FRET biosensors

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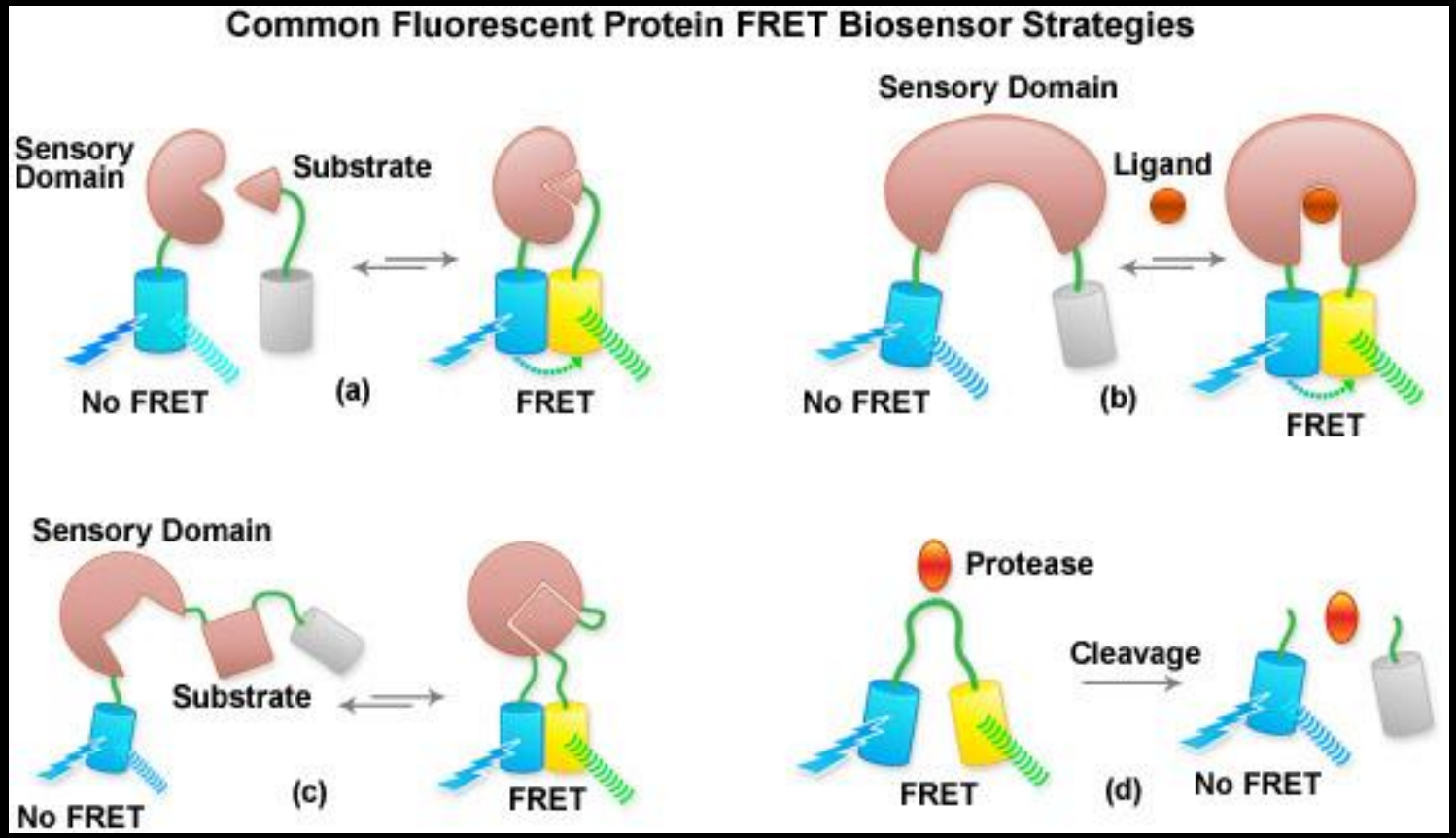

http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/spectralfret.html

Thank you for your attention!