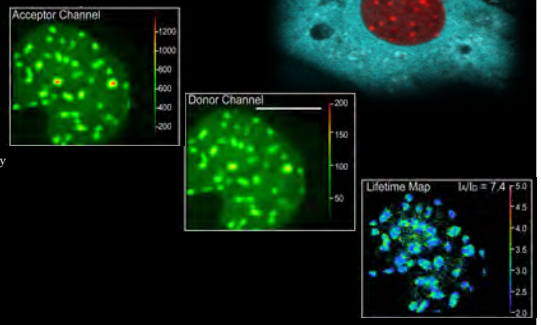
FRET and FLIM: monitoring biosensor activities and protein interactions in living cells



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O'Brien Center Workshop
IUSM
April 12, 2017

Overview

- Förster (Fluorescence) resonance energy transfer (FRET):
  - Why FRET?
  - Spectral overlap and spectral bleedthrough.
- Methods used to detect FRET:
  - FRET by spectral bleedthrough correction.
  - Acceptor photo-bleaching FRET.
  - Fluorescence lifetime imaging microscopy (FLIM).
- ❖ Motive - introduce FLIM method for intravital imaging.

Overview

- Förster (Fluorescence) resonance energy transfer (FRET):
  - Why FRET?

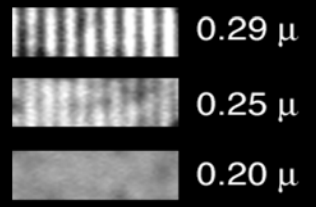
Resolving power of the light microscope

- The wavelength of the illuminating light and the numerical aperture (NA) of the objective limit optical resolution of the *conventional* light microscope to ~200 nm:

Rayleigh equation for resolution:

$r = 1.22 \lambda / 2NA$ or more simply $r = 0.61 \lambda / NA$

where r is the distance separating two adjacent particles that can be resolved.



John Murray in Live Cell Imaging: A Laboratory Manual, 2nd Edition, 2010

What is FRET and why use it?

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- Newer super-resolution techniques offer several fold improvement in resolution (40 – 100 nm; SIM, STED, PALM...).
- Much greater resolution is necessary to detect protein-protein interactions or biosensor conformational changes in living cells.
- FRET microscopy measures the direct transfer of excited state energy from a donor fluorophore to a nearby acceptor that can only occur over a distance less than 80Å.
- FRET microscopy can provide measurements of the spatial relationship between fluorophores on the scale of ångstroms.

FRET measures the spatial relationship between fluorophores

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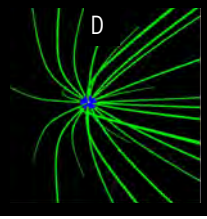
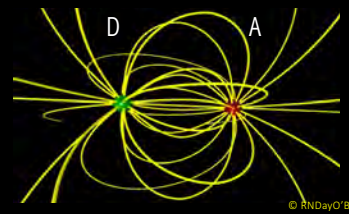
- A fluorophore in the excited-state is an oscillating dipole that creates an electric field (the donor - D).
- If another fluorophore (the acceptor - A) enters the electric field, energy can be transferred directly by dipole-dipole coupling.



❖ No intermediate photon!



Förster resonance energy transfer

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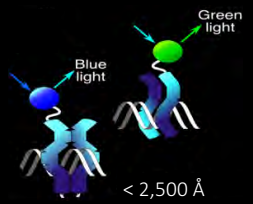
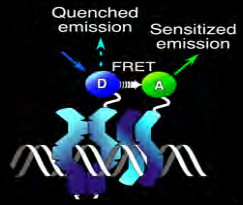
- Theodor Förster described how FRET efficiency decreased as the inverse of the sixth power of the separation distance.

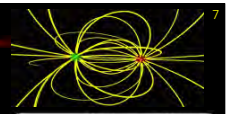


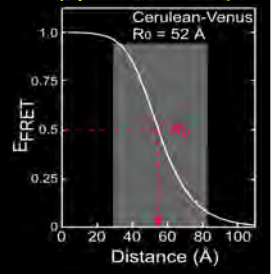
< 2,500 Å



< 80 Å

$$E_{FRET} = \frac{R_0^6}{R_0^6 + r^6}$$





R_0, the Förster distance

❖ The detection of FRET indicates the fluorophores are separated by less than ~ 80Å.

Energiewanderung und Fluoreszenz Naturwissenschaften (1946)
Translated in: Suhling (2012) JBO 17(1):011002

Overview

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- Förster (Fluorescence) resonance energy transfer (FRET):
  - Why FRET?
  - Spectral overlap and spectral bleedthrough.

Spectral overlap is necessary for FRET

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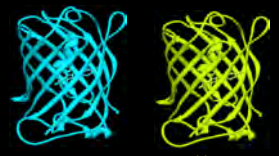
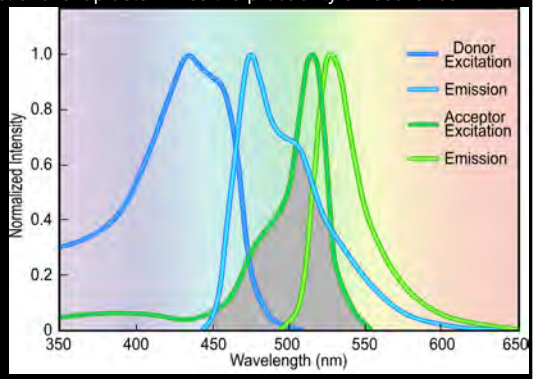
- Förster recognized that the spectral overlap determines the probability of resonance.



- The donor emission spectrum must significantly overlap the absorption spectrum of the acceptor;



- the more overlap → more efficient energy transfer.

Spectral bleedthrough background signals

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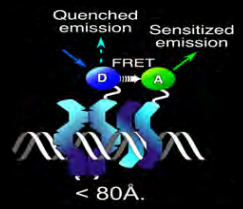
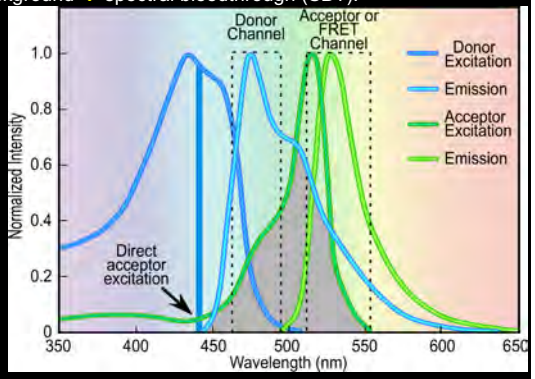
- The more overlap, the more background → spectral bleedthrough (SBT).



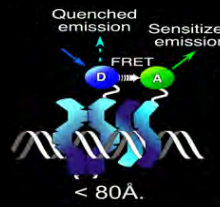
- Acceptor cross-talk: direct acceptor excitation



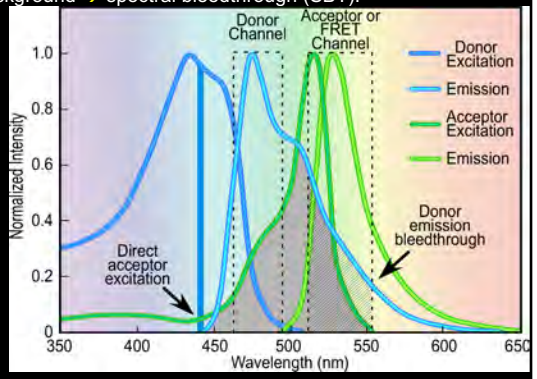
Spectral bleedthrough background signals

11

- The more overlap, the more background → spectral bleedthrough (SBT).



- Acceptor cross-talk: direct acceptor excitation
- Donor bleedthrough: emission in FRET channel



Spectral bleedthrough background signals

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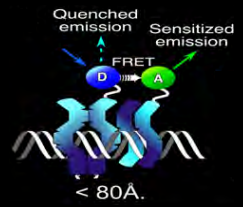
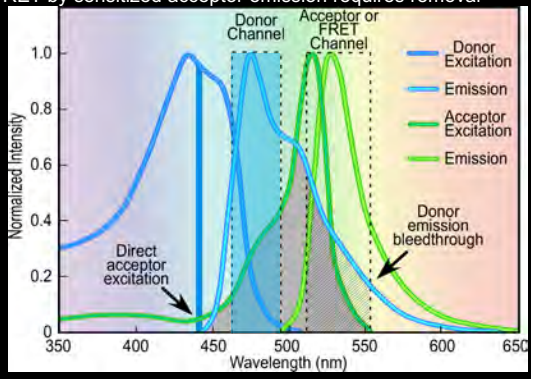
- The accurate measurement of FRET by sensitized acceptor emission requires removal of SBT!



- Alternative methods that detect the effect of FRET on the donor are not subject to SBT.*



* There can be acceptor back-bleedthrough if the donor band-pass is too wide

Overview

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- Förster (Fluorescence) resonance energy transfer (FRET):
  - Why FRET?
  - Spectral overlap and spectral bleedthrough.
- Methods used to detect FRET:

The use and abuse of FRET imaging

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- The genetically encoded FPs led to a dramatic increase in the use of FRET measurements to detect protein interactions in living cells.
- A consequence of this newfound popularity has been the "degradation in the validity of the interpretations" of these experiments.

S. S. Vogel, C. Thaler and S. V. Koushik, Sci STKE, 2006, 2006, re2.

- It is often difficult to directly compare the accuracies of different methods.
  - ❖ Use "FRET Standards" to characterize the experimental system.
  - ❖ Use more than one FRET method to verify the results.

The genetically encoded 'FRET Standards'

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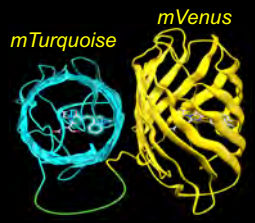
- Numerous methods have been developed to measure FRET, but their accuracy is difficult to determine.
- A simple solution was to develop FRET reference proteins derived from genetically encoded FP fusion proteins.



5-Amino Acid Linker
SGLRS

- Several different FRET methods are used to acquire measurements from cells expressing the fusion proteins to verify their use as standards.
- Model to compare FRET donors and acceptors.

Thaler et al. (2005) Biophys. J 89:2736

Methods used to measure FRET

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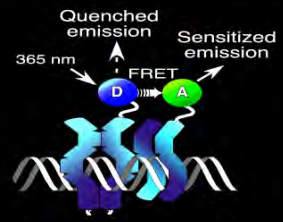
- There are many different ways to measure FRET (here are a few):

1. Spectral bleedthrough correction:
   E = FRET - [Spectral cross-talk]
2. Acceptor photo-bleaching:
   $E = (ID_{post} - ID_{pre})/ID_{post}$
3. Donor lifetime measurements:
   $E = 1 - (\tau_{DA}/\tau_D)$



- ❖ For intravital imaging - ratiometric imaging of biosensors (1:1 donor:acceptor) or lifetime are the most useful methods to measure FRET.

Overview

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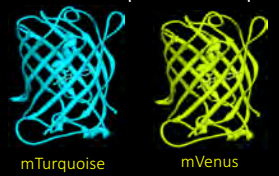
- Förster (Fluorescence) resonance energy transfer (FRET):
  - Why FRET?
  - Spectral overlap and spectral bleedthrough.
- Methods used to detect FRET:
  - FRET by spectral bleedthrough correction.

Spectral overlap is necessary for FRET

18

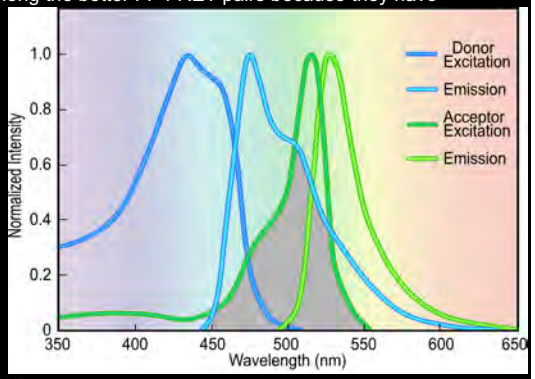
- mTurquoise and mVenus are among the better FP FRET pairs because they have substantial spectral overlap:



mTurquoise mVenus

mTurquoise QY = 0.84
mVenus EC = 92,200 M^{-1} cm^{-1}

$$J(\lambda) = \left(\frac{0.78}{2.52}\right)(92{,}200)(528)^4 = 2.2 \times 10^{15}$$



$$R_0 = 0.211\,[(0.667)(1.4)^{-4}(0.84)(2.2 \times 10^{15})]^{0.167} = 55\text{Å}$$

Spectral bleedthrough correction methods

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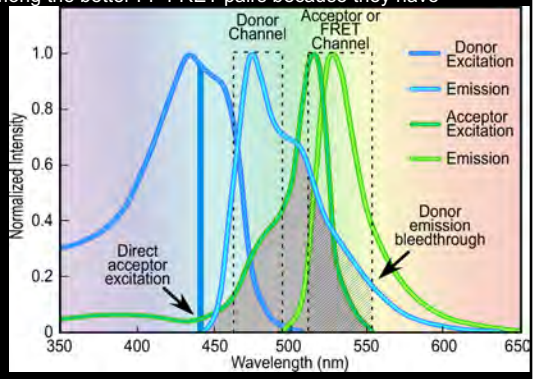
- mTurquoise and mVenus are among the better FP FRET pairs because they have substantial spectral overlap:
- but spectral bleedthrough into the FRET channel is a significant problem.
- Correction techniques are needed to identify and remove spectral bleedthrough from the FRET channel.



FRET by spectral bleedthrough correction

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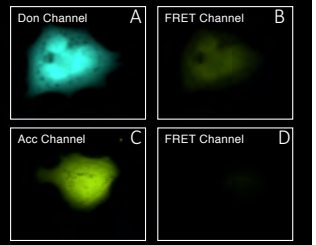
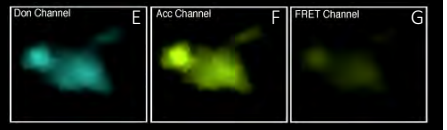
- Most imaging systems come with a computer algorithm for SBT correction (there are Image J plugins).
  - SBT background images:
    - A. Donor alone - Don Channel
    - B. Donor alone - FRET Channel
    - C. Acceptor alone - FRET Channel
    - D. Acceptor alone - Acc Channel



  - Experimental images:
    - E. Experimental - Don Channel
    - F. Experimental - FRET Channel
    - G. Experimental - Acc Channel



FRET by spectral bleedthrough correction

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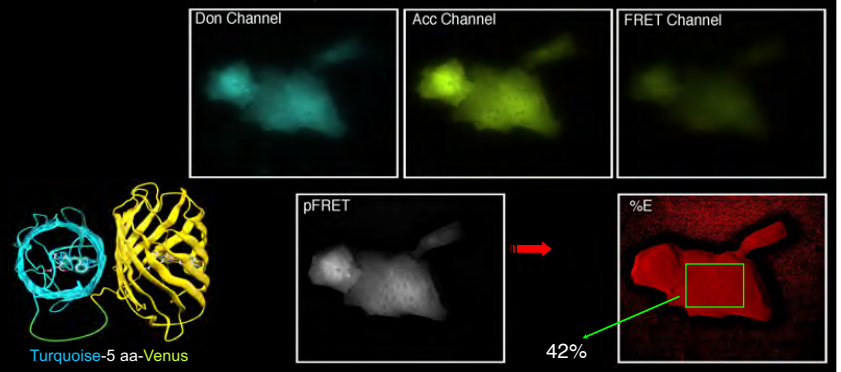
- The two spectral crosstalk components, determined from the control cell measurements, are removed from the FRET image.



FRET by spectral bleedthrough correction: negative control

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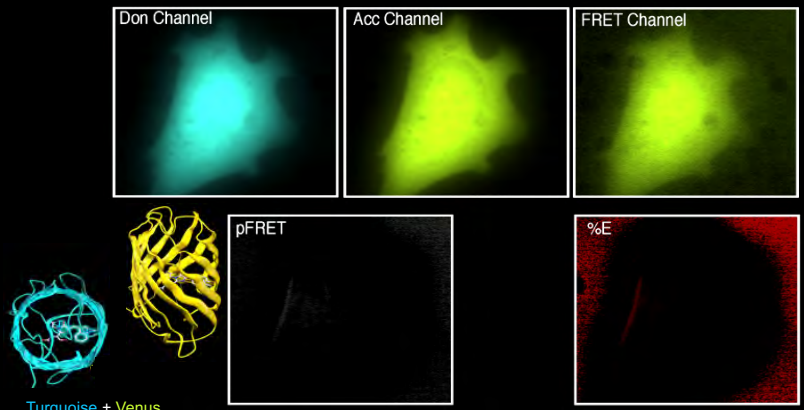
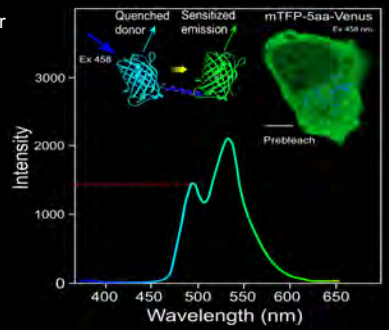


Spectral FRET measurements

23

- Energy transfer results in quenching of donor emission and sensitized emission from the acceptor.
- Exciting the donor and acquiring the complete emission spectrum detects both events.
- This approach is called spectral FRET.



Spectral FRET measurements

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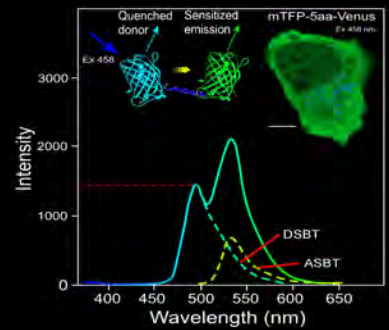
- Spectral FRET uses reference spectra obtained from cells expressing either the donor alone or acceptor alone.
- The method of linear unmixing remove donor spectral bleed through (DSBT).
- Bleedthrough correction removes acceptor spectral bleedthrough (ASBT) from each image pixel.



FRET by spectral bleedthrough correction

25

- Advantages:
  - Simple algorithms available on most imaging systems.
  - Compatible with most types of imaging (difficulties with 2-photon).
- Limitations:
  - Very sensitive to quality of the control data.
  - Subject to artifacts of cell movement.

Overview

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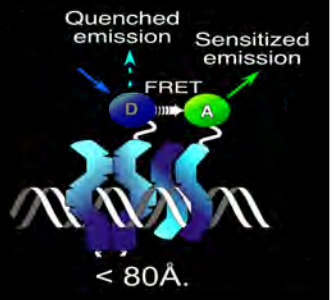
- Förster (Fluorescence) resonance energy transfer (FRET):
  - Why FRET?
  - Spectral overlap and spectral bleedthrough.
- Methods used to detect FRET:
  - FRET by spectral bleedthrough correction.
  - Acceptor photo-bleaching FRET.

Acceptor photo-bleaching

27

- Energy transfer results in quenching of the D emission and sensitized emission from the A.



Acceptor photo-bleaching

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- Energy transfer results in quenching of the D emission and sensitized emission from the A.
- Photo-bleaching the acceptor relieves donor quenching (de-quenching).
- The increase in the donor signal is detected in the donor channel, which is free of SBT background.*

* There can be acceptor back-bleedthrough if the donor band-pass is too wide

Acceptor photo-bleaching in the cell nucleus

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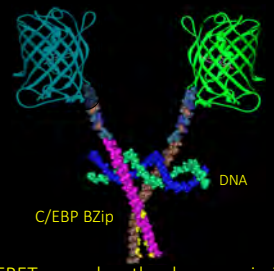
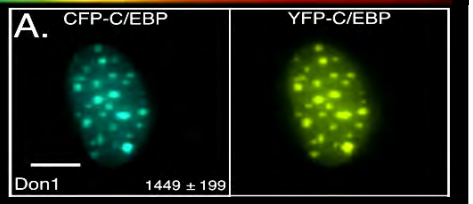
- C/EBPα dimerizes only when bound to DNA in the cell nucleus.
- C/EBPα binds to repeated DNA elements in heterochromatin.





FRET quenches the donor emission

Acceptor photo-bleaching in the cell nucleus

30

- C/EBPα dimerizes only when bound to DNA in the cell nucleus.
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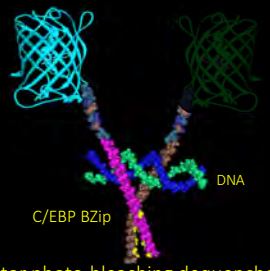

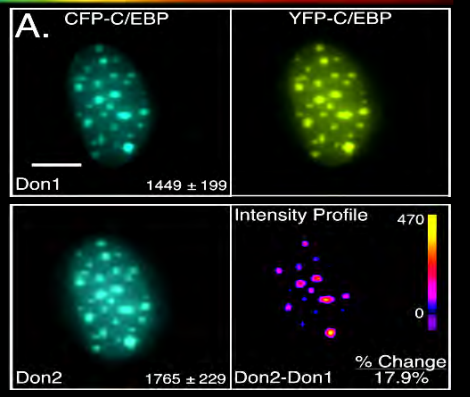

Acceptor photo-bleaching dequenches the donor

Confirming spectral FRET using acceptor photo-bleaching

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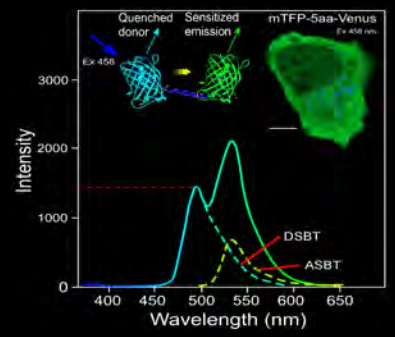
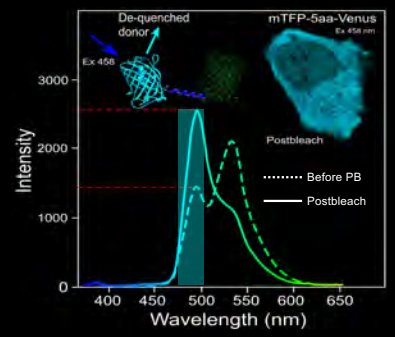
- pb-FRET is commonly used to verify other FRET-based measurements.



Confirming spectral FRET using acceptor photo-bleaching

32

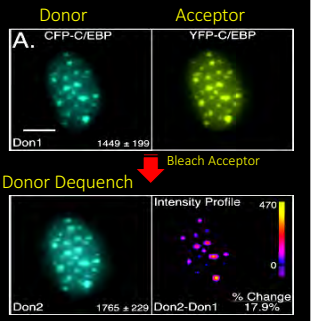
- pb-FRET is commonly used to verify other FRET-based measurements.
- pb-FRET can be used in combination with other FRET methods – e.g., verify SBT correction methods.
- But, pb-FRET is an end point assay.



FRET by acceptor photo-bleaching

33

- Advantages:
  - Simple approach that uses each cell as its own control; it can be very accurate.
  - Commonly used to verify results from other methods.
- Limitations:
  - Requires selective bleaching; dark-states and photo-switching can be problematic.
  - Subject to artifacts of cell movement – limited to stable protein interactions in living cells.
  - Endpoint assay - no dynamics.



Overview

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- Methods used to detect FRET:
  - FRET by spectral bleedthrough correction.
  - Acceptor photo-bleaching FRET.
  - Fluorescence lifetime imaging microscopy (FLIM).

Spectral bleedthrough background signals

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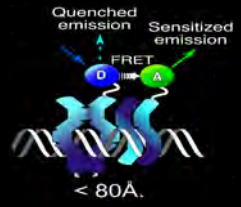
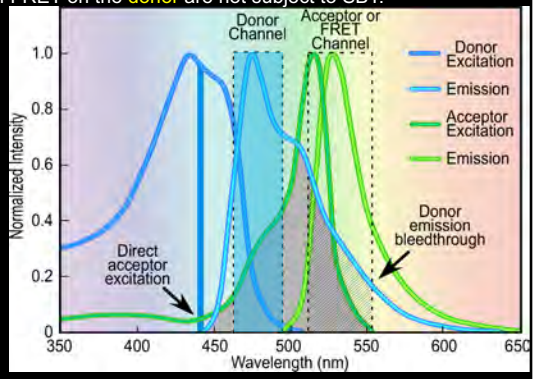
- Methods that detect the effect of FRET on the donor are not subject to SBT.*



- Fluorescence lifetime imaging microscopy (FLIM) measures the effect of FRET on the donor.



* There can be acceptor back-bleedthrough if the donor band-pass is too wide

FRET by fluorescence lifetime imaging microscopy (FLIM)

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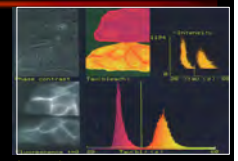
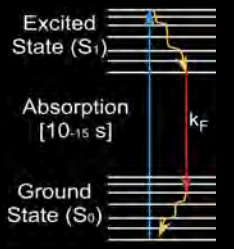
- The Jovin laboratory (1995) was the first to use fluorescence lifetime imaging microscopy (FLIM) to measure FRET.
  Gadella and Jovin (1995) J Cell Biol 129:1543
- Fluorescence lifetime (τ): the average time a population of fluorophores spend in the excited state before returning to the ground state.
- Every fluorophore has an intrinsic lifetime:

Probe	τ_m
Coumarin6 (EtOH)	2.5 ns
mTurquoise FP (cells)	3.9
HPTS (Pyranine) (PB, pH 7.8)	5.3
Fluorescein (PB, pH 7.5)	4.1

- A mixture of these probes with overlapping emissions can be separated by their individual lifetimes!

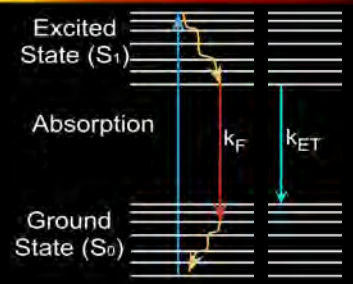




FRET by FLIM

37

- Events in the probe environment that affect the excited state can change the lifetime – molecular stop watch.
- FRET is a quenching event (k_{ET}), allowing transition to the ground state without fluorescence emission.
- Quenching events cause the fluorescence lifetime to shorten.



- Fluorescence lifetime imaging microscopy (FLIM) can accurately measure the change in lifetime resulting from FRET.

FRET by FLIM

38

- To measure FRET by FLIM, only two measurements are required:
  - donor lifetime in the absence of acceptor (τ_D).
  - donor lifetime in the presence of acceptor (τ_{DA}).

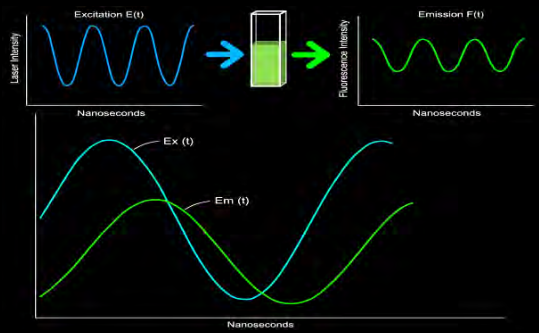
$$E_{FRET} = 1 - (\tau_{DA}/\tau_D)$$

- Two different FLIM methods:
  - Time domain - uses a femtosecond pulsed laser and gated camera, and determines lifetimes by exponential decay fitting methods.
  - Frequency domain - uses excitation light modulated at radio frequencies, and determines lifetimes directly by phasor analysis.
- The physics that underlies the two methods is the same – they only differ in how the signals are analyzed.

Frequency domain FLIM measurements

39

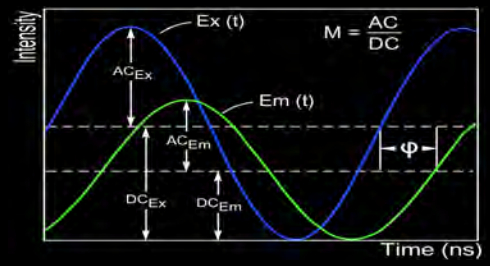
- In frequency domain, a continuous light source is modulated at high radio frequencies (usually 1-400 MHz) to excite the fluorophores.
- The emission signal from the fluorophores will also be modulated:



Frequency domain FLIM measurements

40

- Because of the excited state lifetime, there is a delay in the emission relative to the excitation.
- This causes a phase delay (Φ) and a change in modulation (M) of the emission signal relative to the excitation.



Phase delay: ϕ

Modulation: M = AC/DC

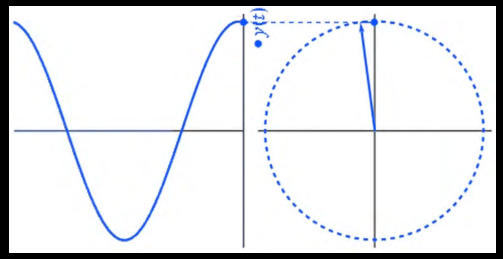
- The Φ and change in M are used to extract the fluorescence lifetime.

Jameson, D.M. (2014). Introduction to Fluorescence, pp 101-110.

Frequency domain data analysis by phasor plot

41

- The phasor plot is used to display frequency characteristics of the emission signal.
- The phasor is the projection of the emission signal waveform as a vector rotating about the origin in a complex plane.



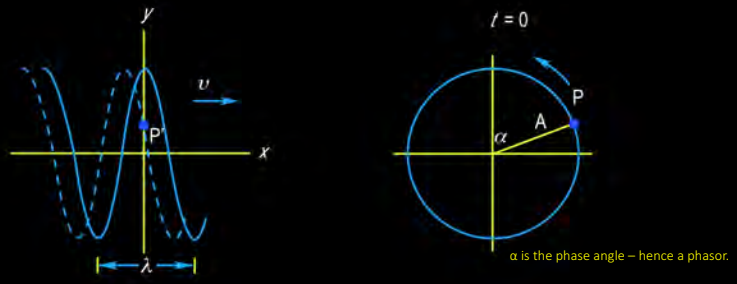
http://upload.wikimedia.org/wikipedia/commons/8/89/Unfasor.gif

Cole & Cole (1941) J Chem Phys 9:341
Jameson et al. (1984) App Spec Rev 20:55
Redford & Clegg (2005) J Fluoresc 15:805

Frequency domain data analysis by phasor plot

42

- The cosine function projects a vector onto the real axis, and the phase angle (α) formed with the real axis measures the phase delay.



α is the phase angle – hence a phasor.

Weber (1981) J Phys Chem 85:949

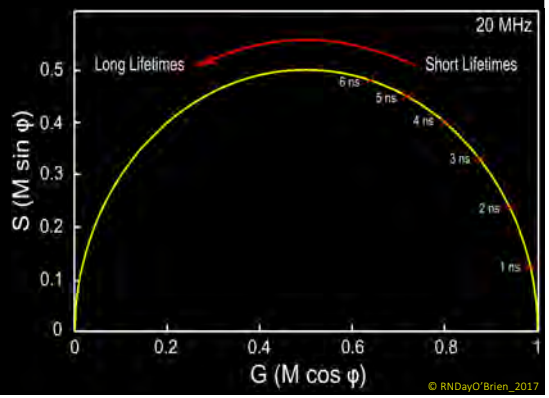
Frequency domain data analysis by phasor plot

43

- The phasor plot maps the Φ and M from every image pixel using the Fourier transform components S and G.
- For fluorophores with single component decays, the relationship:

  M = cos Φ

  describes vectors with an endpoints falling somewhere on a universal semicircle.



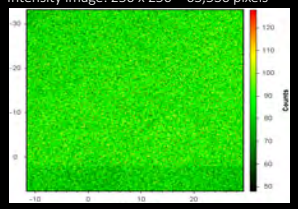
Weber (1981) J Phys Chem 85:949

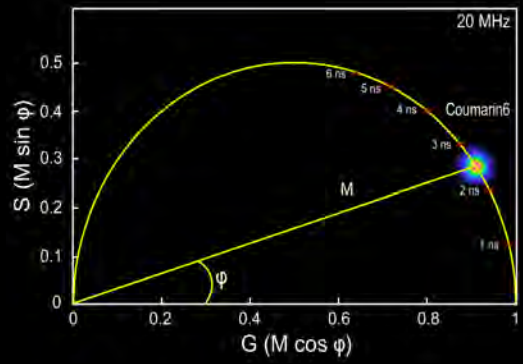
FLIM analysis by phasor plot

44

- Coumarin 6 has a single-component lifetime of 2.5 ns.

Coumarin6
Intensity image: 256 x 256 – 65,536 pixels





- The phasor plot is a vector representation of the Φ and M at every pixel in the image.

Sun et al. (2012) Meth Enzymol 503:372

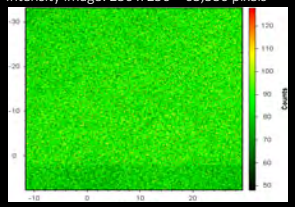
FLIM analysis by phasor plot

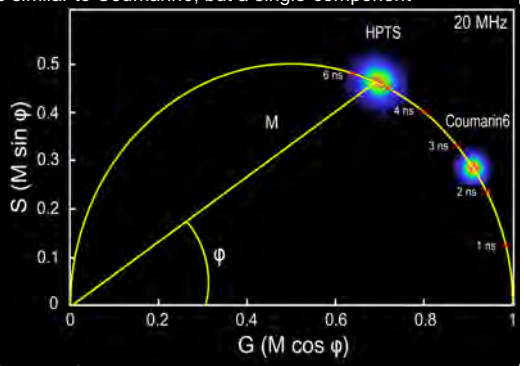
45

- HPTS has spectral characteristics similar to Coumarin6, but a single-component lifetime of 5.3 ns.

HPTS

Intensity image: 256 x 256 – 65,536 pixels





- The phasor plot is made directly from the frequency data at each pixel – *there is no fitting to determine lifetimes.*

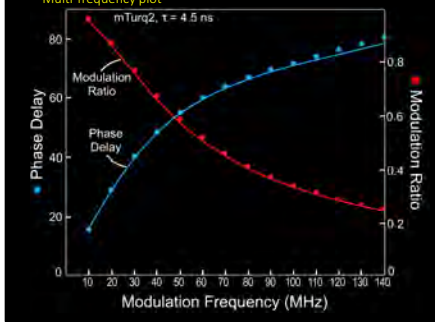
Phasor plot measurements of probe mixtures

46

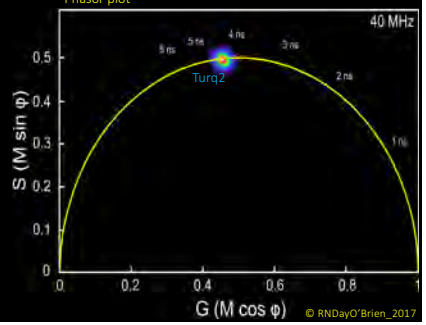
- Cerulean and Turquoise2 have identical spectra, but different lifetimes.
- The lifetime distribution for mTurquoise2 falls on the semicircle, indicating a single component lifetime decay of 4.5 ns.

Purified FPs

Multi-frequency plot



Phasor plot



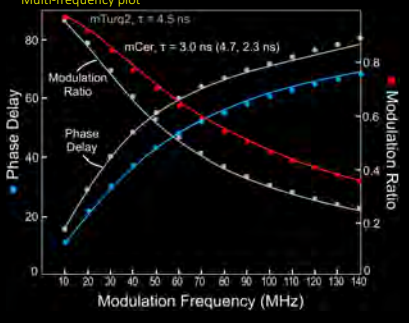
Phasor plot measurements of probe mixtures

47

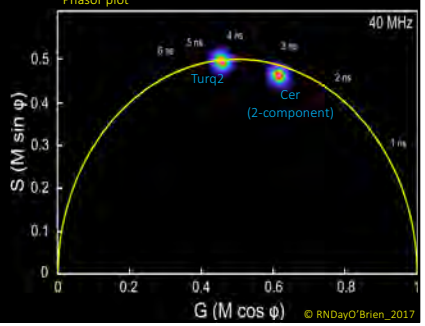
- The lifetime distribution for mCerulean falls inside the universal semicircle, which indicates the presence of more than one lifetime component;
- it is best fit to a two-component decay, with an average lifetime of 3 ns.

Purified FPs

Multi-frequency plot



Phasor plot



Phasor plot measurements of probe mixtures

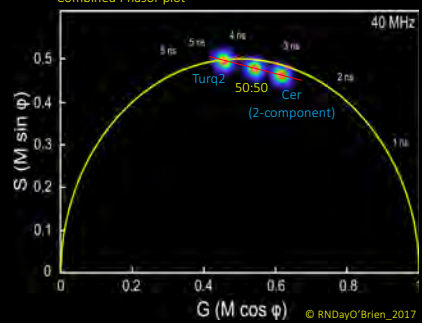
48

- Despite the identical emission profiles for these two FPs, their distinct lifetimes allow phasor analysis to be used to determine the relative contribution of each to a mixture.

Purified FPs

- The lifetime distribution for the mixture falls on a straight line between the distributions for the pure components.
- The position on the line determines their relative contribution.

Combined Phasor plot



Day (2014) Methods 66:200

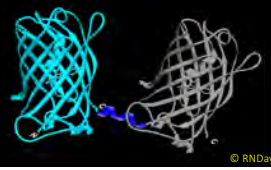
Intramolecular FRET: measurements of the FRET standards

49

- To validate measurements of FRET, FRET standards were generated using Turquoise coupled to Venus through progressively longer linkers.

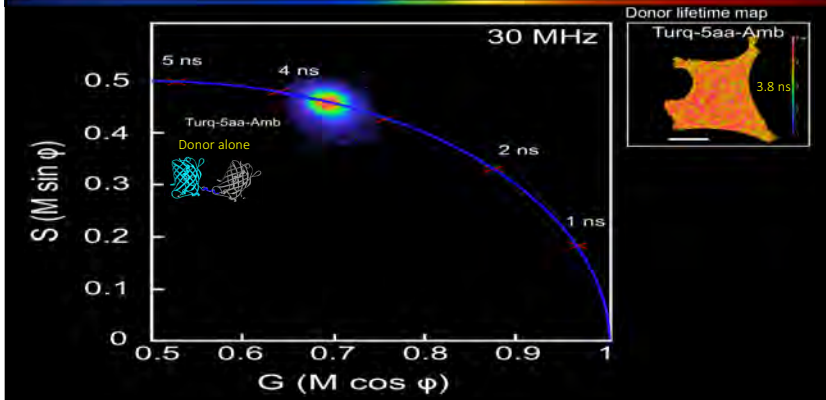
FRET Standard	Linker composition
Turquoise- 5 aa - Amber	-SGLRS-
Turquoise- 5 aa - Venus	-SGLRS-
Turquoise- 10 aa - Venus	-SGLRSPPVAT-
Turquoise- 17 aa - Venus	-SGLRSRAQASNAAVDGT-
Turquoise- 27 aa - Venus	-SGLRSENLYFQGPREFPGGTAGPVATV-
Turquoise- 36 aa - Venus	-SGLRSENLYFQGPREFPGGTGSGRGSGTGTAGPVAT-
Turquoise- 46 aa - Venus	-SGLRSENLYFQGPREFPGGTGSGRGSGTGTGSGRGSGTGTAGPVAT-

- Fusions were also created with Amber - the Y66C mutant of Venus that folds correctly, but does not act as a FRET acceptor.



FLIM analysis by phasor plot: FRET standards in living cells

50

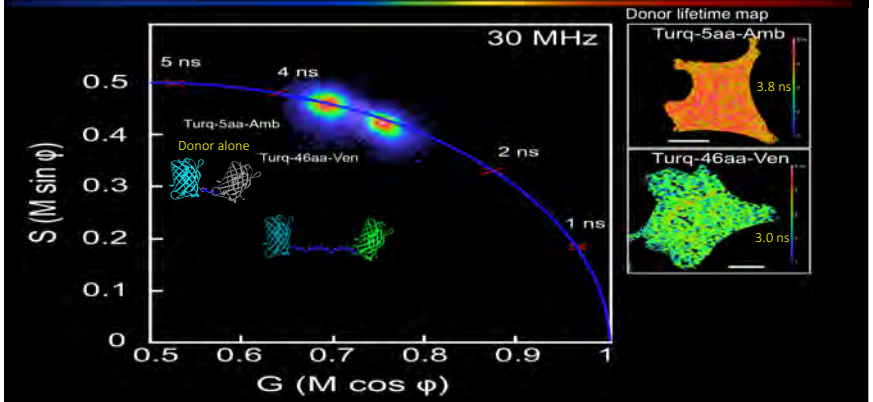


- Turq-5aa-Amber has a single lifetime of 3.8 ns.

* Ven Y66C (Amb) folds but doesn't absorb or emit – control for probe environment

FLIM analysis by phasor plot: FRET standards in living cells

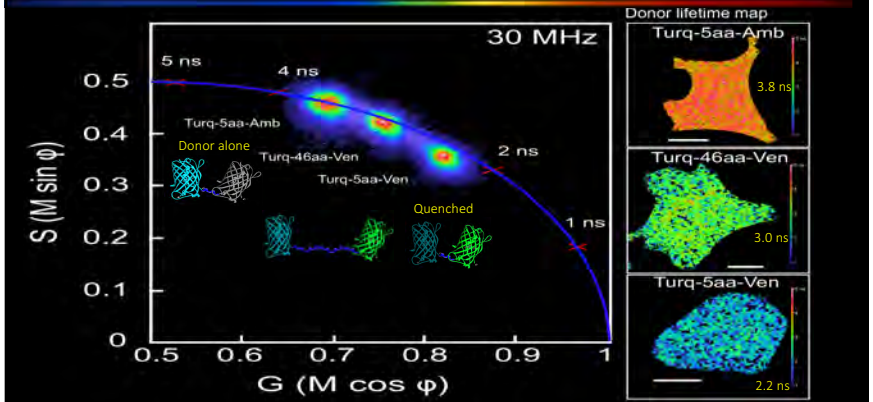
51



- Turq-46aa-Ven is weakly quenched: $E_{FRET} = 1 - (\tau_{DA}/\tau_D) = 24\%$

FLIM analysis by phasor plot: FRET standards in living cells

52

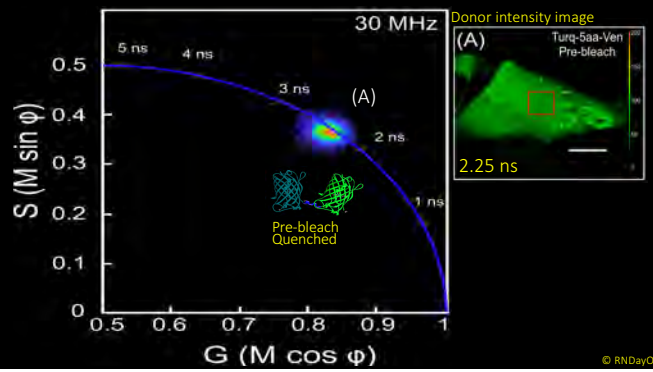


- Turq-5aa-Ven is strongly quenched: $E_{FRET} = 1 - (\tau_{DA}/\tau_D) = 43\%$

Verifying FRET with acceptor photo-bleaching

53

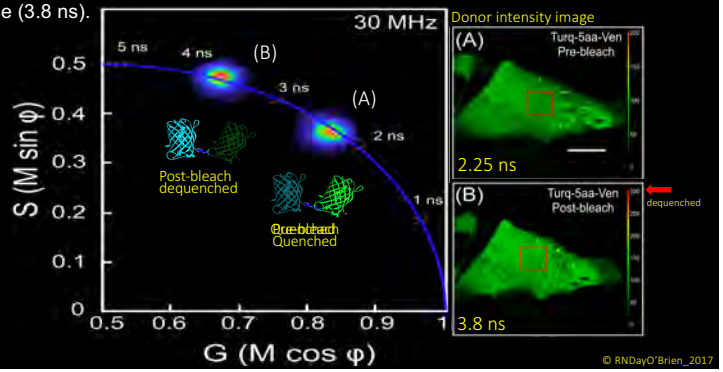
- The Turquoise-5aa-Venus is highly quenched because of FRET.



Verifying FRET with acceptor photo-bleaching

54

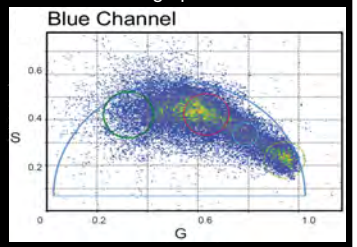
- The Turquoise-5aa-Venus is highly quenched because of FRET.
- Photobleaching Venus results in de-quenching and a return to the radiative lifetime of mTurquoise (3.8 ns).



FRET by FLIM

55

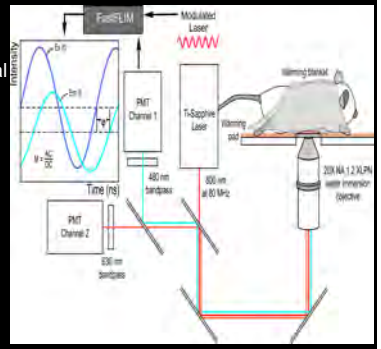
- Advantages:
  - Time-resolved measurements are not affected by excitation intensity, probe concentration, or light scatter.
  - The frequency characteristics are directly measured from each image pixel - there is no fitting, and no *a priori* knowledge of the biology is required.
  - Quenched and unquenched probe populations can be quantified.
  - Label-free intravital imaging of metabolic states.
- Limitations:
  - System and analysis are complex.
  - Photon-intensive - but only if you need to separate the component contributions – the phasor plot provides a method to visualize cell physiology.



Intravital Imaging

56

- Goal: To use intravital imaging of the kidney to evaluate the molecular and cellular basis of physiology and pathophysiology.
- Challenges:
  - Extreme heterogeneity – vascular and epithelial components with highly coordinated functions.
  - Unique structure-function relationships that are dynamic.
  - Study of pathophysiology and disease require longitudinal measurements.
  - Probe delivery.



- Intravital FLIM imaging of complex cellular environments can overcome these challenges.