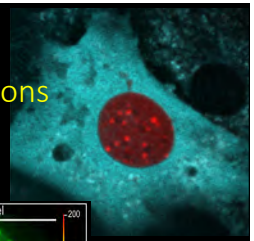
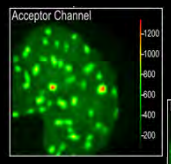
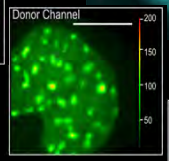
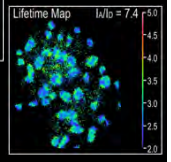
Förster resonance energy transfer microscopy: application and limitations









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O'Brien Center Workshop
IUSM
April 29, 2015

Overview: Application and limitations of FRET

- Förster resonance energy transfer (FRET):
  - A brief history of FRET.
  - Optical resolution.
  - A brief history of FRET microscopy.
  - Problems with spectral crosstalk.
  - Genetically encoded FRET Standards.
- FRET by ratiometric imaging:
  - Measurements of biosensor activity.
  - 2PE Intravital measurement of biosensor activities.
- FRET by Frequency Domain (FD) FLIM:
  - Lifetime measurement and data analysis.
  - FRET-FLIM measurements of intermolecular interactions:

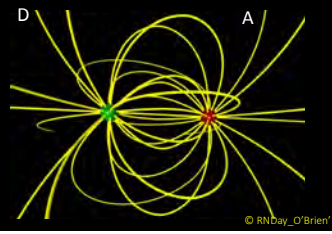
    Homologous protein interactions - C/EBPα Bzip domain.
    Heterologous interactions - HP1α.

Overview: Application and limitations of FRET

- Förster resonance energy transfer (FRET):
  - A brief history of FRET.

Förster resonance energy transfer

- FRET is the direct transfer of excited state energy from a donor fluorophore to a nearby acceptor.
- A fluorophore in the excited-state is an oscillating dipole that creates an electric field (the donor - D).
- If another fluorophore enters the electric field, energy can be transferred directly to that fluorophore (the acceptor - A).
- No intermediate photon!



A brief history of FRET

- Cario and Franck illuminated a mixture of mercury and thallium vapors at a wavelength absorbed only by mercury and observed fluorescence emission from both atoms.
  Z. Physik. 11, 161 (1922)

- Jean-Baptiste Perrin recognized that energy could be transferred from an excited donor molecule to nearby acceptor molecules through direct electric field interactions.
  C.R. Acad. Sci. (Paris) 184, 1097 (1927)
  1926 Nobel prize in Physics for establishing the reality of atoms and molecules

- Perrin's son Francis supplied a quantum mechanical theory describing excitation energy transfer.
  Ann. Chim, Physique 17, 283 (1932)
  In a letter to Einstein, J.B. Perrin said Francis "will be a better physicist than his father".

Clegg, RM (2006) *Reviews in Fluorescence*:1-145

FRET: the distance requirement

- J.B. Perrin realized that the rate of energy transfer depends on the separation of the dipoles (*r*).
- He defined the distance R_0 as when 50% of the energy is radiated from the donor, and 50% of the energy is transferred to the acceptor.
- He assumed *exact resonance* between the molecules and estimated:

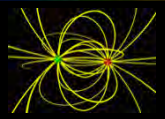
$R_0 \approx 1.2\ \lambda$ If λ = 500 nm (green), then $R_0 \approx$ 600 nm
This was off by 2 orders of magnitude!

- F. Perrin realized that spectra naturally occur over a wide frequency range.
- The probability that donor and acceptor molecules will have the same frequency at the same time is small.

Clegg, RM (2006) *Reviews in Fluorescence*:1-145

FRET: the distance requirement

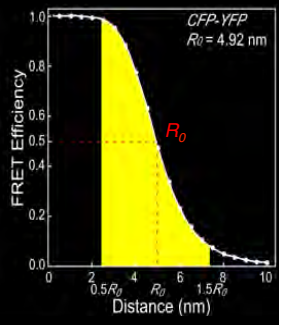
- Electric field interactions:



- Theodor Förster recognized the probability of energy transfer would be low, and quantitatively described how FRET efficiency varies as the inverse of the sixth power of the distance.
  Naturwissenschaften (1946) 33:166
  Translated in J Biomed Optics (2012) 17:011002

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

R_0, the Förster distance



Förster resonance energy transfer (FRET)

Overview: Application and limitations of FRET

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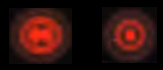


Optical resolution in microscopy

9

- The lateral (x-y) resolution of an optical image is defined as the distance by which two objects must be separated to be distinguished as separate objects:

$$r = \frac{(0.61)\,\lambda}{NA}$$

$$\frac{(0.61)\ 440\ nm}{1.2} = 225\ nm$$

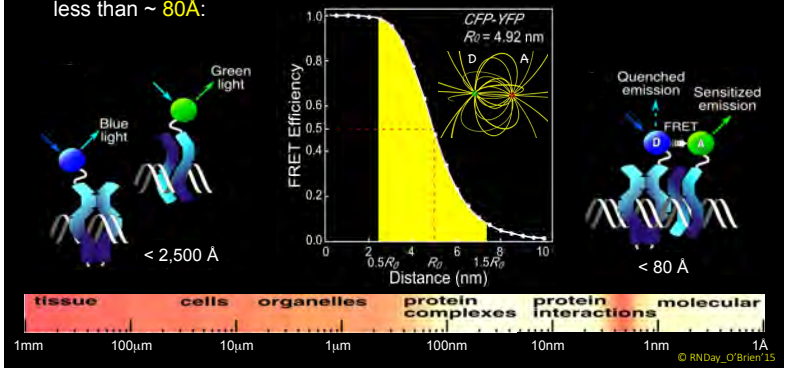
  - Rayleigh criterion: Theoretical limit for an ideal specimen - your results may differ.

- Newer optical techniques offer several fold improvement in resolution (40 – 100 nm; SIM, STED, PALM...).
- Much greater resolution is necessary to detect protein-protein interactions in living cells.
- FRET microscopy can provide measurements of the spatial relationship between fluorophores on the scale of less than 80 ångstroms.

FRET measures the spatial relationship between the probes

10

- The optical resolution of the conventional light microscope is limited to ~ 250 nm (2,500 Å).
- The detection of FRET indicates the fluorophores are separated by less than ~ 80Å:



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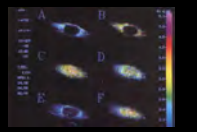
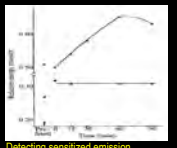


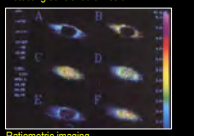
A brief history of FRET microscopy

12

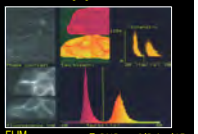
- Microscopy methods that measure FRET have become valuable tools for investigating biochemical networks in living cells.
- Fernandez and Berlin (1976) were the first to use a microscope to detect FRET to measure temporal variations in cell surface receptors.
  Fernandez and Berlin (1976) Nature 264:411


Detecting sensitized emission

- The Tsien laboratory (1991) was the first to FRET imaging to measure the activation of PKA by cAMP in single living cells.
  Adams et al. (1991) Nature 349:694


Ratiometric imaging

- The Jovin laboratory (1995) was the first to use fluorescence lifetime imaging microscopy (FLIM) to measure FRET from EGF receptors.
  Gadella and Jovin (1995) J Cell Biol 129:1543


FLIM

Overview: Application and limitations of FRET

13

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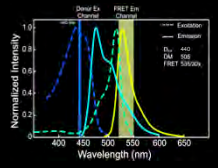
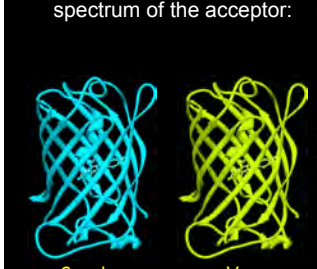
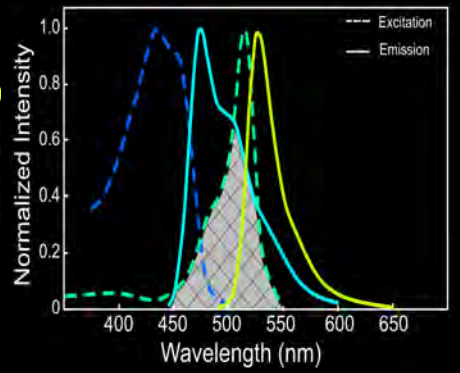


FRET requires strong spectral overlap

14

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



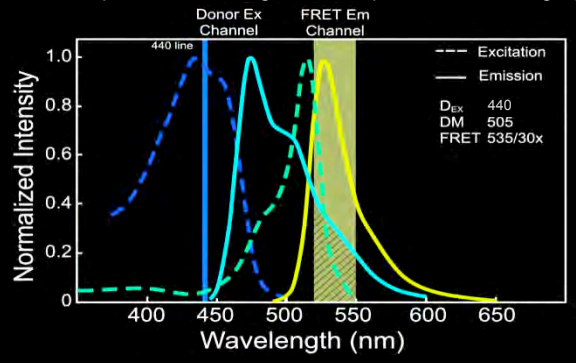


- Förster: The spectral overlap determines the probability that the frequency dependent oscillators will be in resonance.

Spectral bleedthrough background signals

15

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



Spectral bleedthrough background signals

16

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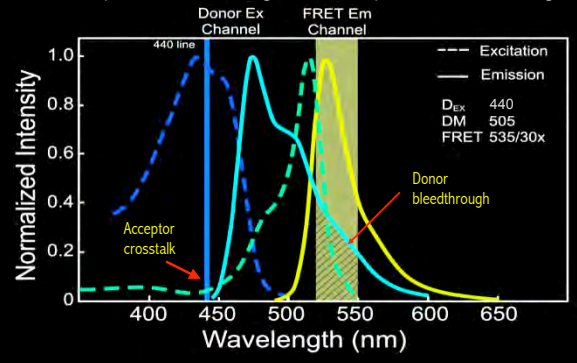
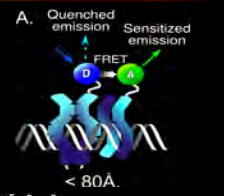


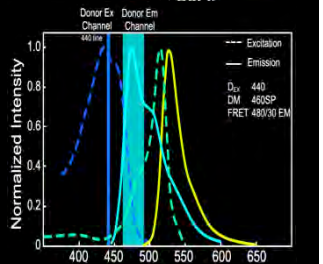
- ❖ The accurate measurement of FRET by sensitized acceptor emission requires removal of SBT!

Spectral bleedthrough background signals

17

- There are several ways to deal with SBT background:
  1. Use correction algorithms based on separate control cells to remove acceptor and donor SBT.
  2. If donor and acceptor are at a fixed ratio, use ratiometric imaging – SBT will be constant.
  3. The donor is quenched by FRET, use acceptor photobleaching and measure donor de-quenching.
  4. Directly measure the effect of FRET on the donor, use fluorescence lifetime imaging microscopy to measure the change in donor lifetime.
- How accurate are these methods?





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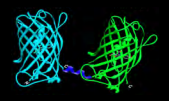
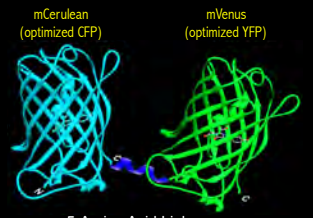


Measurements in living cells: FRET standards

19

- How do you verify FRET measurements obtained by from living cells by different laboratories?
- A simple solution was to develop genetically encoded FRET reference proteins - "FRET standards".



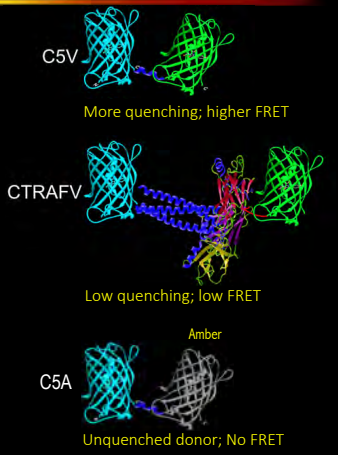
- The FRET standards have been produced in a variety of cell systems, and measurements by several different methods confirmed their utility.

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

Cerulean-Venus FRET standards

20

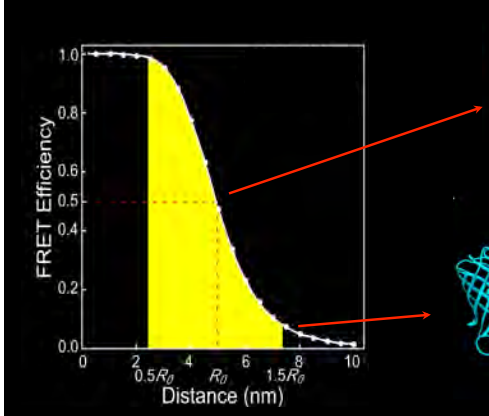
- The Vogel lab developed a series of fusion proteins based on the optimized Cerulean and Venus FPs.
- The fusions have progressively longer linkers between the Cerulean and Venus.
- Amber - the Y66C mutant of Venus that folds correctly, but does not act as a FRET acceptor – an important control for donor environment in FLIM.

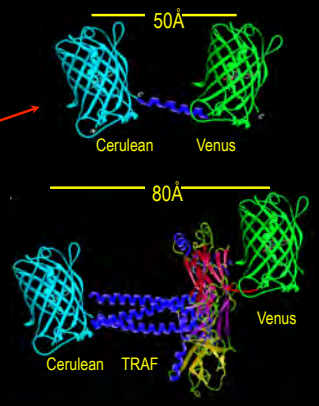


Koushik et al. (2006) Biophys. J 91:L99

The linked mCerulean-mVenus 'FRET Standards'

21





Available from Addgene: www.addgene.org

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

Overview: Application and limitations of FRET

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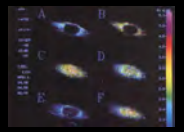
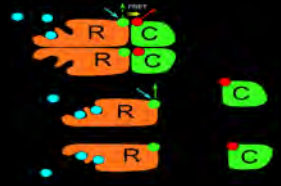


FRET by ratiometric imaging

23

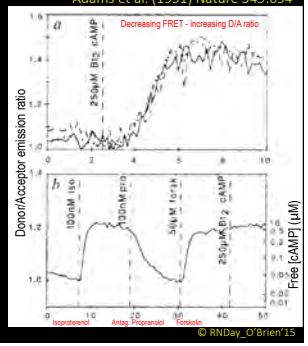
- The Tsien laboratory (1991) used ratiometric imaging to measure the change in FRET upon cAMP binding to PKA:

Adams et al. (1991) Nature 349:694



The first "biosensor"

- They purified the separate regulatory and catalytic subunits, labeled with fluorescein (D) or rhodamine (A), and microinjected the combined holoenzyme into cells (1:1 ratio).
- Ratio imaging (D/A) measured the loss of FRET that occurred with cAMP binding.



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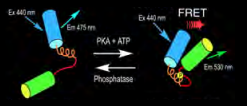
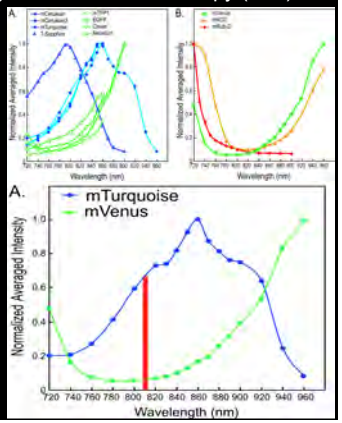


O'Brien Probe Development Core

25

- The goal of the core is to identify promising fluorescent protein (FP) candidates for biosensor probes with utility for intravital microscopy (IVM).
- Evaluated many different FPs for their 2PE characteristics, and their suitability when paired for FRET-based biosensor probe applications.
- mTurquoise, a cyan FP with improved quantum yield and photostability, paired with mVenus was most useful for the development of 2PE biosensor probes for IVM.



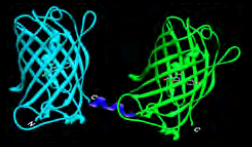
Tao, Dunn, Day et al. in preparation

Biosensor probes

26

- The FRET standards serve as starting point for the development of FRET-based biosensor probes.
- The genetically encoded biosensor probes consist of donor and acceptor FPs connected by a sensing unit:
- The sensing unit contains a substrate linker that is modified by a cellular activity and a sensor linker that binds to the modification:
- A kinase activity reporter (AKAR) is a reporter of intracellular PKA activity.

FRET Standard



FRET-based Biosensor probe

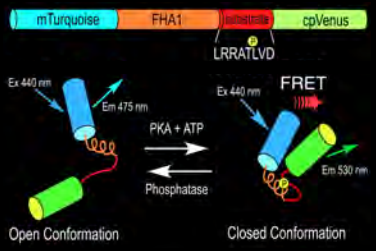
Zhou et al. (2012) Methods in Enzymol 504:317

Improved FRET-based biosensor probes

27

- AKAR4.1 consists of mTurquoise and circular permuted Venus coupled through a low affinity phospho-substrate binding domain, FHA1, and a PKA specific substrate.
- The phosphorylation of the AKAR substrate by PKA causes the sensing unit to change conformation, altering the distance between the FRET pair.
- The changing intramolecular FRET signal reports the spatiotemporal dynamics of protein activity inside living cells.

AKAR4.1

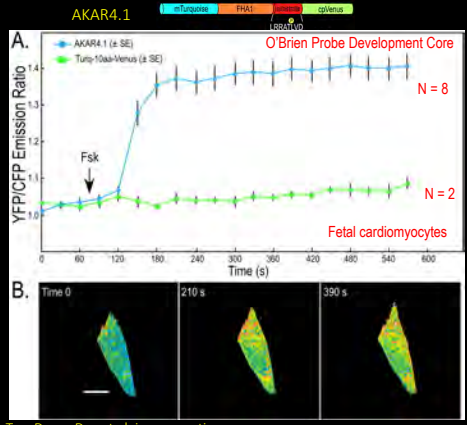


Can intravital microscopy monitor FRET-based biosensor activities?

28

- Goal: To develop biosensor probes with utility for intravital microscopy (IVM).
- The 2PE FRET imaging (Ex 810 nm) of isolated primary mouse fetal cardio-myocytes expressing the AKAR4.1 biosensor or the mTurquoise-10AA-Venus FRET standard.
- The addition of Fsk results in a rapid increase in the YFP/CFP emission ratio for AKAR4.1, but not the FRET standard.

AKAR4.1



Tao, Dunn, Day et al. in preparation

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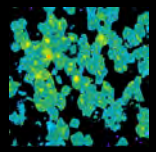
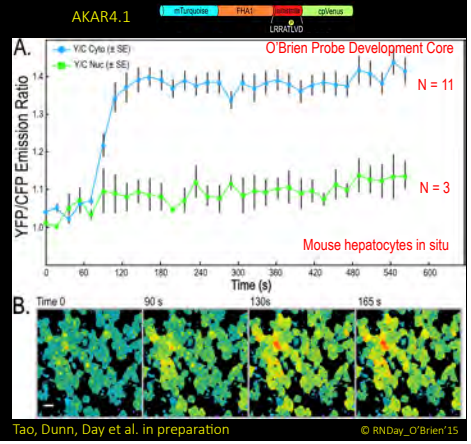


Intravital microscopy monitors FRET-based biosensor activities

30

- Goal: To develop biosensor probes with utility for intravital microscopy (IVM) in *living animals*.
- The AKAR4.1 adenoviral vector was introduced by tail vein injection and imaged by IVM after 7 days.
- 3D image volumes were collected from the left lateral lobe of the liver prior to and following IP injection of glucagon (0.2 mg/kg).
- The YFP/CFP emission ratio within 1.5 min of glucagon administration (time 0).

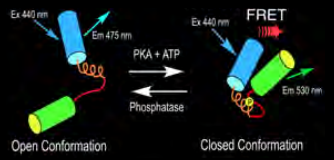


Tao, Dunn, Day et al. in preparation

Ratio imaging of biosensor probes

31

- Strength -
  - Simple approach - bleed-through background is constant (1:1).
  - Large scale screening applications.
  - With viral delivery it can be used for 2PE IVM.
- Weakness -
  - Limited to linked probes;
  - Limited dynamic range.
  - Function difficult to predict.



- Optimized FPs for biosensor probes:
  - Optimize donor for method – mTurquoise (2PE ratio or lifetime).
  - Circularly permuted (cp) FPs – optimize acceptor orientation.

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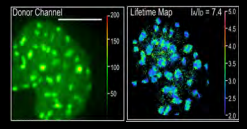
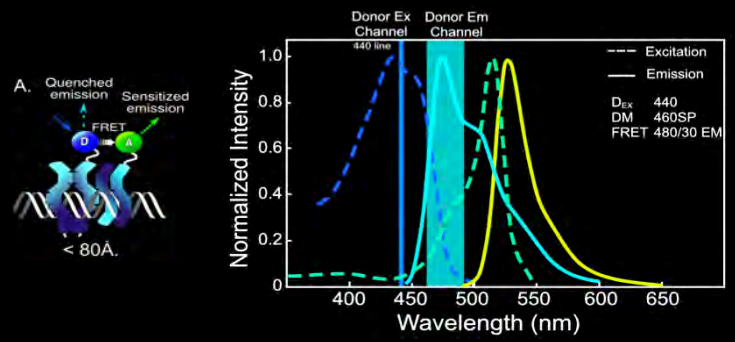
  - Genetically encoded FRET Standards.
- FRET by ratiometric imaging:
  - Measurements of biosensor activity.
  - 2PE Intravital measurement of biosensor activities.
- FRET by Frequency Domain (FD) FLIM:
  - Lifetime measurement and data analysis.



Spectral bleedthrough background signals

33

- Methods that detect changes in donor fluorescence because of FRET are not subject to SBT.



❖ Fluorescence lifetime imaging microscopy (FLIM) measures the change in donor fluorescence lifetime because of FRET.

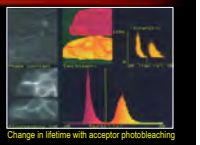
FRET by fluorescence lifetime imaging microscopy (FLIM)

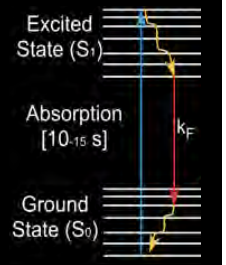
34

- The Jovin laboratory (1995) was the first to use fluorescence lifetime imaging microscopy (FLIM) to measure FRET from EGF receptors.
  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, typically on the order of a few nanoseconds:

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

http://www.iss.com/resources/reference/data_tables/LifetimeDataFluorophores.html

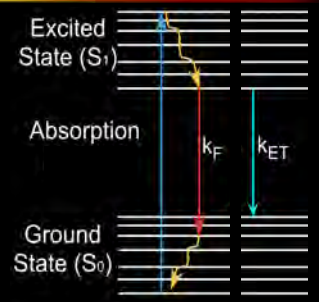




FRET by FLIM

35

- 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.



- Only two measurements are required to determine FRET efficiency (E_{FRET}):

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

❖ Fluorescence lifetime imaging microscopy (FLIM) can accurately measure the change in lifetime.

FLIM measurements: Frequency domain

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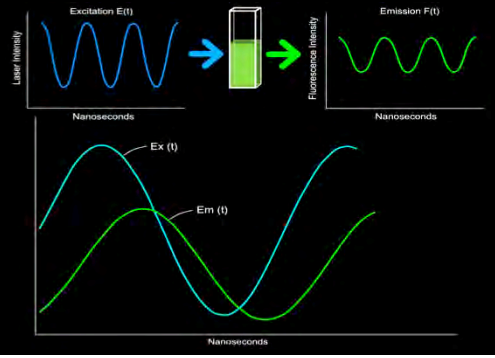
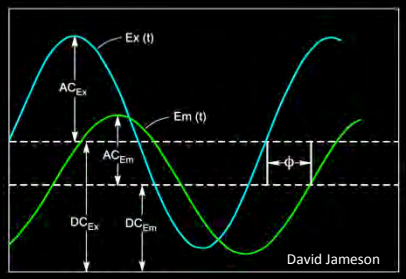
- In frequency domain, a continuous light source is modulated at high frequency (1-200 MHz) to excite the fluorophores:
- The emission signal from the fluorophores will also be modulated:



FLIM measurements: Frequency domain

37

- 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:

$M = \frac{AC}{DC}$

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

FD FLIM analysis by phasor plot

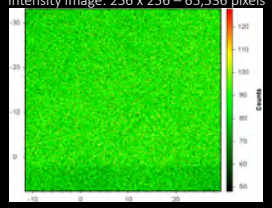
38

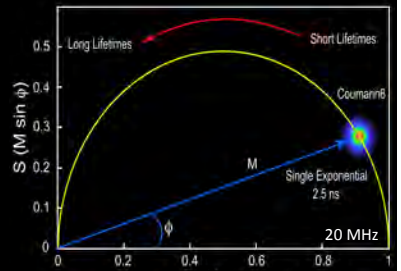
- The phasor plot is a simple geometric representation of the frequency characteristics of the signals resulting from repetitive excitations.

Redford & Clegg (2005) J Fluoresc 15:805
Jameson et al. (1984) App Spec Rev 20:55

- The phasor plot maps the frequency characteristics of the emission signals from each image pixel using a vector representation with angle determined by the phase delay (Φ) and length determined by the modulation (M).

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





G and S are the Fourier transform components of the fluorescence impulse response I(t)

FLIM analysis by phasor plot

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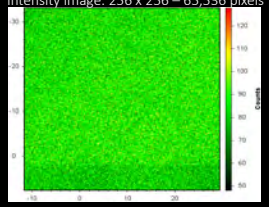
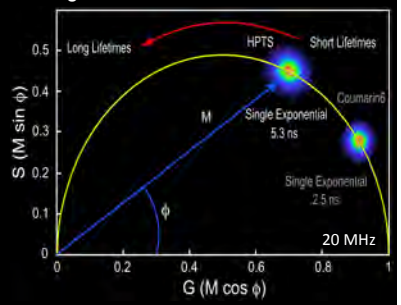
- The distribution of lifetimes for a species with a short lifetime will fall to the right on the semicircle, while those with longer lifetimes will move to the left along the semicircle.

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



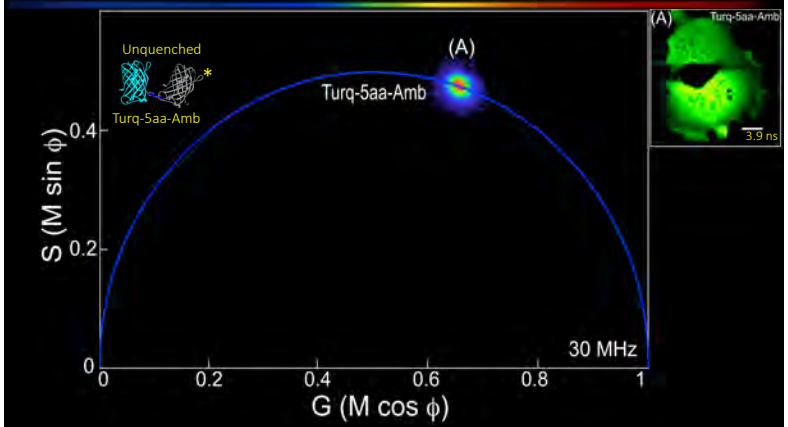


❖ The phasor plot is made directly from the frequency data at each pixel – *it requires no assumption or a priori knowledge of the system.*

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

FLIM analysis by phasor plot: FRET standards in living cells

40

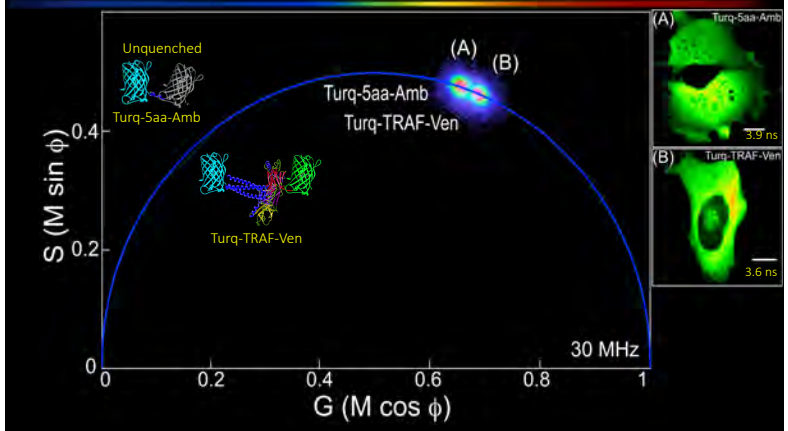


- Turq-5aa-Amber has a single lifetime of 3.9 ns.

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

FLIM analysis by phasor plot: FRET standards in living cells

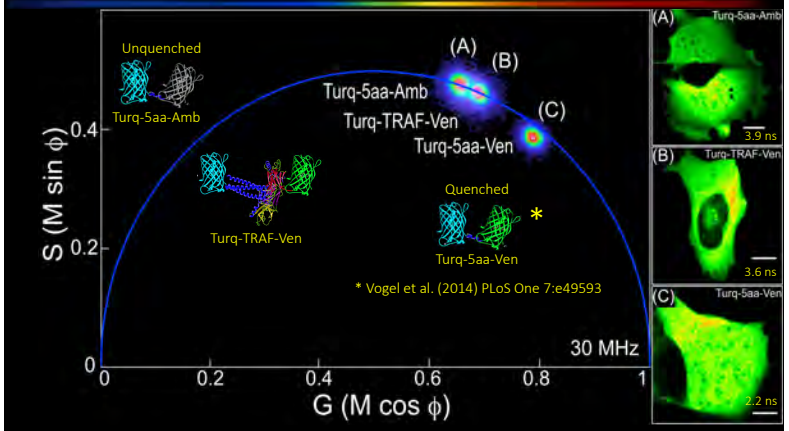
41



- Turq-TRAF-Ven is weakly quenched: $E_{FRET} = 1 - (\tau_{DA}/\tau_D) = 9\%$

FLIM analysis by phasor plot: FRET standards in living cells

42

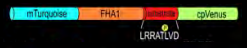


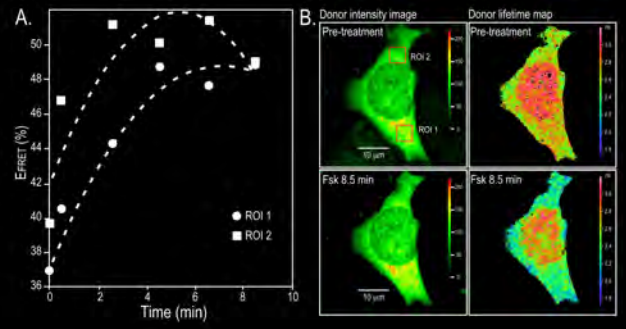
- Turq-5aa-Ven is strongly quenched: $E_{FRET} = 1 - (\tau_{DA}/\tau_D) = 43\%$

FLIM measurements of AKAR biosensor probe activities

43

- FLIM measures the changing lifetime of the AKAR4.1 biosensor probe after treatment with Forskolin (Fsk):





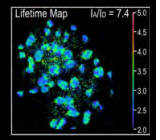
- The pixel resolution lifetime map shows where the probe lifetimes are changing.

Overview: Application and limitations of FRET

44

- Förster resonance energy transfer (FRET):
  - A brief history of FRET.
  - Optical resolution.
  - A brief history of FRET microscopy.
  - Problems with spectral crosstalk.
  - Genetically encoded FRET Standards.
- FRET by ratiometric imaging:
  - Measurements of biosensor activity.
  - 2PE Intravital measurement of biosensor activities.
- FRET by Frequency Domain (FD) FLIM:
  - Lifetime measurement and data analysis.
  - FRET-FLIM measurements of intermolecular interactions:

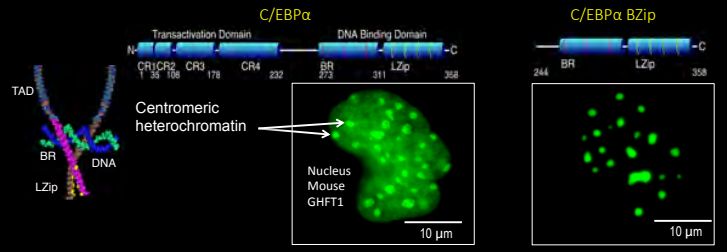
    Homologous protein interactions - C/EBPα Bzip domain.
    Heterologous interactions - HP1α.



Measuring *intermolecular* protein interactions with FRET-FLIM

45

- C/EBPα localizes binds to DNA as an obligate dimer.



- The C/EBPα BZip domain alone is sufficient for DNA binding and localization to centromeric heterochromatin.
- Centromeric heterochromatin is analogous to an endogenous DNA array allowing visualization of protein interactions associated with chromatin.
  - ❖ Can FRET-FLIM detect C/EBPα dimer formation on DNA?

FRET-FLIM analysis of BZip domain interactions

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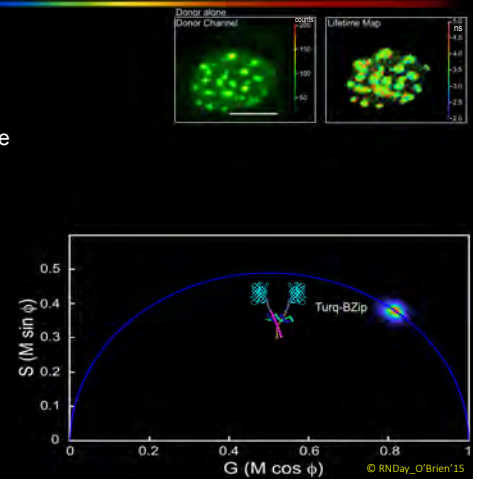
- The lifetime map shows the probe lifetime in specific regions.
- The phasor plot represents the lifetime distribution in the entire image.



FRET-FLIM analysis of BZip domain interactions

47

- The lifetime map shows the probe lifetime in specific regions.
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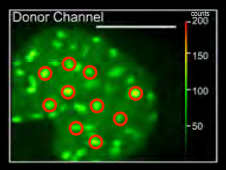
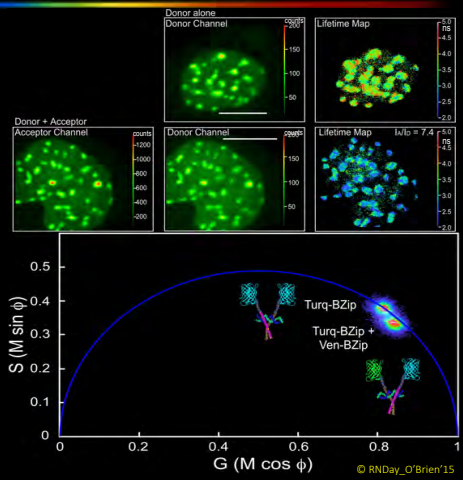


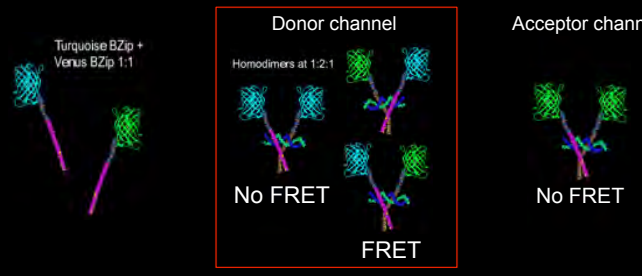


- The lifetime information can be acquired from individual regions of interest (ROI).

Measuring intermolecular protein interactions with FRET-FLIM

48

- To detect FRET, FLIM measurements need only made in the donor channel.



- Because the Acceptor- and Donor-labeled proteins are encoded by different plasmids, every transfected cell will have a different A:D ratio.
  - ❖ The A:D ratio influences the FRET efficiency (E_{FRET}).

FRET-FLIM analysis of BZip domain interactions

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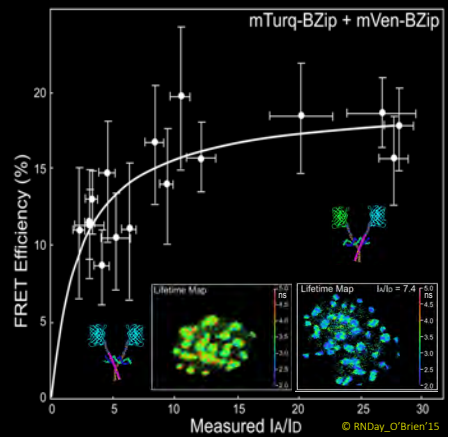
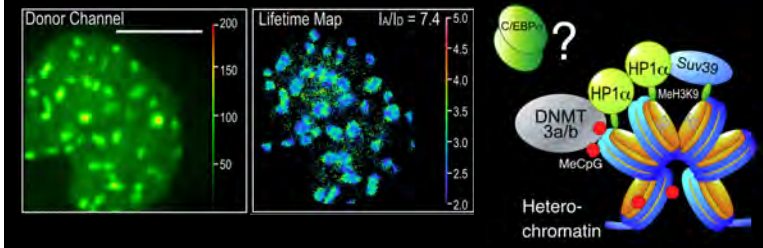
- How can we use the cell-to-cell variability in the Acceptor-to-Donor ratio (I_A/I_D)?
- FLIM was used to determine the E_{FRET} for multiple ROI in cells expressing Turquoise- and Venus-labeled BZip proteins.
- The E_{FRET} for 16 different cells (~10 ROI per cell) are plotted as a function of the I_A/I_D ratio.



C/EBPα localizes to heterochromatin

50



- Does C/EBPα interact with the HP1α protein network?

Co-IP of HP1α and C/EBPα

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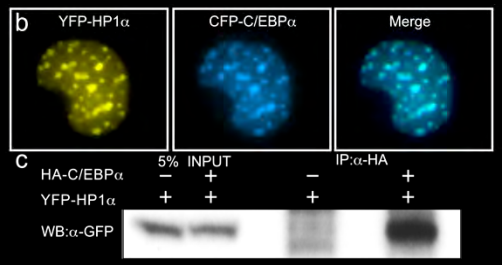
- HP1α in binds to methylated K9 of H3 in heterochromatin where it functions to assemble macromolecular complexes.



- Do C/EBPα and HP1α interact in living cells?

Heterologous interactions between HP1α and C/EBPα

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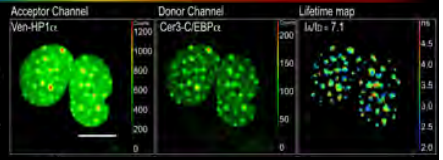
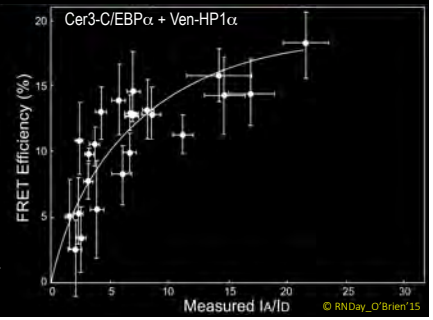


- FLIM was used to measure the interactions of Cer3-C/EBPα and Venus-HP1α.
- The average E_{FRET} and the I_A/I_D ratio was determined in 5-10 different ROI for each cell (n=23).
- The E_{FRET} is plotted as a function of the I_A/I_D for each cell.

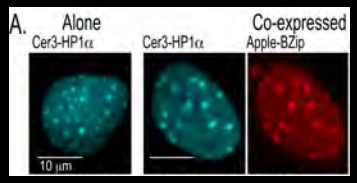


Siegel et al. (2013) J Biomed Optics 18:025002

Heterologous interactions between HP1α and C/EBPα

53

- The C/EBPα BZip domain alone is sufficient for DNA binding and localization to centromeric heterochromatin.



- Does the BZip domain of C/EBPα interact with HP1α?

Heterologous interactions between HP1α and C/EBPα

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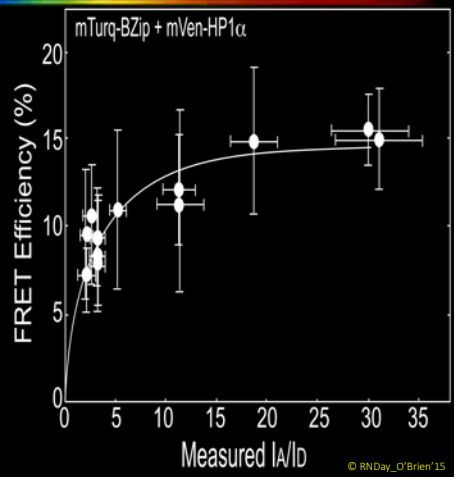
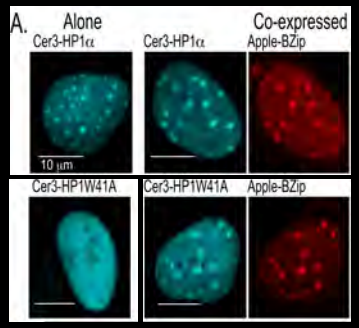
- The BZip domain is sufficient for the interaction in regions of heterochromatin.



Heterologous interactions between HP1α and C/EBPα

55

- What is required for the interaction?



- The HP1α^{W41A} mutation disrupts the interaction with MeK9H3;
- but when co-expressed with the BZip it was recruited to heterochromatin.

Heterologous interactions between HP1α and C/EBPα

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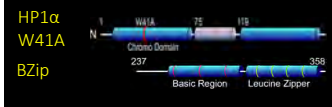
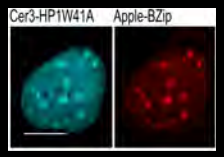
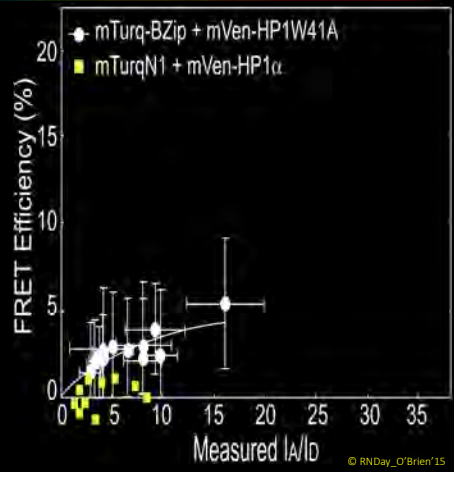




- Disrupting HP1α interactions with chromatin alters the interaction with the BZip domain.

Siegel et al. (2013) J Biomed Optics 18:025002



ISS Alba system: Dynamics (FCS, FCCS), interactions (FRET-FLIM), nanoimaging

Present:
Jing Qi, Ph.D.
Wen Tao, Ph.D.
Past:
Amanda Siegel, Ph.D.
Katie Summers
Corey Ariss
Nikki Hays
Ignacio Demarco, Ph.D.
Ty Voss, Ph.D.
John Enwright, Ph.D.
Meg Crook, M.D.
Cindy Booker

Collaborators:
Mike Davidson, FSU
Fred Schaufele, UCSF
Ammasi Periasamy, Keck Center for Cellular Imaging, UVA
Ken Dunn, IUSM, Indiana Center for Biological Microscopy

Support
NIH DK 43701-18, IUSM, NIH O'Brien Center (P30DK079312)

FLIM Demonstration MS 369
Wednesday April 29
Session 1: 1:00 to 2:45
Session 2: 3:15 to 5:00