Förster Resonance Energy Transfer:
monitoring protein interactions in living cells

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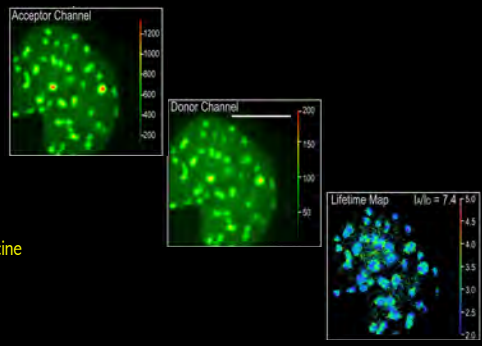

O'Brien Center Workshop
Indiana University School of Medicine
May 7, 2013

Overview: Application and limitations of FRET

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- What is FRET?
- What are the requirements for FRET?
- Problems with spectral crosstalk.
- FRET by Frequency Domain (FD) FLIM:
  - Data analysis by phasor plots.
  - Genetically encoded FRET Standards.
  - Biosensor probes.
- Monitoring nuclear protein interactions with FLIM:
  - FRET-FLIM measurements of intermolecular interactions: Homologous protein interactions - C/EBPα Bzip domain.
  - Heterologous interactions - HP1α.

The expanding fluorescent protein (FP) palette

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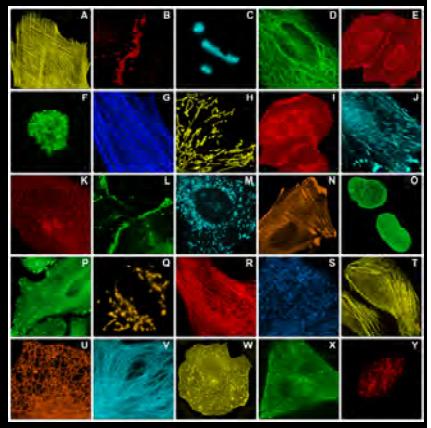
- FPs that emit across the visible spectrum are now available.
- Many new FPs have been optimized for expression in a variety of systems.
- Some of the FPs have characteristics suitable for Förster (fluorescence) resonance energy transfer (FRET) microscopy.



Day and Davidson (2009) Chem. Soc. Rev 38:

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.

What is FRET and why use it?

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

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

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

- Newer optical techniques offer several fold improvement in resolution, but 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 ångstroms.

What is FRET?

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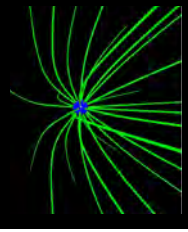
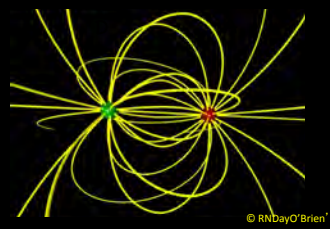
- FRET is the direct transfer of excited state energy from a donor fluorophore to a nearby acceptor.
- A fluorophore in the excited-state is an oscillating dipole that creates an electric field (the donor - D).
- If another fluorophore enters the electric field, energy can be transferred directly to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





Förster's contribution

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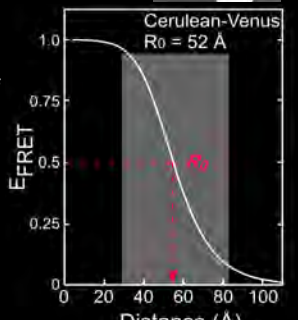
- Electric field interactions:

- Theodor Förster recognized the probability of energy transfer would be low, and quantitatively described how FRET efficiency decreased as the inverse of the sixth power of the distance.

"Energiewanderung und Fluoreszenz" Naturwissenschaften (1946) 36:166-175

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

R_0, the Förster distance



Förster resonance energy transfer (FRET)

FRET measures the spatial relationship between the FPs

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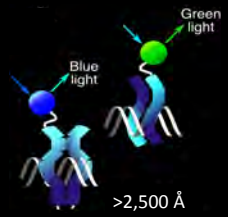
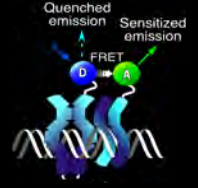
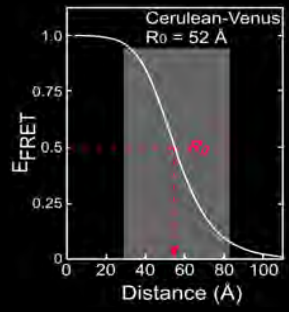
- The optical resolution of the light microscope is limited to 250 nm.
- The detection of FRET indicates the fluorophores are less than ~ 8 nm (80Å) apart:



>2,500 Å



< 80 Å



Overview: Application and limitations of FRET

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The spectral overlap requirement

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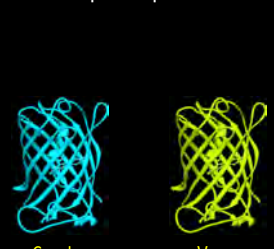
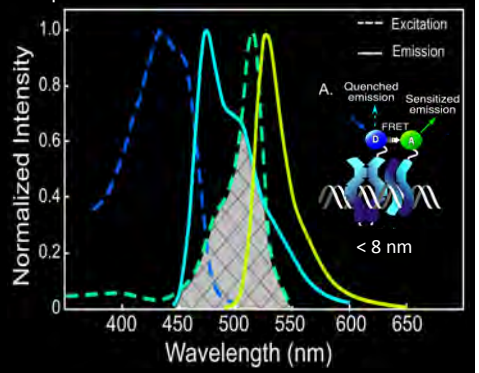
- The donor emission spectrum must significantly overlap the absorption spectrum of the acceptor.





- More spectral overlap → more spectral bleedthrough.

Spectral bleedthrough background signals

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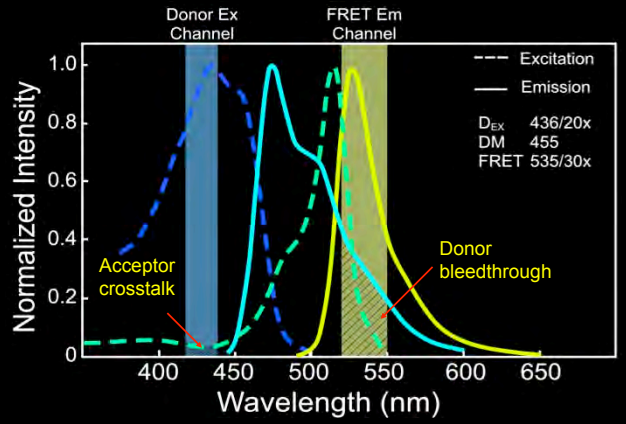
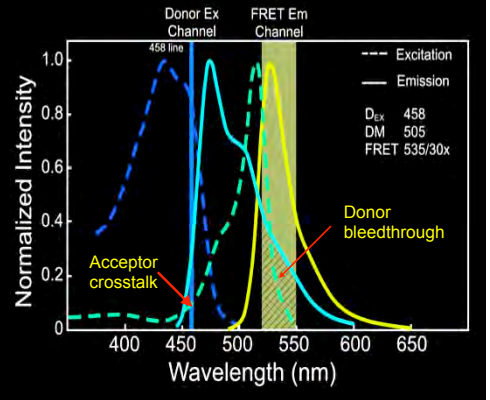
- Spectral bleedthrough - the more overlap, the more background.



Spectral bleedthrough background signals

12

- This is still a problem when using LSCM –



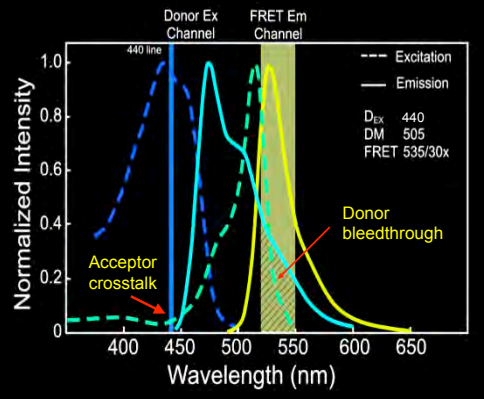
Spectral bleedthrough background signals

13

- This is still a problem when using LSCM –

  even with diode lasers:

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



Spectral bleedthrough background signals

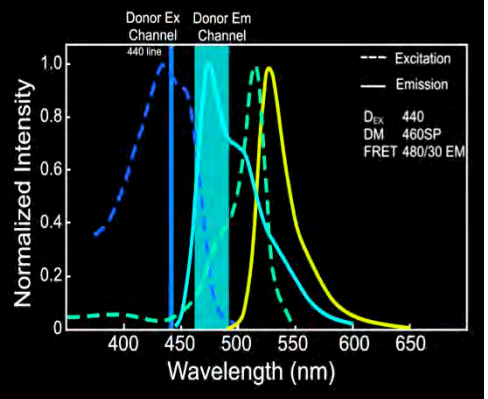
14

- This is still a problem when using LSCM –

  even with diode lasers:

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

- ❖ Alternatively, methods that detect changes in donor fluorescence are typically not affected by SBT, and can be most accurate.



Overview: FRET methods

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- Methods for measuring FRET:

1. Sensitized acceptor emission:
   - E = FRET - [Spectral cross-talk]

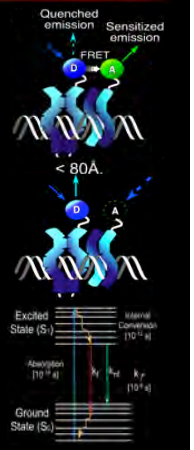
✔ Cross-talk algorithms available on most systems;
✘ Relies on data from separate control cells - limits accuracy.

2. Acceptor photobleaching:
   - E = 1 - (I_{DA}/I_D)

✔ Simple approach – uses each cell as its own control;
✘ Requires selective acceptor bleaching – end point assay.

3. Donor lifetime measurements:
   - E = 1 - (τ_{DA}/τ_D)

✔ Detects shortening of donor lifetime – direct measurement.
✔ Model independent measurements – most accurate.
✘ Complex systems requiring long acquisition times.



Overview: Application and limitations of FRET

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- FRET by Frequency Domain (FD) FLIM:

Fluorescence Lifetime

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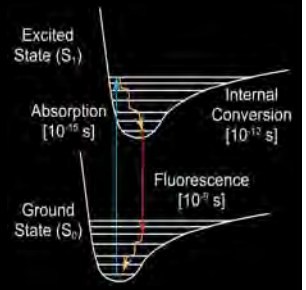
- Fluorescence lifetime (τ): the average time a population of fluorophores spend in the excited state before returning to the ground state.
- Every fluorophore has a characteristic lifetime, typically on the order of a few nanoseconds:

Probe	τ_m
Coumarin6 (EtOH)	2.5 ns
Cerulean FP (cells)	3.1
HPTS (Pyranine) (PB)	5.3
Fluorescein (PB)	4.1
mEGFP (cells)	2.9
Alexa 594 (PB)	3.9



Fluorescence Lifetime Imaging Microscopy (FLIM)

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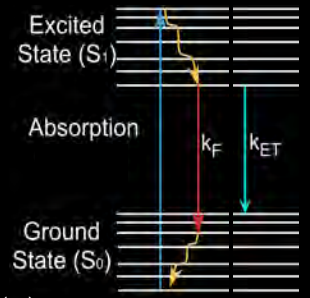
- FRET is a quenching pathway that directly influences the excited state:
- FRET will cause the fluorescence lifetime to shorten - this can be accurately measured microscopically.
- Quantifying FRET efficiency (E_{FRET}) by FLIM requires two measurements:
  - donor lifetime in the absence of acceptor (τ_D).
  - donor lifetime in the presence of acceptor (τ_{DA}).

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



FLIM measurements: Frequency domain

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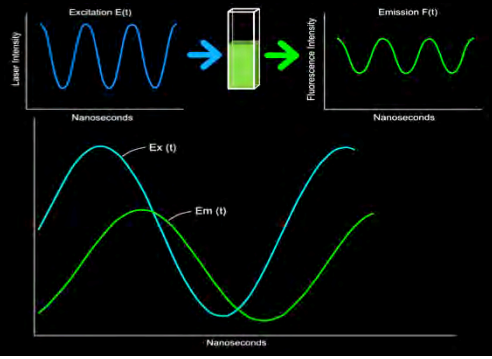
- In frequency domain, a continuous light source is modulated at high frequency (1-200 MHz) to excite the fluorophores:
- The emission signal from the fluorophores will also be modulated:



FLIM measurements: Frequency domain

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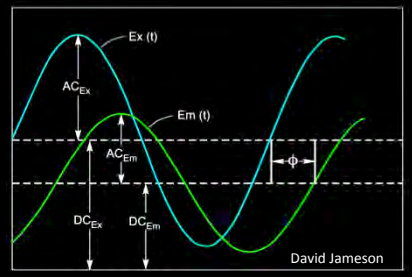
- Because of the excited state lifetime, there is a delay in the emission relative to the excitation.
- This causes a phase delay (Φ) and a change in modulation (M) of the emission signal relative to the excitation:



Phase delay: ϕ

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

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

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- FRET by Frequency Domain (FD) FLIM:
  - Data analysis by phasor plots.

Frequency domain data analysis: the phasor (polar) plot

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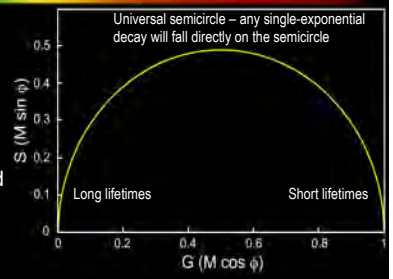
- The phasor plot was developed to analyze and display frequency characteristics.
  Cole & Cole (1941) J Chem Phys 9:341
  Jameson et al. (1984) App Spec Rev 20:55
- It is applicable to any system excited with repetitive perturbations.
  Redford & Clegg (2005) J Fluoresc 15:805



- The phasor plot is a geometric representation of the phase delay (Φ) and relative modulation (M) of the emission signal.
- The frequency characteristics at every image pixel are represented by a vector with the polar coordinates:
  G = M × cos Φ
  S = M × sin Φ

FLIM analysis by phasor plot

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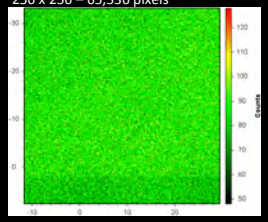
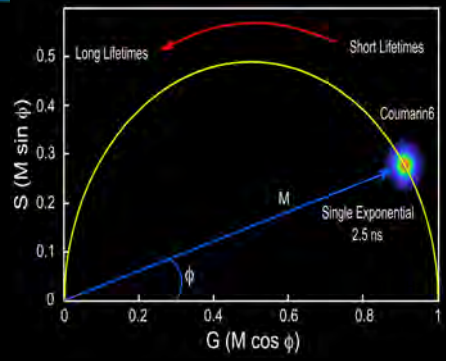
256 x 256 – 65,536 pixels





- Coumarin6 has spectral characteristics similar to the CFPs and a lifetime of 2.5 ns.

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

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

FLIM analysis by phasor plot

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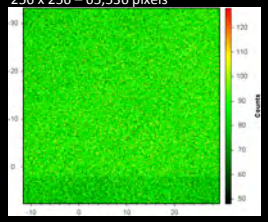
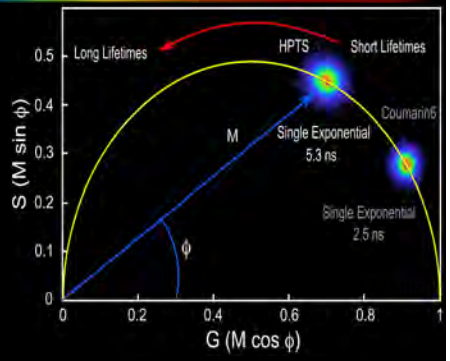
256 x 256 – 65,536 pixels





- HPTS also has spectral characteristics similar to the CFPs, and a lifetime of 5.3 ns.

❖ Both of these dyes have only one lifetime component, so the distribution of lifetimes for each population falls directly on the semicircle.

Lifetime measurements for purified FPs:

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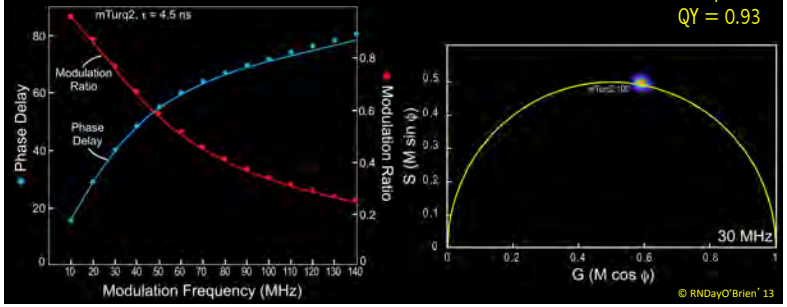
- FPs with 6x-His tag are expressed in bacteria, purified on metal affinity resin, concentrated, and analyzed by FLIM.
- Turquoise2 and Cerulean are CFPs with identical spectra, but different lifetimes.

mTurquoise2
QY = 0.93



Lifetime measurements for purified FPs:

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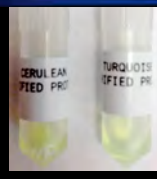
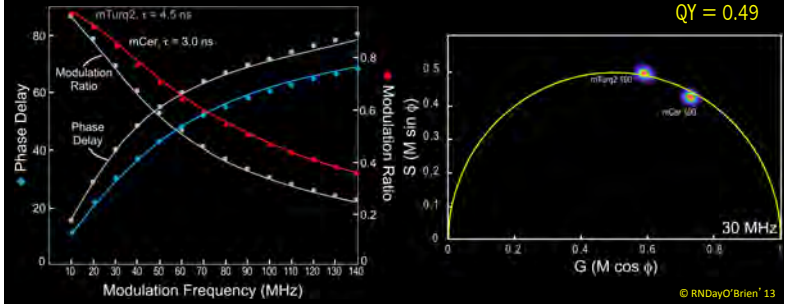


- Lifetime measurements clearly distinguish the two species;

mCerulean
QY = 0.49



Lifetime measurements for purified FPs:

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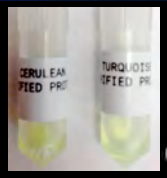
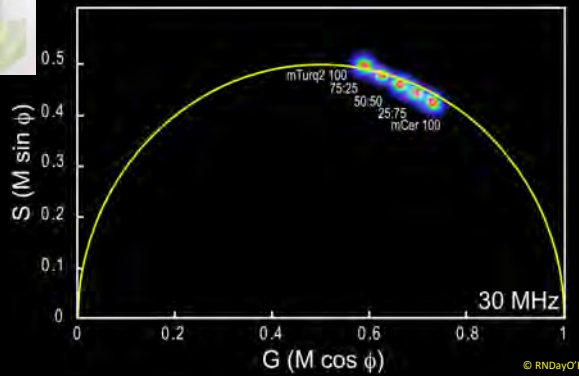


- Lifetime measurements clearly distinguish the two species;
- FLIM determines their relative concentrations in mixtures:



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Measurements in living cells: FRET standards

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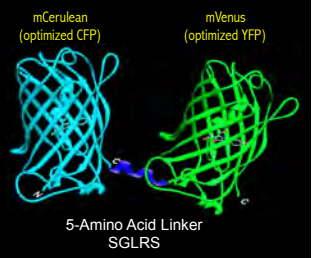
- How do you verify FRET measurements obtained by from living cells by different laboratories?
- A simple solution was to develop genetically encoded FRET reference proteins - "FRET standards".



- 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 developed by S. Vogel (NIH)

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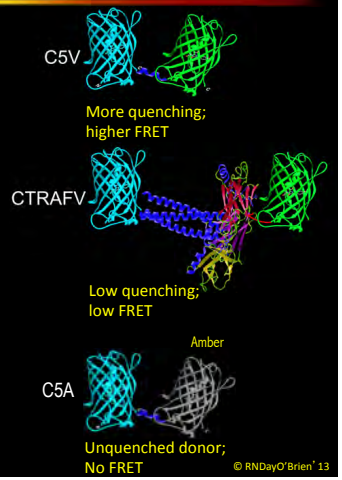
- The Vogel lab developed a series of fusion proteins based on the optimized Cerulean and Venus FPs.
- The fusions have progressively longer linkers between the Cerulean and Venus.
- Amber - the Y66C mutant of Venus that folds correctly, but does not act as a FRET acceptor – an important control for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



Thaler *et al.* (2005) *Biophys. J* 89:2736;
Koushik et al. (2006) Biophys. J 91:L99

FLIM analysis by phasor plot: FRET standards in living cells

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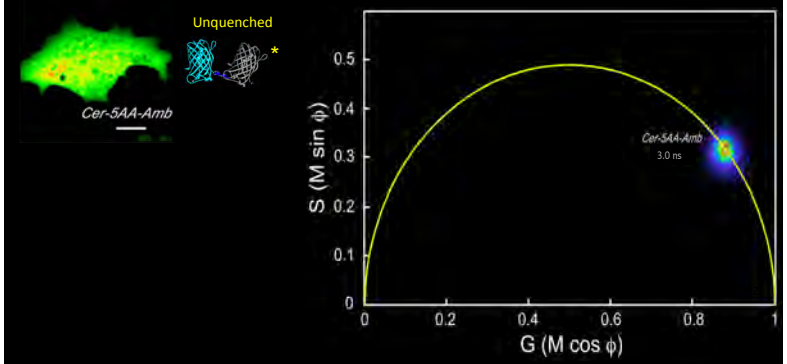

- Cer-5AA-Amb has a lifetime of ~ 3 ns - unquenched donor.

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

FLIM analysis by phasor plot: FRET standards in living cells

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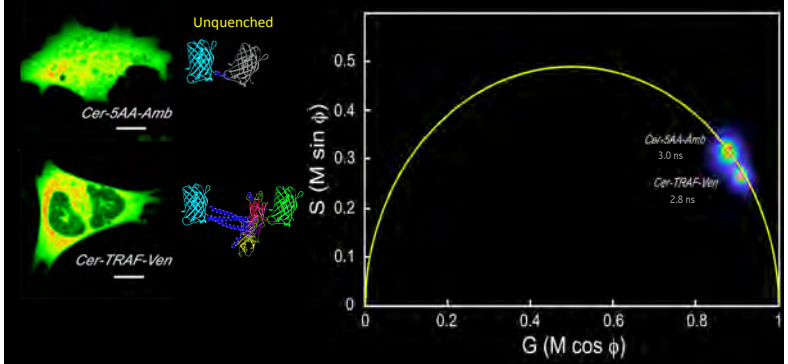

- For Cer-TRAF-Ven, Cer is quenched; $E_{FRET} = 1 - (\tau_{DA}/\tau_D) = 7\%$

FLIM analysis by phasor plot: FRET standards in living cells

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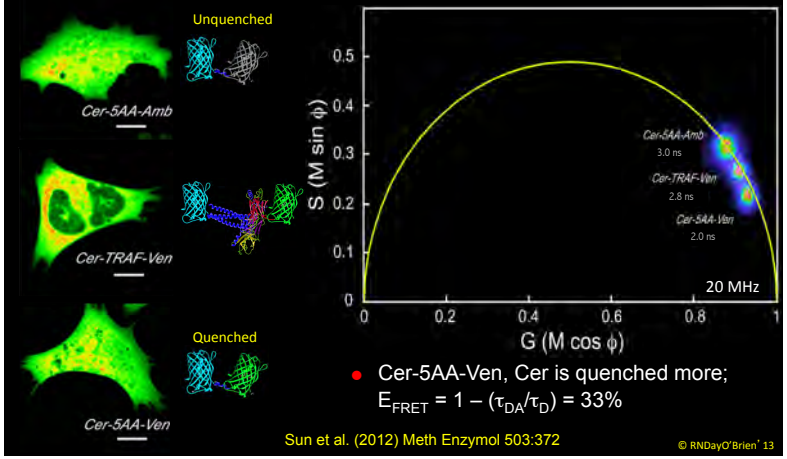

- Cer-5AA-Ven, Cer is quenched more; $E_{FRET} = 1 - (\tau_{DA}/\tau_D) = 33\%$

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

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FRET-based biosensor probes

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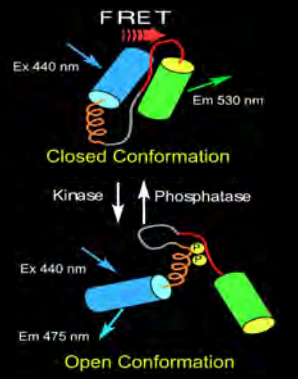
- The genetically encoded biosensor probes consist of donor and acceptor FPs connected by sensing unit:
  - The sensing unit includes an element that is modified by the biological event,
  - and a binding motif that recognizes the modification.
- The biological event causes the sensing unit to change conformation, altering the distance between the FRET pair.



- The changing intramolecular FRET signal reports the spatiotemporal dynamics of protein activity inside living cells.

Detecting Scr kinase activity

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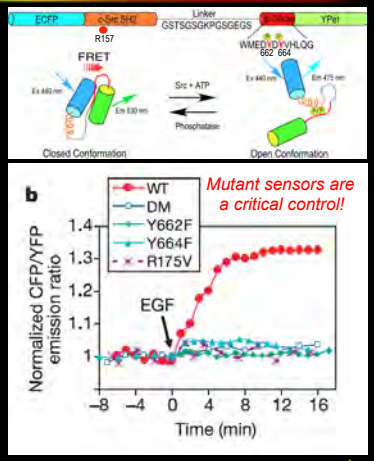
- The sensing unit of the Src biosensor consists of the c-Src substrate peptide from p130 cas, and the c-Src SH2 domain:
  - The probe is in a "closed conformation" before phosphorylation → high FRET.
  - Phosphorylation by c-Src induces a conformational change → low FRET.
  - Ratiometric imaging reports the changes in biosensor conformation on the time scale of seconds.



Wang et al. (2005) Nature 434:1041

Lifetime imaging of biosensor probe activities

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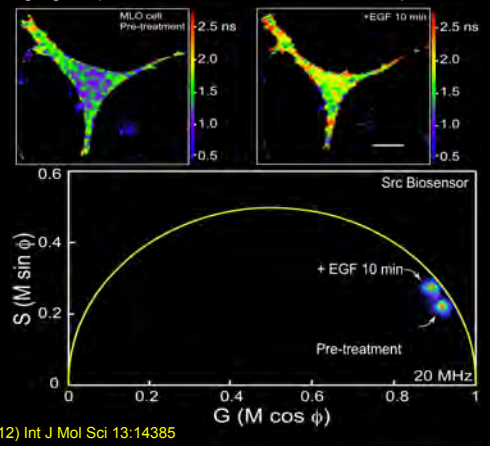
- Lifetime imaging maps the subcellular distribution of probe activities:



Julia Hum
Fred Pavalko

Hum et al. (2012) Int J Mol Sci 13:14385

Measuring FRET using FLIM

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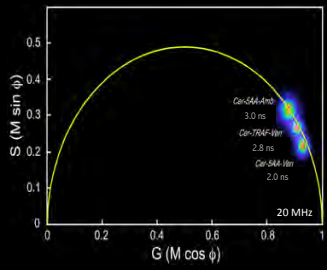
- Strength -
  - Measurements are not influenced by intensity or probe concentration;
  - Quenched (bound) and unquenched donor populations can be quantified.
  - Independent method to verify intensity measurements.



- Weakness -
  - System and analysis are complex;
  - Photon-intensive - measurements can take many seconds to acquire.

Overview: Application and limitations of FRET

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- What is FRET?
- What are the requirements for FRET?
- Problems with spectral crosstalk.
- FRET by Frequency Domain (FD) FLIM:
  - Data analysis by phasor plots.
  - Genetically encoded FRET Standards.
  - Biosensor probes.
- Monitoring nuclear protein interactions with FLIM:

Heterochromatin maintains genome integrity

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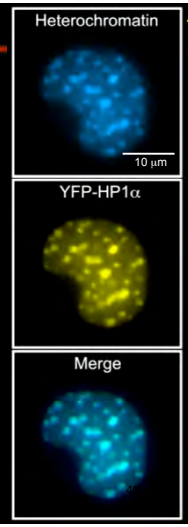
- We're using live cell imaging approaches to determine how networks of protein interactions are coordinated inside the cell nucleus.
- Pericentric heterochromatin provides a structural scaffold that maintains genome integrity.
- The formation of heterochromatin requires heterochromatin protein 1 (HP1α) through its direct interactions with histones and chromatin modifying proteins.
- There is increasing evidence that HP1α plays roles in the regulation of gene transcription, DNA replication, and repair.



HP1α coordinates networks of protein interactions

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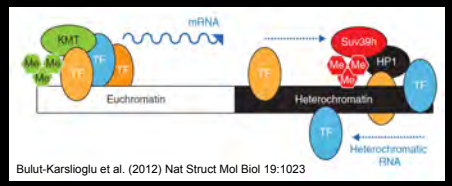
- Recent studies indicate that HP1α interacts with sequence-specific transcription factors (TF).



Bulut-Karslioglu et al. (2012) Nat Struct Mol Biol 19:1023

- Our studies demonstrated that the transcription factor CCAAT/enhancer binding protein alpha (C/EBPα) interacts with HP1α.
- C/EBPα directs programs of cell differentiation, regulates genes involved in energy metabolism, and localizes to methylated promoters in regions of heterochromatin.

The intranuclear distribution of C/EBPα

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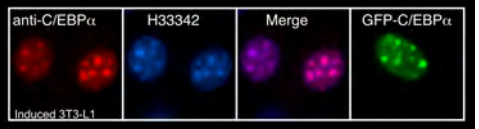
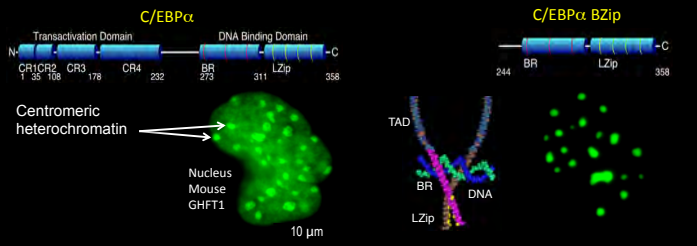
- C/EBPα localizes to regions of heterochromatin in 3T3-L1 adipocyte cells (ICC staining):



- GFP-tagged C/EBPα localizes to regions of heterochromatin in mouse cells:



- The C/EBPα BZip domain binds to DNA as an obligate dimer.

C/EBPα localizes to heterochromatin

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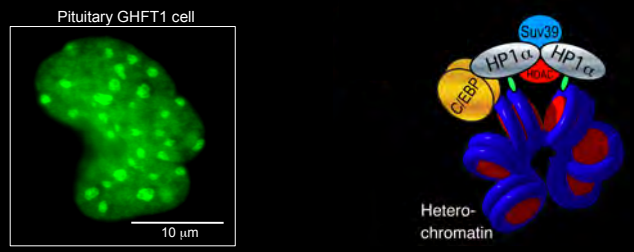
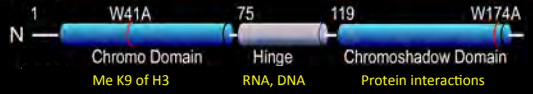


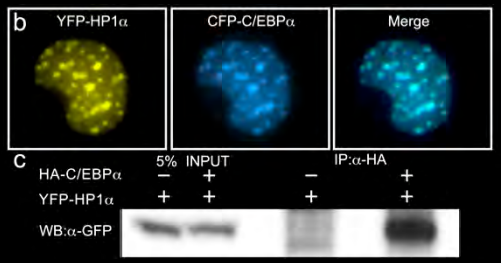
- Does C/EBPα specifically interact with HP1α in regions of heterochromatin?
- If so, what protein domains are involved, and what is the functional significance?

Heterochromatin protein 1 (HP1α)

44



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



- How dynamic is HP1α?

PA-GFP: an optical marker

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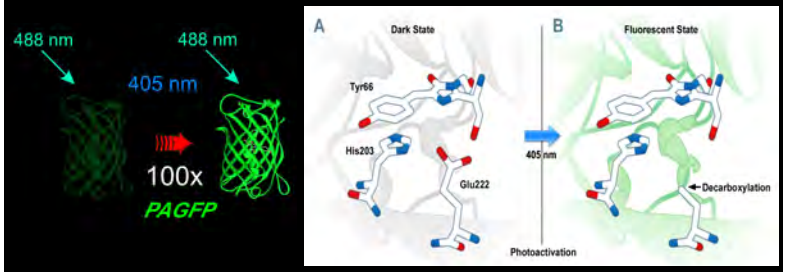
- Intense 405 nm illumination is thought to cause decarboxylation of the glutamic acid at position 222 near the chromophore;
- this converts the chromophore from a neutral to an anionic state:



PA-GFP: an optical marker to measure protein mobility

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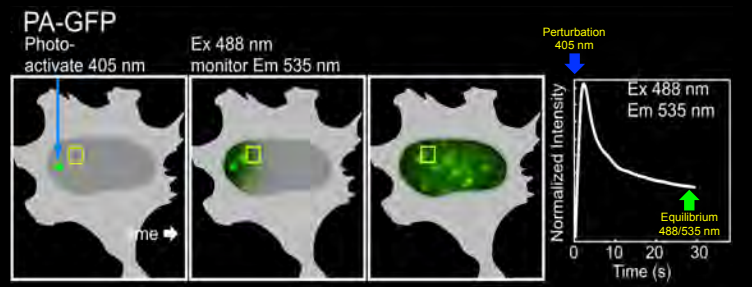
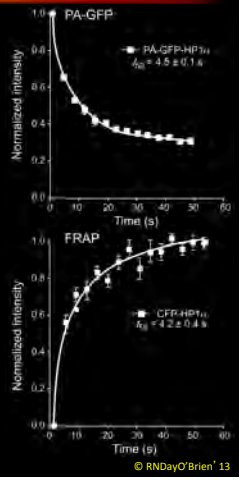
- Photoactivate PA-GFP in a region of interest in the cell.
- Monitor the diffusion of the newly fluorescent protein over time.



HP1α is highly mobile within the cell nucleus

47



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

Demarco et al. (2006) Mol. Cell. Biol. 26:8087

Overview: Application and limitations of FRET

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- FRET by Frequency Domain (FD) FLIM:
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- Monitoring nuclear protein interactions with FLIM:
  - FRET-FLIM measurements of intermolecular interactions: Homologous protein interactions - C/EBPα Bzip domain.

The intranuclear distribution of C/EBPα

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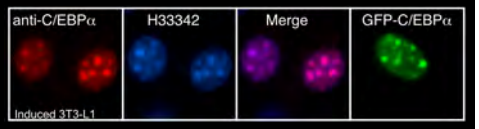
- C/EBPα localizes to regions of heterochromatin in 3T3-L1 adipocyte cells (ICC staining):



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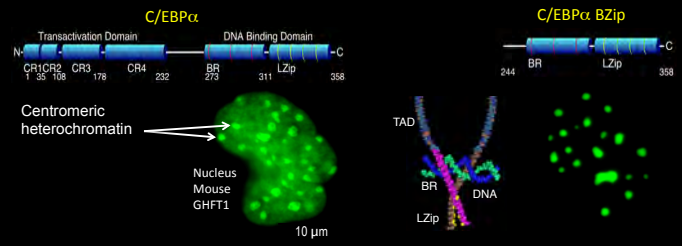
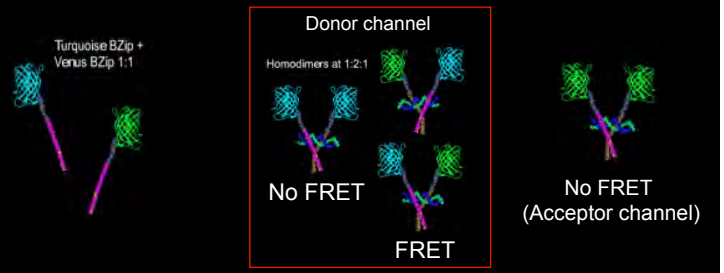


- The C/EBPα BZip domain binds to DNA as an obligate dimer.

FRET-FLIM: measuring intermolecular protein interactions

50

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



  - ❖ Changing the input ratio influences D:A levels.

BZip domain interactions analyzed in cell populations

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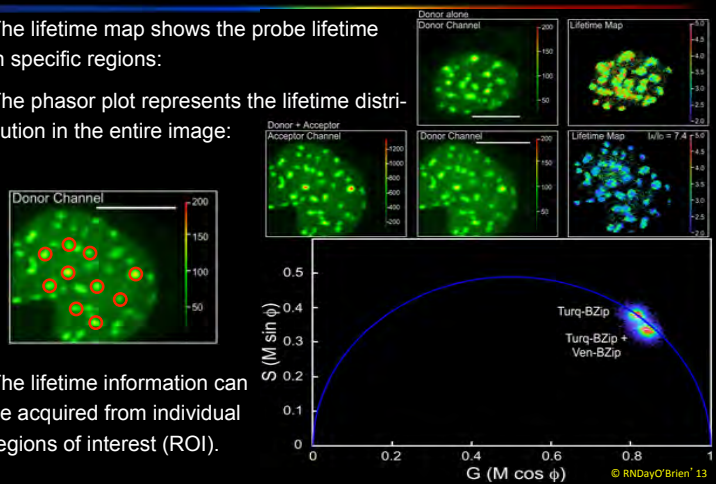
- The lifetime map shows the probe lifetime in specific regions:
- The phasor plot represents the lifetime distribution in the entire image:



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

BZip domain interactions analyzed in cell populations

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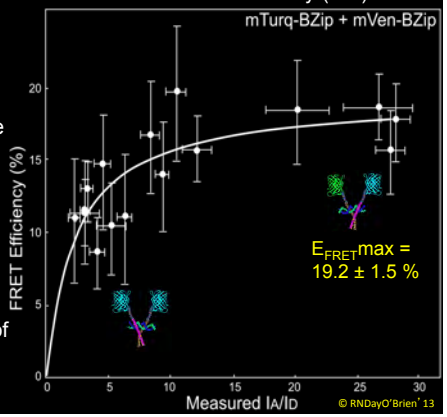
- Because the D- and A-labeled proteins were expressed independently, each cell has a different A:D ratio, and this affects the FRET efficiency (E%).

"Variation is your friend" - FS

- FLIM-FRET was used to measure the interactions of the Turquoise- and Venus-labeled BZip domains:
- The E% and mean lifetime for 16 different cells (~10 ROI per cell) are plotted as a function of the A/D intensity ratio.



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  - Heterologous interactions - HP1α.

Intermolecular FRET measurements in living cells

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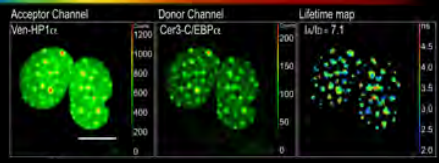
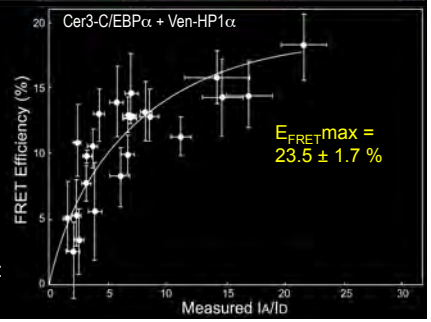
- FLIM was used to measure the interactions of Cer3-C/EBPα and Venus-HP1α:



- The average E_{FRET} and the acceptor to donor intensity ratio (I_A/I_D) was determined in 5-10 different ROI for each cell (n=23).
- The results are plotted as a function of the I_A/I_D for each cell:

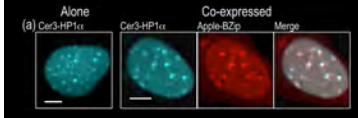


Siegel et al. (2013) J Biomed Optics 18:0250

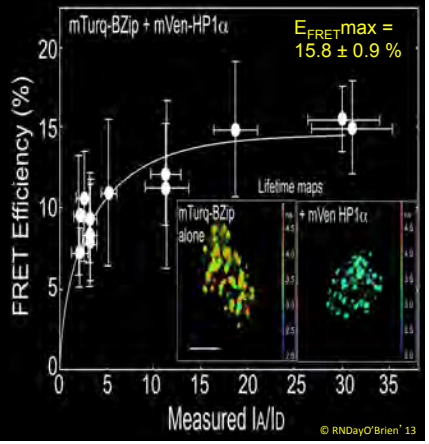
The interactions between HP1α and the BZip domain

55

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



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



The interactions between HP1α and the BZip domain

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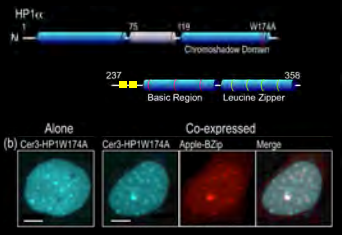
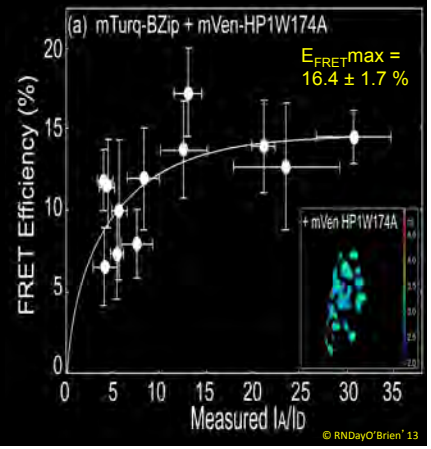
- Many proteins interacting with HP1α have a common PxVxL motif – the HP1α^{W174A} disrupts these interactions:



- There are two PxVxL-like sequences in the BZip domain;
- they do not appear to mediate the interaction.



The interactions between HP1α and the BZip domain

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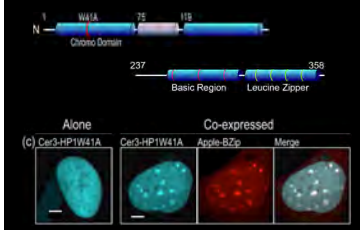
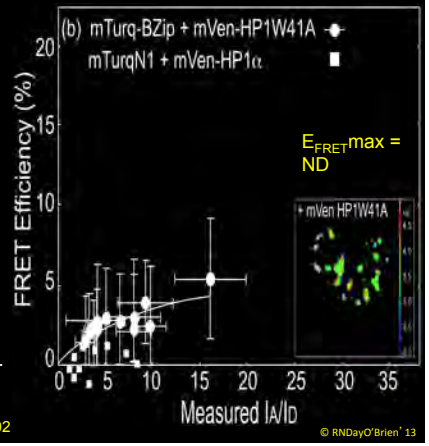
- The W41 residue in the chromo domain is necessary for interaction with chromatin – the HP1α^{W41A} disrupts this:





E_{FRET}max = ND

- Disrupting HP1α interactions with chromatin does alter the interaction with the BZip domain.

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

Conclusions

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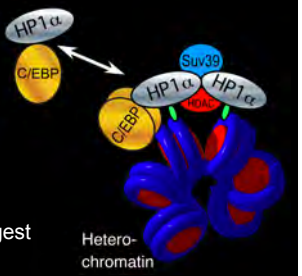
- These studies demonstrate that the BZip transcription factor C/EBPα interacts with HP1α when associated with regions of heterochromatin.
- The BZip domain alone is sufficient for the interaction, and the interaction does not require a canonical PxVxL motif;
- but the association of HP1α with MeK9 of H3 is necessary for a strong interaction with the BZip domain protein.
- Taken together, the results demonstrate the interaction between a transcription factor and HP1α in regions of heterochromatin, and suggest a mechanism by HP1α might regulate transcription.



Prospective: in vivo imaging of metabolic states

59

- Multiphoton microscopy enables high-resolution imaging deep in living tissues.
  - There is a wealth of information in the intrinsic fluorescence signals.
  - 2PE microscopy cannot assign the intrinsic fluorescence signals to specific molecular sources.
- Because 2PE uses a pulsed laser source, it is straightforward to implement FLIM for measurements of probe lifetimes deep in tissues.
- 2PE lifetime measurements analyzed by phasor plot allows the identification of the molecular species -
  and measures their coexistence in the same pixel of the image

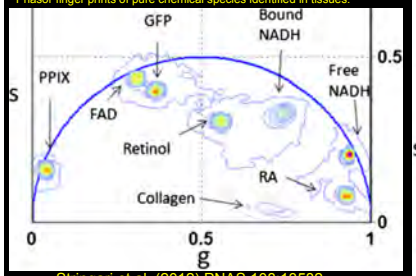
❖ This approach can identify the molecular source of intrinsic fluorescence signals.

Prospective: in vivo imaging of metabolic states

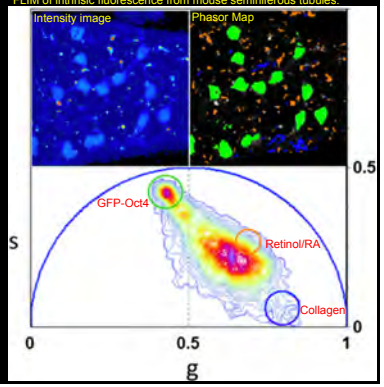
60

- Phasor map of intrinsic fluorescence signals from Oct-4 GFP mouse seminiferous tubules:
  - GFP identifies undifferentiated germ cells.
  - Blue identifies collagen.
  - Orange is retinol/RA - retinoids are a signature of Sertoli cells.

Phasor finger prints of pure chemical species identified in tissues:



FLIM of intrinsic fluorescence from mouse seminiferous tubules:



Stringari et al. (2012) PNAS 108:13582