# Measuring Protein Network Interactions Inside Living Cells

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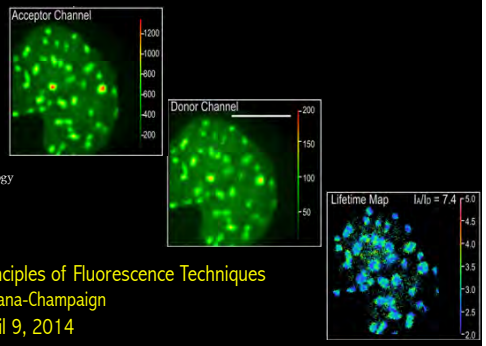
Department of Cellular & Integrative Physiology




Principles of Fluorescence Techniques
Urbana-Champaign
April 9, 2014

## Overview:



- The biological model:
  - Proteins controlling heterochromatin formation – HP1α.
  - Liaisons with a transcription factor – C/EBPα.
  - Why this is important.
- Monitoring protein dynamics in living cells:
  - Fluorescence recovery after photobleaching (FRAP).
  - Photoactivatable-GFP (PA-GFP).
  - Fluorescence Correlation Spectroscopy (FCS).
- Monitoring nuclear protein interactions in living cells:
  - FRET, fluorescence lifetime imaging microscopy (FLIM), phasor plots.
  - FRET standards and biosensor probes.
  - FRET-FLIM measurements of C/EBPα-HP1α interactions.
  - Fluorescence Cross-Correlation Spectroscopy (FCCS).



## Overview:



- The biological model:
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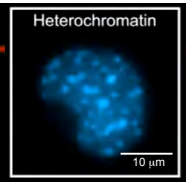
  - Why this is important.



## Imaging protein interaction networks






- Understanding the control of gene expression requires the analysis of networks of protein interactions in their natural environment inside living cells.
- We're using live cell imaging approaches to determine how networks of protein interactions are coordinated inside the cell nucleus.

## Imaging protein interaction networks

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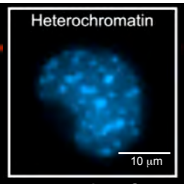
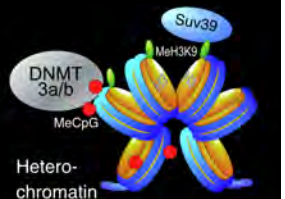



- Understanding the control of gene expression requires the analysis of networks of protein interactions in their natural environment inside living cells.
- We're using live cell imaging approaches to determine how networks of protein interactions are coordinated inside the cell nucleus.
- Centromeric heterochromatin provides the structural integrity to maintain the genome.
- Heterochromatin is formed by the coordinated actions of DNA methyltransferases (DNMT) and histone methyltransferases (e.g., SUV39).






## Heterochromatin protein 1 alpha (HP1α)

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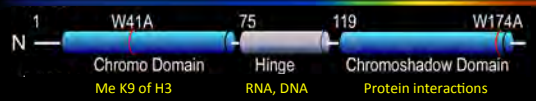
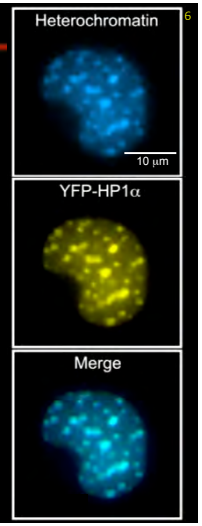



- The formation of heterochromatin also requires heterochromatin protein 1 (HP1α), which interacts with histones and chromatin modifying proteins.
- HP1α in binds to methylated K9 of H3 in heterochromatin where it functions to assemble macromolecular complexes.




## HP1α coordinates networks of protein interactions

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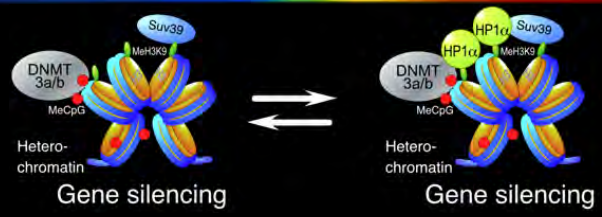


- HP1α serves to translate histone methylation into DNA methylation by recruiting DNMT 3a/b.
  Smallwood et al. (2007) Genes & Dev. 21:1169
- There is increasing evidence that HP1α plays roles in the regulation of gene transcription, DNA replication, and repair.
  Kwon & Workman (2011) Bioessays 33:280



## HP1α coordinates networks of protein interactions

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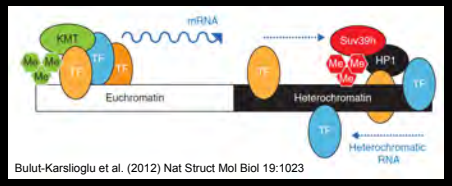
- Recent studies show that HP1α interacts with sequence-specific transcription factors (TF).




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

- Our studies demonstrate that HP1α interacts with the transcription factor CCAAT/enhancer binding protein alpha (C/EBPα).

❖ What is the functional significance of the interaction between C/EBPα and HP1α?

## *Unexpected* activities of C/EBPα

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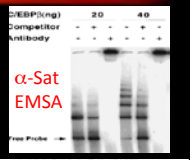
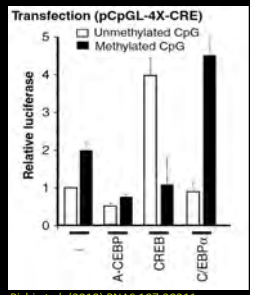
- C/EBPα activates programs of gene expression required for cell differentiation (e.g., adipocytes).
  Lin & Lane (1994) PNAS 91:8757
- C/EBPα binds to α-satellite DNA repeat elements in regions of centromeric heterochromatin.
  Tang & Lane (1999) Genes & Dev. 13:2231
- C/EBPα requires DNA methylation to optimally bind to DNA elements in gene promoters.
- This may allow C/EBPα to activate a subset of differentiation specific genes.




Rishi et al. (2010) PNAS 107:20311



## The intranuclear distribution of C/EBPα

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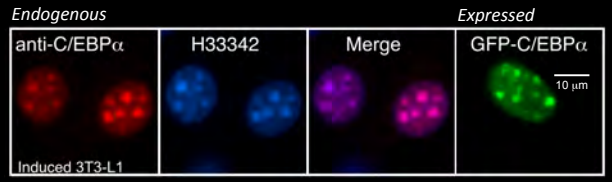
- The endogenous C/EBPα localizes to regions of heterochromatin in differentiated 3T3-L1 adipocyte cells (ICC staining).




- The expressed GFP-C/EBPα has precisely the same pattern of localization.
- Taken together, this suggests that C/EBPα, a transcription factor that initiates programs of cell differentiation, can gain access genes in heterochromatin.



## Why this is important

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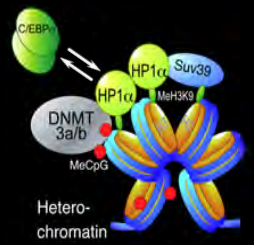
- Because HP1α plays a crucial role in the formation of heterochromatin, we propose that functional interactions between HP1α and C/EBPα could mediate access to genes that are silenced.
- To test this hypothesis, we use biochemical and molecular analysis in combination with the microscopic imaging of living cells.




❖ We're using live cell imaging to monitor protein dynamics and to quantify protein interactions that underpin C/EBPα transcriptional activity.



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  - Why this is important.
- Monitoring protein dynamics in living cells:
  - Fluorescence recovery after photobleaching (FRAP).
  - Photoactivatable-GFP (PA-GFP).
  - Fluorescence Correlation Spectroscopy (FCS).

## Monitoring protein dynamics in living cells: FRAP

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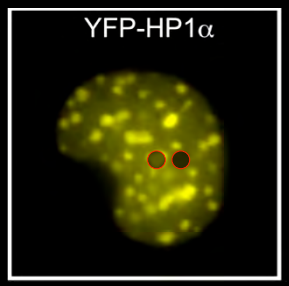
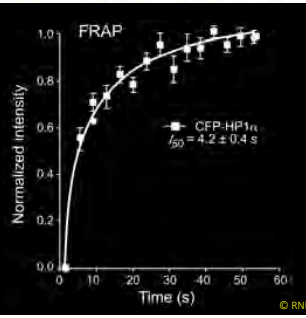
- FRAP uses photobleaching of a small region of fluorescence, and then monitors that region over time to follow the return to a steady state:

FRAP
Fluorescence recovery after photobleaching




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



## Monitoring protein dynamics in living cells: PA-GFP

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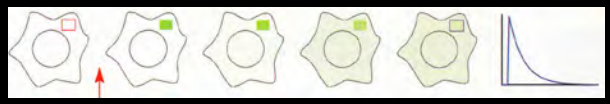
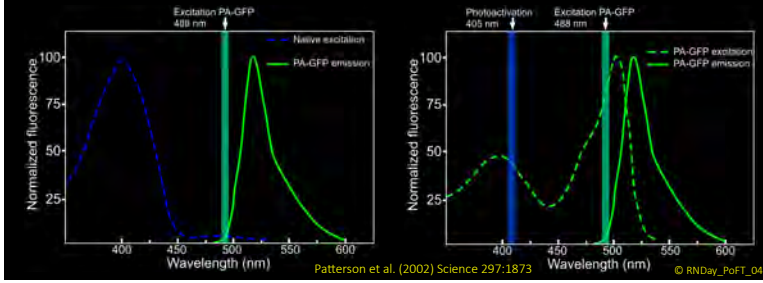
- The photoactivatable GFP (PA-GFP) is converted from a dark state to a bright fluorescent state by photoactivation.

PA-GFP

Photoactivation



- Intense 405 nm illumination switches excitation peak to 504 nm:




Patterson et al. (2002) Science 297:1873



## PA-GFP: an optical marker

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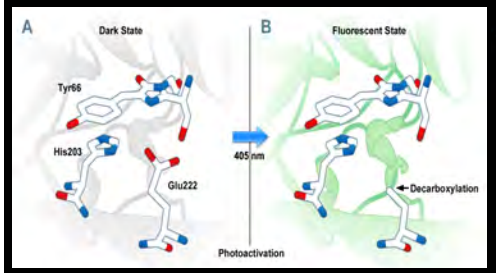
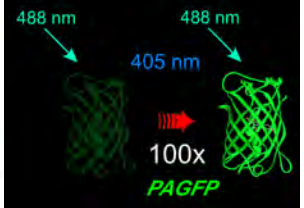
- Intense 405 nm illumination is thought to cause decarboxylation of the glutamic acid at position 222 near the chromophore.
- This converts the chromophore from a dark state to a bright fluorescent state:






## Imaging protein dynamics with PA-GFP

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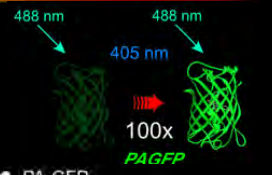
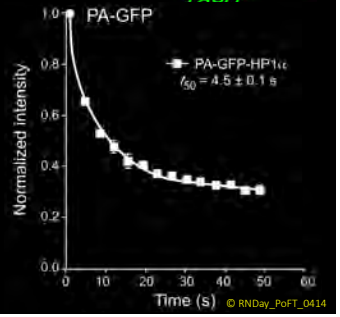
- Photoactivate PA-GFP with 405 nm laser in a region of interest in the cell.
- Monitor the diffusion of the newly fluorescent protein with 488 nm excitation over time.






❖ Qualitative measures of protein diffusion.

## Monitoring protein dynamics in living cells: FCS

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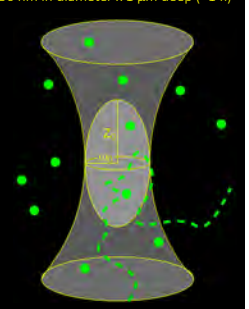
- FCS monitors fluctuations in fluorescence intensity inside a small optically defined confocal volume.
- FCS is a single-molecule detection technique that directly quantifies diffusion and interactions within the observation volume inside living cells.
- FCS requires extremely low* probe concentration.
- FCS is an ideal tool for the analysis of:
  - Molecular mobility (diffusion, flow, exchange).
  - Molecular interactions (complements FRET).
  - Kinetics.
  - Photophysics.




* 1 fl, 1 nM solution = 1 molecule at a time



## Imaging protein dynamics with FCS

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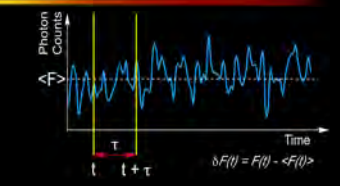
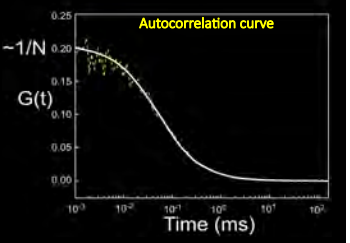
- Fluorescence fluctuations within the focal spot are recorded photon by photon and deviations from the average (<F>) are measured.
- The time series is correlated with itself after the lag time τ, and normalized by the average intensity:

$$G(t) = \frac{\langle \delta F(t)\, \delta F(t+\tau) \rangle}{\langle F(t)^2 \rangle}$$

- The fluctuations provide information about the number of molecules (N), brightness, and rate of diffusion.






## Calibration for FCS

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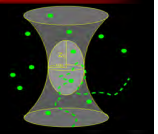
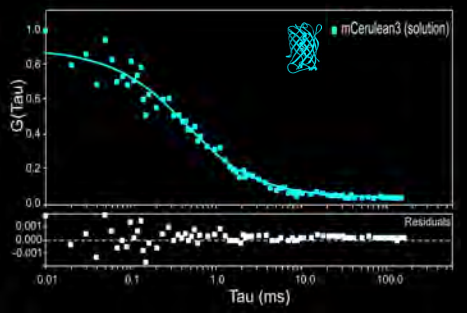
- Calibration standards: measurements of a pure dye (Coumarin 6 or Alexa 568) with a known diffusion coefficient determines the axial and radial dimensions ($z_0/w_0$).



- The diffusion coefficient for purified mCerulean3 in solution is 82 ± 7 $\mu m^2 s^{-1}$.



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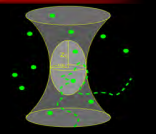
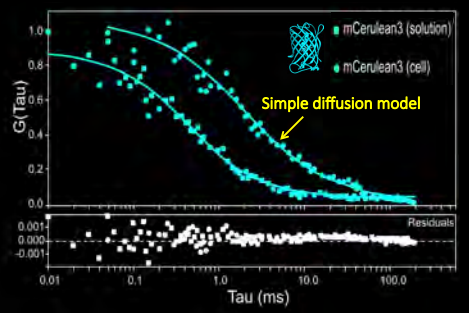


- The diffusion coefficient for purified mCerulean3 in solution is 82 ± 7 $\mu m^2 s^{-1}$.
- In living cells, the diffusion coefficient is 26 ± 4 $\mu m^2 s^{-1}$.
- Cerulean3 is well suited for FCS studies.



## FCS measurements of Cerulean3-HP1α

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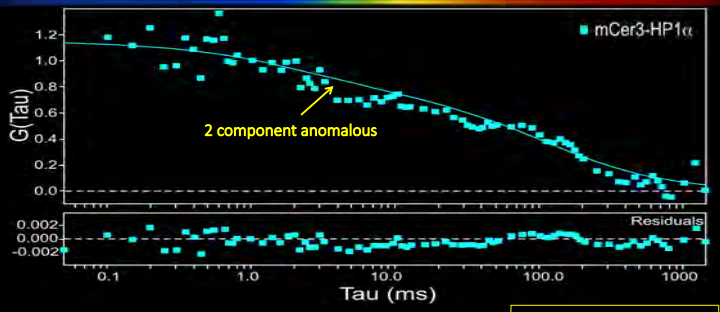


- The diffusion of HP1α is best fit to a 2-component model of diffusion while interacting with nuclear structure.

| Diffusion coefficient |
| --- |
| HP1α |
| 4.8 ± 1.1 $\mu m^2 s^{-1}$ |

- FCS quantifies the mobility of HP1α inside the living cell nucleus – enabling comparative studies with other proteins.

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



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  - FRET, fluorescence lifetime imaging microscopy (FLIM), phasor plots.



## Förster resonance energy transfer (FRET) microscopy

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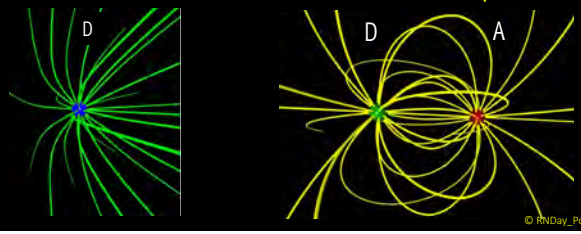
- FRET is the direct transfer of excited state energy from a donor fluorophore to a nearby acceptor.
- A fluorophore in the excited-state is an oscillating dipole that creates an electric field (the donor - D).
- If another fluorophore enters the electric field, energy can be transferred directly to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






## FRET measures the spatial relationship between the FPs

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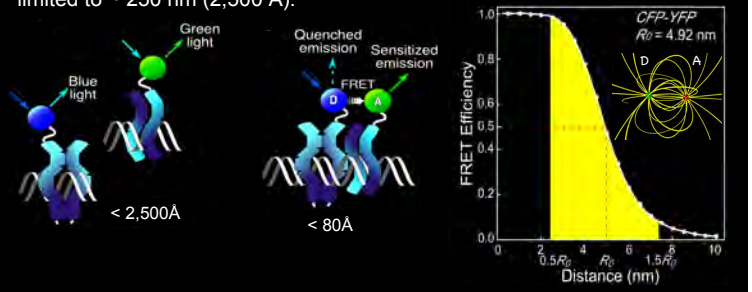
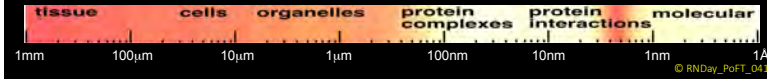
- The optical resolution of the conventional light microscope is limited to ~ 250 nm (2,500 Å).




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



## The spectral overlap requirement

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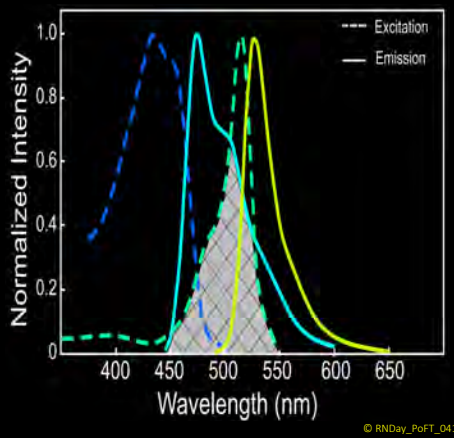
- The donor emission spectrum must significantly overlap the absorption spectrum of the acceptor.




## Spectral bleedthrough background signals

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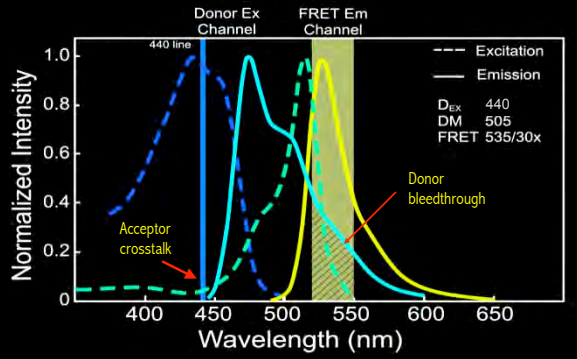
- The more overlap, the more background → Spectral bleedthrough (SBT).




- Methods that measure sensitized emission from the acceptor must *accurately* correct for SBT.



## Spectral bleedthrough background signals

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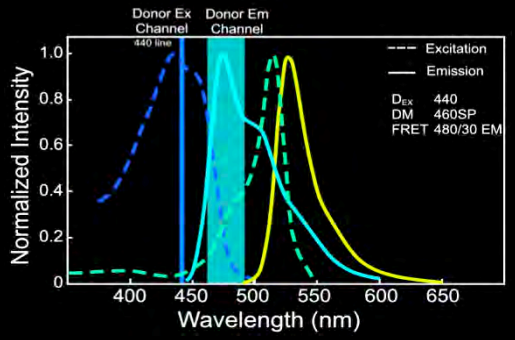
- Alternatively, methods that detect changes in donor fluorescence due to FRET are not subject to SBT.




- Fluorescence lifetime imaging microscopy (FLIM) measures quenching of the donor when FRET occurs.



## Fluorescence Lifetime

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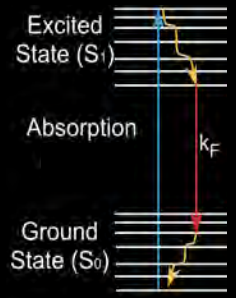
- Fluorescence lifetime ($\tau$): the average time a population of fluorophores spend in the excited state before returning to the ground state.
- Every fluorophore has a characteristic lifetime, typically on the order of a few nanoseconds:

| Probe | $\tau_m$ |
|---|---|
| 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 |




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

## FRET by FLIM

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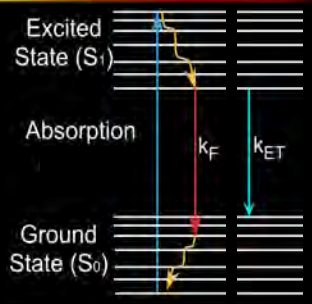
- FRET is a quenching event ($k_{ET}$), allowing transition to the ground state without fluorescence emission.
- Quenching events cause the fluorescence lifetime to shorten.




- 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.



## FRET by Frequency Domain (FD) FLIM

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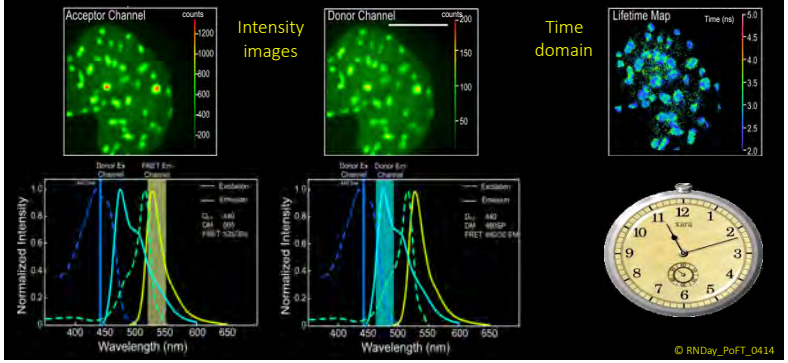
- The FLIM system is a confocal microscope that acquires intensity images in two different channels simultaneously:
- The FLIM system also collects information in the time-domain:






## Frequency domain (FD) FLIM measurements

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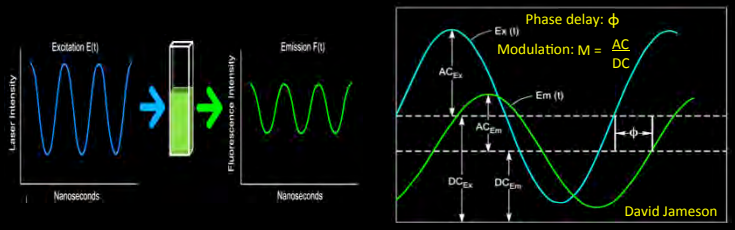
- A modulated light source is used to excite the probe. Because of the excited state lifetime, there is a delay in the emission relative to the excitation.
- FD FLIM measures the phase delay (Φ) and change in modulation (M) of the emission signal relative to the excitation wave.




- The Φ and M are used to determine the fluorescence lifetime at every pixel in an image.



## FD FLIM analysis by phasor plot

32

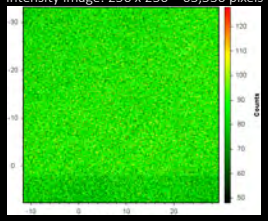
- The phasor plot was developed to analyze and display frequency characteristics.

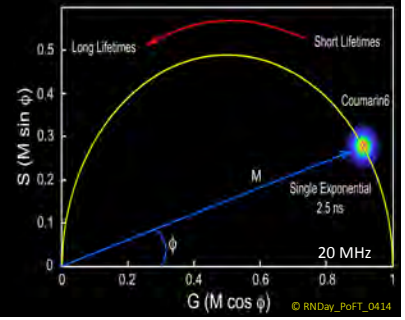
Redford & Clegg (2005) J Fluoresc 15:805
Jameson et al. (1984) App Spec Rev 20:55

- The phasor plot is a vector representation of the phase delay (Φ) and relative modulation (M) at every pixel in the image.

Coumarin6

Intensity image: 256 x 256 – 65,536 pixels

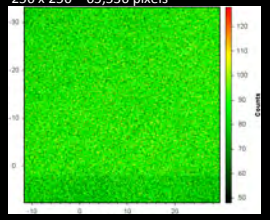


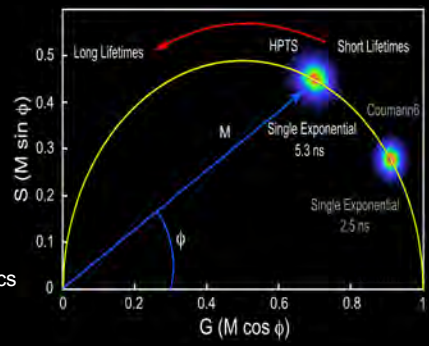


## FLIM analysis by phasor plot

33

HPTS
256 x 256 – 65,536 pixels






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

❖ 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



## Overview:

34

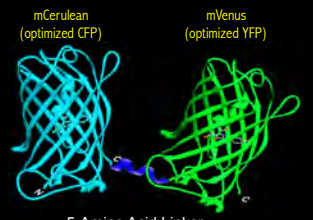
- The biological model:
  - Proteins controlling heterochromatin formation – HP1α.
  - Liaisons with a transcription factor – C/EBPα.
  - Why is it important.
- Monitoring protein dynamics in living cells:
  - Fluorescence recovery after photobleaching (FRAP).
  - Photoactivatable-GFP (PA-GFP).
  - Fluorescence Correlation Spectroscopy (FCS).
- Monitoring nuclear protein interactions in living cells:
  - FRET, fluorescence lifetime, frequency domain FLIM, phasor plots.
  - FRET standards and biosensor probes.



## Measurements in living cells: FRET standards

35

- 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.

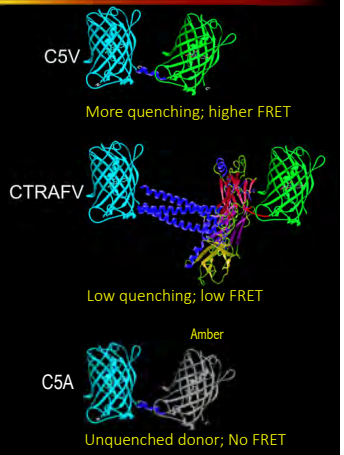
Thaler et al. (2005) Biophys. J 89:2736



## Cerulean-Venus FRET standards

36

- 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

## Optimized Donors for FLIM

37

- FLIM measurements are greatly simplified if the donor fluorophore has a single-component lifetime.

| Donor[a] | IB[b] | Photo-stablity | 2 component lifetime (fraction) | Tau(f)[c] lifetime (ns ± SD) | $\chi^2$ (± SD)[d] |
|---|---|---|---|---|---|
| Cerulean | 14 | ++ | 2.2 ± 0.2 (0.66)<br>4.6 ± 0.4 (0.34) | 3.0 ± 0.05 | 1.1 ± 0.37 |
| Cerulean3 | 24 | +++ | 3.9 ± 0.06 (0.99) | 3.9 ± 0.06 | 5.7 ± 2.5 |
| Turquoise | 25 | +++ | 3.9 ± 0.03 (0.99) | 3.9 ± 0.06 | 3.6 ± 2.0 |
| Turquoise2 | 28 | ++ | 4.1 ± 0.04 (0.99) | 4.1 ± 0.04 | 3.2 ± 1.6 |

[a] Expressed in cells at 37°, n = 10 or more.
[b] Intrinsic brightness is ε x QY.
[c] tau fraction, average lifetime.
[d] for 12 frequencies.

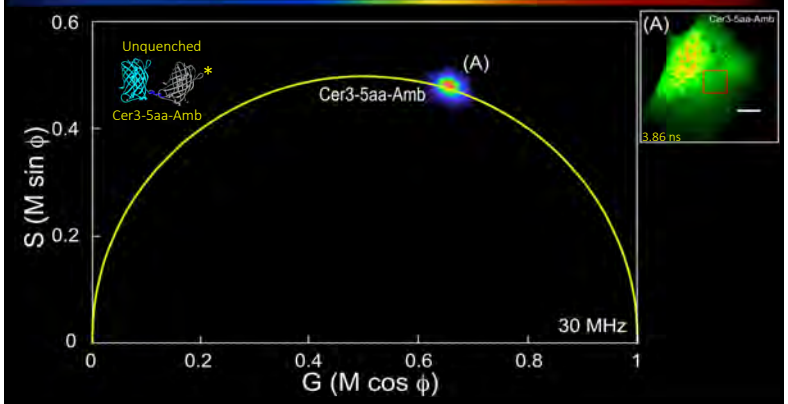
Day and Davidson (2014) *In Press*

- Cerulean has a 2-component lifetime.
- The newer variants of Cerulean are better fit by a single-component lifetime (and are a better choice for FLIM).



## FLIM analysis by phasor plot: FRET standards in living cells

38



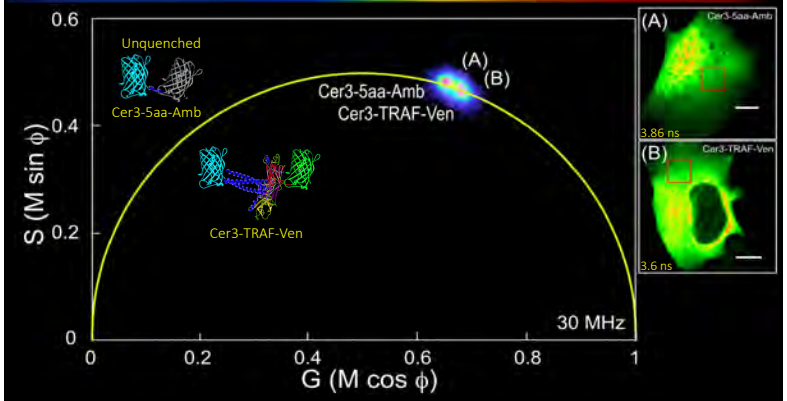

- Cerulean3 has a single lifetime of ~ 3.9 ns.

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



## FLIM analysis by phasor plot: FRET standards in living cells

39

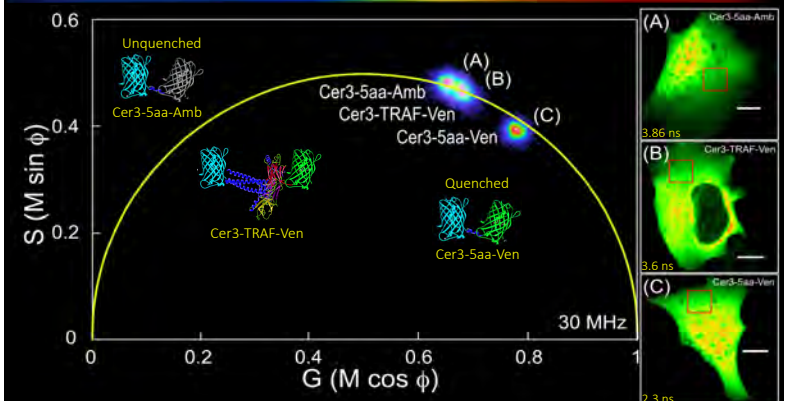



- For Cer3-TRAF-Ven, there is weak quenching;
  $E_{FRET} = 1 - (\tau_{DA}/\tau_D) = 7\%$



## FLIM analysis by phasor plot: FRET standards in living cells

40




- Cer3-5AA-Ven, Cer3 is quenched more;
  $E_{FRET} = 1 - (\tau_{DA}/\tau_D) = 40\%$

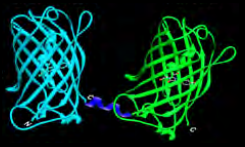
Day (2013) Methods/j.ymeth.2013.06.017

## Biosensor probes

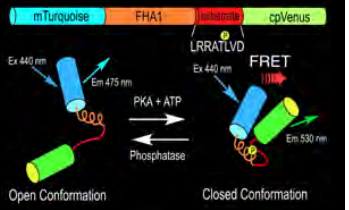
41

- 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 sensing unit:
- A kinase activity reporter (AKAR) is a reporter of intracellular PKA activity.

FRET Standard



FRET-based Biosensor probe




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

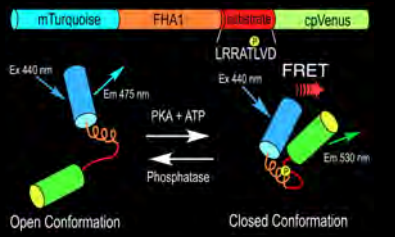


## FRET-based biosensor probes

42

- The phosphorylation of the AKAR substrate by PKA causes the sensing unit to change conformation, altering the distance between the FRET pair.
  - mTurquoise and circular permuted Venus;
  - coupled through a low affinity phospho-substrate binding domain, FHA1, and a PKA specific substrate.

AKAR4.1




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

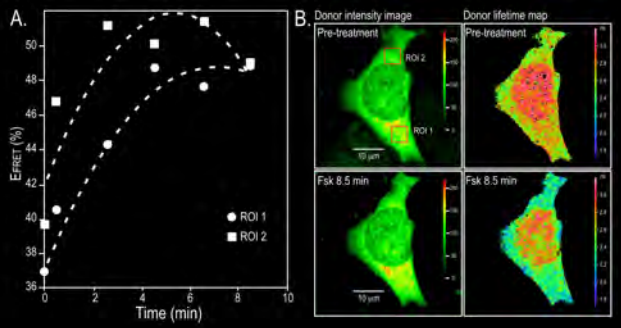
- The changing intramolecular FRET signal reports the spatiotemporal dynamics of protein activity inside living cells.



## FLIM measurements of AKAR biosensor probe activities

43

- FLIM measures the changing lifetime of the AKAR biosensor probe over time after treatment with Forskolin (Fsk):




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



## Overview:

44

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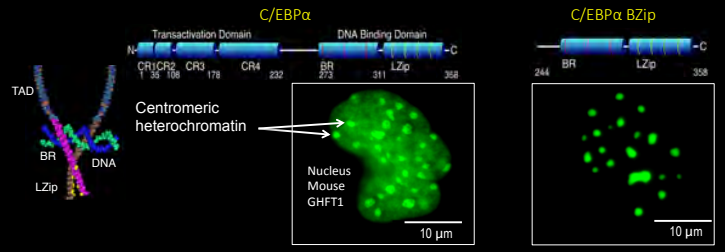
  - Fluorescence Correlation Spectroscopy (FCS).
- Monitoring nuclear protein interactions in living cells:
  - FRET, fluorescence lifetime, frequency domain FLIM, phasor plots.
  - FRET standards and biosensor probes.
  - FRET-FLIM measurements of C/EBPα-HP1α interactions.

## Measuring *intermolecular* protein interactions with FRET-FLIM

45

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



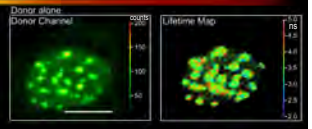

- The C/EBPα BZip domain alone is sufficient for DNA binding and localization to centromeric heterochromatin.
- Centromeric heterochromatin is analogous to an endogenous DNA array allowing visualization of protein interactions associated with chromatin.
  - ❖ Can FRET-FLIM detect C/EBPα dimer formation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

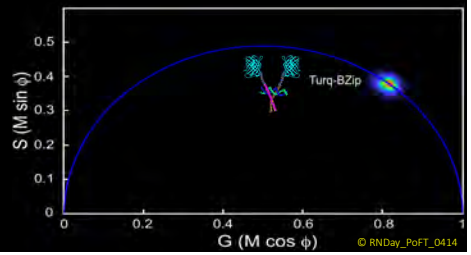


## FRET-FLIM analysis of BZip domain interactions

46

- 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

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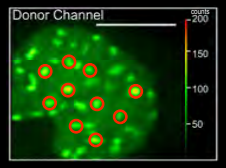
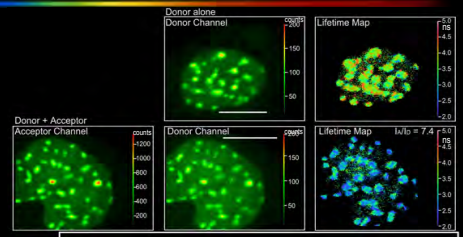
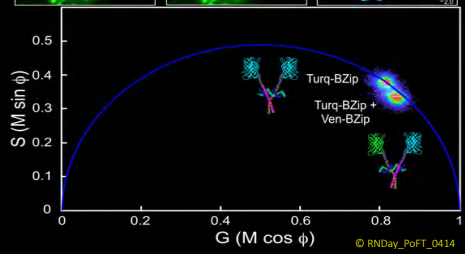



- The lifetime information can be acquired from individual regions of interest (ROI).



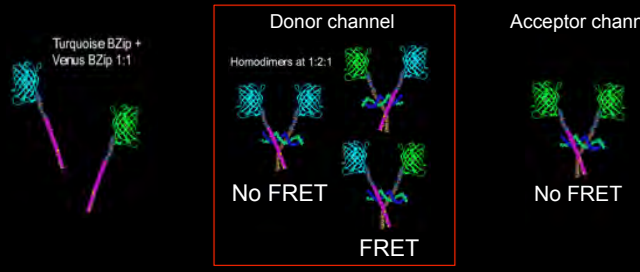






## Measuring intermolecular protein interactions with FRET-FLIM

48

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

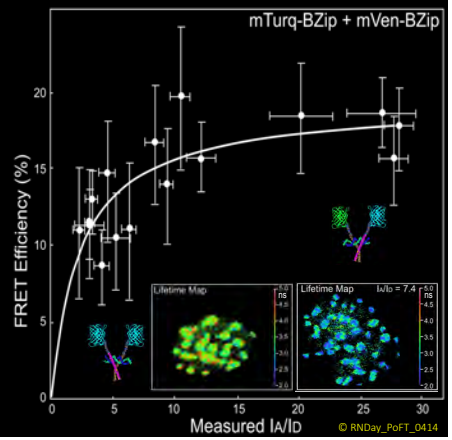



- Because the Acceptor- and Donor-labeled proteins are encoded by different plasmids, every transfected cell will have a different A:D ratio.
  - ❖ The A:D ratio influences the FRET efficiency ($E_{FRET}$).

## FRET-FLIM analysis of BZip domain interactions

49

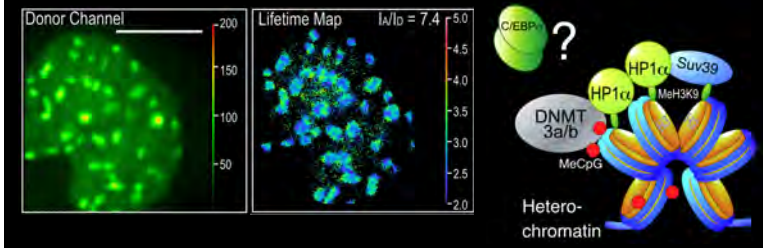
- How can we use the cell-to-cell variability in the Acceptor-to-Donor ratio ($I_A/I_D$)?
- FLIM was used to determine the $E_{FRET}$ for multiple ROI in cells expressing Turquoise- and Venus-labeled BZip proteins.
- The $E_{FRET}$ for 16 different cells (~10 ROI per cell) are plotted as a function of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






## C/EBPα localizes to heterochromatin

50

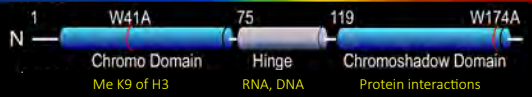



- Does C/EBPα interact with the HP1α protein network?

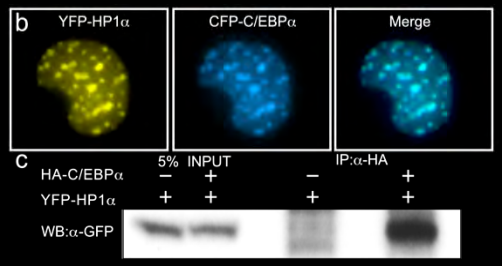


## Co-IP of HP1α and C/EBPα

51




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

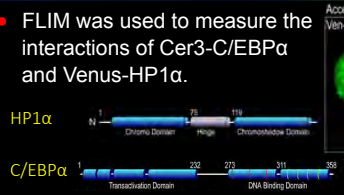


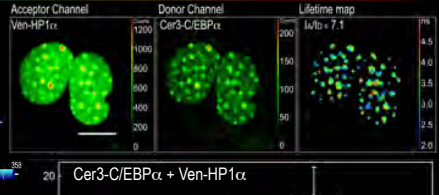



## Heterologous interactions between HP1α and C/EBPα

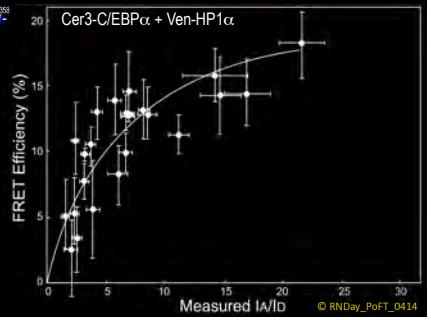
52

- 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.

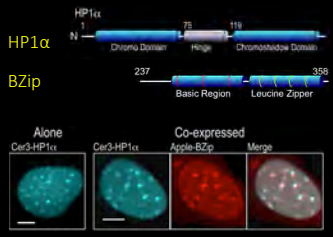


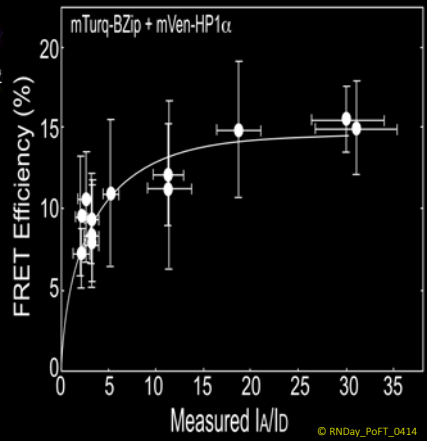

Siegel et al. (2013) J Biomed Optics 18:025002

## Heterologous interactions between HP1α and C/EBPα

53

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




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

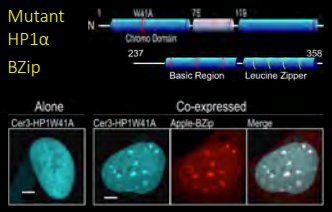
Siegel et al. (2013) J Biomed Optics 18:025002

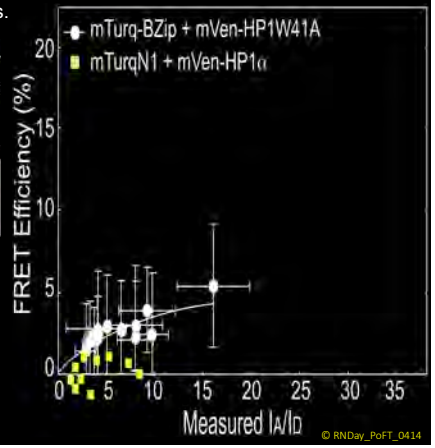


## Heterologous interactions between HP1α and C/EBPα

54

- The W41 residue in the chromodomain is necessary for interaction with chromatin – the HP1α$^{W41A}$ mutation disrupts this.




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

❖ Do they move in a common complex?

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



## Overview:

55

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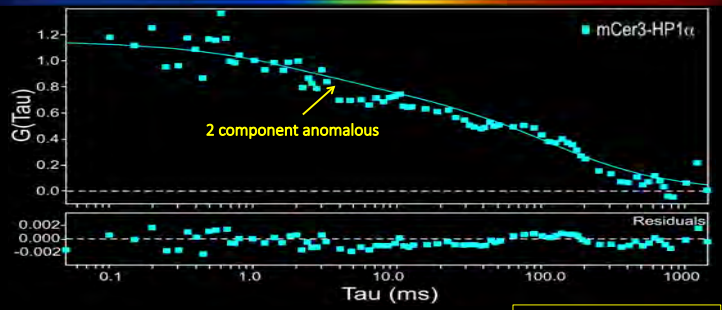
  - FRET standards and biosensor probes.
  - FRET-FLIM measurements of C/EBPα-HP1α interactions.
  - Fluorescence Cross-Correlation Spectroscopy (FCCS).



## FCS measurements of Cerulean3-HP1α

56



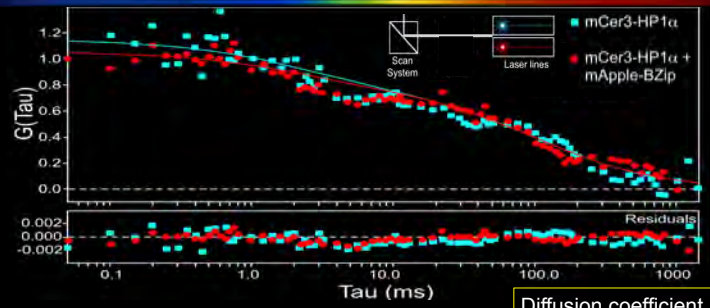

- The diffusion of HP1α is best fit to a 2-component model of diffusion while interacting with nuclear structure.

| Diffusion coefficient |
| --- |
| HP1α |
| 4.8 ± 1.1 μm$^2$s$^{-1}$ |

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

## FCS of Cerulean3-HP1α alone and with mApple-BZip

57




- When co-expressed, the diffusion of either HP1α or BZip was not affected by the other, suggesting the proteins do not interact in the mobile fraction.
- We can't rule out a small fraction of interacting proteins.

Diffusion coefficient
HP1α
4.8 ± 1.1 $\mu m^2 s^{-1}$

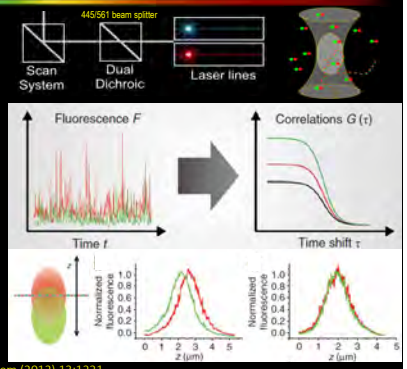
C/EBP BZip domain
7.1 ± 1.7 $\mu m^2 s^{-1}$



## Fluorescence cross-correlations spectroscopy (FCCS)

58

- FCCS can determine the auto-correlation for the two different labeled proteins, as well as their cross-correlation.
- The axial detection volume overlap and correction for crosstalk is critical.
- The crosstalk is spectral bleed-through (cyan into red channel), the number of fluorophores, and the brightness ratio.




Bacia and Schwille (2007) Nat Methods 2:2842; ChemPhysChem (2012) 13:1221

- Ideal fluorophore properties are required, and this is difficult to achieve with the red FPs.

Siegel et al. (2013) Int. J. Mol. Sci. 14:20304



## Controls for FCCS measurements

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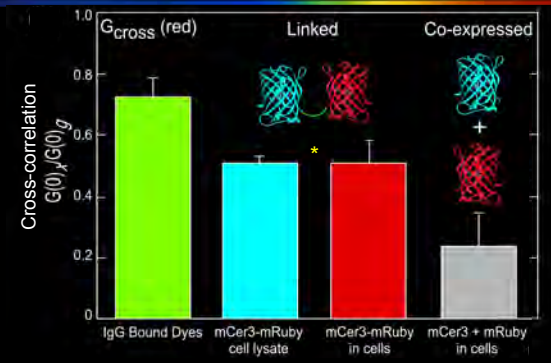


* Non-ideal behavior of the red FPs? (dark state, triplet state)

- The positive controls are a mixture of IgG bound dyes (Alexa 488 and Texas Red), and expression of the linked FPs.
- The negative control is the co-expression of the individual FPs.



## FCCS: Do C/EBPα and HP1α interact in the mobile phase?

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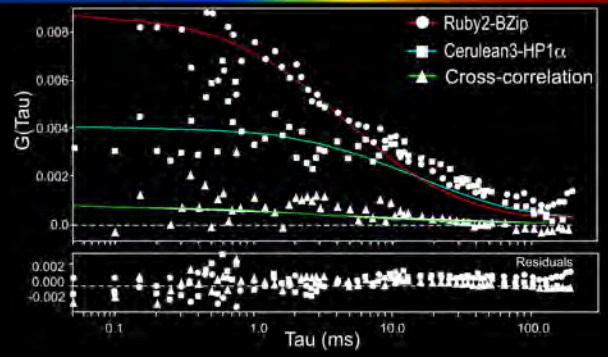


- FCCS confirms the C/EBP BZip proteins diffuse at a faster rate than the co-expressed HP1α.
  - These proteins only interact when bound to chromatin.

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

## Conclusions

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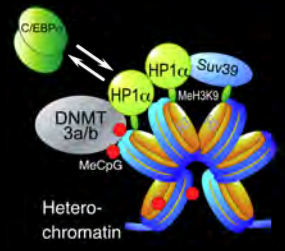
- How is the HP1α network of protein interactions regulated?
- The BZip transcription factor C/EBPα interacts with HP1α when associated with regions of heterochromatin.
- The BZip domain alone is sufficient for the interaction, and the interaction does not require a canonical PxVxL motif;
- but the association of HP1α with MeK9 of H3 is necessary for a strong interaction with the BZip domain protein.
- What is the functional significance of the interaction between C/EBPα and HP1α?






## Future directions

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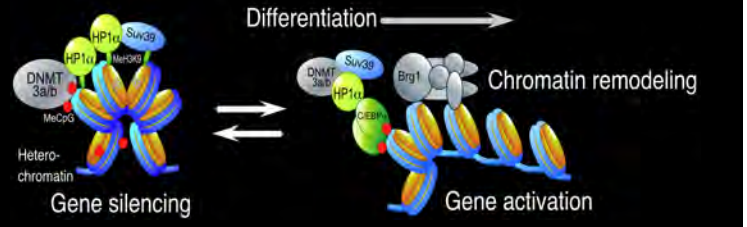
- The results suggest a mechanism by which C/EBPα might regulate HP1α and allow activation of genes localized to regions of heterochromatin:




- C/EBPα binds to methylated DNA elements where it interacts with HP1α.
- This might function to disrupt its association with methyltransferases, while recruiting Brg1 and the SWI/SNF chromatin-remodeling complex to allow activation of genes required for differentiation.