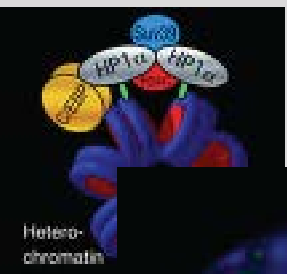
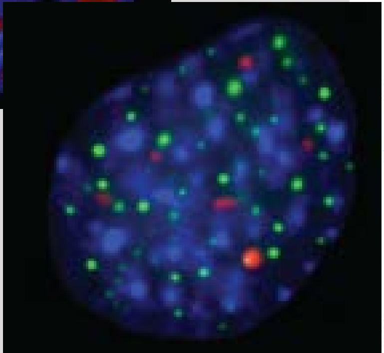
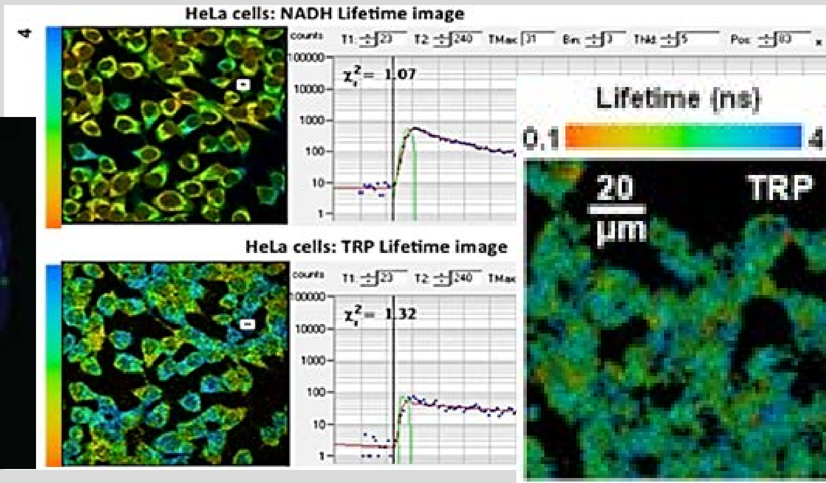
Investigation of Protein-Protein Interactions in a Single Living Cell: 2- and 3-color FLIM-FRET Microscopy

ADFLIM

July 26-28, 2017







W.M. Keck Center for Cellular Imaging

Ammasi Periasamy, Ph.D.

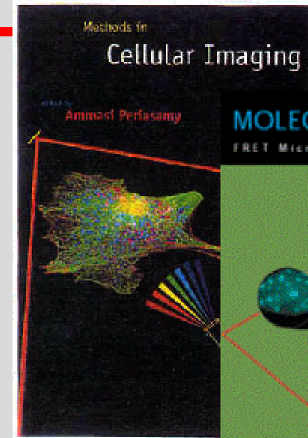
Center Director

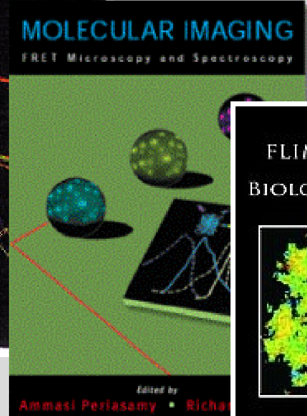
Professor of Biology and Biomedical Engineering

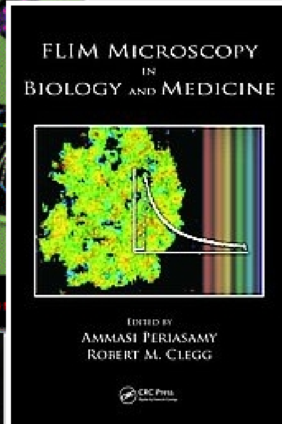
University of Virginia, Charlottesville, USA

Technology Development and Applications

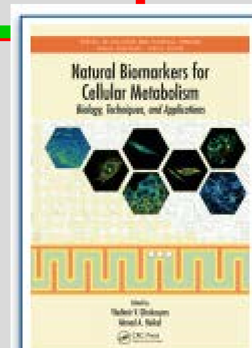
* Fluorescence Lifetime Imaging Microscopy (FLIM)
* Förster Resonance Energy transfer Microscopy (FRET)
* FLIM-FRET Microscopy –
• Workshop Since 2002

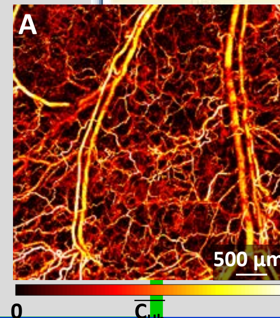


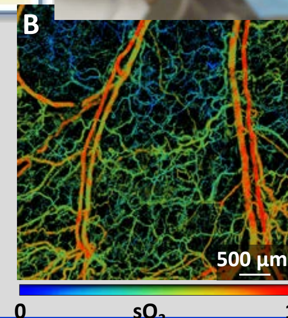


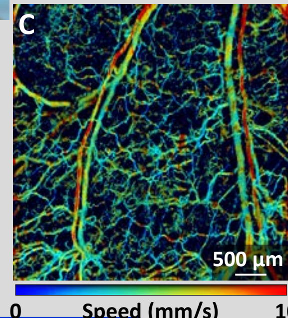


* Endogenous Molecular Imaging NADH, FAD, Tryptophan Optical Redox Ratio
* Energy Metabolism – Photoacoustic FLIM Imaging
* Intravital Imaging –
* Multiphoton Photoacoustic Imaging Microscopy (M-PAM)









$MRO_2 = \varepsilon \cdot C_{Hb} \cdot s_aO_2 \cdot OEF \cdot BF$

* Cervical & Leukemia Cancer
* Prostate cancer

http://www.kcci.virginia.edu/workshop-2017

17th Annual Workshop on

FLIM and FRET Microscopy

Imaging Protein-Protein Interactions

March 5-9, 2018

- Hands-on instructions on 10+ systems
- 10 internationally recognized faculty
- Best imaging and analysis solutions
- Personal attention for a maximum of 25 participants
- Individual problem solving

Since 2002

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Guest Lecturers

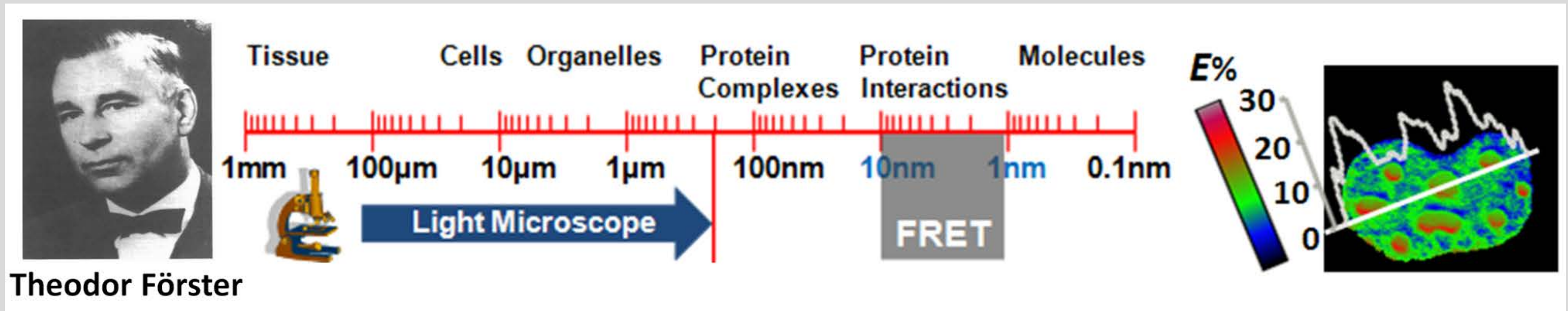
Dr. M. Stanley, Chroma Tech.

Dr. P. So, MIT

15th Annual Workshop on FLIM and FRET Microscopy – March 7-11, 2016

What is FRET?

RET or FRET



Förster Resonance Energy Transfer
Or
Fluorescence Resonance Energy Transfer
FRET

FRET can tell us about dynamic behavior of biological molecules and biological systems

History and Basics of FRET

Förster Resonance Energy Transfer

Theory of FRET in the 1920s

Energy transfer by electrodynamical dipole-dipole interaction proposed by Jean Perrin and Francis Perrin.

Their equation for distance dependence gave 15-25 nm as the required separation for FRET.

Equation underestimated the dependence on separation distance. i.e. interacting molecules need to be closer than they thought.

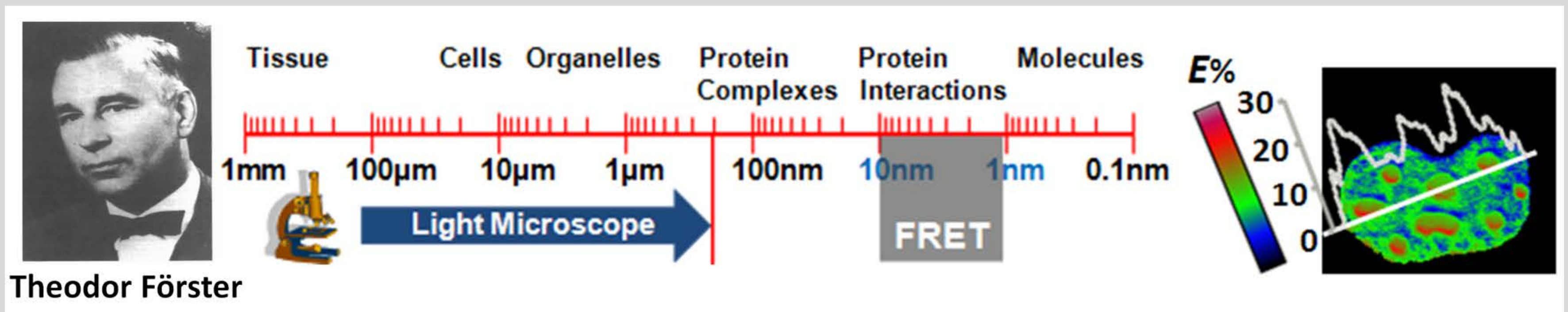
Theodor Förster and FRET in the 1940s

Förster derived new equations consistent with experiments

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

R_0 – Förster distance; the distance between donor and acceptor at which energy transfer is 50%

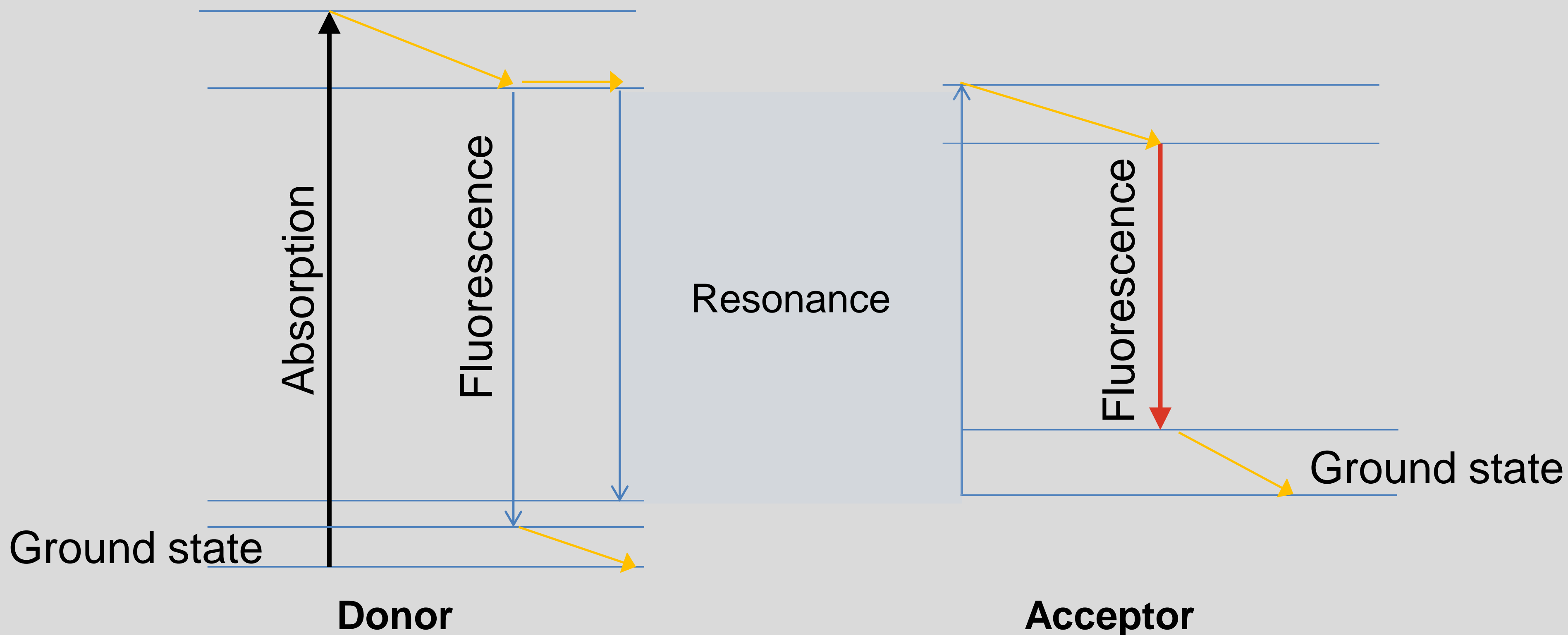
$$R_0^6 = \frac{9000(ln10)\kappa^2 \varnothing_D}{N_A 128\pi^2 n^4}\left[\frac{\int_0^\infty \varepsilon_A(\bar{v})F(\bar{v})\bar{v}^{-4}d\bar{v}}{\int_0^\infty F(\bar{v})d\bar{v}}\right]$$



Förster Resonance Energy Transfer happens at distances of 1~10 nm. This is good for looking at protein-protein interactions.

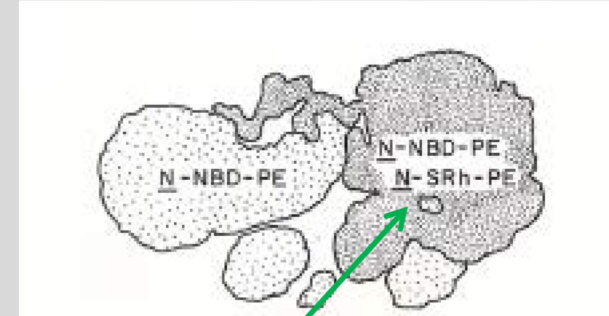
What is FRET?

FRET is a process by which radiationless transfer of energy occurs from a donor fluorophore in the excited state to an acceptor molecule in the ground state in close proximity.



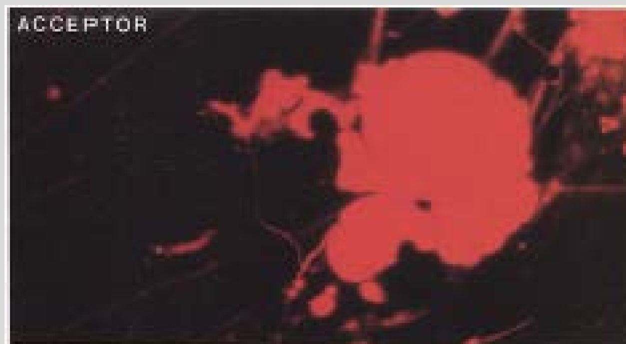
Sensitized emission is a decrease in donor emission with increase in acceptor emission

First FRET Microscopy images using video camera systems



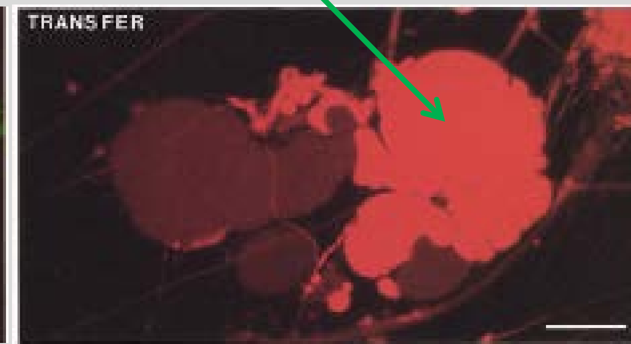
- N-NBD-PE (donor, fluorescein) is excited at 436 nm and emits at 515-565 nm
- N-SRh-PE (acceptor, rhodamine) is excited at 546 nm and emits at 610 nm.

If excitation at 436 nm, we see emission at 610 nm if and, only if, the donor and the acceptor are close together.



(λ_{ex} = 546 nm, λ_{em} = LP610 nm)

(λ_{ex} = 436 nm, λ_{em} = 515-565 nm)



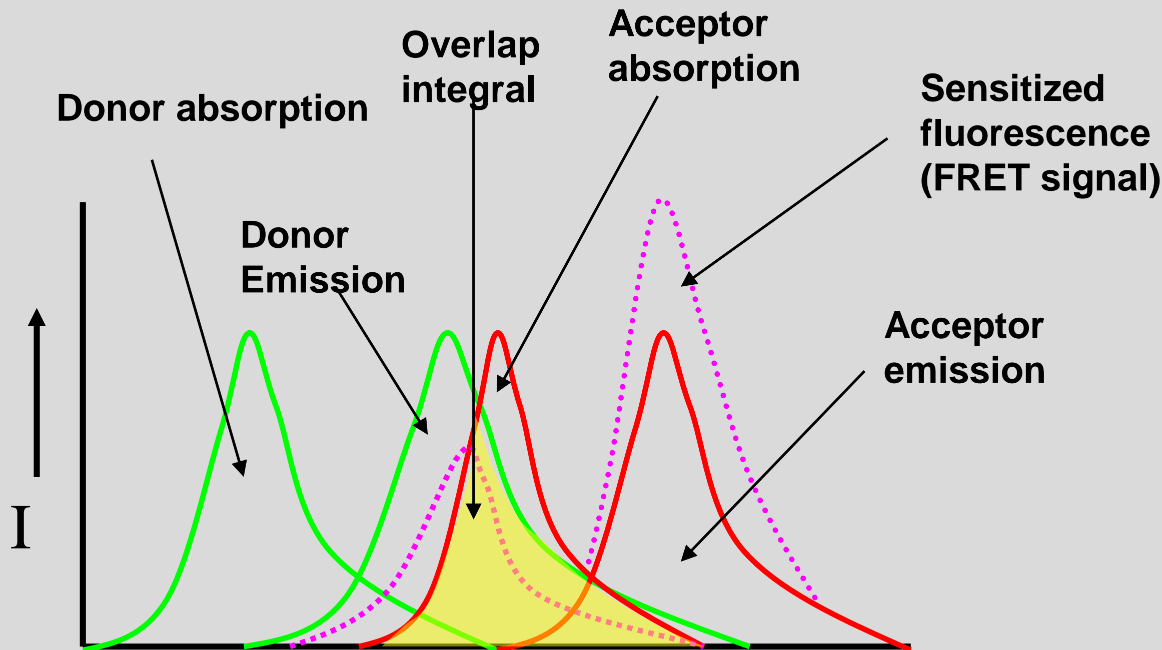
(λ_{ex} = 436 nm, λ_{em} = LP610 nm)

Uster and Pagano, *J Cell Biol 103:1221-1234, 1986*

Three conditions for FRET to occur

Condition 1: Spectral overlap >30%

FRET can occur when the emission spectrum of a donor fluorophore significantly overlaps (>30%) the absorption spectrum of an acceptor.



No FRET if the spectrum is not overlapped.

$$R_0^6 = \frac{9000(ln10)\kappa^2 \emptyset_D}{N_A 128\pi^2 n^4}\left[\frac{\int_0^\infty \varepsilon_A(\bar{v})F(\bar{v})\bar{v}^{-4}d\bar{v}}{\int_0^\infty F(\bar{v})d\bar{v}}\right]$$

Condition 2. Distance between molecules < 10 nm

Inverse relationship between energy transfer efficiency (*E*) and sixth power of distance between donor and acceptor (*r*).

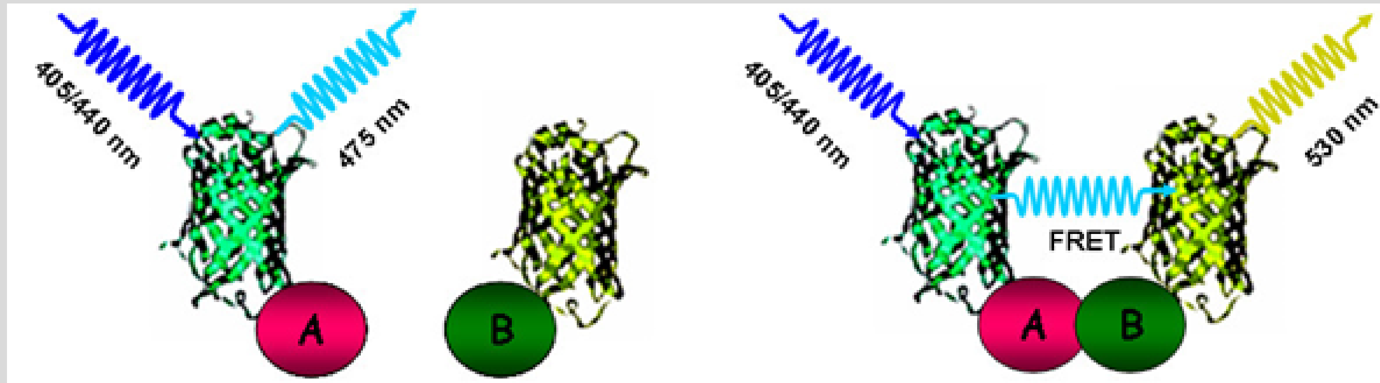
$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

$$r = R_0 \left(\frac{1}{E} - 1\right)^{\frac{1}{6}}$$

r – distance between donor and acceptor

R_0 – Förster distance

E – Energy transfer efficiency



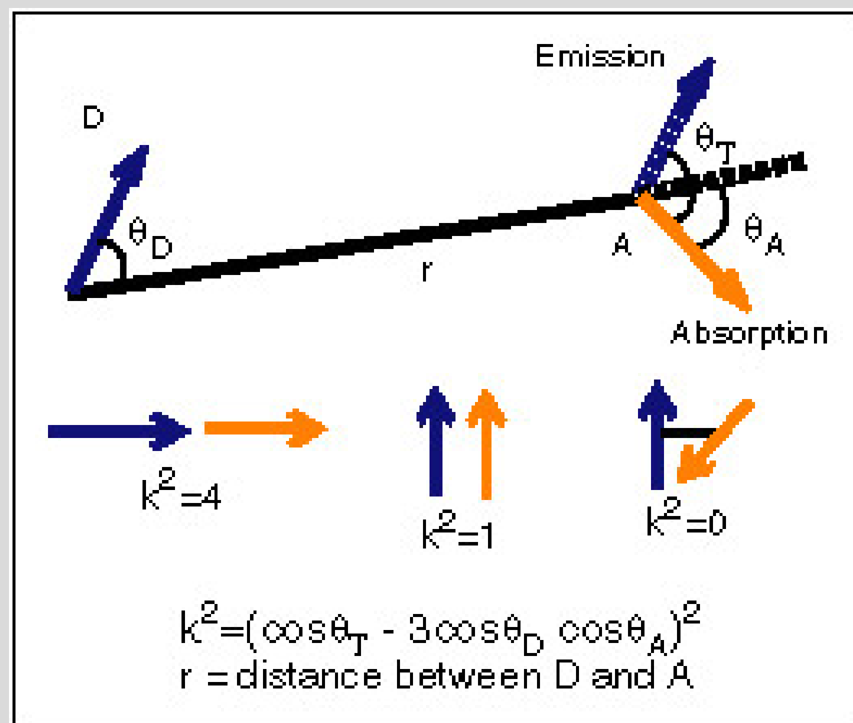
No FRET **Close enough for FRET**

Condition 3: Favourable dipole orientation

$$R_0^6 = \frac{9000(ln10)\kappa^2 \emptyset_D}{N_A 128\pi^2 n^4}\left[\frac{\int_0^\infty \varepsilon_A(\bar{v})F(\bar{v})\bar{v}^{-4}d\bar{v}}{\int_0^\infty F(\bar{v})d\bar{v}}\right]$$

κ^2 is the orientation factor
κ^2 is 0 if the dipoles are perpendicular to each other.
κ^2 is at its maximum, 4, when the dipoles are in series
Any κ^2 between 1 and 4 is okay.

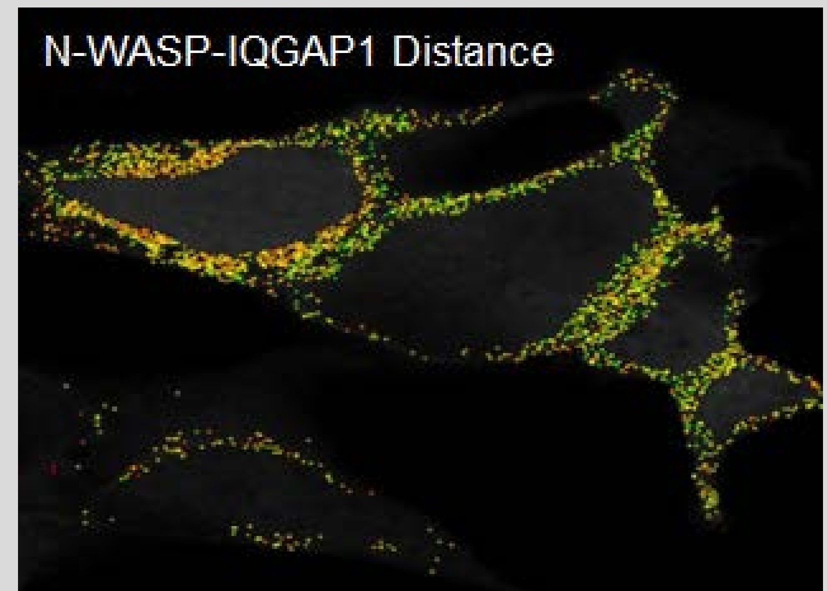
If dipoles are parallel, far field fluorescence happens instead



FRET microscopy enables study of protein interactions in live cells

Benefits of FRET microscopy

- Live specimens
- Single cell assay
- Investigate subcellular compartments
- Spatial and temporal information of the interacting proteins



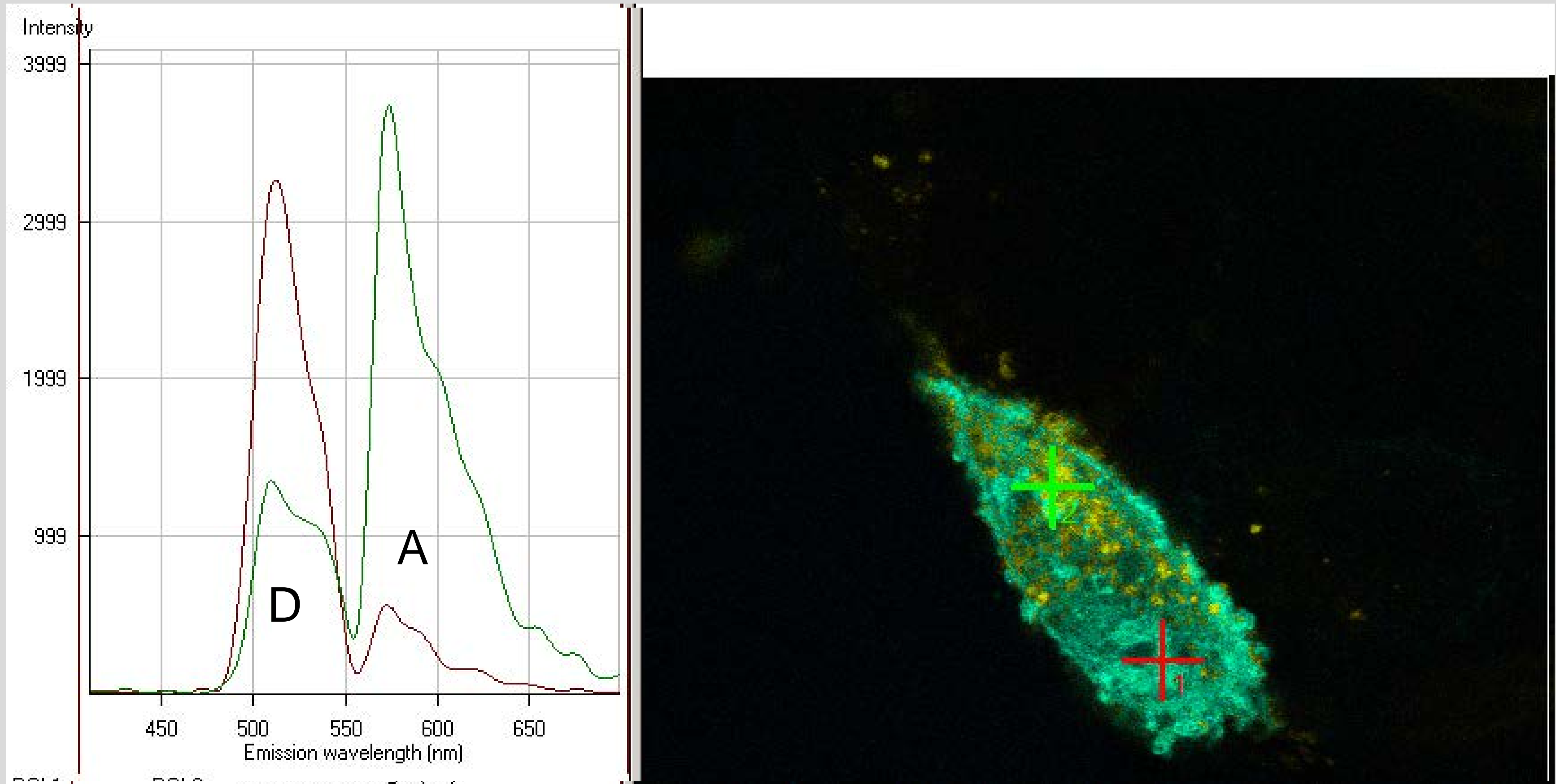
Plotting distance information rather than E% shows that the interacting proteins are closer together at some sites than others.
(Wallrabe *et al.* Cytoskeleton, 2013)

Available FRET microscopy techniques

- **Wide-field or digitized video FRET (*WF-FRET*)**
- **Laser Scanning Confocal FRET (*C-FRET*)**
- **Spectral Imaging FRET (psFRET)**
- **Multiphoton excitation FRET (2p-FRET)**
- **Acceptor Photobleaching FRET (apFRET)**
- **Lifetime Imaging FRET (FLIM-FRET)**
- **Photo-quenching FRET (PQ-FRET)**
- **Label-Free FRET Microscopy (NADH-TRP)**

Spectral FRET Microscopy

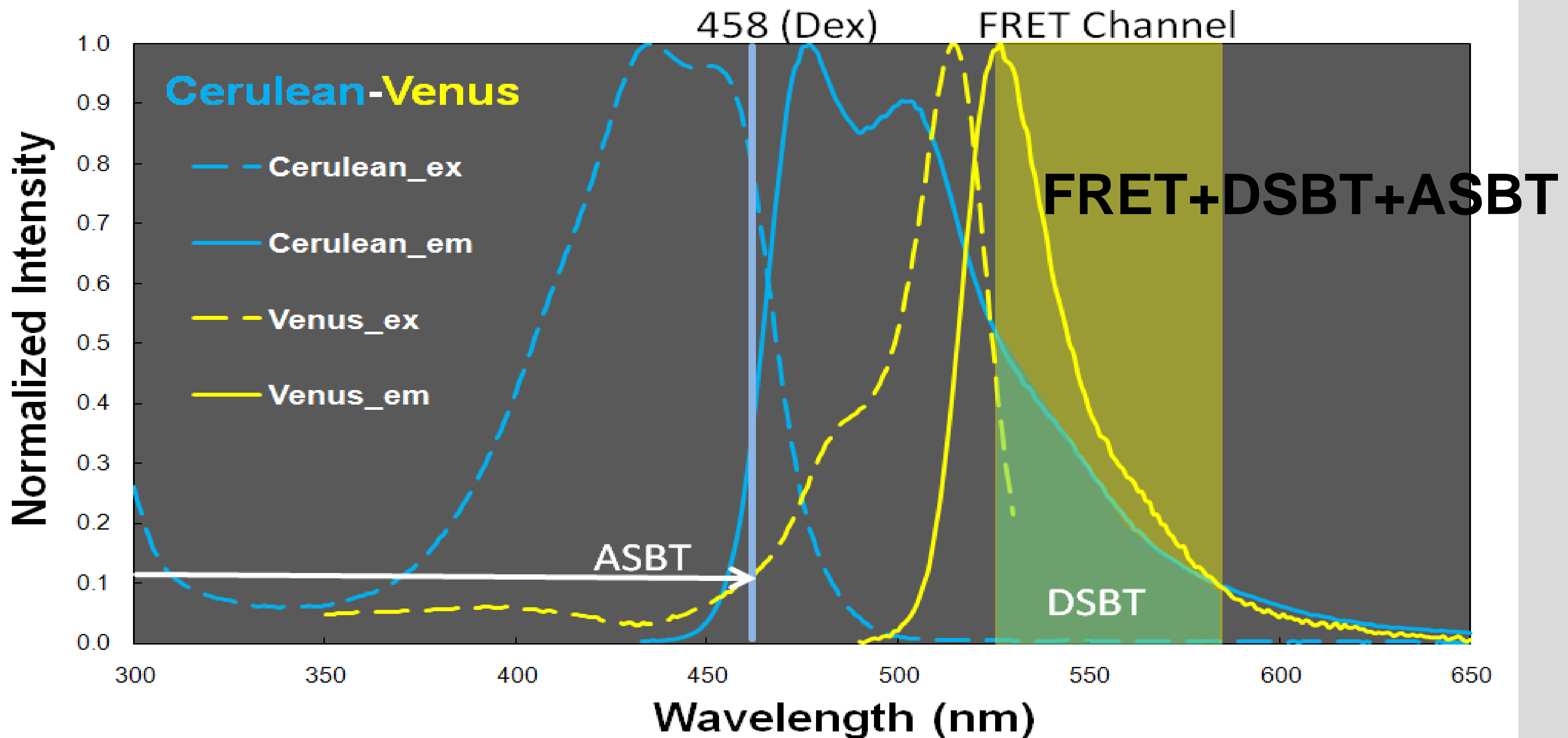
Interactions of eGFP-Rac1 and IQGAP1-mOrange in MDCK cells



Zeiss 510META, 63x Oil, Ex 488 & 561 nm

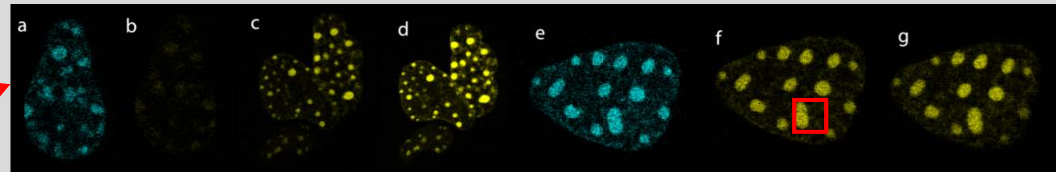
2-color FRET

Major Contaminations in the FRET Channel



- **Donor Spectral Bleedthrough (DSBT) – donor molecules excited by donor excitation wavelength yield its fluorescence into the FRET channel. What we call it as cross-talk.**
- **Acceptor Spectral Bleedthrough (ASBT) – acceptor molecules excited by donor excitation wavelength yield its fluorescence detected in the FRET channel.**

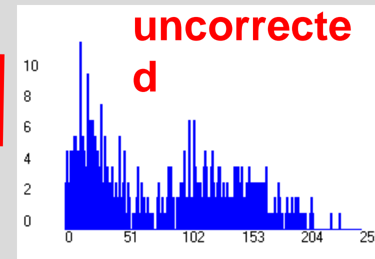
Data Analysis—PFRET Plugin

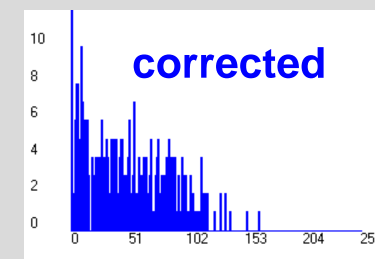


$$E = 1 - (I_{DA} / I_D)$$

$$E_{PFRET} = 1 - I_{DA} / [I_{DA} + PFRET^*((\Psi_{dd} / \Psi_{aa})^* (Q_d/Q_a))$$

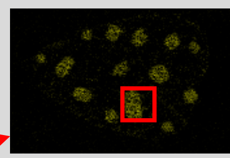
ugh Images
pecimen
bg_DA8-458-d-[e].tif
f: Dex_Aem bg_DA8-514-a-[g].tif
g_DA8-458-a-[f].tif
ted BSBT
en
0
Remove Calibrated ASBT
Acceptor Only Specimen
c: Dex_Aem c
d: Aex_Aem d
Threshold 1
Range 10
OK Cancel

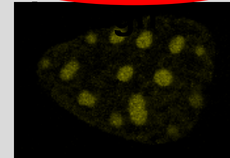




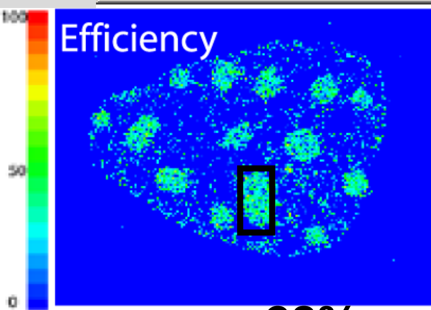
Bleed-

PFRET

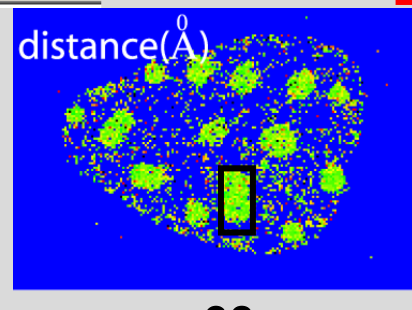




No correction



28%



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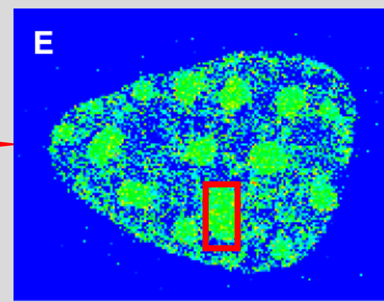
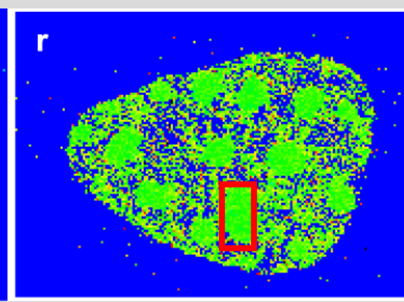


49%



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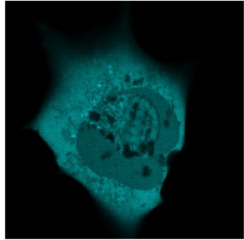
Contamination or bleedthrough correction

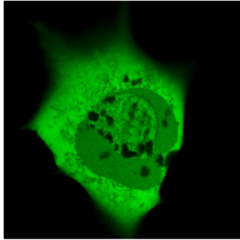
$$E = [1 - (I_{DA}/I_D)]$$

Ratio Imaging

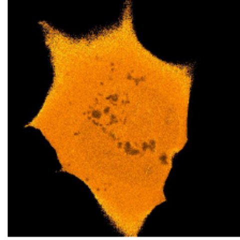
Quenched Donor

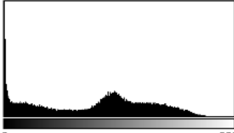


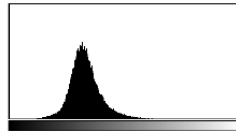
Uncorrected FRET



FRET

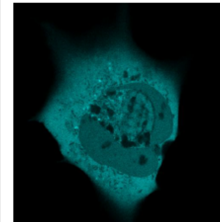




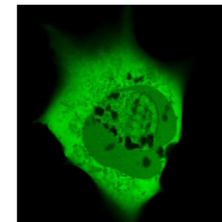


PFRET Analysis

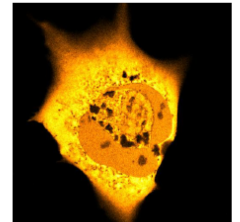
Quenched Donor

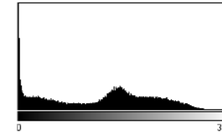


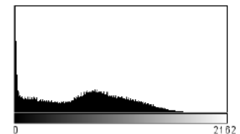
Uncorrected FRET



PFRET



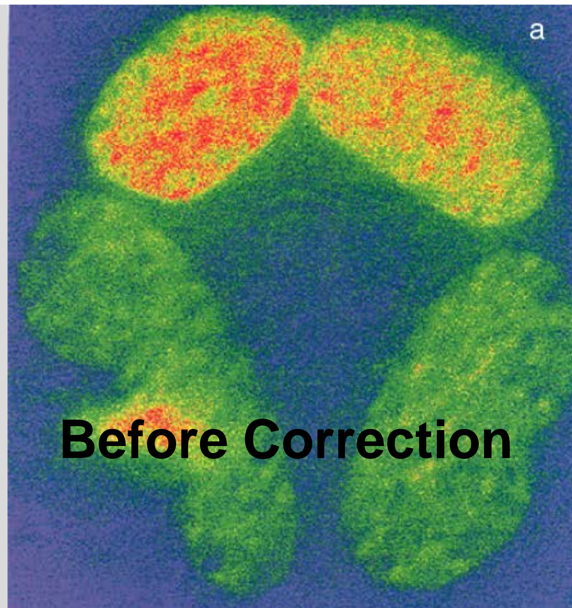


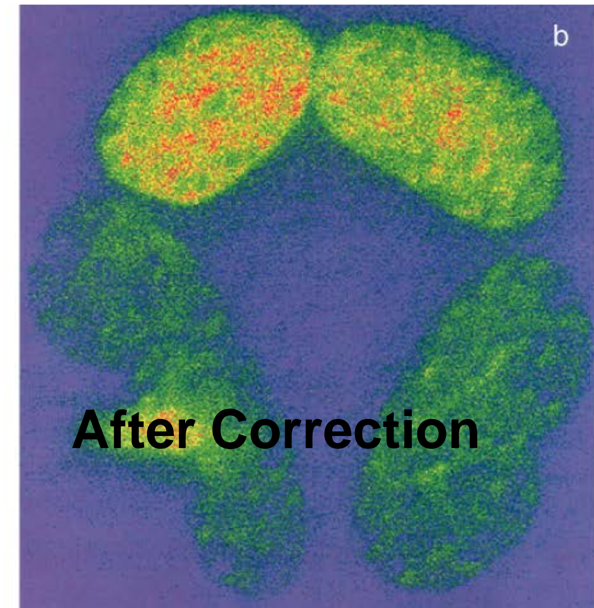


E% = 54.810%

$$E_n = 1 - \{I_{DA} / [I_{DA} + PFRET * (\psi_{dd} / \psi_{aa})* (Q_d / Q_a)] \}$$

BFP-GFP-Pit-1 protein dimerization





Confocal FRET microscopy using the PFRET method

Dr. Yuansheng Sun

Donor (D) Channel
Acceptor (A) Channel
FRET Channel
pFRET
'A / D' Ratio
E%
10 µm
5 4 3 2 1 0
40 30 20 10 0

Representative selected ROIs

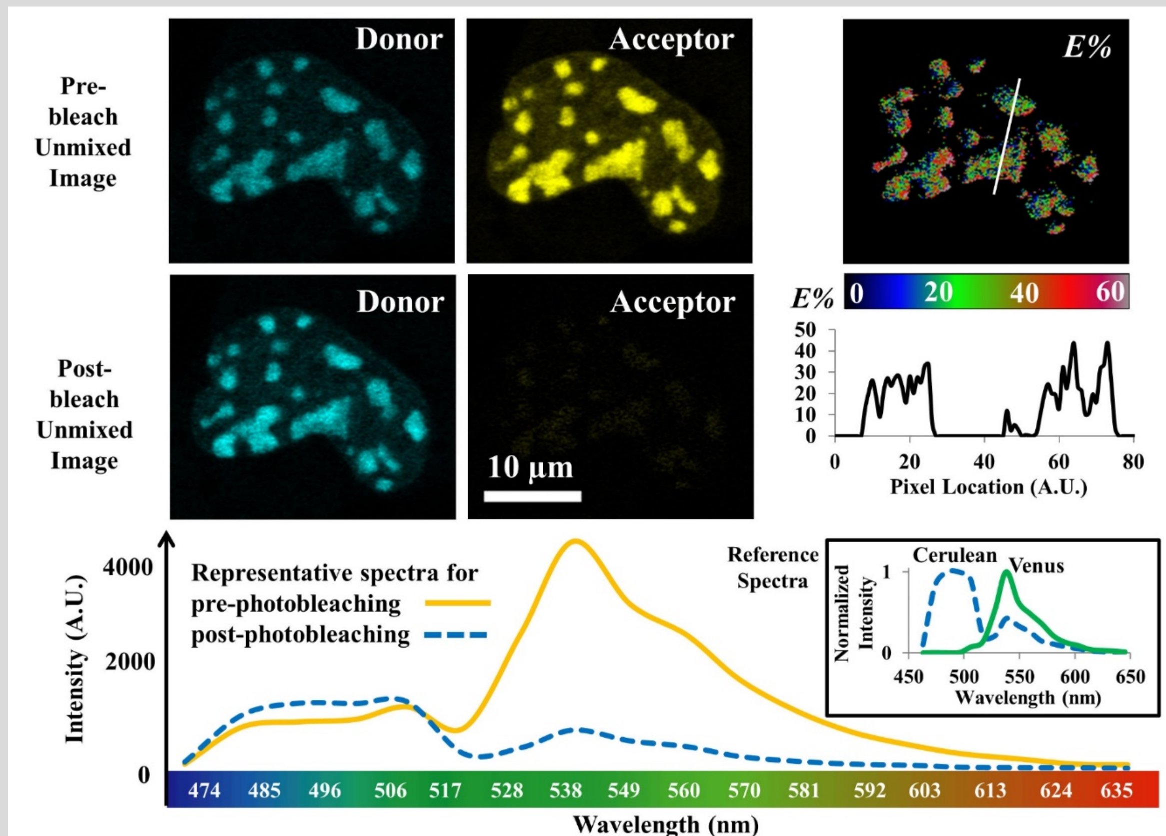
E%
30 25 20 15 10 5
0 1 2 3 4 5
Linear Correlation R: 0.8
'Acceptor -to- unquenched Donor' Ratio

Images of live cells co-expressing Cerulean- (donor) and Venus- (acceptor) tagged bZip were acquired in the Donor, FRET and Acceptor imaging channels. The graph shows an increasing trend of E% with an increased 'acceptor -to- unquenched donor' ratio, by either a 2nd order polynomial (solid) or linear (dashed) curve fitting.

(Zeiss 510 Meta; 63X / 1.4NA oil immersion; Donor Ex458 nm, Em470-500 nm; FRET : Dex 458 nm Em535-590 nm; Acceptor Ex 514nm)

Sun et al Cytometry A 83A:780-793, 2013

Acceptor photobleaching spectral FRET microscopy

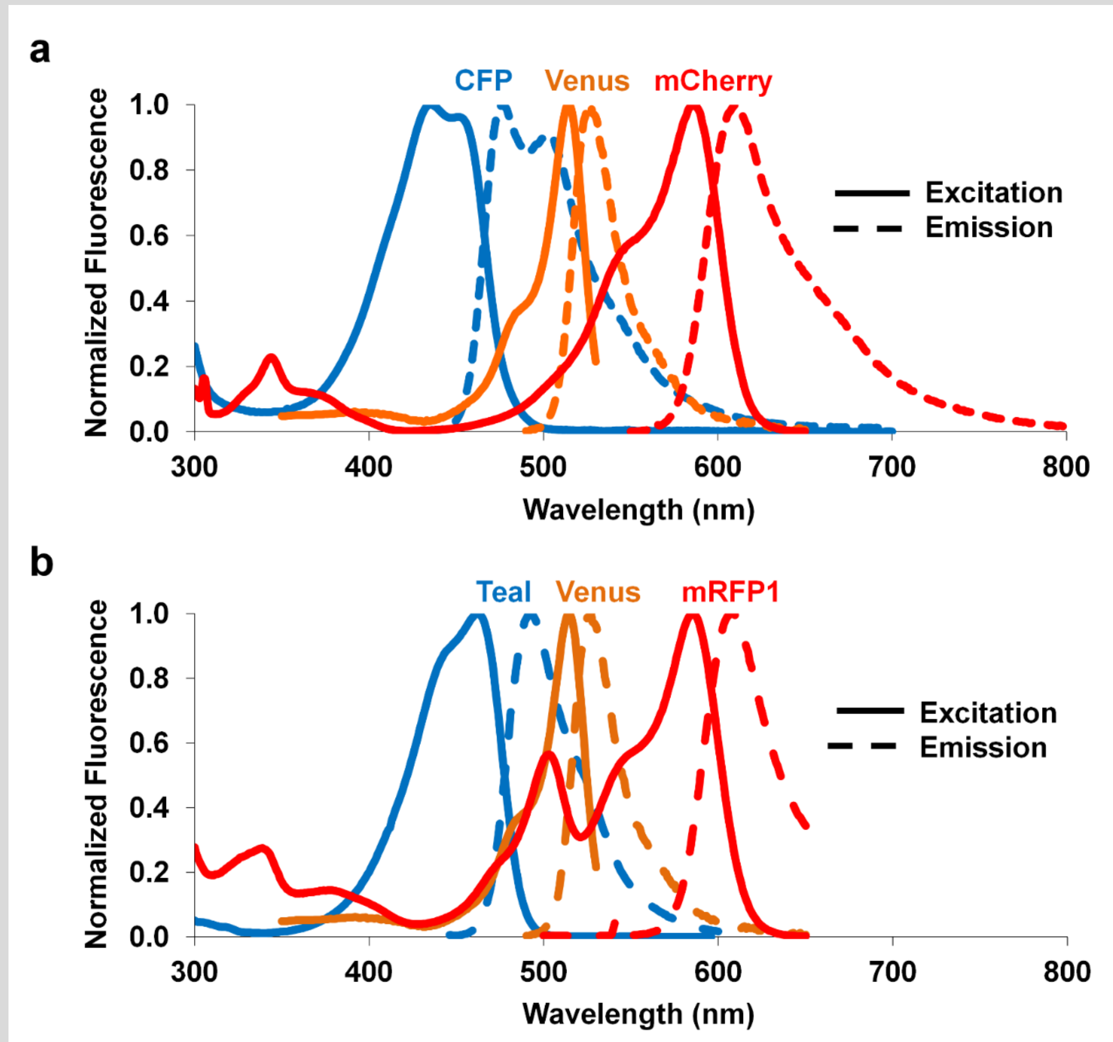


Live cells co-expressing Cerulean (donor) and Venus (acceptor) tagged bZip were excited by a 458 nm laser line, and spectral images (λ-stack for 470 ~ 640 nm) were acquired at the same imaging conditions, before and after photobleaching the acceptor with the 514 nm laser line (bleaching time: ~120 seconds).

Sun et al Cytometry A 83A:780-793, 2013

3-color FRET Microscopy

3-color FRET Microscopy

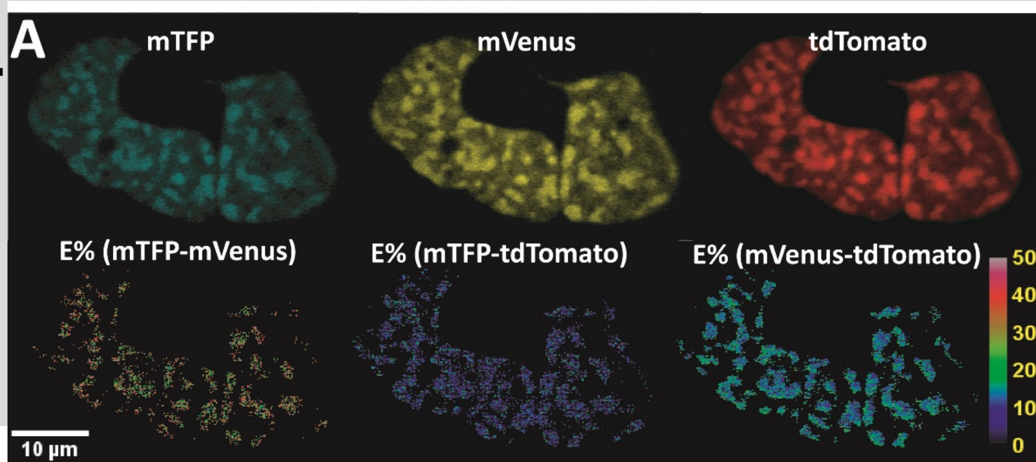


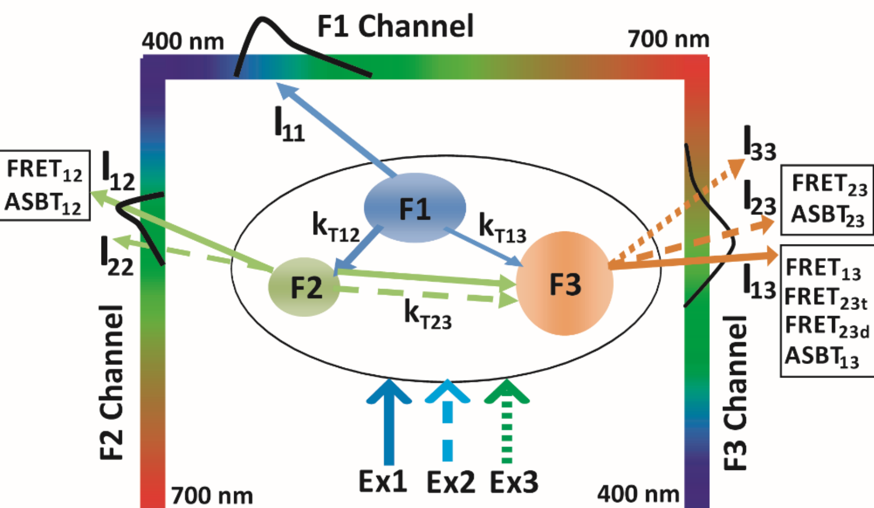
Wallrabe et al., Cytoskeleton 70: 819-836, (2013)

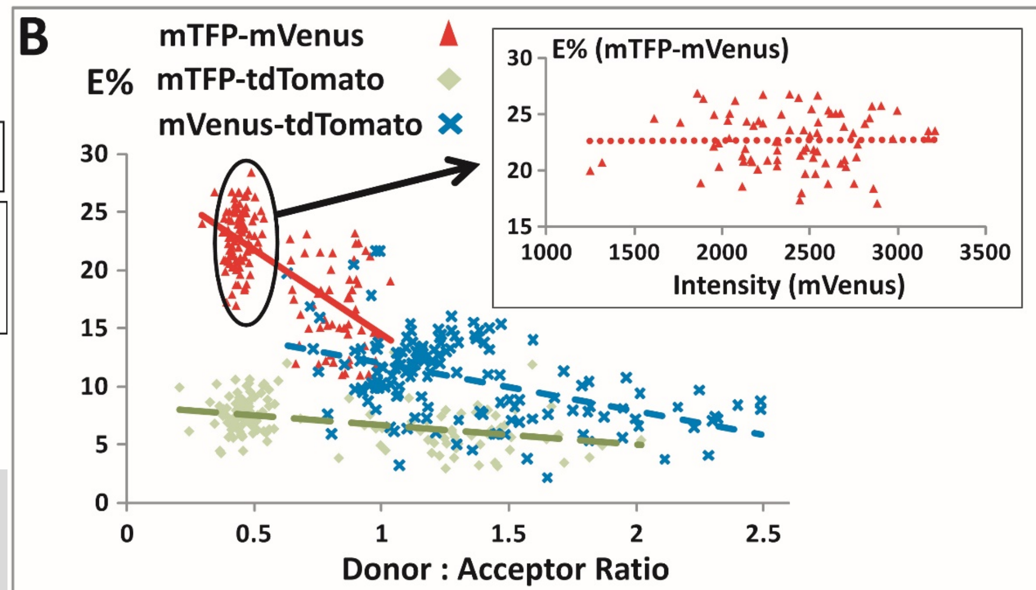
Dr. Yuansheng Sun

Biophysical Journal **99, 1274-1283 (2010)**

3-Color FRET







Demonstration of the homo-dimerization of C/EBPα and its interaction with H1Pα in live mouse pituitary GHFT1 cells by 3-color spectral FRET (3sFRET) Microscopy.

(Zeiss 510 Meta, 63 X / 1.4 NA Oil). Biophysical J. Vol. 99, 1274-1283, (2010).

Horst Wallrabe

Representative images of cells co-expressing Teal-N-WASP, Venus-IQGAP1 and mRFP1-Cdc42, and exhibiting 3-color FRET. Single fluorescence and composite images are shown, along with ROI's and E%'s for the indicated protein pairs that exhibited FRET within each ROI.

Teal-N-WASP | Venus-IQGAP1 | mRFP1-Cdc42

10 µm

Composite | Cell-Cell ROIs | Peripheral ROIs

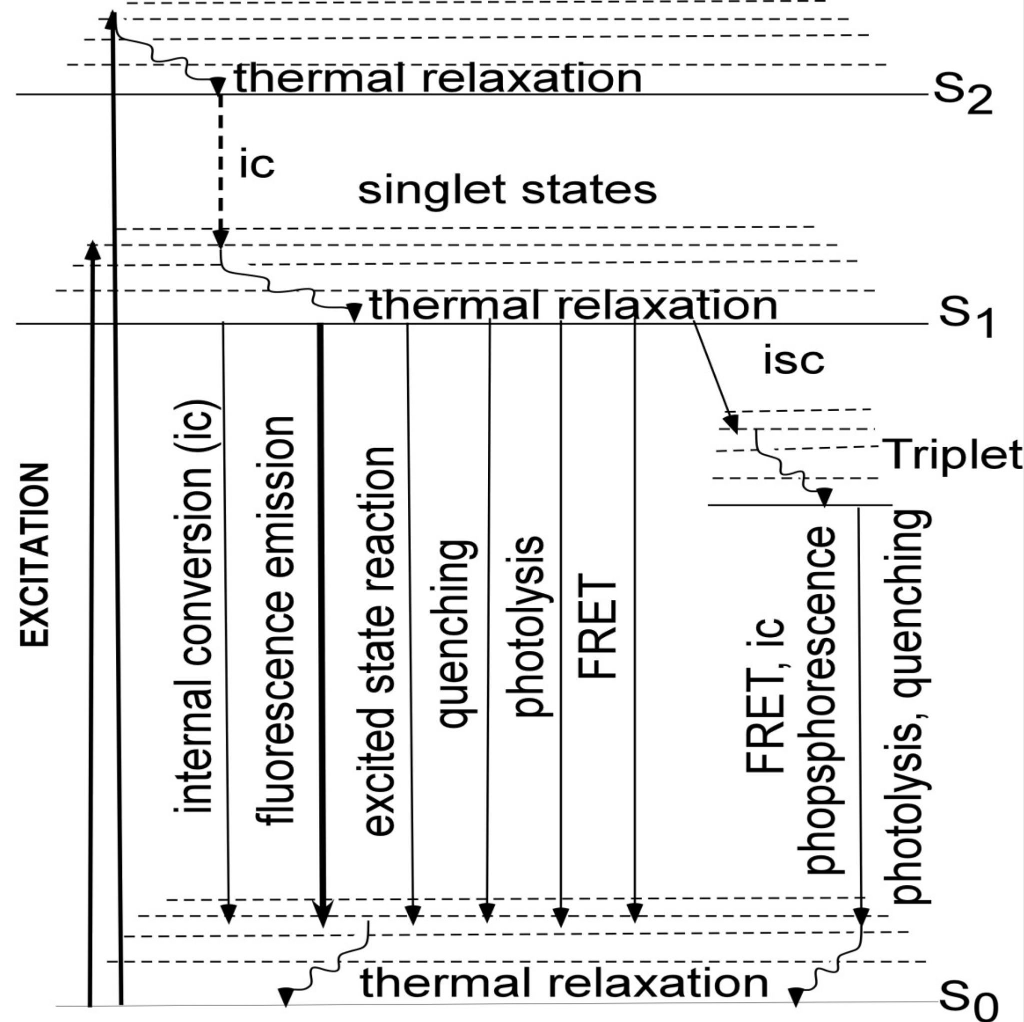
N-WASP-IQGAP1 E% | N-WASP-Cdc42 E% | IQGAP1-Cdc42 E%

E% 0 20 40 60 80

Leica SP5X, 63x 1.2 W;

Cytoskeleton 70: 819-836, (2013)

FLIM Microscopy



Perrin-Jablonski Energy Level Diagram of a fluorescent molecule

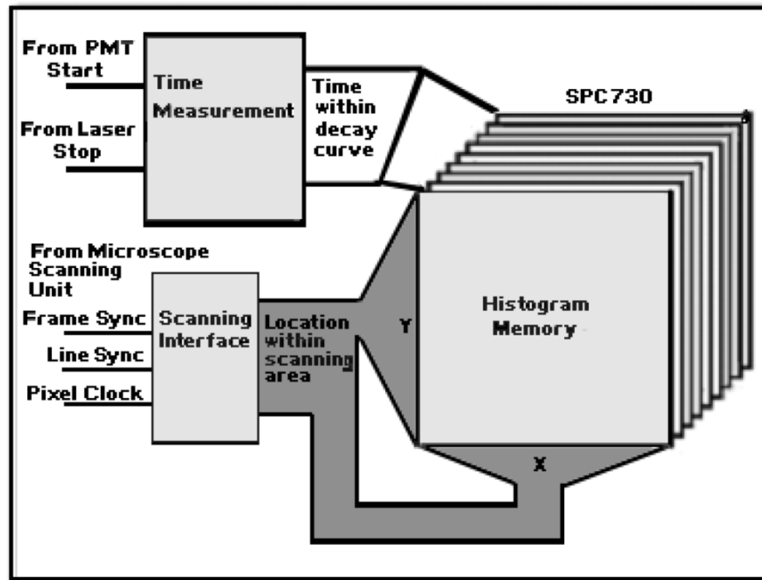
WHAT'S FLUORESCENCE LIFETIME?

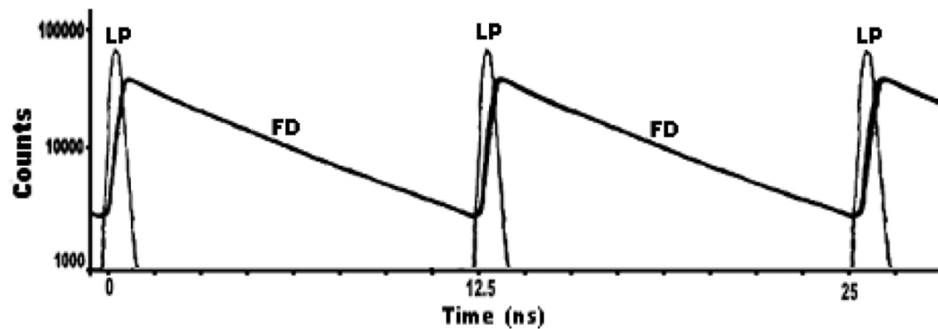
The Fluorescence Lifetime is the average time that a molecule remains in the excited state prior to return to the ground state.

Lifetime generally falls in the range of 1 to 100 ns.

***Independent of change in excitation light intensity, probe concentrations, and light scattering, but highly dependent on the local environment of the fluorophore.**

Two-photon FLIM Microscopy

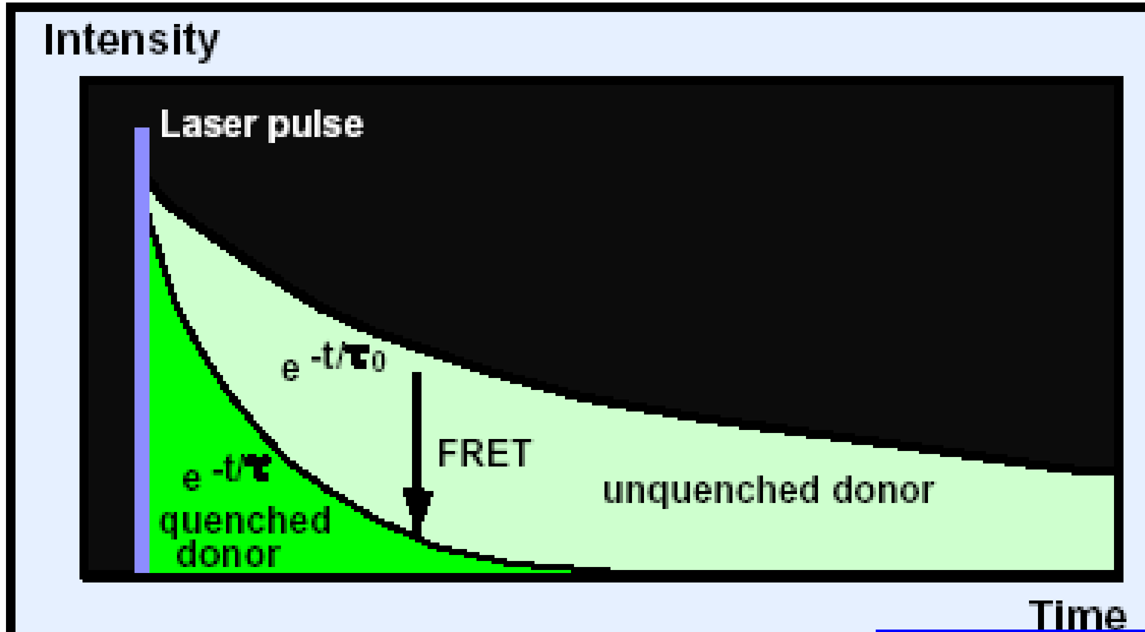




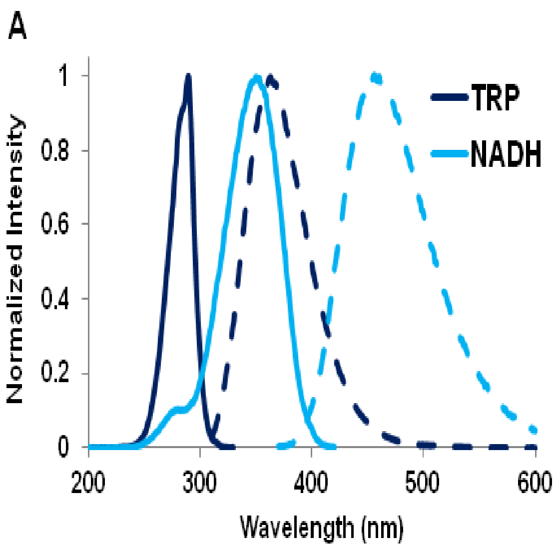
Synchronization of various signals

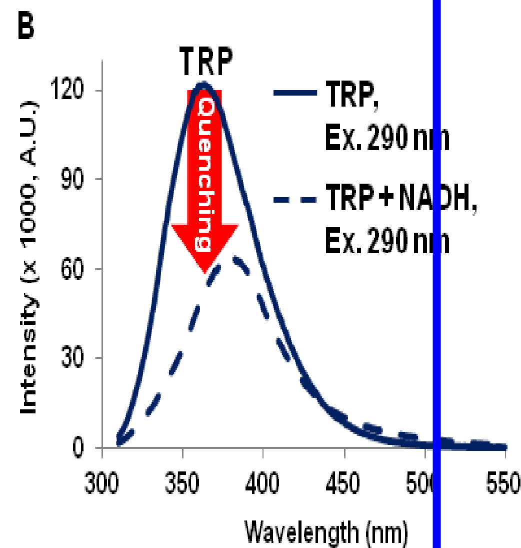
Chen and Periasamy, Molecular Imaging:FRET Microscopy and Spectroscopy, Chapter 13, Oxford University Press, 2005.

FLIM-FRET Microscopy



Donor – Tryptophan
Acceptor – NAD(P)H





$$E = R_0^6 / (R_0^6 + r^6) \quad [1]$$

$$E = 1 - (\tau_{DA} / \tau_D) \quad [2]$$

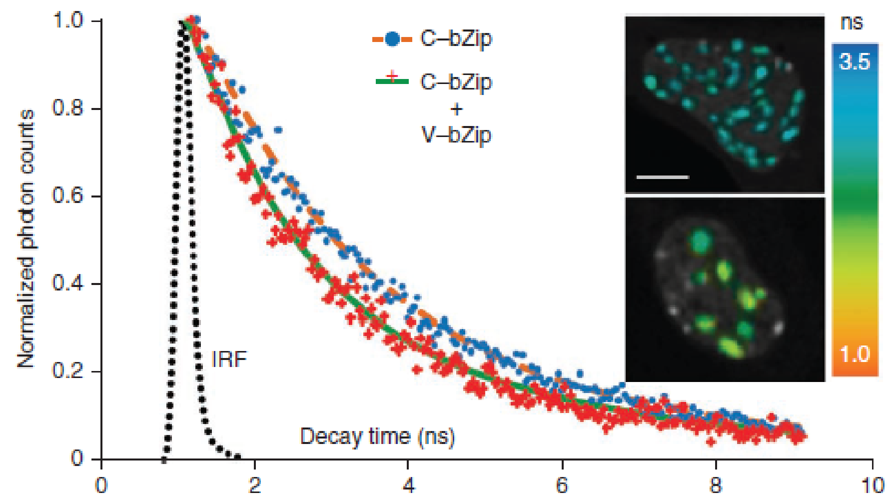
$$k_T = (1/\tau_D)(R_0/r)^6 \quad [3]$$

$$r = R_0\{(1/E) - 1\}^{1/6} \quad [4]$$

$$R_0 = 0.211\{\kappa^2 n^{-4} Q_D J(\lambda)\}^{1/6} \quad [5]$$

Dr. Yuansheng Sun

...erized C/EBPα-bZip in living cell nucleus using ...EBPα-bZip was tagged with either Cerulean (C) ...fetime decay kinetics for the C–bZip ...d the presence of V–bZip (FRET acceptor) ...measured decay data into a single or double- ...ectively, with the measured instrument ...f a threshold allowed fitting to the pixels in ...hromatin of the cell nucleus. The comparison between the representative measured decay data points, the fitting curves and the lifetime (overlaid with intensity) images of the two cases clearly shows that the C in cells expressing both C–bZip and V–bZip decayed faster (or has a shorter lifetime) than that in cells expressing C–bZip alone, indicating that the C attached to bZip was quenched by the V attached to bZip because of FRET. Scale bar, 10 μm.



Investigating protein-protein interactions in living cells using fluorescence lifetime imaging microscopy

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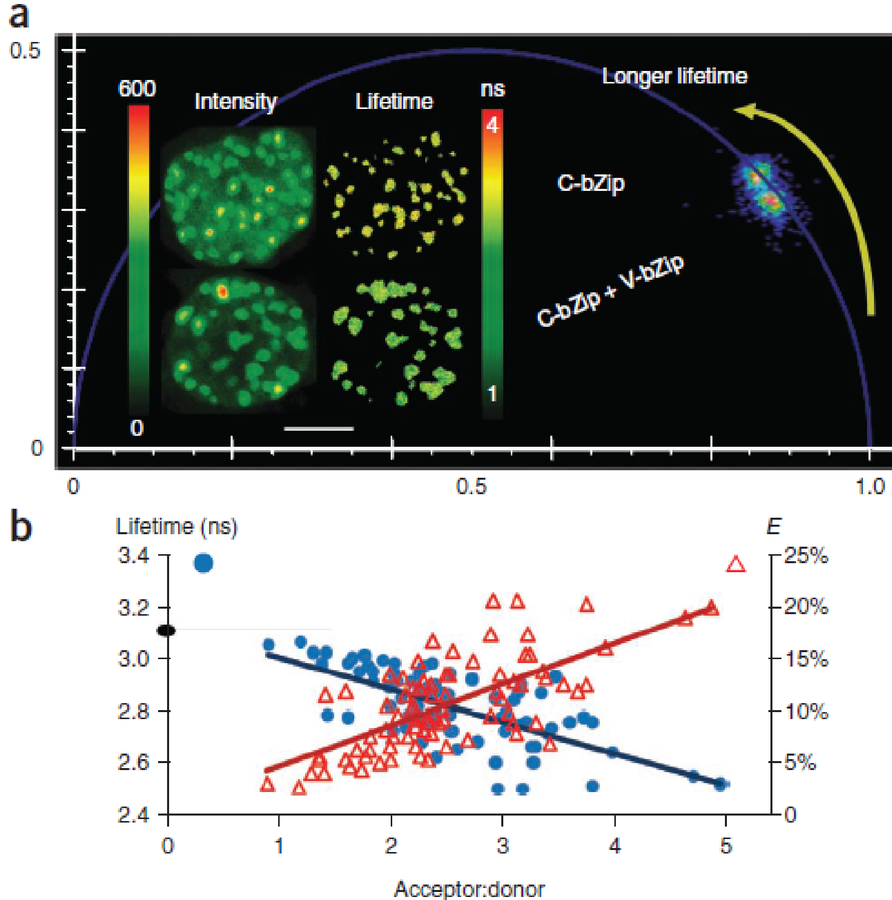
Nature Protocols 6: 1324-1340, 2011

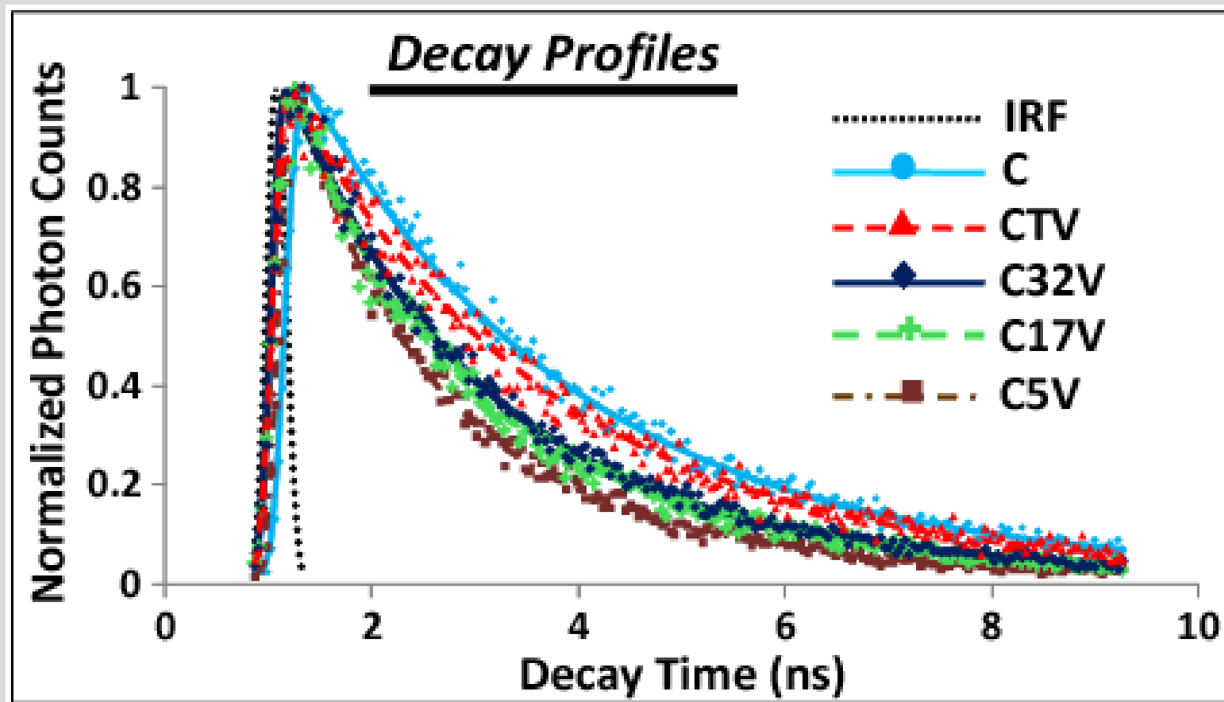
Fluorescence lifetime imaging microscopy (FLIM) is now routinely used for dynamic measurements of signaling events inside living cells, including detection of protein-protein interactions. An understanding of the basic physics of fluorescence lifetime measurements is required to use this technique. In this protocol, we describe both the time-correlated single photon counting and the frequency-domain methods for FLIM data acquisition and analysis. We describe calibration of both FLIM systems, and demonstrate how they are used to measure the quenched donor fluorescence lifetime that results from Förster resonance energy transfer (FRET). We then show how the FLIM-FRET methods are used to detect the dimerization of the transcription factor CCAAT/enhancer binding protein-α in live mouse pituitary cell nuclei. Notably, the factors required for accurate determination and reproducibility of lifetime measurements are described. With either method, the entire protocol including specimen preparation, imaging and data analysis takes ~2 d.

Figure 7 | Investigation of the dimerization of C/EBPα-bZip in living cell nucleus using FD FLIM-FRET microscopy. bZip was tagged with either Cerulean (C) or Venus (V). (**a**) The intensity and lifetime images of representative cells, which only express C–bZip (donor-alone control) and that co-express C–bZip (FRET donor) and V–bZip (FRET acceptor), are compared. The FD FLIM data acquired at the fundamental modulation frequency (20 MHz) is displayed on the phasor plot. The comparison demonstrates a shorter lifetime of Cerulean in the cell that expresses both C–bZip and V–bZip. The lifetimes of C–bZip in the absence and the presence of V–bZip were estimated by single- and double-exponential fittings, respectively. (**b**) The average donor lifetime, obtained from ten cells that expressed only C–bZip, was 3.15 ns (indicated by the black dot). The apparent lifetimes for 80 regions of interest (ROIs) identified in ten cells coexpressing C–bZip and V–bZip were then determined, and the range was from 2.5 to 3.05 ns, resulting in a variety of energy transfer efficiencies (E) calculated on the basis of equation (3). To investigate how the quenched C–bZip lifetimes were influenced by the acceptor-to-donor ratio, we roughly determined the ratio using the intensities obtained in the acceptor and donor channels for each ROI. With all 80 ROIs, the lifetime (blue dots with a dark blue trend line: R = 0.35) or E (red triangles with a dark red trend line: R = 0.19) shows a negative or positive dependency on the acceptor-to-donor ratio, respectively (scale bar, 10 μm).

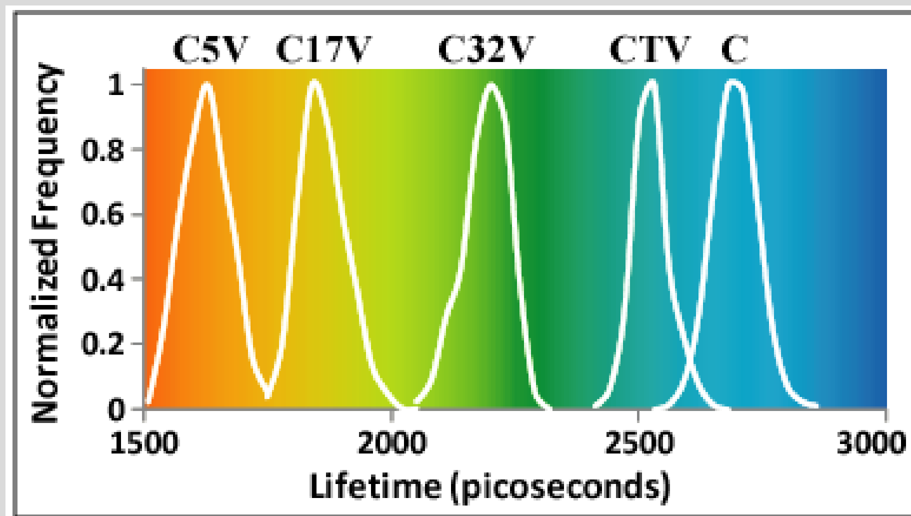


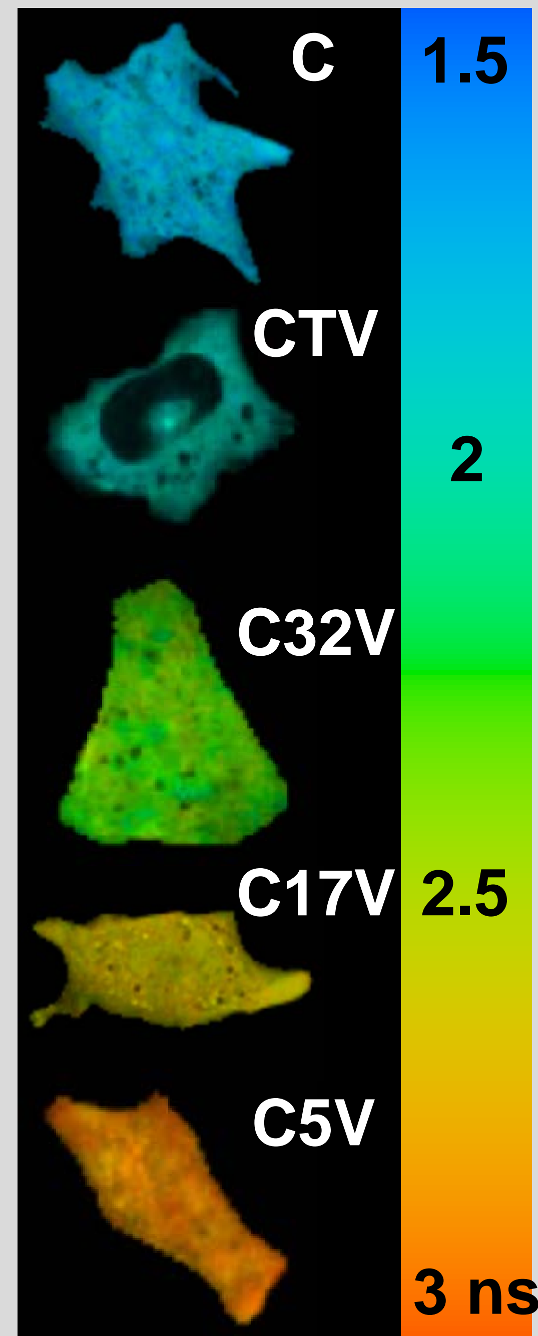
Sun et al Nature Protocols 6: 1324-1340, 2011

TCSPC FLIM-FRET Microscopy



FRET Standards Donor & Acceptor linked with amino acids (aa)



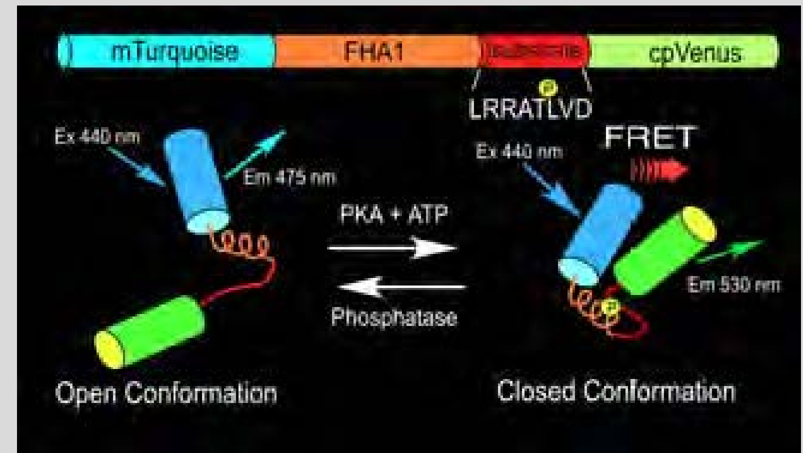


Biosensor Probes

The genetically encoded biosensor probes enable noninvasive detection of spatial and temporal characteristics of *specific* cell signaling or metabolic events.

Biosensors typically contain reporter modules consisting of donor and acceptor FPs directly linked by a sensing unit that detects a specific cellular event.

FRET based Biosensors



- **If the biosensor conformation puts the acceptor (A) fluorophore close (<80A) to the donor, energy can be transferred directly by Förster resonance energy transfer (FRET).**

Applications –
Ratio Biosnsor
Calcium biosensors
Intravital imaging

Which fluorophore to choose? Not a trivial question!

Fluorophores broadly fall into 3 categories

- Organic dyes, such as FITC, Rhodamine, Alexa and Cy varieties (secondary antibody-labeling, ligand-dye conjugates, etc)
- GFP-type fluorophores, (Visible fluorescent proteins) such as Cerulean, Teal, Venus, RFP, mCherry, mOrange, etc
- Semiconductor nanocrystals, widely called Quantum Dots (QDs)

…….each with their advantages and disadvantages

Important fluorophore properties

- **Size of fluorophore;** Organic dyes are small, GFP mutants are ~28-30 kd, QDs 1- >6 nm
- **Quantum Yield (QY)**, particularly the donor in FRET (QY changes from the published value upon conjugation or fusion)
- **Molar extinction coefficient**, the higher-the better
- **Relative brightness**, linked to above, particularly relevant for GFP-type fluorophores
- **pH stability**, particularly relevant for almost all FRET pairs Alexas shows excellent pH stability
- **Photo-stability**; how quickly do they bleach?

R_0 –value for FRET-partners

FRET applications

If you conduct fluorescence microscopy … you can do FRET - with some extra steps.

Qualitative or Quantitative FRET Microscopy

Qualitative FRET microscopy

- **Do cellular components interact – yes/no?**
- **Is there a conformational change – yes/no?**
- **Have two 'fretting' components separated –Y/N?**

Quantitative FRET microscopy

- **Are we observing specific or random interactions**
- **Relative distances between cellular components, i.e. is 'A' closer to 'B' or 'C'**
- **By quantifying 'Regions of Interest' (ROIs), we investigate the distribution, effects etc of D & A**

Protein	Color	Peak Ex	Peak Em	Brightness	Photo-Stability	Reference	Source
EBFP2	Blue	383	448	18	++	Ai et al. 2007	Dr. Robert Campbell
Cerulean3	Cyan	433-445	475-503	24	+++ *	Markwardt et al. 2011	Dr. Mark Rizzo
mTurquoise2	Cyan	433-445	475-503	28	+++ *	Goedhart et al. 2012	Dr. Theo Gadella
mTFP	Teal	462	492	54	+++ *	Ai et al. 2006	Allele Biotech
EmGFP	Green	487	509	39	++++	Cubitt et al. 1999	Invitrogen
Clover	Green	505	515	84	++	Lam et al. 2012	Addgene
mNeonGrn	Green	506	517	92	++++	Shaner et al. 2013	Dr. Nathan Shaner
Venus Citrine	Yellow/Grn	515 516	528 529	53 58	+ +++	Nagai et al. 2002 Griesbeck et al. 2001	Dr. Atsushi Miyawaki Dr. Roger Tsien
Amber†	None			0		Koushik et al. 2006	Addgene
mKO2 (Kusabira)	Orange	551	565	39	+++	Karasawa et al. 2004; Sakaue-Sawano 2008	MBL International
mTagRFP-T	Orange	555	584	33	++++	Merzlyak et al. 2007; Shaner et al. 2008	Evrogen
tdTomato	Orange	554	581	95	+++	Shaner et al. 2004	Dr. Roger Tsien
mRuby2	Red	559	600	43	+++	Lam et al. 2012	Addgene
mCherry	Red	587	610	17	+++	Shaner et al. 2004	Clontech
mKate2 (Katushka)	Deep Red	588	633	25	++++	Shcherbo et al. 2009	Evrogen

* Depends on illumination source;

† Y66C mutant folds, but does not absorb or emit - important control for FRET-FLIM.

Summary

* Three major conditions for FRET to occur –spectral overlap (>30% D to A), distance between molecules (1-10nm), dipole moment orientation (1-4).

* For 2-color FRET requires 2 fluorophores for 3-color FRET 3 fluorophores are required

* Spectral bleedthrough correction is required for quantitative FRET data analysis

* Intensity based FRET can be implemented in any fluorescence microscopy system by selecting appropriate filters for the selected FRET pair.

* In FLIM-FRET method, one should follow the change in lifetime of the donor in the presence (D+A) and absence of acceptor (Donor alone).

* FLIM data analysis varies depending on the biological interest.

* Preparation of the FRET pair or labeling the proteins is important.

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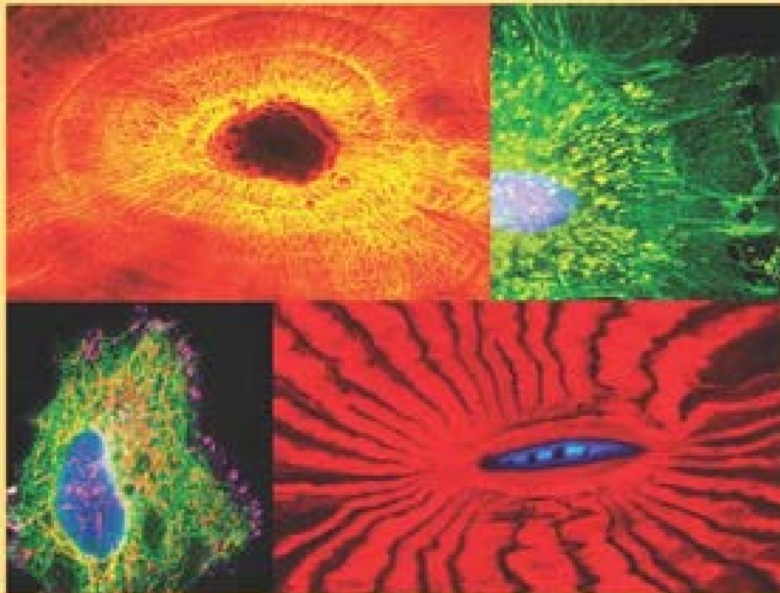
The Fluorescent Protein Revolution



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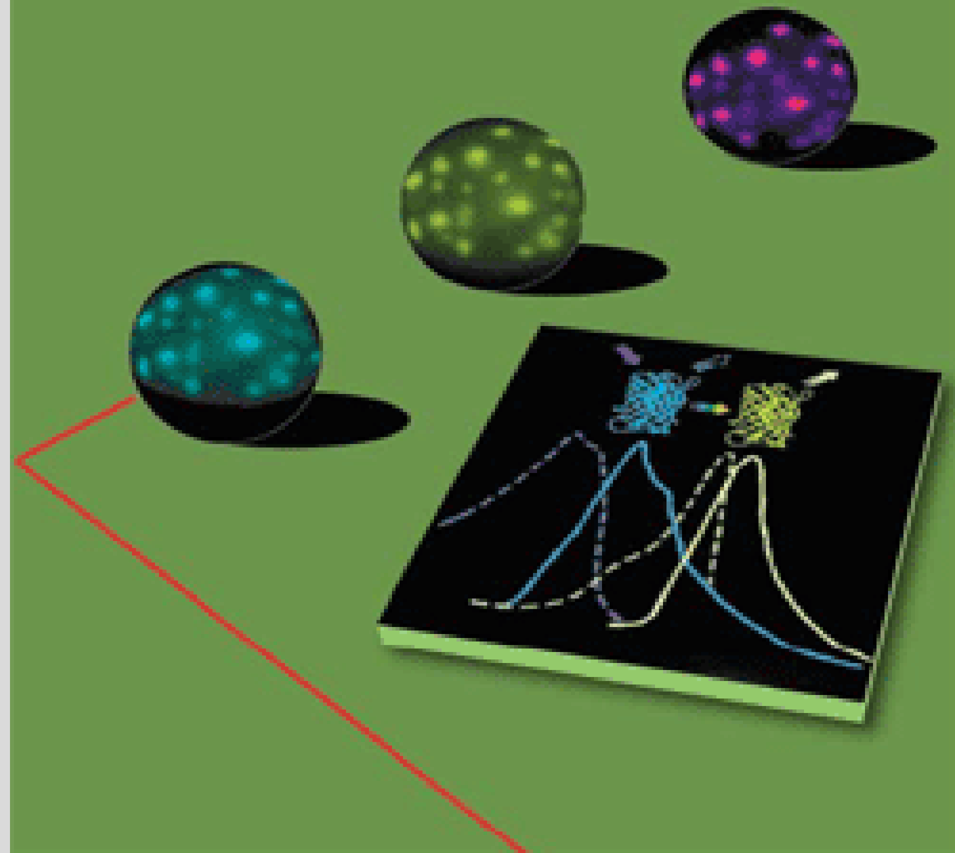
FRET Microscopy and Spectroscopy



Edited by
Ammasi Periasamy • Richard N. Day

Oxford University Press, 2005

Society of Photo-Optical Instrumentation Engineers (SPIE)
Photonics West 2015, San Francisco, CA, USA

January 28 - February 2, 2017

Visit SPIE web site http://www.spie.org/ for BiOS 2016

Multiphoton Microscopy in the Biomedical Sciences XVI

Abstract due date July 18, 2016

Conference Chairs: Ammasi Periasamy, University of Virginia
Karsten König, Saarland University, Germany
Peter So, MIT

JenLab Young Investigator Award

- **Graduate students or postdocs or scientists who are not more than 32 years old . Award Prize: US $2000 cash award**

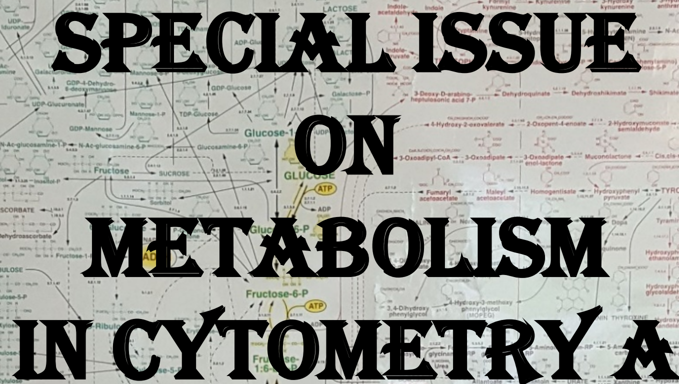
Students Poster Session Competition (SPSC-MP):
Graduate students and Postdocs are welcome to participate. US $500 award each, maximum 4 poster awards.

Topics Includes- Multiphoton Technology Development, SHG, THG, CARS, SRS, FCS, NADH, FAD, FRET, & FLIM Microscopy techniques & applications.

SPIE-Photonics West Multiphoton Microscopy

JenLab Award
$2000

Poster Awards-$500 each



SPECIAL ISSUE ON METABOLISM IN CYTOMETRY A

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THANK YOU!!