Methods to Study Protein-Protein Interactions

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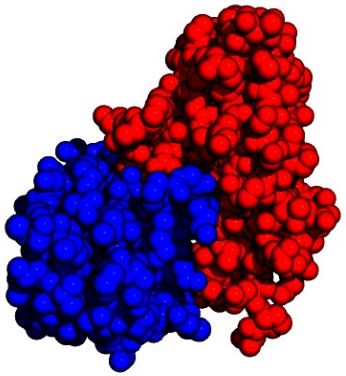
Protein-Protein Interactions: The "Interactome"

2 challenges:

- find which proteins interact (the partners)

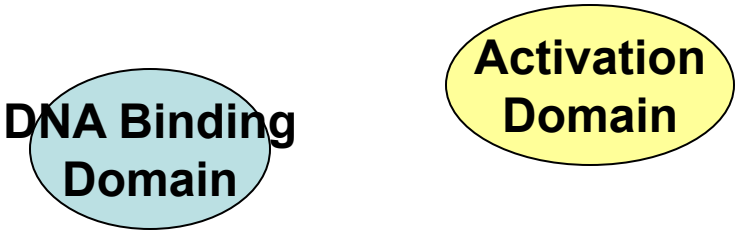


- find which residues participate in the interactions

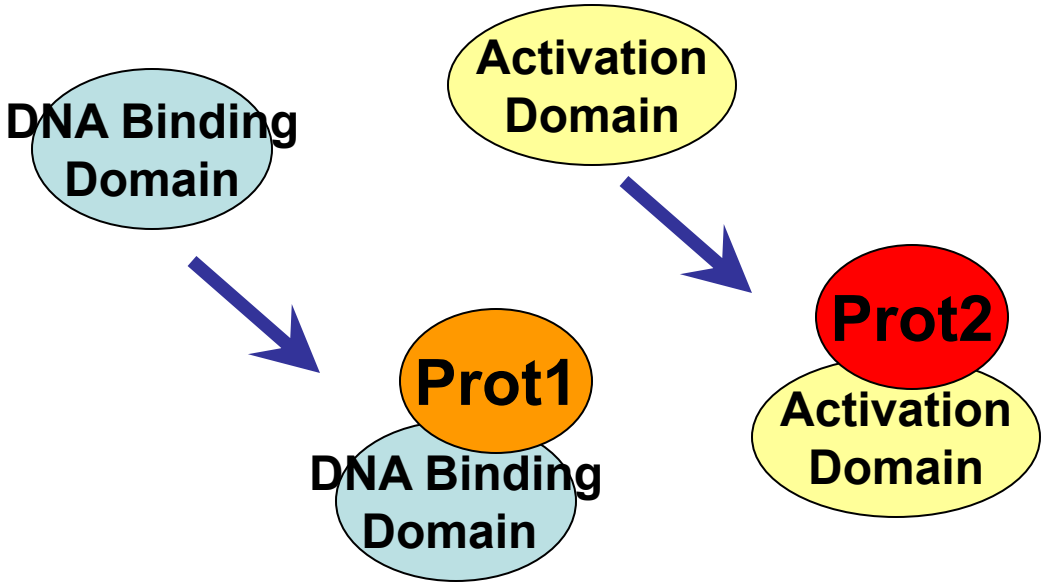
Studying protein-protein interactions

- 1. 2-hybrid system
- 2. Antibody-array
- 3. Pull-down
- 4. Immunoprecipitation
- 5. FRET

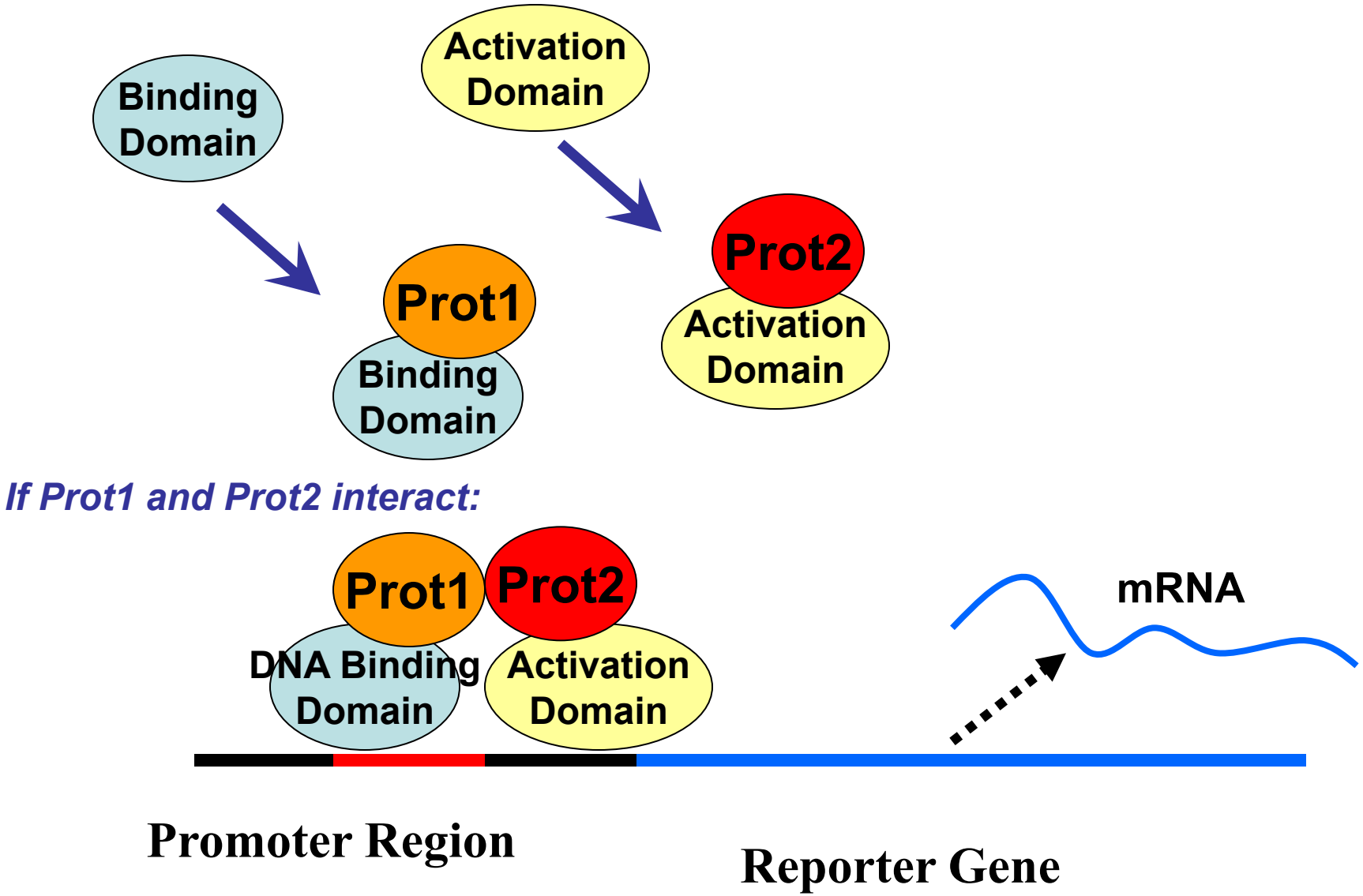
Yeast 2-hybrid system



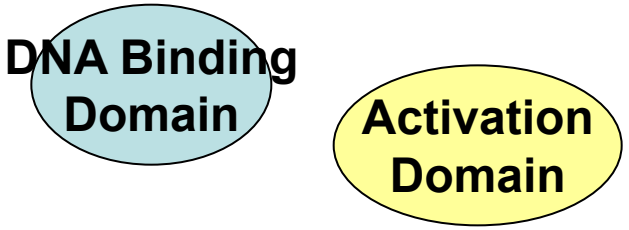
Yeast 2-hybrid system



Yeast 2-hybrid system



Yeast 2-hybrid system



- A transcription factor is split into 2 domains

- 2 hybrid proteins are designed, each containing one of the two proteins that are tested

- If the two proteins interact, the two domains from the transcription factor will interact, causing expression of a (detectable) reporter gene

- The reporter can be:
  - essential, in which case the yeast colony dies if the 2 proteins do not interact

  - reversely, the reporter gene can be attached to a green fluorescent protein

Unfortunately, the rate of false positive is high (estimated > 45%)

Mammalian 2-hybrid system

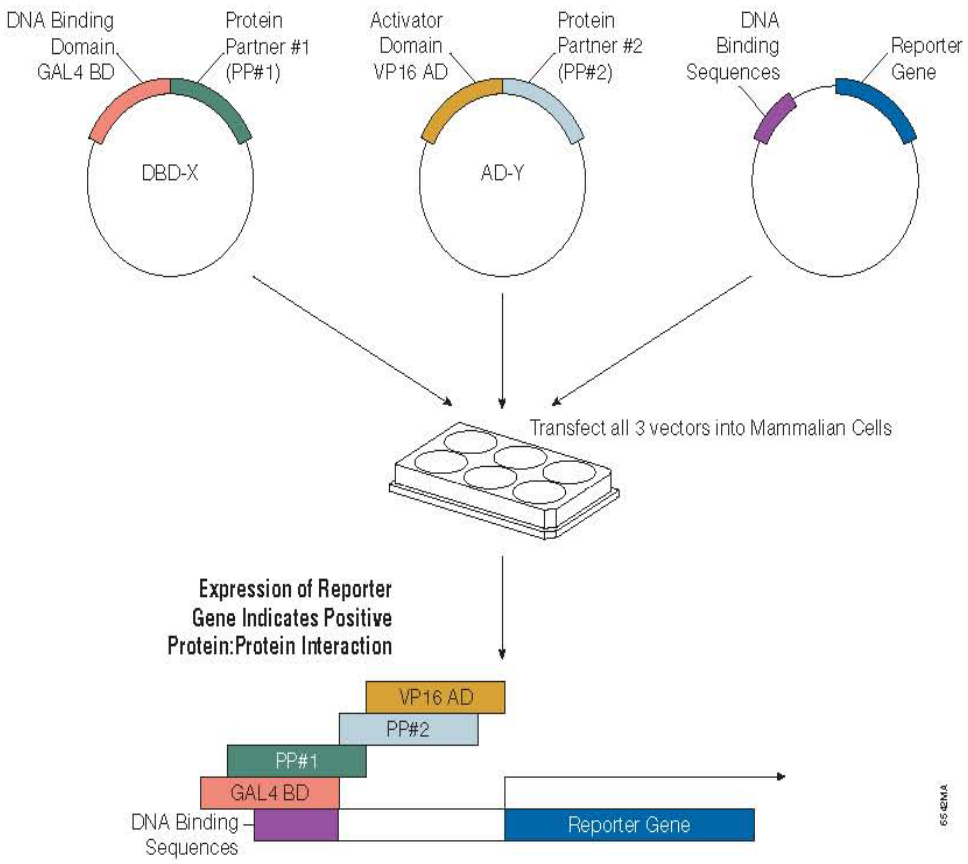


Figure 2. Principle of the mammalian two-hybrid system. The protein coding sequence for the bait protein is cloned into a vector that contains the DNA binding sequence (DBD-X (bait) fusion). The protein coding sequence for the prey protein is cloned into a vector that contains the sequences for transcriptional activation (AD-Y (prey) fusion). The vectors also must contain the necessary elements for growth and protein expression in mammalian cells. The recombinant vectors are then transfected along with a third plasmid containing the appropriate DNA binding sites upstream of a reporter gene. Only if proteins X and Y physically interact are the DBD and AD brought together to reconstitute a functionally active factor that binds to upstream sequences and activate expression of the reporter gene.

Mammalian two-hybrid system formats

The mammalian two-hybrid system allows characterization of mammalian protein:protein interactions within a cellular environment that mimics native conditions. Yeast and mammalian cells differ in patterns of post-translational modification, such as glycosylation, phosphorylation and acylation, as well as in the intracellular localization of proteins. These types of protein modifications, as well as other unique factors or modulators present in mammalian cells, may influence the ability of protein domains to interact.

Another advantage of the mammalian two-hybrid system is that the assay is less time-consuming than the yeast two-hybrid system. Instead of waiting 3–4 days for yeast colonies to grow to a reasonable size for a blue-color assay, typical reporter assays in the mammalian system can be performed within 48 hours of transfection.

The most common format of the mammalian two-hybrid system consists of one vector containing the DBD of the GAL4 protein, another vector containing the AD of the herpes simplex virus VP16, and a third vector containing 4-5 GAL4 binding sites upstream of a specific reporter gene.

The primary difference between the various systems is the reporter gene used for detection of positive interactions. The three most commonly used reporter genes are luciferase, β-galactosidase and secreted alkaline phosphatase (SEAP).

Antibody-Array Interaction Mapping

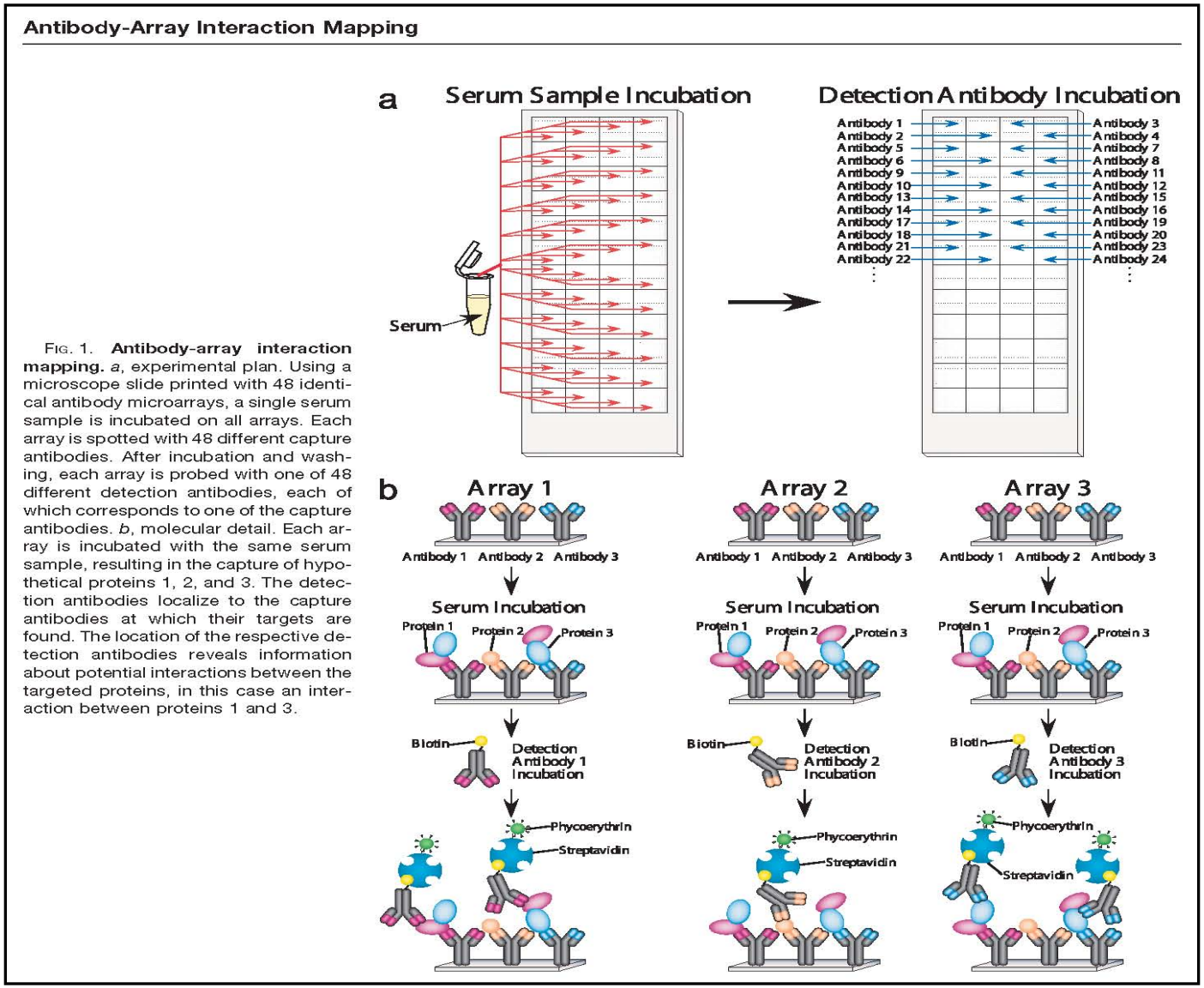


FIG. 1. **Antibody-array interaction mapping.** *a*, experimental plan. Using a microscope slide printed with 48 identical antibody microarrays, a single serum sample is incubated on all arrays. Each array is spotted with 48 different capture antibodies. After incubation and washing, each array is probed with one of 48 different detection antibodies, each of which corresponds to one of the capture antibodies. *b*, molecular detail. Each array is incubated with the same serum sample, resulting in the capture of hypothetical proteins 1, 2, and 3. The detection antibodies localize to the capture antibodies at which their targets are found. The location of the respective detection antibodies reveals information about potential interactions between the targeted proteins, in this case an interaction between proteins 1 and 3.

Pull-down assay *in vitro*

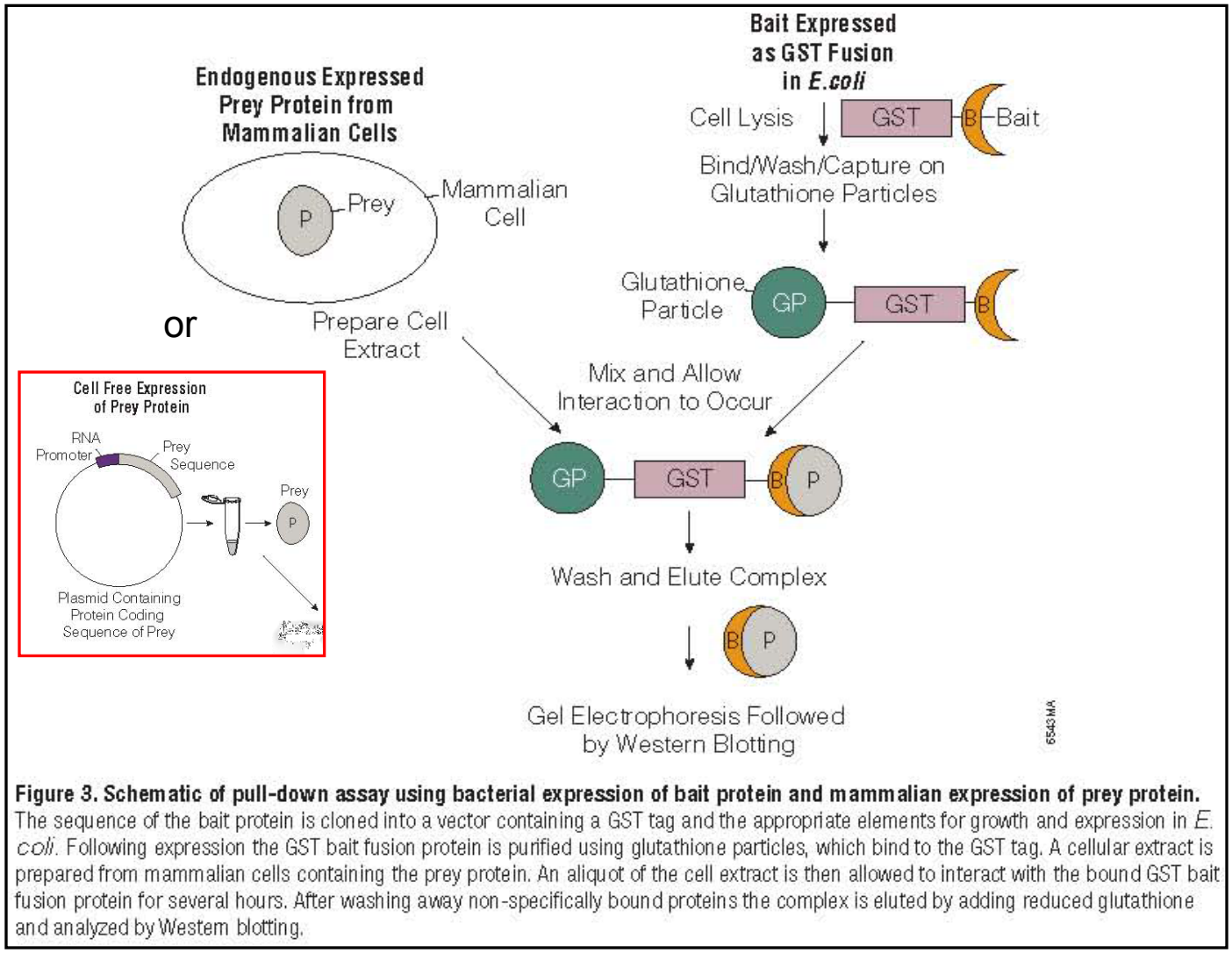


Figure 3. Schematic of pull-down assay using bacterial expression of bait protein and mammalian expression of prey protein. The sequence of the bait protein is cloned into a vector containing a GST tag and the appropriate elements for growth and expression in *E. coli*. Following expression the GST bait fusion protein is purified using glutathione particles, which bind to the GST tag. A cellular extract is prepared from mammalian cells containing the prey protein. An aliquot of the cell extract is then allowed to interact with the bound GST bait fusion protein for several hours. After washing away non-specifically bound proteins the complex is eluted by adding reduced glutathione and analyzed by Western blotting.

Immunoprecipitation of proteins expressed in the cell

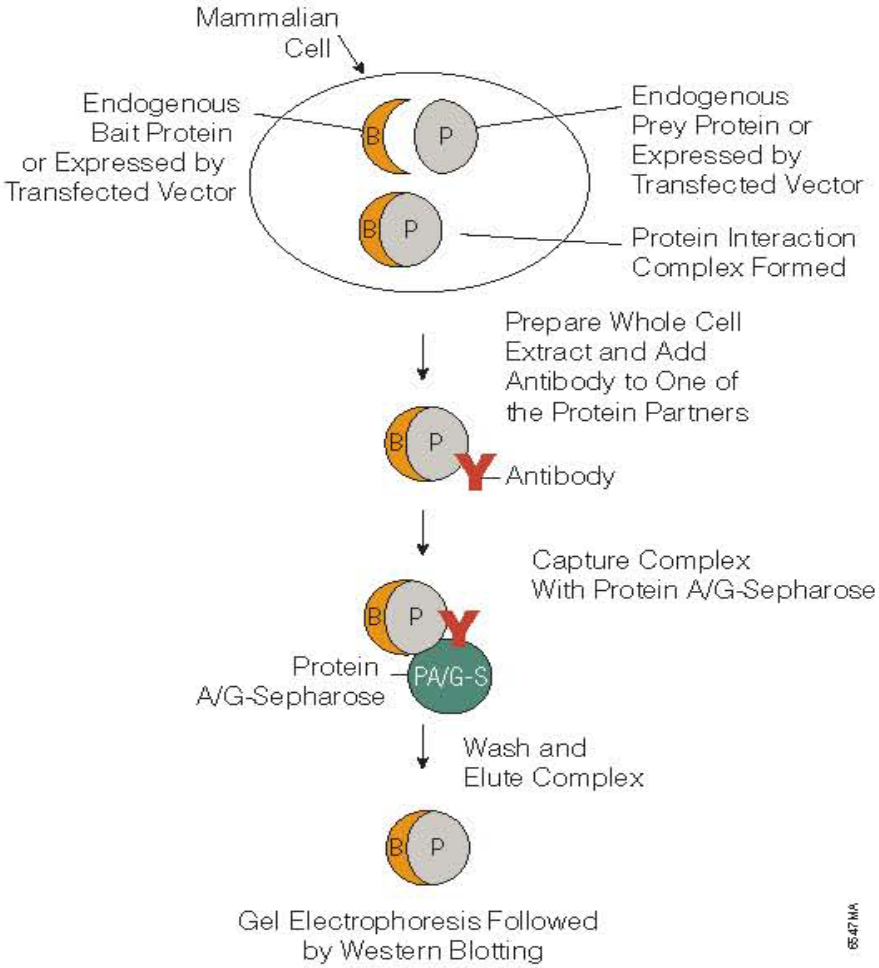
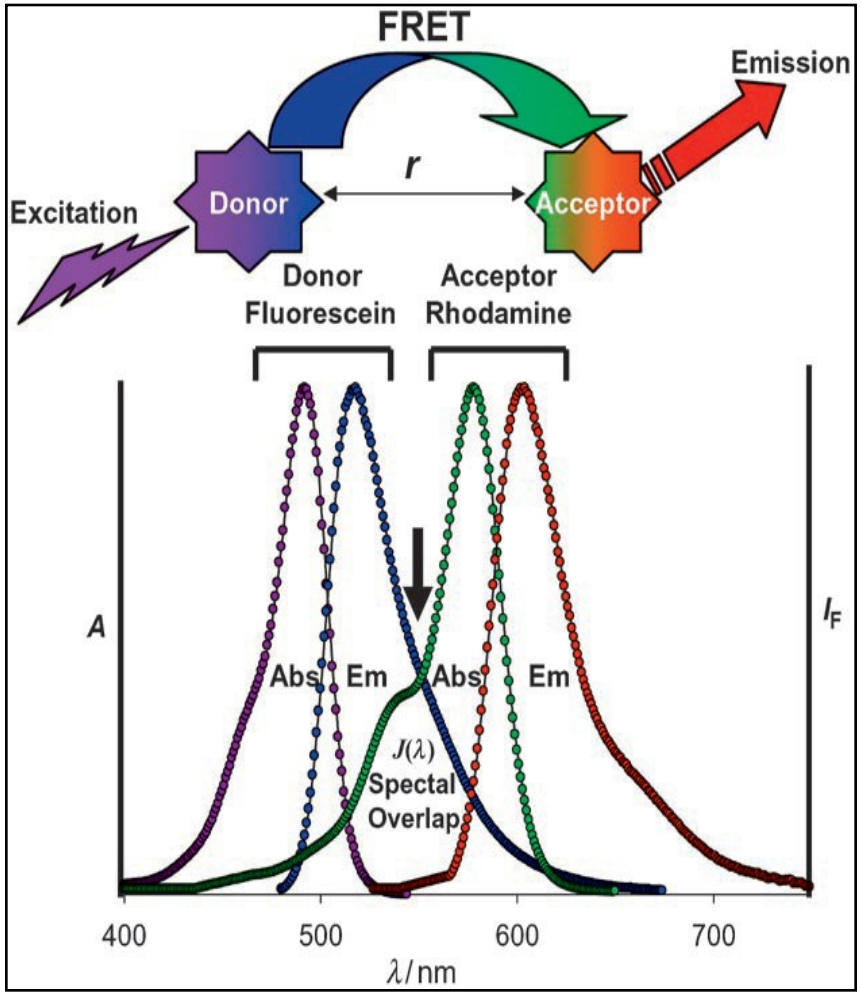
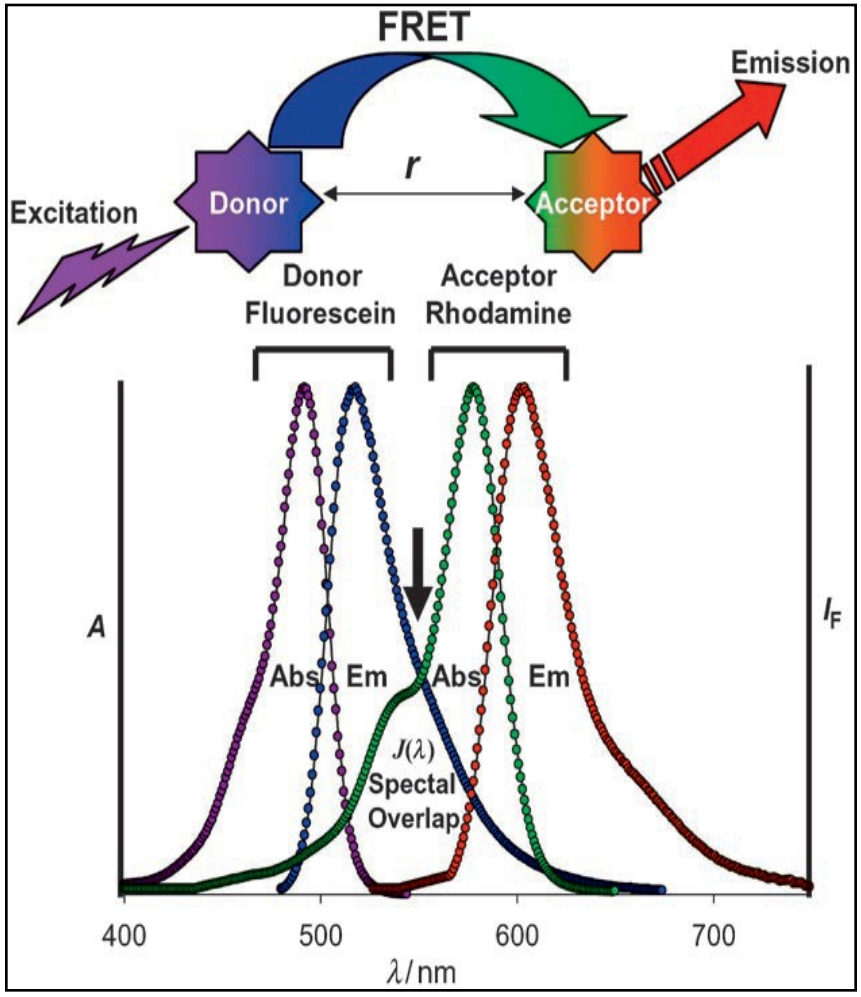


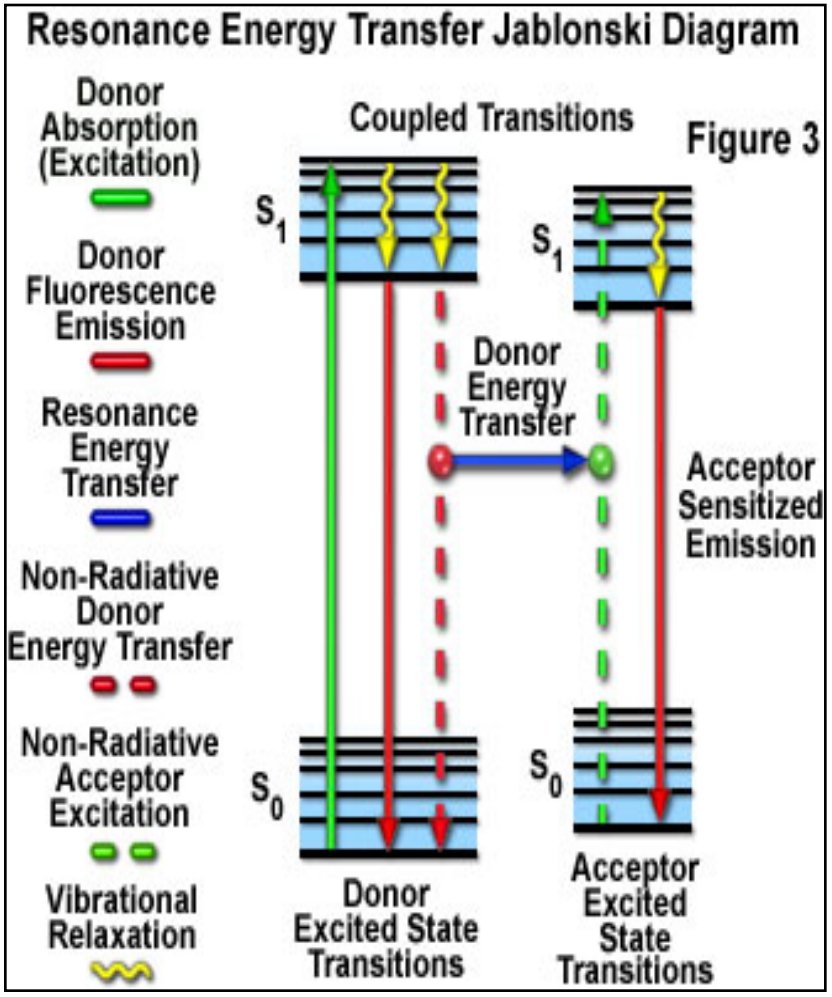
Figure 7. Schematic of immunoprecipitation from mammalian cells. Mammalian cells are cultured using conditions that stimulate the endogenous expression and complex formation of prey and bait protein partners. A whole cell extract is prepared using conditions to maintain the integrity of the complex. Antibodies to either partner are added and the complex is captured using protein A or protein G-sepharose. After washing to remove non-specific proteins, the bait and prey proteins are eluted and analyzed by Western blotting using antibodies to either partner.

FRET(Fluorescence Resonance Energy Transfer)

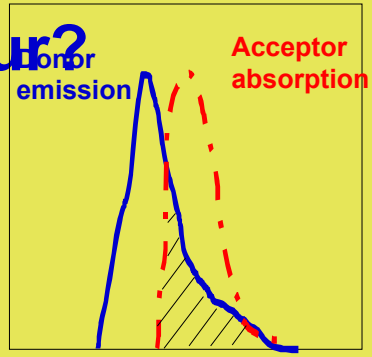


FRET (Fluorescence Resonance Energy Transfer)



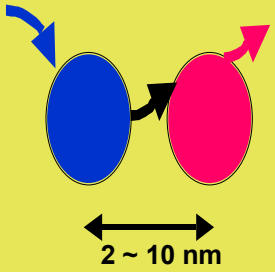


When will FRET occur?

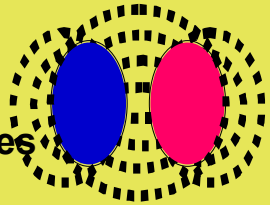


1) Spectral overlap

Donor emission spectrum must significantly overlap the absorption spectrum of the acceptor (>30%)



2) Distance between the donor and acceptor is between 2 - 10 nm



3) Favorable orientation of fluorophores

FRET-efficiency (E) depends inversely on the sixth power of the donor – acceptor distance (r) :

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

The distance R_0 at which the FRET-efficiency equals 0.5 is called Förster radius

R_0 depends on the spectroscopic properties of the donor – acceptor pair; typical values for R_0 are 20-50 Å

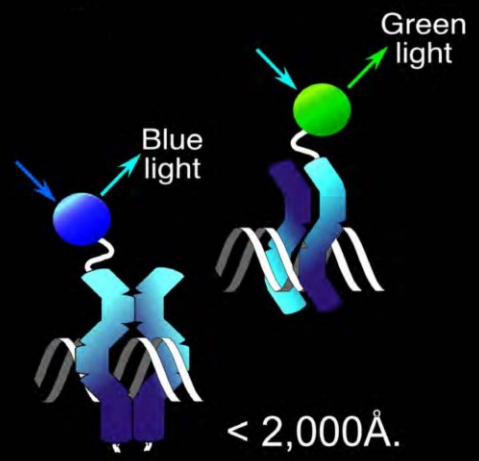
Aequorea victoria Green Fluorescent Protein (GFP)

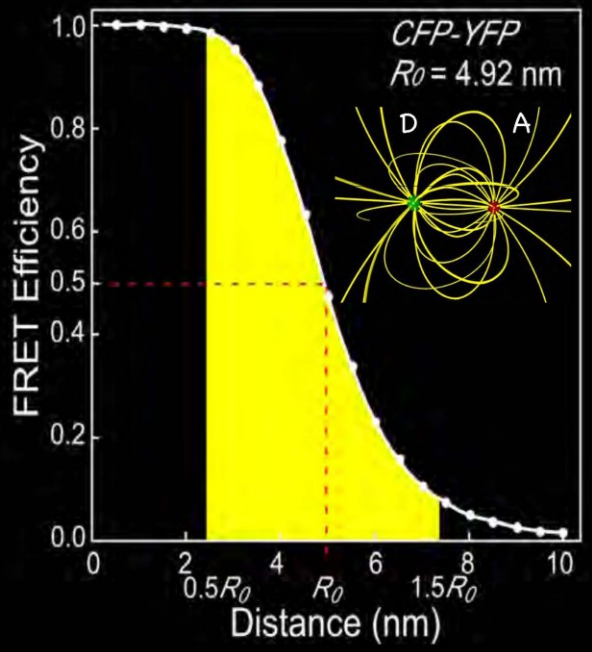
- *Aequorea victoria* makes the chemiluminescent protein aequorin, which emits blue light.

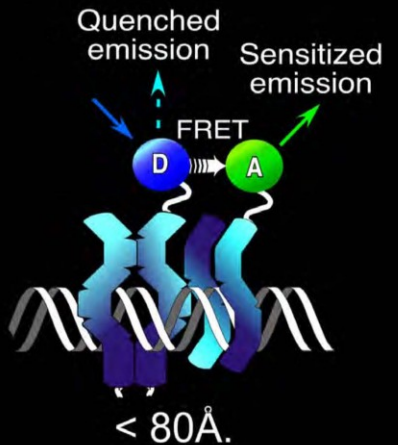
- GFP absorbs the blue light and shifts the emission to green light.

- The cloning of GFP caused a *revolution* in cell biology - allowing genetically encoded fluorescence labeling.

- The detection of FRET indicates the fluorophores are less than ~ 80Å apart:

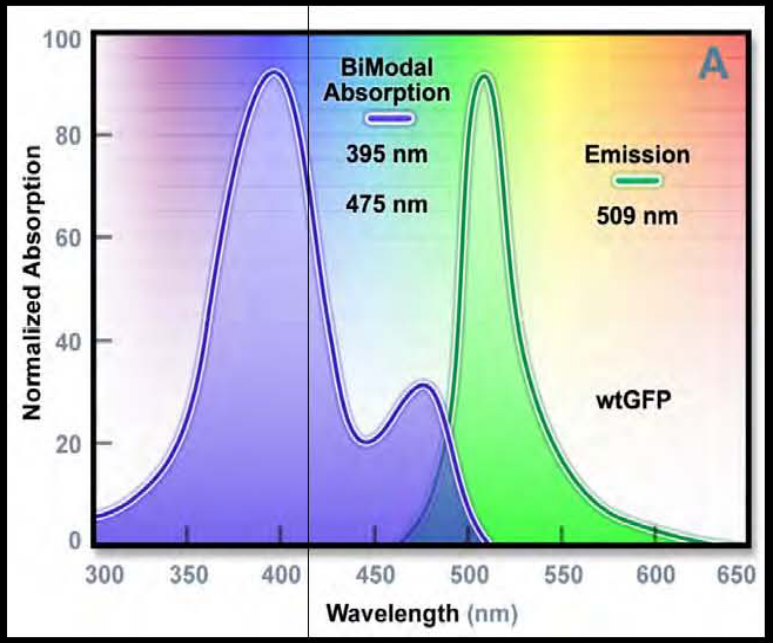






General characteristics of GFP

- The wild type GFP displays a complex absorption spectrum:

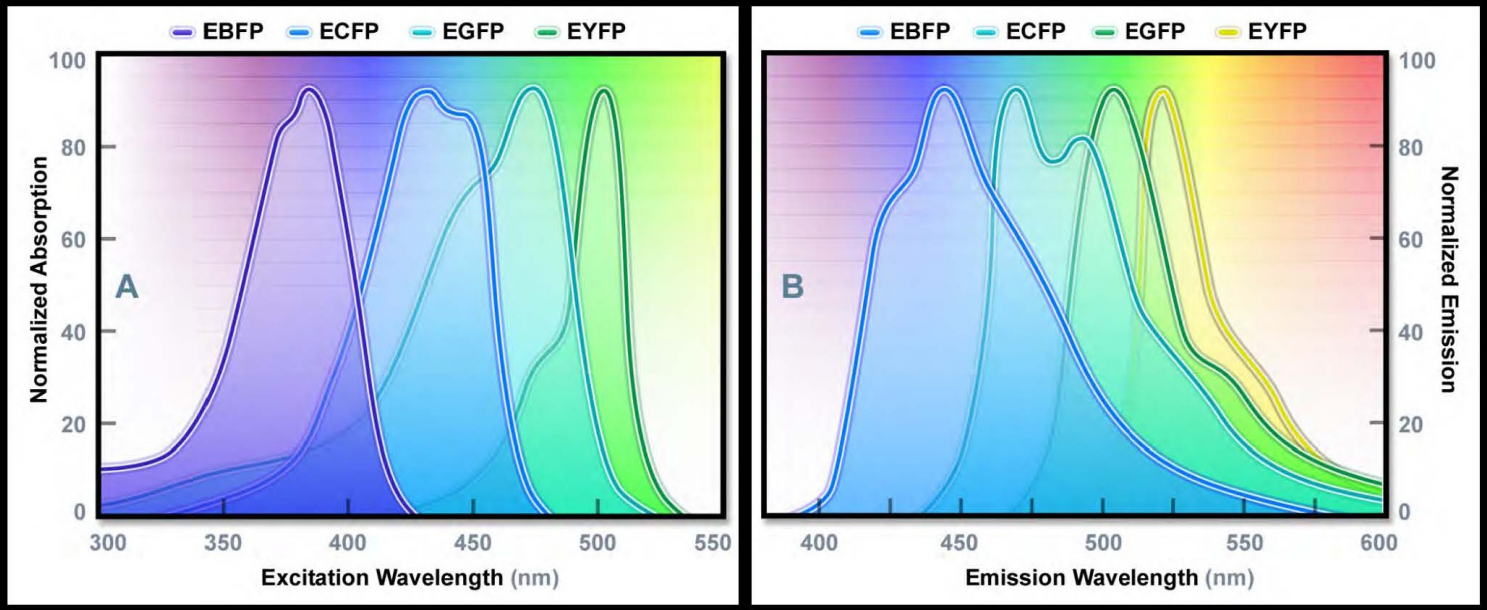


M_1….VTTF-$S_{65}Y_{66}G_{67}$-VQCFS…K_{238}

- The Tyr66 is protonated, and absorbs strongly at 397 nm.
- A charged intermediate accounts for the secondary absorption at 476 nm.

Mutant color variants of *A.v.* GFP

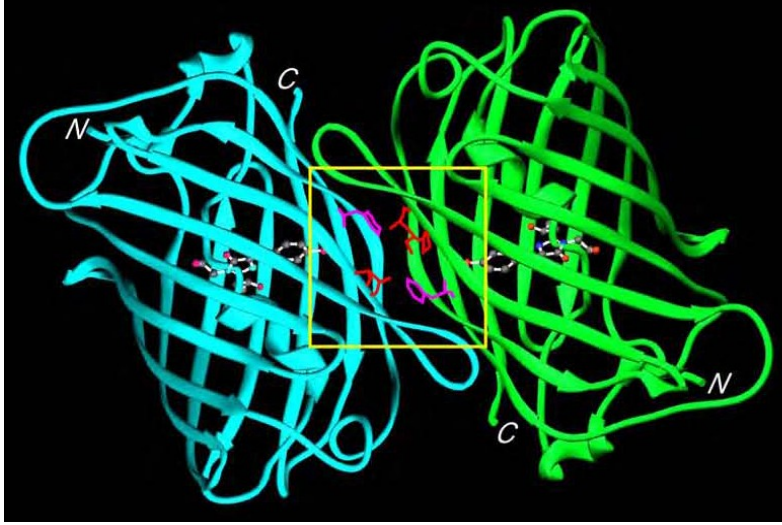
- *A.v.*-base FP color variants from blue to yellow:



- The 530 nm emission of YFP was the most red-shifted of the color variants derived from *A.v.* GFP.

Aequorea FPs and dimer formation

- Dimerization is not typically observed when the proteins are free to diffuse within the cell;
- but, the expression of FPs at high concentrations in a diffusion limited volume can lead to the formation of dimers.



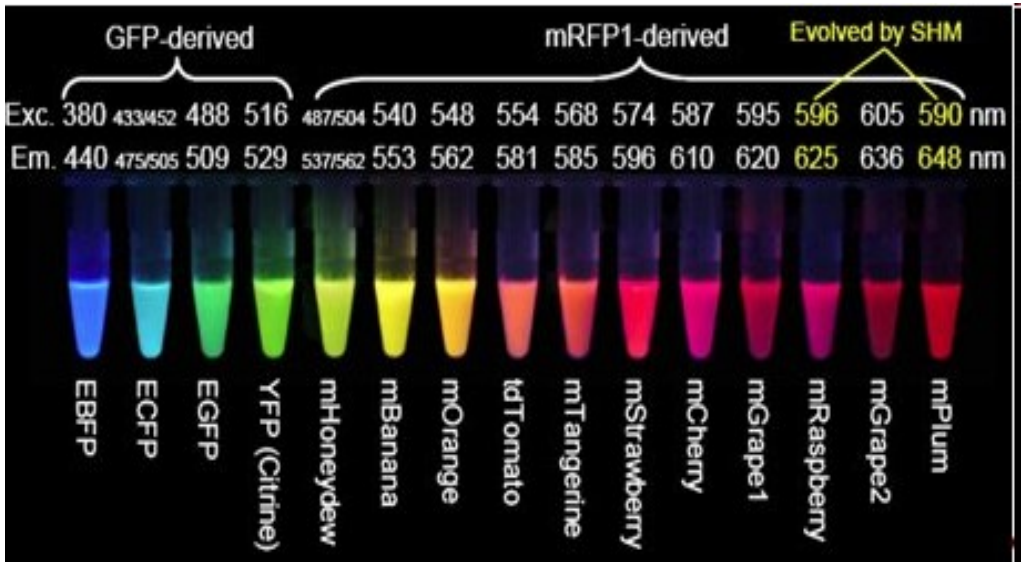
- The substitution of alanine^{206} with lysine (*A206K*) prevents dimer formation.

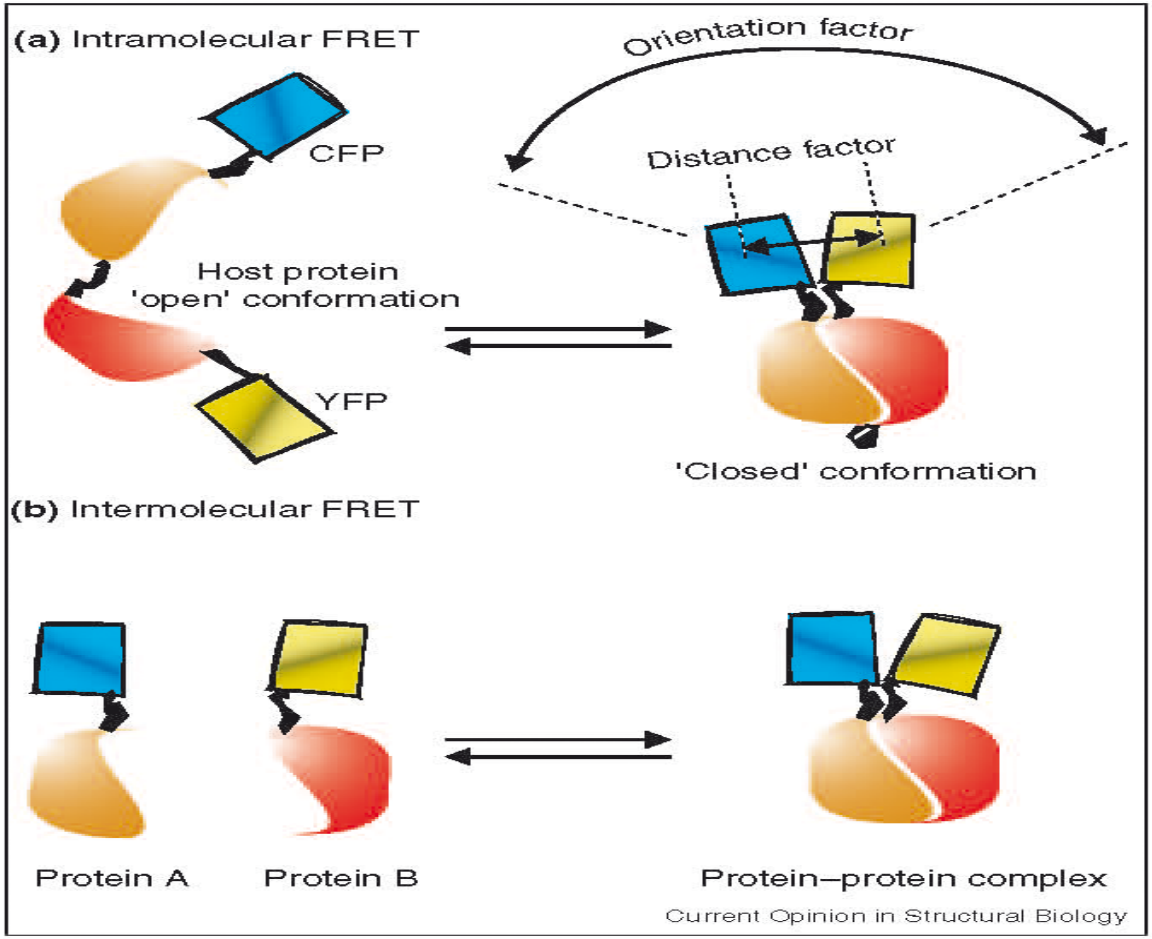
  Zacharias et al (2002) *Science* **296**:913; Kenworthy (2002) *TBCS* **27**:435

- This is *especially* important for FRET-based imaging methods.

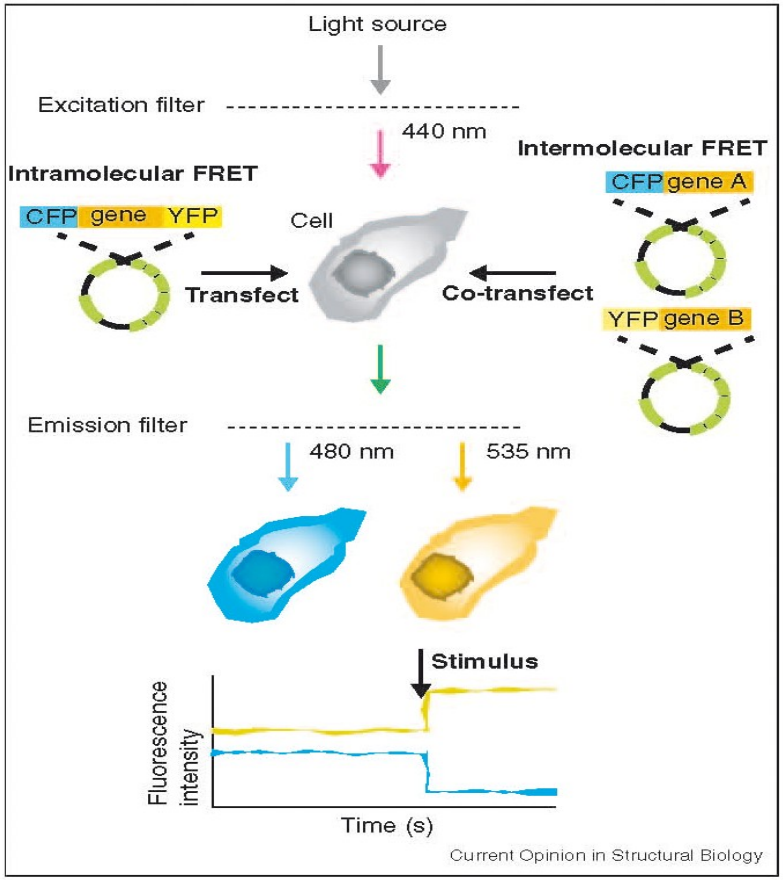
Feasible chromophore-pairs in FRET studies

FRET					
Donor			Acceptor		
Protein[a]	*Excitation peak (nm)*	*Emission peak (nm)*	*Protein*[a]	*Excitation peak (nm)*	*Emission peak (nm)*
BFP	**383**	**448**	***GFP***	**488**	**507**
CFP	**433/445**	**475/503**	***YFP***	**513**	**527**
GFP[2]	**395/475**	**510**	***YFP***	**513**	**527**
YFP	**513**	**527**	***DsRed***	**558**	**583**

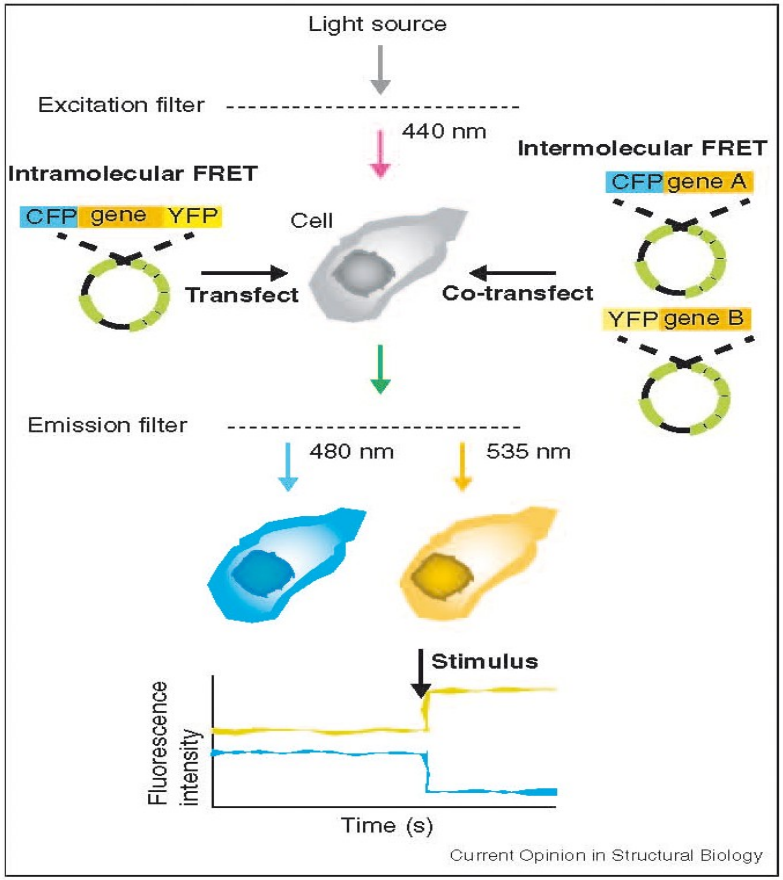




Intramolecular and intermolecular FRET. **(a)** Intramolecular FRET can occur when both the donor and acceptor chromophores are on the same host molecule, which undergoes a transition, for example, between 'open' and 'closed' conformations. In each square box corresponding to CFP or YFP (shown in cyan or yellow, respectively), a diagonal line represents the chromophore. The amount of FRET transferred strongly depends on the relative orientation and distance between the donor and acceptor chromophores: the parallel orientation and the shorter distance (<100 Å) generally yield larger FRET. **(b)** Intermolecular FRET can occur between one molecule (protein A) fused to the donor (CFP) and another molecule (protein B) fused to the acceptor (YFP). When the two proteins bind to each other, FRET occurs. When they dissociate, FRET diminishes.



FRET imaging microscopy experiment. In FRET experiments, a single transfection (intramolecular FRET) or co-transfection (intermolecular FRET) of the constructs must first be performed. The occurrence of FRET can be observed by exciting the sample at the donor excitation wavelengths while measuring the fluorescence intensities emitted at wavelengths corresponding to the emission peaks of the donor versus those of the acceptor. If the acceptor and donor are at a favorable distance and orientation, donor emission intensity decreases (CFP, cyan) while the acceptor emission (YFP, yellow) intensity increases.



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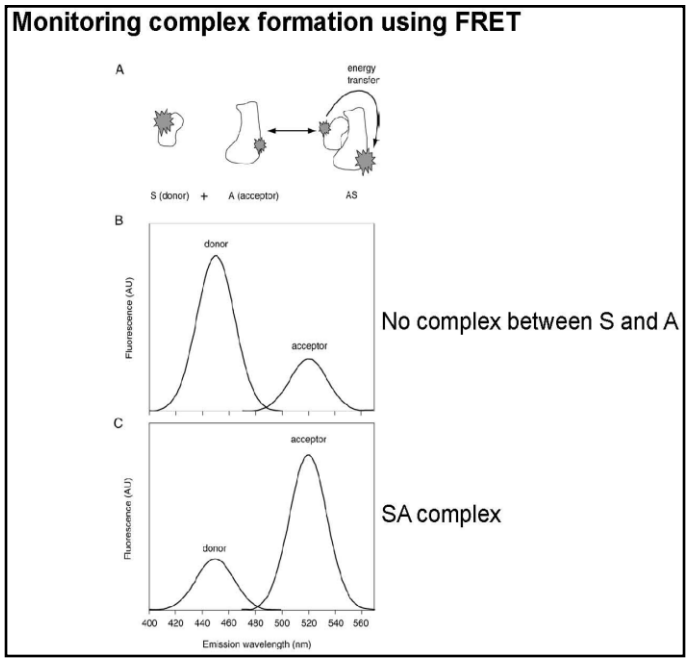

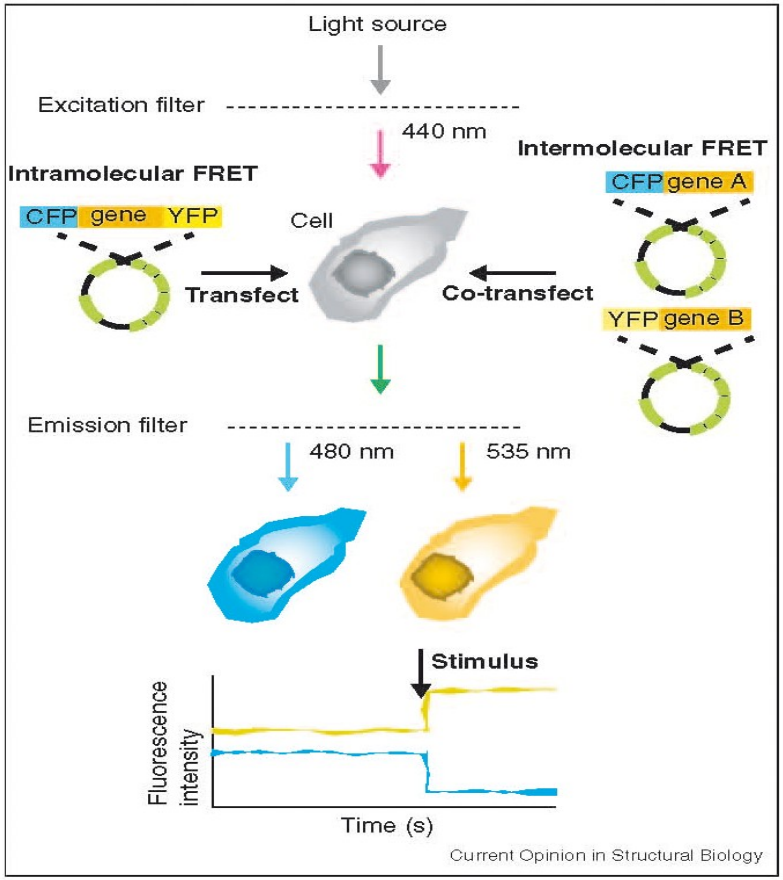

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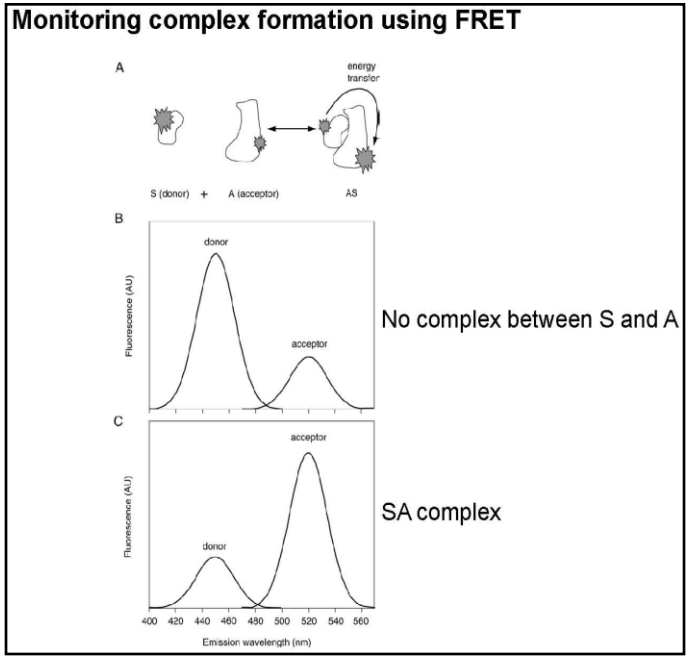


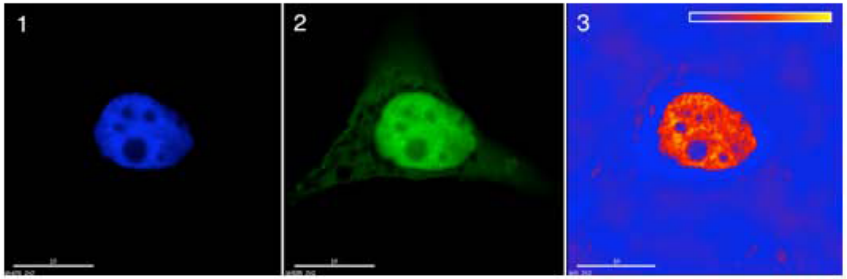


Figure 8. The FRET pair CFP (donor) and YFP (acceptor) were used to label 2 nuclear proteins co-localized to interchromatin granules. This was done on a widefield fluorescence microscope using standard CFP/YFP filter sets (available from Chroma). FRET efficiency varied throughout the cell, with most FRET occurring in the nucleus.

FLIM (Fluorescence Lifetime Imaging Microscopy)- FRET

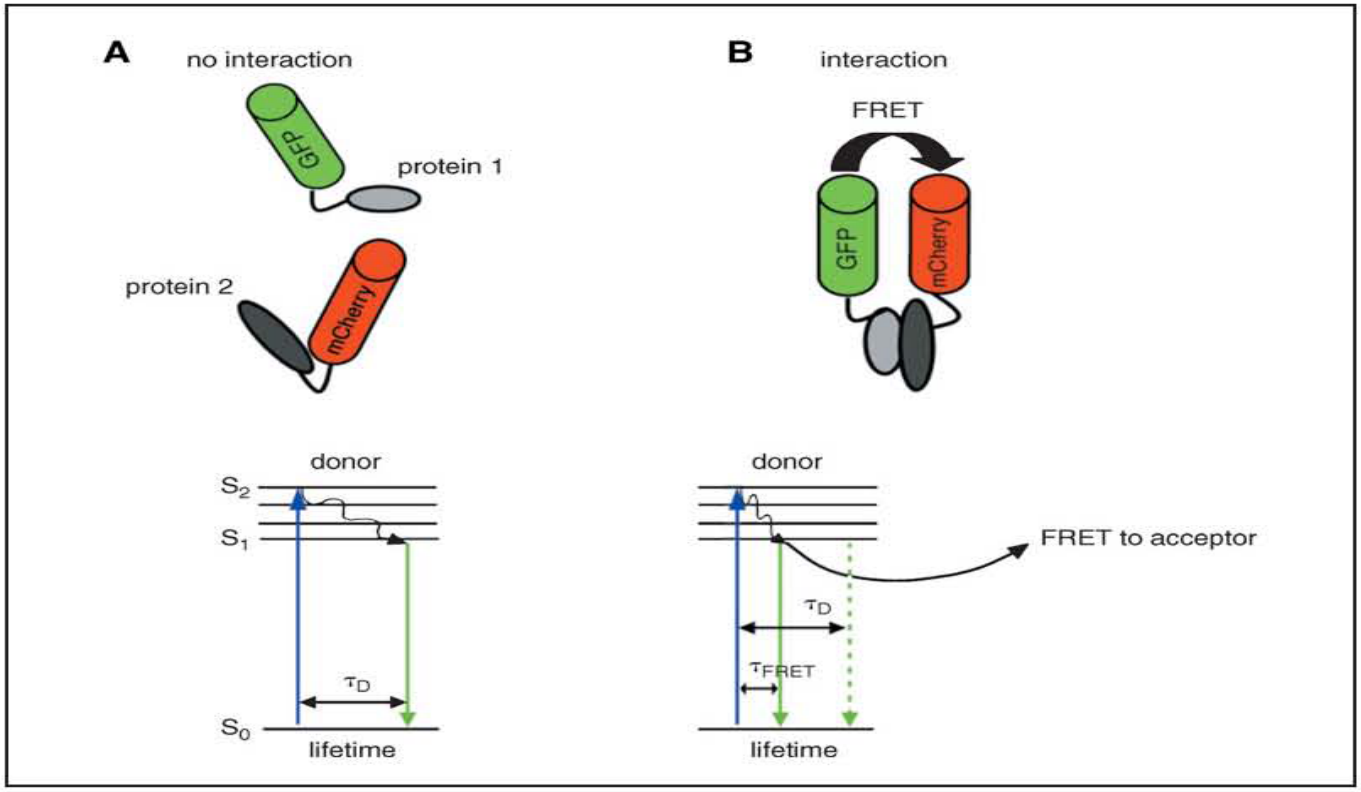


Figure 12.10.2 Detection of fluorescence resonance energy transfer (FRET) by fluorescence lifetime imaging microscopy (FLIM). (**A**) The two fluorescent fusion proteins do not interact. On absorbing light, the donor fluorophore changes from ground state (S_0) to the excited state (S_2), as illustrated in the simplified Jablonski energy-level diagram (**A**, bottom). This is followed by emission of a photon (fluorescence) during the next few nanoseconds (τ_D). (**B**) The two fluorescent fusion proteins interact illustrating the effect of energy transfer on donor fluorescence lifetime. As the Jablonski diagram shows (**B**, bottom), deactivation from the donor excited state can occur either by fluorescence (downward-pointing arrow), or through the radiationless transfer of energy to the acceptor by FRET. The occurrence of FRET is detectable by a decrease in the donor fluorescence lifetime (τ_{FRET}).

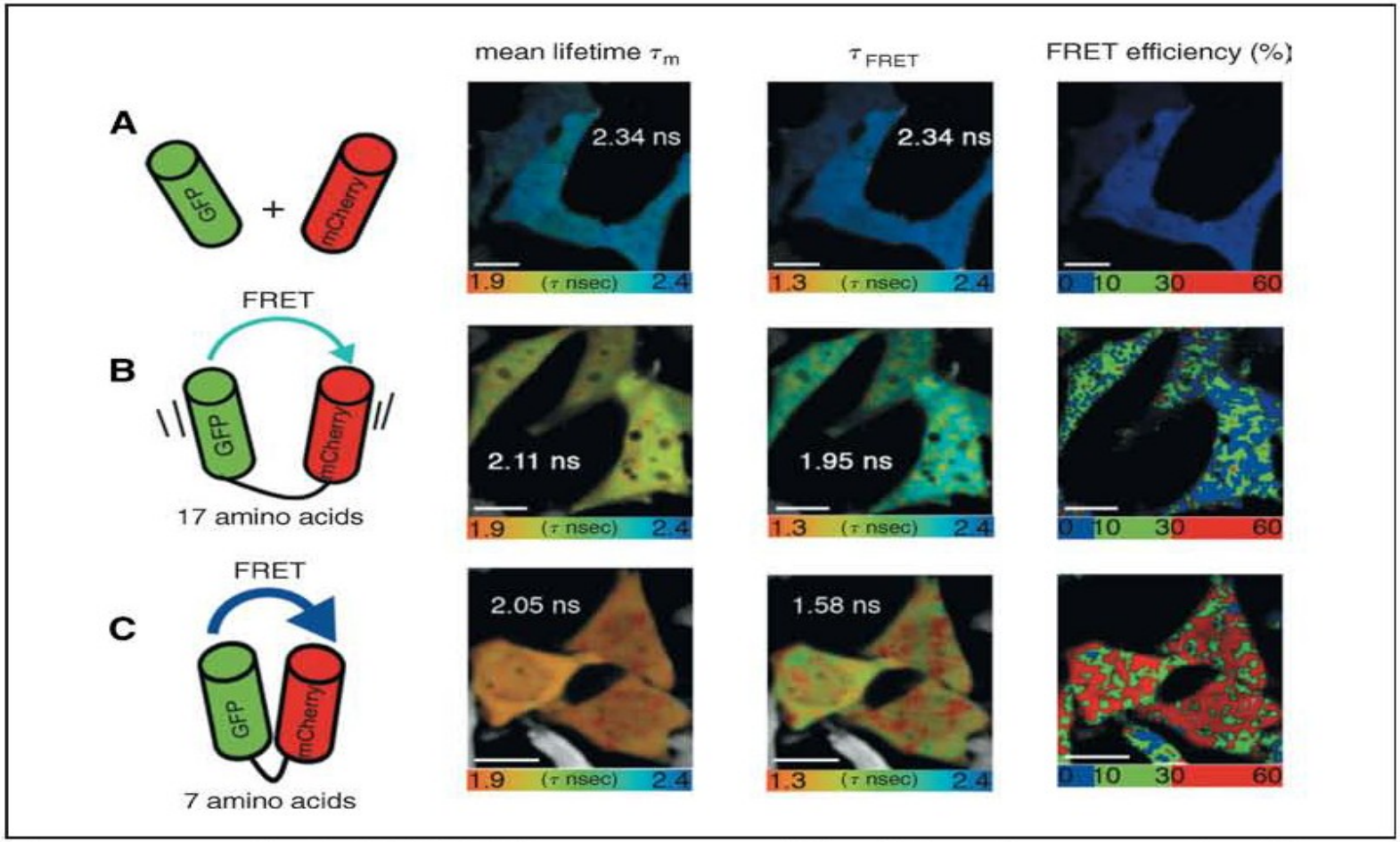


Figure 12.10.7 In vivo FLIM-FRET measurements. Living HeLa cells co-expressing either unfused, free EGFP and unfused, free mCherry (**A**), or GFP-coupled directly to mCherry through a 17-amino-acid linker (**B**), or GFP-coupled directly to mCherry through a 7-amino-acid linker (**C**) were imaged by using the multiphoton scanning microscope. The fluorescence lifetime was analyzed by using the SPCImage software. For each panel, the spatial distribution of the mean fluorescence lifetime (τ_m) and of the fluorescence lifetime of the donor molecules interacting with the acceptor (τ_{FRET}) is shown throughout the cells. The FRET efficiencies were calculated for each pixel as E_{FRET} (%) $= (1 - \tau_{FRET}/\tau_D) \times 100$. Color scale shown covers the range of E_{FRET} values from 0% to 60%. Bars, 10 μm. For a color version of this figure, see *http://www.currentprotocols.com*

FRET and Flow-Cytometry

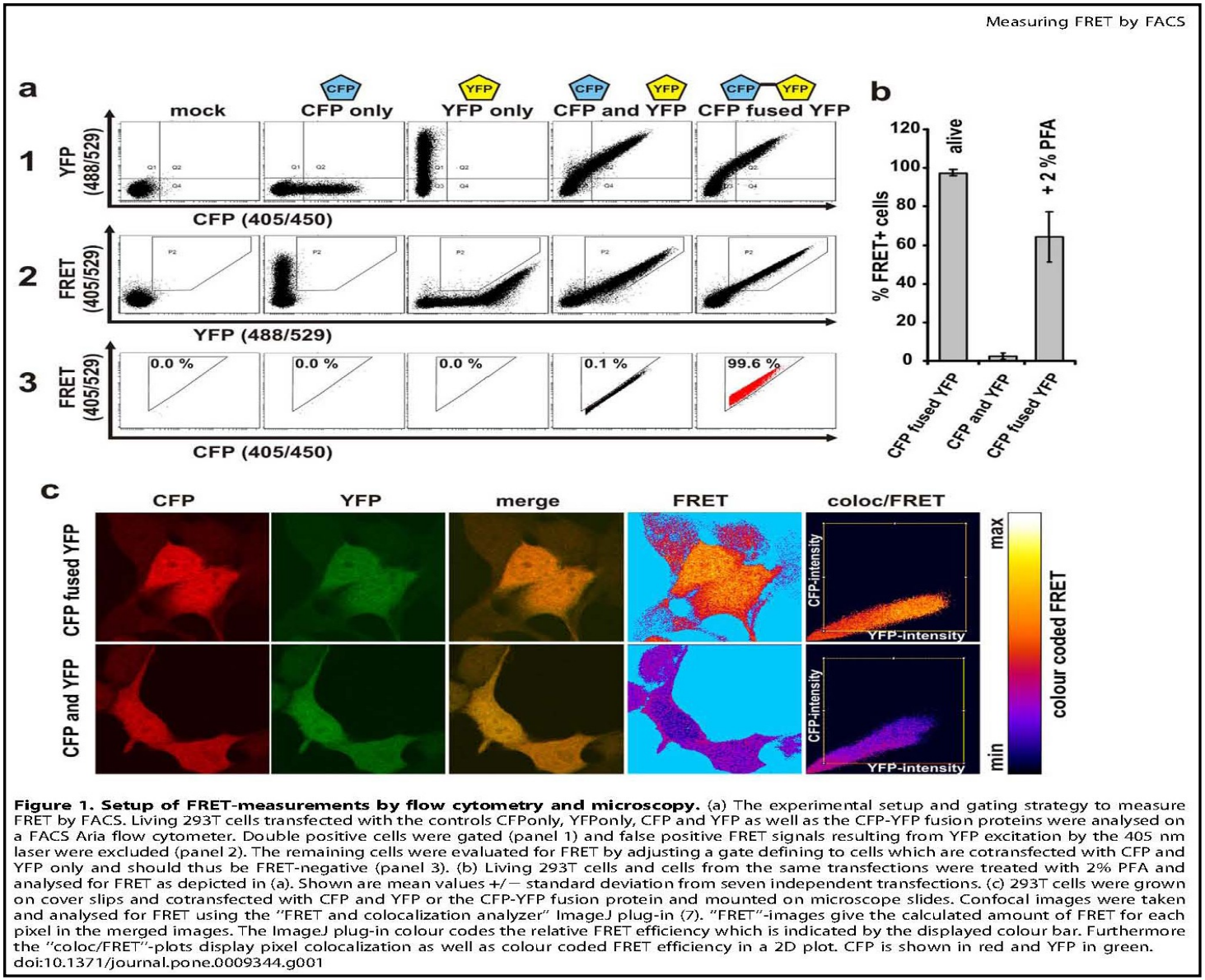
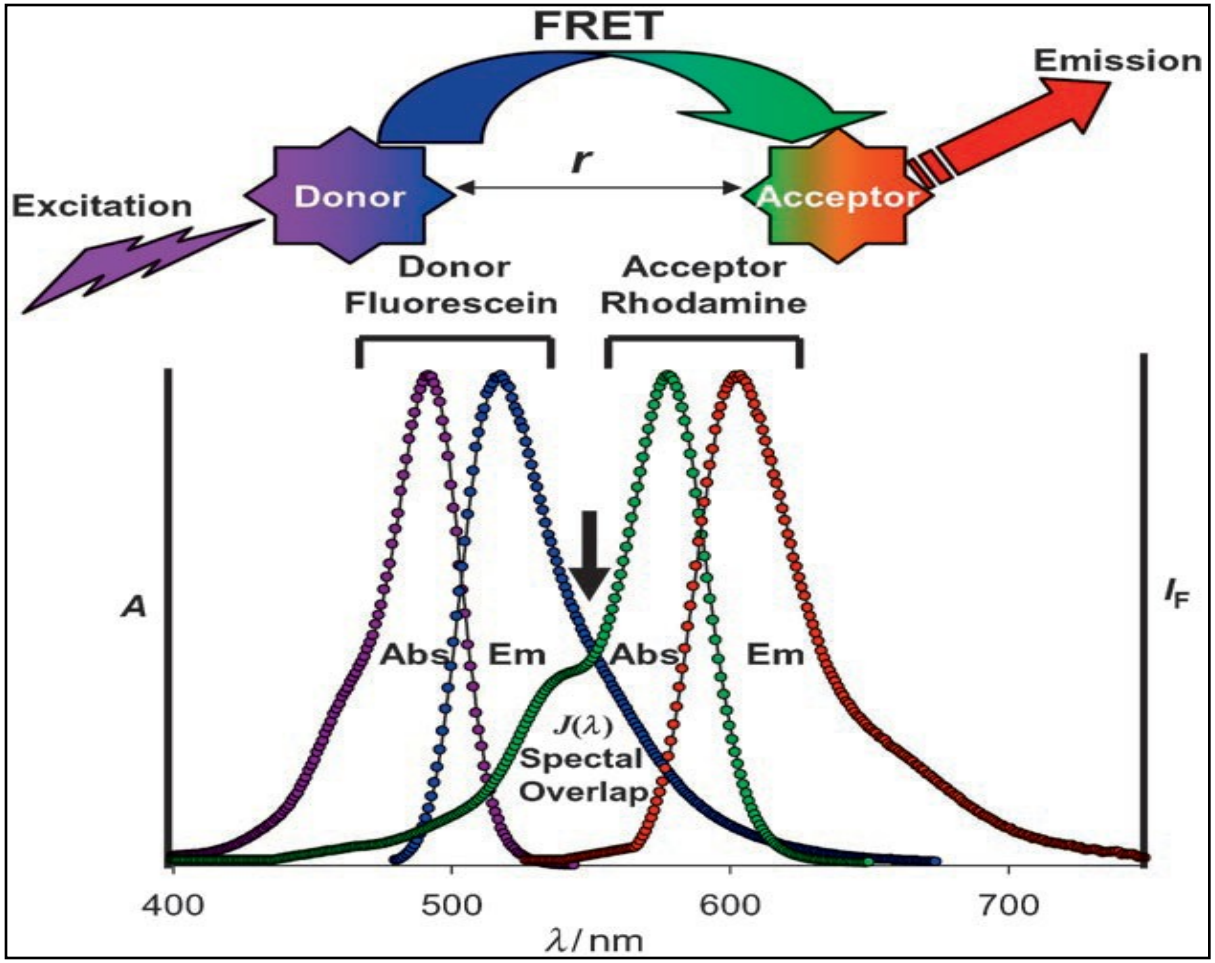
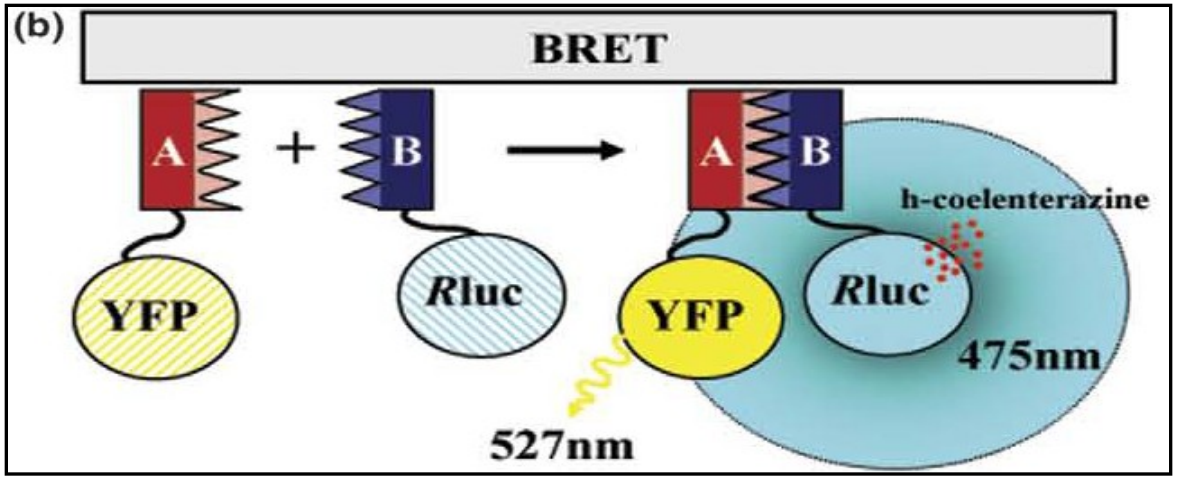
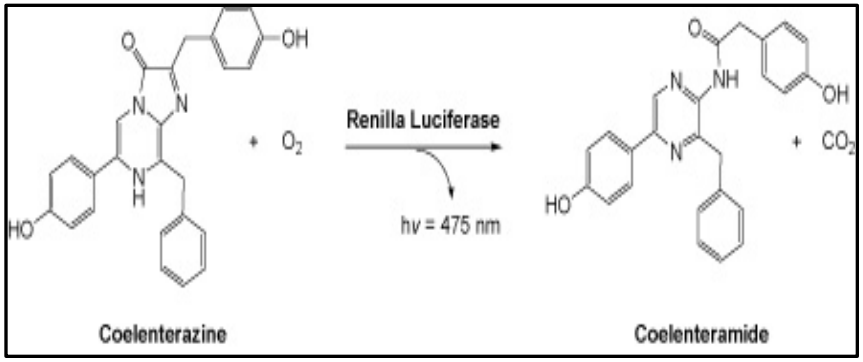


Figure 1. Setup of FRET-measurements by flow cytometry and microscopy. (a) The experimental setup and gating strategy to measure FRET by FACS. Living 293T cells transfected with the controls CFPonly, YFPonly, CFP and YFP as well as the CFP-YFP fusion proteins were analysed on a FACS Aria flow cytometer. Double positive cells were gated (panel 1) and false positive FRET signals resulting from YFP excitation by the 405 nm laser were excluded (panel 2). The remaining cells were evaluated for FRET by adjusting a gate defining to cells which are cotransfected with CFP and YFP only and should thus be FRET-negative (panel 3). (b) Living 293T cells and cells from the same transfections were treated with 2% PFA and analysed for FRET as depicted in (a). Shown are mean values +/− standard deviation from seven independent transfections. (c) 293T cells were grown on cover slips and cotransfected with CFP and YFP or the CFP-YFP fusion protein and mounted on microscope slides. Confocal images were taken and analysed for FRET using the "FRET and colocalization analyzer" ImageJ plug-in (7). "FRET"-images give the calculated amount of FRET for each pixel in the merged images. The ImageJ plug-in colour codes the relative FRET efficiency which is indicated by the displayed colour bar. Furthermore the "coloc/FRET"-plots display pixel colocalization as well as colour coded FRET efficiency in a 2D plot. CFP is shown in red and YFP in green.
doi:10.1371/journal.pone.0009344.g001


FRET
Emission
Excitation
Donor
r
Acceptor
Donor
Fluorescein
Acceptor
Rhodamine
A
Abs
Em
Abs
Em
I_F
$J(\lambda)$
Spectal
Overlap
400
500
600
700
λ / nm

Bioluminiscence Resonance Energy Transfer (BRET)





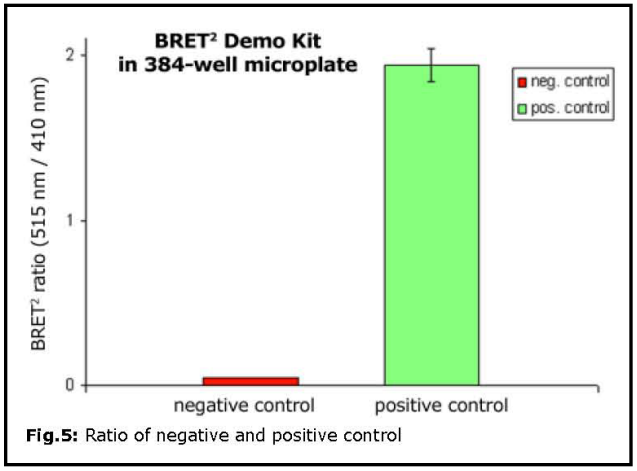
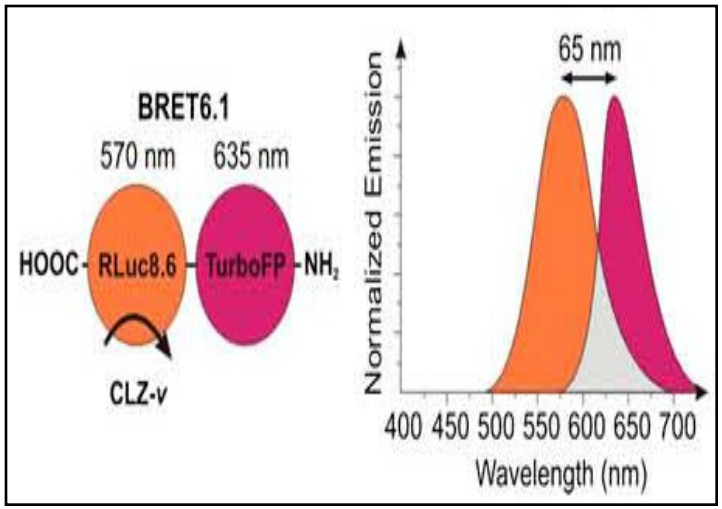


Fig.5: Ratio of negative and positive control

BRET for protein-protein interactions imaging in deep-tissues in living mice

Bioluminescence images of HT1080 cells stably expressing BRET fusion proteins accumulated in the lungs of nude mice (tail vein injection). Injection of luciferase substrate at 1.5 h after cell injection (imaged using sequentially open/donor/akceptor filters)



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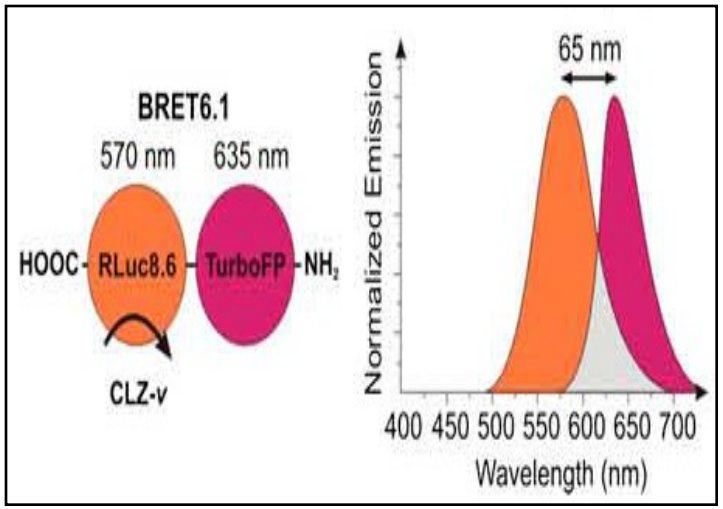

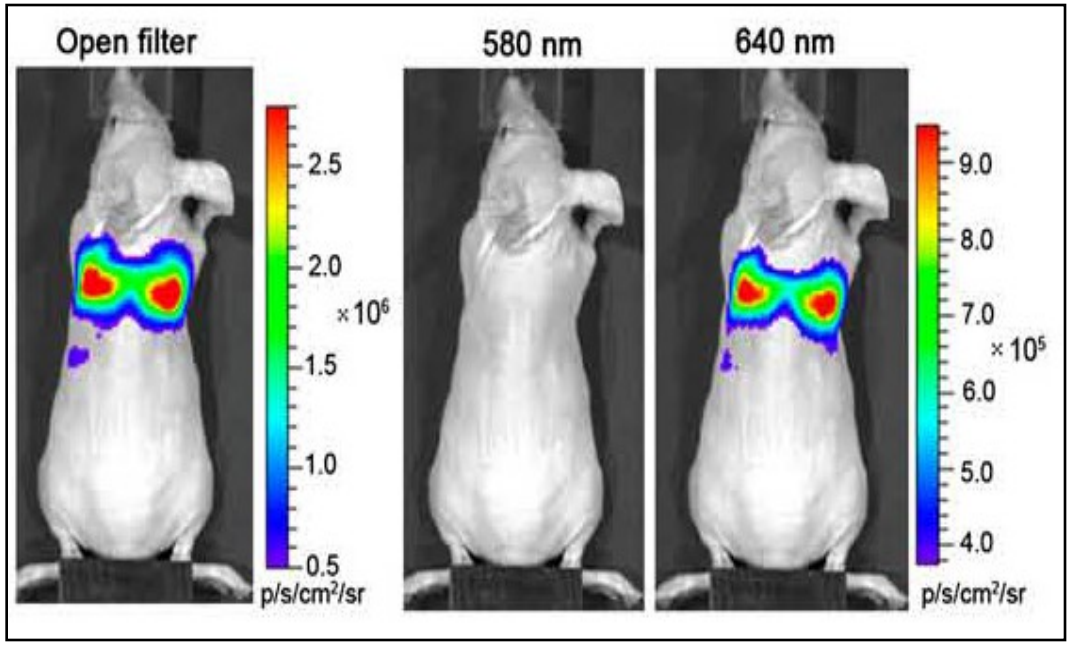

FRAP (Fluorescence Recovery After Photobleaching)

Fluorescence recovery after photobleaching is a quantitative fluorescence technique that can be used to measure the dynamics of molecular mobility in 2D by taking advantage of the fact that most fluorophores are irreversibly bleached by incident light of very high intensity.

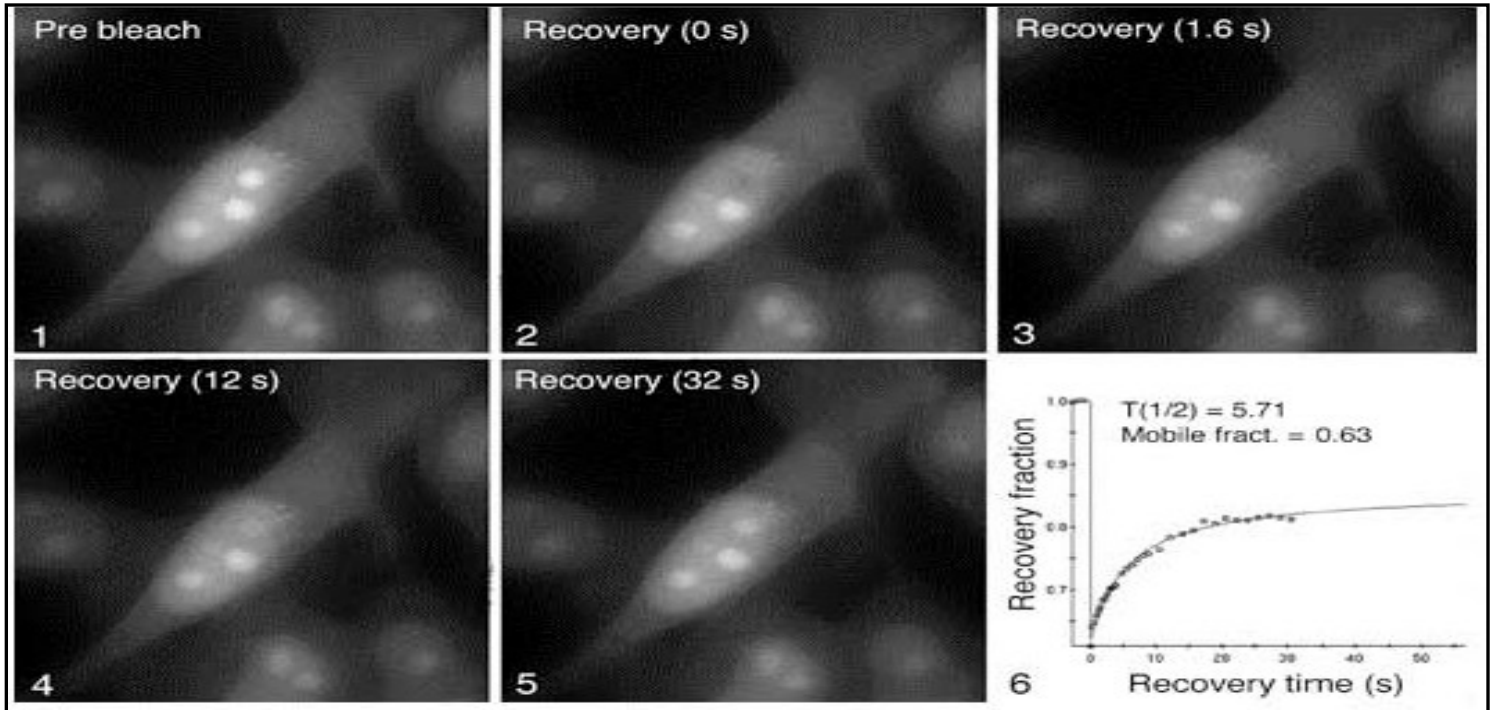

Figure 3. An image is taken prior to photobleaching (1), a region of interest is bleached to approximately 50% of its original intensity (2), images are acquired after the photobleach period (3-5). For qualitative FRAP, it may be enough to simply plot fluorescence intensity over time (known as the recovery curve), or just evaluate the time course images obtained. The function of true FRAP analysis, however, is to fit the recovery curve to a predefined model (6). The mobile fraction represents the fraction of recovered fluorescence. T(1/2) is the point at which the half-height recovery intersects the recovery curve. T(1/2) can then be used to calculate the diffusion coefficient (see Axelrod, 1976, for a review of FRAP analysis).