UniversitätsSpital Zürich

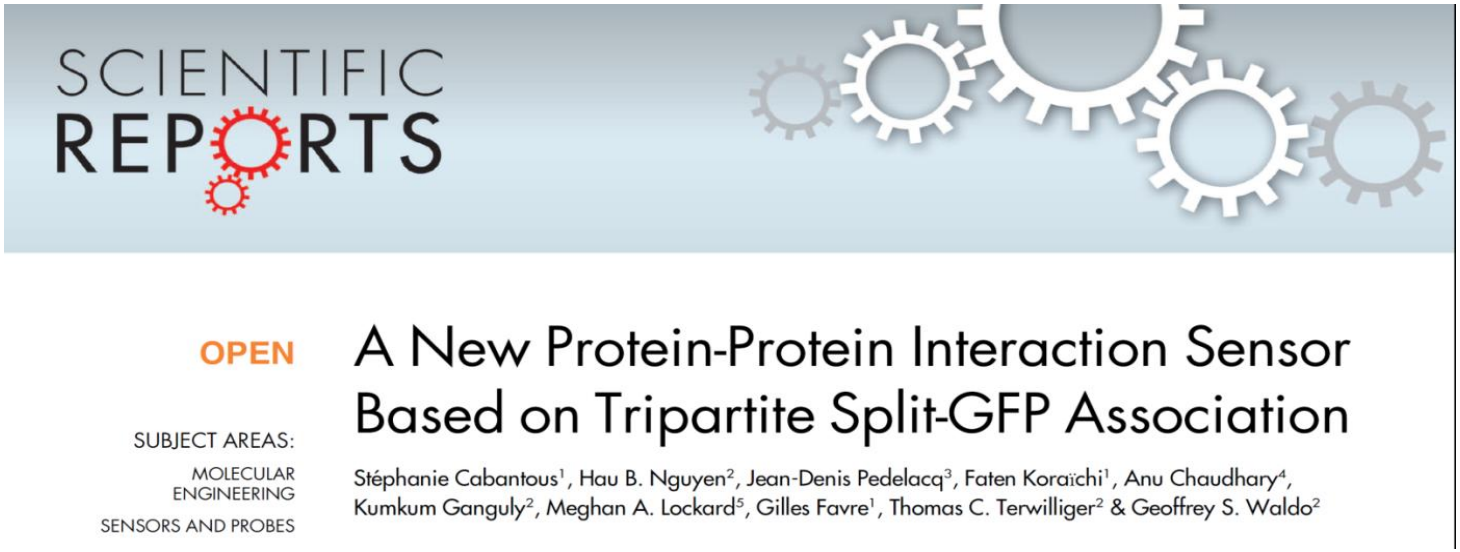
Studying protein-protein interactions by Bimolecular Fluorescence Complementation

Assunta Senatore

November 12th 2013

Outline

- Principle of the technique
- Examples of applications
- Critical points and limitations
- Evolvution of the BiFC



Principle of Bimolecular Fluorescent Complementation (BiFC)

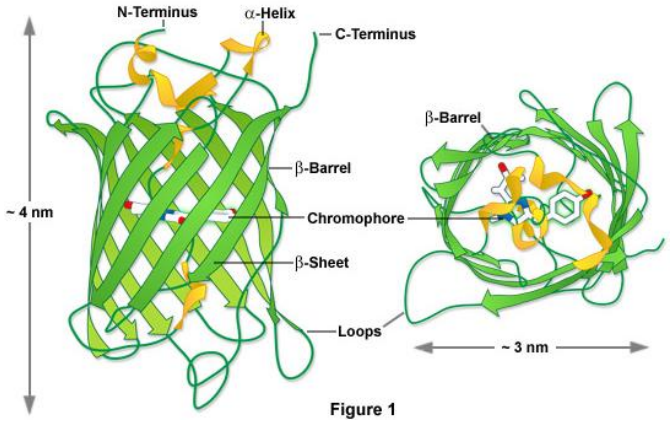
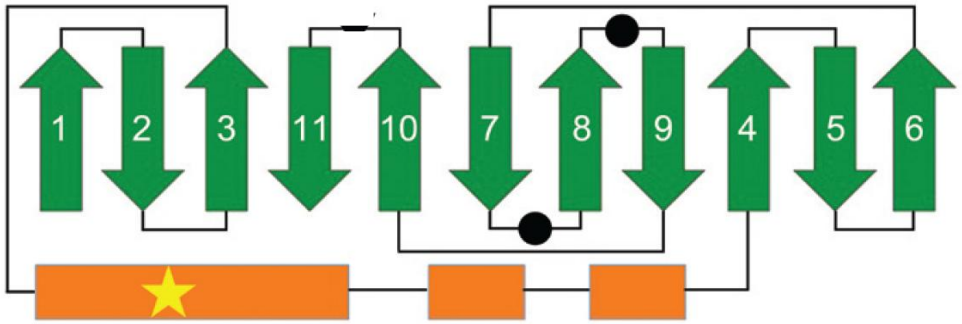
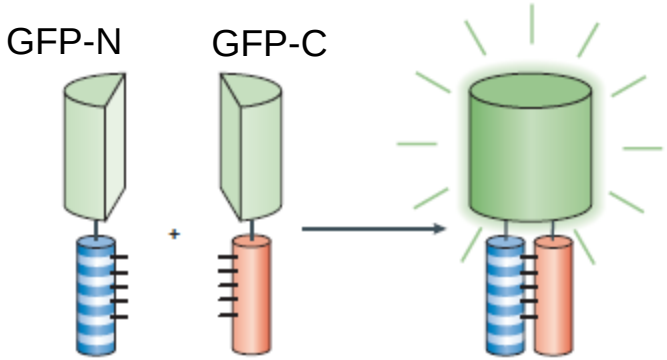


Figure 1



Kodama Yand Hu CD, *BioTechniques* , 2012

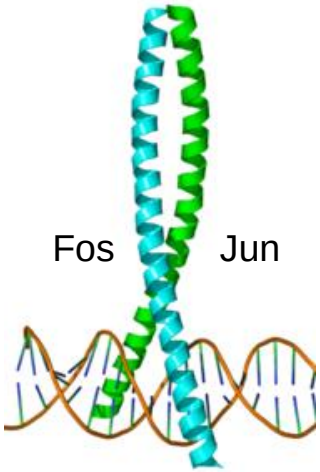


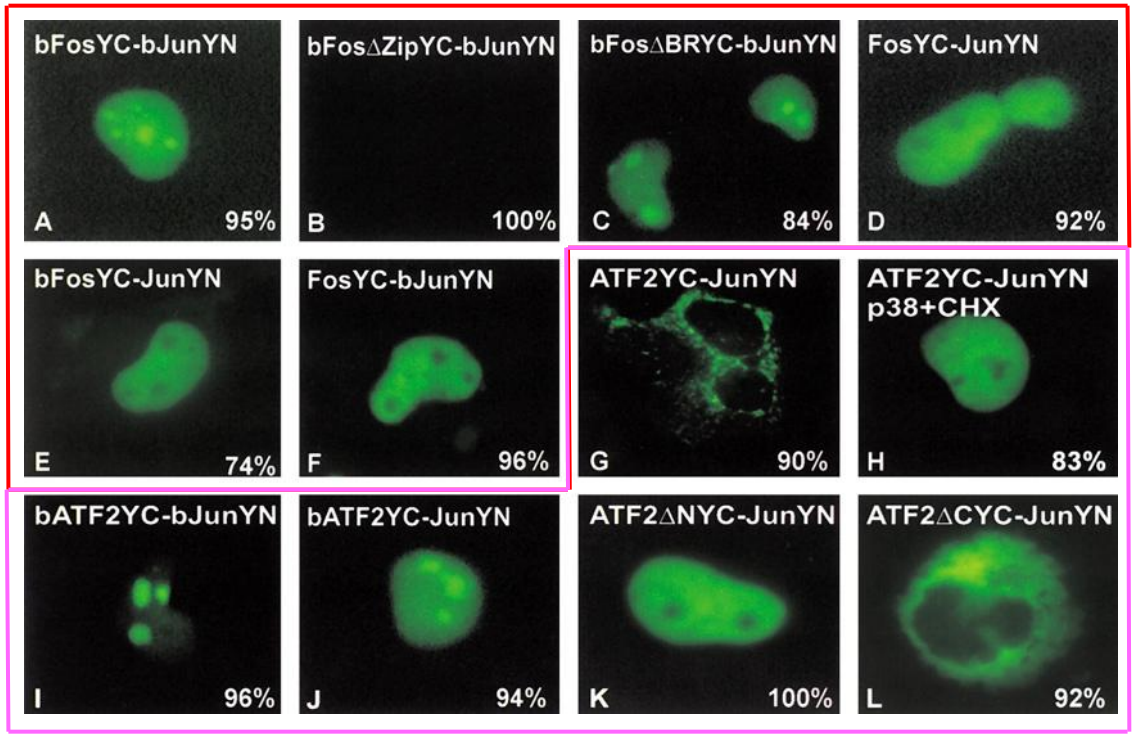
Bimolecular fluorescence complementation (BiFC) relies on the reconstitution of fluorescent proteins and enables both the analysis of protein-protein interactions and the visualization of protein complex formations *in vivo*.

Interactions between the bZIP domains in cells

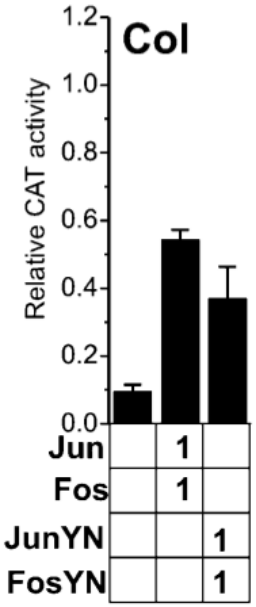
HU CD., Chichenov Y. and Kerppola T, *Molecular cell*, 2002

AP-1





AP-1 responsive Collagenase reporter



Interaction between bZIP domains of Fos and Jun detected by BiFC of bFosYC and bJunYN (A).
No fluorescence complementation upon deletion in the leucine zipper that prevents Fos-Jun dimerization (B).
Mutation of the basic regions of bFos and bJun had no effect on bFos-bJun heterodimer localization (C).
Cells coexpressing either full length FosYC or JunYN or both exhibited predominantly nuclear fluorescence that was excluded from the nucleoli (D, E and F).

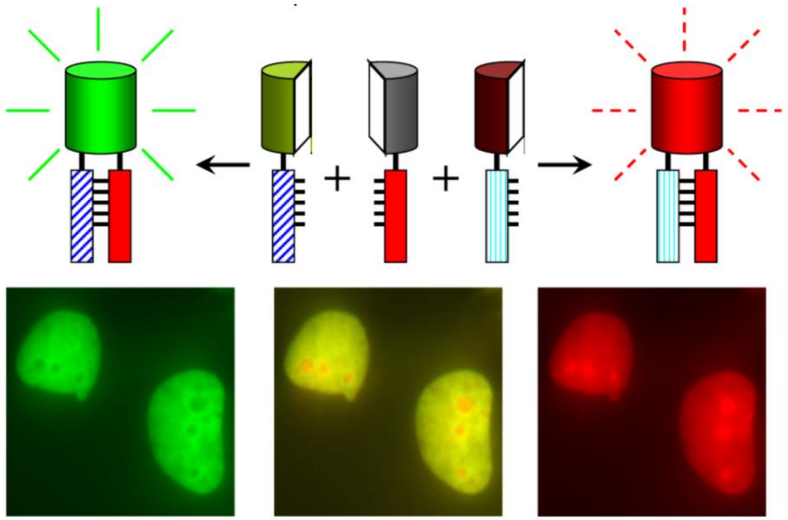
Full-length ATF2YC and JunYN dimerization localizes in the perinuclear region (G).
Dimers formed by the bZIP domain of ATF2 with full lenght Jun or bJun display nucleolar localization (I and J).
Deletion of the N terminus in ATF2 results in nucleoplasmic localization of the ATF2ΔNYC-JunYN (K) while ATF2ΔCYC-JunYN is excluded from the nucleus.

Visualization of multiple protein interactions by multicolor BiFC

Table 1. List of fluorescent proteins used in BiFC assays.

Fluorescent protein	Excitation Peak (nm)[1]	Emission Peak (nm)[1]	Cell type or organism in the first use
EBFP	382*	448*	Mammalian (COS-1)
Cerulean	439	479	Mammalian (COS-1)
ECFP	452	478	Mammalian (COS-1)
EGFP	488	512	Bacteria (*E. coli*)
GFP-S65T	489*	510	Plant (Onion epidermis)
frGFP	485*	510*	Bacteria (*E. coli*)
sfGFP	503*	518*	Mammalian (HeLa)
Dronpa	503*	518*	Mammalian (HEK293)
EYFP	514/515	527	Mammalian (COS-1)
Venus	515	528	Mammalian (COS-1)
Citrine	516	529	Mammalian (COS-1)
mRFP	549*	570*	Plant (Tobacco BY2 and Onion epidermis)
DsRed monomer	556*	556*	Plant (Onion epidermis)
mCherry	587*	610*	Mammalian (Vero)
mKate	587*	621*	Mammalian (COS-7)

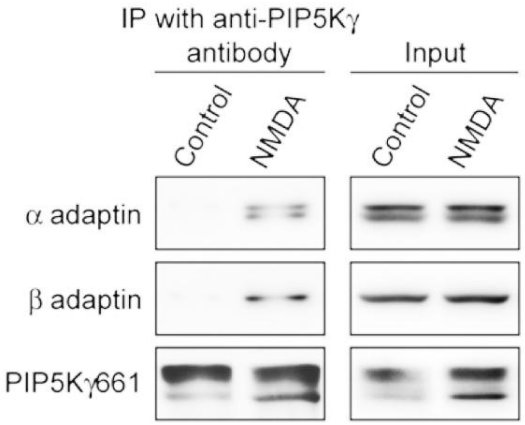
Kodama Yand Hu CD, *BioTechniques* , 2012

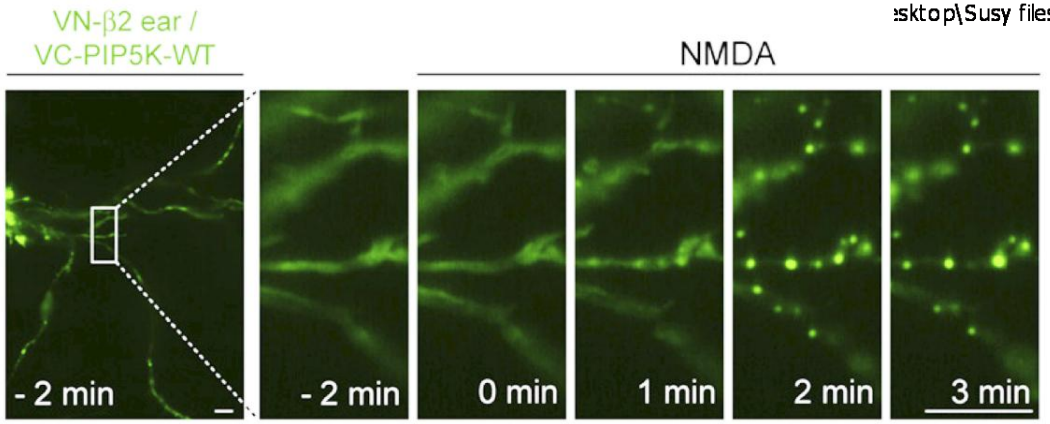


Kerppola T., *Nat Rev Mol Cell Biol*. 2006

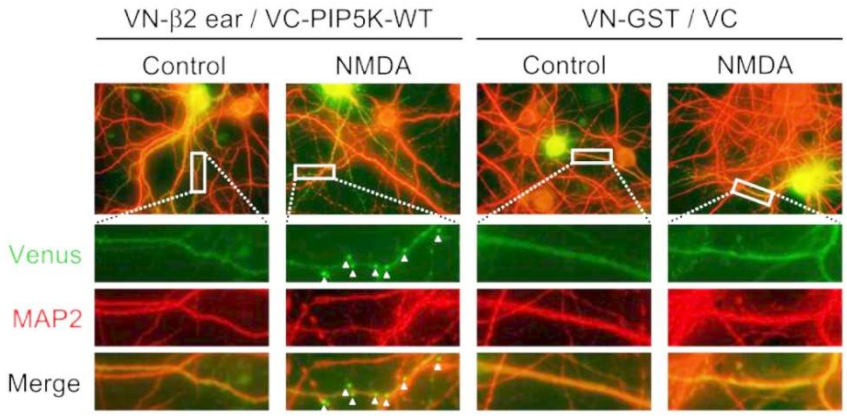
Formation of bimolecular fluorescent complexes with different spectra is possible through interactions between proteins that are fused to different fluorescent protein fragments. These complexes can be independently visualized by using different excitation and emission wavelengths. The panels at the bottom show multicolor BiFC analysis of two different complexes in the same cells.

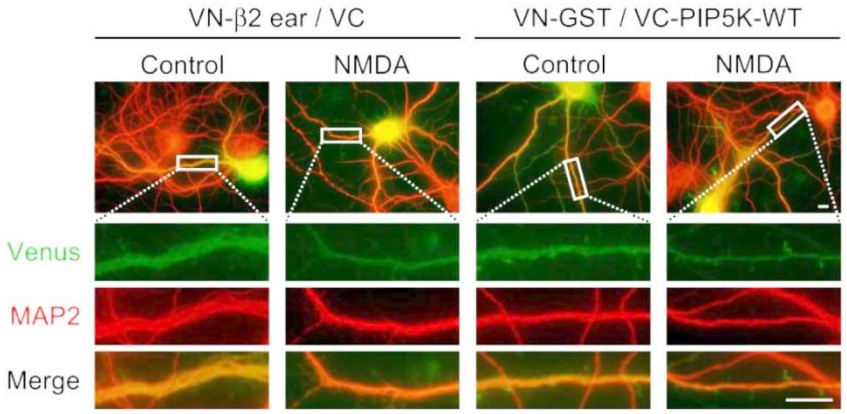
Time-lapse monitoring of protein-protein interaction in neurons





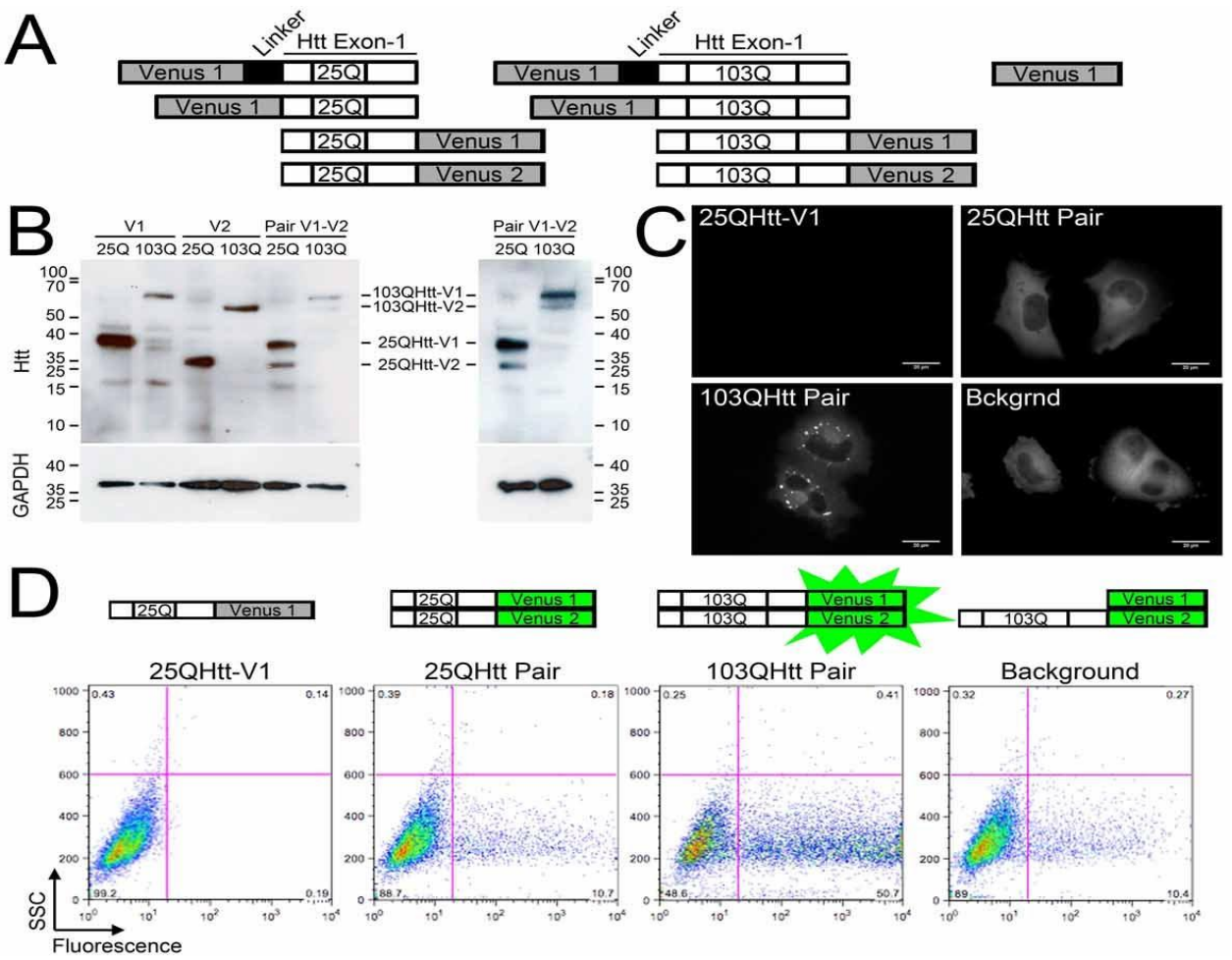
! Imaging at 32°C





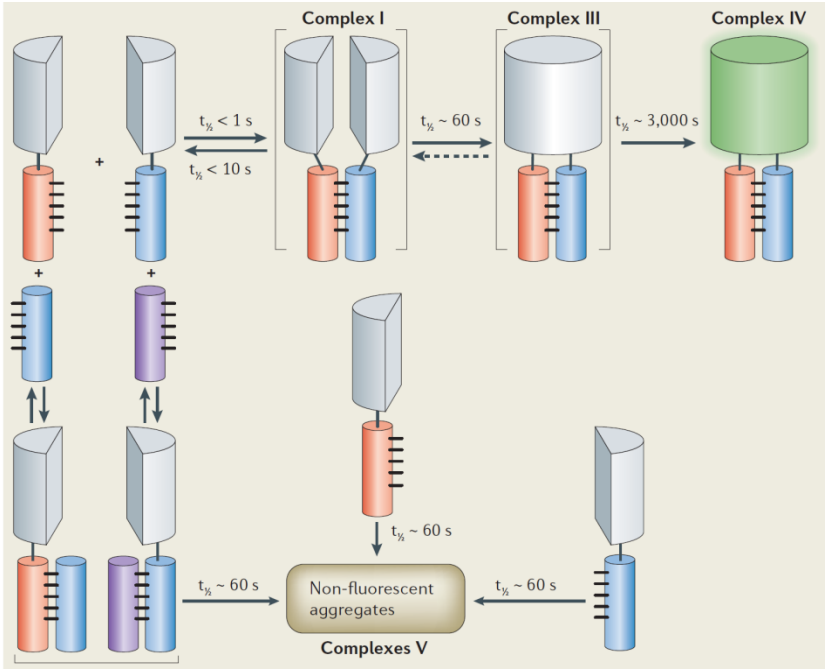
Unoki T. et al., Neuron, 2012

Visualization of mutant hungtintin oligomers



Herrera F., *Plos Currents Huntington Disease*, 2011

Critical points and limitations of BiFC



Kerppola T, *Nature Reviews Molecular Cell Biology,* 2006

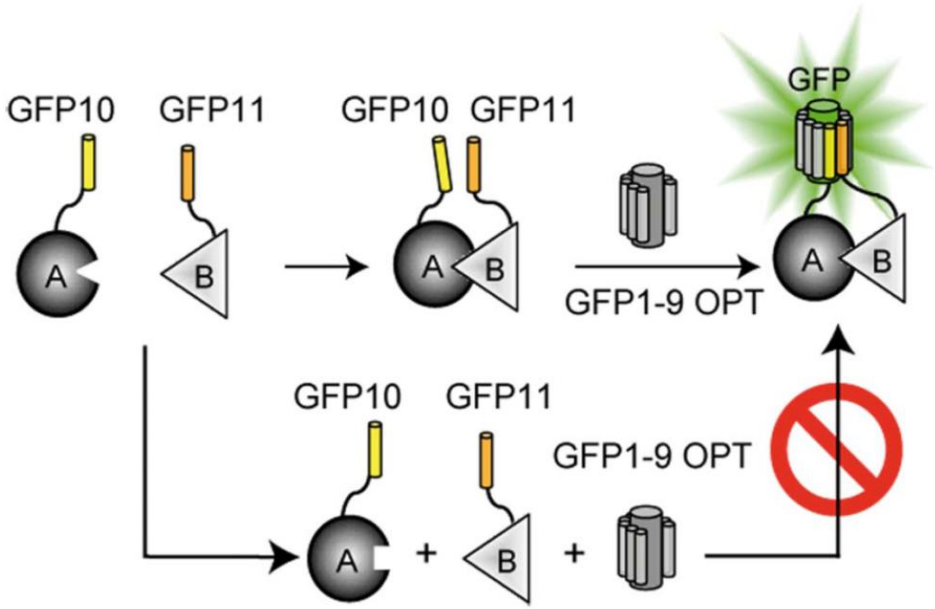
- **Poor real-time detection**
- **Irreversible BiFC formation**: pros and contra
- **False positive**: independent fluorescent protein fragment associations
- **Use of fusion proteins**: Altering protein structure and steric hindrance
- **Temperature dependence**
- **Unknown relationship of the interaction:** direct or indirect**?**

SCIENTIFIC REPORTS

OPEN

A New Protein-Protein Interaction Sensor Based on Tripartite Split-GFP Association

Cabantous S. et al., *Scientific reports*, October 2013



Directed evolution of GFP

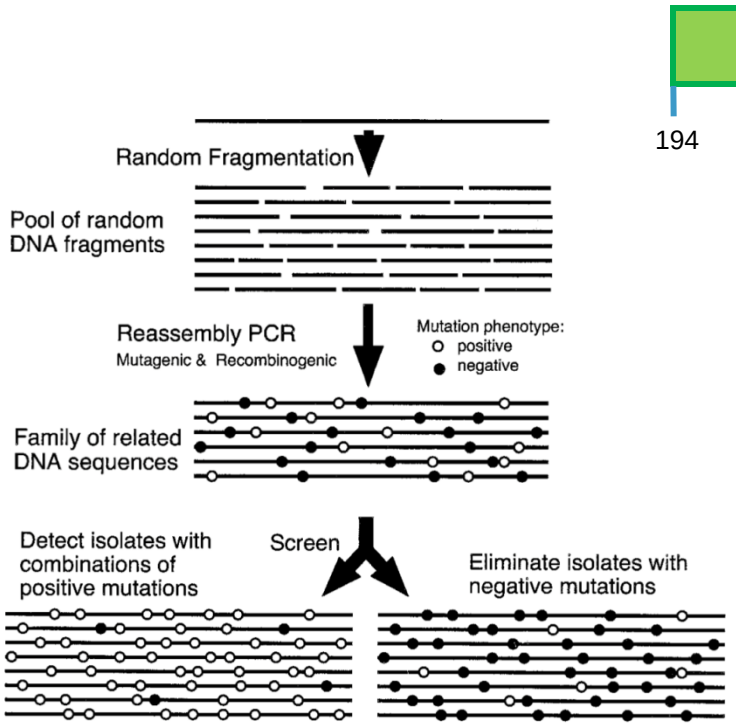
1) Mutation: The gene encoding GFP is mutated and/or recombined at random to create a large library of gene variants by PCR and DNA shuffling.

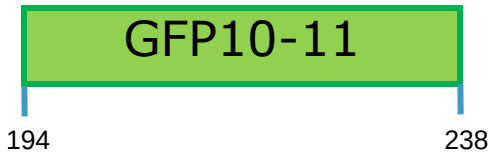
2) Selection: The library is tested for the presence of mutants (variants) with:

•Higher solubility
•Efficient complementation with GFP1-9

3) Sequencing: The variants identified in the screen are sequenced.

DNA shuffling of GFP10-11 cassette





- fragmentation with DNase I: fragments of 10-50 bp are produced
- reassembly by thermocycling in the presence of a DNA polymerase: the fragments prime each other based on homology
- amplification of reassembled products by a conventional PCR.

Point mutations may be generated during each of these steps.

β strand — linker — β strand

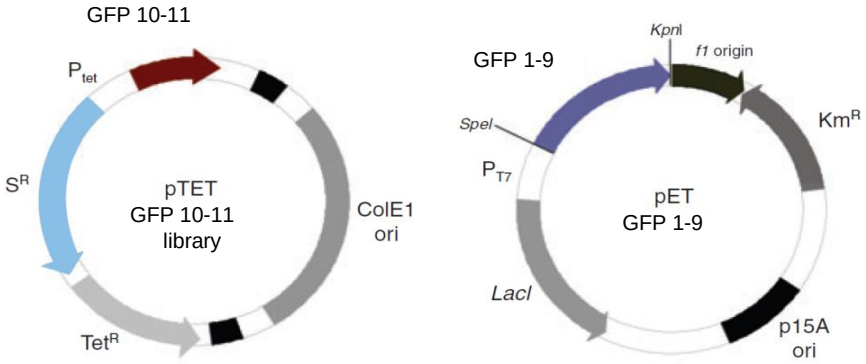
GFP10 *NdeI* *BamHI* **GFP11**

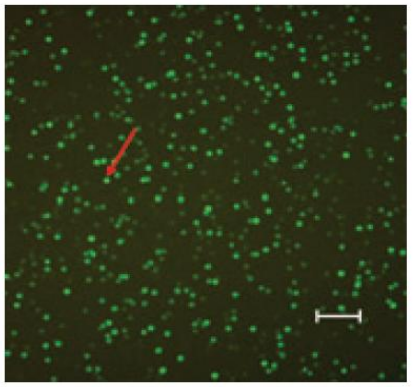
```
WT    YTMGLPDNHYLSTQSVLSKDPNGTGGSGGGSHMGGGSGSGGGSGGGSTSEKRDHMVLLEFVTAAGITGAS*
SM1   YTMDLPDNHYLSTQTILLKDLNGTGVGSGGGSHMGGGSGSGGGSGGGSTSEKRDHMVLLEYVTAAGITDAS*
SM2   YTMDLPDNHYLSTQTILLKDLNGTGVGSGGGSHMGGGSGSGGESGGGSTGEKRDHMVLLEYVTAAGITGAS*
SM3   YTMDLPDNHYLSTQTILLKDLNGTGVGSGGGSHMGGGSGSGGGSGGGSTSEKRDHMVLLEYVTAAGITDAS*
SM4   YTMDLPDNHYLSTQTILLKDLNGTDVGSGGGSHMGGGSGSDGGSGGGSTGEKRDHMVLLEYVTAAGITGAS*
SM5   YTMDLPDNHYLSTQTILLKDLNGTGVGSGGGSHMGGGSGSGGGSGGGSTSEKRDHMVLLEYVTAAGITDAS*
SM6   YTMDLPDNHYLSTQTILLKDLNGTGGGSGDGCHMDGGSGSGGGSGGGSTGEKRDHMVLLEYVTAAGITGAS*
```

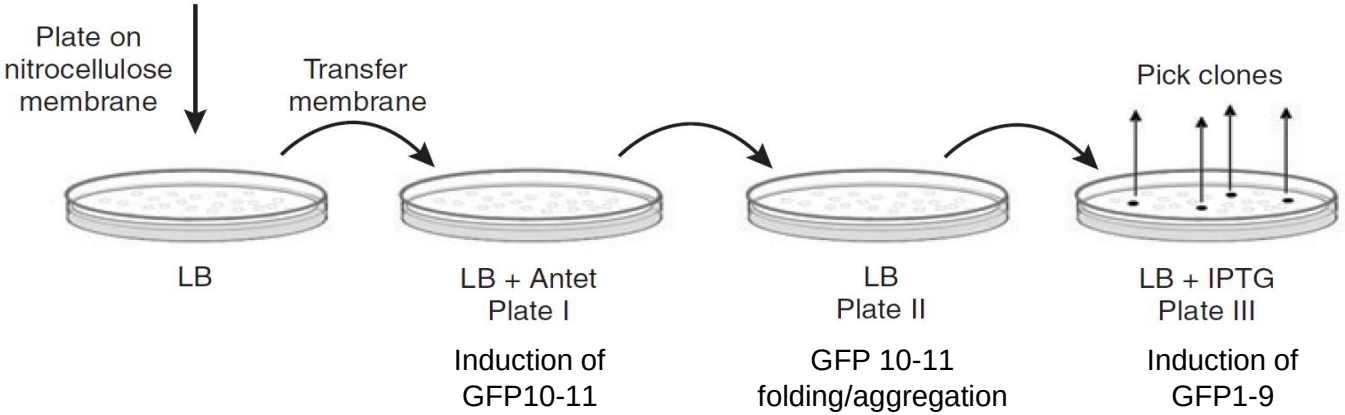
Selection of GFP10-11 for complementation with with GFP1-9

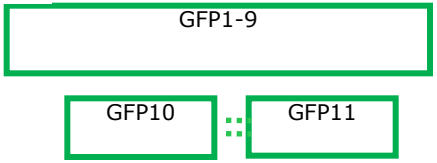
Sequential induction protocol

Modified version from Cabantous S., NATURE METHODS 2006







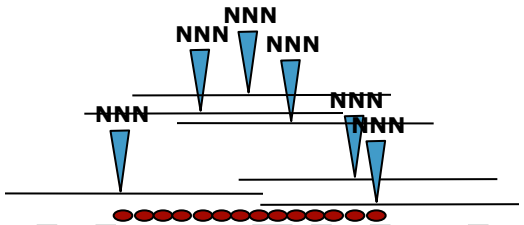


Optimization of GFP10 solubility

- **Primer doping mutagenesis** of GFP10 by 14 Oligo containing an NNN coding degeneracy at the central target aa of the GFP10 M1 domain

- A 60% soluble **protein HSP** was inserted in the NdeI:BamHI cloning site

- **GFP10-l1-HSP-l2-GFP 11** variants expressed in pTET SpecR vector are screened for the *in vivo* complementation assay by sequential induction protocol



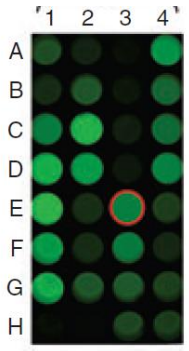
GFP10 M1 YTMDLPDNHYLSTQTILLKDLNGTGVGSGGGSHM-(HPS)-GSGGGSGGGSTSEKRDHMVLLEYVTAAGITDAS*

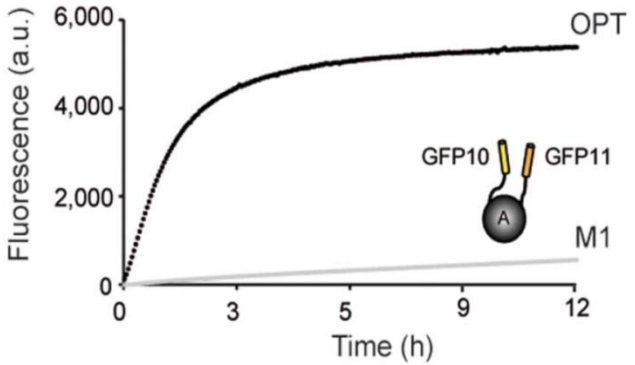
GFP10 M2 YTMDLPD**D**HYLSTQTIL**S**KDLNGT**D**VGSGGGSHM-(HPS)-GSGGGSGGGSTSEKRDHMVLLEYVTAAGITDAS*

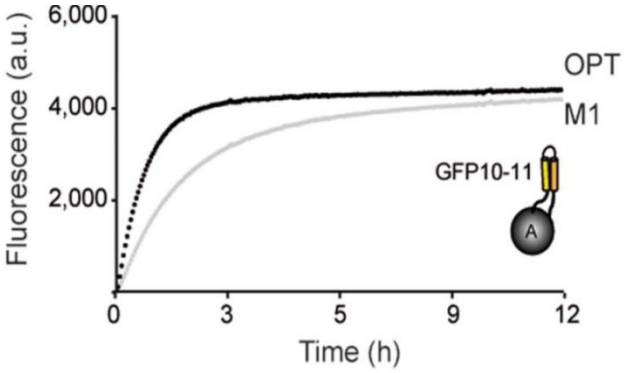
Evolution of GFP 1-9 and *in vitro* selection

- library of GFP 1-9 in pTE p15 vector
- recGFP 1-9 production
- recovery from inclusion bodies and refolding
- mix of equal amount of GFP 1-9 refolded pellet fractions with soluble GFP10-A-GFP11 fusion or GFP10-11 peptide
- fluorescence kinetics measurement over time in a plate reader

```
                    10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GFP1-9 SF           MSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPVPWPTL
GFP1=9 M1           .R.............I...........F..............S...................
GFP1=9 OPT          .R.............I...........F..........I...S...................
                    70        80        90        100       110       120
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GFP1-9 SF           VTTLTYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLV
GFP1=9 M1           ....................................................................
GFP1=9 OPT          ...........................................Y........................
                    130       140       150       160       170       180
                    ....|....|....|....|....|....|....|....|....|....|....|....|
GFP1-9 SF           NRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFKIRHNVEDGSVQLAD
GFP1=9 M1           ...........................................................T........
GFP1=9 OPT          ..............................K.........N.......T................
                    190
                    ....|....|....|.
GFP1-9 SF           HYQQNTPIGDGPVLLP*
GFP1=9 M1           ................*
GFP1=9 OPT          ................*
```



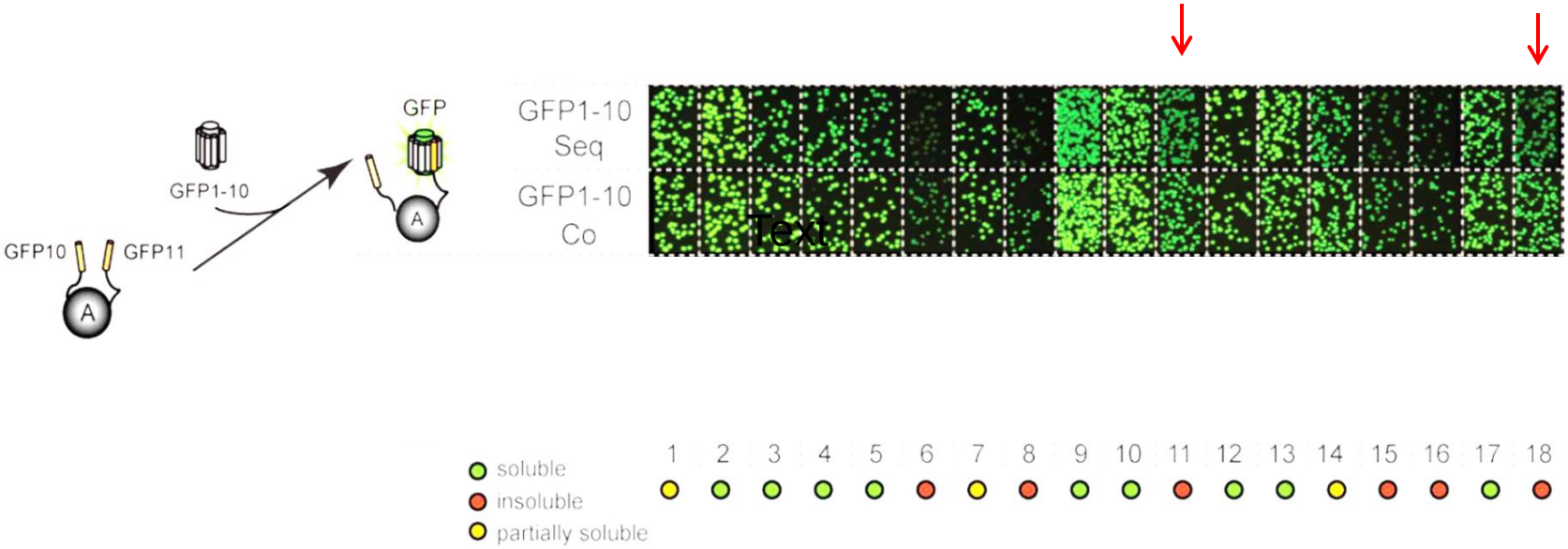




Complementation curves of GFP 1-9 M1 and GFP 1-9 OPT with GFP10-proteinA-GFP11 fusion protein or GFP10/11 hairpin

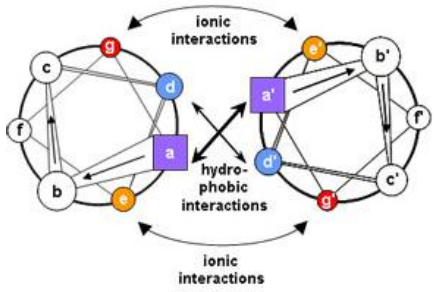
Complementation of the tripartite split GFP *in vivo*

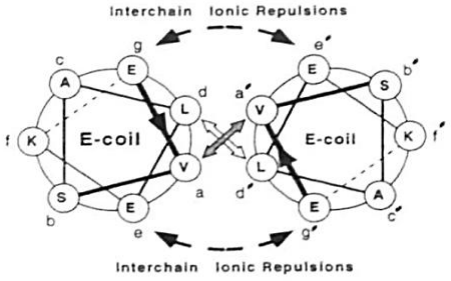
- Cloning of 18 *P. aerophilum* test protein of known solubility as GFP10-POI-GFP11 "sandwiches" in pTET vector (ANTET induction)
- in vivo solubility assay by complementation with GFP 1-9

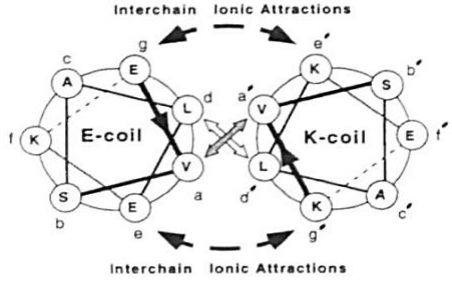


No false positive detected by GFP 1-9 OPT

Coiled-coil heterodimerization test to validate the tripartite GFP as a protein-protein interactor sensor







Attractive coiled coil pair

K1 coil KVSALKENVSALKEKVSALTEKVSALKEKVSALKE

E1 coil KVSALENEVSALEKEVSVLEKEVSALEKEVRALEK

Repulsive coiled coil pair

E1 coil KVSALENEVSALEKEVSVLEKEVSALEKEVRALEK

E1 coil KVSALENEVSALEKEVSVLEKEVSALEKEVRALEK

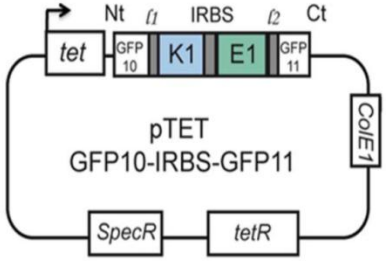
Coiled-coil heterodimerization test to validate the tripartite GFP as a protein-protein interactor sensor

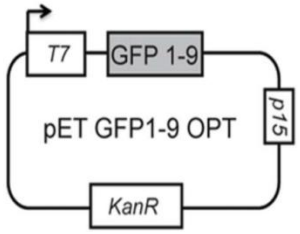
Pair-wise combination of

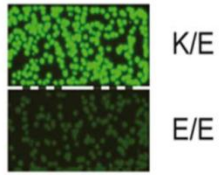
GFP10-K1 | E1-GFP11

or

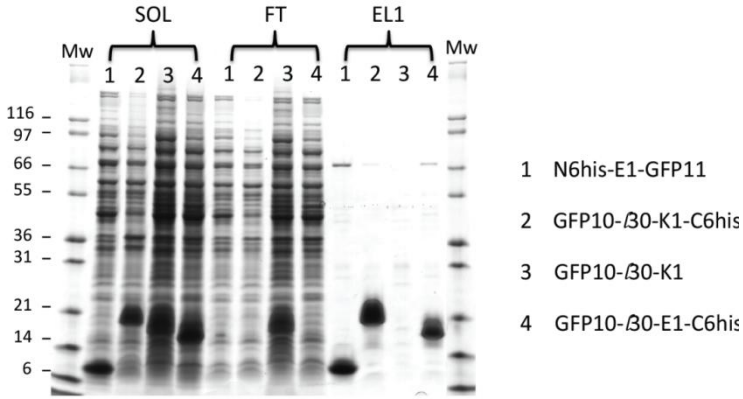
GFP10-E1 | E1-GFP11



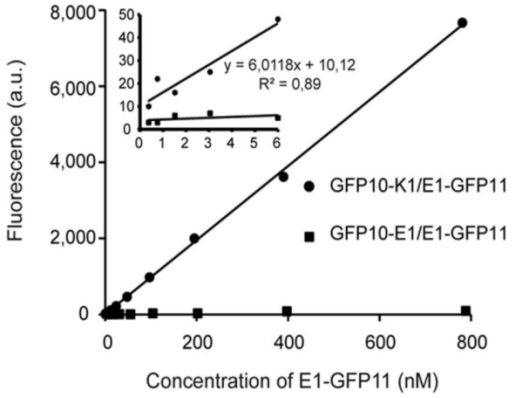


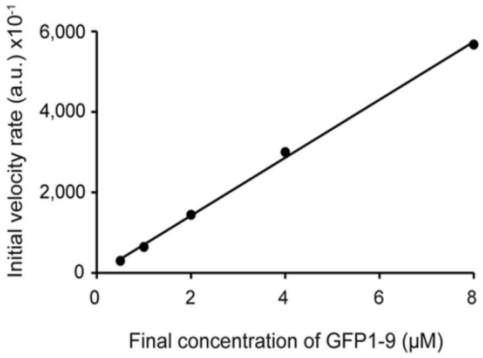


Specific detection of interacting partners



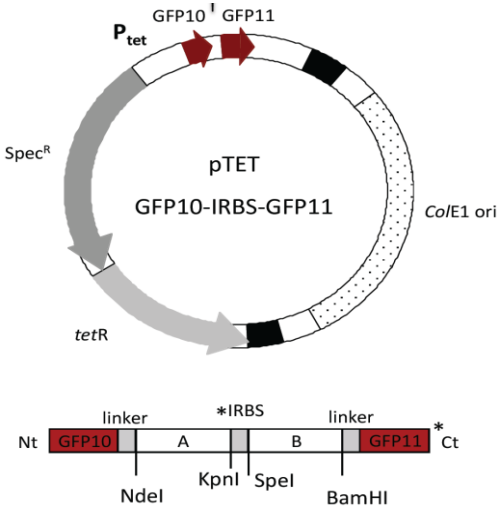
His-tagged protein purification by metal affinity resin

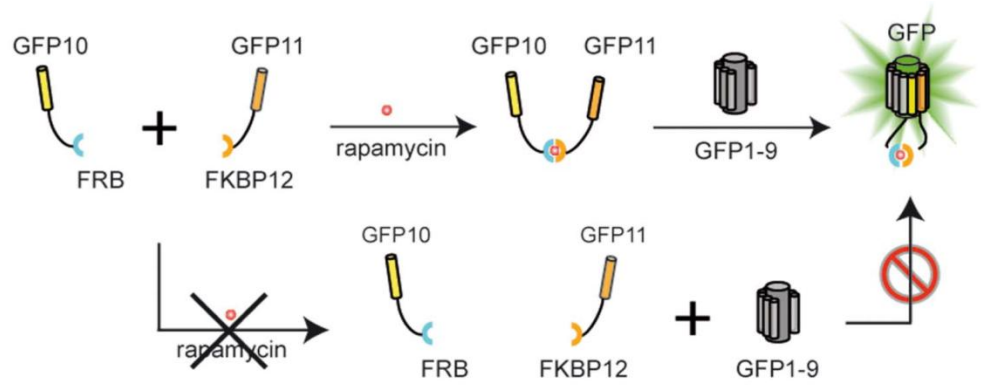




Sensitivity down to 1 nM E1/K1 coil

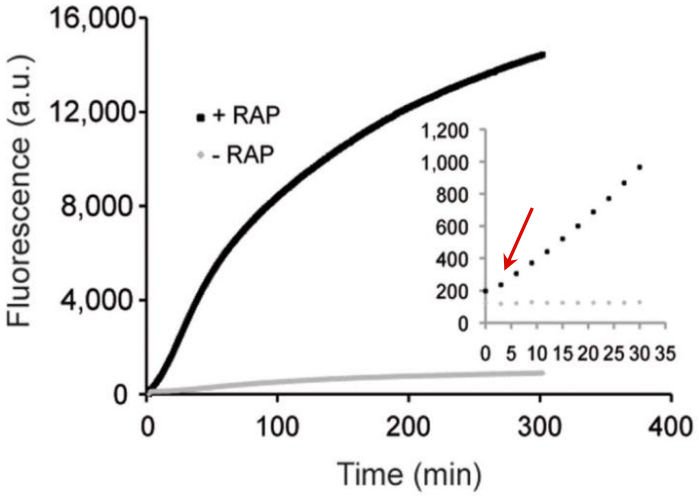
Does the tripartite GFP sensor detect dynamic protein-protein interactions?





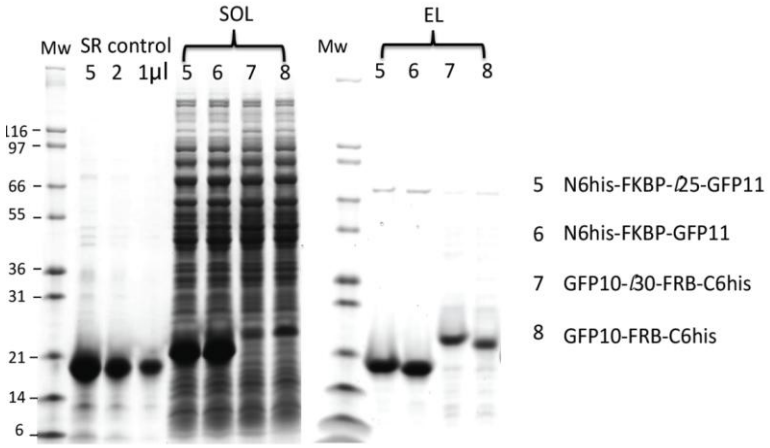
In Vitro complementation assay

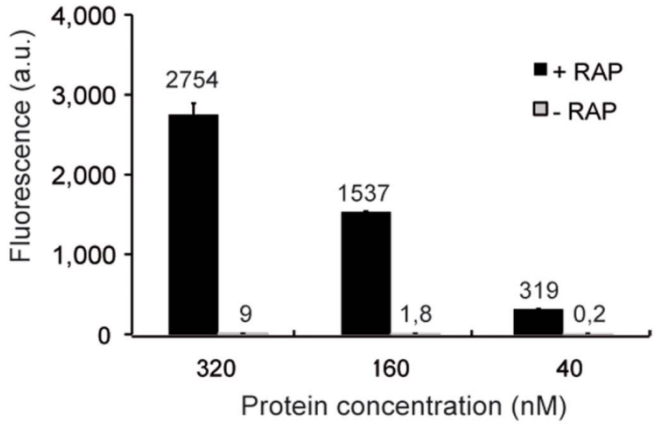
Soluble extracts of *E. Coli* cells expressing GFP10-FRB and FKBP12-GFP11 fusion proteins
+
purified GFP 1-9
+
Rapamycin

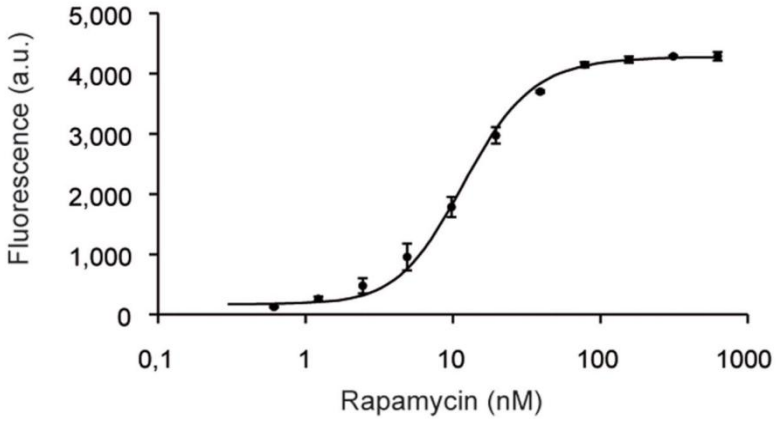


Rapid detection of rapamycin-induced FRB-FKBP interaction

Quntitative analysis of rapamycin-induced protein-protein interaction by the tripartite GFP sensor

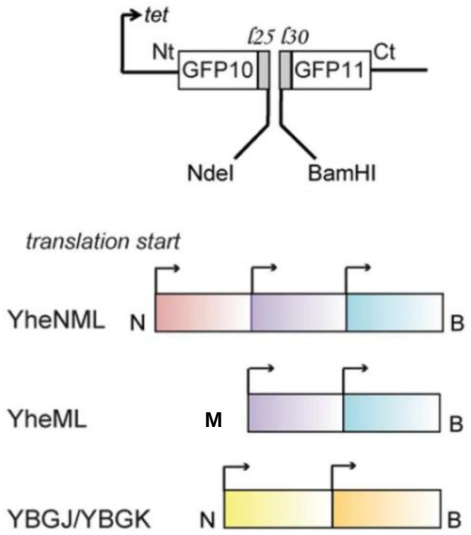


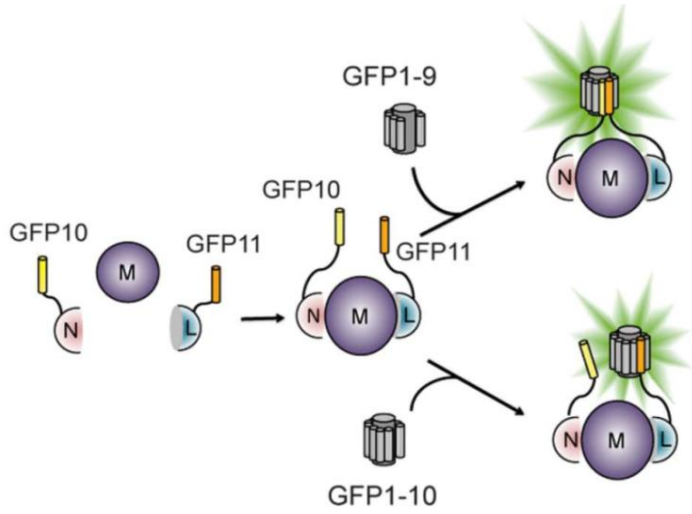


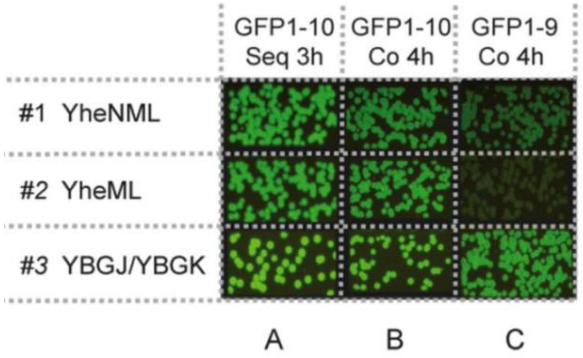


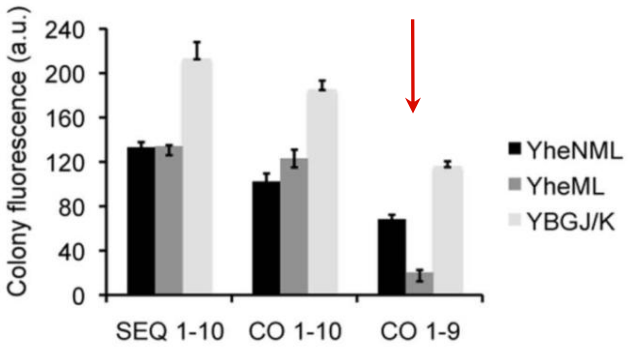
Rapamycin dose-response-induced FRB-FKBP interaction

Monitoring protein complex involving multiple interacting partners





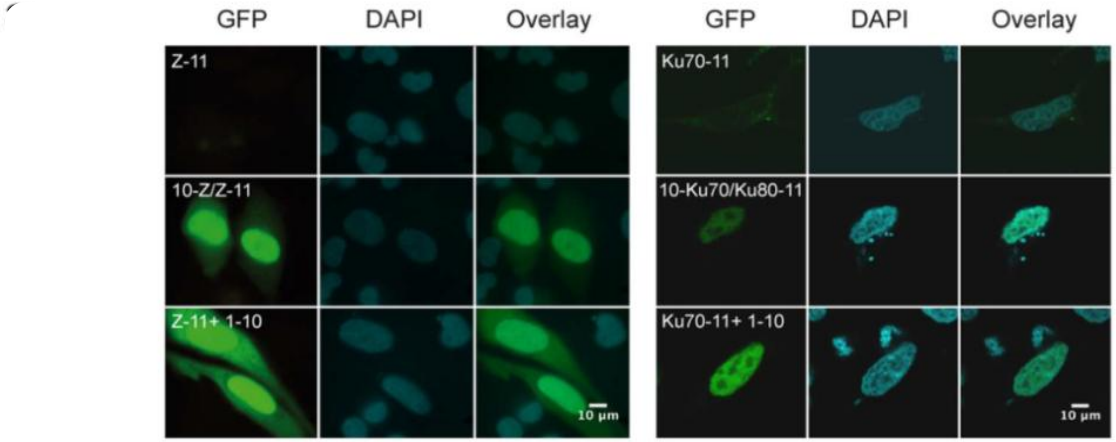


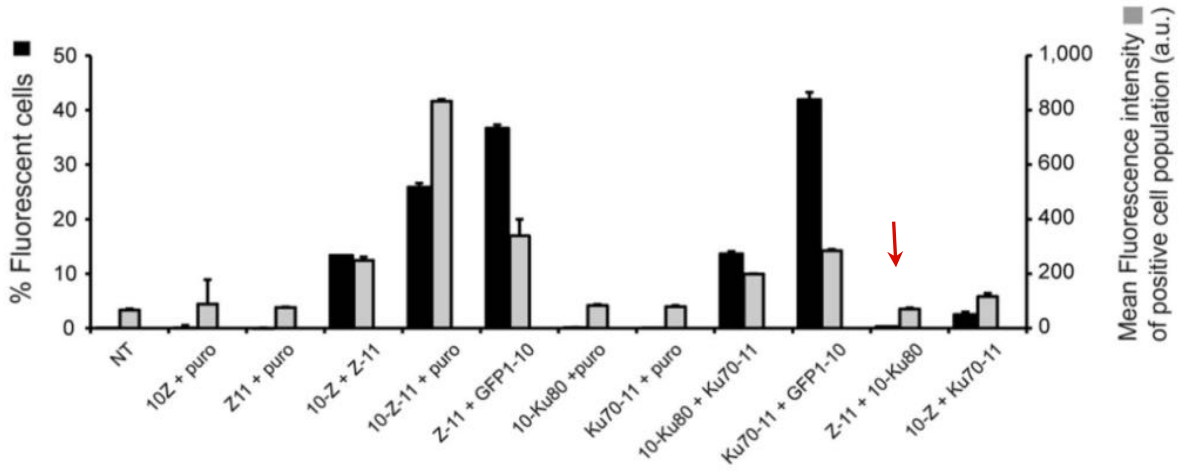


Visualization of protein-protein interaction by tripartite GFP in mammalian cells (1)

Yeast **GCN4 leucin zipper** coiled-coil heterodimerization in CHO cells expressing GFP 1-9

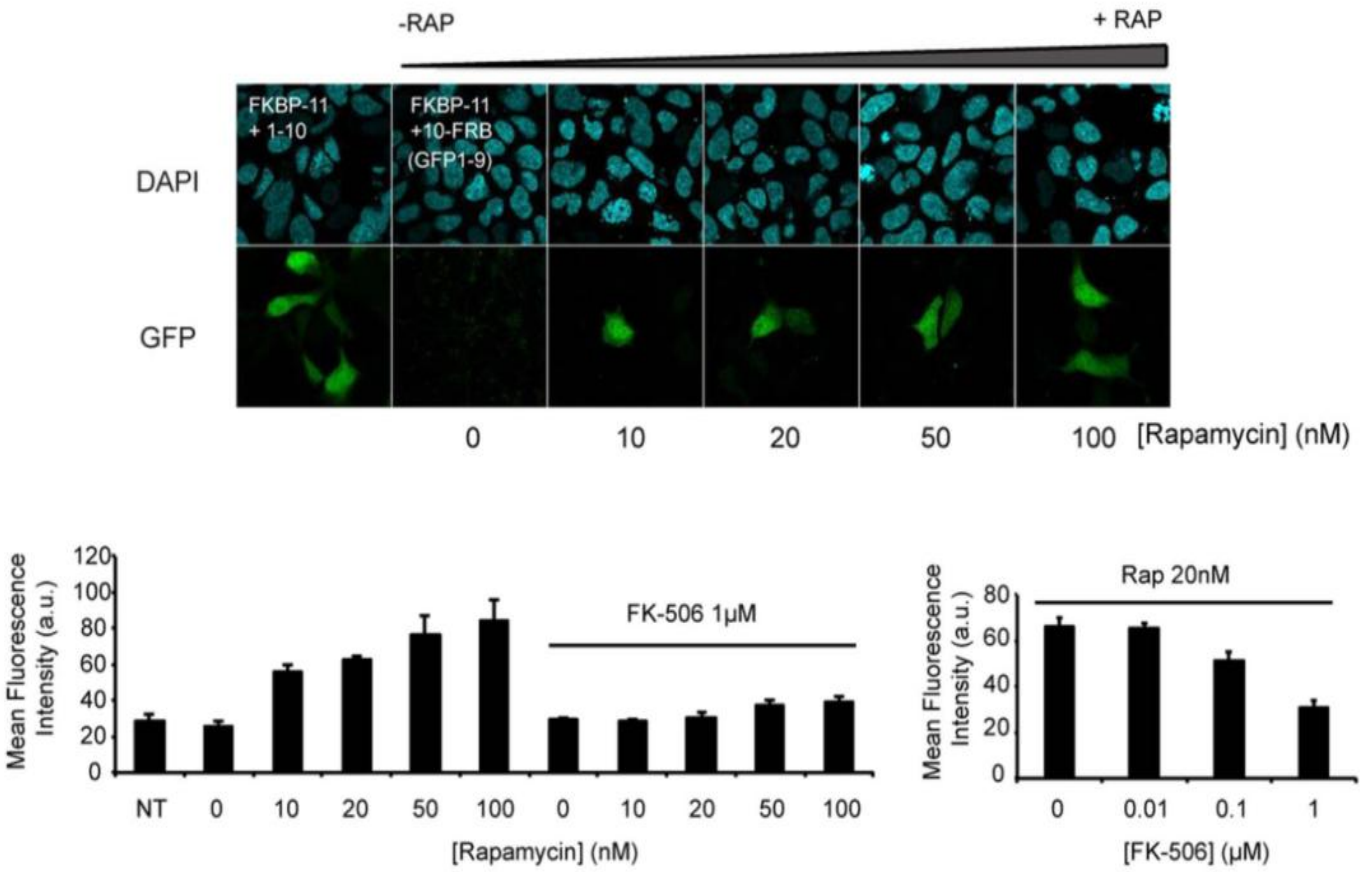
Ku70-GFP11 and **Ku80-GFP10** in HEK 293 cells expressing GFP1-9





FACS analysis

Visualization of protein-protein interaction by tripartite GFP in mammalian cells (2)



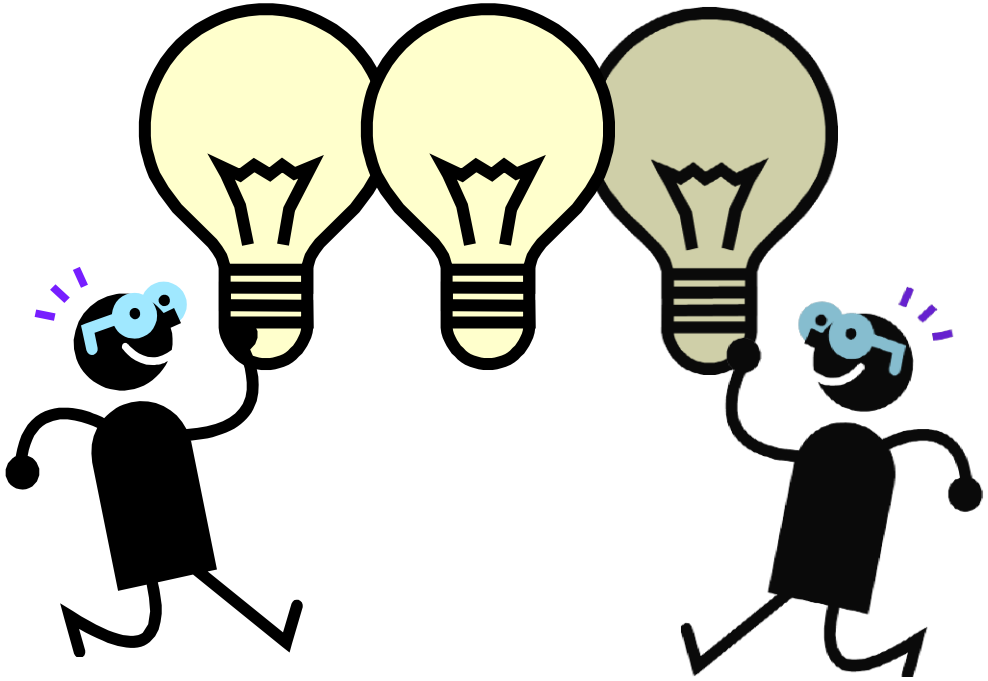
Tripartite split GFP complementation in monitoring inhibitors of protein-protein interaction by small molecules

“Advancement” in BiFC

- **Small engineered β strands (20 aa): minimize protein interference and aggregation**
- **High sensitivity**
- **Induced-interaction can be detectable within a few minutes**
- **Soluble tagging system: production of high yield of fusion proteins in *E. Coli* at 37°C.**
- **No temperature dependent**

- **Something more? Detection of protein protein interaction within the intracellular compartment…**

People interaction generating ideas



Thank you for your attention!