Interazioni proteina-proteina

Organisms are complex systems of chemical compounds which, through interactions with each other and the environment, play a wide variety of roles.

Protein-protein interactions

- Genetic approach
  - Yeast 2 Hybrid (Y2H)
- Biochemical approach
  - Co-immunoprecipitation (Co-IP)
  - Fusion protein affinity chromatography
- Cell-biology approach
  - Fluorescence resonance energy transfer (FRET)
  - Bimolecular Fluorescence complementation (BiFC)

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Y2H allows to:

- Test interactions between two known proteins
- Screen for interactors of a known protein

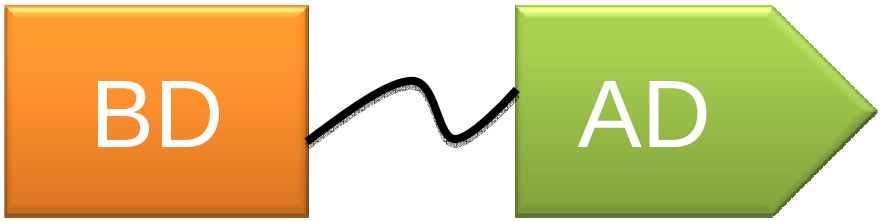
What kind of interactions can you test?

- Interactions involving cytosolic and nuclear proteins, protein fragments or protein domain

Y2H-I

Based on the fact that some transcription factors (TF) have two domains: one that binds to a promoter DNA sequence (Binding Domain = **BD**) and another that activates transcription (Activation Domain = **AD**) by interacting with the RNA polymerase II complex.

Transcription factor can not activate transcription unless DNA-binding domain is physically associated with an activating domain (Fields and Song,1989) .



Y2H-II

Screen based on auxotrophic selection!!!

Auxotrophy= the inability of an organism to synthesize a particular organic compound required for its growth.

Most yeast strains used for 2H have a lesion in either *URA3*, *LEU2*, *HIS3*, *TRP1* and/or *ADE2* which allows selection for yeast cells that were transformed with plasmids that carry the corresponding gene by growth in the absence of the appropriate amino acids.

HIS3 reporter - *Screen on His $^{-}$ media* (usually need to add 3-amino-1,2,4-triazole [3-AT] to increase selectivity)

TRP1 reporter - *Screen on Trp $^{-}$ media*

MET15 reporter - *Screen on Met $^{-}$ media*

LEU2 reporter - *Screen on Leu^{-} media*

HIS3, TRP1, MET15, LEU2, are genes that encode essential enzymes for *de novo* synthesis of the amino acids L-histidine, L-leucine, L-tryptophan, and L-methionine, respectively.

ADE2 = phosphoribosylaminoimidazole carboxylase, which catalyzes the sixth step in the *de novo* biosynthesis of purine nucleotides - *Screen on Ade $^{-}$ media.*

URA3 = orotidine-5-phosphate decarboxylase, an essential enzyme in pyrimidine biosynthesis in Saccharomyces cerevisiae - *Screen on Ura $^{-}$ media* (can do negative selection by adding FOA)

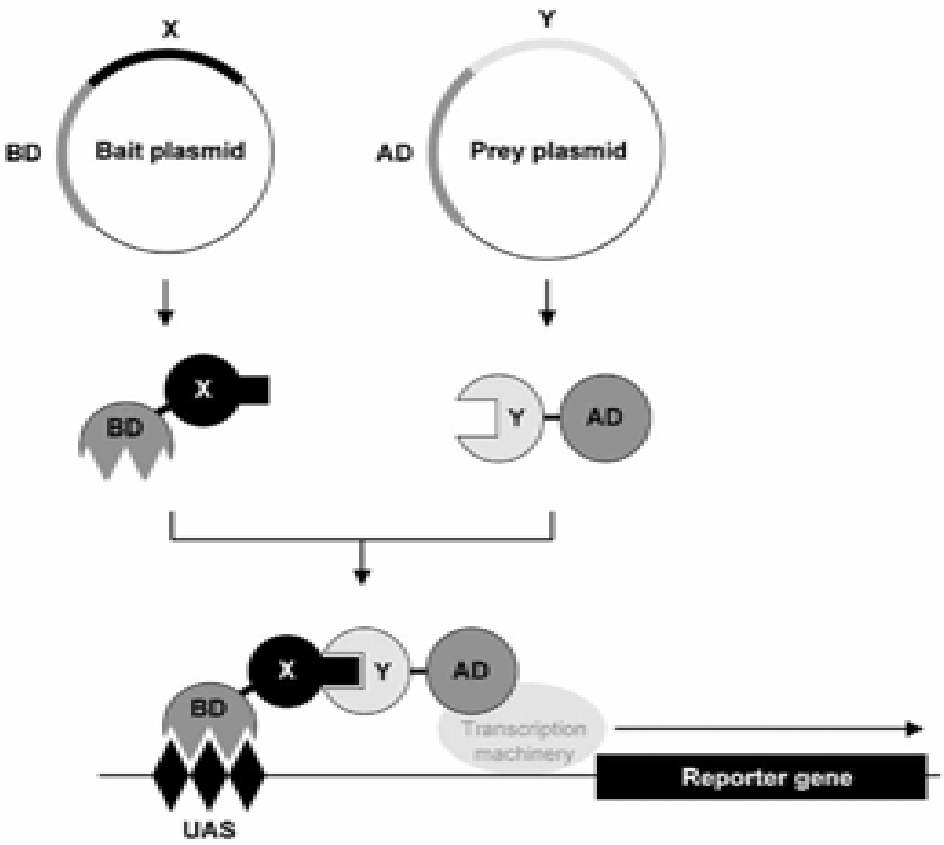
How does the system work?

Protein you are studying (**X**) = Bait
Fused to the DNA binding domain of a TF

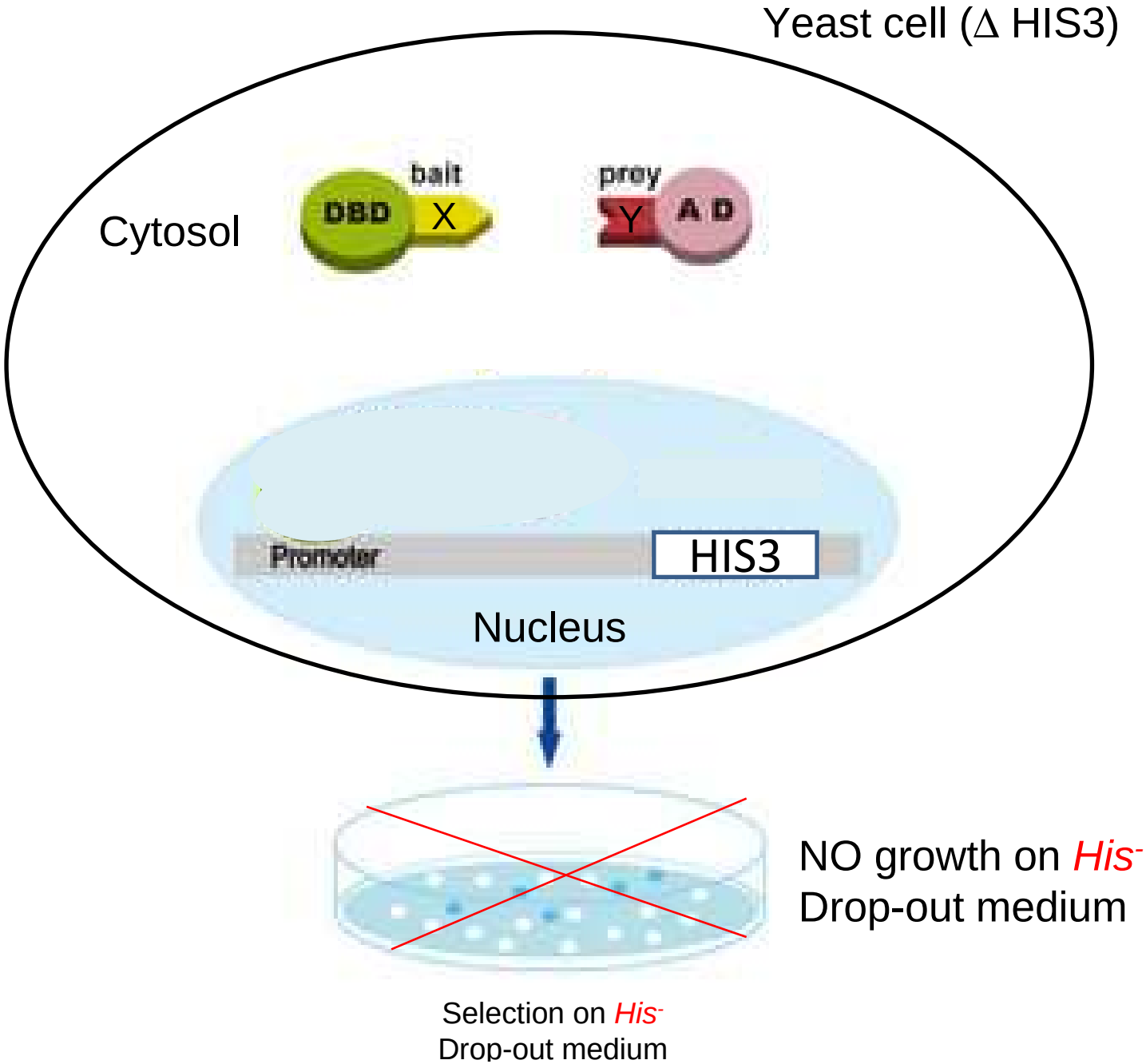
Protein(s) you are screening (**Y, Z**, etc,) = Prey
Fused to the activation domain of a TF

- **Transform** Bait and Prey plasmids into **yeast**

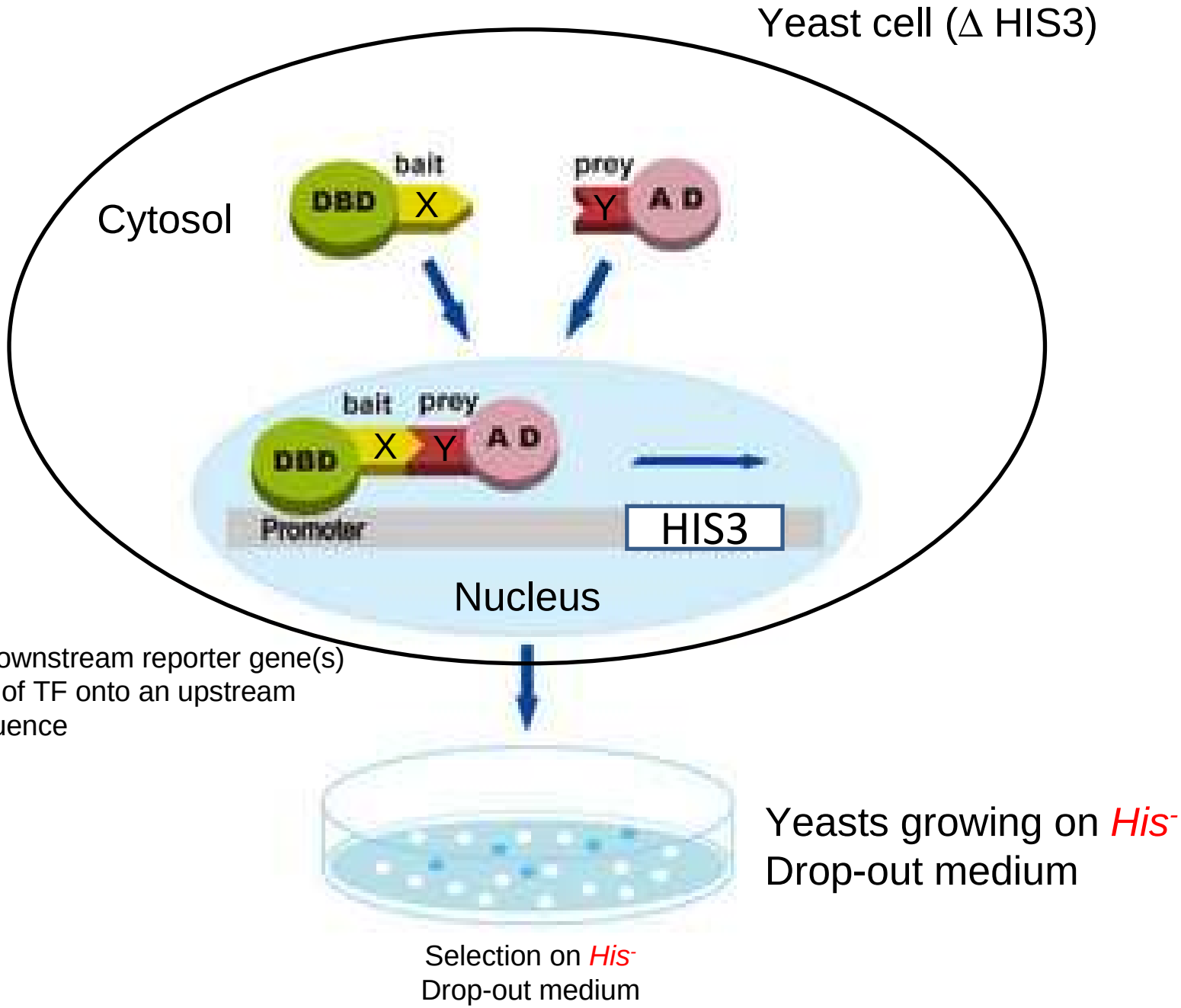
- **Measure** the **expression** of a **reporter** gene.



NO interaction between X and Y



Interaction between X and Y

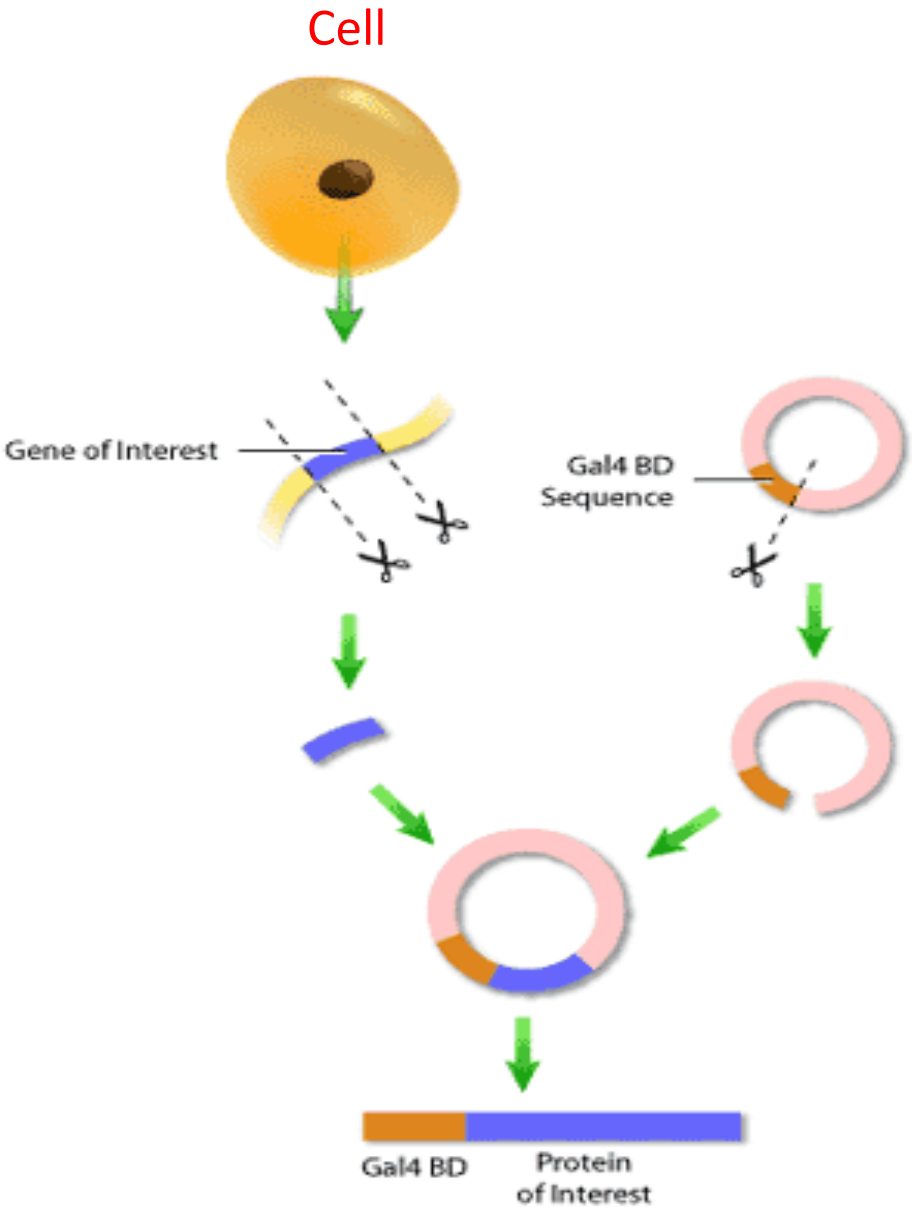


Activation of downstream reporter gene(s) by the binding of TF onto an upstream activating sequence

Yeasts growing on *His*$^{-}$ Drop-out medium

Selection on *His*$^{-}$ Drop-out medium

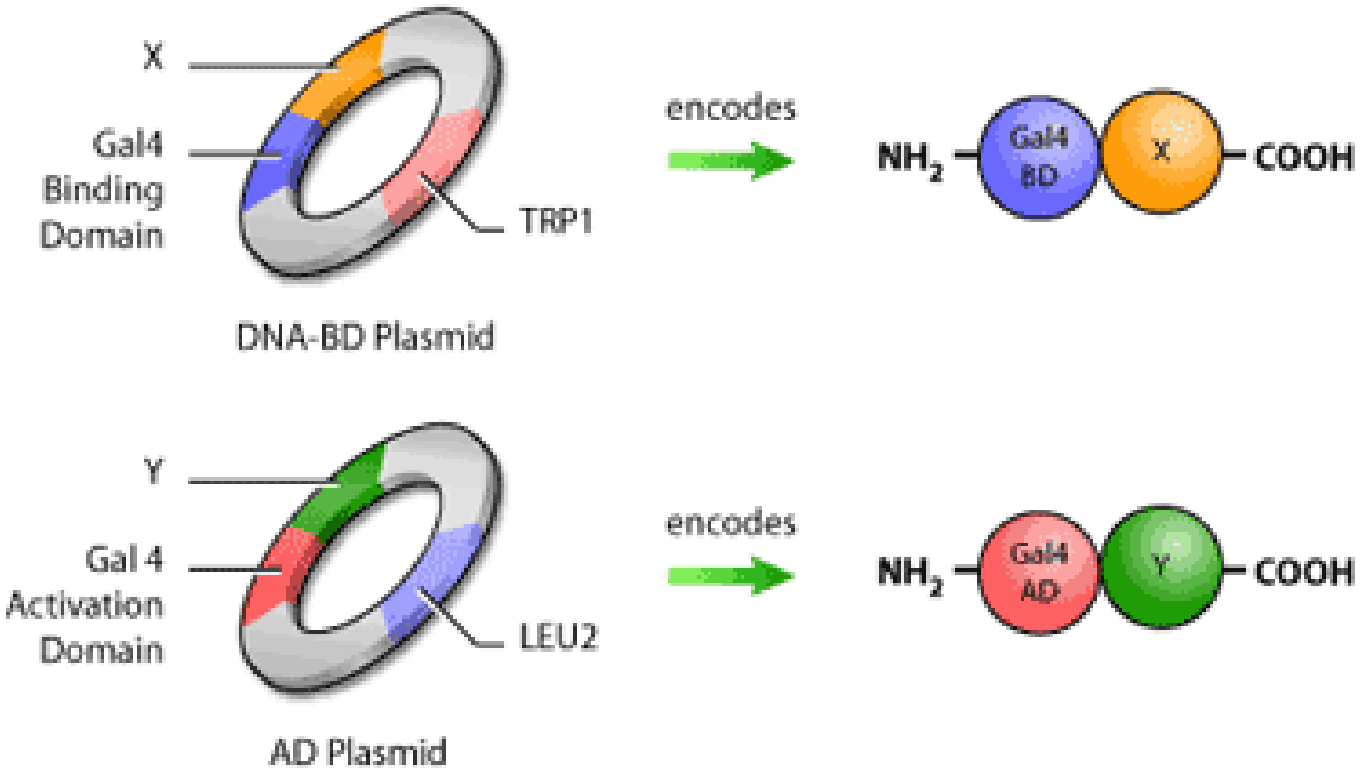
GAL4-based Y2H assay



Yeast TF Gal4

Plasmid construction. The '**bait**' and '**prey**' fusion proteins are constructed in the same manner. The 'bait' or 'prey' DNA is isolated and inserted into a plasmid adjacent to the GAL4 BD or AD DNA, respectively.

In addition to the fusion proteins, these plasmids also contain selection genes, or genes encoding proteins that contribute to a cell's survival in a particular environment.

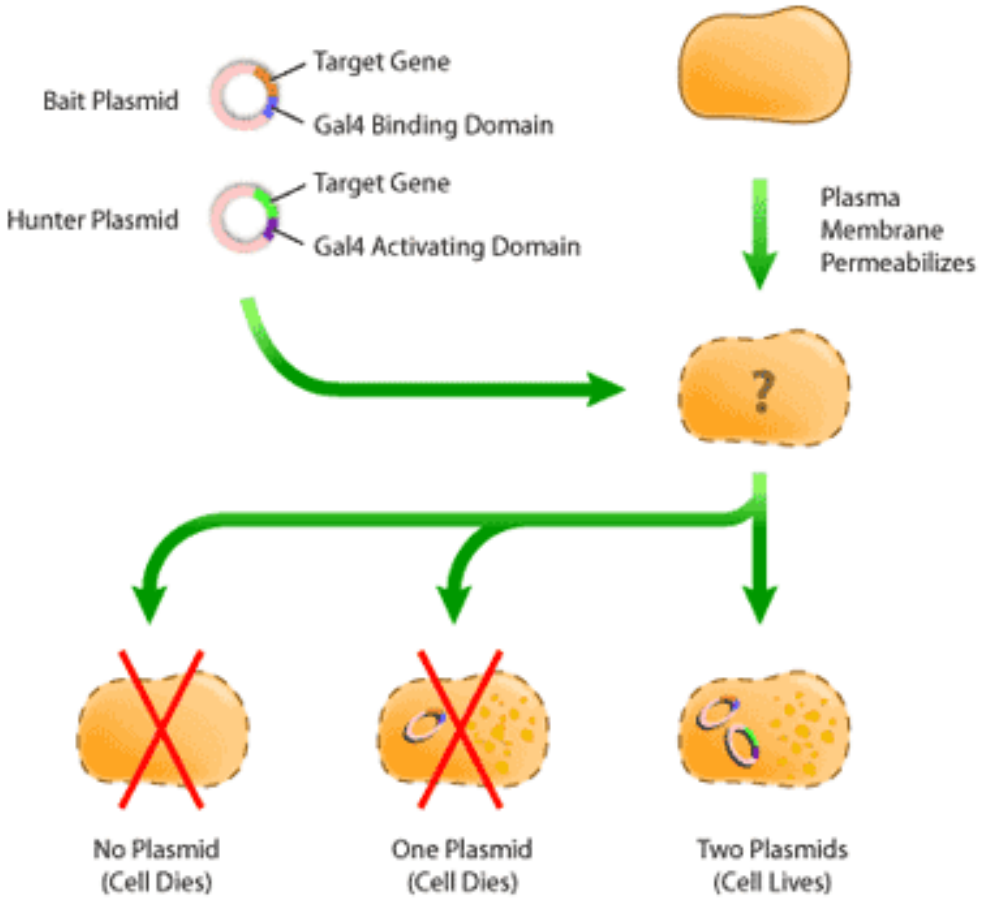


Yeast cells (Δ **LEU2**, Δ **TRP1**, Δ HIS3)

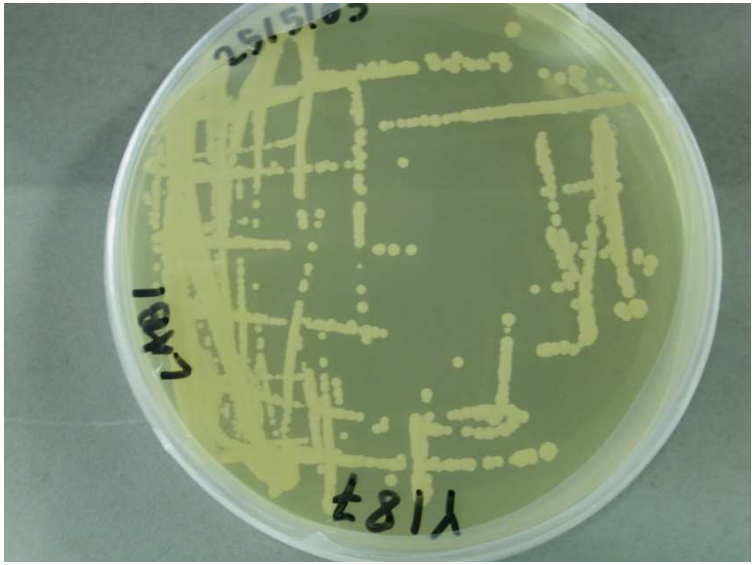
Transformation. The ‘bait’ and ‘prey’ plasmids are introduced into yeast cells.

Once transfection has occurred, cells containing both plasmids are selected on minimal media.

Only cells containing both plasmids have both genes encoding for missing nutrients, and consequently, are the only cells that will survive.

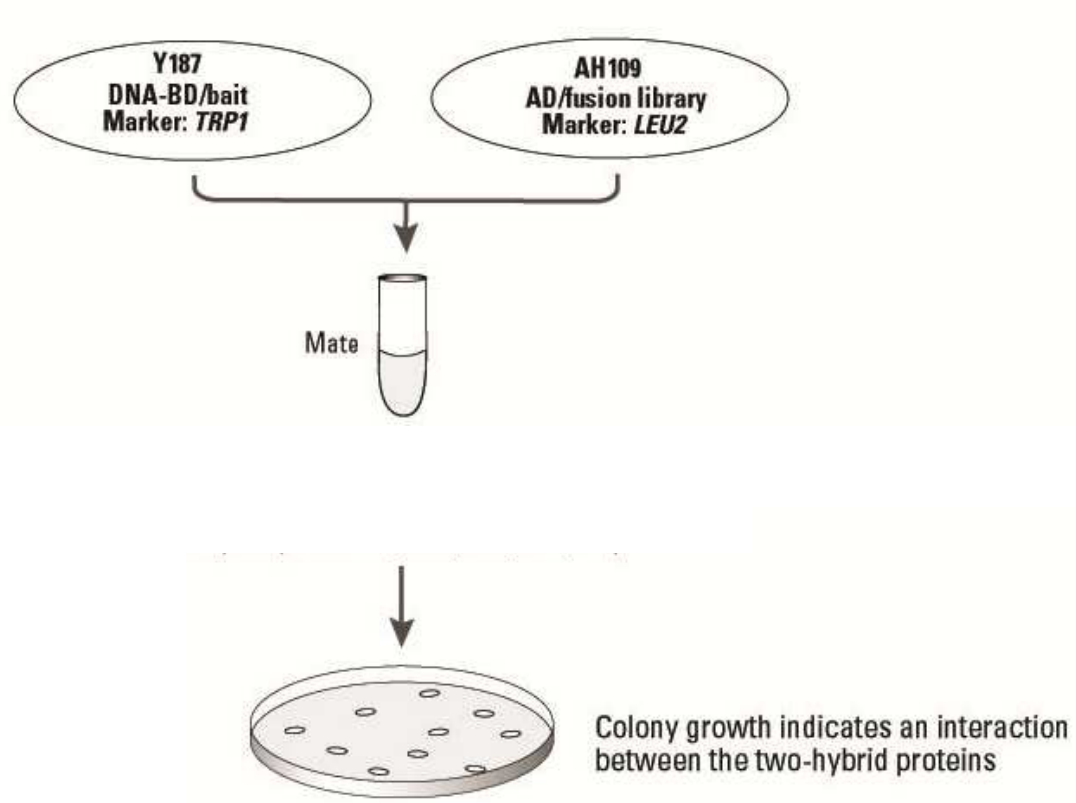


Selection on Trp^-/Leu^-
Drop-out medium



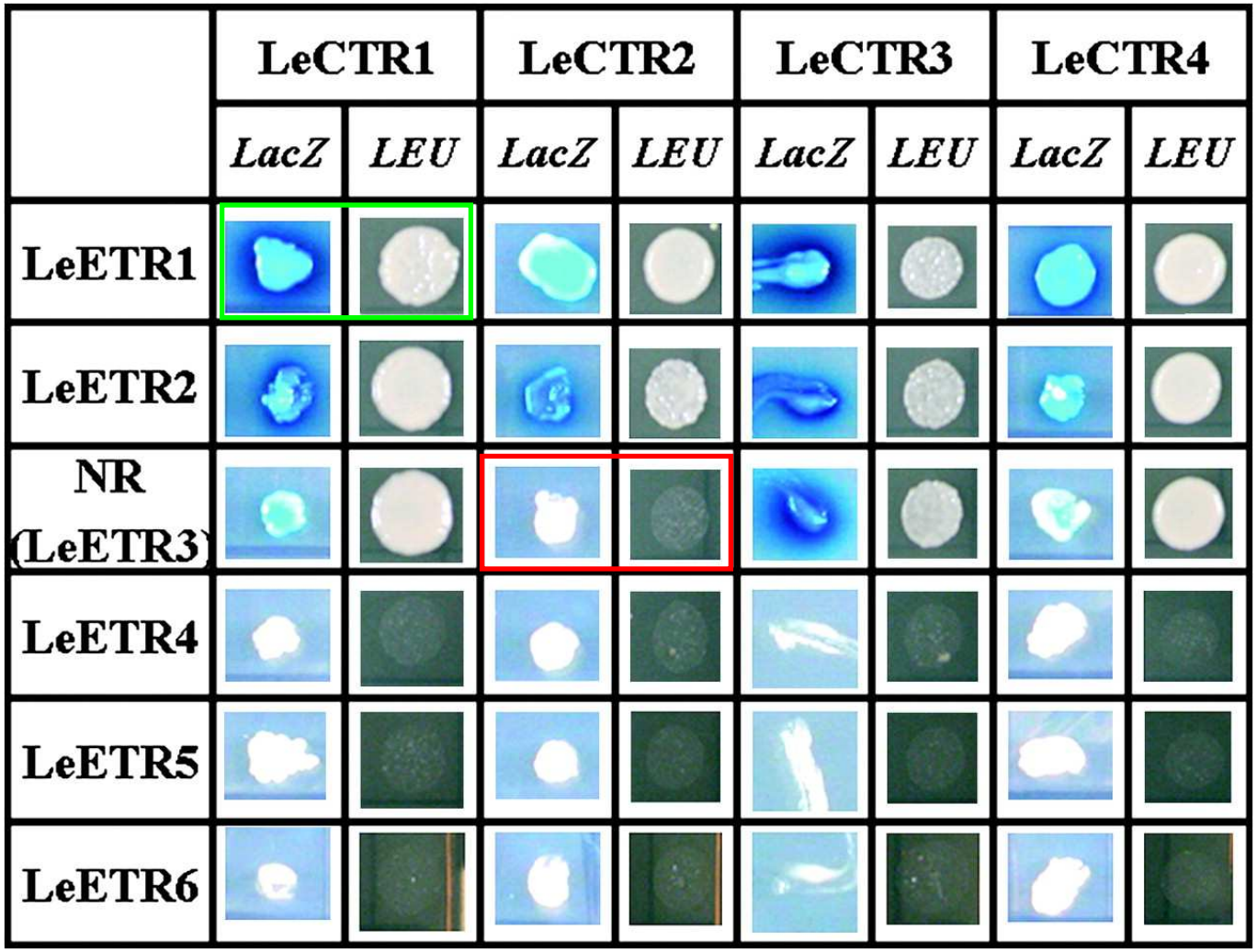
If bait (protein X) and prey (protein Y) interact,…
downstream reporter genes, for example HIS3 or a color marker like LacZ (β-galactosidase) is transcribed, resulting in histidine prototrophy or blue coloration of the yeast cells.

Yeast cells
(Δ **LEU2**, Δ **TRP1**, Δ **HIS3**)



Selection on Trp^-/Leu^- /His^-
Drop-out medium

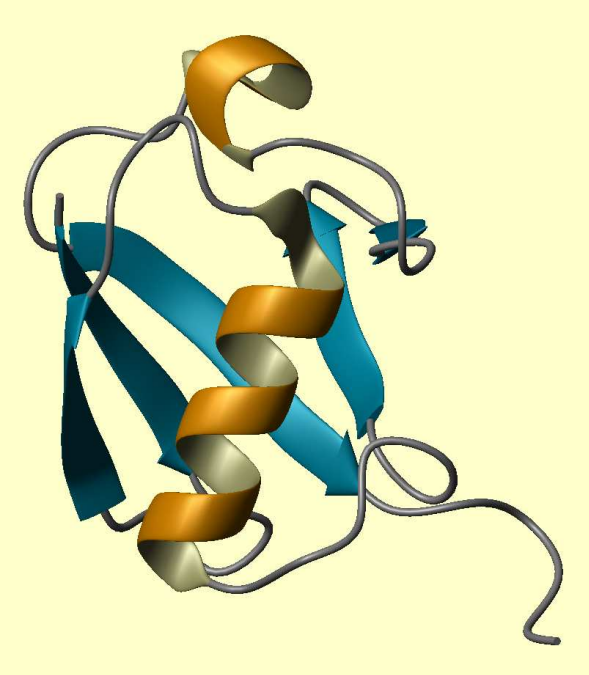
Yeast two-hybrid assay for interaction between tomato ethylene receptors and LeCTRs



Limits….

Not suitable for interactions involving integral membrane proteins or membrane-associated proteins

Split ubiquitin (UBQ) system for integral membrane proteins

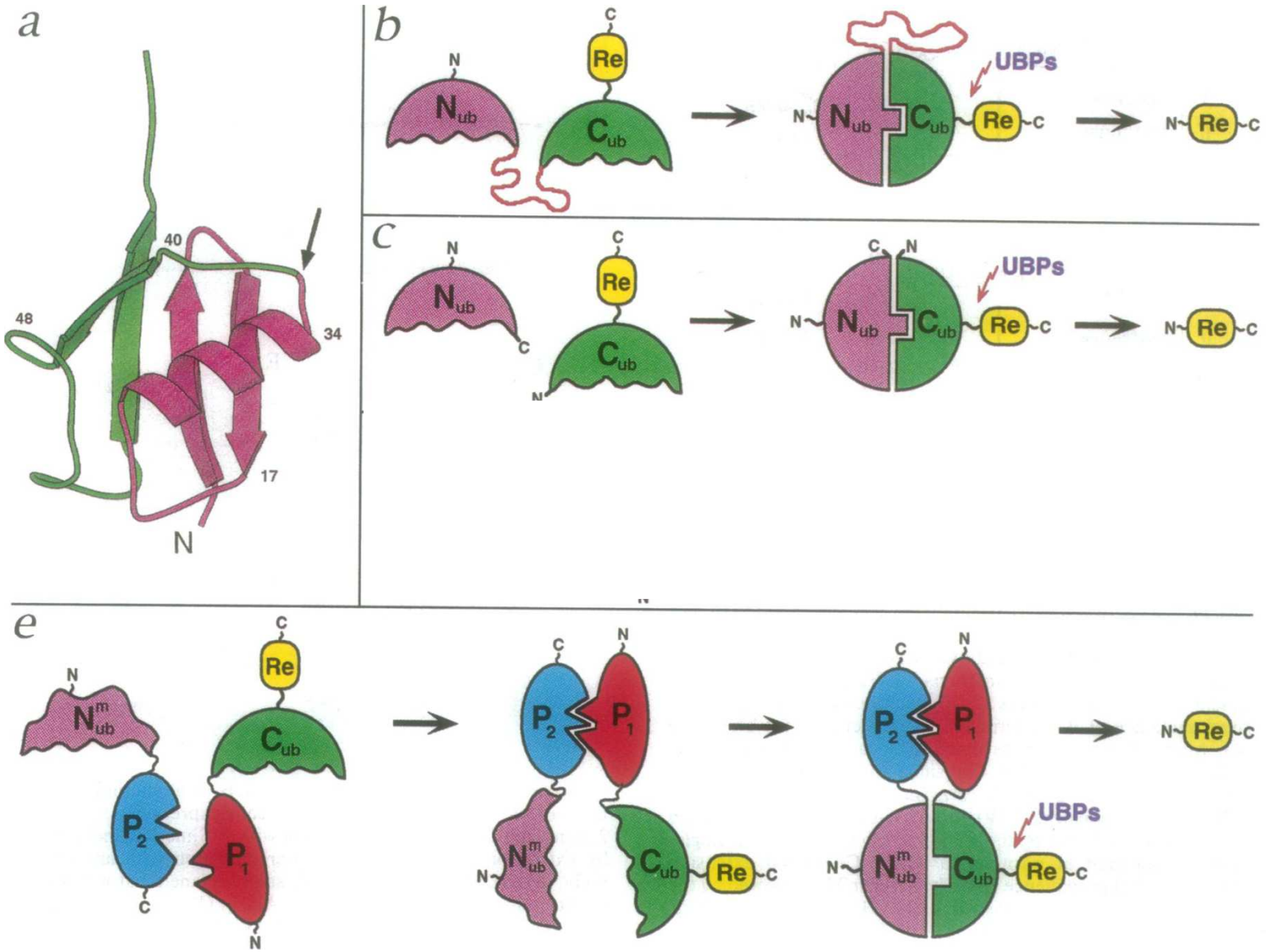


•UBQ is a small protein of 76 aa

•It acts as a “tag” for protein degradation

•Proteins fused to ubiquitin are rapidly cleaved *in vivo* by UBQ -specific proteases (UBPs)

•UBQ can be expressed in yeast as an N-terminal half (Nub) as well as a C-terminal half (Cub)



Insertion (wavy red line)
UBQ N-terminal (Nub; pink) and C-terminal (Cub; green) subdomains
Reporter protein (Re; yellow).
The insertion did not detectably interfere with the Ub folding, which was required for the in vivo cleavage of the fusion by Ub-specific proteases (UBPs; red lightning arrow), yielding the free reporter.

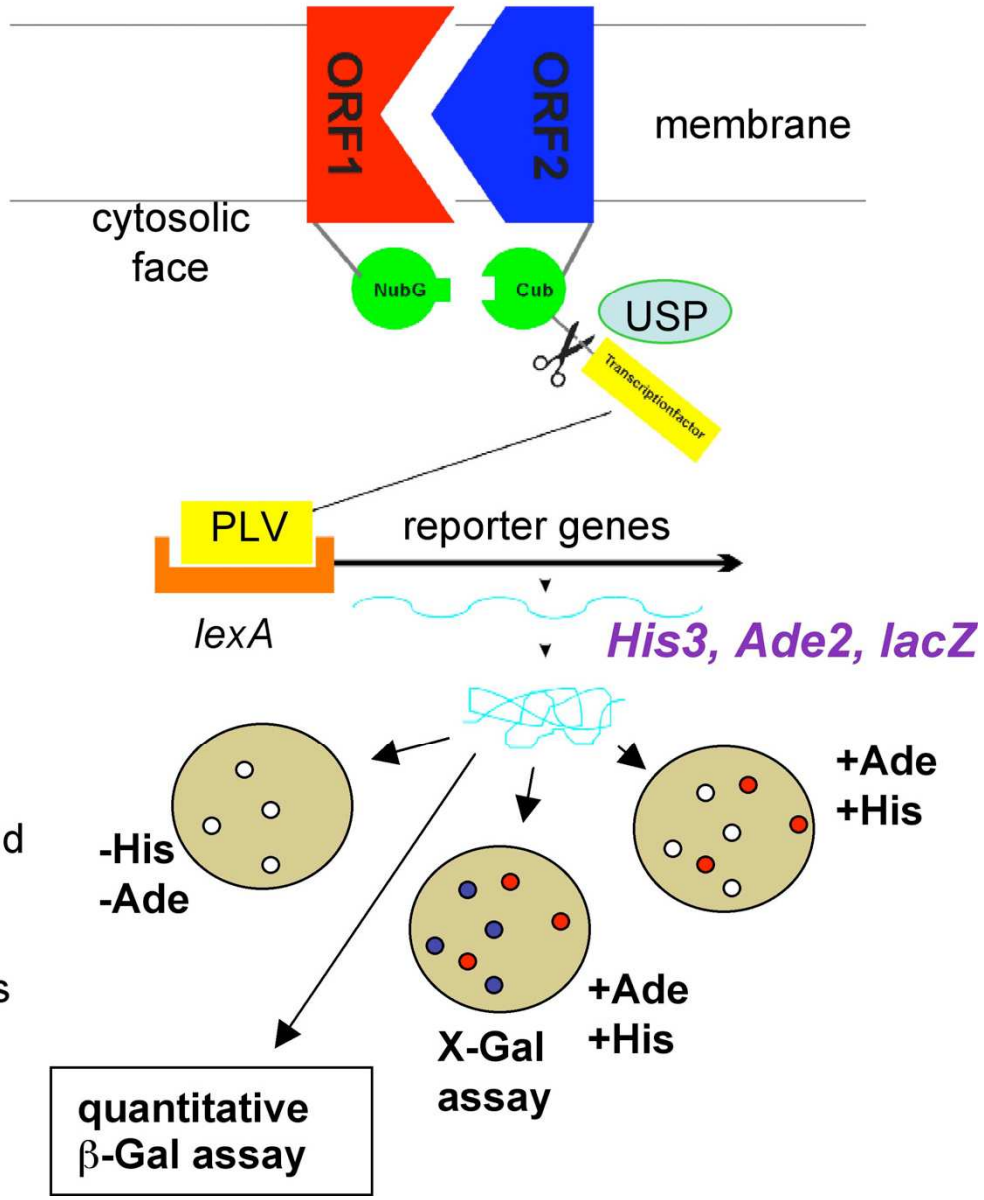
mbSUS: detection of interactions

1. N_{ubG}- and C_{ub}-fusions reconstitute at the membrane in yeast

2. USPs (Ub specific proteases) cleave PLV

3. PLV activates *lexA*-regulated reporter genes in the nucleus

4. Reporter genes ***Ade2***, ***His3*** and ***lacZ*** allow detection of interactions by growth, X-Gal staining, and red/white analysis of cells.



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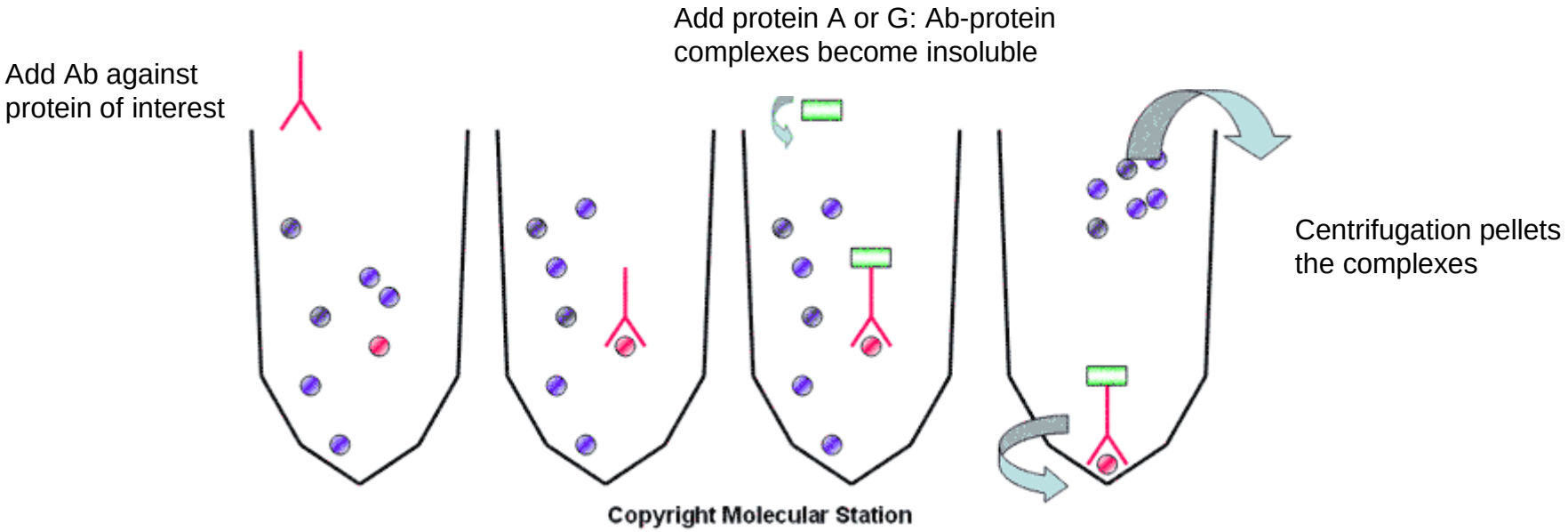
Immunoprecipitation (IP) uses the specificity of antibodies (Ab_s) to isolate target protein (antigen) out of a complex sample mixture

1) An antibody for the protein of interest is incubated with a cell extract so that the antibody will bind the protein in solution

2) The antibody/antigen complex will then be pulled out of the sample using protein A/G-coupled agarose beads.

3) The sample can then be separated by SDS-PAGE for Western blot analysis



1) Sample preparation

The choice of lysis buffer is critical and dependent on the nature of the protein to be studied. NP-40, a non-ionic detergent, is the most commonly used detergent in cell lysis buffers. Increasing the salt concentration, decreasing the detergent concentration, or changing the detergent to Triton X-100, Saponin, Digitonin, CHAPS etc. are steps that can be taken to optimize conditions for immunoprecipitation.

2) Preclearing

The preclearing step is incorporated into the procedure to lower the amount of non-specific contaminants in the cell lysate and to remove proteins with high affinity for Protein G or Protein A (from Streptoccocal bacteria).

3) Antibody incubation/formation of antibody-antigen complexes

The success of immunoprecipitation depends on the affinity of the antibody for its antigen as well as for Protein G or Protein A.

4) Precipitation

The strength of interaction between the mAb and Protein G or Protein A is an important factor in the decision of which slurry to use. Protein G coupled to some insoluble matrix (e.g. sepharose beads) binds well to most subclasses of rat immunoglobulins and mouse IgG1, while Protein A binds much better to mouse IgG2a, IgG2b, and IgG3.

5) Analysis by SDS-PAGE

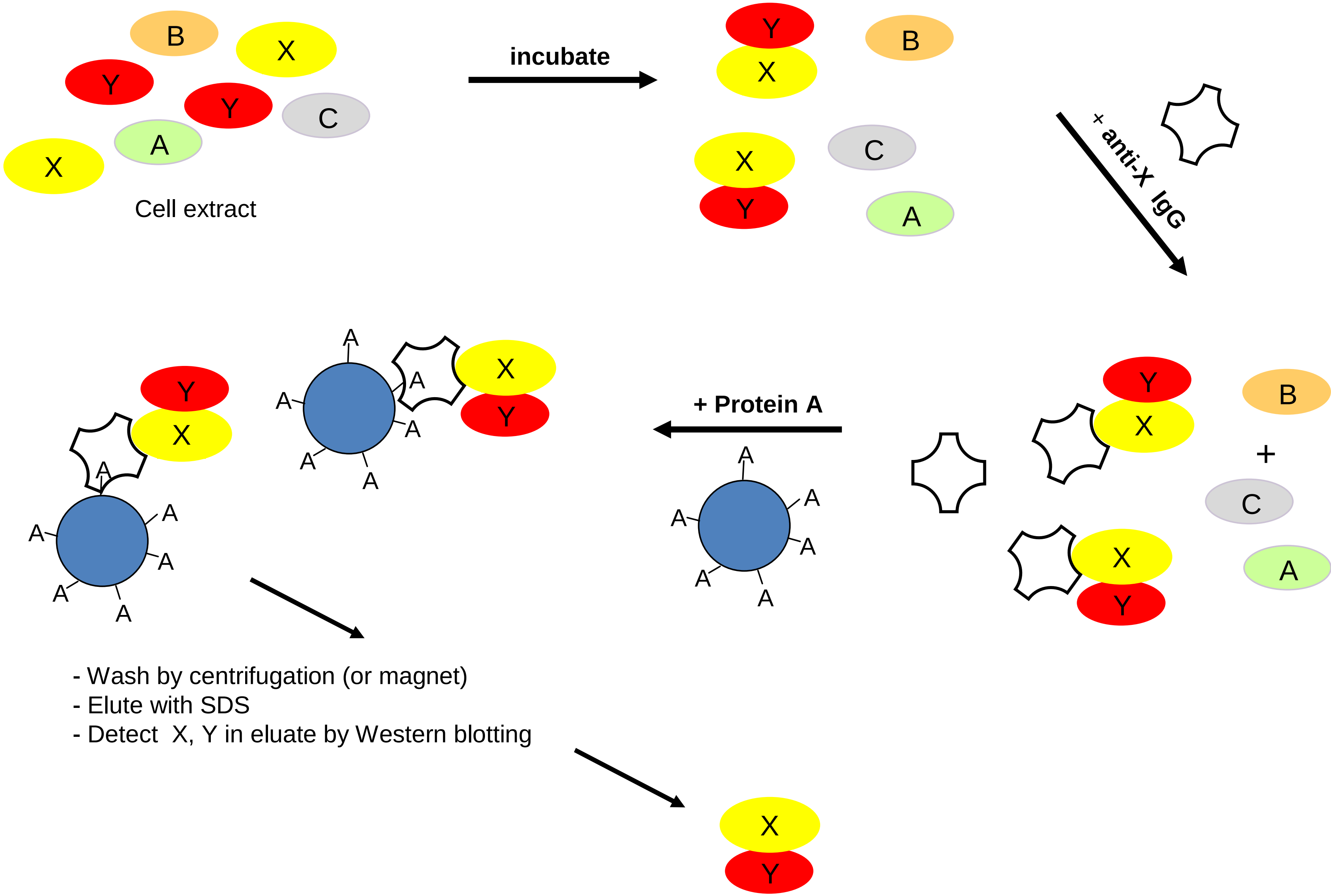
Co-immunoprecipitation (Co-IP)

- For protein interaction discovery

- It is conducted in essentially the same manner as an IP

- However, in a co-IP the target antigen precipitated by the antibody “co-precipitates” a binding partner/protein complex from a lysate

Does X interact with Y in the cell?



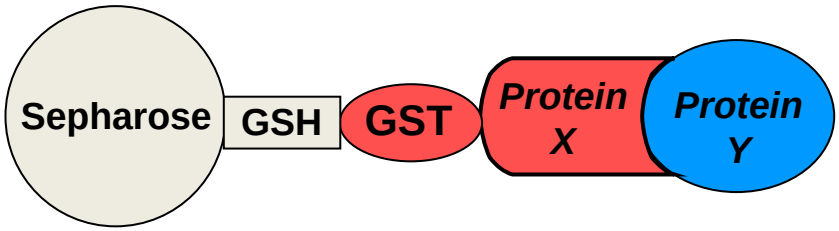
Fusion protein affinity chromatography

- Express the protein of interest as a fusion protein
  - 6-8X His residues
  - Glutathione S-transferase (GST)
  - Other “tags”

- Bind and purify the protein of interest
  - Poly His residues will bind Ni^{2+}
  - GST will bind glutathione

GST pull-down assay

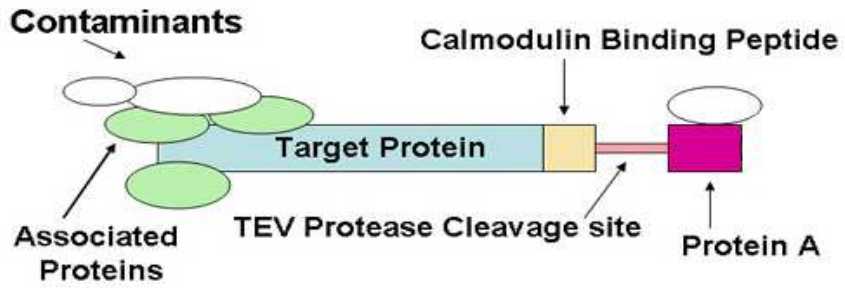
- Incubate with cell extract or purified protein



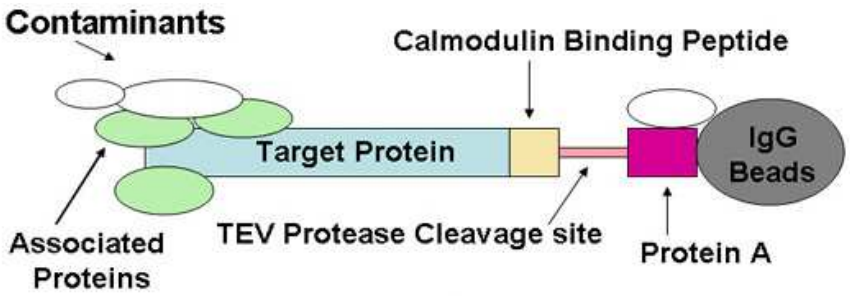
- Wash;
- Elute with SDS or excess glutathione (GSH)

- Analyze eluant by SDS-PAGE
- Identify *Protein Y* by western blotting or mass spec.

Tandem affinity purification (TAP)



- 1st affinity column: IgG beads



- Wash; cleave with TEV protease
- Apply eluant to 2nd affinity column: calmodulin beads

- Native elution with EGTA

Difficulties when using biochemical approaches

- Stability of protein:protein interactions
  - Many are not stable enough to survive purification!
- Fusion protein functionality
  - Many times fusions will not be functional!
- Quality of the antibody
  - Is it good enough to precipitate enough protein for analysis?

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Fluorescence Resonance Energy Transfer (FRET)

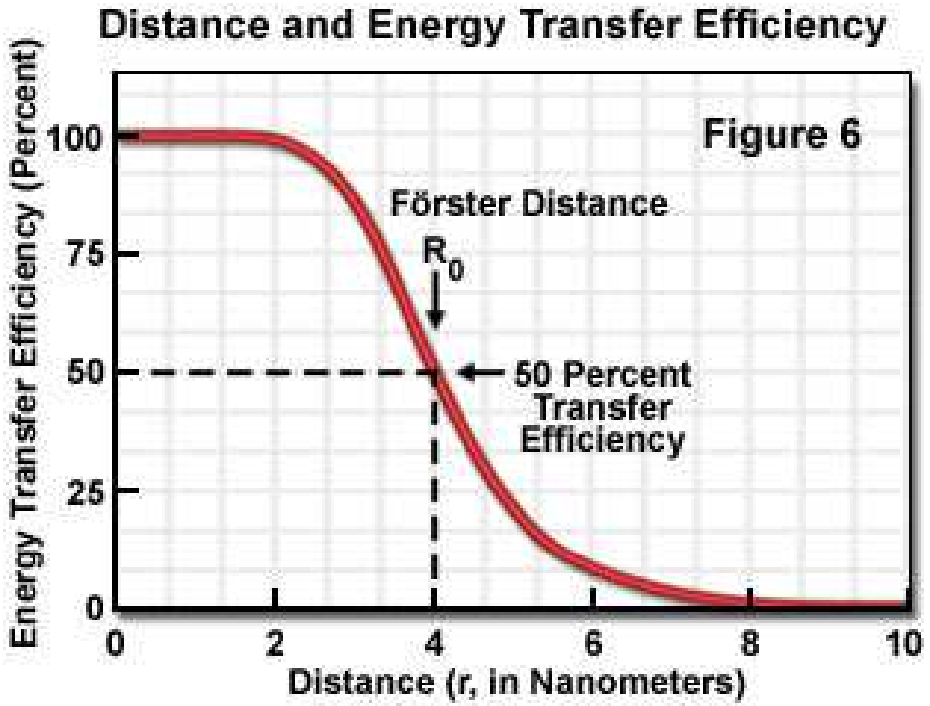
• FRET occurs between 2 fluorescent molecules

• Energy flows form one molecule to the other by non-radiative transfer (long range dipole-dipole coupling mechanism)

• FRET can be used to show that molecules are within 5-10 nm of each other: is a sort of “molecular ruler”!

FRET efficiency varies as the sixth power of the distance between the two molecules !

The efficiency of this energy transfer depends upon:
• the distance between acceptor and donor
• the degree of spectral overlap between the emission spectra of the donor and absorption of spectra of the acceptor
• the relative parallel orientation of donor and acceptor dipoles.



The distance at which the energy transfer is 50% efficient is called the Forster Radius (Ro)

FRET Pairs

Donor	Acceptor	F&#65533;rster Distance (Nanometers)
Tryptophan	Dansyl	2.1
IAEDANS (1)	DDPM (2)	2.5 - 2.9
BFP	DsRFP	3.1 - 3.3
Dansyl	FITC	3.3 - 4.1
Dansyl	Octadecylrhodamine	4.3
CFP	GFP	4.7 - 4.9
CF (3)	Texas Red	5.1
Fluorescein	Tetramethylrhodamine	4.9 - 5.5
Cy3	Cy5	>5.0
GFP	YFP	5.5 - 5.7
BODIPY FL (4)	BODIPY FL (4)	5.7
Rhodamine 6G	Malachite Green	6.1
FITC	Eosin Thiosemicarbazide	6.1 - 6.4
B-Phycoerythrin	Cy5	7.2
Cy5	Cy5.5	>8.0

(1) 5-(2-iodoacetylaminoethyl)aminonaphthalene-1-sulfonic acid
(2) N-(4-dimethylamino-3,5-dinitrophenyl)maleimide
(3) carboxyfluorescein succinimidyl ester
(4) 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene

Experimentally measured Förster critical distances, which were ascertained from the spectral overlap of several popular donor-acceptor fluorophore pairs.

The rate of energy transfer depends upon the extent of spectral overlap between the donor emission and acceptor absorption spectra.

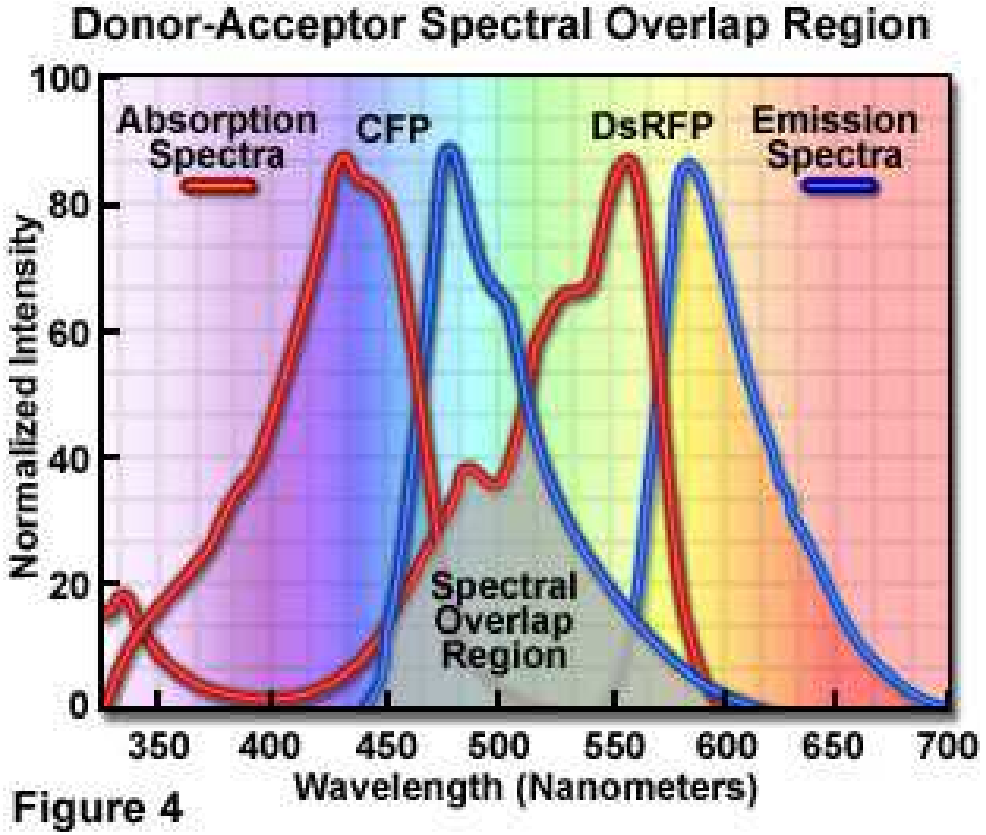
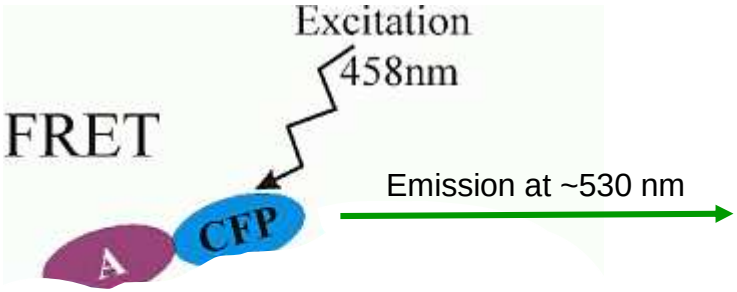
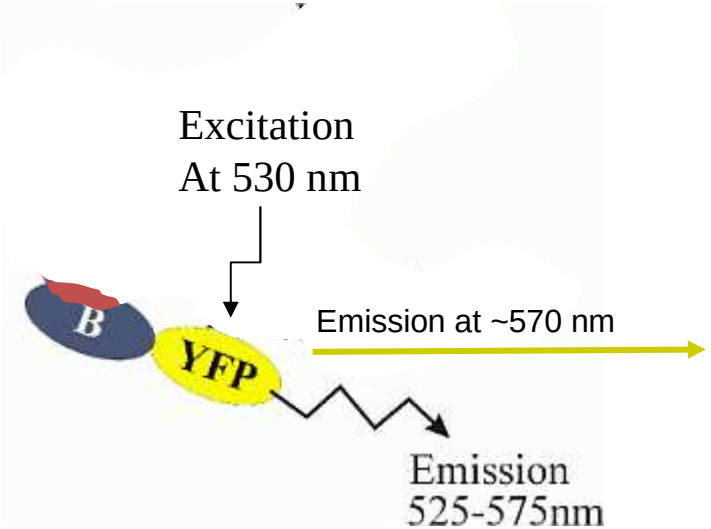
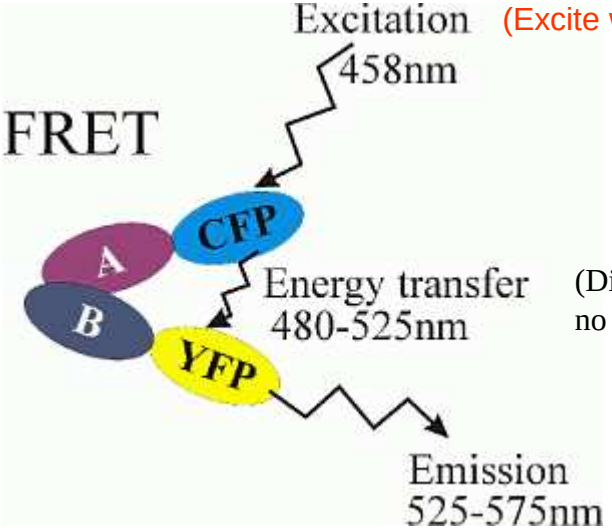


Figure 4

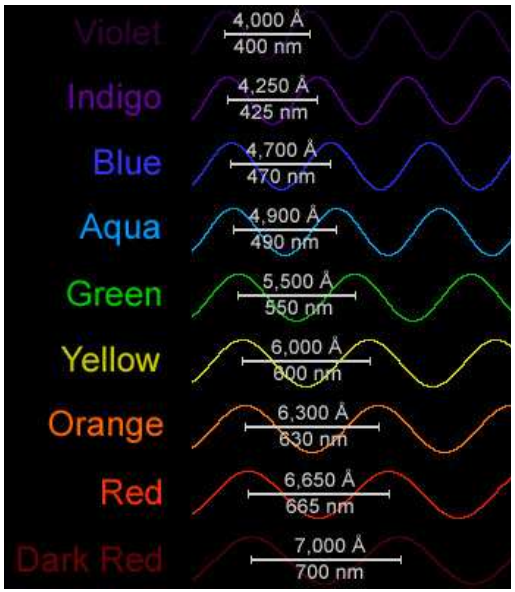
FRET







(Measure this)



YFP = Yellow fluorescent protein
CFP = Cyan fluorescent protein

Bimolecular fluorescence complementation (BiFC)

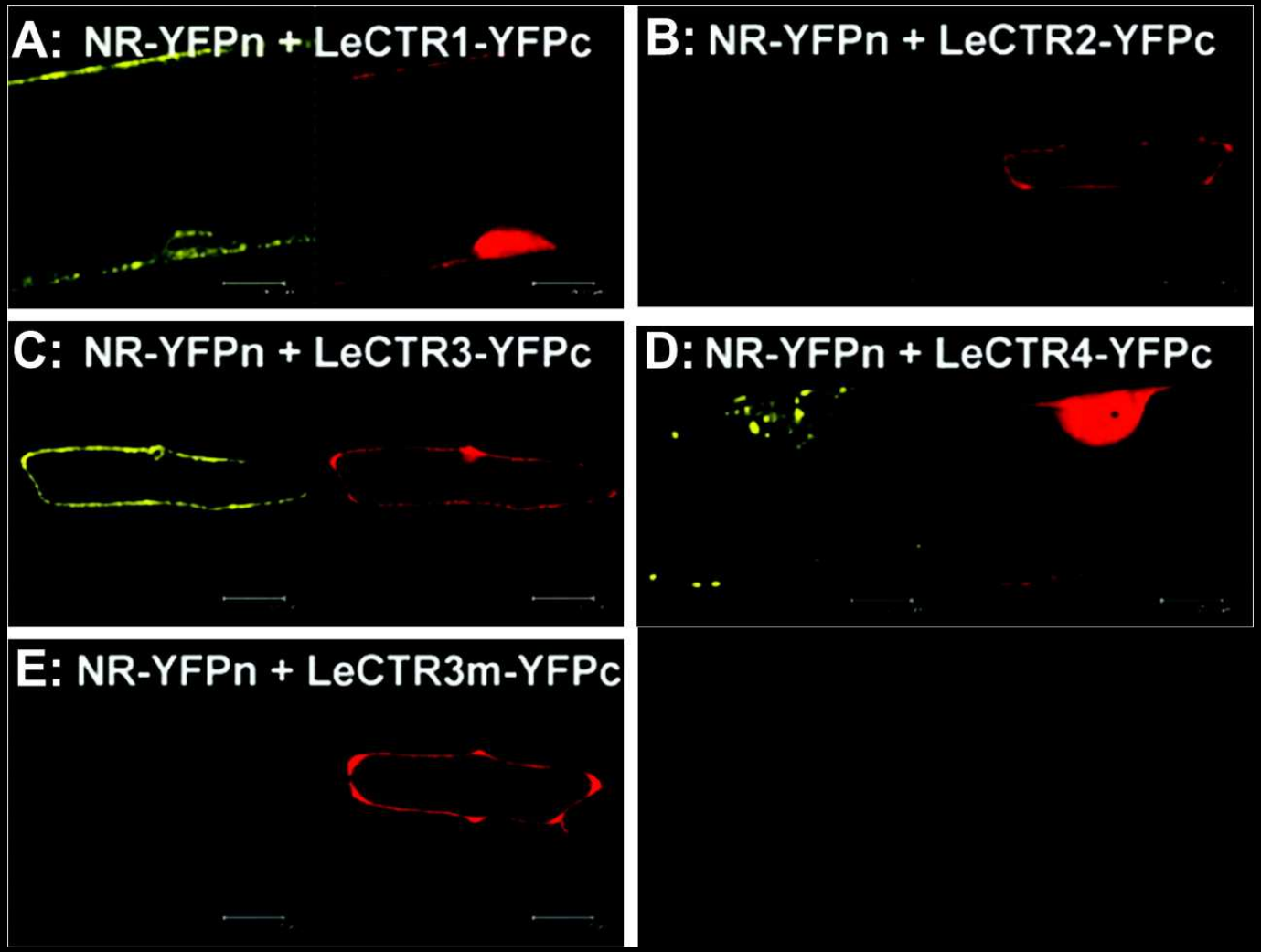
BiFC uses reconstitution of fluorescence from a "split fluorophore" to visualize interaction between two tagged proteins

For example: the yellow fluorescent protein (YFP) is split into two non-overlapping N-terminal (YN) and C-terminal (YC) fragments. Each fragment is cloned in-frame to a gene of interest, enabling expression of fusion proteins.

Reconstitution of fluorescence takes place only when the two fragments of the split chromophore are brought together by protein–protein interactions.

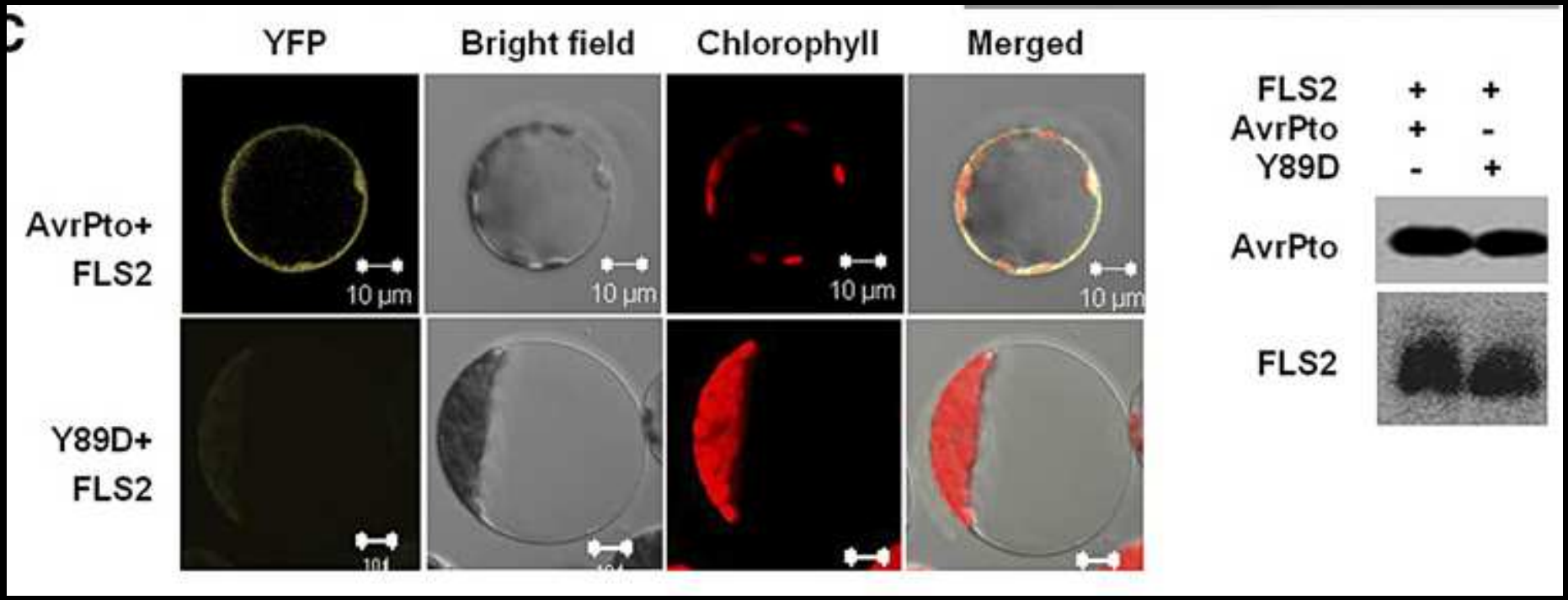
BiFC has several advantages including absence of background signal, high specificity and high stability of the reconstituted chromophore complexes

BiFC



Images of the onion epidermal cells expressing the NR and LeCTR BiFC constructs (false colour yellow) plus the red fluorescent protein mRFP1 (false colour red).
Combinations of these constructs were then co-bombarded into onion epidermal cells together with mRFP1 as transformation control and a cytoplasmic marker.

BiFC


C
YFP
Bright field
Chlorophyll
Merged
AvrPto+
FLS2
Y89D+
FLS2
10 µm
FLS2 + +
AvrPto + -
Y89D - +
AvrPto
FLS2