Cds in Scienze e Tecnologie Biologiche

AA 2019-2020

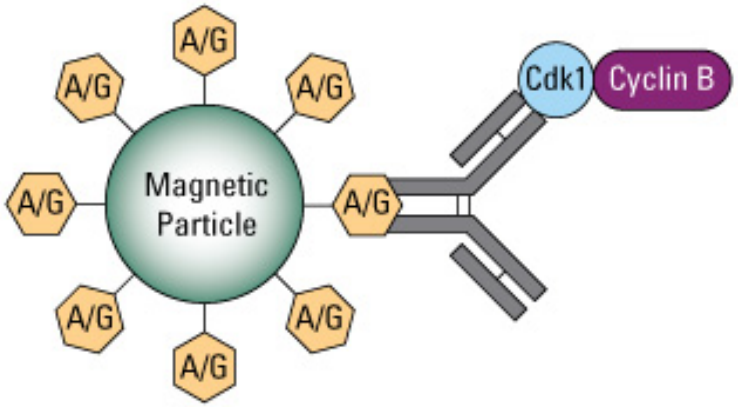
Corso di Laboratorio di Biologia Cellulare

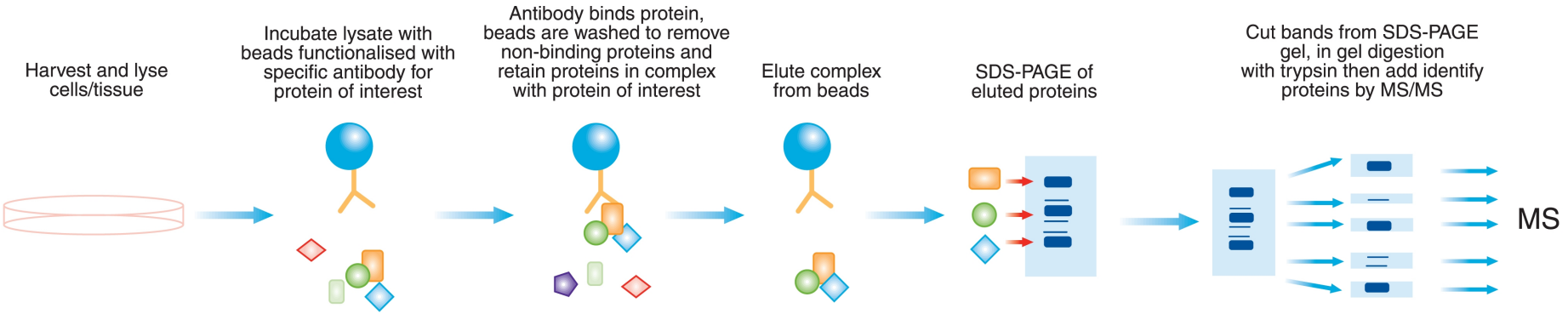
Lezione 10

Analisi dell'interazione proteina-proteina

L'analisi dell'interazione di specifiche proteine cellulari con altre macromolecole permette di studiarne in dettaglio la funzione e all'occorrenza di mofidificarla.

Analisi dell'interazione proteina-proteina da lisato cellulare: co-immunoprecipitazione





Se le proteine partner sono note, si analizza mediante WB

Se non sono note, si sottopongono le bande dell'interattoma all'analisi mediante spettrometria di massa.

Alternativa: pulldown con proteine di fusione-TAG (es. GST)

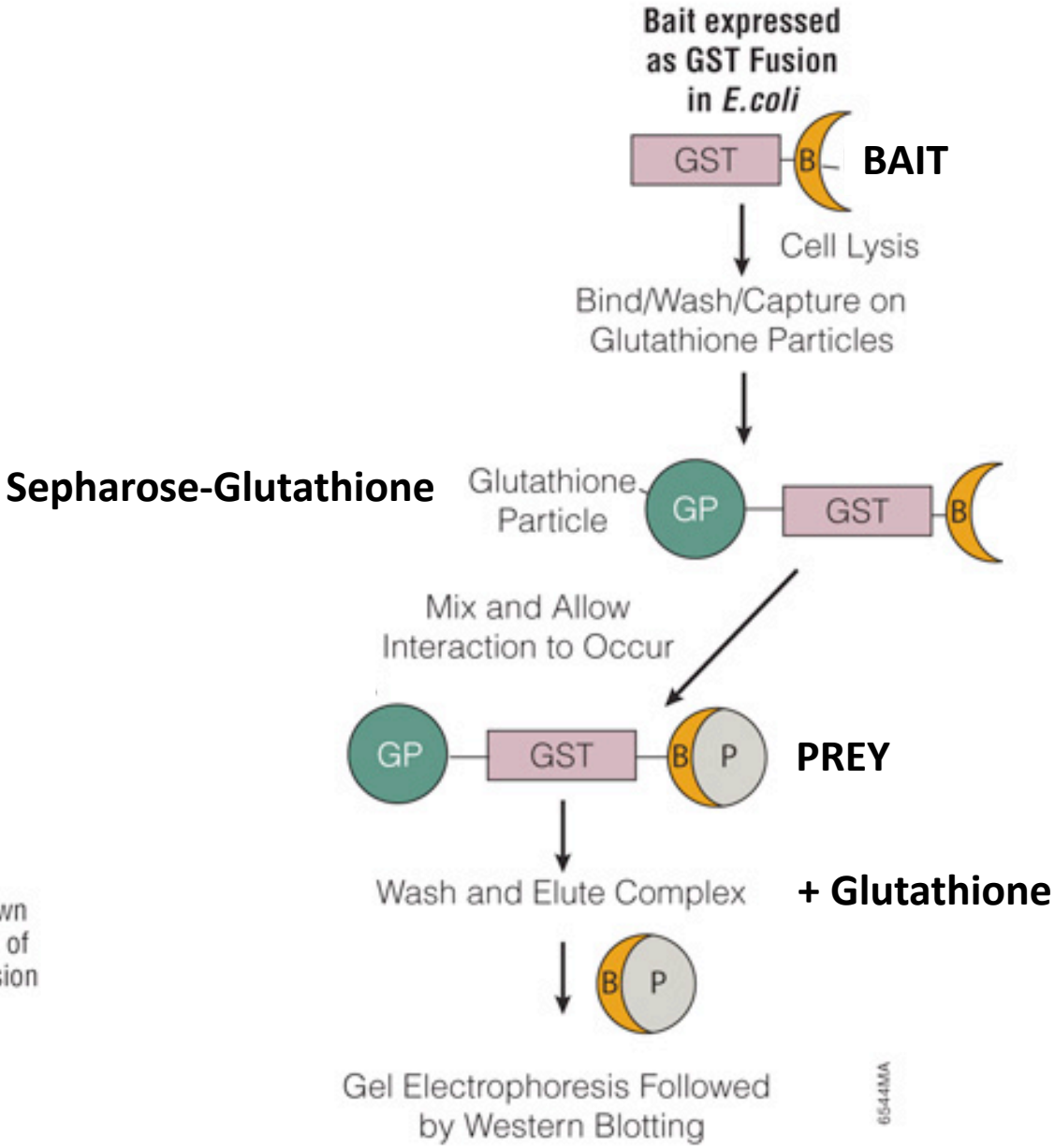
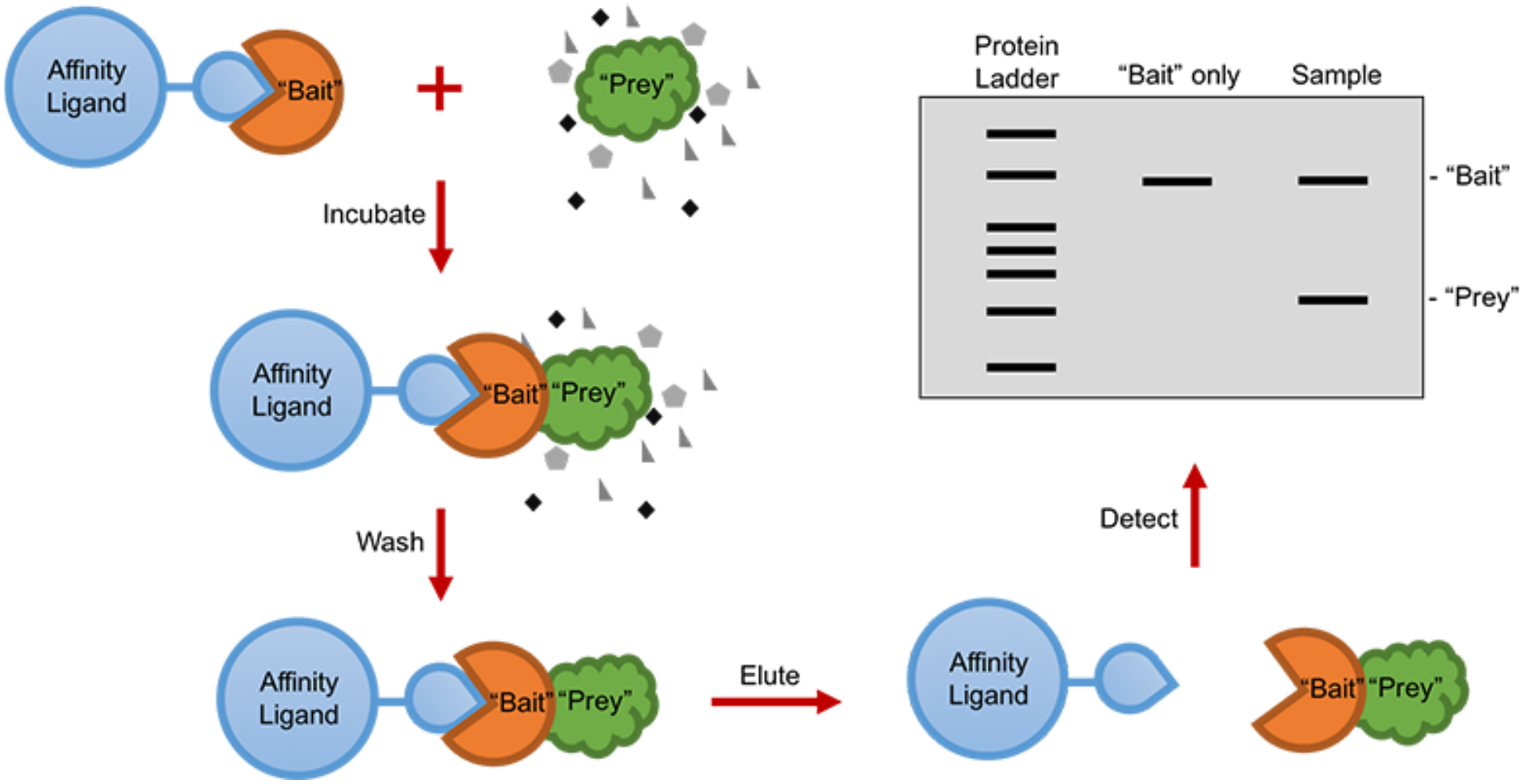
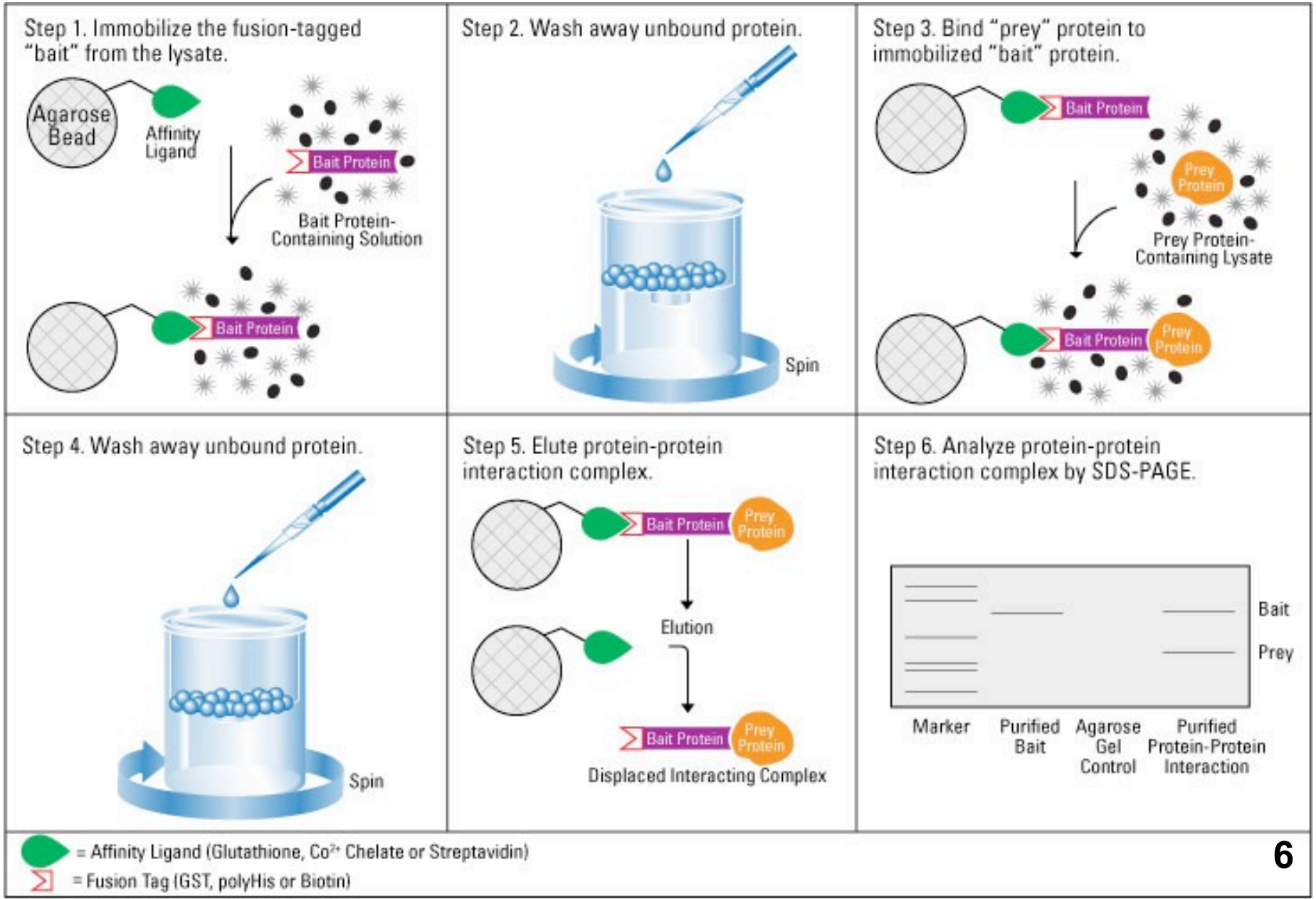


Figure 1. Schematic of pull-down assay using bacterial expression of bait protein and cell-free expression for the prey protein.


Affinity Ligand
"Bait"
"Prey"
Incubate
Affinity Ligand
"Bait"
"Prey"
Wash
Affinity Ligand
"Bait"
"Prey"
Elute
Affinity Ligand
"Bait"
"Prey"
Detect
Protein Ladder
"Bait" only
Sample
- "Bait"
- "Prey"

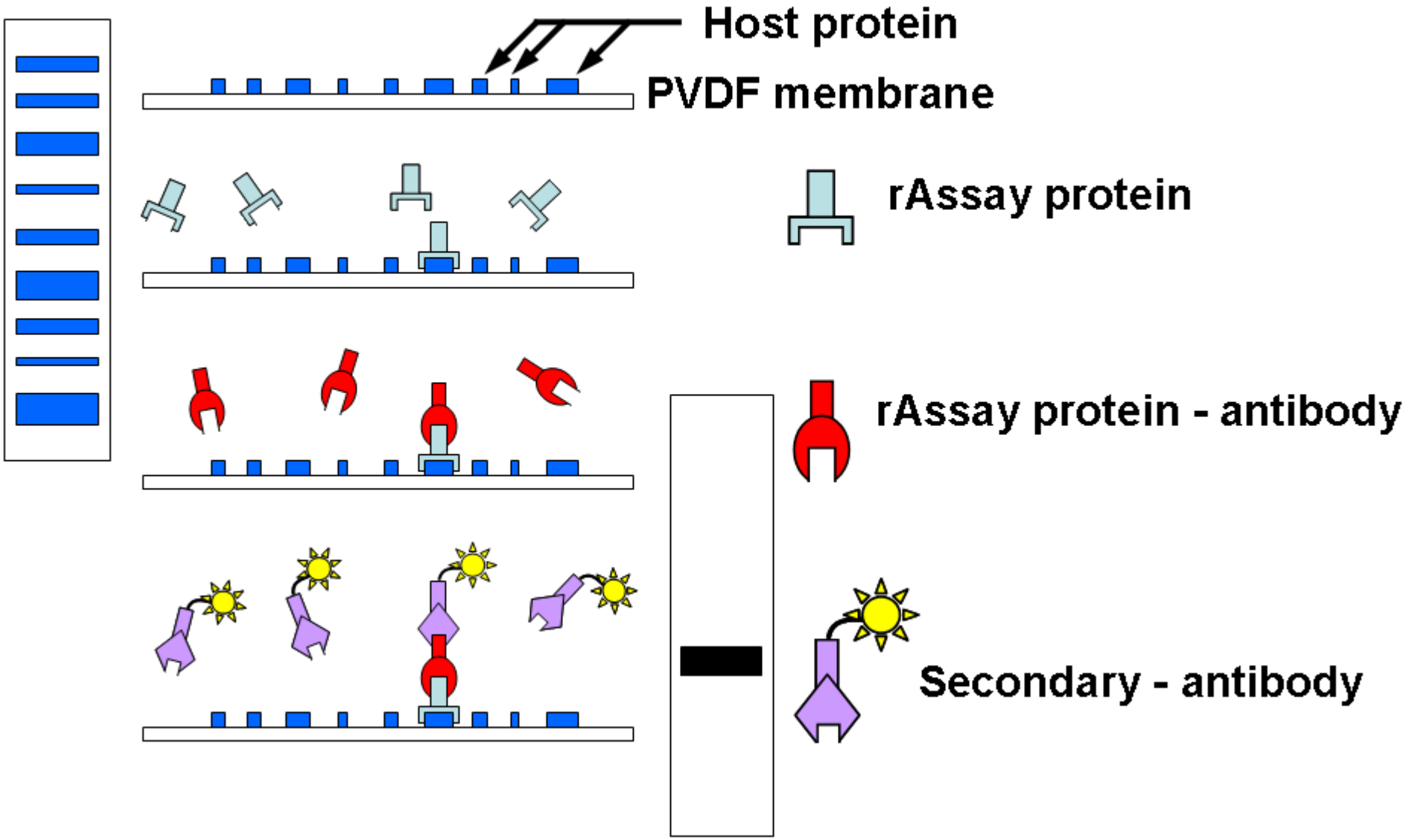
pulldown con proteine di fusione-tag (es. GST)



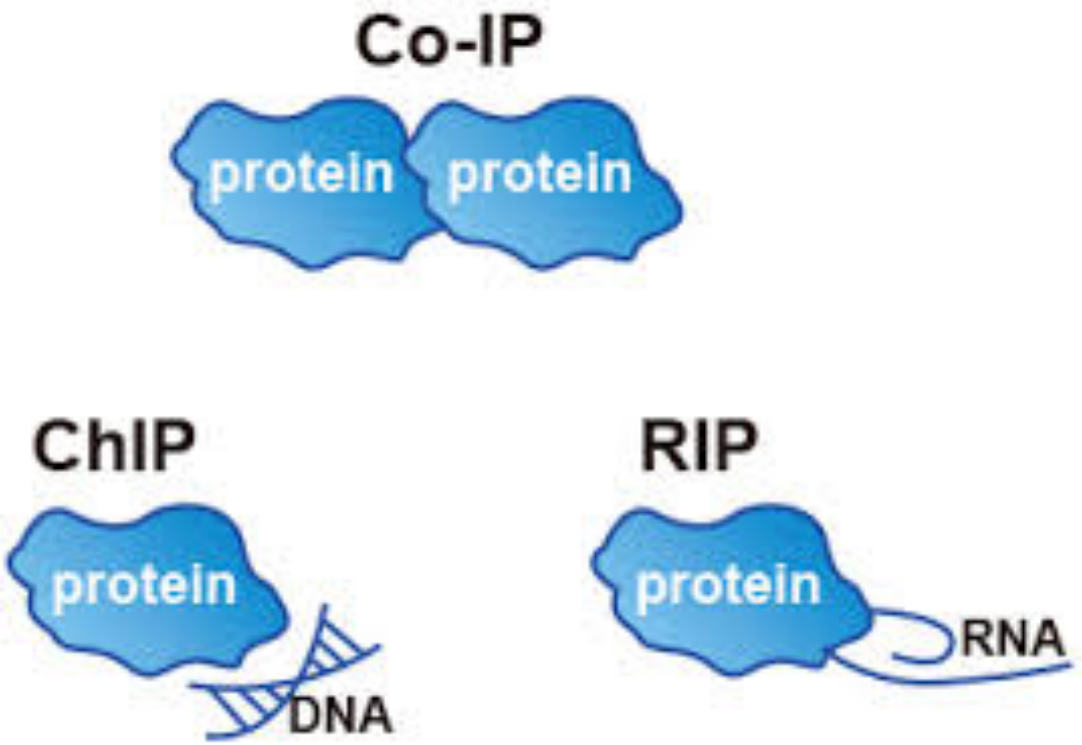
Comparison of Co-IP with endogenous proteins versus tagged proteins

	Endogenous proteins	Tagged proteins (pull-down assay)
Main advantages	**Protein complexes are isolated in a relatively natural state.**	**An *N*- or *C*-terminal tag is likely available for antibody binding after complex formation. Antibody binding is unlikely to interfere with complex formation.**
Issues to consider	**The epitope may be buried upon complex formation. Antibody binding may interfere with complex formation.**	**The expression levels of recombinant proteins are substantially higher than those of their endogenous counterparts, which may result in artifactual results.**

Analisi di interazione DIRETTA proteina-proteina mediante FAR WESTERN:



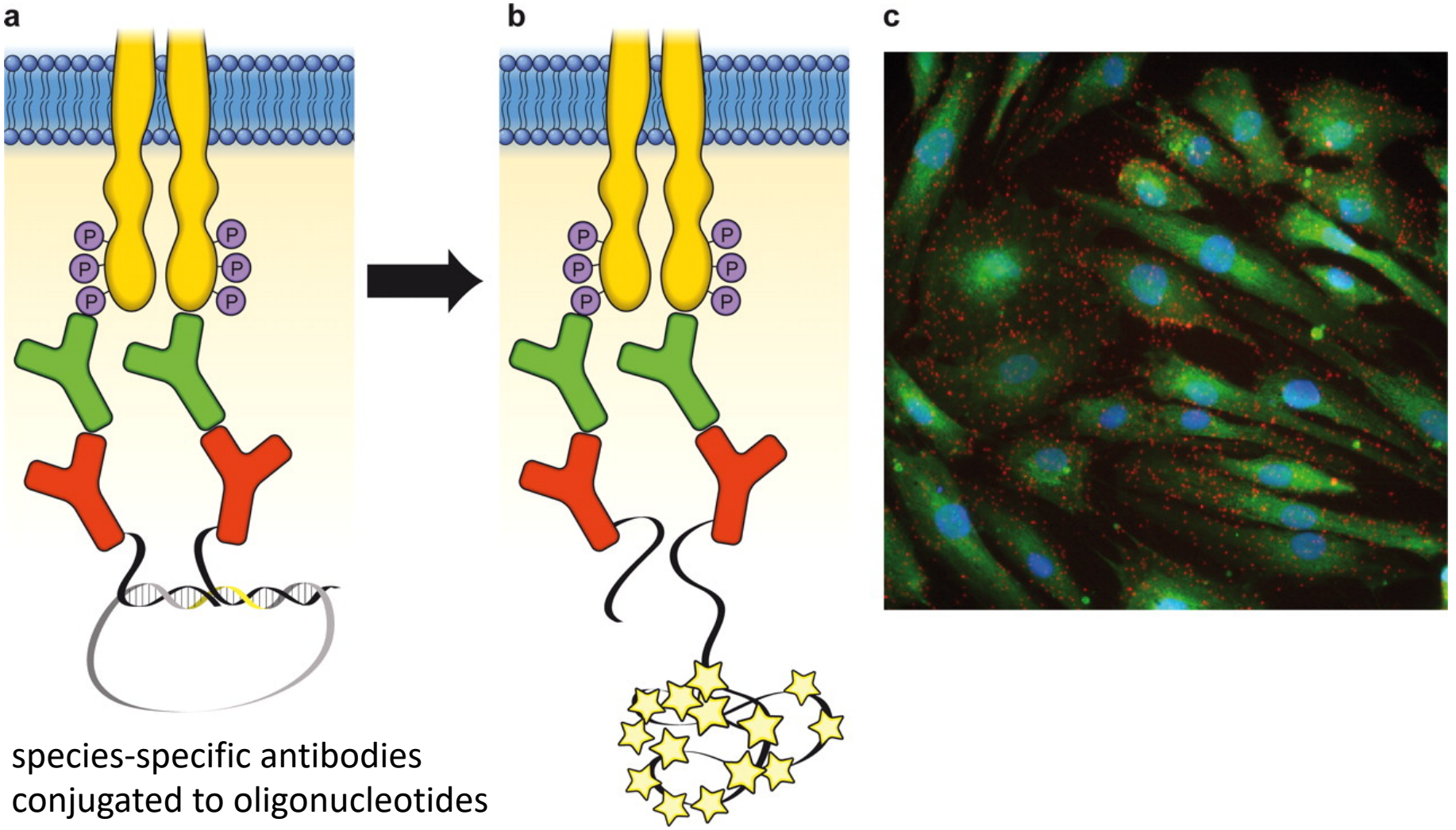
Analisi dell'interazione di una proteina con altre macromolecole: diversi tipi di immunoprecipitazione



TECNICHE per l'analisi dell'interazione proteina-proteina IN SITU:

Proximity Ligation Assay PLA

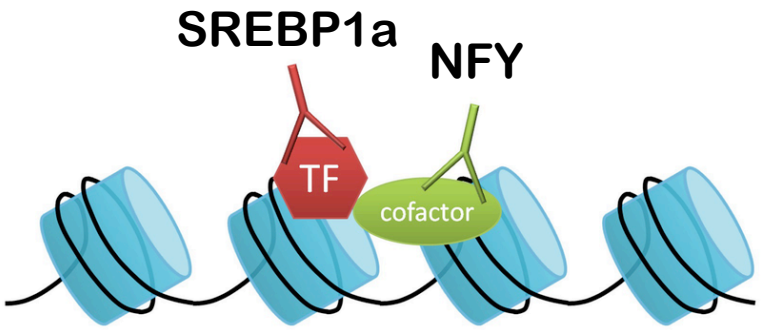
In situ proximity ligation assay PLA



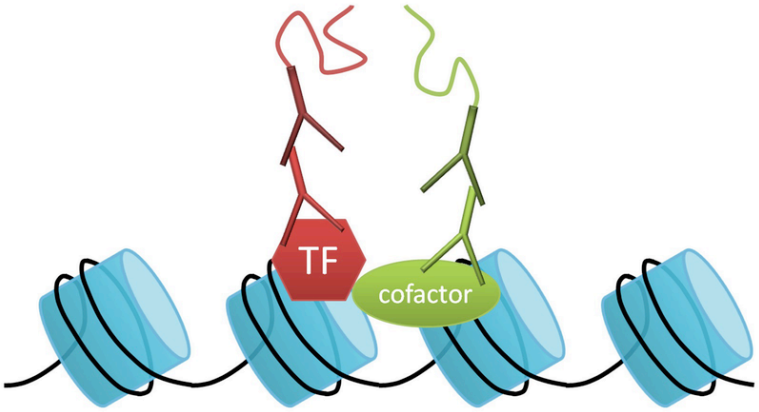
species-specific antibodies conjugated to oligonucleotides used as templates for the joining of two additional linear oligos into a DNA circle

DNA amplification
hybridize with fluorescent probe

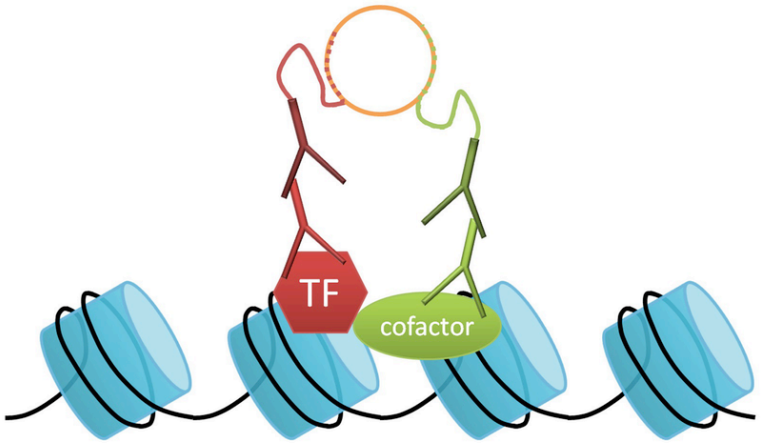
In situ PLA per visualizzare l'interazione tra un TF e un cofattore



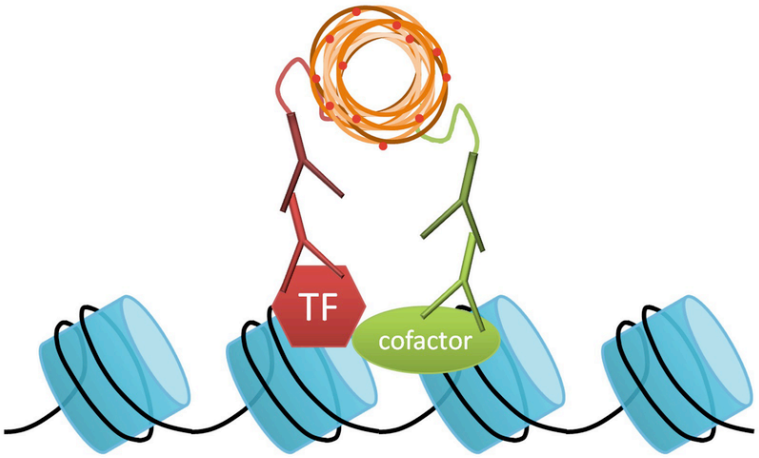
Primary antibodies



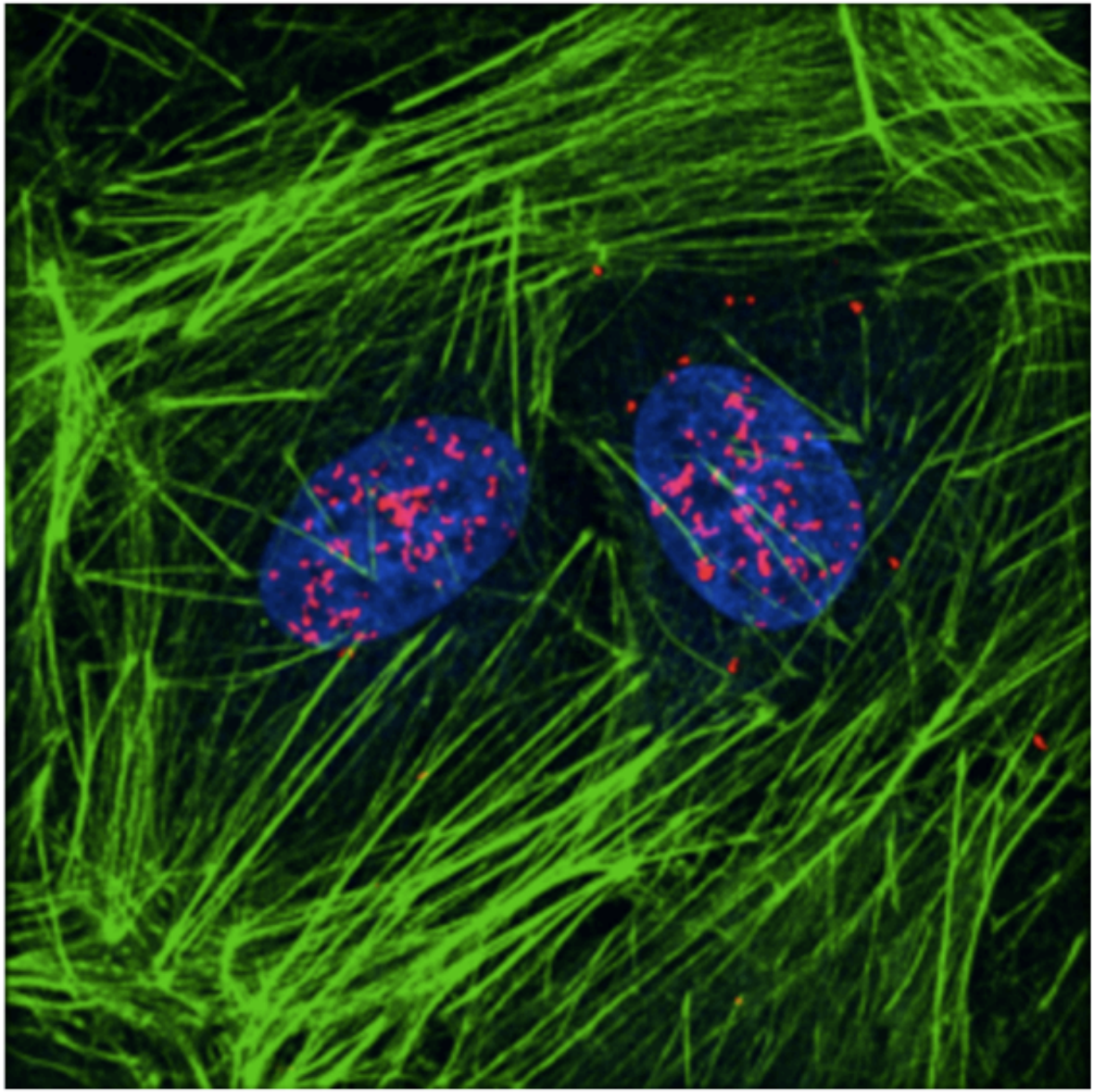
Secondary antibodies



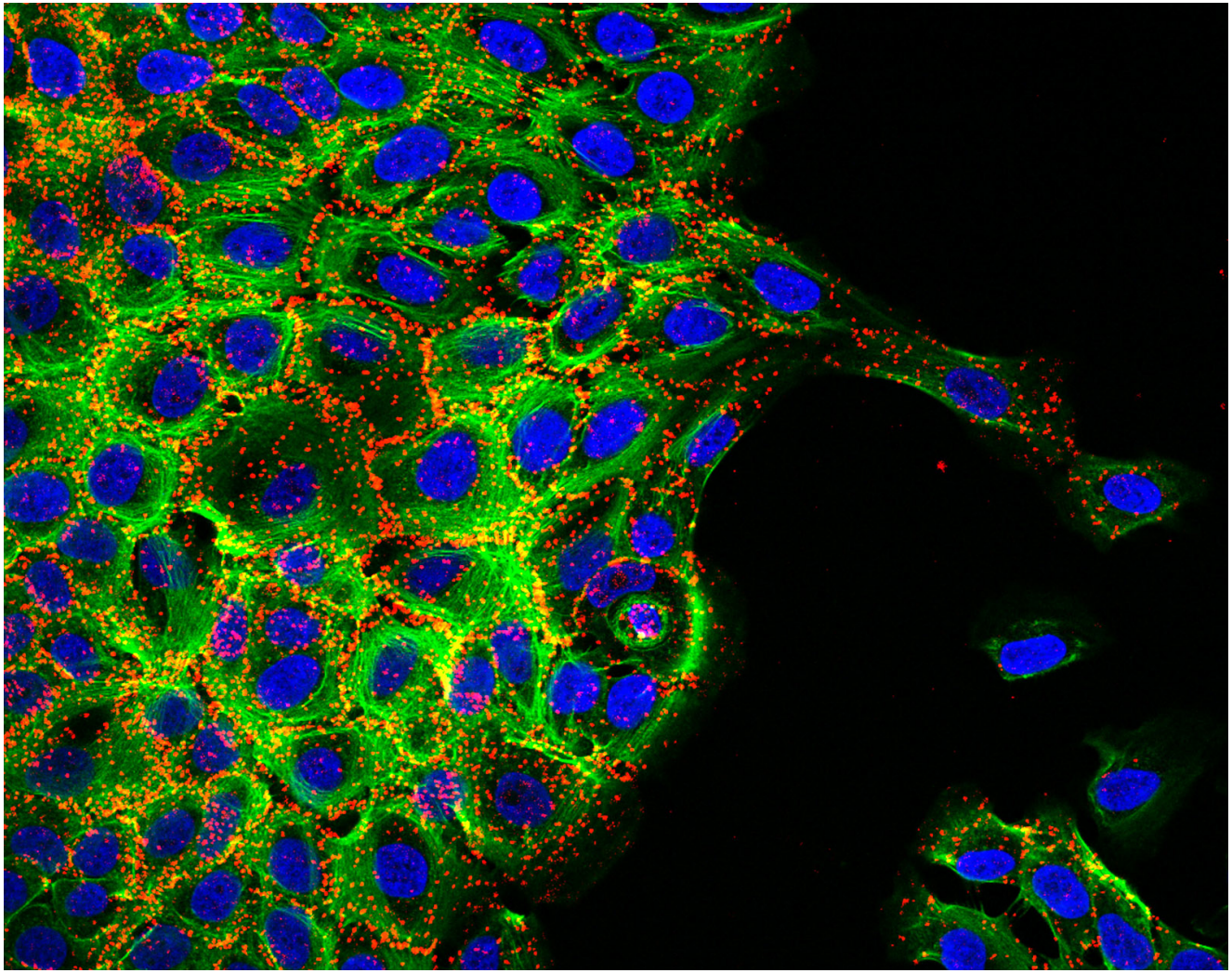
Ligation



Amplification



Proximity Ligation Assay in showing SREBP1a-NFY nuclear complexes (red) in cells co-stained with phalloidin (green) and DAPI (blue).



In situ proximity ligation assay for E-cadherin and p120ctn (red) in MCF10A-ER-Src cells stained with Phalloidin to mark actin filaments (green) and DAPI

Sistemi reporter

Geni reporter

Geni la cui espressione ectopica può essere facilmente identificata e/o misurata mediante:

- visualizzazione diretta
- saggio di attività enzimatica

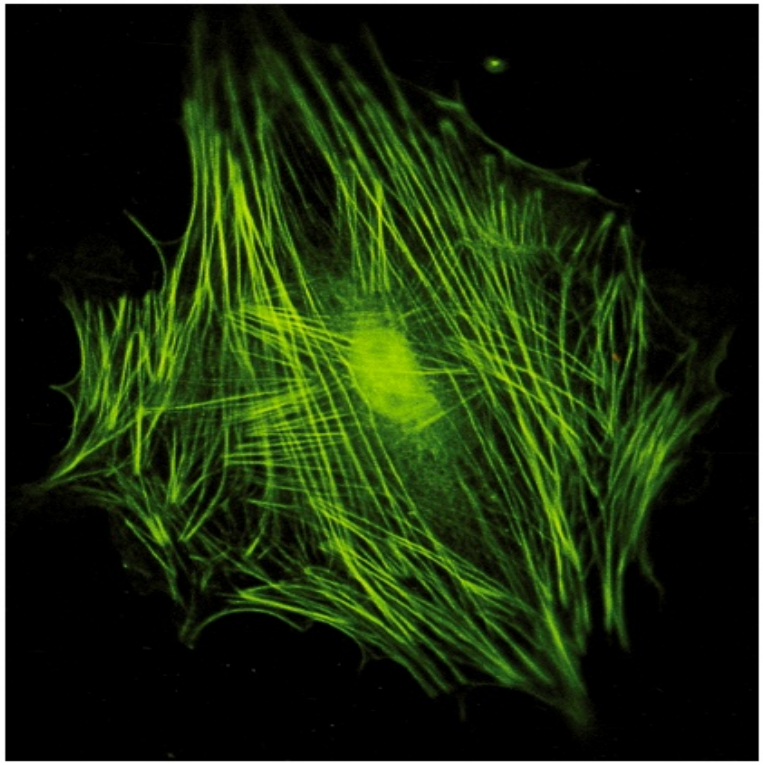
I geni reporter possono essere espressi a partire da un promotore:

- costitutivo (utilizzo il reporter per visualizzare una cellula o una proteina)
- regolato (utilizzo il reporter per visualizzare o misurare l'attività del promotore)

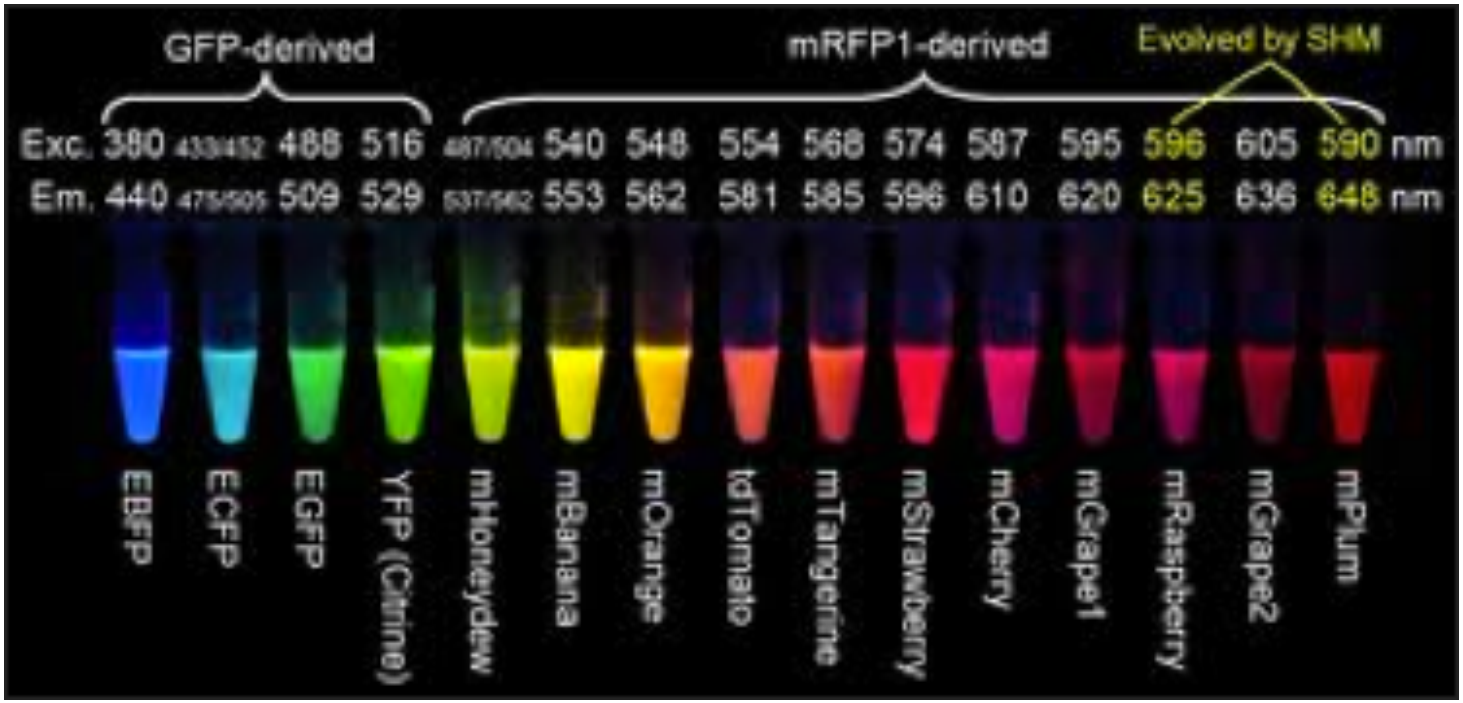
PROBLEMA:

DOVE (in quale organello-cellula- tessuto) è espressa una proteina di interesse? VISUALIZZARE LA PROTEINA IN SITU

Soluzione: FUSIONE del cDNA con un gene reporter facilmente visualizzabile (es. GFP)



Per la visualizzazione diretta in situ
mediante tecniche di imaging
oppure per applicazioni med. tecniche di FACS
si utilizzano
reporters fluorescenti (fotoproteine)



Limite: non posso quantificarne l'espressione con precisione

GFP = Green Fluorescent Protein

- Proteina di circa 27 Kda isolata dalla medusa *Aequora victoria*

- Ha FLUORESCENZA INTRINSECA:

  Il cromoforo è un

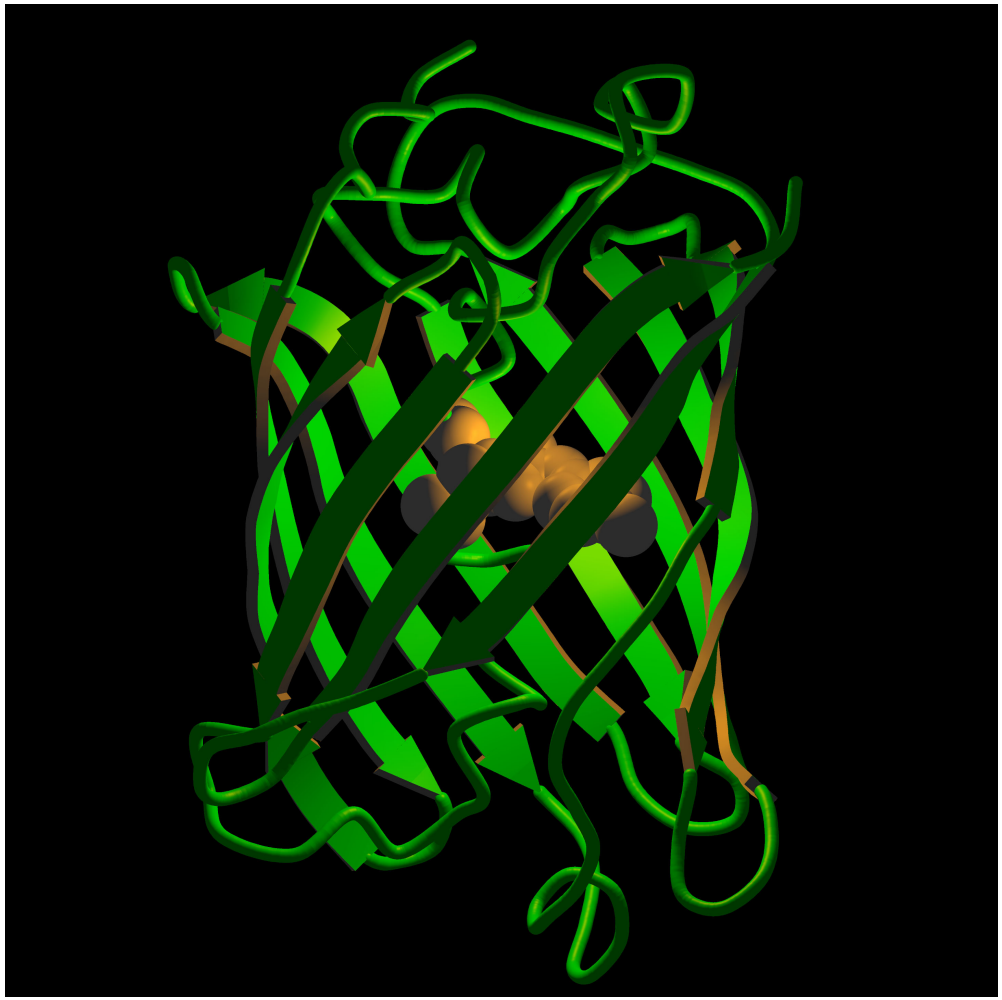
  TRIPEPTIDE CICLICO

  codificato nella

  sequenza primaria,

  che si forma mediante

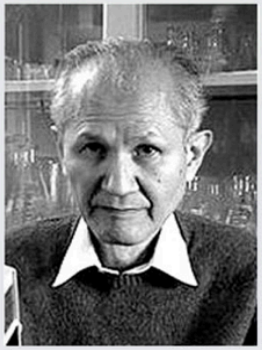
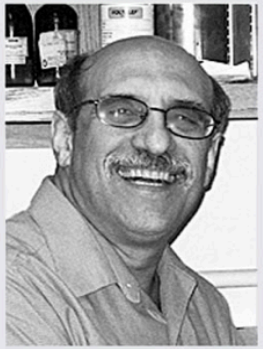
  reazione autocatalitica



- Assorbe ed emette nel visibile

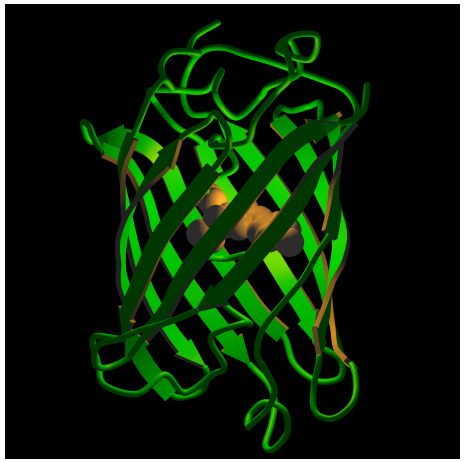
Osservazione al microscopio
a fluorescenza
Ex: 475 nm
Em: 525 nm

Imaging con reporters fluorescenti (fotoproteine)


Premio Nobel per la Chimica 2008

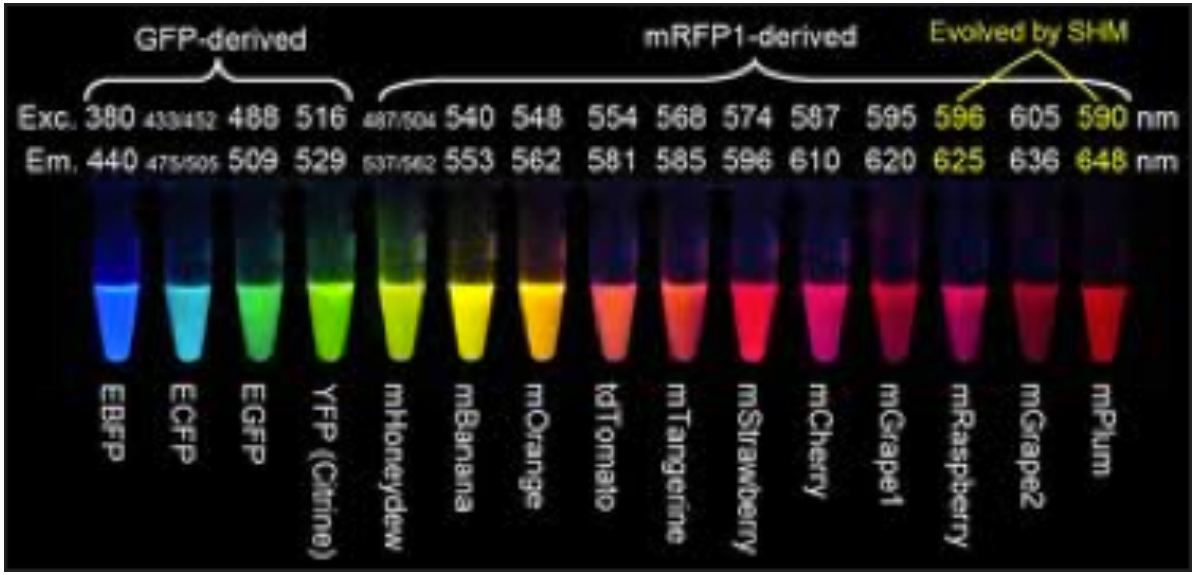
Osamu Shimomura Martin Chalfie Roger Y. Tsien



GFP

↓

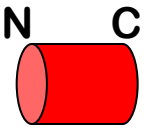
EGFP etc.



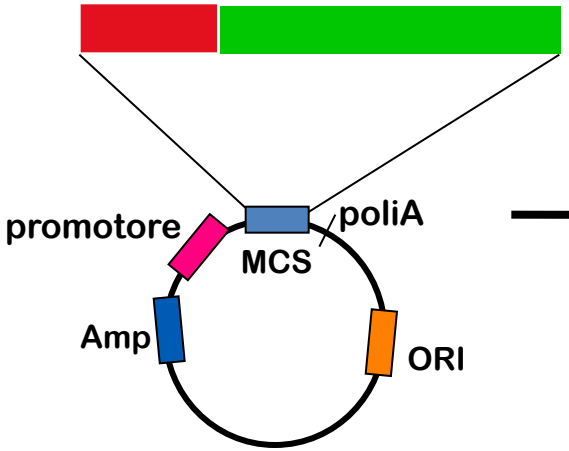
Utilizzo di fotoproteine per determinare la LOCALIZZAZIONE di una proteina di interesse (espressa in fusione)

Gene X

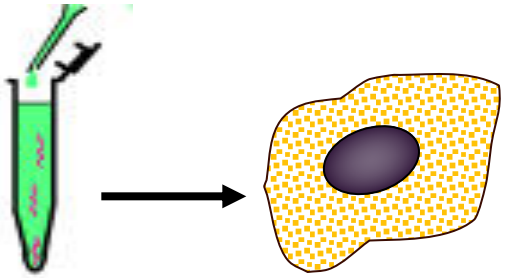
Codifica per la proteina di interesse X



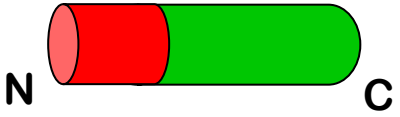
Gene X **GFP**



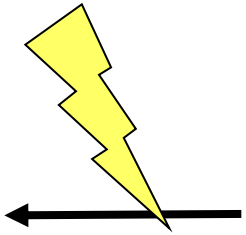
Trasfezione



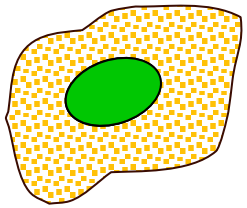
Trascrizione e traduzione: espressione della proteina di fusione nelle cellule



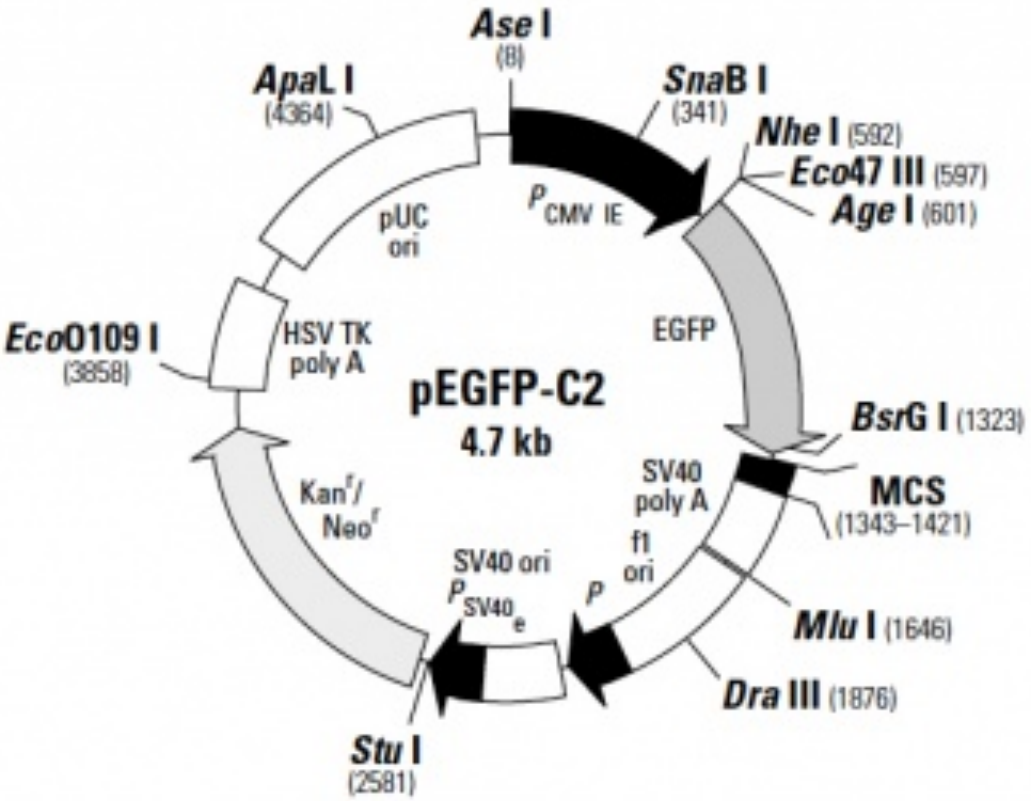
La proteina X assume la sua tipica localizzazione intracellulare

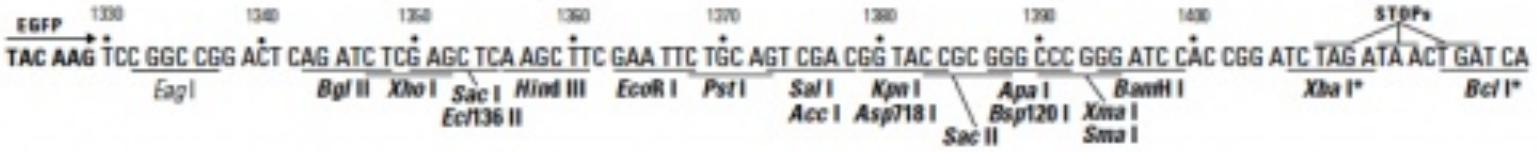


Osservazione al microscopio a fluorescenza
Ex: 475 nm
Em: 525 nm



Visualizzazione in situ

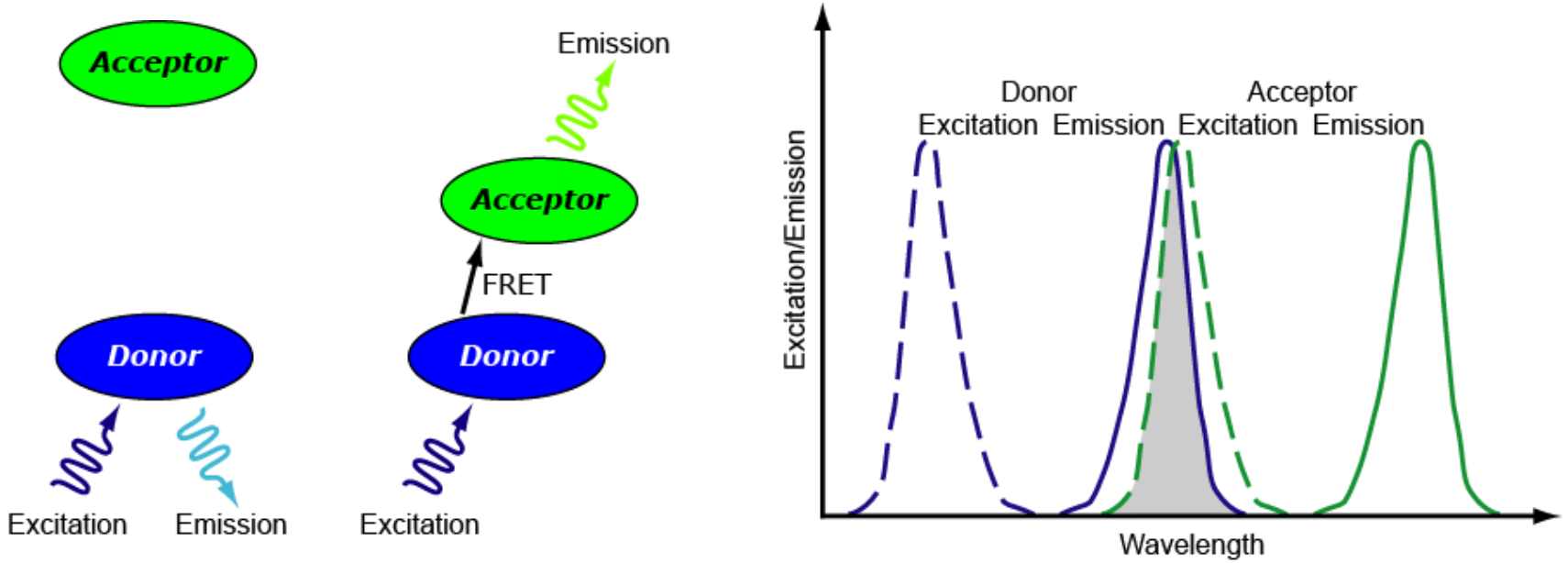

Ase I
(8)
ApaL I
(4364)
SnaB I
(341)
Nhe I (592)
Eco47 III (597)
Age I (601)
P CMV IE
pUC ori
EGFP
Eco0109 I
(3858)
HSV TK poly A
pEGFP-C2
4.7 kb
BsrG I (1323)
SV40 poly A
MCS
(1343–1421)
Kanr/ Neor
SV40 ori
P SV40e
f1 ori
P
Mlu I (1646)
Dra III (1876)
Stu I
(2581)


EGFP
1330
1340
1350
1360
1370
1380
1390
1400
STOPs
TAC AAG TCC GGC CGG ACT CAG ATC TCG AGC TCA AGC TTC GAA TTC TGC AGT CGA CGG TAC CGC GGG CCC GGG ATC CAC CGG ATC TAG ATA ACT GAT CA
EagI
Bgl II
Xho I
Sac I
Ecl136 II
Hind III
EcoR I
Pst I
Sal I
Acc I
Kpn I
Asp718 I
Sac II
Apa I
Bsp120 I
BamH I
Xma I
Sma I
Xba I*
Bcl I*

TECNICHE per l'analisi dell'interazione proteina-proteina IN SITU:

Fluorescence Resonance Energy Transfer
FRET

Förster Resonance Energy Transfer



Emission and excitation spectrum must significantly overlap

Es. CFP/YFP

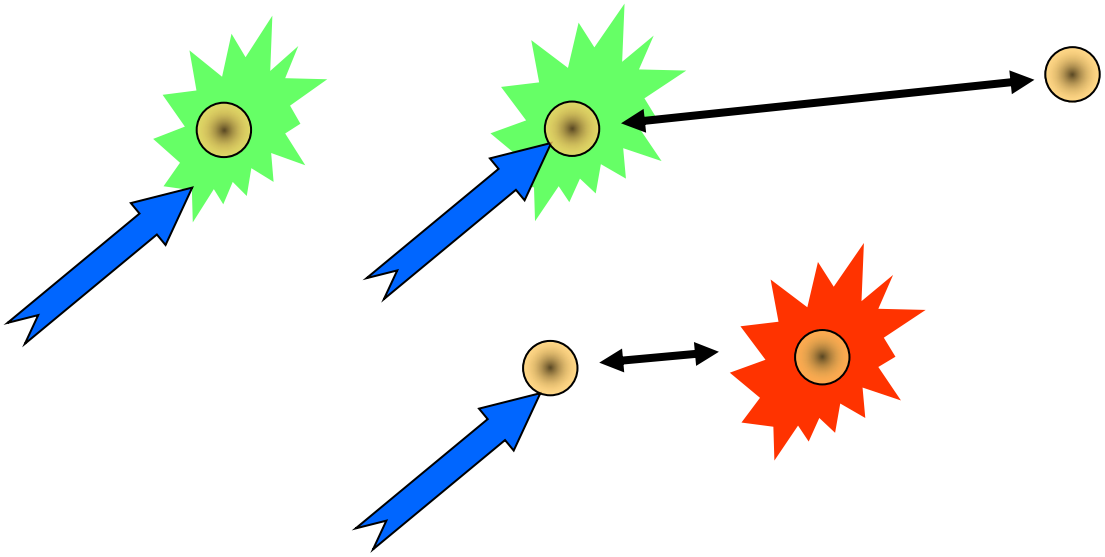
Fluorescein/Rhodamine

Cy3/Cy5

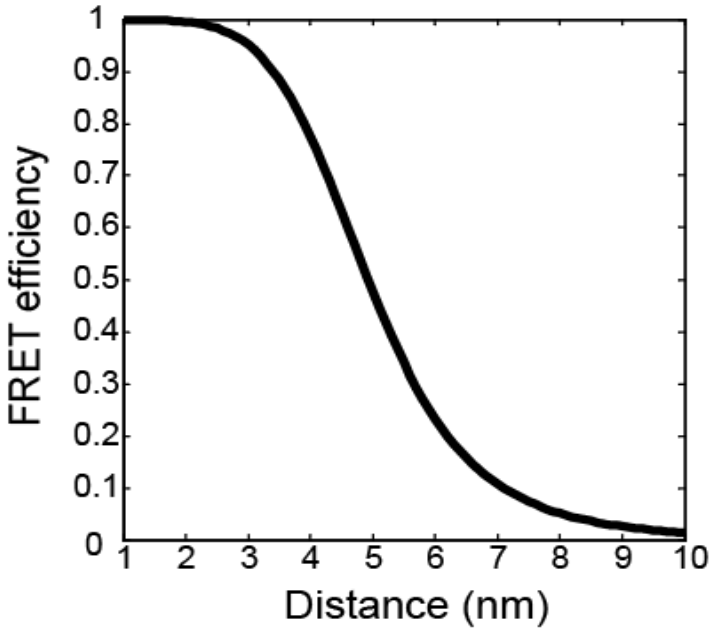
La FRET è efficiente per piccole distanze

Isolated donor

Donor distance too great



Donor distance correct

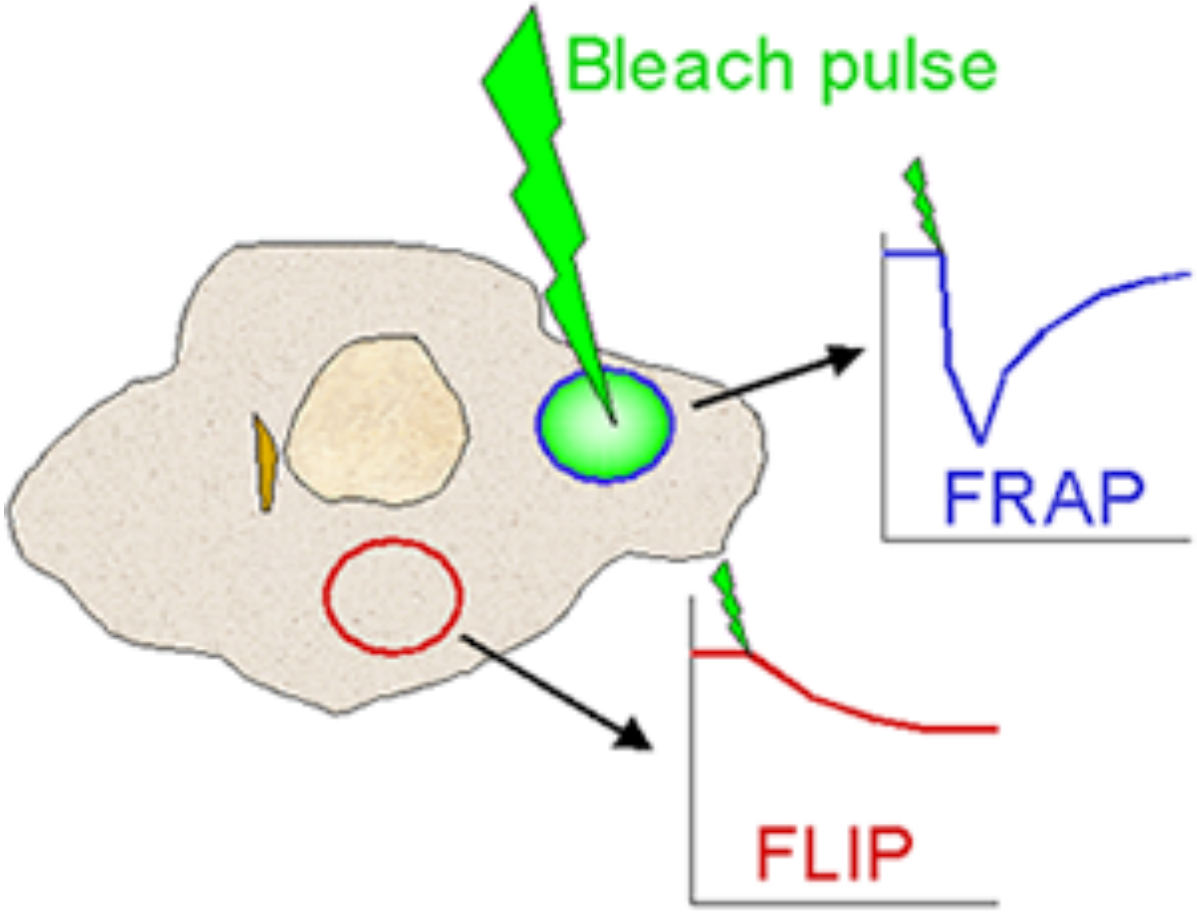


For CFP-YFP,
50% transfer at R_0 = 4.9 nm

È efficiente per distanze inferiori a 10 nm,
NB: è inferiore al limite di risoluzione del microscopio a fluorescenza!!!

Sfruttare il photo-bleaching per studiare la mobilità intracellulare

Illuminazione di una regione definita con un laser (luce molto intensa)



Photobleaching: la luce induce la conversione chimica permanente del fluoroforo in una molecola non fluorescente.

FRAP

Fluorescence Recovery after Photo-bleaching

- **Photobleaching** (also termed **fading**) occurs when a fluorophore permanently loses the ability to fluoresce due to photon-induced chemical damage and covalent modification.
- 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.
- A defined region of the sample is illuminated with high intensity light causing the fluorophore within that region to become photobleached. This creates a darker, bleached region, within the sample. Photobleached molecules are subsequently replaced by nonbleached molecules over time, and this results in an increase in fluorescence intensity in the bleach region.
- Recovery of fluorescence into the bleached area occurs as a result of the **diffusional exchange** between bleached and unbleached molecules. The fraction of fluorescent molecules that can participate in this exchange is referred to as the mobile fraction.

FRAP

Fluorescence Recovery after Photo-bleaching

Domanda: visualizzare la mobilità di una molecola nella cellula

Idea: sbiancare la molecola in un'area della cellula

e osservare il recupero della fluorescenza mediante trasporto della molecola

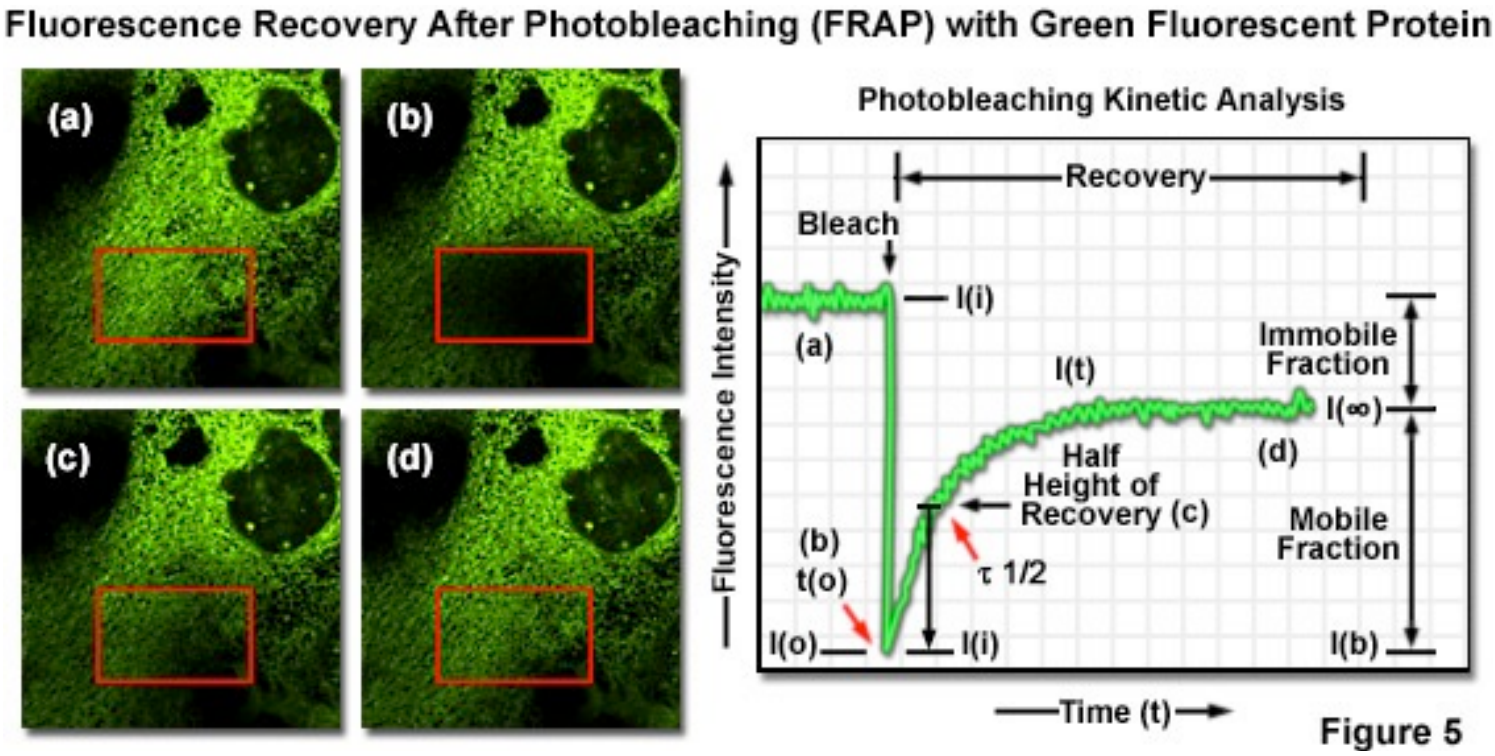


Figure 5

FLIP
Fluorescence Loss in Photo-bleaching

Domanda: visualizzare le connessioni tra compartimenti cellulari

Idea: sbiancare la molecola in un'area della cellula

e osservare la diminuzione del segnale in aree connesse