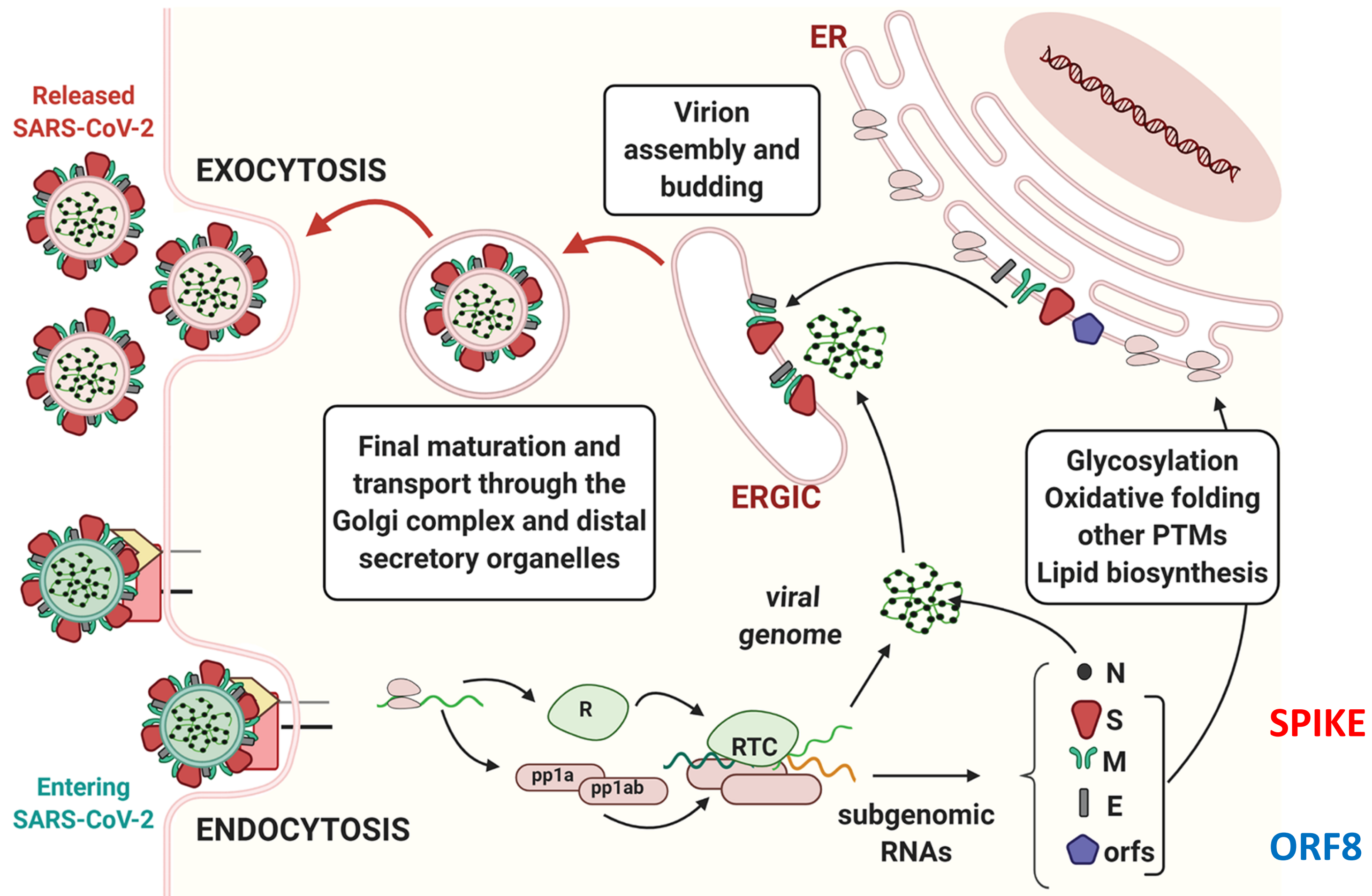
Cds in Scienze e Tecnologie Biologiche

AA 2020-2021

Corso di Laboratorio di Biologia Cellulare

Lezione 6

Esperimento 1:
LE PROTEINE SPIKE E ORF8 LOCALIZZANO LUNGO LA VIA SECRETORIA



SPIKE

ORF8

PER STUDIARE LE FUNZIONI CELLULARI DELLE PROTEINE VIRALI POSSIAMO CHIEDERCI CON QUALI PROTEINE CELLULARI ESSE INTERAGISCANO:
LA RISPOSTA SI OTTIENE MEDIANTE L’ANALISI DELL’ INTERATTOMA DELLE PROTEINE VIRALI

ANALISI DELL'INTERAZIONE PROTEINA-PROTEINA MEDIANTE AFFINITY PURIFICATION

A SARS-CoV-2-Human Protein-Protein Interaction Map Reveals Drug Targets and Potential Drug-Repurposing

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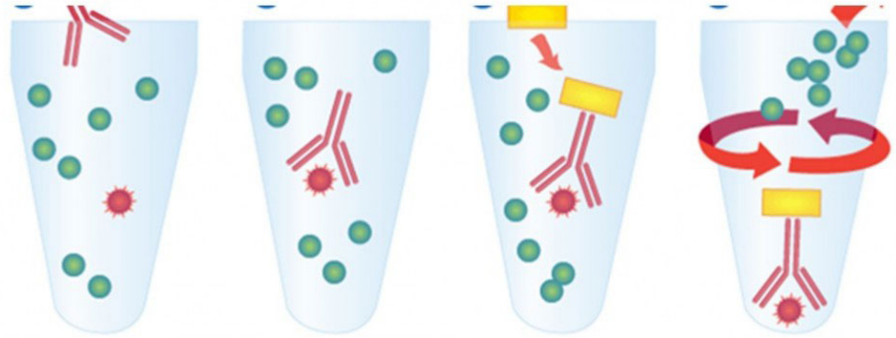
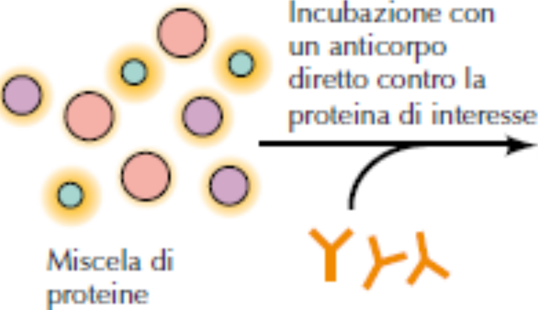
ABSTRACT

An outbreak of the novel coronavirus SARS-CoV-2, the causative agent of COVID-19 respiratory disease, has infected over 290,000 people since the end of 2019, killed over 12,000, and caused worldwide social and economic disruption[1,2]. There are currently no antiviral drugs with proven efficacy nor are there vaccines for its prevention. Unfortunately, the scientific community has little knowledge of the molecular details of SARS-CoV-2 infection. To illuminate this, we cloned, tagged and expressed 26 of the 29 viral proteins in human cells and identified the human proteins physically associated with each using affinity-purification mass spectrometry (AP-MS), which identified 332 high confidence SARS-CoV-2-human protein-protein interactions (PPIs). Among these, we identify 67 druggable human proteins or host factors targeted by 69 existing FDA-approved drugs, drugs in clinical trials and/or preclinical compounds, that we are currently evaluating for efficacy in live SARS-CoV-2 infection assays. The identification of host dependency factors mediating virus infection may provide key insights into effective molecular targets for developing broadly acting antiviral therapeutics against SARS-CoV-2 and other deadly coronavirus strains.

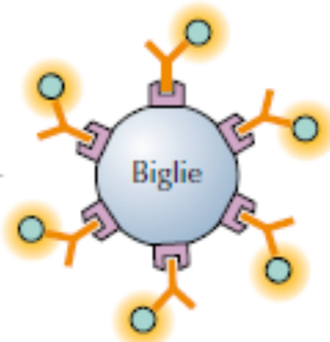
Immunoprecipitazione: tecnica per la purificazione di proteine mediante anticorpi specifici



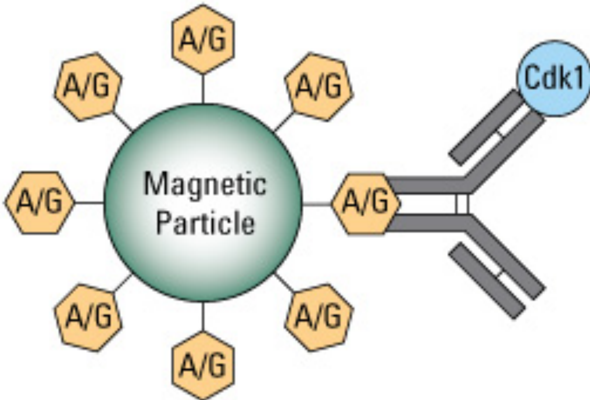
Lisato cellulare

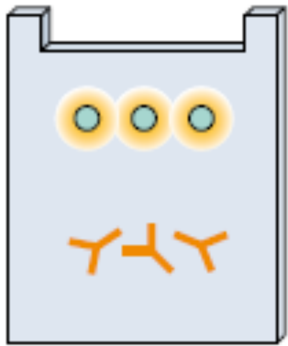


Raccolta dei complessi antigene-anticorpo

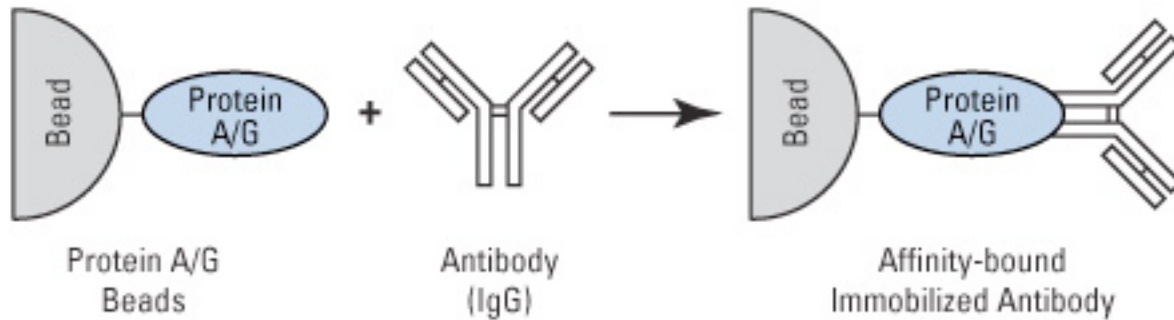


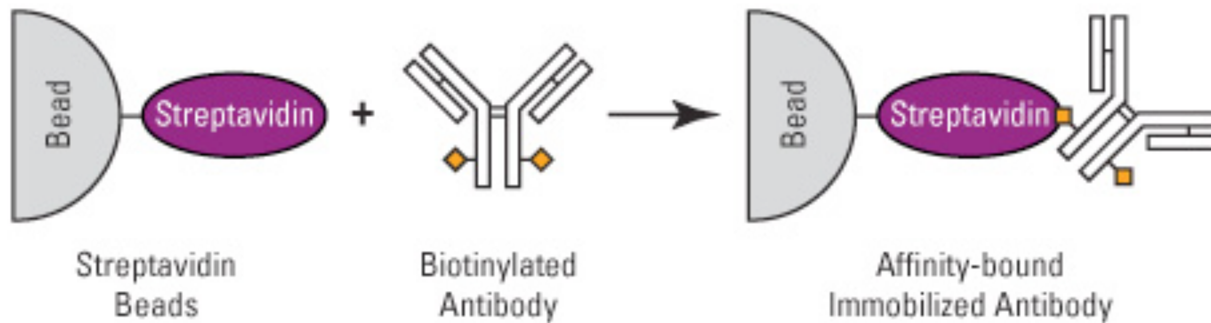
Legame a un anticorpo specifico per la proteina di interesse

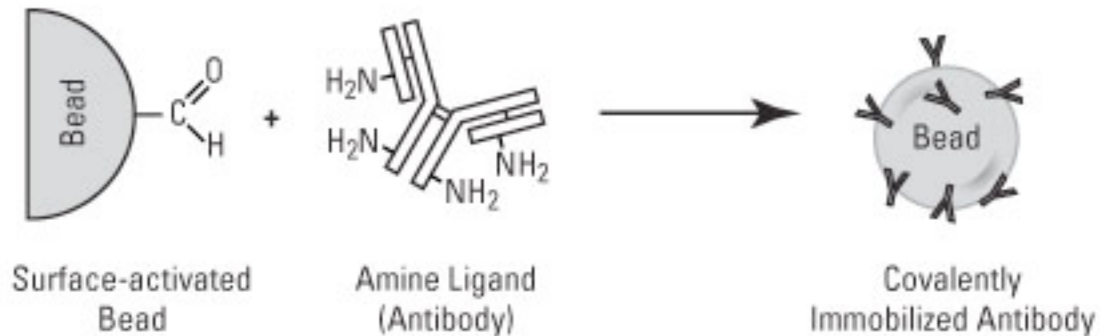


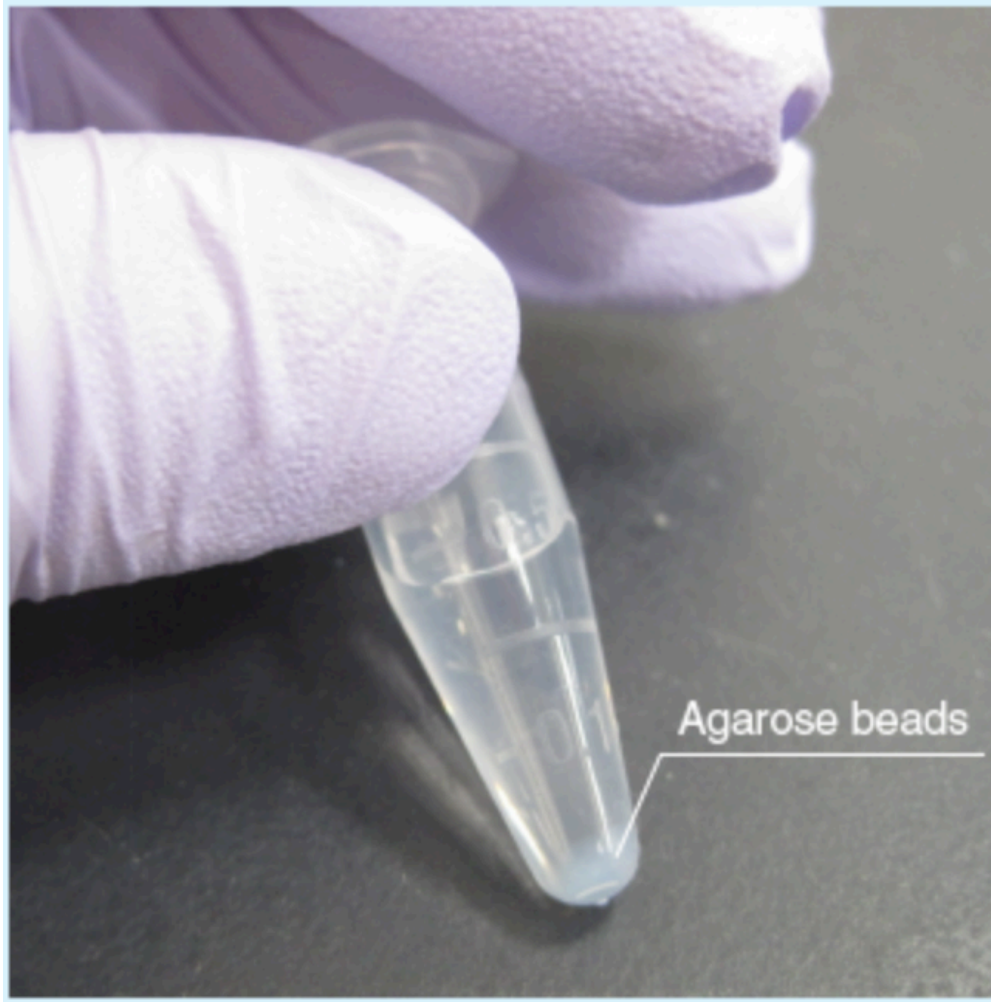


Legame degli anticorpi alle beads

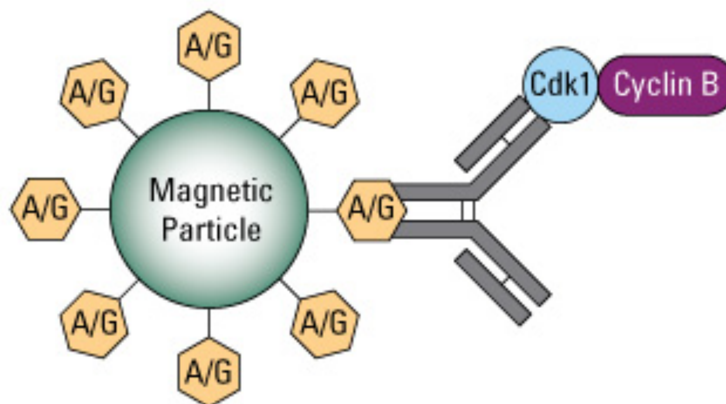


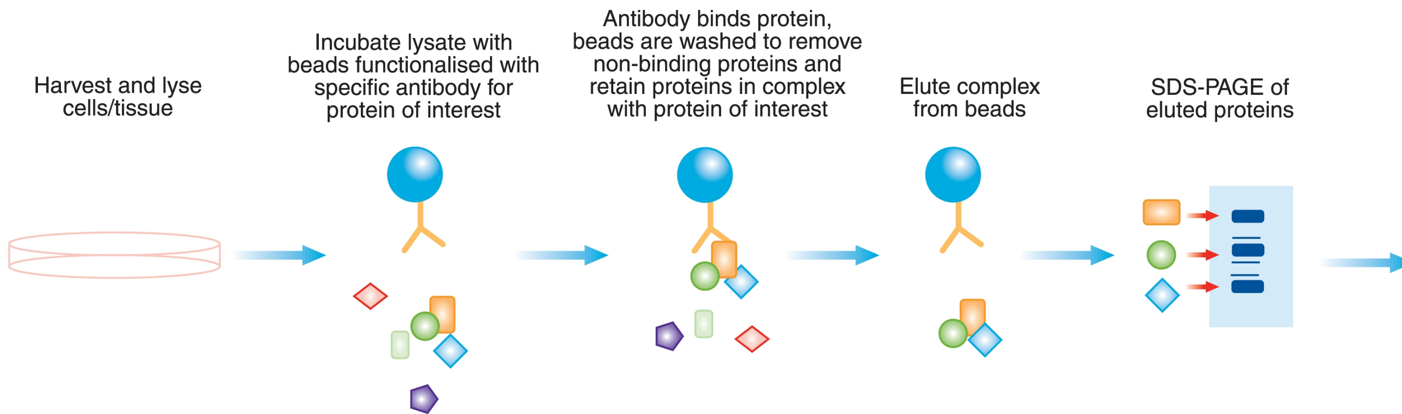





Agarose beads

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





Se le proteine partner sono candidati noti, si analizzano mediante WB

Visualizzazione delle proteine immunoprecipitate: western blotting

Tecnica che prevede il riconoscimento mediante anticorpi specifici di proteine previamente sottoposte ad elettroforesi.

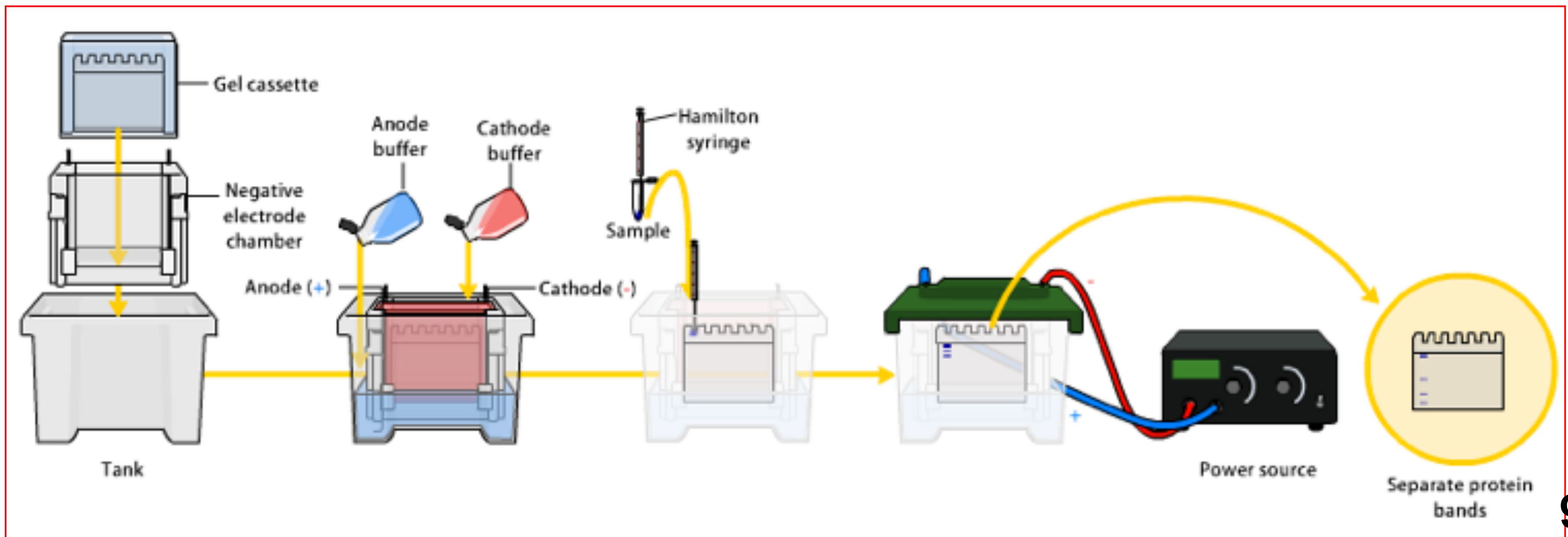
Permette di ottenere informazioni su:

Massa molecolare (velocità di migrazione)

Livelli di espressione

Modificazioni post-traduzionali (Ab specifici)

Interazioni con altre proteine

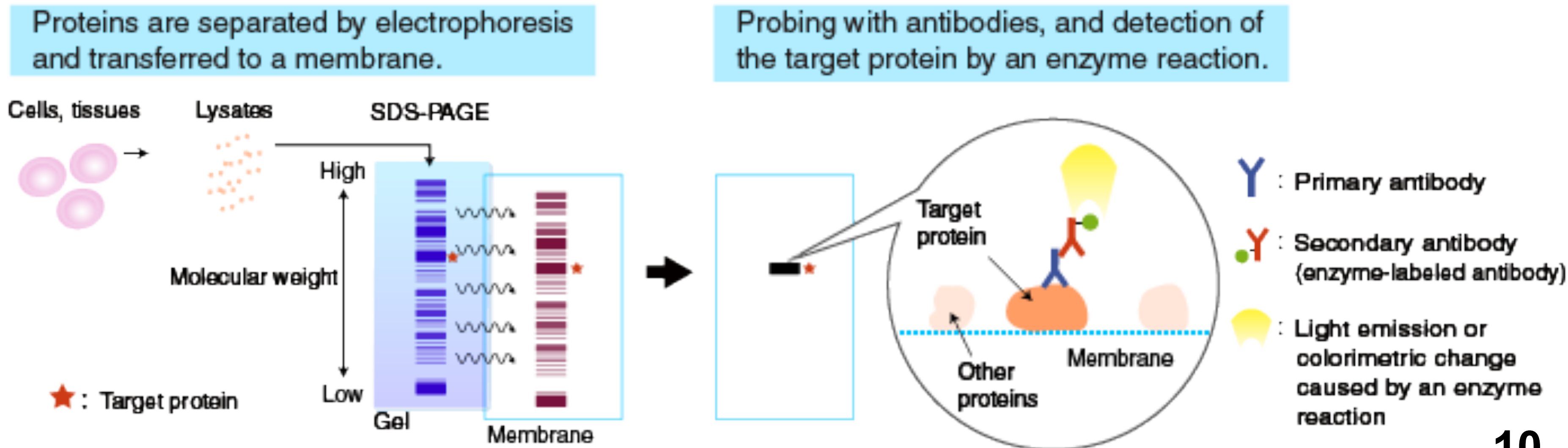


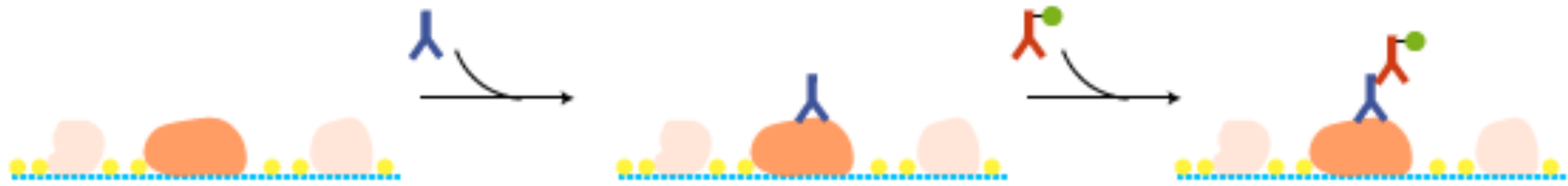
Poichè i gel di poliacrilamide sono supporti poco stabili e impenetrabili agli Ab , è necessario trasferire le bande proteiche su un supporto che le renda accessibili: di solito una membrana di nitrocellulosa, mediante un campo elettrico trasversale.

Il legame alla membrana è stabile e consente di effettuare un'incubazione in fase liquida con un anticorpo primario specifico, che riconoscerà specificamente la banda dell'antigene

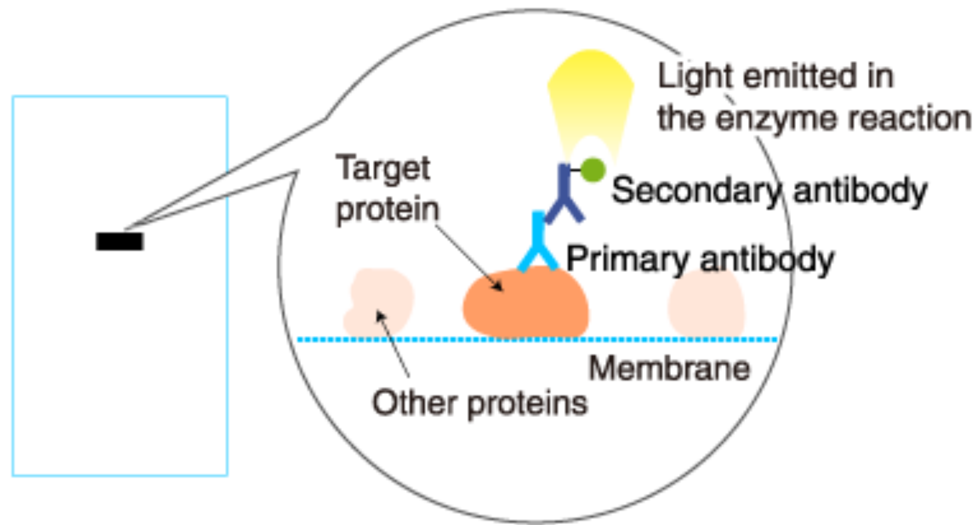
Si effettua successivamente un'incubazione con un anticorpo secondario coniugato con un ENZIMA

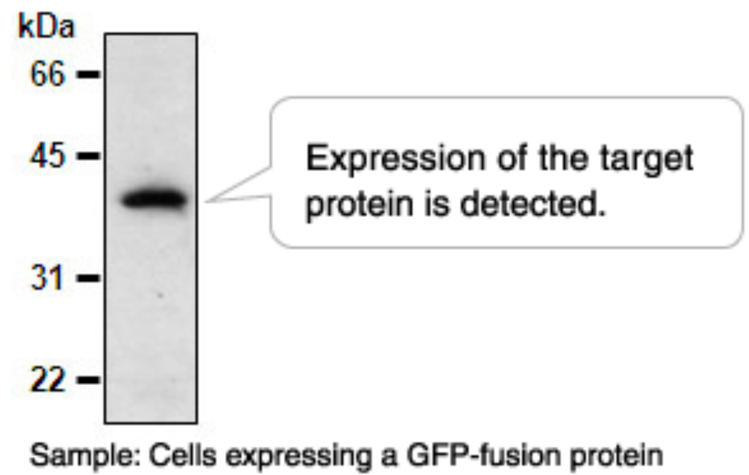
Reazione di SVILUPPO: incubazione con il substrato che sviluppa un prodotto colorato o chemiluminescente





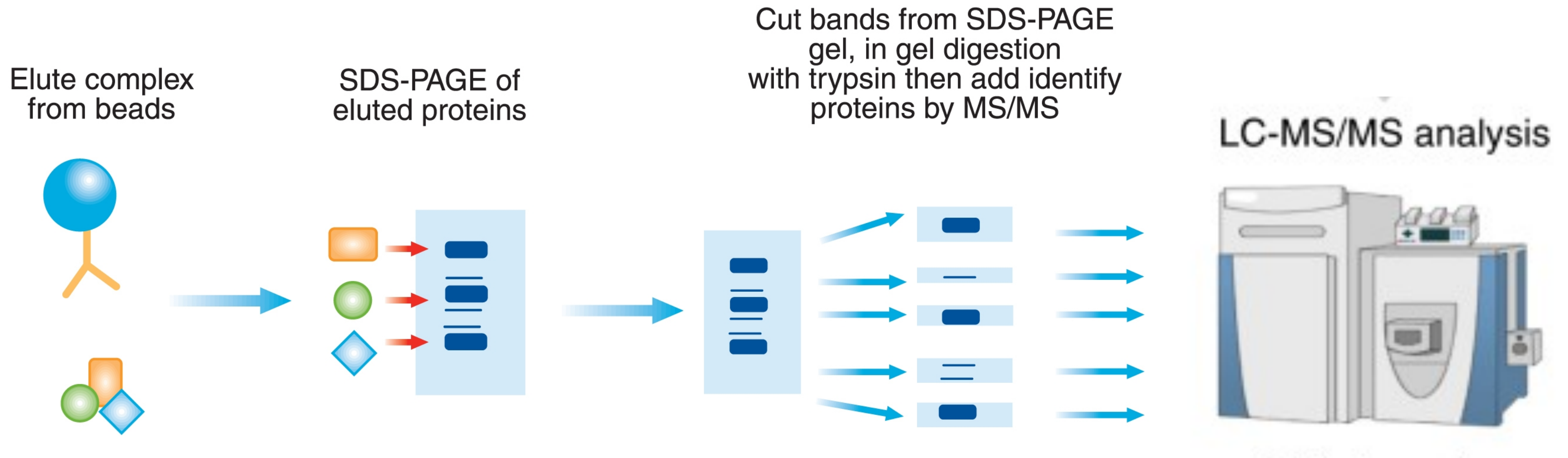
Enzyme name	Chromogenic substrate	Chemiluminescence substrate
HRP (Horseradish peroxidase)	DAB and TMB	Luminol-based (ECL)
AP (Alkaline phosphatase)	BCIP/NBT and pPNPP	Dioxetane-based (CDP-star®)



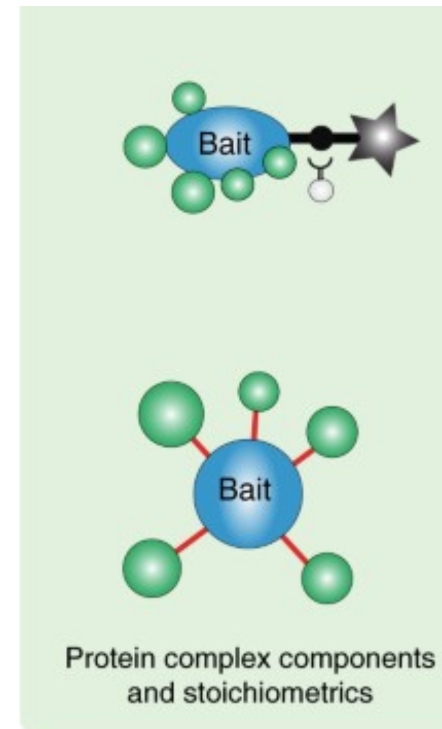


Sample: Cells expressing a GFP-fusion protein

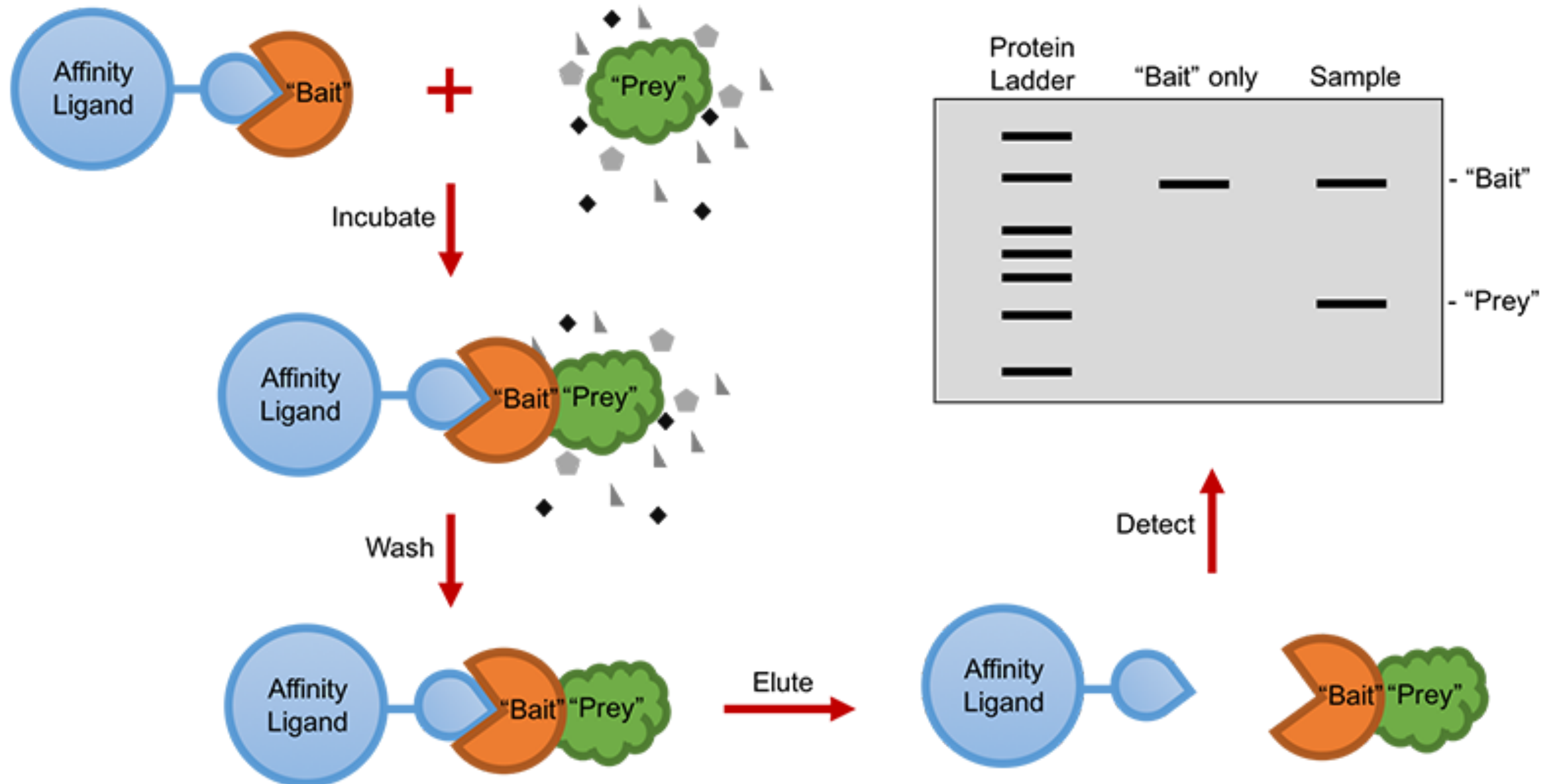
Analisi dell'interattoma mediante spettrometria di massa



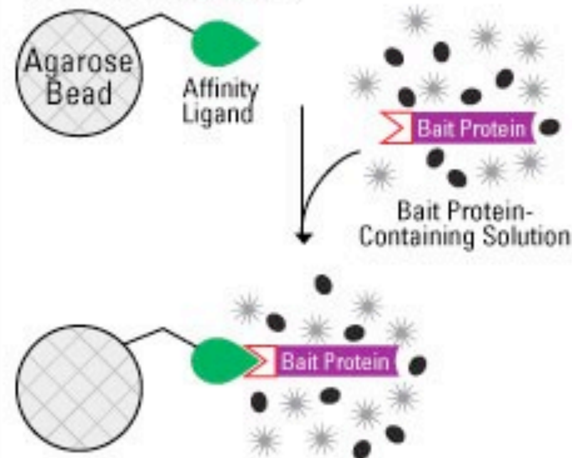
Se le proteine partner non sono note, si sottopongono le bande dell'interattoma all'analisi mediante spettrometria di massa.



Tecniche alternative:
AP-pulldown di proteine di fusione (BAIT) espresse in batteri o cellule eucariotiche

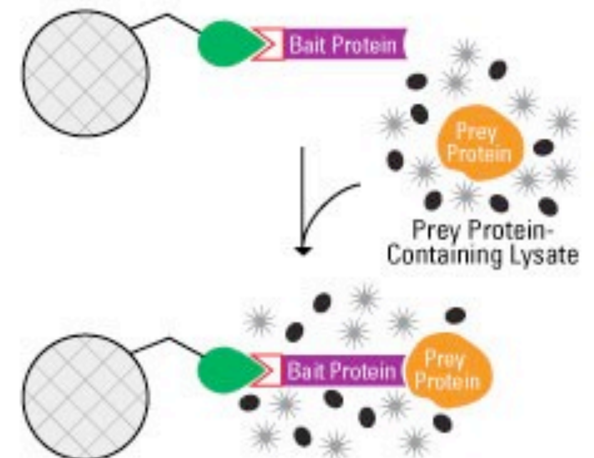


Step 1. Immobilize the fusion-tagged "bait" from the lysate.



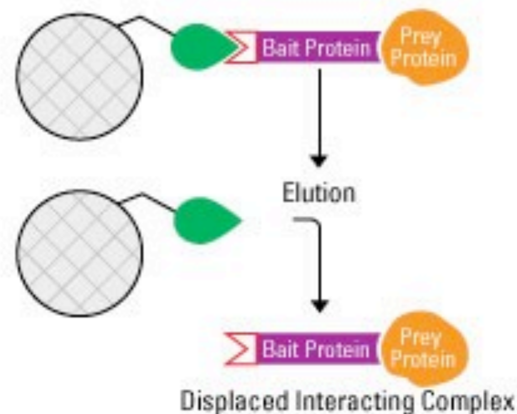
Step 2. Wash away unbound protein.

Step 3. Bind "prey" protein to immobilized "bait" protein.

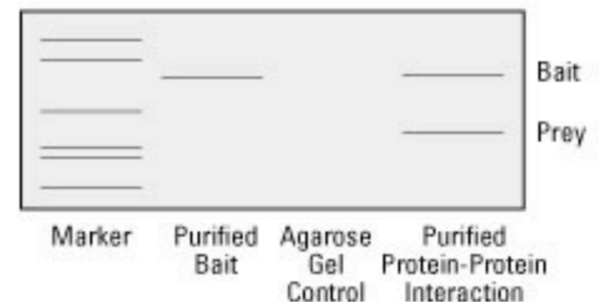


Step 4. Wash away unbound protein.

Step 5. Elute protein-protein interaction complex.



Step 6. Analyze protein-protein interaction complex by SDS-PAGE.



= Affinity Ligand (Glutathione, Co^{2+} Chelate or Streptavidin)

= Fusion Tag (GST, polyHis or Biotin)

GST-pulldown di proteine di fusione con GST espresse in batteri

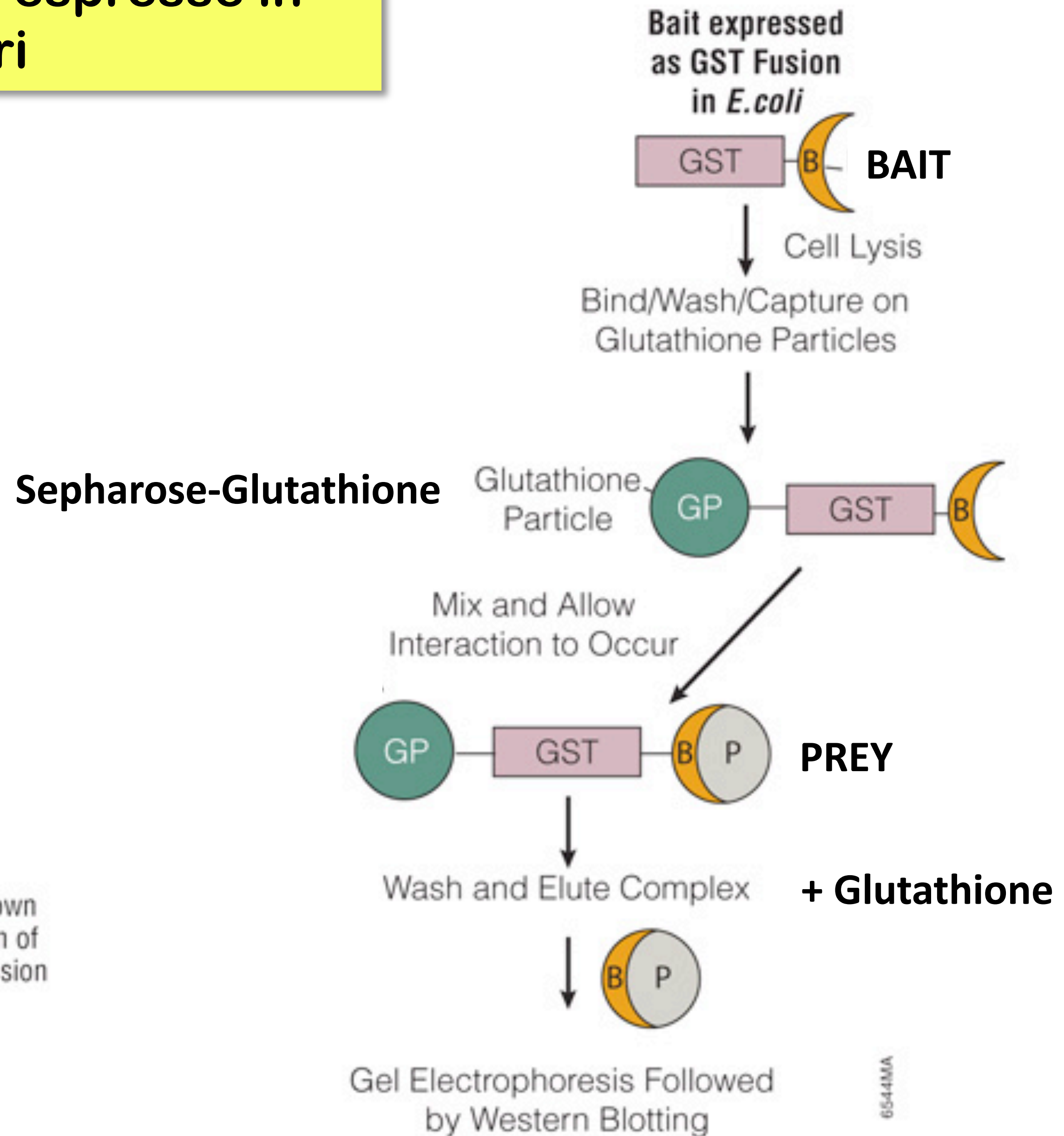
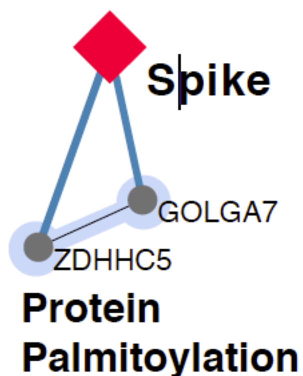


Figure 1. Schematic of pull-down assay using bacterial expression of bait protein and cell-free expression for the prey protein.

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.**

Rappresentazione dell’interattoma



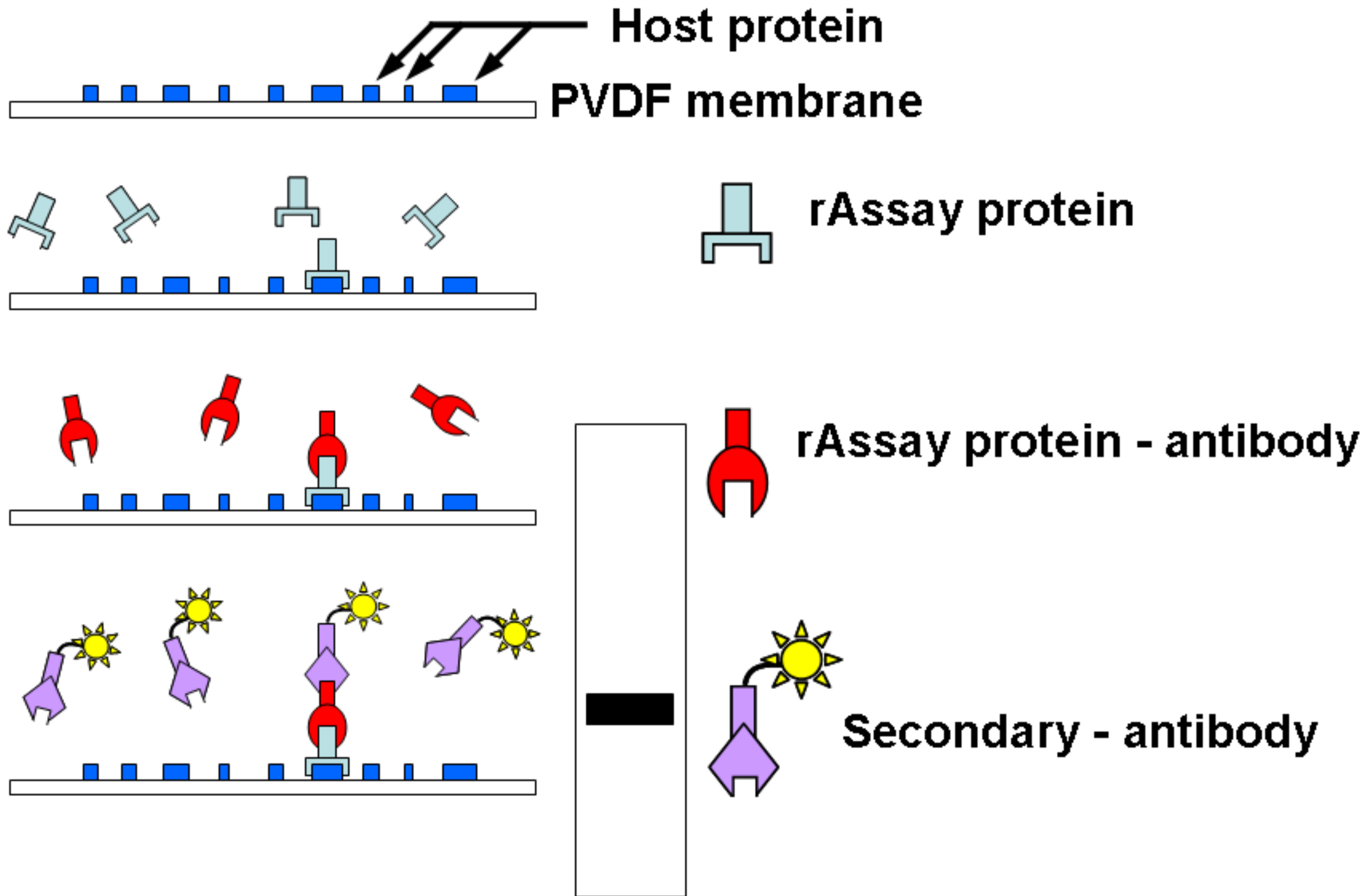
ER Protein Quality Control

EDEM3
ERLEC1
OS9
UGGT2
ERO1B
SIL1
HYOU1
NGLY1
TOR1A
FOXRED2
SDF2

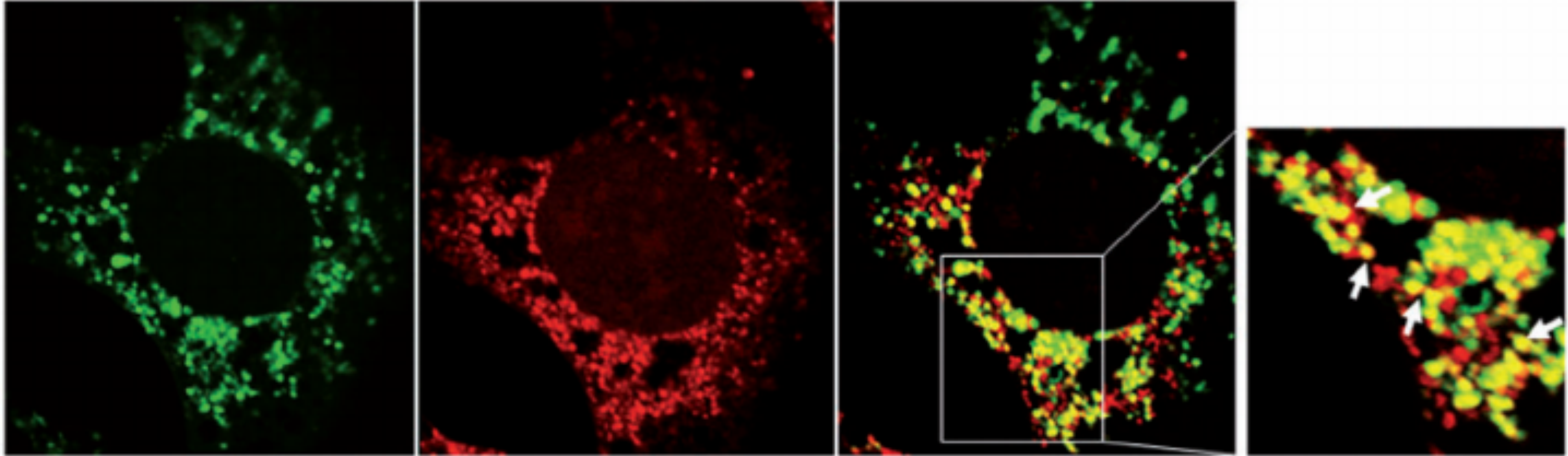
Orf8

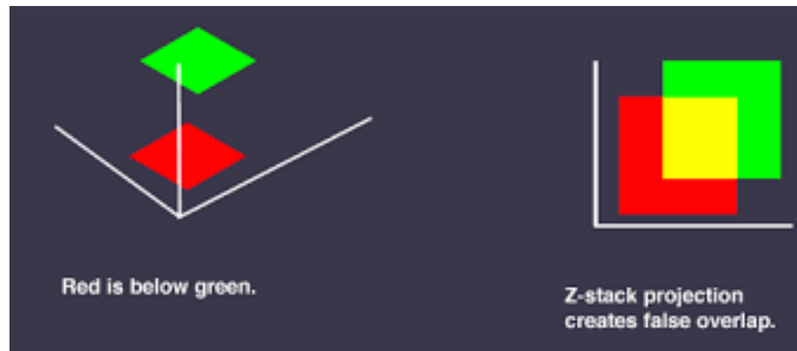
FBXL12
POGLUT3
PLEKHF2
CISD3
INHBE
GDF15
SMOC1
NEU1
PLAT
POGLUT2
STC2
NPTX1
POFUT1
HS6ST2
MFGE8
ERP44
CHPF2
EMC1
DNMT1
ITGB1
ADAM9
PCSK6
PLD3
PUSL1
IL17RA
GGH
FKBP7
FKBP10
PLOD2
PVR
COL6A1
LOX
CHPF
NPC2
TM2D3
ADAMTS1

VALIDAZIONE DELLE INTERAZIONI (1):
Analisi di interazione DIRETTA proteina-proteina mediante FAR WESTERN:



VALIDAZIONE DELLE INTERAZIONI (2): microscopia confocale





NB: è necessario l'impiego della microscopia confocale

VALIDAZIONE DELLE INTERAZIONI (3)

Analisi dell'interazione proteina-proteina IN SITU:

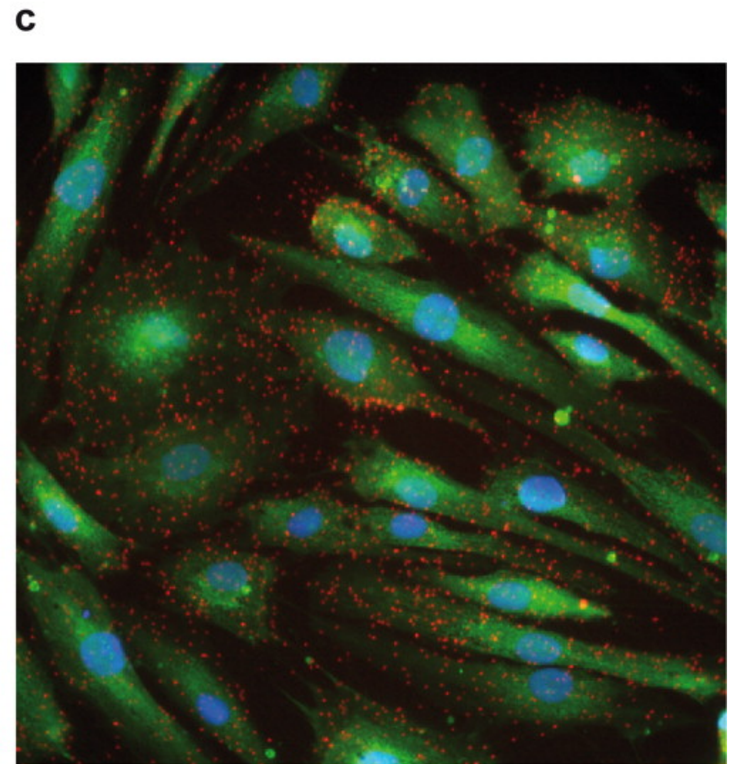
Proximity Ligation Assay PLA

In situ proximity ligation assay PLA

a

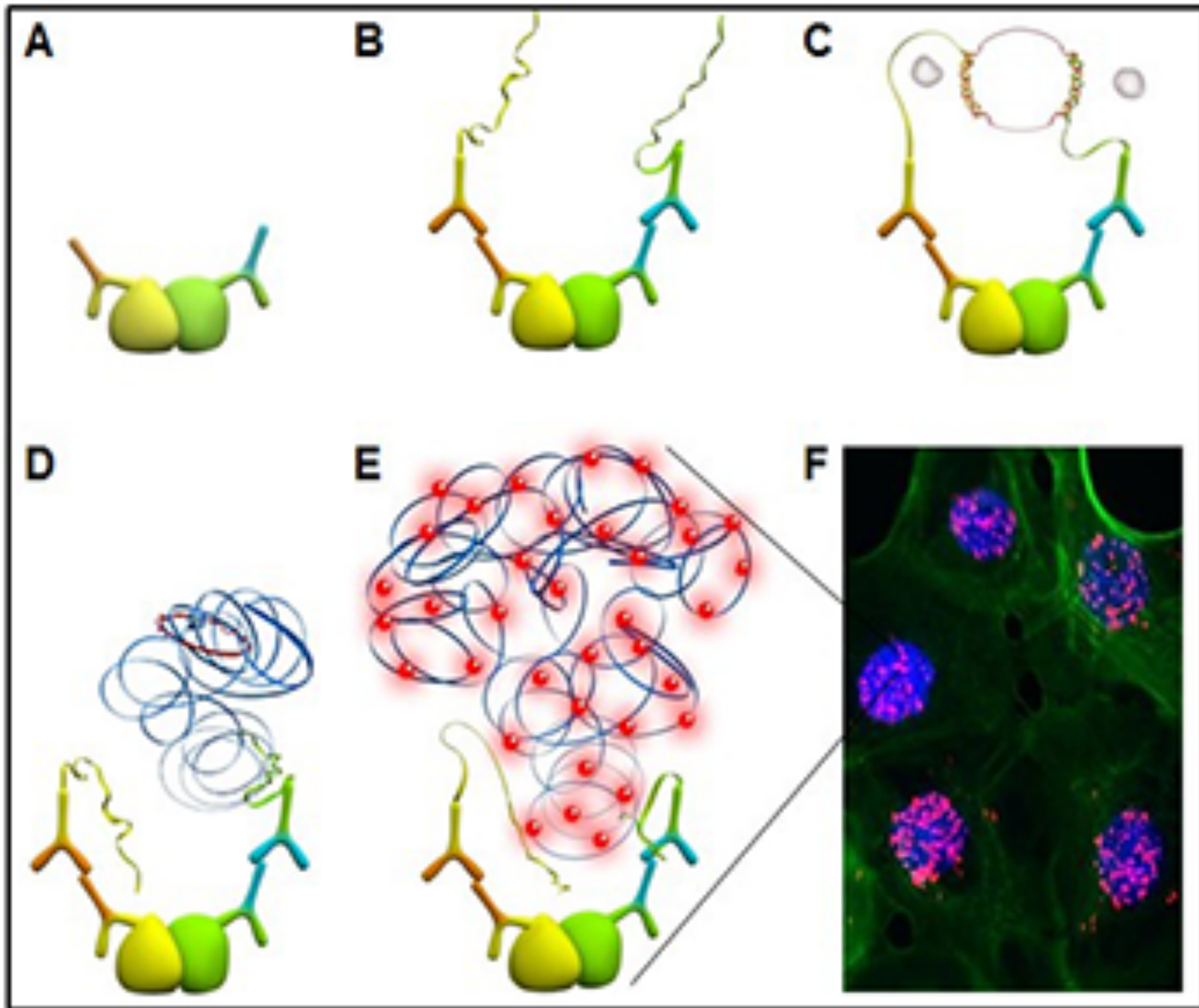
b

c

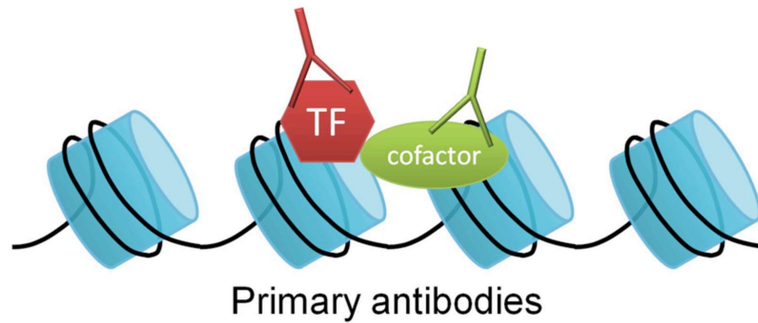


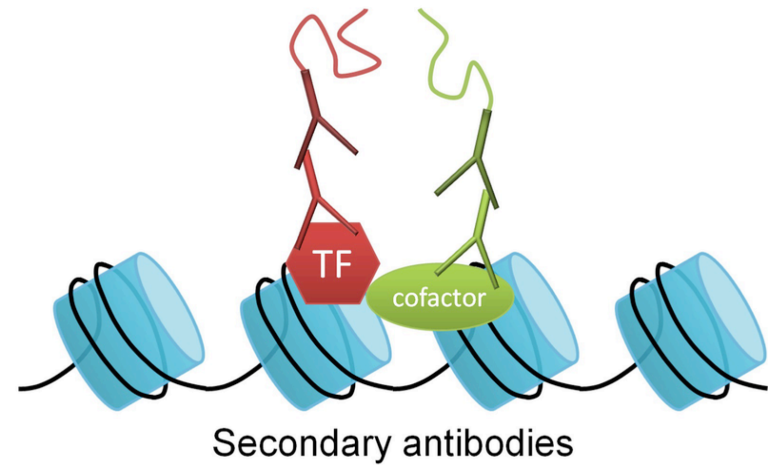
species-specific antibodies conjugated to oligonucleotides used as templates for the joining of two additional linear oligos into a DNA circle

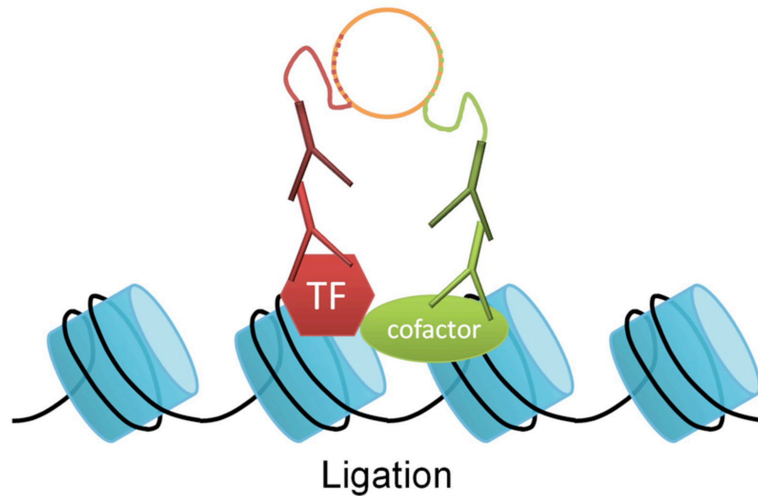
DNA amplification
hybridize with fluorescent probe

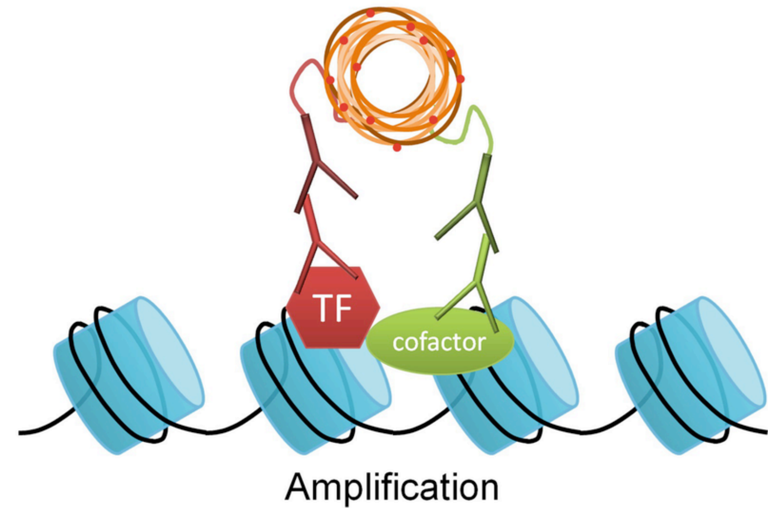

A
B
C
D
E
F

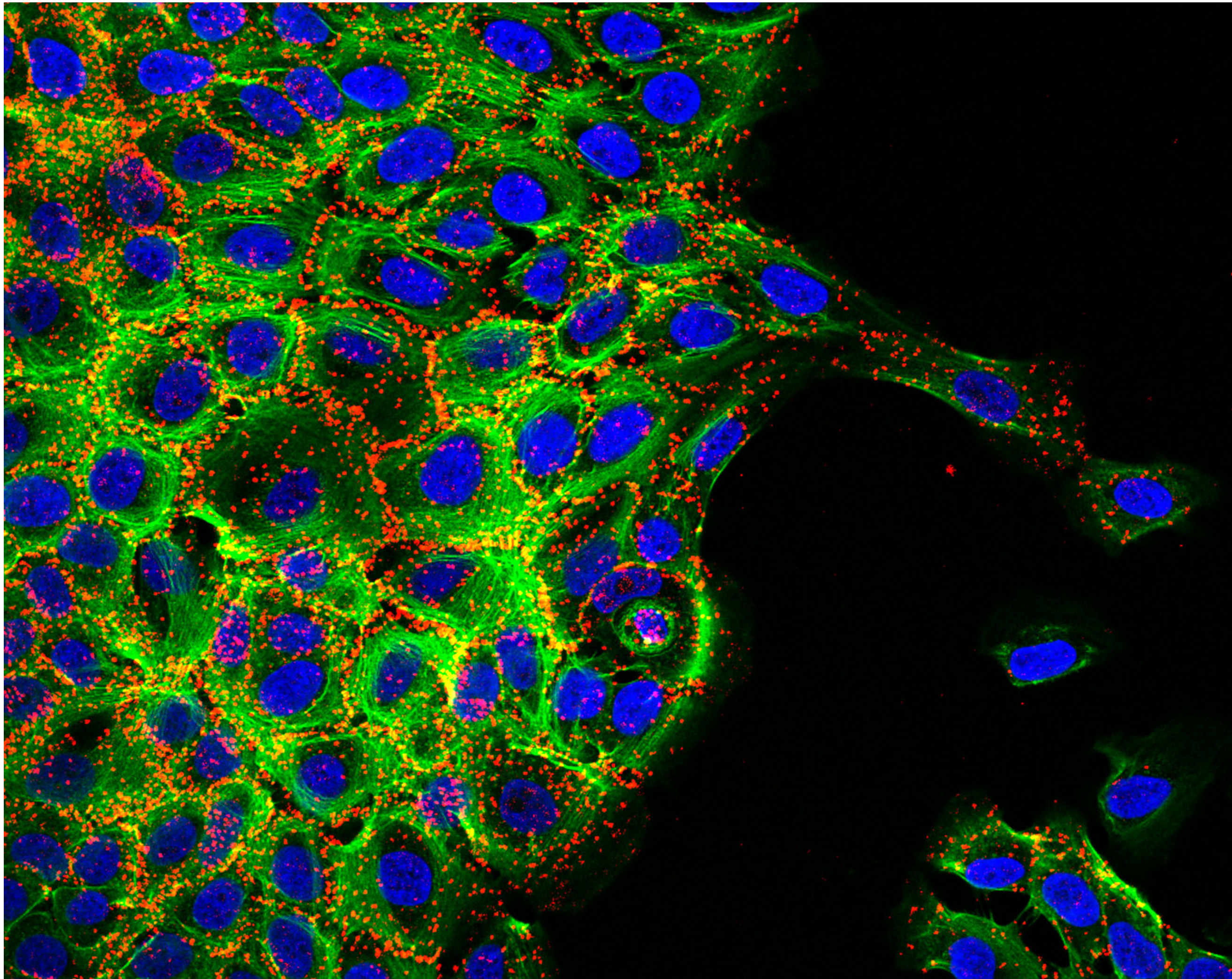
In situ PLA per visualizzare l'interazione tra un TF e un cofattore









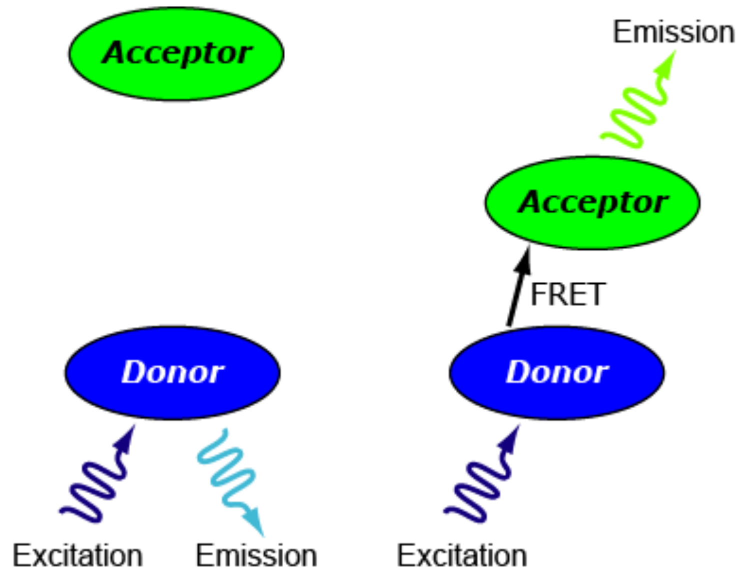


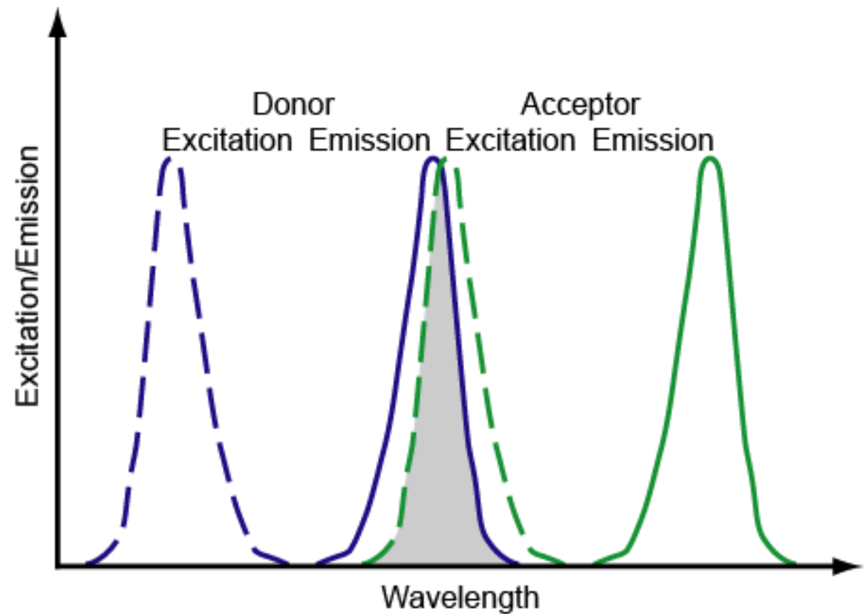
In situ proximity ligation assay for E-cadherin and p120ctn (red) in MCF10A-ER-Src cells stained with Phalloidin to mark actin filaments (green) and DAPI

VALIDAZIONE DELLE INTERAZIONI (4)

Analisi dell'interazione proteina-proteina IN SITU mediante
Fluorescence Resonance Energy Transfer
FRET

Förster Resonance Energy Transfer





Emission and excitation spectrum must significantly overlap

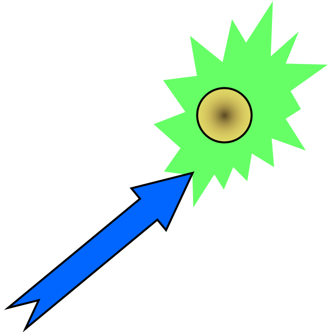
Es. CFP/YFP

Fluorescein/Rhodamine

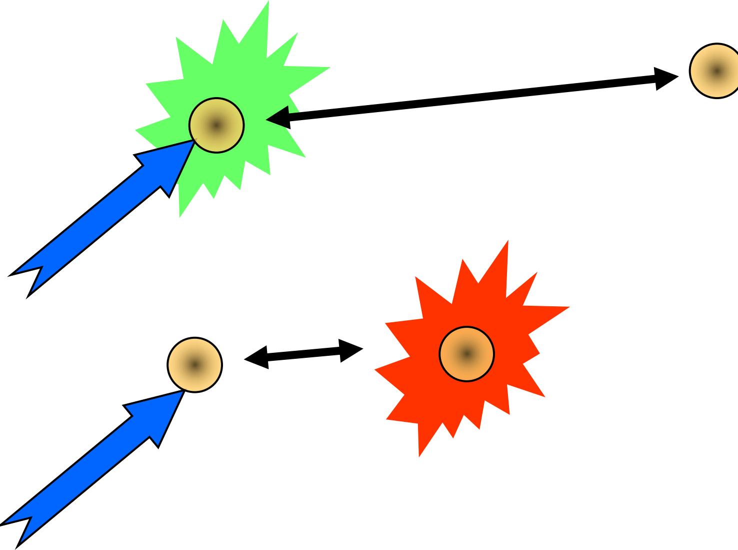
Cy3/Cy5

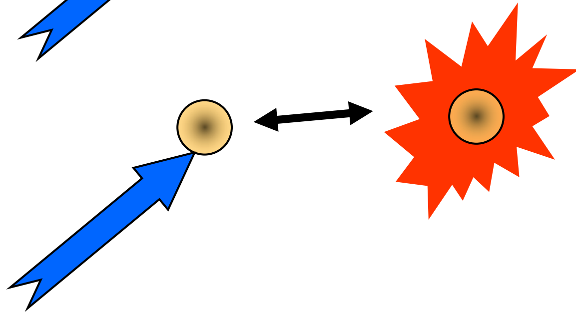
La FRET è efficiente per piccole distanze

Isolated donor

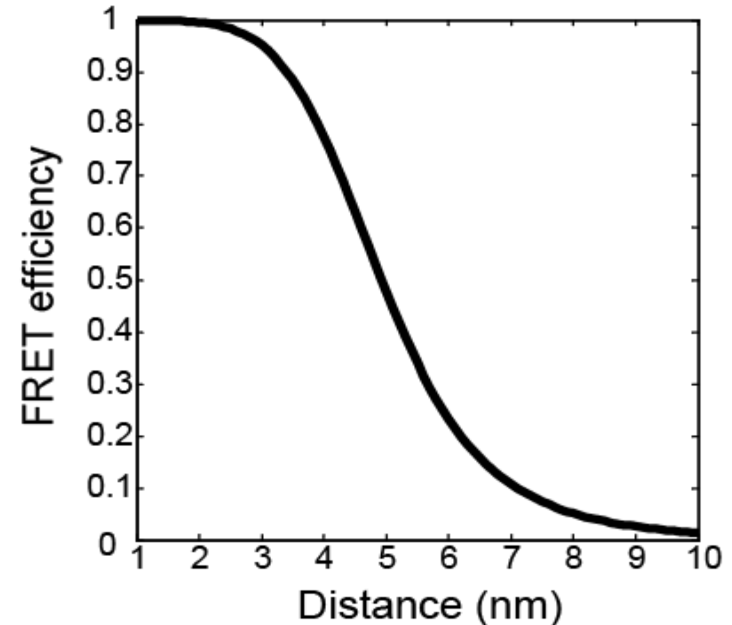


Donor distance too great





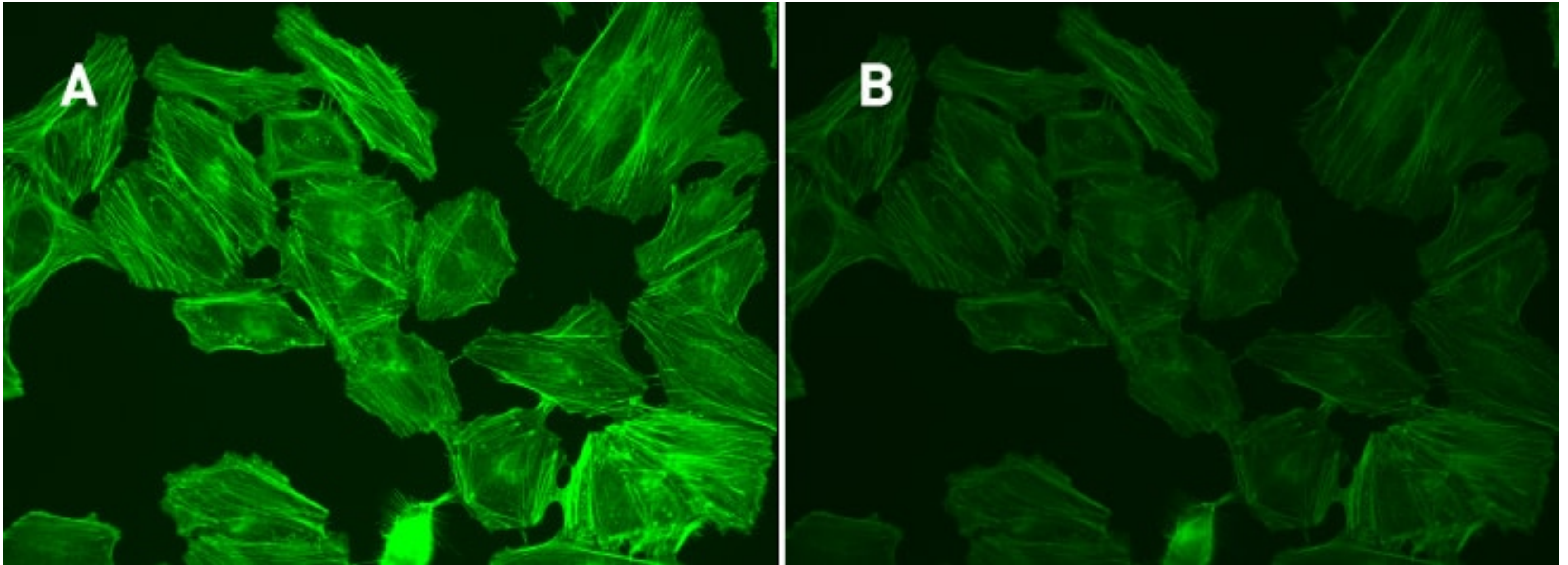
Donor distance correct



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!!!

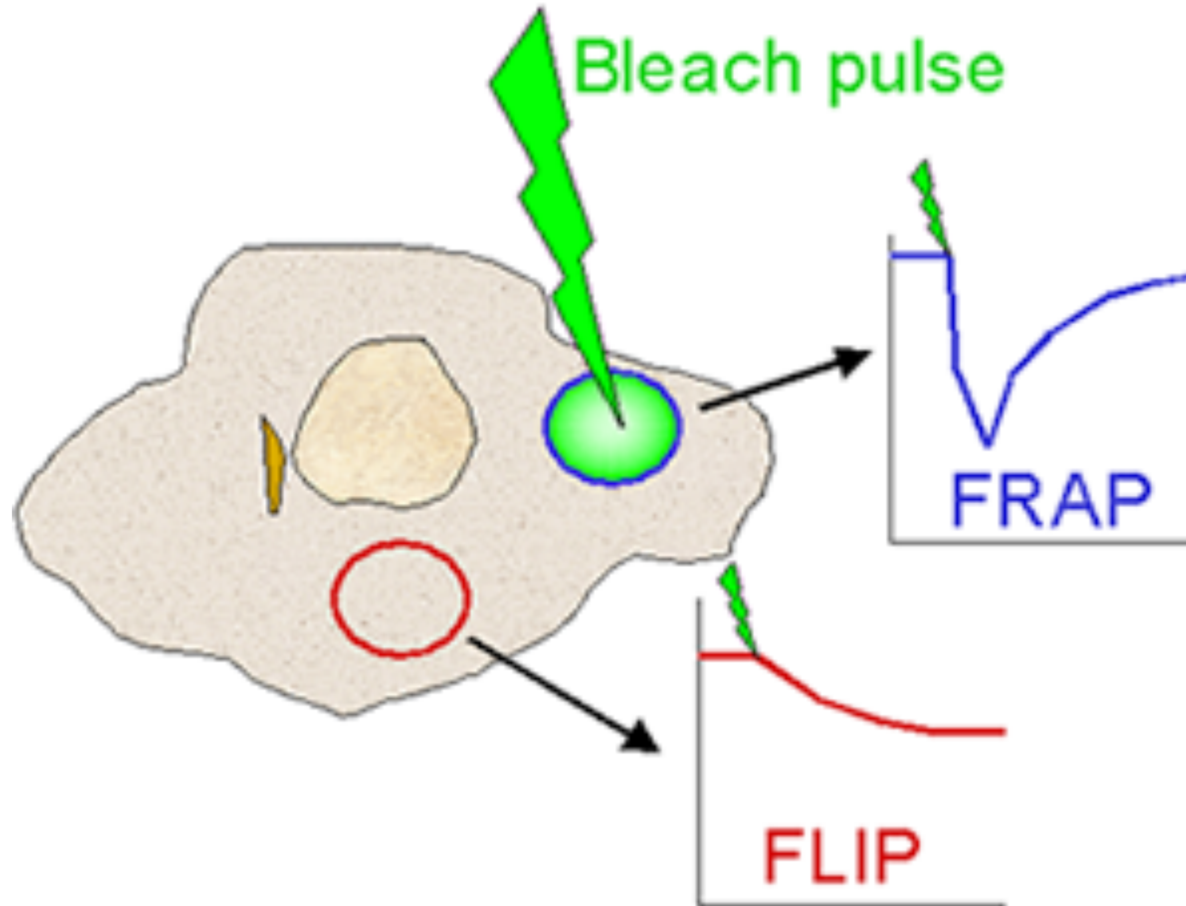
Photo-bleaching



La luce di eccitazione induce la conversione chimica permanente del fluoroforo in una molecola non fluorescente. Dopo un tempo (variabile) di esposizione si ha la progressiva perdita della fluorescenza.

Sfruttare il photo-bleaching per studiare la mobilità intracellulare

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



FRAP

Fluorescence Recovery after Photo-bleaching

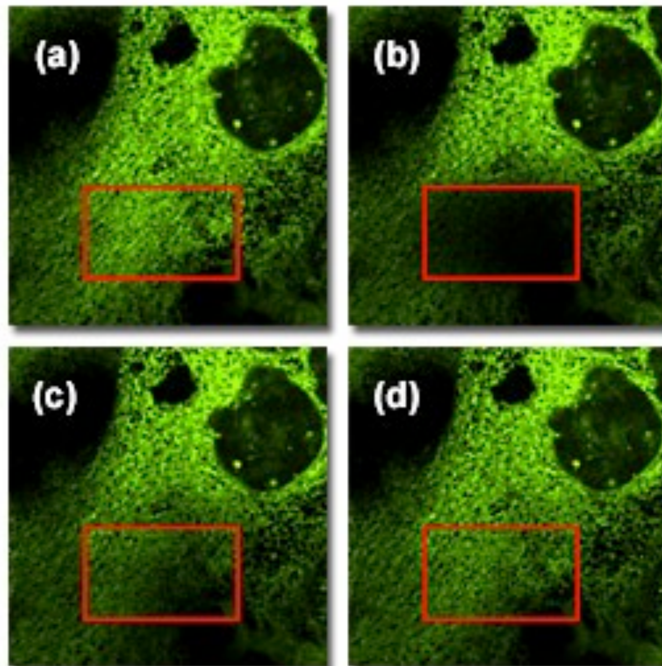
- **Photobleaching** (also termed **fading**) occurs when a fluorophore permanently loses the ability to fluoresce due to photon-induced chemical damage and covalent modification.
- Fluorescence recovery after photobleaching is a **quantitative fluorescence technique** that can be used to **measure the 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 causato dalla diffusione della molecola

Fluorescence Recovery After Photobleaching (FRAP) with Green Fluorescent Protein



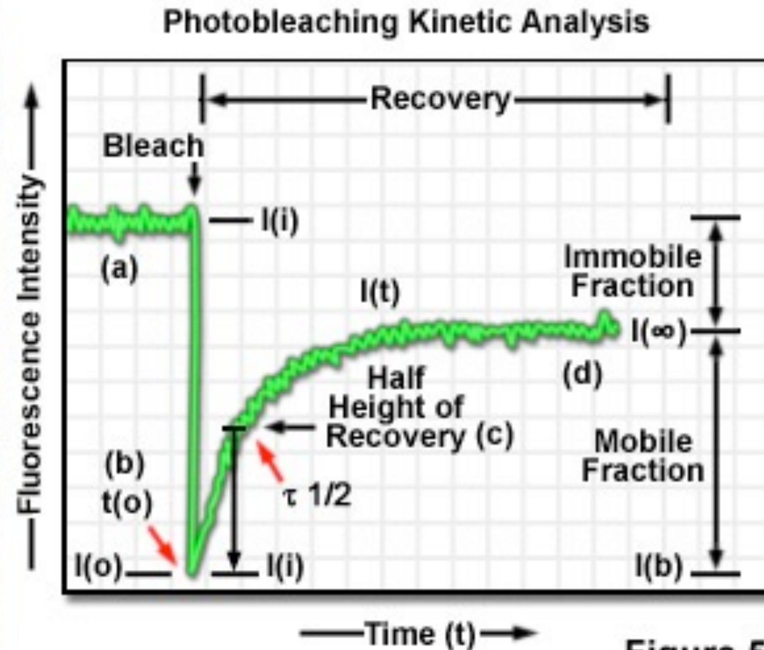


Figure 5

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