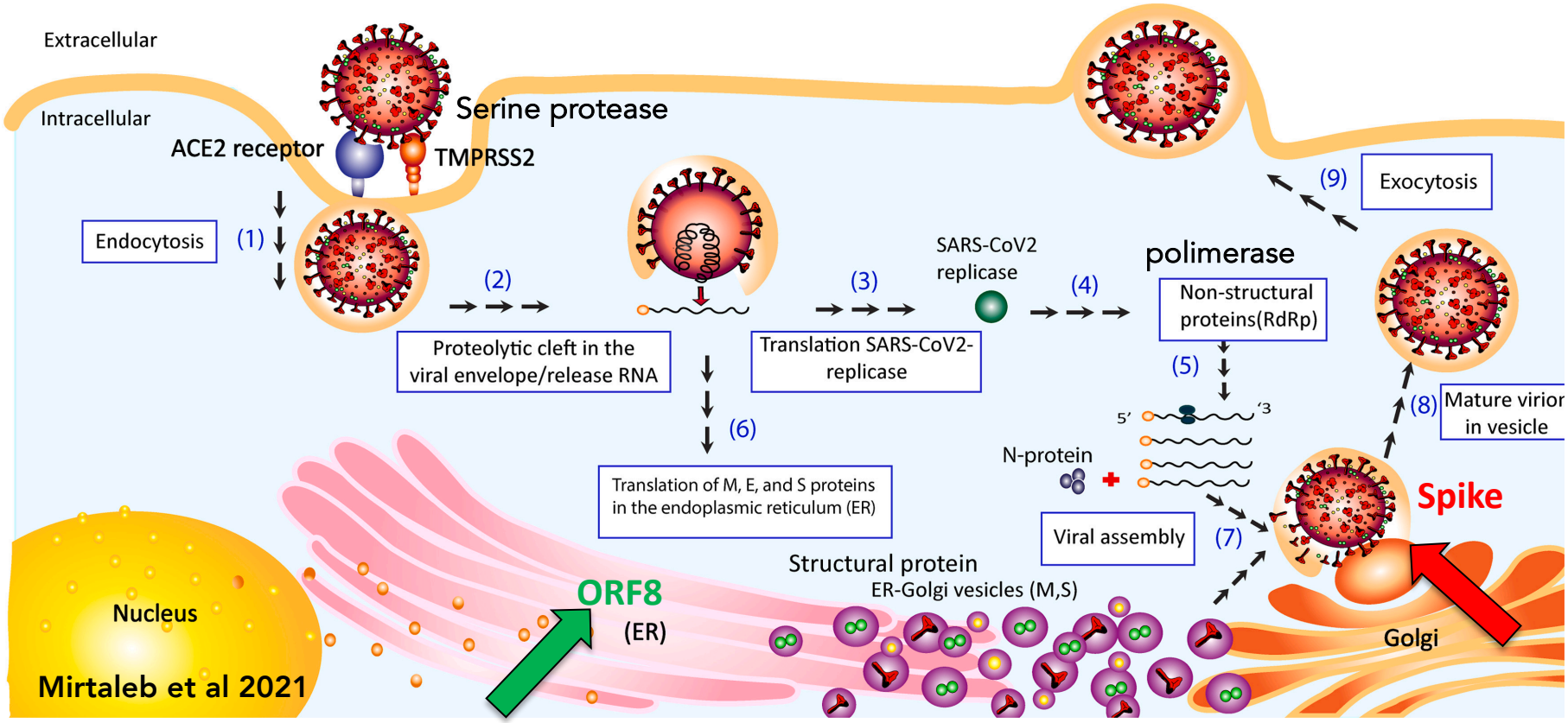
Cds in Scienze e Tecnologie Biologiche

AA 2024-2025

Corso di Biotecnologie Cellulari

Lezione 5

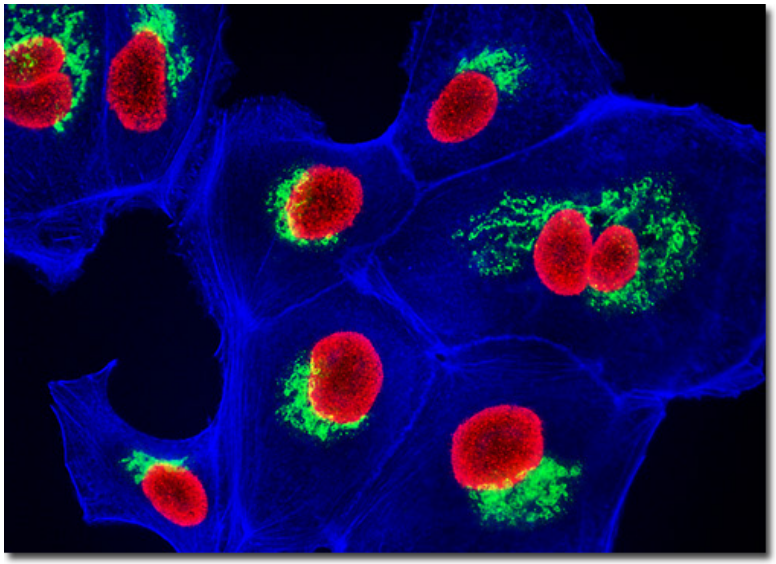
1. **sovraespressione delle proteine virali Spike e ORF8 (in fusione con TAG HA) in un modello cellulare;**
2. **Analisi della localizzazione subcellulare mediante immunofluorescenza;**
3. **Analisi dell'interattoma (esperimento virtuale).**

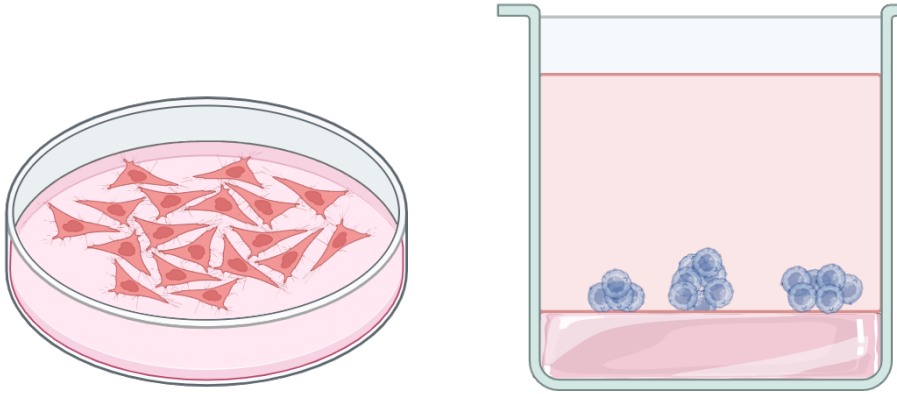


APPROCCIO SPERIMENTALE: DETTAGLIO

1. SCELTA DEL MODELLO CELLULARE

VALUTARE PRATICITA' VS RILEVANZA FISIOLOGICA





EPITELIALE O MESENCHIMALE?

2D o 3D?

STRATEGIA SPERIMENTALE: DETTAGLIO

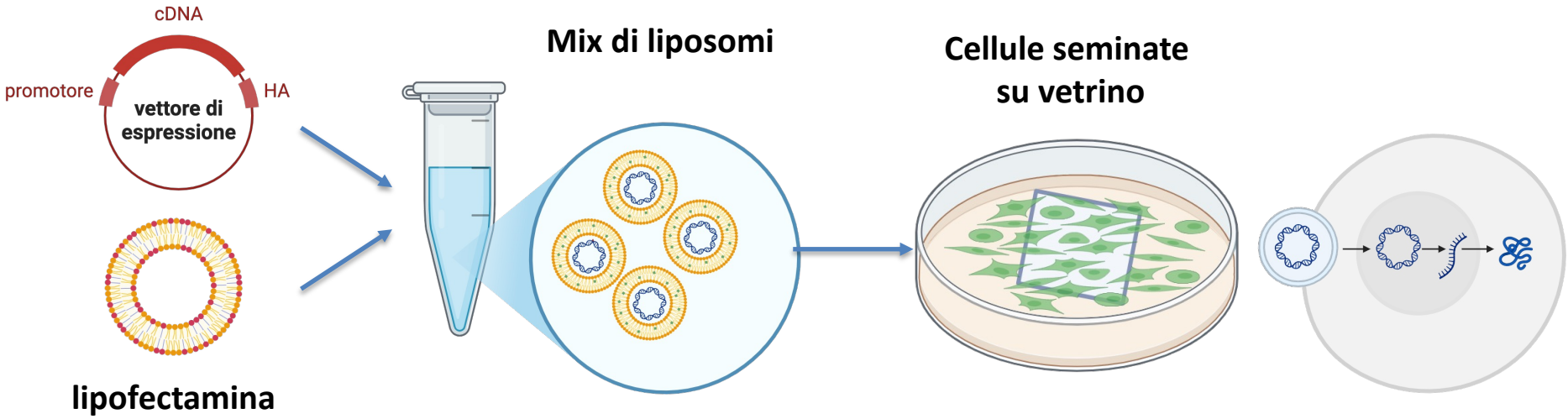
1. **SCELTA DEL MODELLO CELLULARE:**

   - **MODELLO 2D – es. LINEA CELLULARE EPITELIALE UMANA di polmone**
   - **MODELLO 3D – es. ORGANOIDI DERIVATI DA POLMONE (modello animale o UMANO)**
   - **MODELLO epiteliale – es. COLTURE ALI**

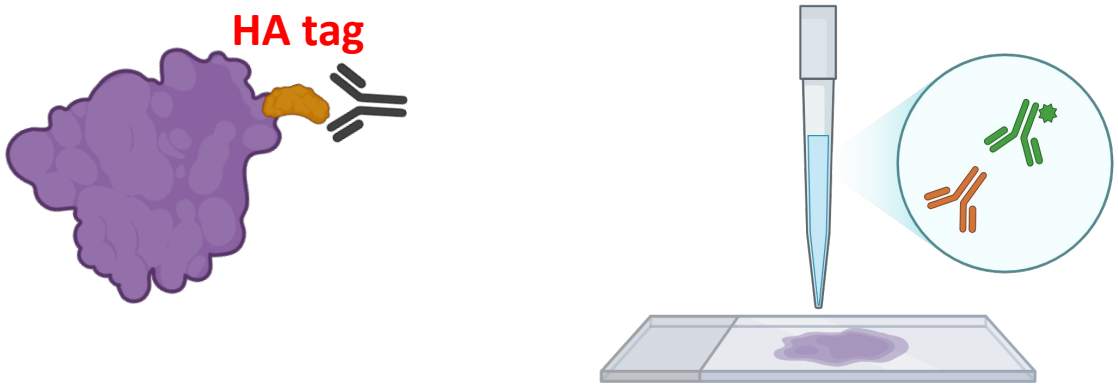
APPROCCIO SPERIMENTALE: DETTAGLIO

1. SCELTA DEL MODELLO CELLULARE
2. DISEGNO DELL'ESPERIMENTO
3. SCELTA DEI SAGGI
4. SCELTA DEGLI STRUMENTI

Trasfezione di cellule in coltura

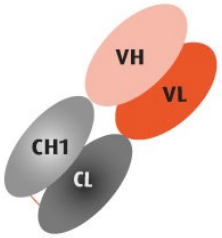


Riconoscimento di proteine espresse in maniera ectopica mediante immunofluorescenza con anticorpi speficifi per il TAG



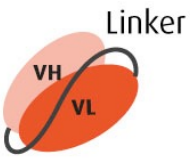
Anticorpi camelidi, nanobodies, single chain Fv, sono prodotti in vitro con tecniche di biologia molecolare

Camelidi = Heavy chain-only



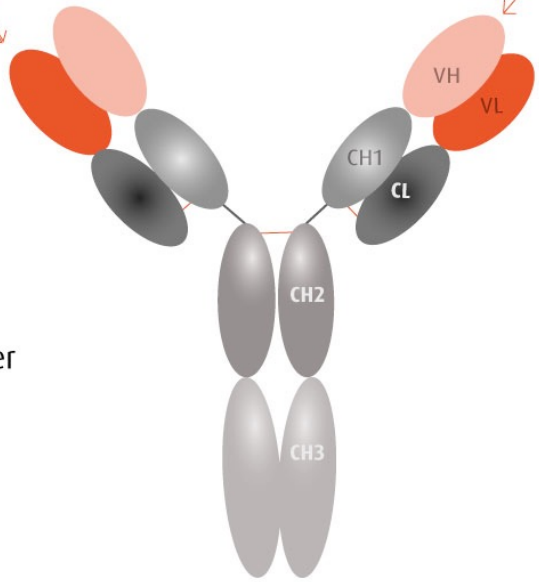
Fab

≃ 50 kD



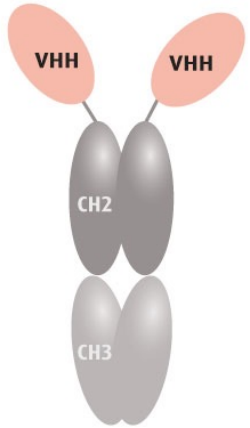
scFv
Single chain Fv fragment

≃ 25 kD



Human IgG Immunoglobulin

≃ 150 kD

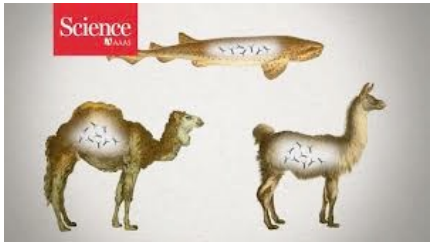


Llama Immunoglobulin

≃ 80 kD

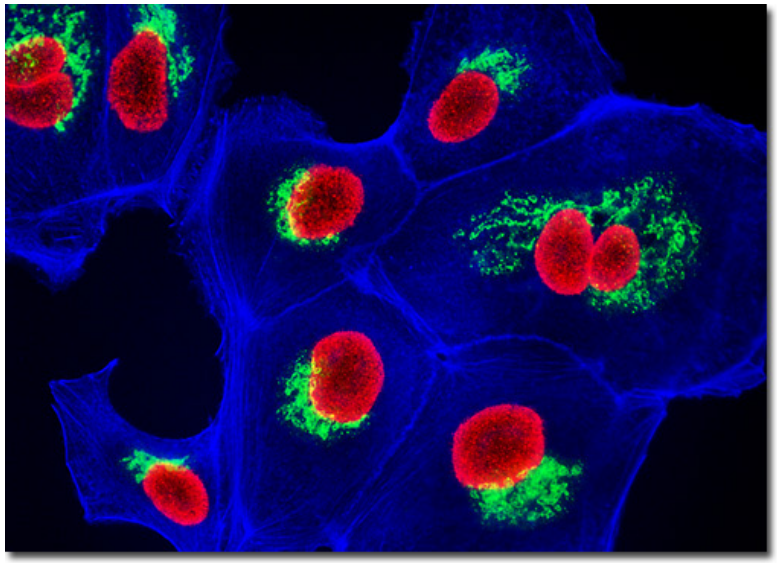
VHH single domain antibody

> 15 kD

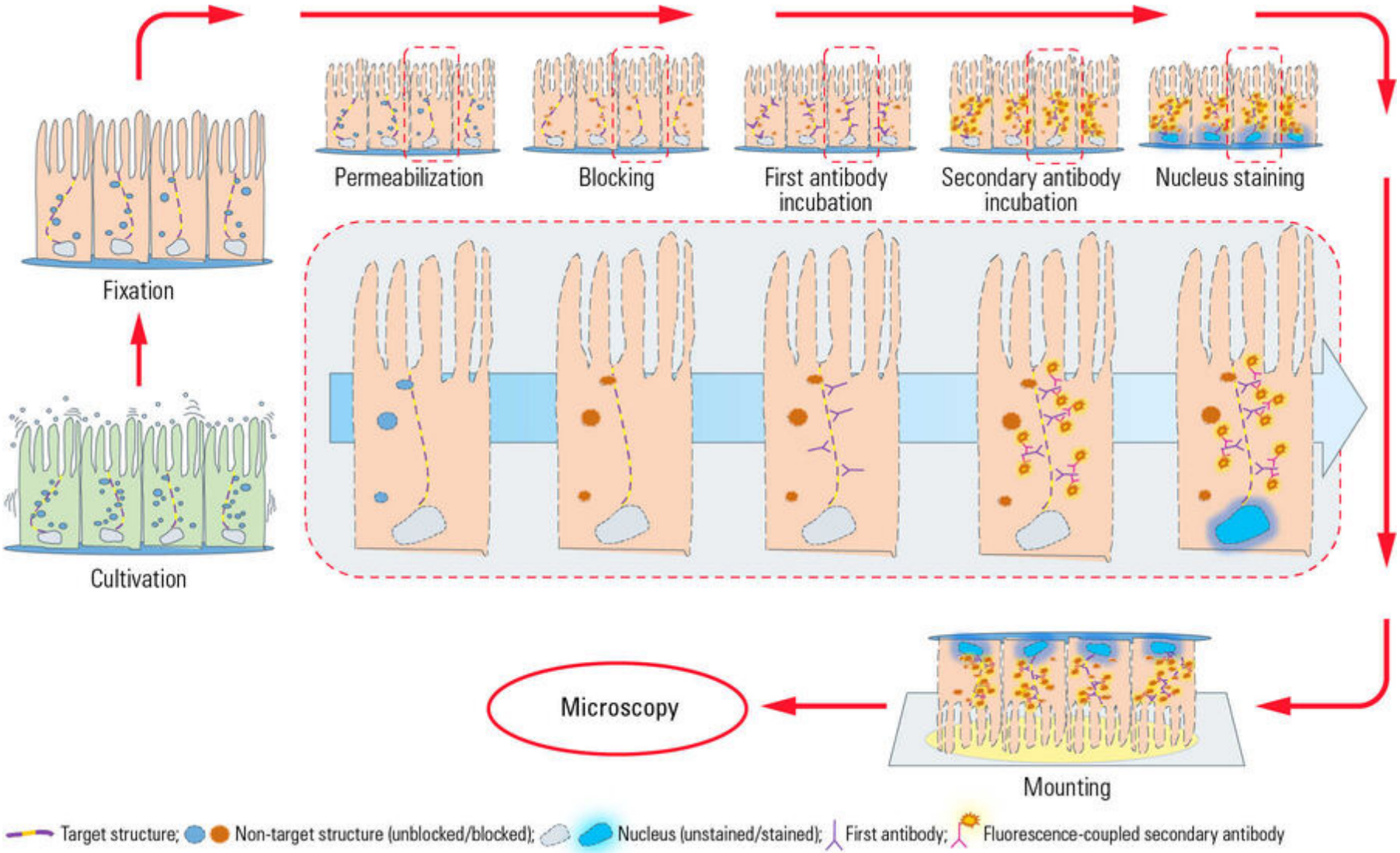


APPROCCIO SPERIMENTALE: DETTAGLIO

1. SCELTA DEL MODELLO CELLULARE
2. DISEGNO DELL'ESPERIMENTO
3. SCELTA DEI SAGGI
4. SCELTA DEGLI STRUMENTI



IMMUNOFLUORESCENZA: PROCEDURA

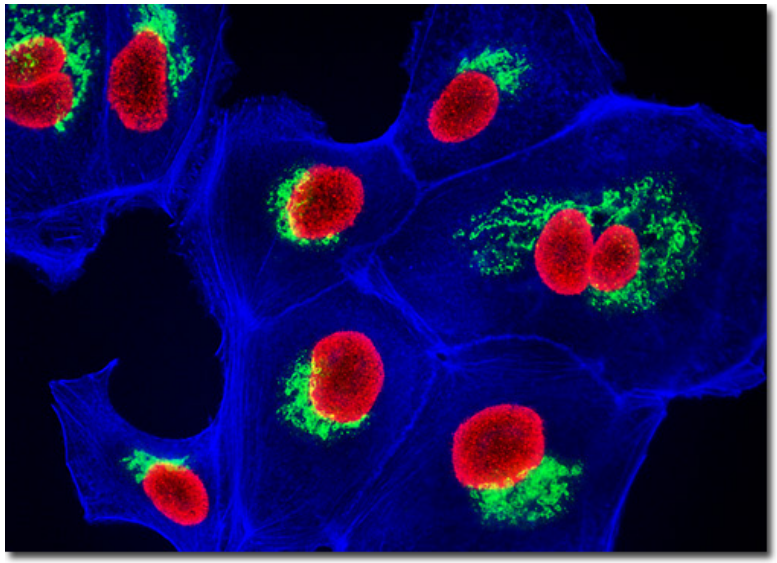


https://www.leica-microsystems.com/science-lab/life-science/how-to-prepare-specimen-for-immunofluorescence-microscopy/14

APPROCCIO SPERIMENTALE: DETTAGLIO

1. SCELTA DEL MODELLO CELLULARE
2. DISEGNO DELL'ESPERIMENTO
3. SCELTA DEI SAGGI
4. SCELTA DEGLI STRUMENTI

Microscopia a epifluorescenza widefield o confocale?



ESPERIMENTO VIRTUALE:
Analizzare l'interattoma (proteine cellulari associate) delle proteine virali

STRATEGIA SPERIMENTALE: DETTAGLIO

1. **SCELTA DEL MODELLO CELLULARE:**

   - **MODELLO 2D – es. LINEA CELLULARE EPITELIALE UMANA di polmone**
   - **MODELLO 3D – es. ORGANOIDI DERIVATI DA POLMONE (modello animale o UMANO)**
   - **MODELLO epiteliale – es. COLTURE ALI**

STRATEGIA SPERIMENTALE: DETTAGLIO

1. SCELTA DEL MODELLO CELLULARE:
2. DISEGNO DELL'ESPERIMENTO - SCELTA DELL'APPROCCIO SPERIMENTALE
3. SCELTA DEGLI STRUMENTI
4. ANALISI DEI RISULTATI

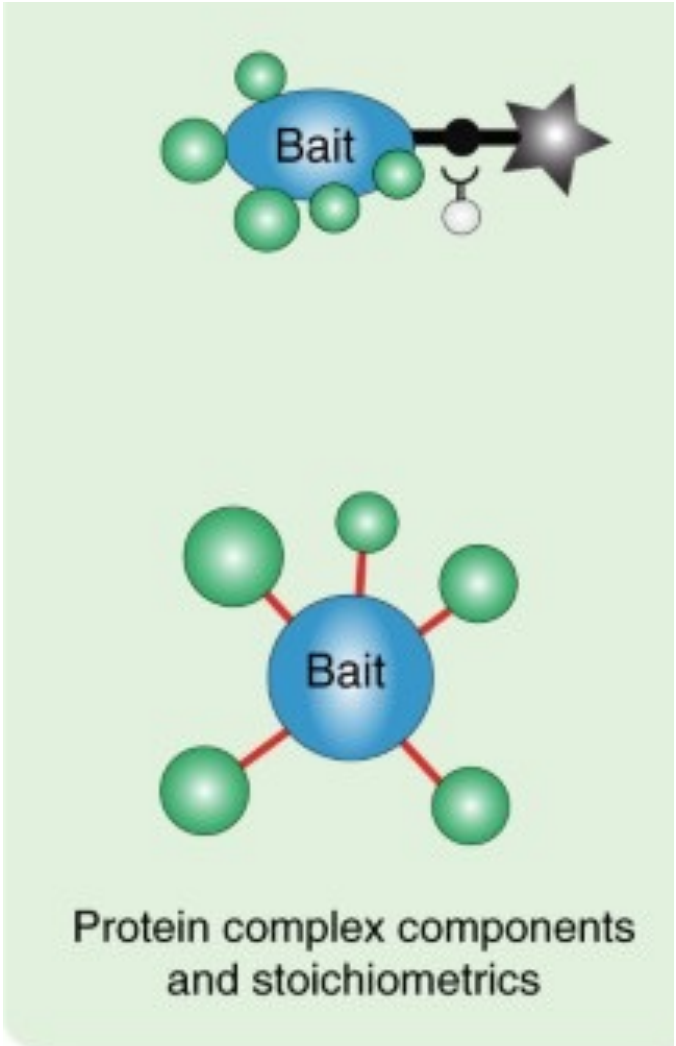
DISEGNO DELL'ESPERIMENTO:

a) **Sovraesprimere separatamente le ORFs VIRALI nelle cellule modello**

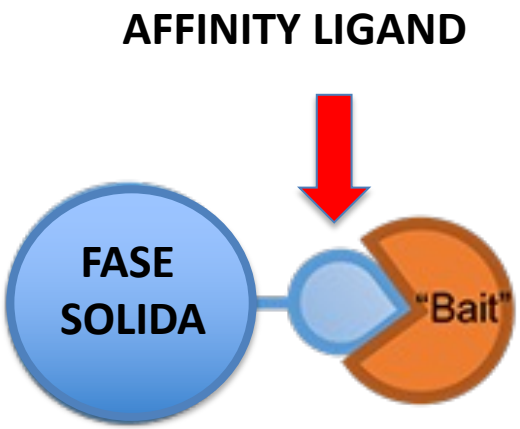
b) **Analizzare l'INTERATTOMA (proteine cellulari associate) delle proteine virali**

APPROCCIO SPERIMENTALE:

Utilizzo di tecniche che permettono di isolare proteine endogene associate a una proteina «ESCA» sovraespressa

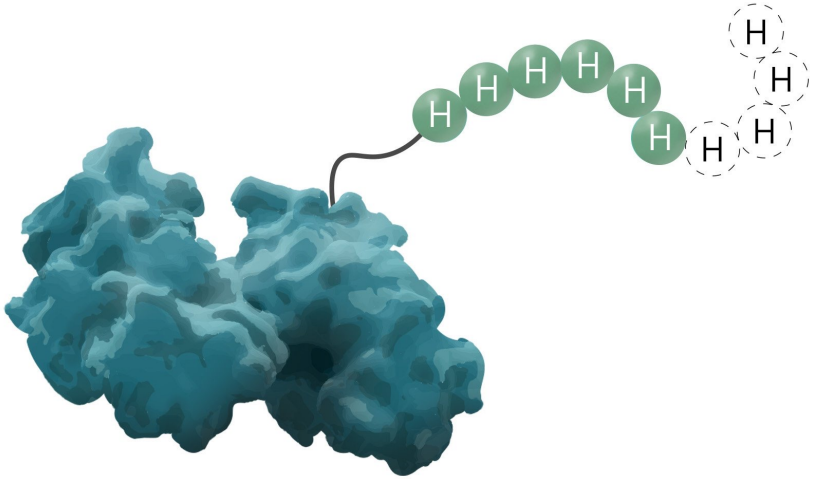


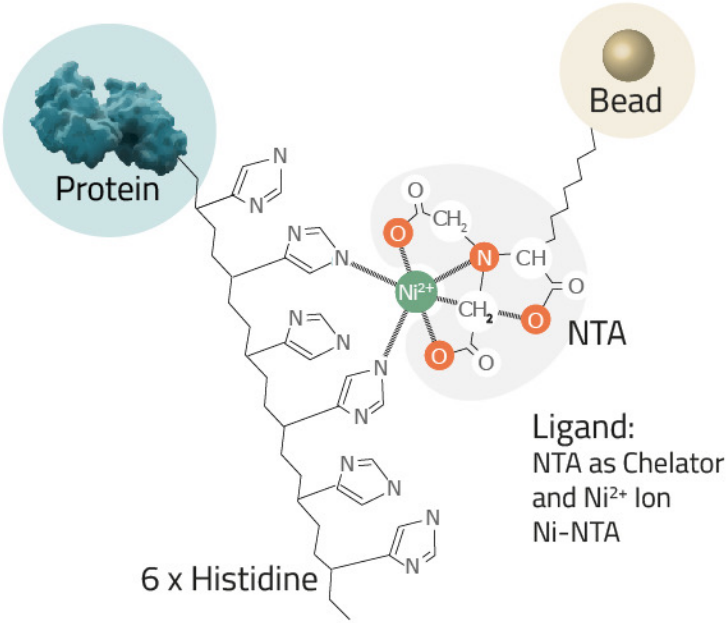
APPROCCIO #1: PURIFICAZIONE DI PROTEINE DI FUSIONE PER AFFINITA'



Le BAIT sono PROTEINE DI FUSIONE (prodotte in batteri o in cellule eucariotiche) che vengono purificate mediante un LIGANDO ad alta affinità coniugato ad una fase solida

Produzione e purificazione della proteina ORF8-His

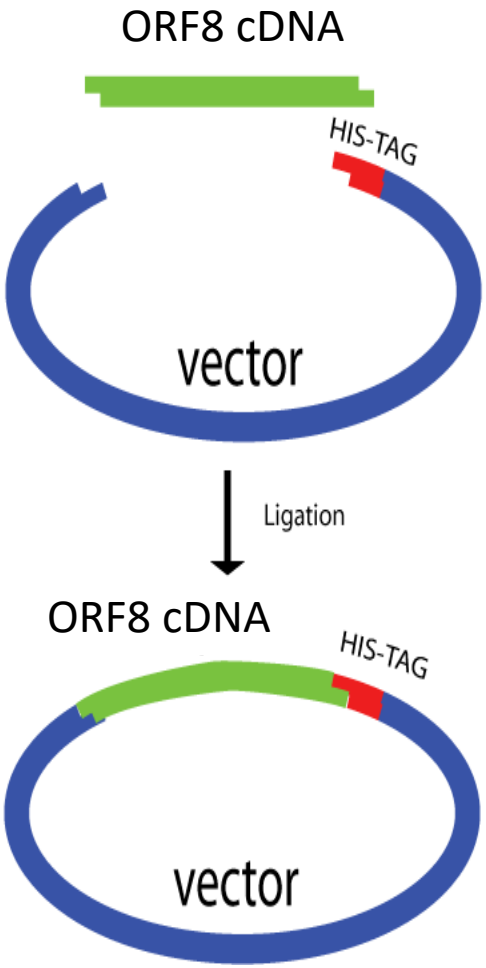




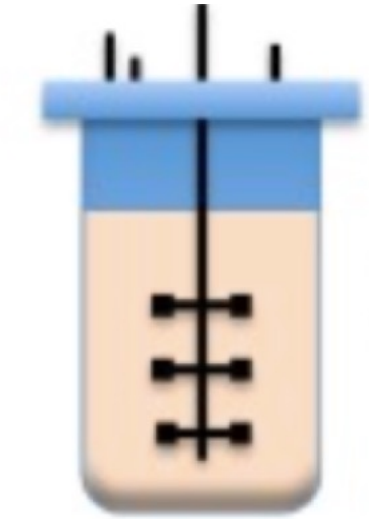
https://cube-biotech.com/knowledge/protein-purification/his-tag/

Produzione e purificazione della proteina ORF8-His

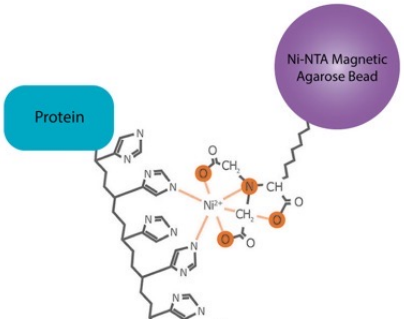
- **Clonaggio**



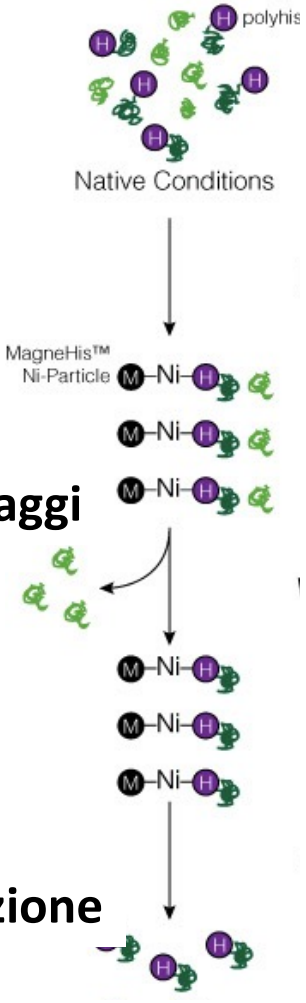
- **Trasformazione batterica**
- **Selezione**
- **Coltura**



- **Lisi dei batteri**



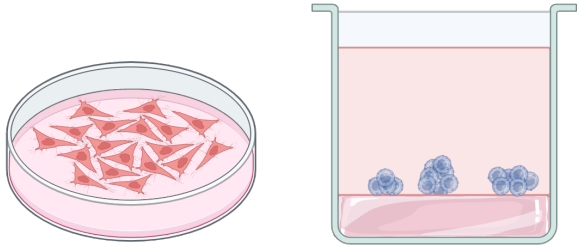
- **Legame alla resina**



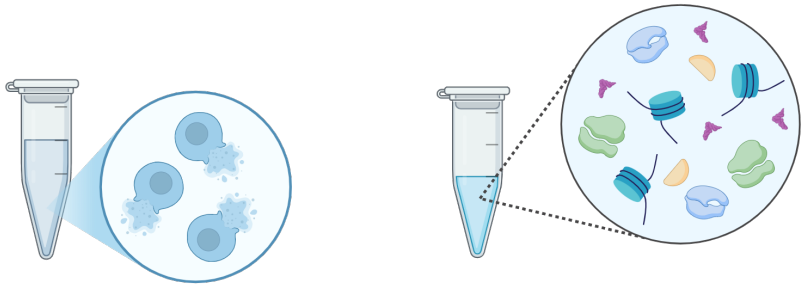
- **Lavaggi**
- **Eluizione**

ANALISI DELL'INTERAZIONE PROTEINA-PROTEINA mediante PURIFICAZIONE

Modello cellulare



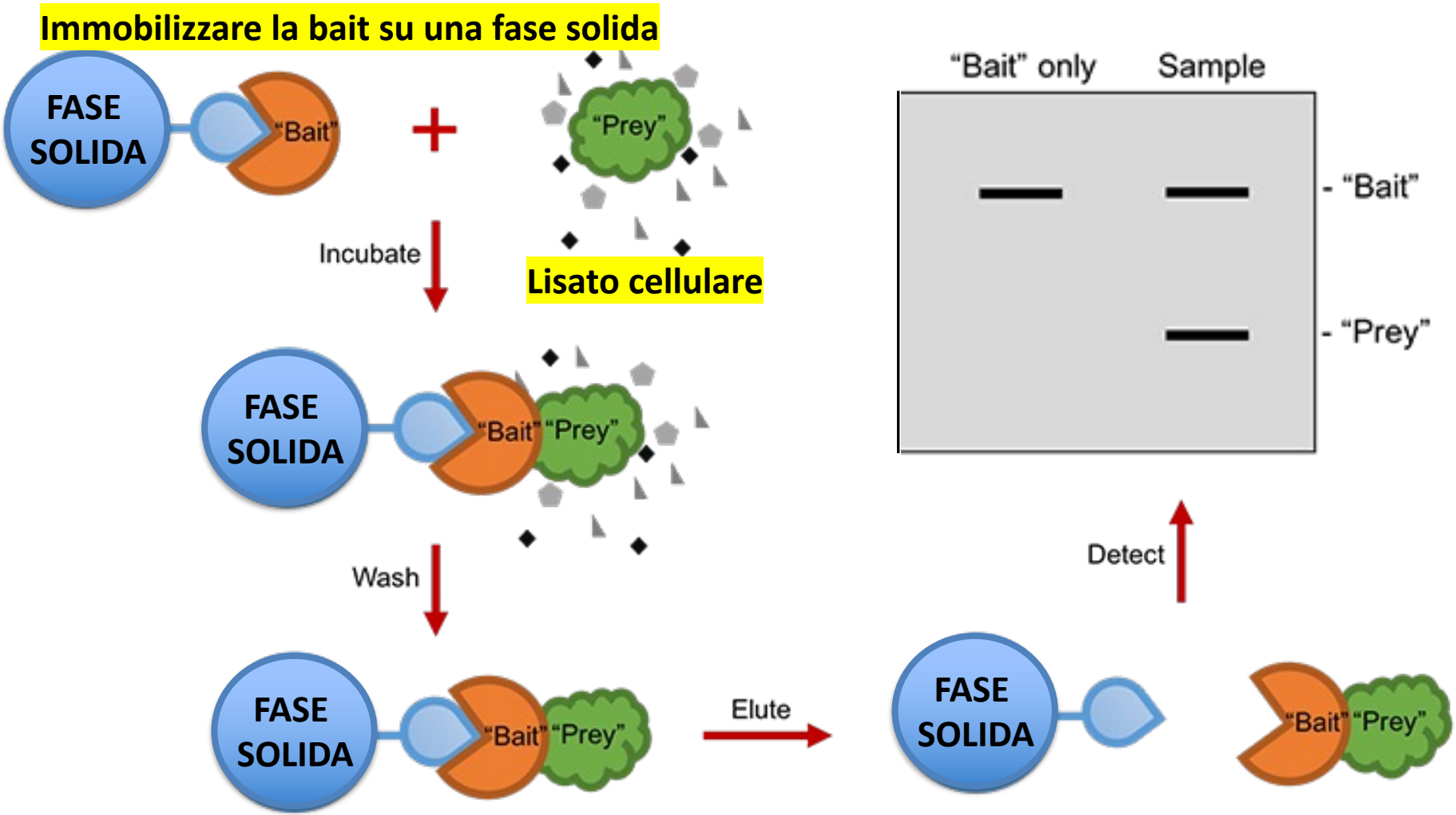
Ottenimento di un lisato cellulare

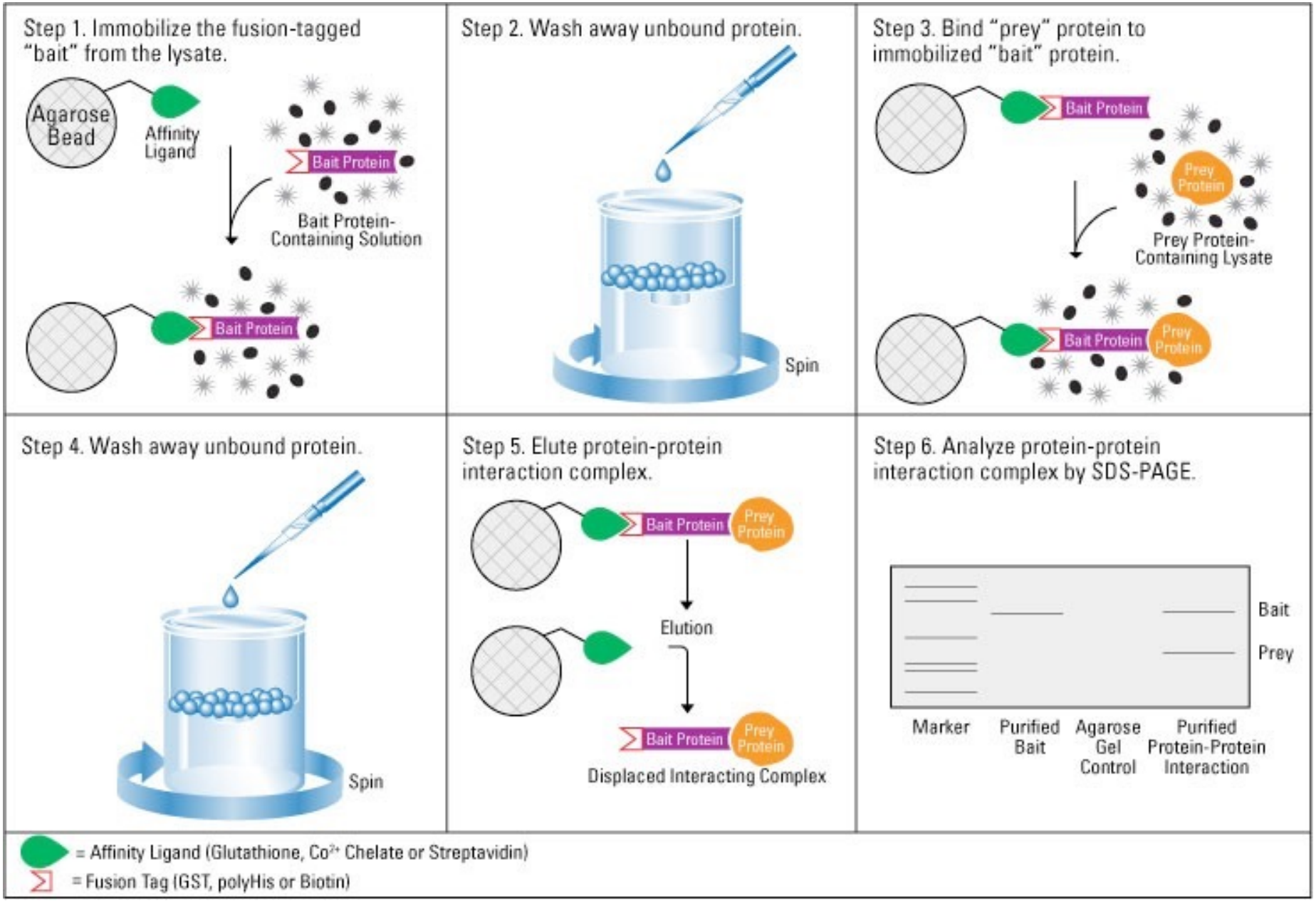


bait immobilizzata su fase solida

Incubazione con lisato cellulare

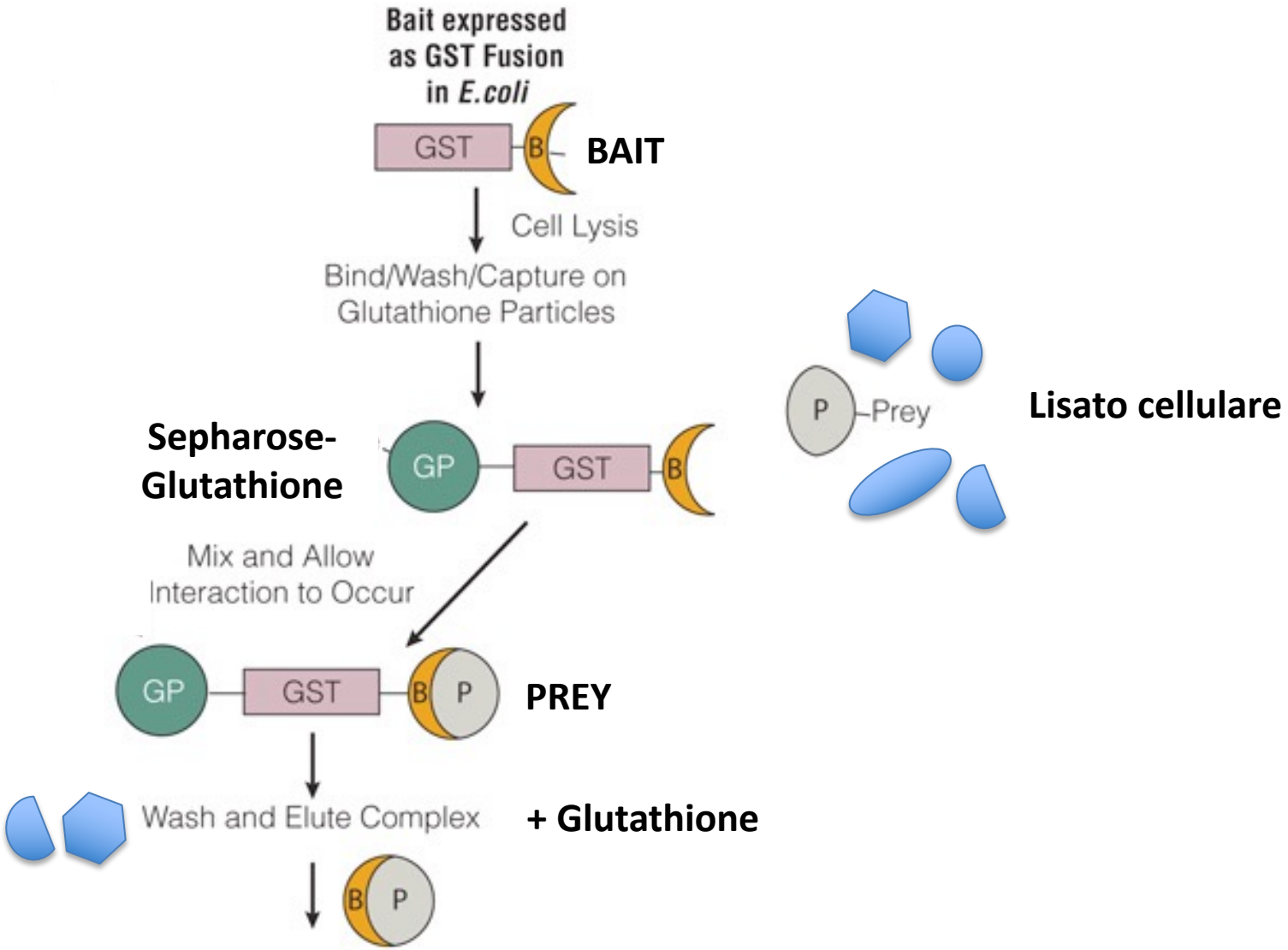
ANALISI DELL'INTERAZIONE PROTEINA-PROTEINA mediante PURIFICAZIONE




Step 1. Immobilize the fusion-tagged "bait" from the lysate.
Agarose Bead
Affinity Ligand
Bait Protein
Bait Protein-Containing Solution
Bait Protein
Step 2. Wash away unbound protein.
Spin
Step 3. Bind "prey" protein to immobilized "bait" protein.
Bait Protein
Prey Protein
Prey Protein-Containing Lysate
Bait Protein
Prey Protein
Step 4. Wash away unbound protein.
Spin
Step 5. Elute protein-protein interaction complex.
Bait Protein
Prey Protein
Elution
Bait Protein
Prey Protein
Displaced Interacting Complex
Step 6. Analyze protein-protein interaction complex by SDS-PAGE.
Bait
Prey
Marker
Purified Bait
Agarose Gel Control
Purified Protein-Protein Interaction
= Affinity Ligand (Glutathione, Co2+ Chelate or Streptavidin)
= Fusion Tag (GST, polyHis or Biotin)

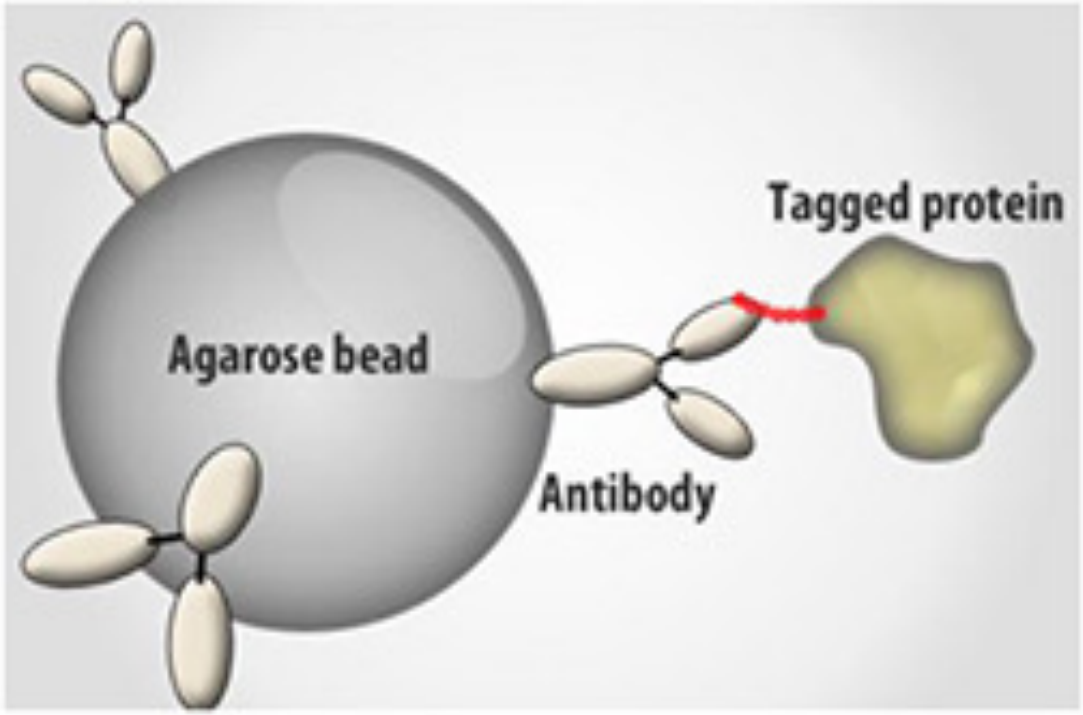
GST-pulldown

Utilizza proteine di fusione con GST (espresse in batteri)



APPROCCIO #2:

PURIFICAZIONE DI PROTEINE SOVRAESPRESSE O ENDOGENE mediante IMMUNO-PRECIPITAZIONE

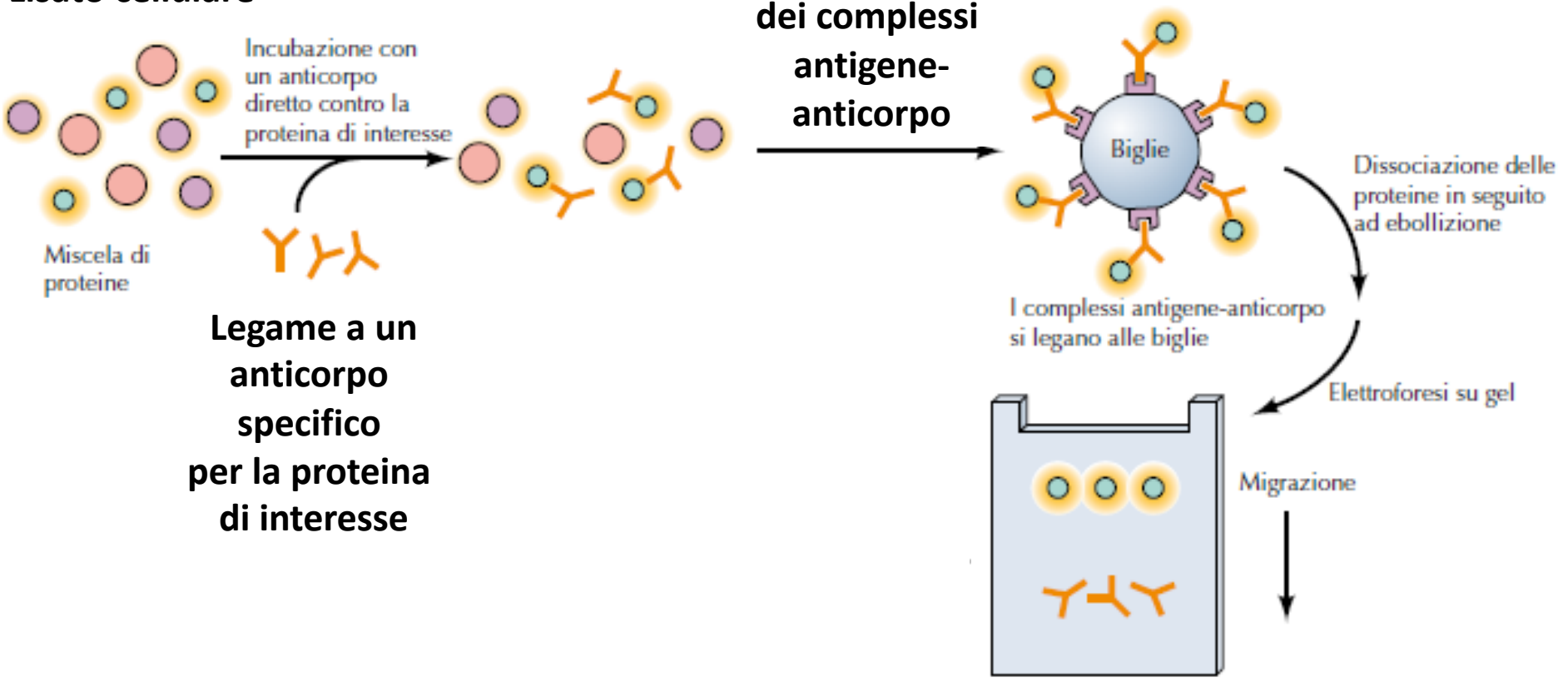


IMMUNOPRECIPITAZIONE:

purificazione di proteine mediante anticorpi specifici

Immunoprecipitazione

Lisato cellulare



Legame a un anticorpo specifico per la proteina di interesse

Raccolta dei complessi antigene-anticorpo

Immobilizzazione degli anticorpi sulla resina

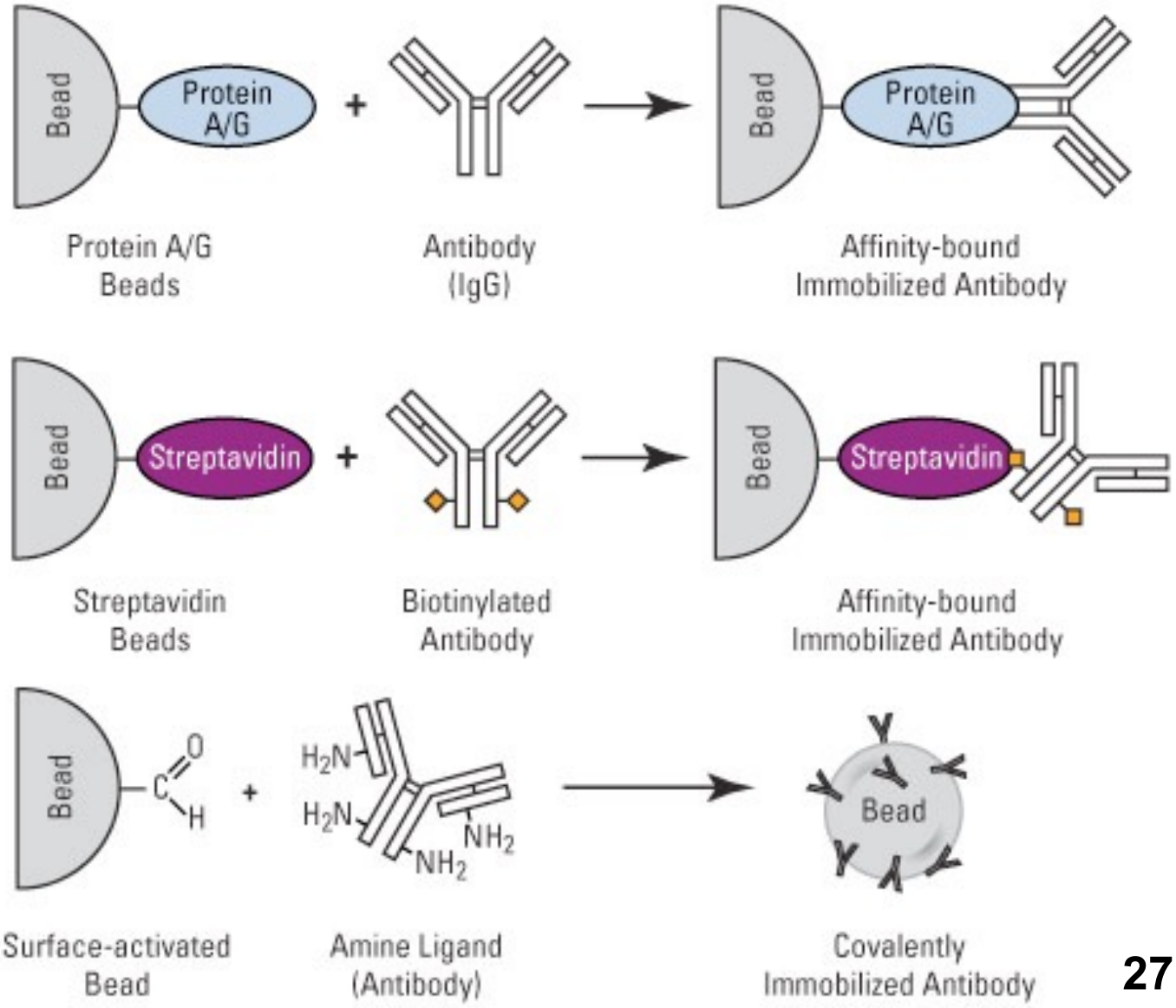
https://www.cd-bioparticles.com/t/Protein-Isolation_48.html

IgG biding proteins

Protein A
Staphilococcus

Protein G
Streptococcus

Protein A/G
Recombinant fusion protein

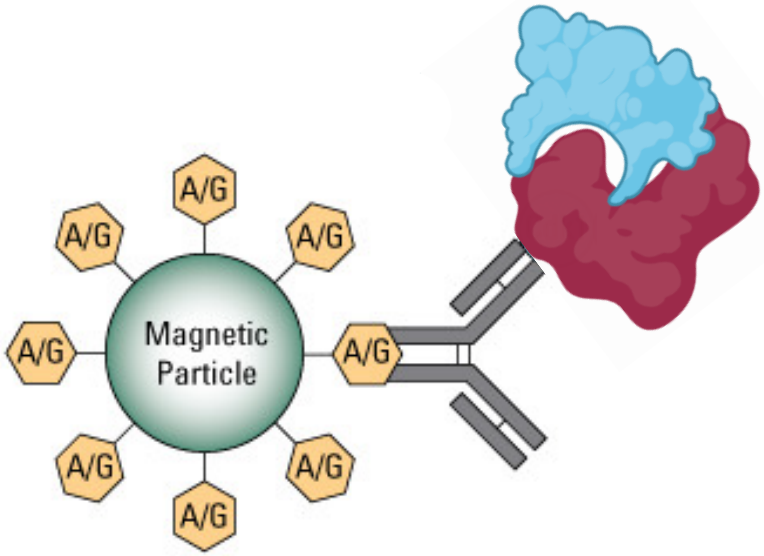


Confronto dei 2 approcci proposti

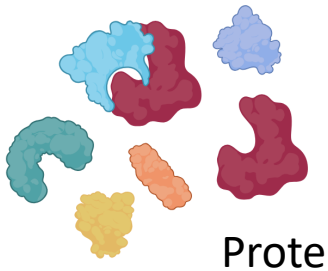
Comparison of Co-IP with endogenous proteins versus tagged proteins

	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.**

4) Analisi dell'interazione proteina-proteina da lisato cellulare: identificazione degli interattori

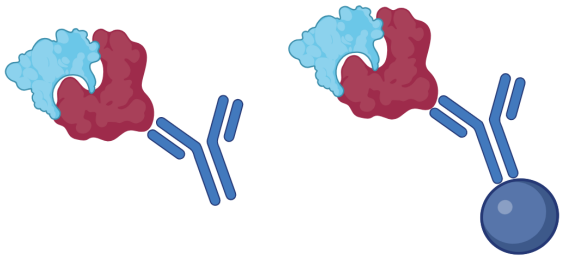


1) Lisato cellulare



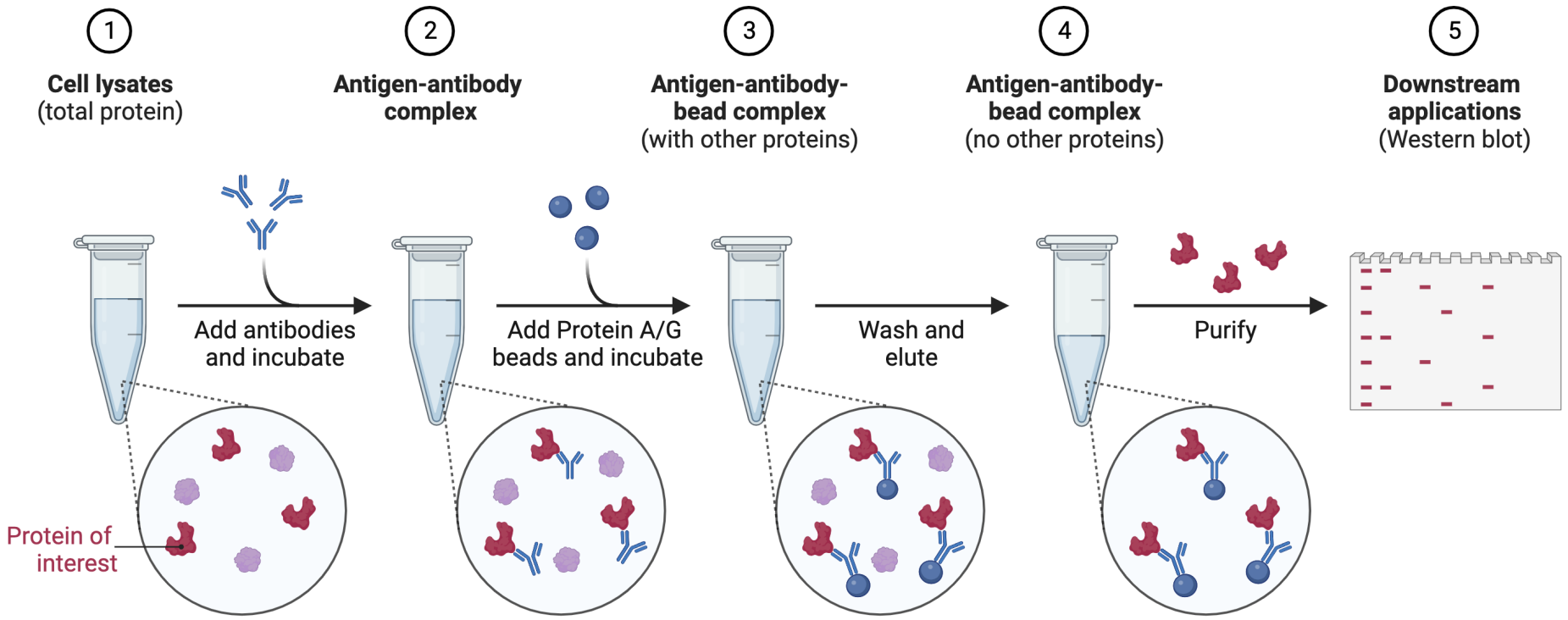
Proteina
di interesse
(esca)

2) Purificazione dell'esca



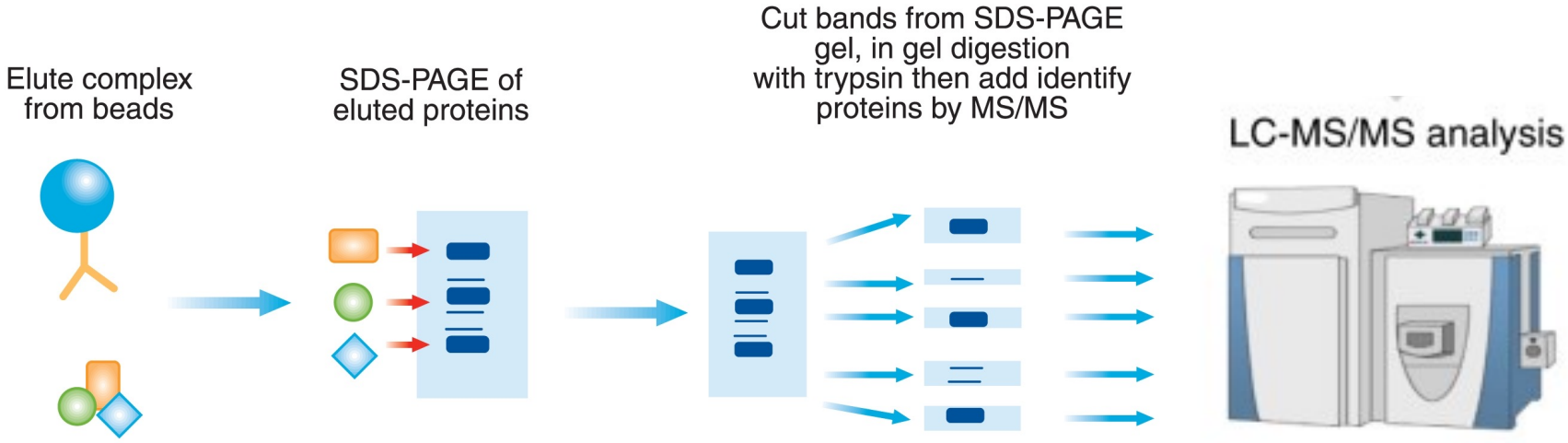
3) Analisi delle proteine co-purificate

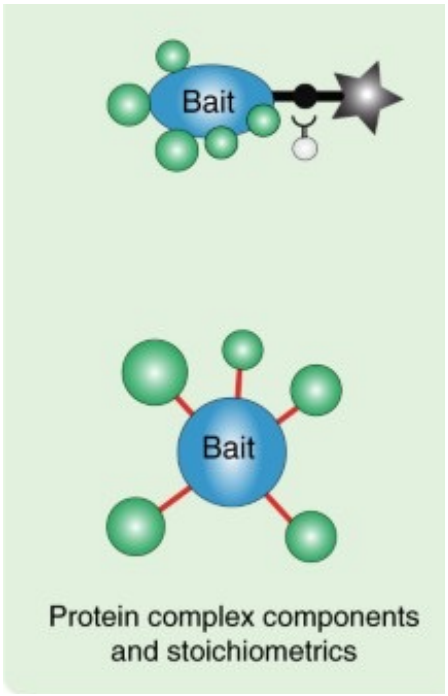
SCELTA DEI SAGGI PER L'ANALISI DELL'INTERATTOMA #1



Se le proteine partner sono candidati noti,
si possono analizzare mediante Western Blot

SAGGI E STRUMENTI PER L'ANALISI DELL'INTERATTOMA #2





Analisi dell'interattoma mediante spettrometria di massa

Se le proteine partner NON sono note, si sottopongono le bande proteiche dell'interattoma a digestione e successiva analisi mediante spettrometria di massa.

5) ANALISI DEI RISULTATI

DELL'INTERATTOMA DELLE PROTEINE DI SARS-COV-2 IN CELLULE UMANE

Article

Nature | Vol 583 | 16 July 2020 | 459

A SARS-CoV-2 protein interaction map reveals targets for drug repurposing

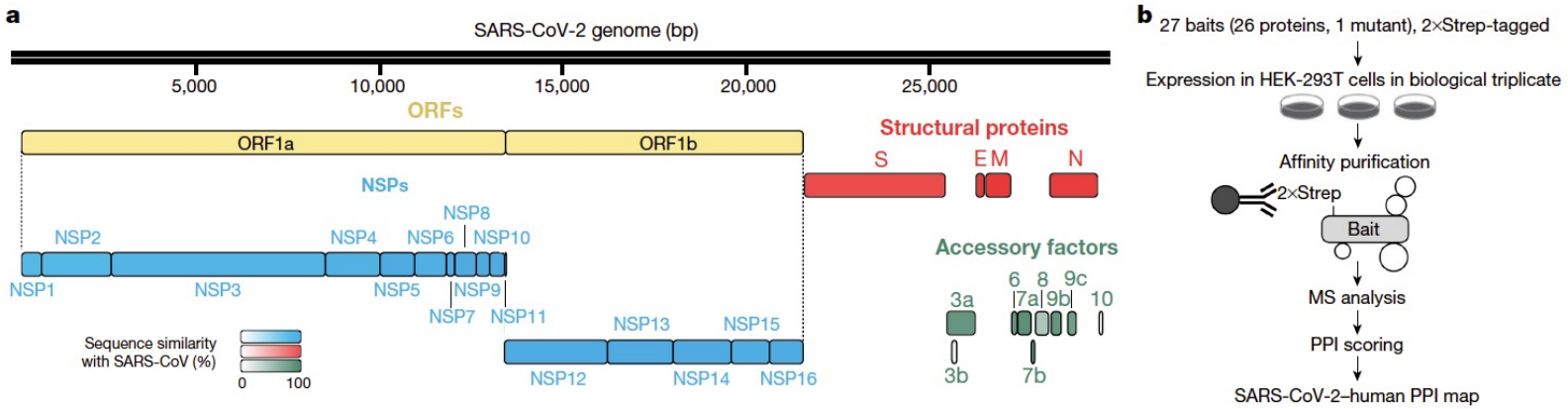
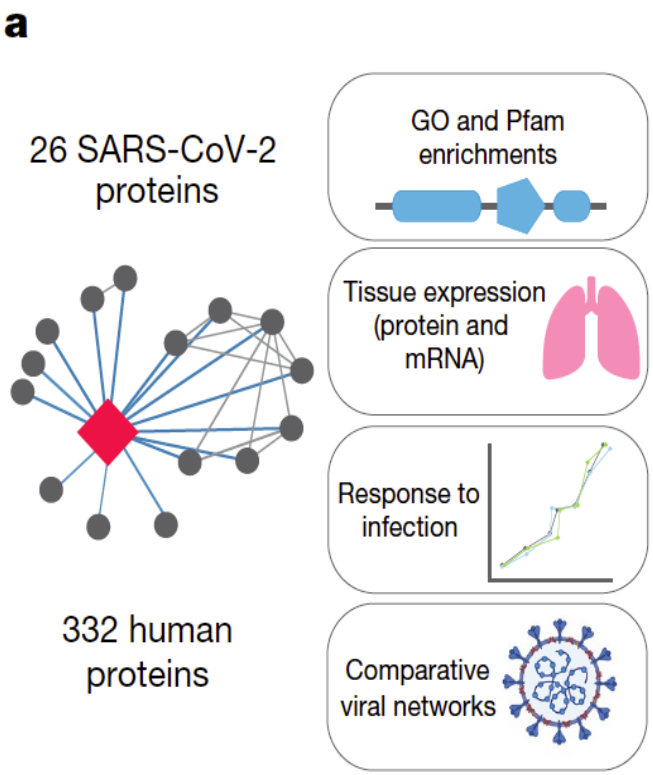
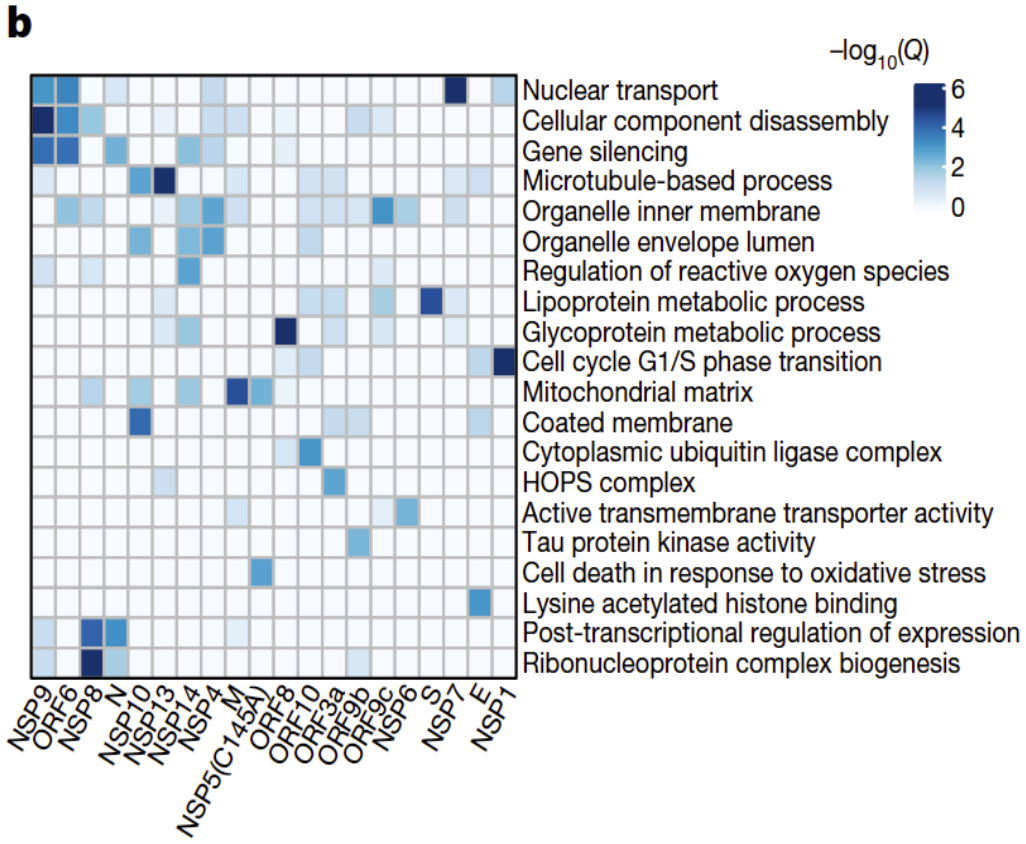


Fig. 1 | AP-MS workflow for the identification of SARS-CoV-2–host protein–protein interactions. a, SARS-CoV-2 genome annotation. The colour intensity is proportional to the protein sequence similarity with SARS-CoV homologues (when homologues exist). $n = 4$ structural proteins; $n = 16$ NSPs; $n = 9$ accessory factors. **b**, Experimental workflow for AP-MS studies. MS, mass spectrometry; PPI, protein–protein interaction.

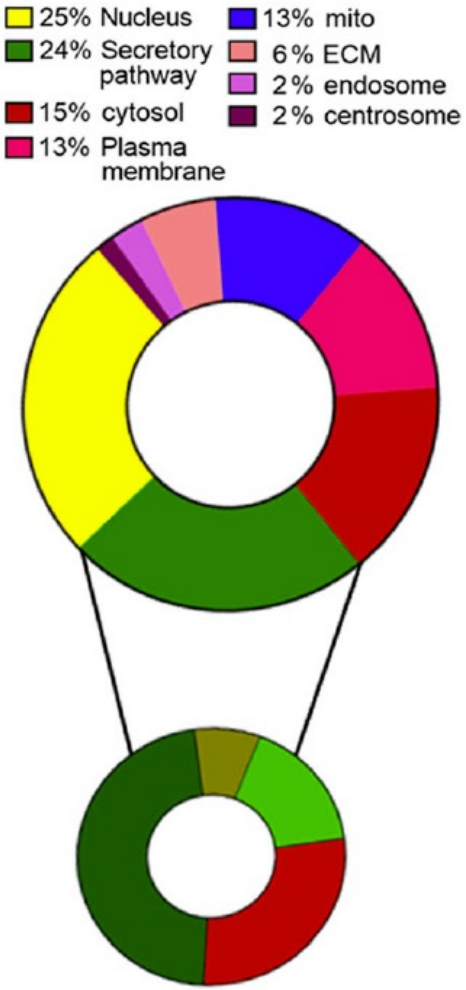
5) ANALISI DEI RISULTATI

DELL'INTERATTOMA DELLE PROTEINE DI SARS-COV-2 IN CELLULE UMANE

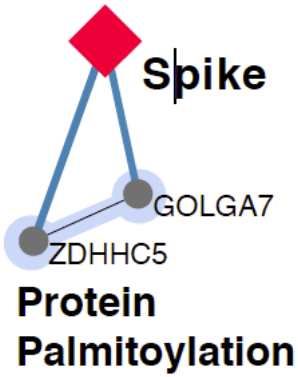


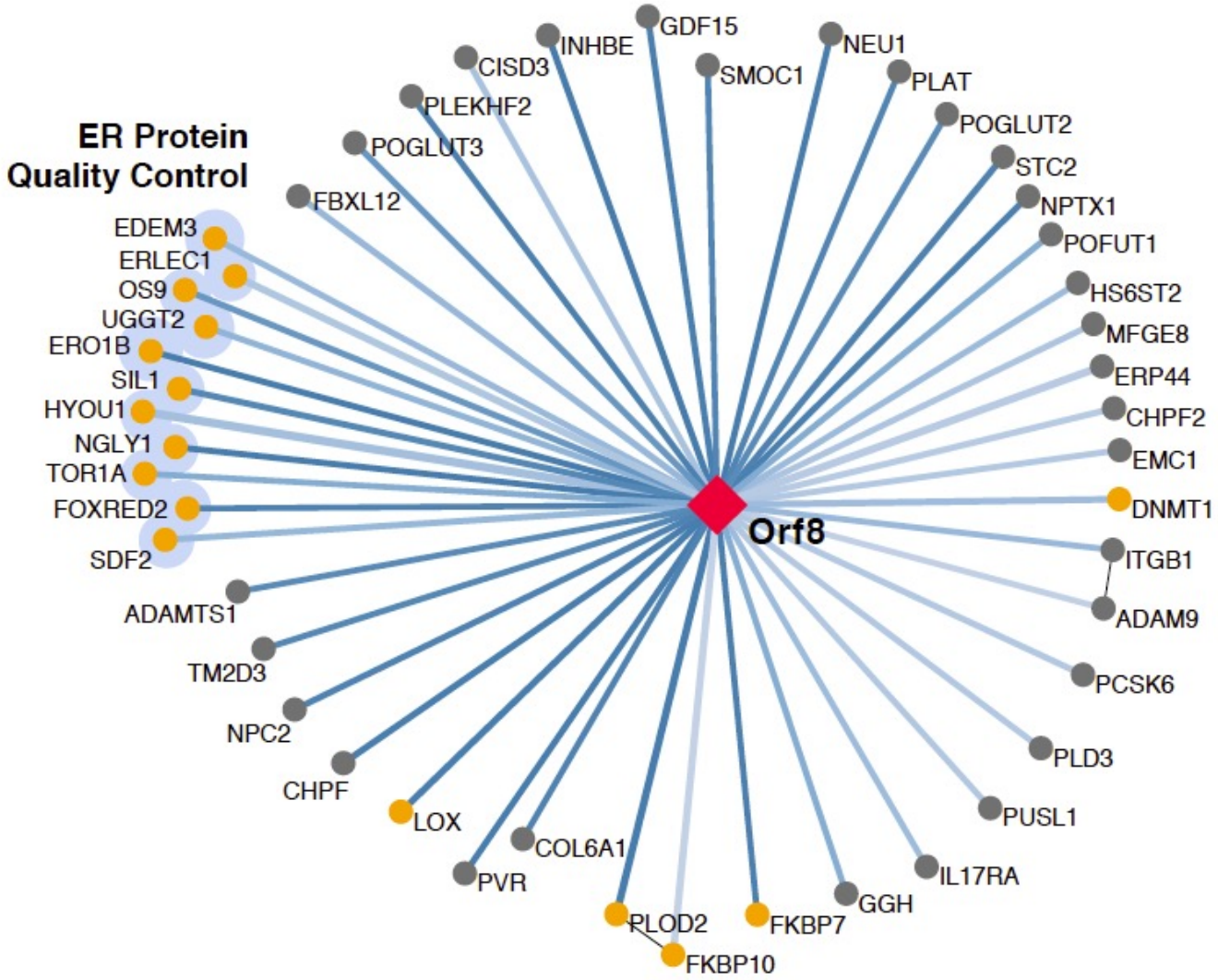


ANALISI DELL'INTERATTOMA DELLE PROTEINE DI SARS-COV-2 IN CELLULE UMANE

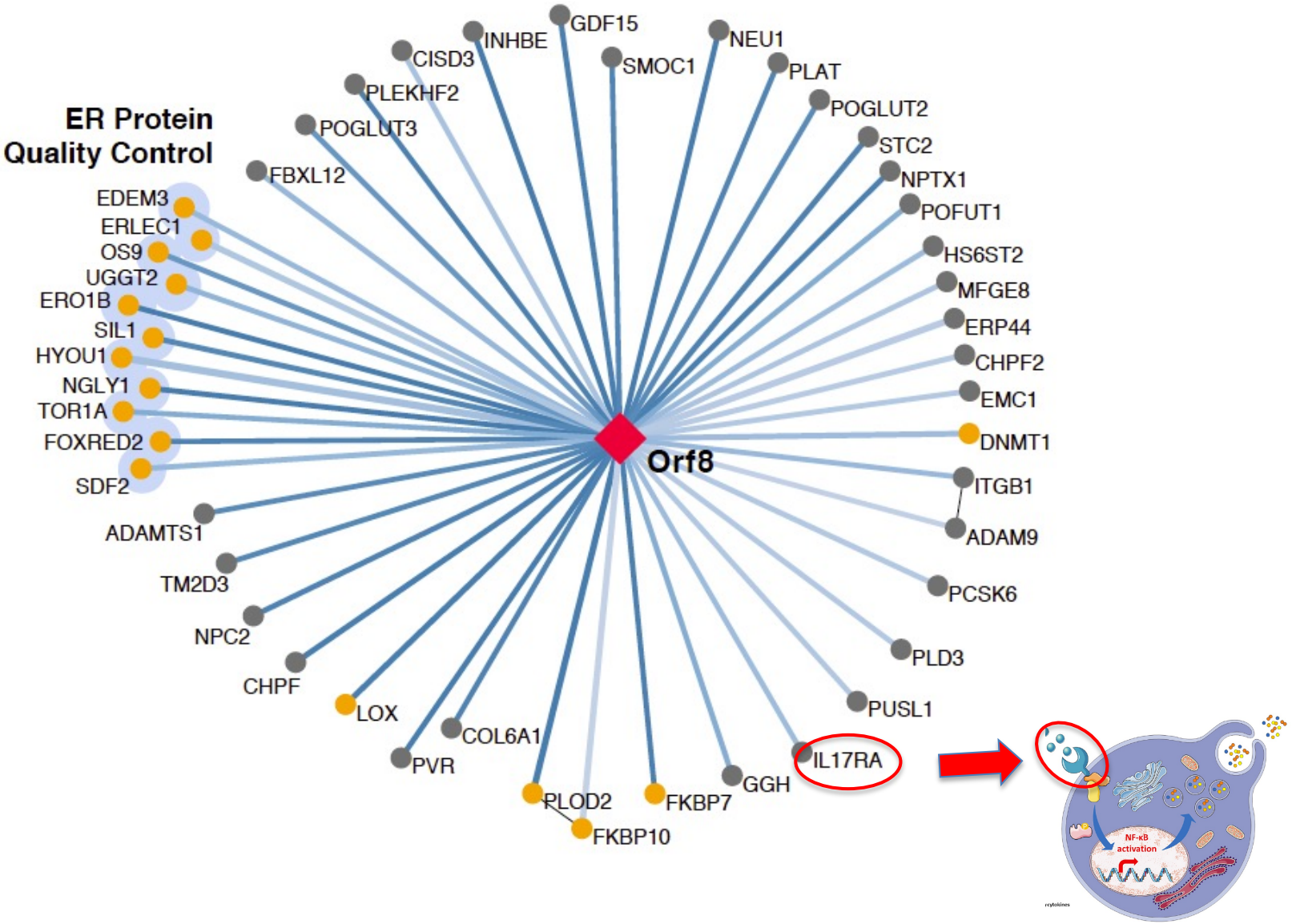


Rappresentazione dell'interattoma





Interattoma di ORF8



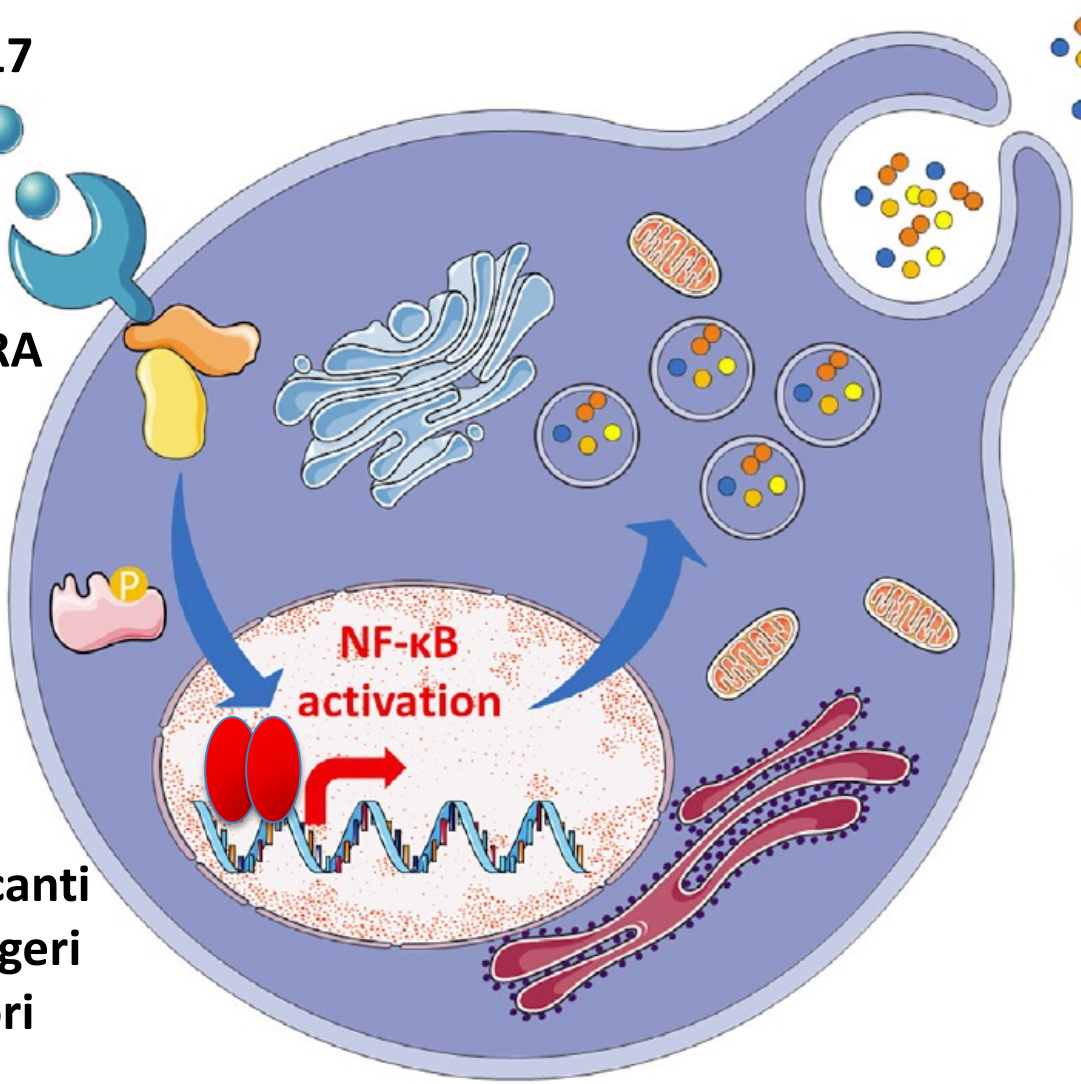
IL-17 RA è un recettore coinvolto nell'infiammazione

Secrezione di messaggeri infiammatori

IL-17

IL-17RA

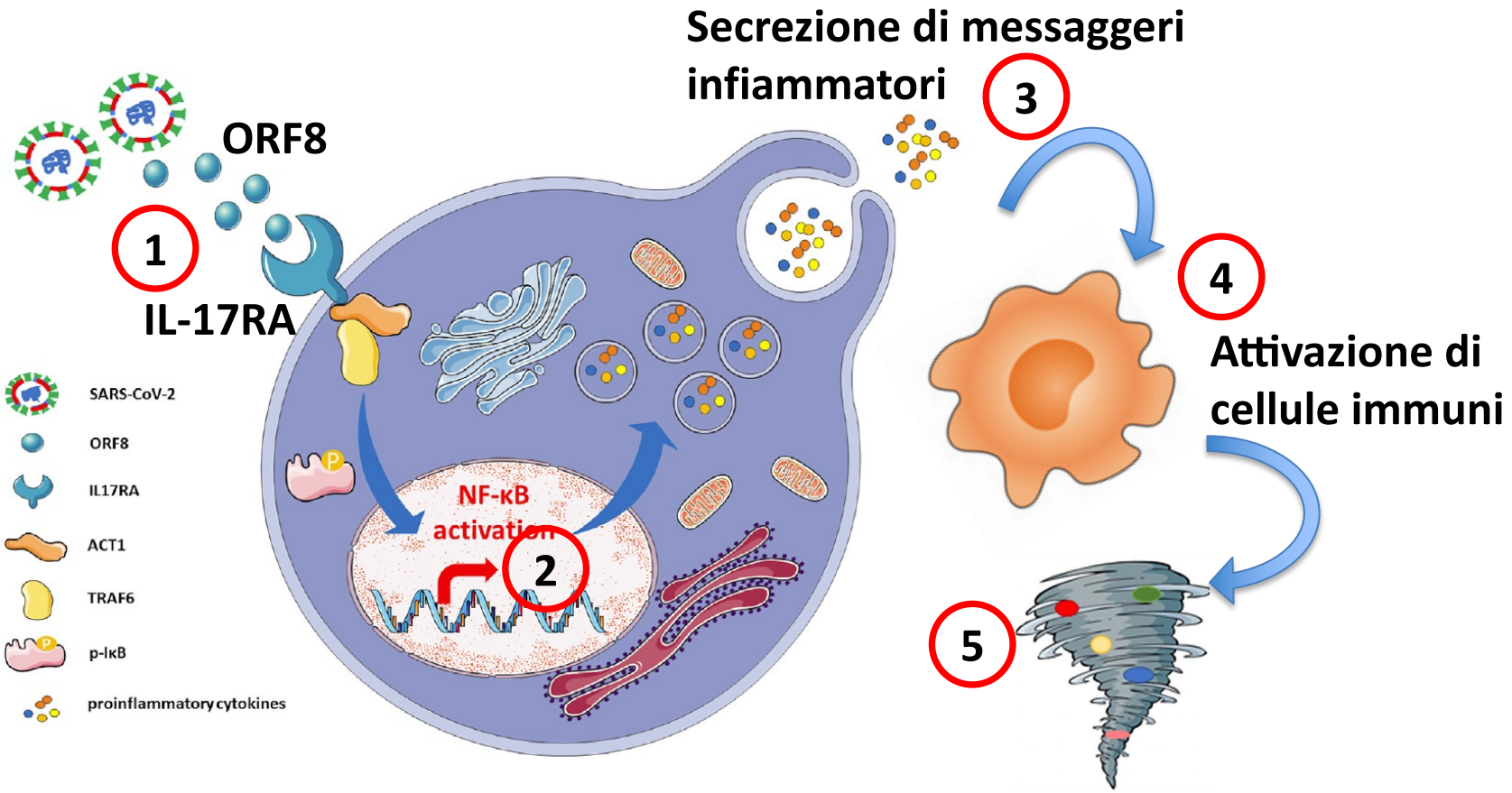
TNFα
IL-1
IL-6
IL-12



Geni codificanti per messaggeri infiammatori

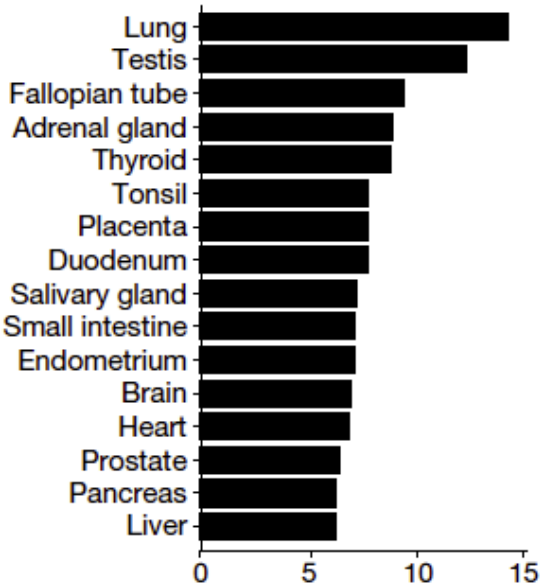
Attivazione di cellule immuni/ infiammatorie

IPOTESI: ORF8 lega e attiva il recettore IL-17RA innescando una risposta infiammatoria



È plausibile che IL-17 RA sia implicato nell'infiammazione indotta da SARS-COV2?

Analisi dell'espressione di IL-17 RA (mRNA e proteina) in diversi tessuti



Espressione differenziale

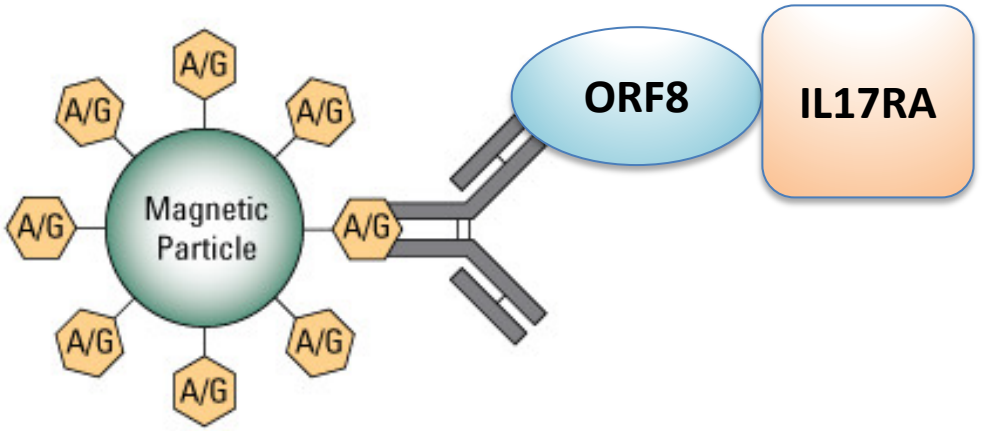
È plausibile che IL-17 RA sia implicato nell'infiammazione indotta da SARS-COV2?

È necessario una conferma (validazione) dell'interazione tra le proteine ORF8 e IL17RA di tipo sia biochimico che biologico

Validazione delle interazioni (1): co-IP oppure AP e western blotting

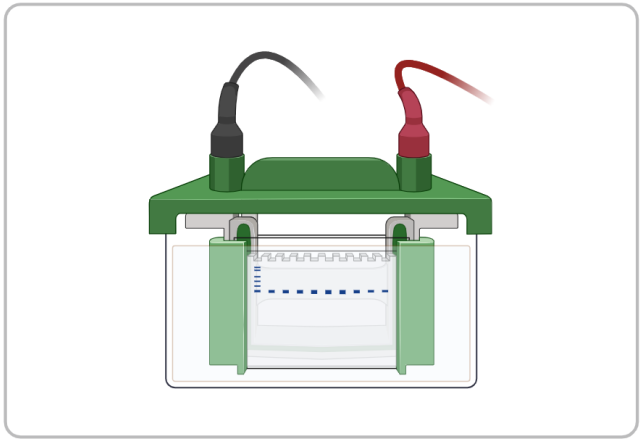
È utile anche per effettuare una MAPPATURA delle porzioni delle proteine coinvolte nell'interazione
e per stabilire in QUALI CONDIZIONI essa avviene

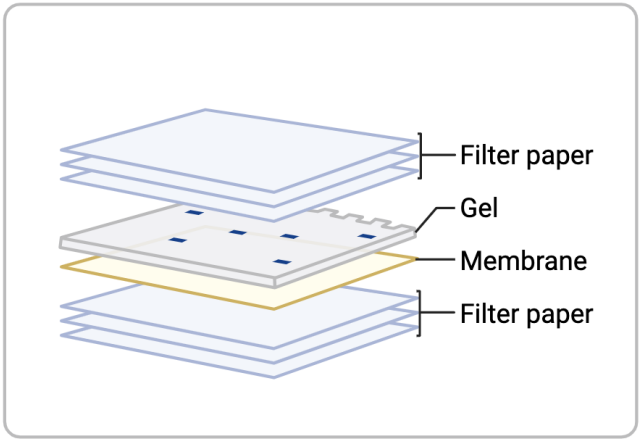
Lisato cellulare

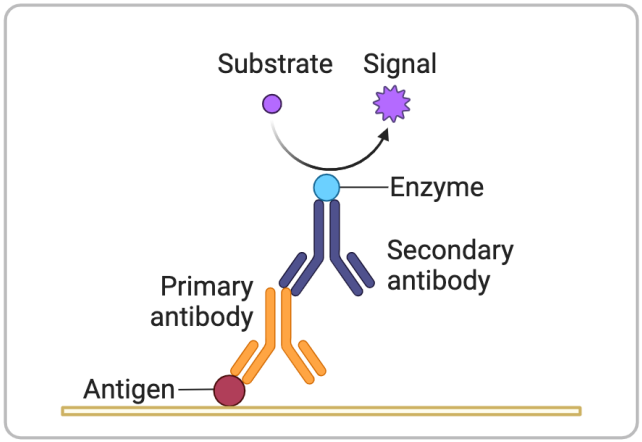


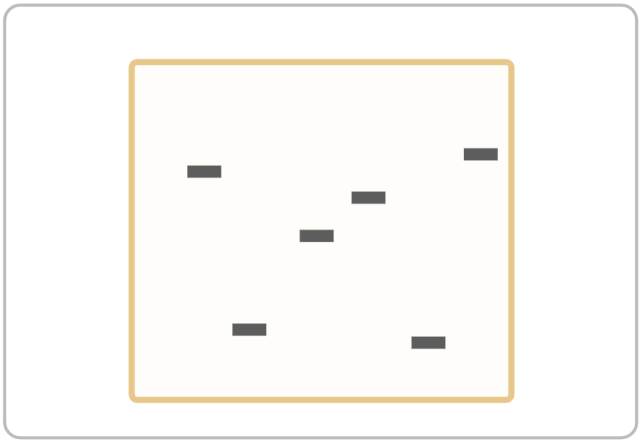
Le proteine partner sono candidati noti,
quindi si possono analizzare mediante WB

Validazione delle interazioni (1): co-IP e western blotting





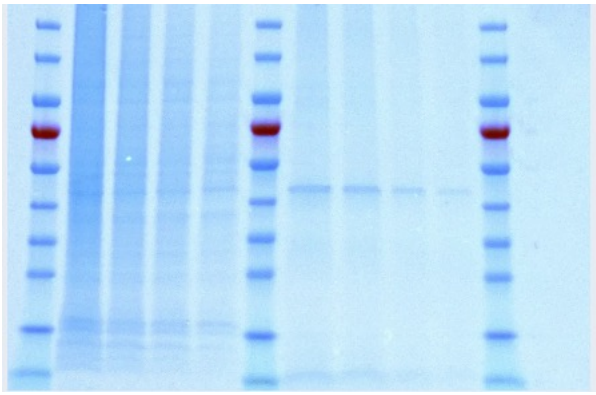




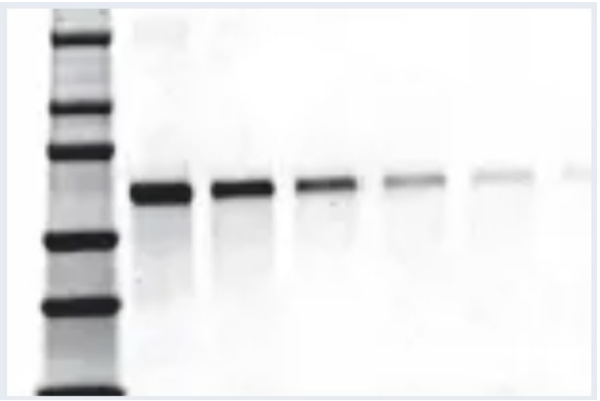
QUALI STRUMENTI
UTILIZZARE PER LA RILEVAZIONE?

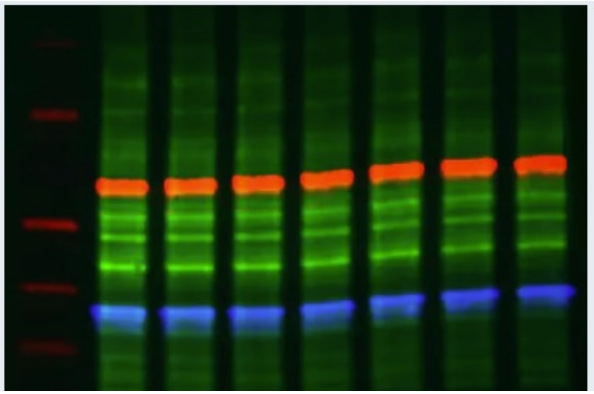
GEL IMAGER

visibile

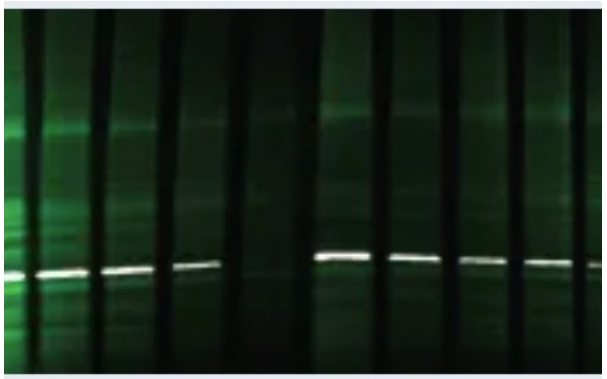


chemiluminescenza



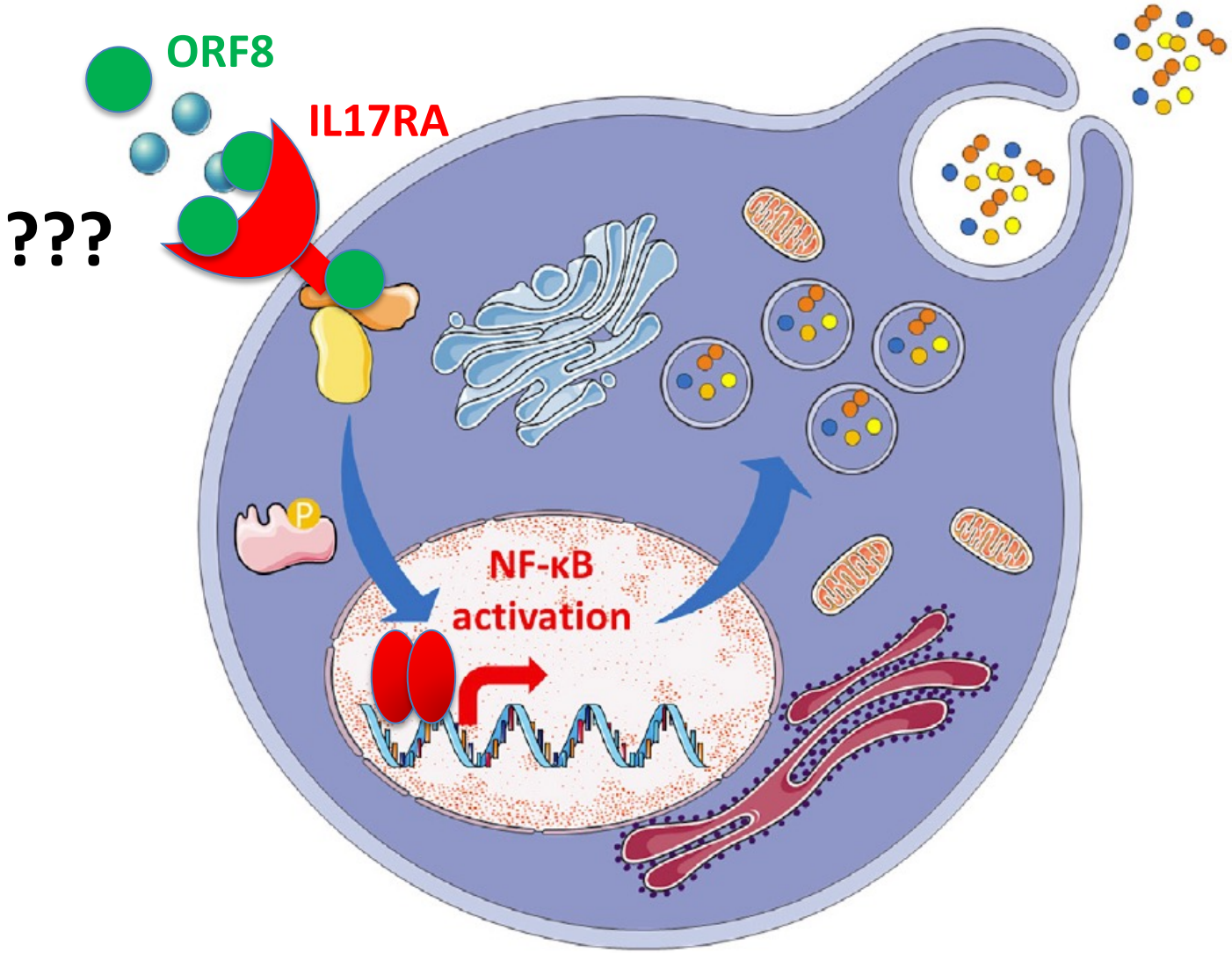


UV



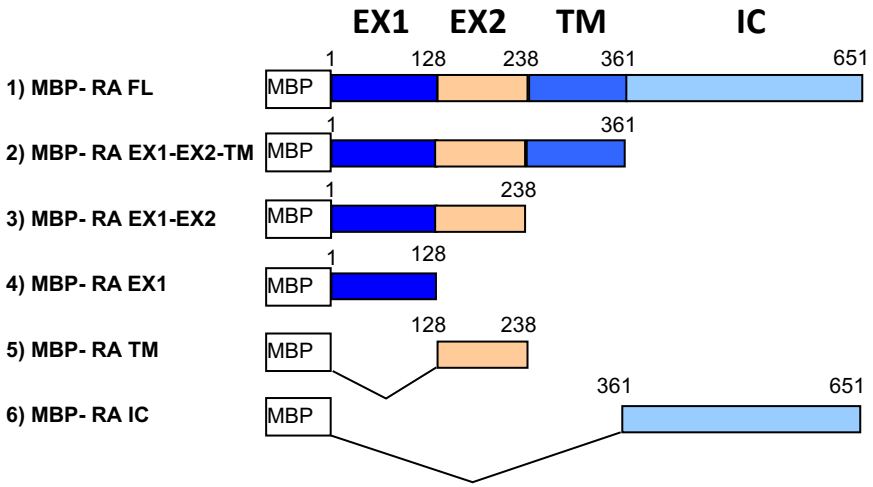
fluorescenza

MAPPATURA DELLE INTERAZIONI

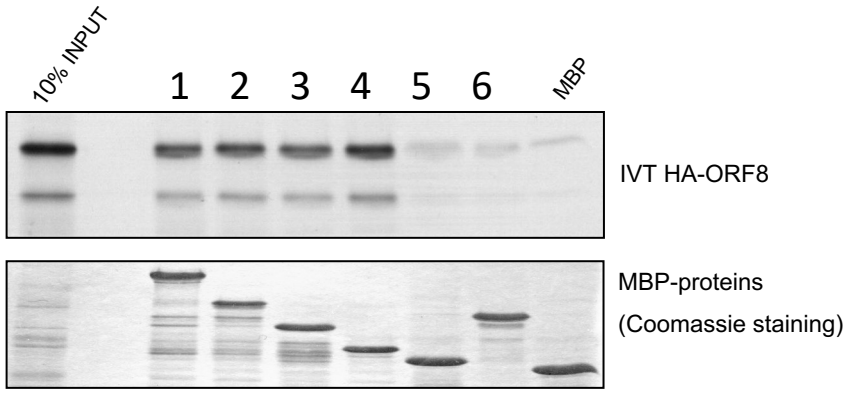


MAPPATURA DELLE INTERAZIONI

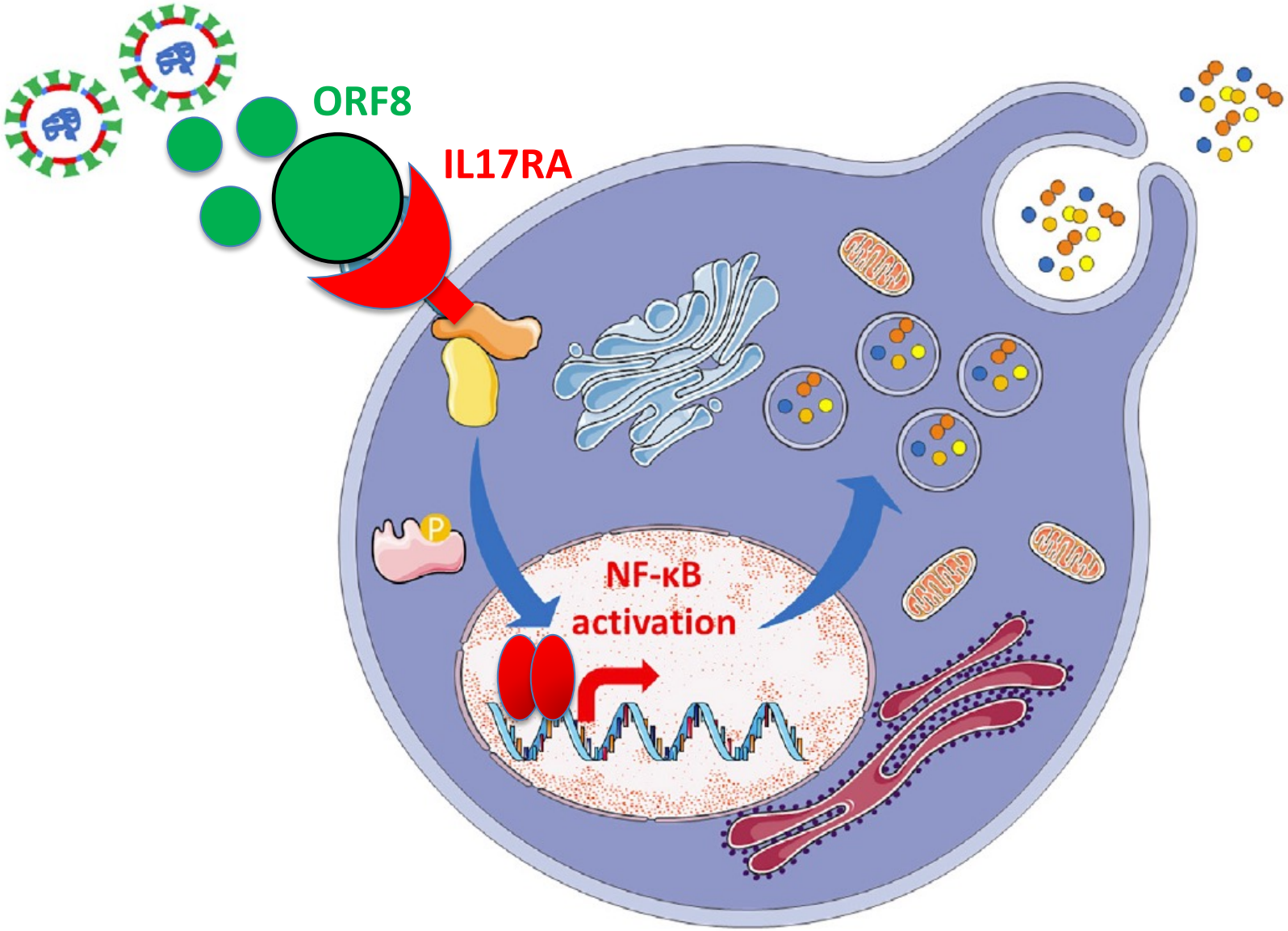
Mediante CO-IP/AP e WB/FAR-WESTERN con PORZIONI (mutanti di delezione) delle proteine



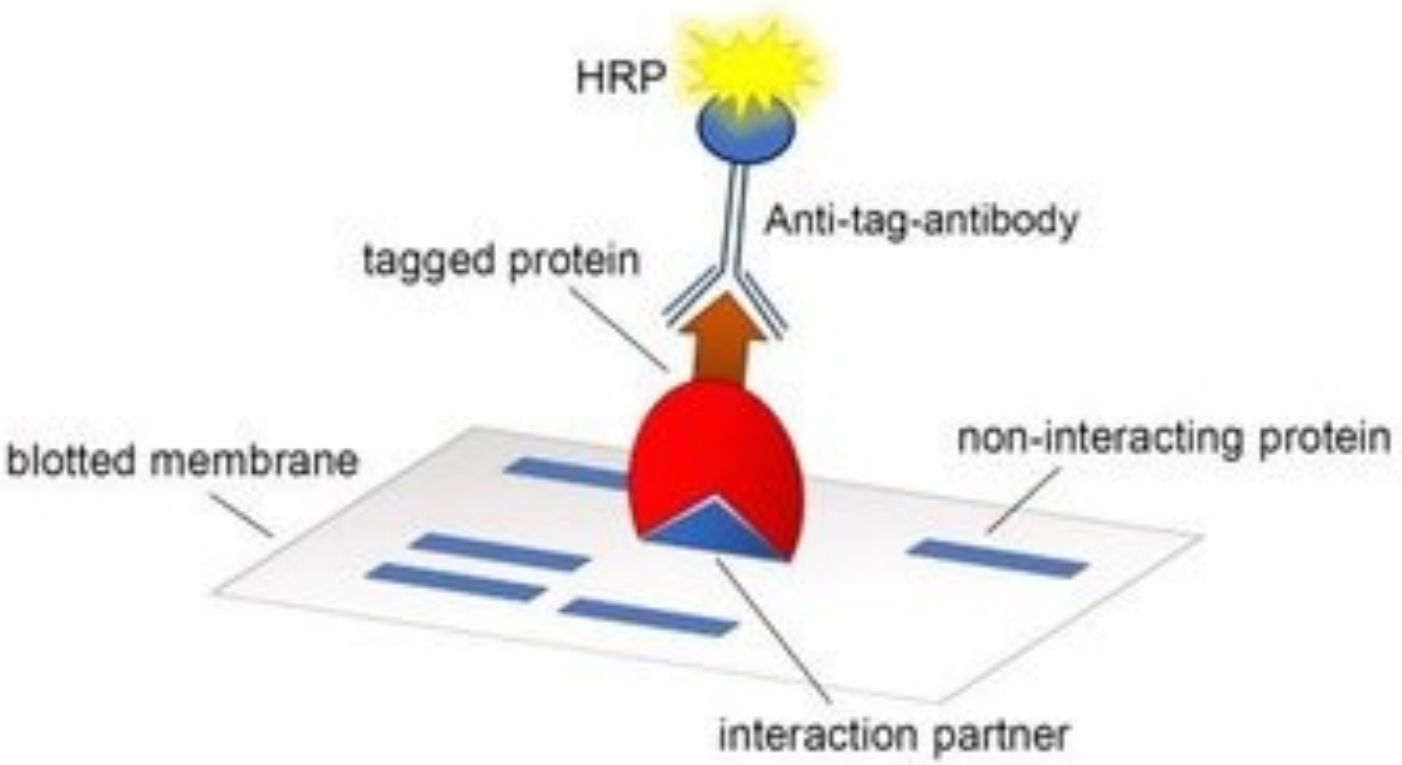
Pulldown of MBP-RA deletion proteins with IVT HA-ORF8



ORF8 lega la porzione extracellulare del recettore IL-17

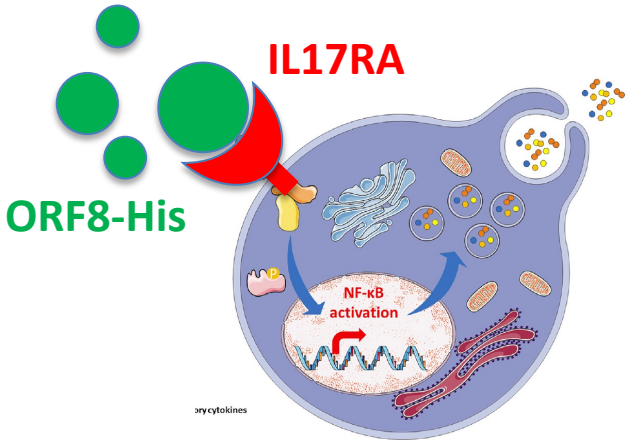


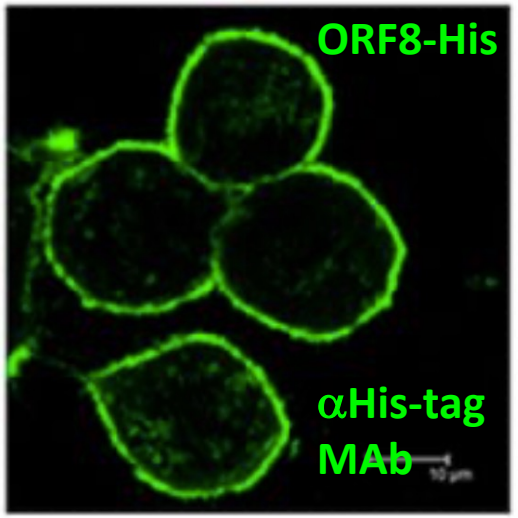
VALIDAZIONE DELLE INTERAZIONI (2): Analisi di interazione DIRETTA proteina-proteina mediante FAR WESTERN:

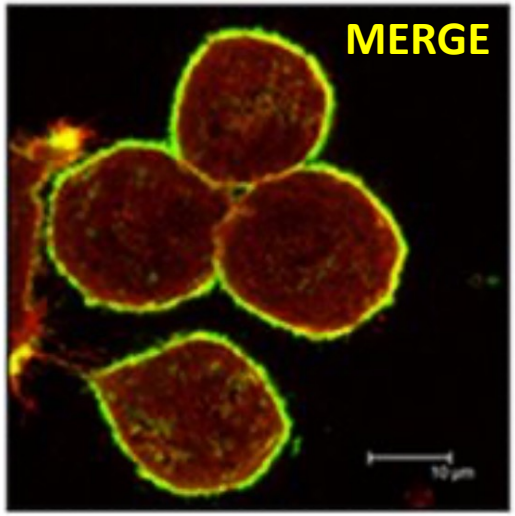


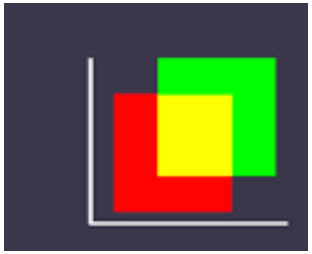
VALIDAZIONE DELLE INTERAZIONI (3): immunofluorescenza e microscopia confocale

- **Aggiunta di ORF8-His alla coltura di cellule H1299**







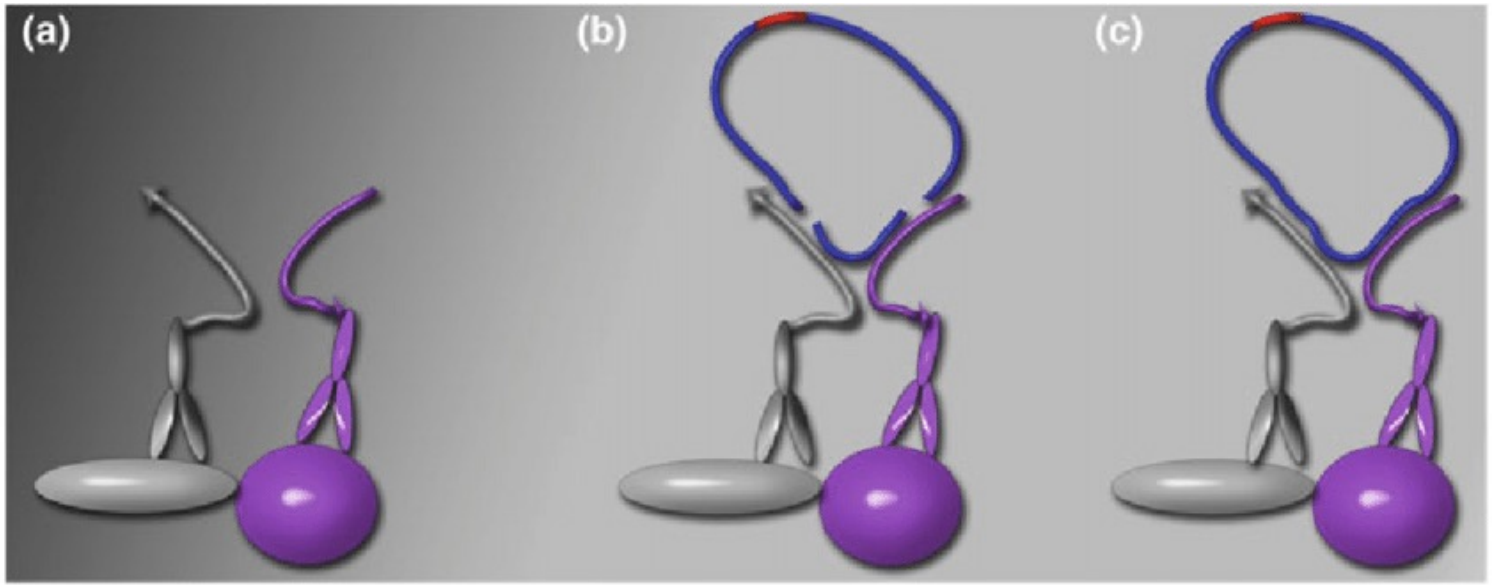


VALIDAZIONE DELLE INTERAZIONI (4)

Analisi dell'interazione diretta proteina-proteina IN SITU:

Proximity Ligation Assay PLA

IN SITU Proximity Ligation Assay PLA

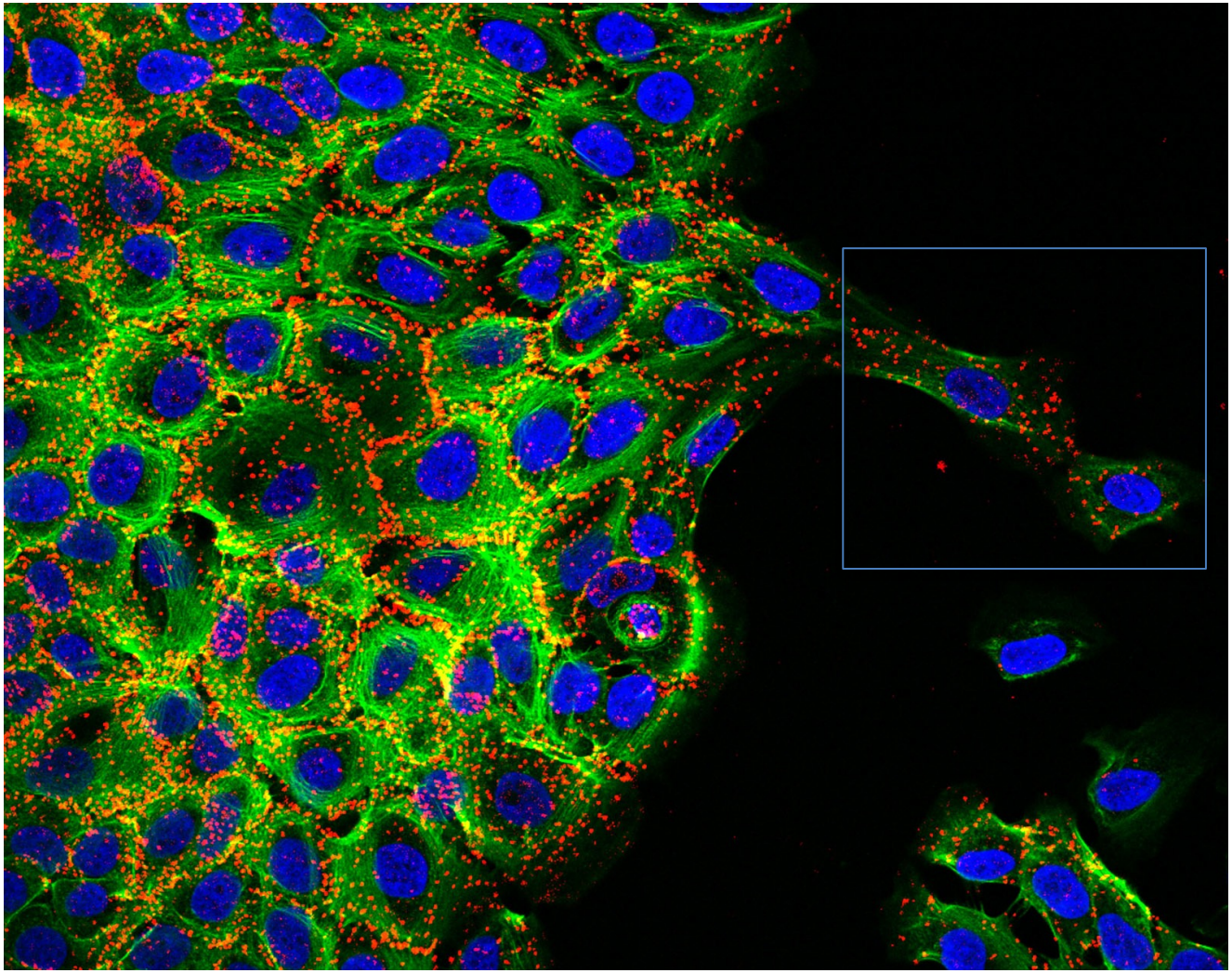


Secondary antibodies conjugated to oligonucleotides

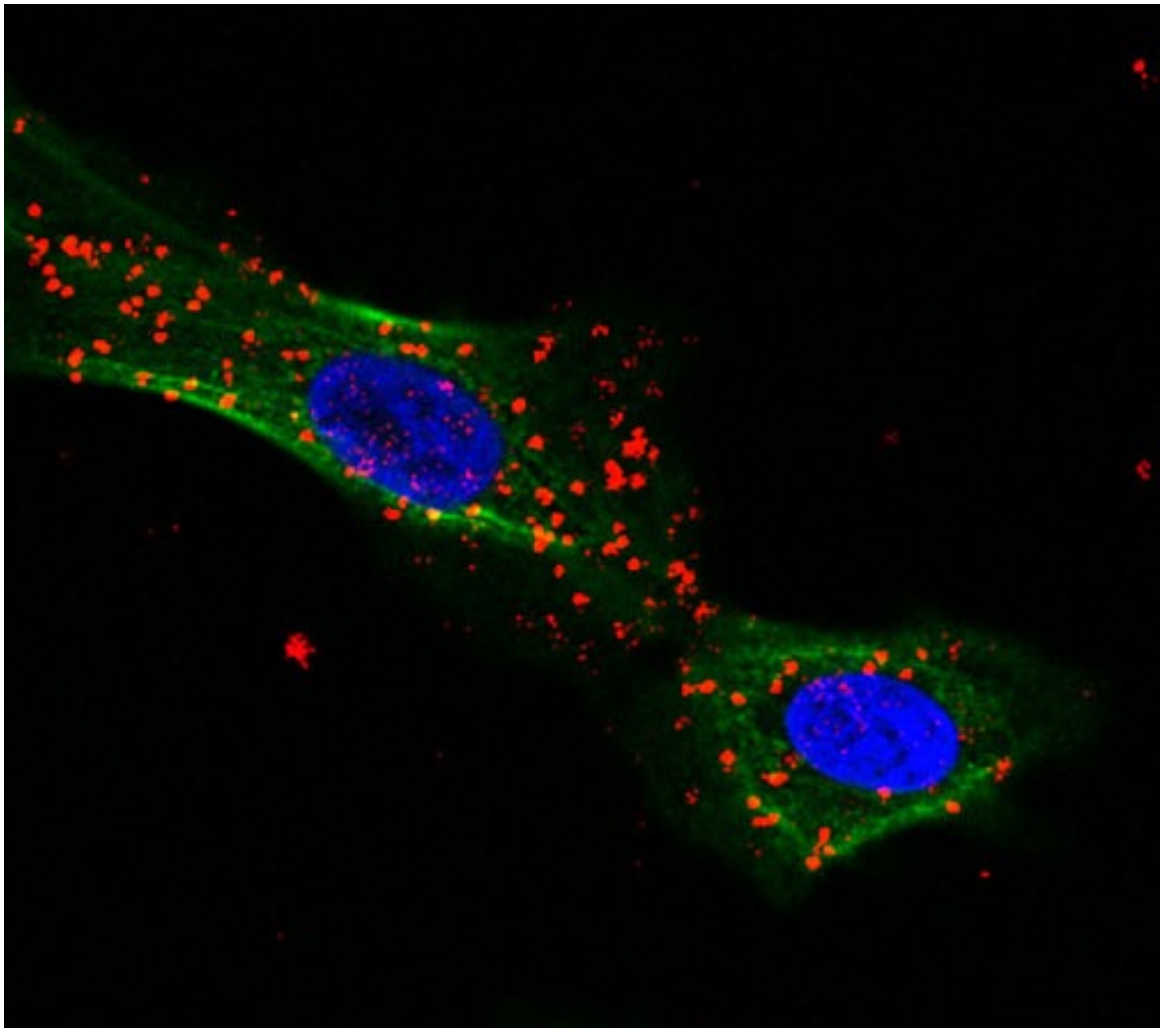
used as templates for the joining of linear oligos into a DNA circle

DNA amplification hybridize with fluorescent probe

NB il saggio evidenzia la PROSSIMITA': le proteine devono essere distanti meno di 40 nm



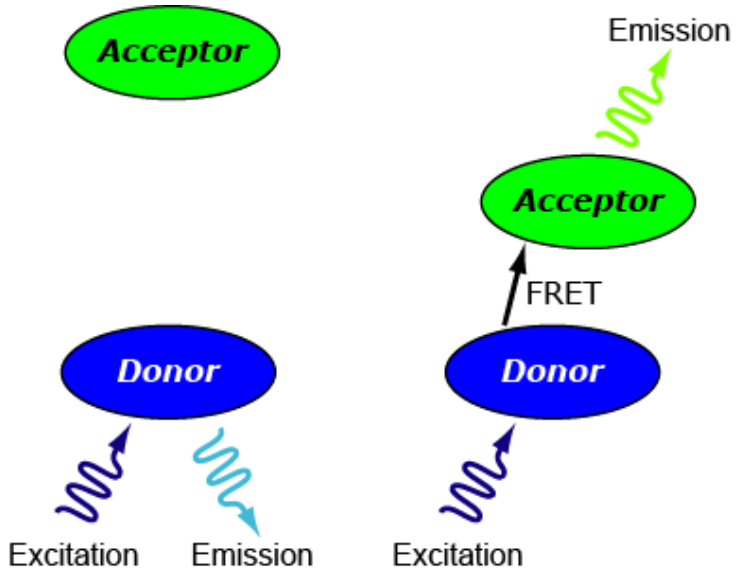
In situ proximity ligation assay for E-cadherin and p120ctn (red) in MCF10A-ER-Src cells stained with Phalloidin to mark actin filaments (green) and DAPI

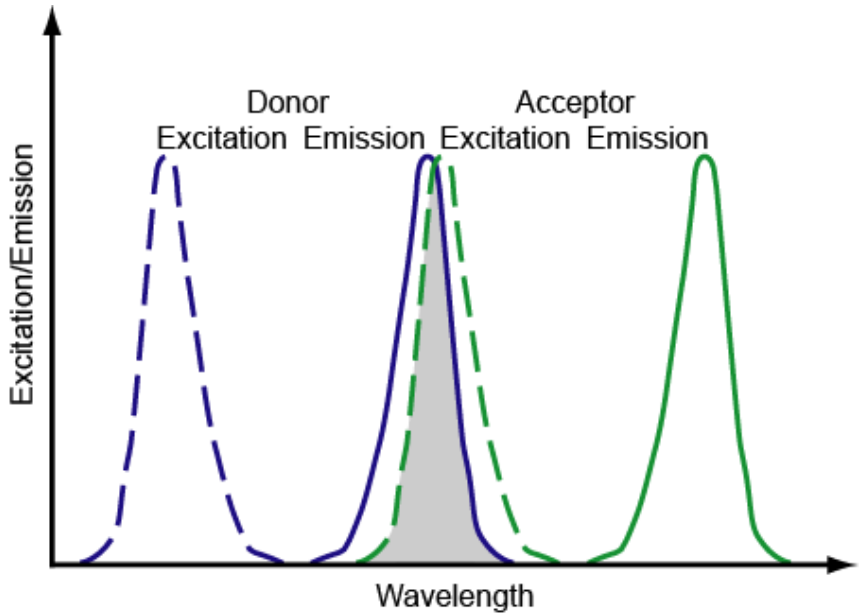


VALIDAZIONE DELLE INTERAZIONI (5)

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

Förster Resonance Energy Transfer





Emission (donor) and excitation (acceptor) spectra must significantly overlap

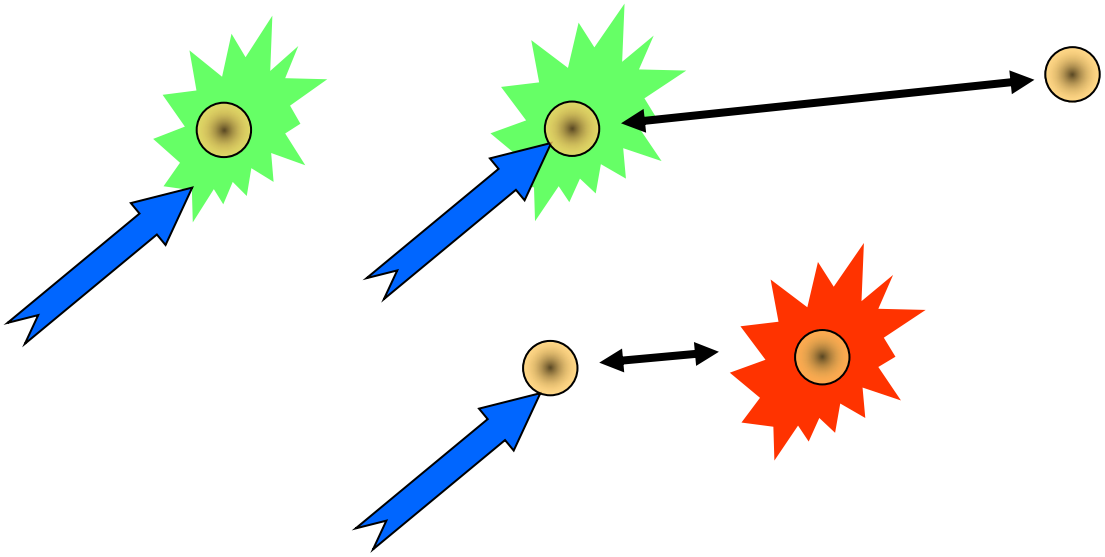
Es. CFP/YFP

Cy3/Cy5

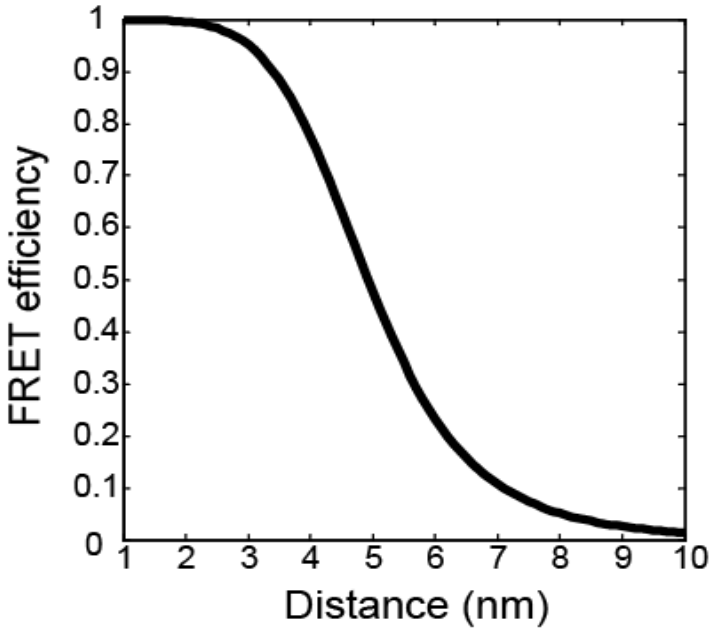
La FRET è efficiente su piccole distanze

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
minore del limite di risoluzione del microscopio a fluorescenza!