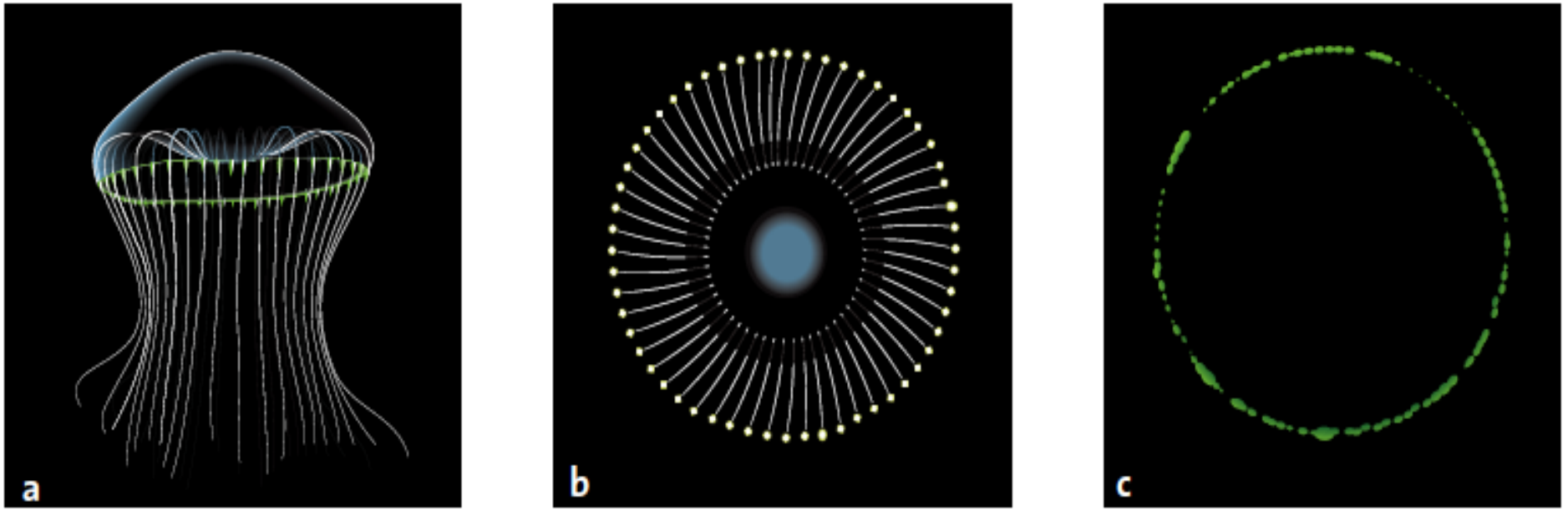
PLS *Virtual Summer School* per Studenti (PVS3) – 7 settembre 2021

Tavolozze biologiche
La proteina fluorescente GFP e i suoi usi nella ricerca biomedica

Nicola Zambrano
zambrano@unina.it

Green Fluorescent Protein

La **green fluorescent protein** (**GFP**) fu scoperta nel 1962 in una medusa, l'*Aequorea victoria*, così chiamata perché raccolta nella baia dell'isola Victoria in Canada.



The jellyfish Aequorea victoria *lives in the sea off the west coast of North America (a). The jellyfish's bioluminescent organ is located along the edge of the "umbrella" (b and c).*

Green Fluorescent Protein

La medusa ***Aequorea victoria*** se ne serve come richiamo, o per spaventare eventuali predatori.

La GFP, se colpita ed eccitata da una radiazione ad una specifica lunghezza d'onda, è in grado di riemettere luce di colore verde acceso.

Grazie alla sua proprietà di fluorescenza, alle sue modeste dimensioni è diventata negli ultimi decenni un diffuso strumento per esperimenti e tecniche di biologia molecolare

Green Fluorescent Protein

Aequorea victoria

?

Il Premio Nobel per la Chimica 2008 è stato assegnato a Roger Tsien, Martin Chalfie, e a Osamu Shimomura, "**for the discovery and development of the green fluorescent protein, GFP**"

8 October 2008

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Osamu Shimomura, Martin Chalfie, Roger Y. Tsien

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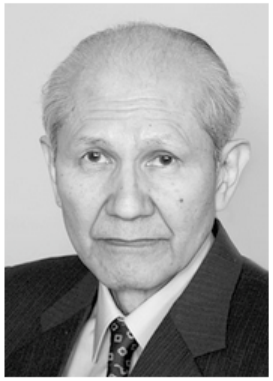

Photo: U. Montan

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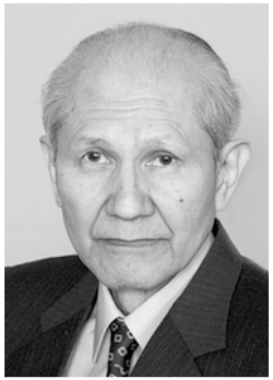

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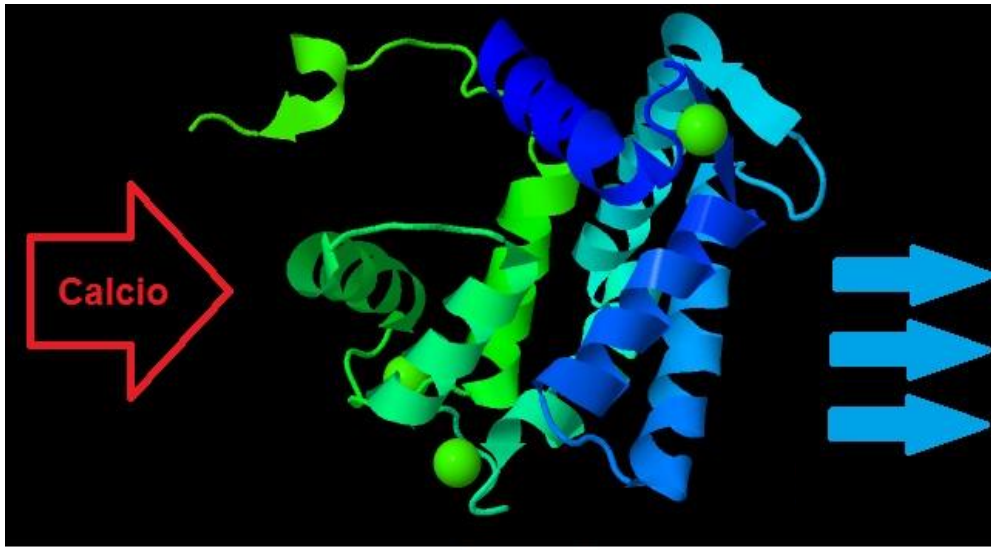
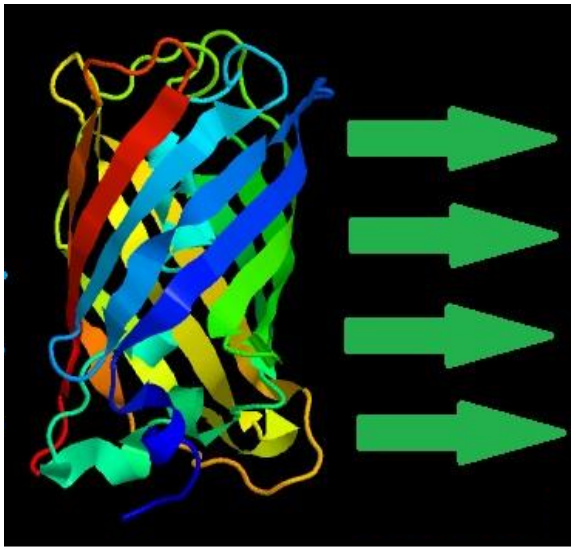
Il Sistema Aequorin/GFP



Aequorina

Luce Azzurra

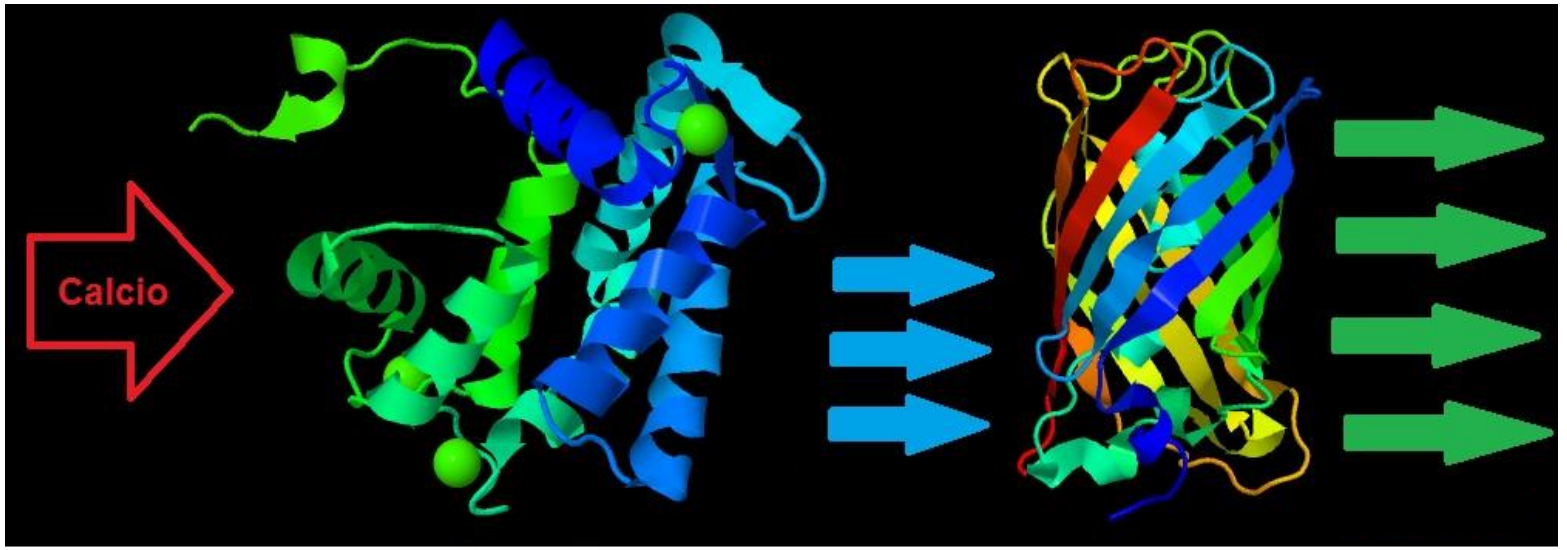
Il Sistema Aequorin/GFP



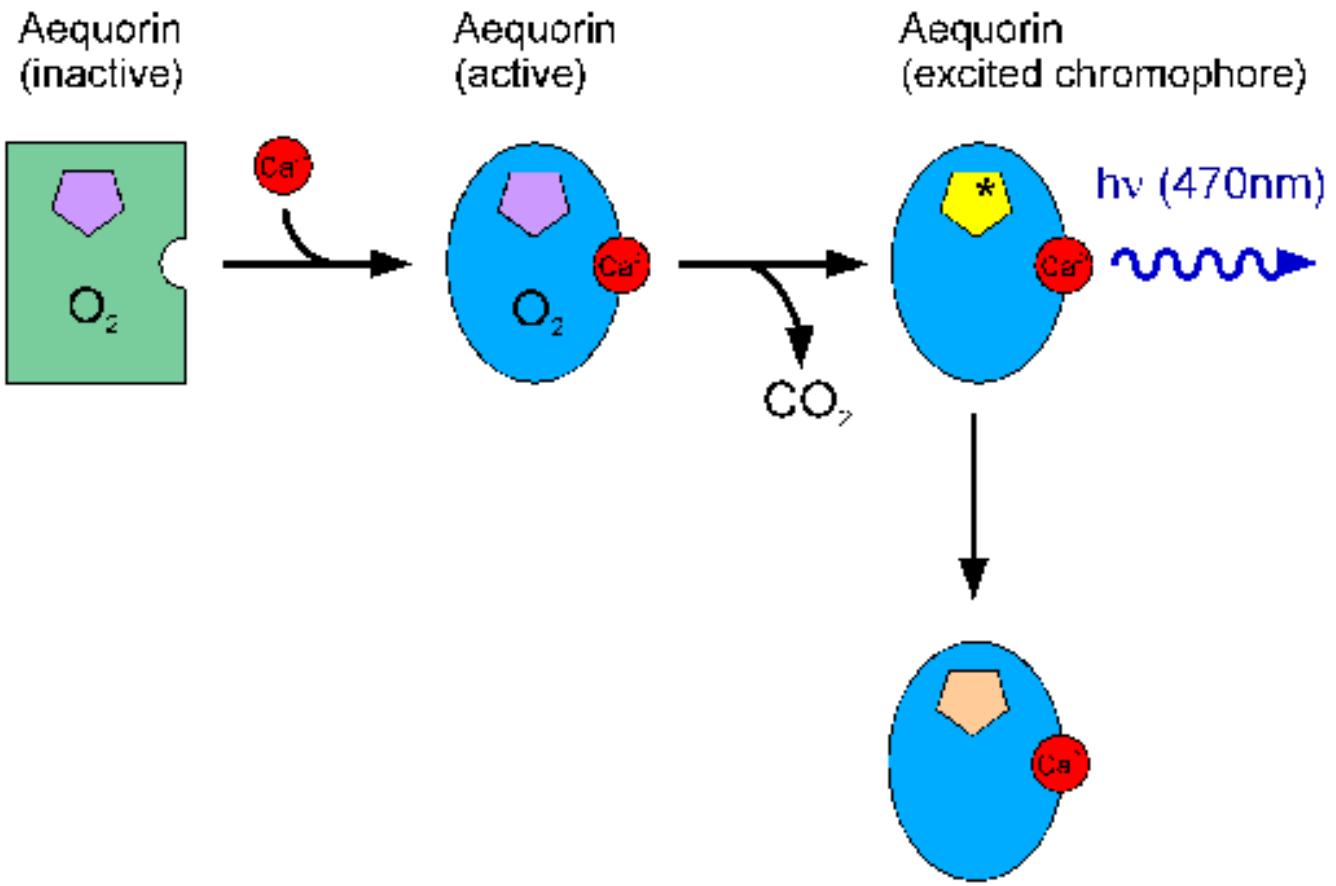
GFP

Luce Verde

Il Sistema Aequorin/GFP

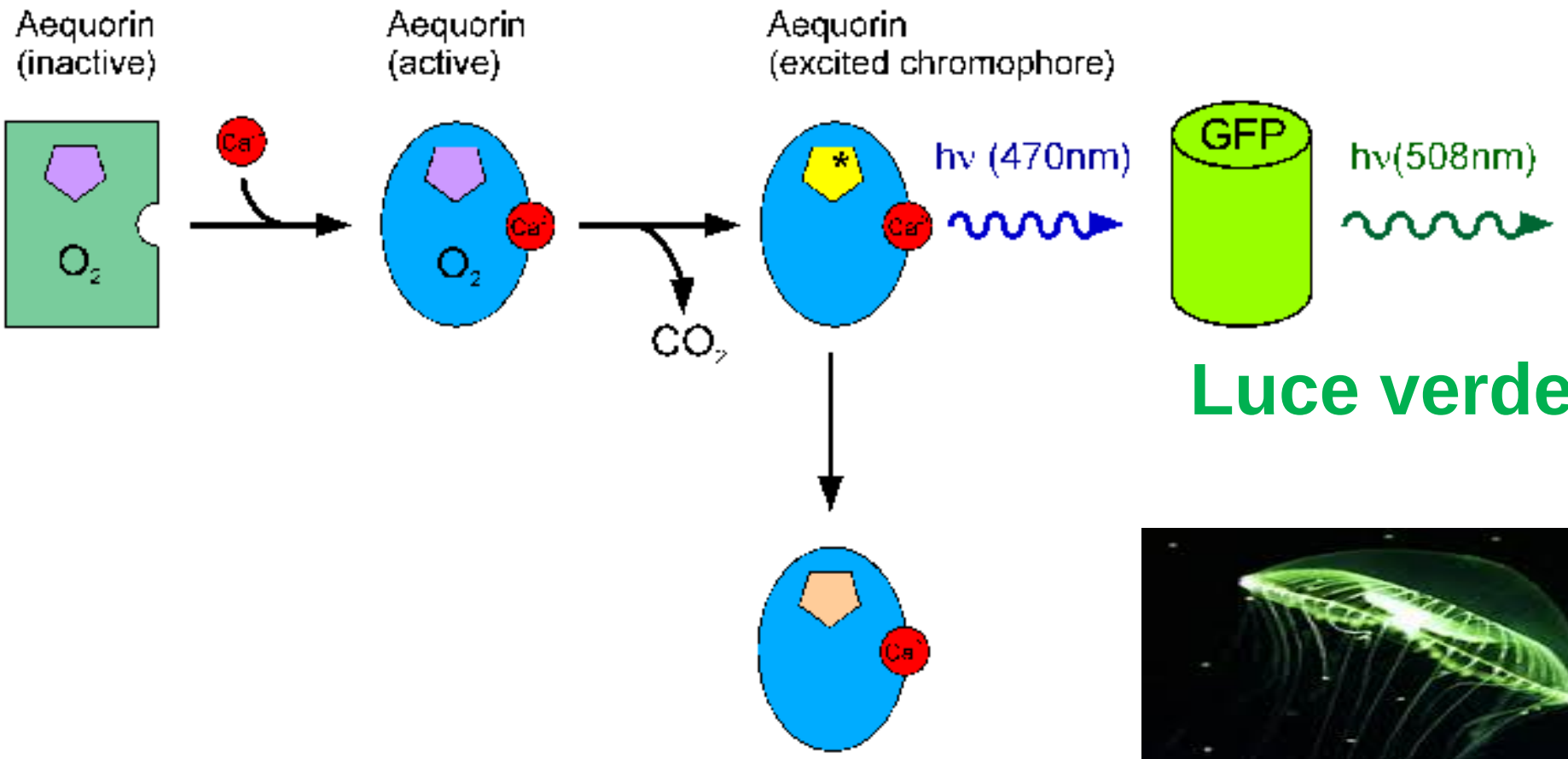


Il Sistema Aequorin/GFP



Luce blu

Il Sistema Aequorin/GFP



Luce verde

Il Sistema Aequorin/GFP

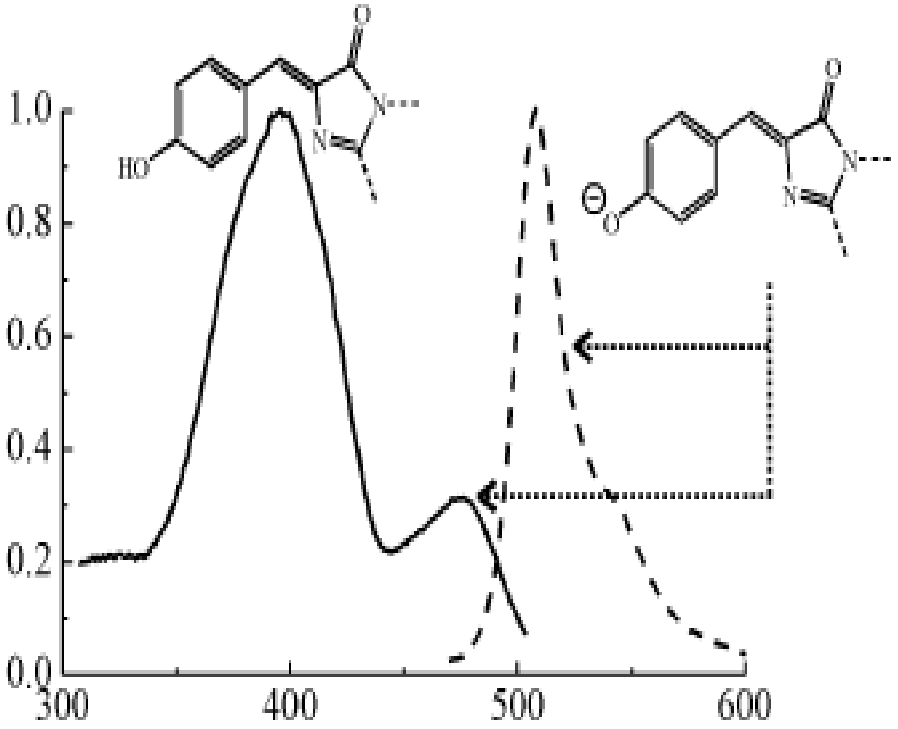
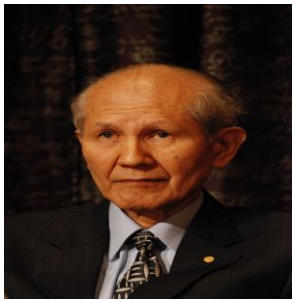


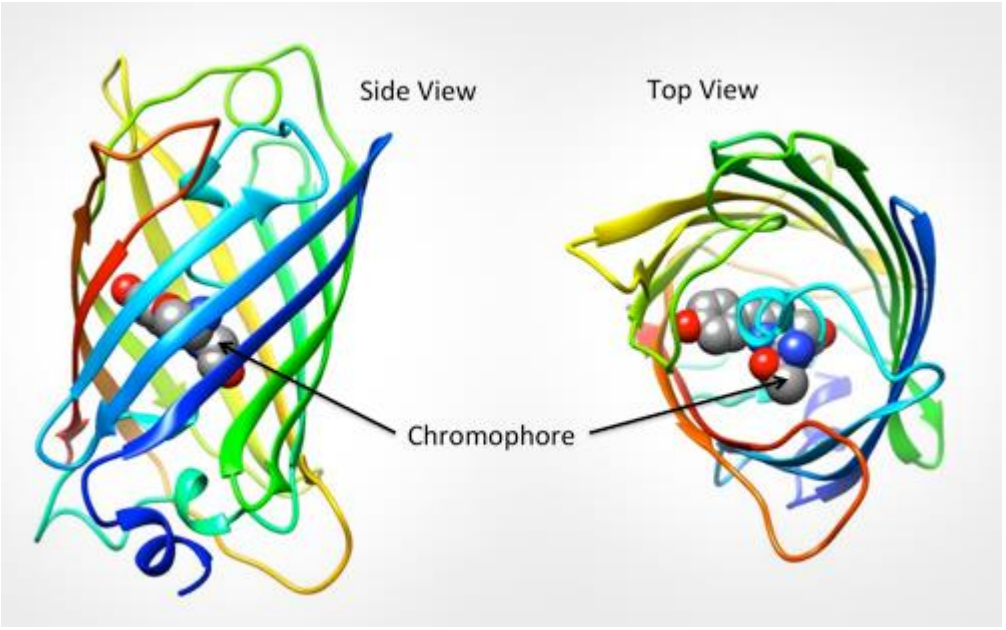
Figure 2. Fluorescence excitation (full-line curve) and emission (dashed curve) spectra of native GFP from *Aequorea victoria* (Tsien et al., 1998).

Green Fluorescent Protein



Osamu Shimomura è stato il primo a isolare la proteina GFP e a scoprire la sua fluorescenza verde.

La GFP è costituita da 238 amminoacidi e ha un PM di 27.000 Dalton. È costituita da 11 foglietti beta disposti in circolo a formare una struttura denominata barile-β (β-barrel o β-can). Inoltre sono presenti due segmenti ad alfa elica, uno alla base del barile, l'altro lungo il suo asse centrale. Quest'ulima elica contiene il fluoroforo, ovvero la porzione in grado di assorbire la luce ed emettere fluorescenza.

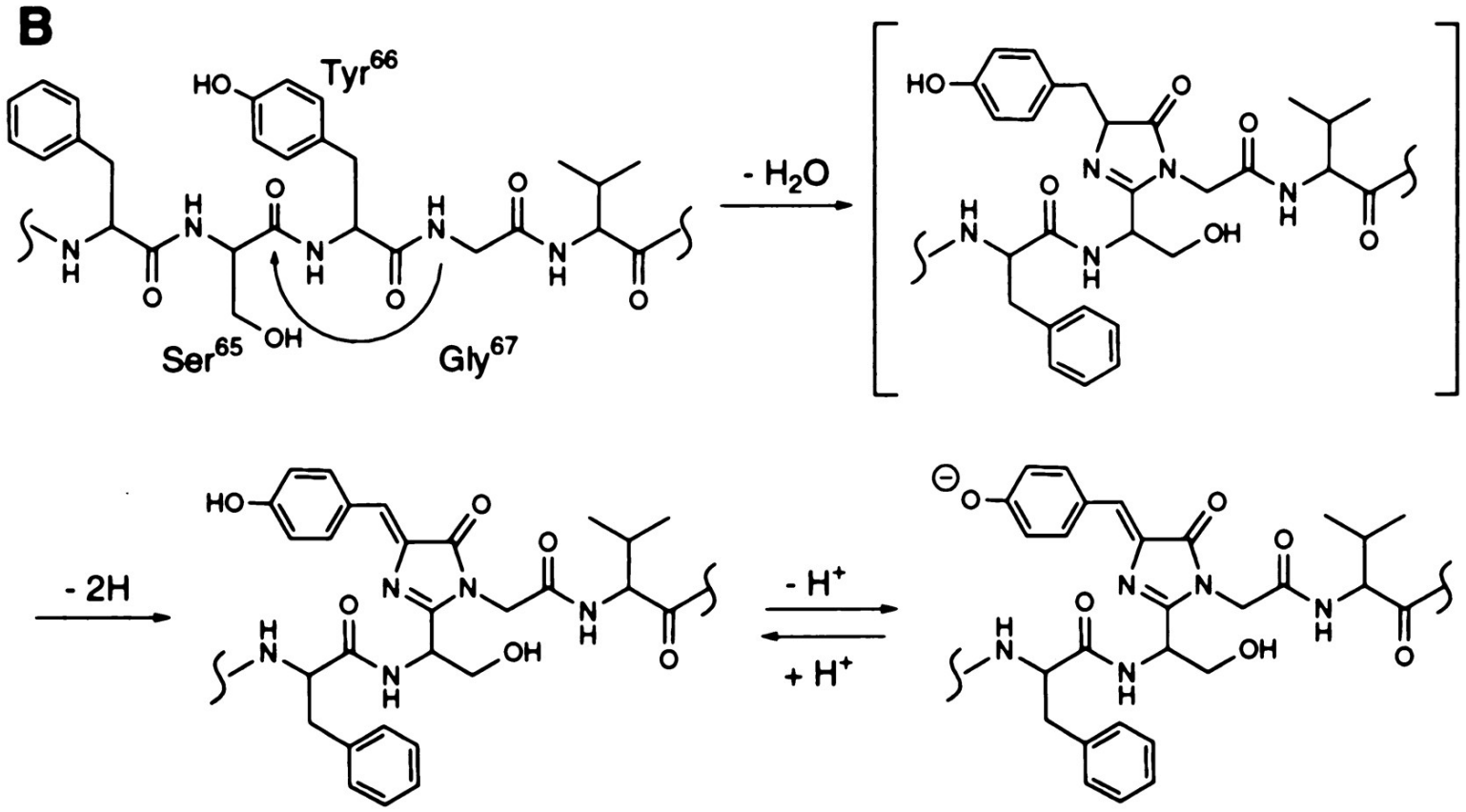


Il Cromoforo Ser_{65}-Tyr_{66}-Gly_{67}

A

```
1         .         .         .         .         .
MSKGEELFTGVVPILVELDGDVNGQKFSVSGEGEGDATYGKLTLKFICTT
          .         .         .         .          238
GKLPVPWPTLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPE.....DELYK
```

B



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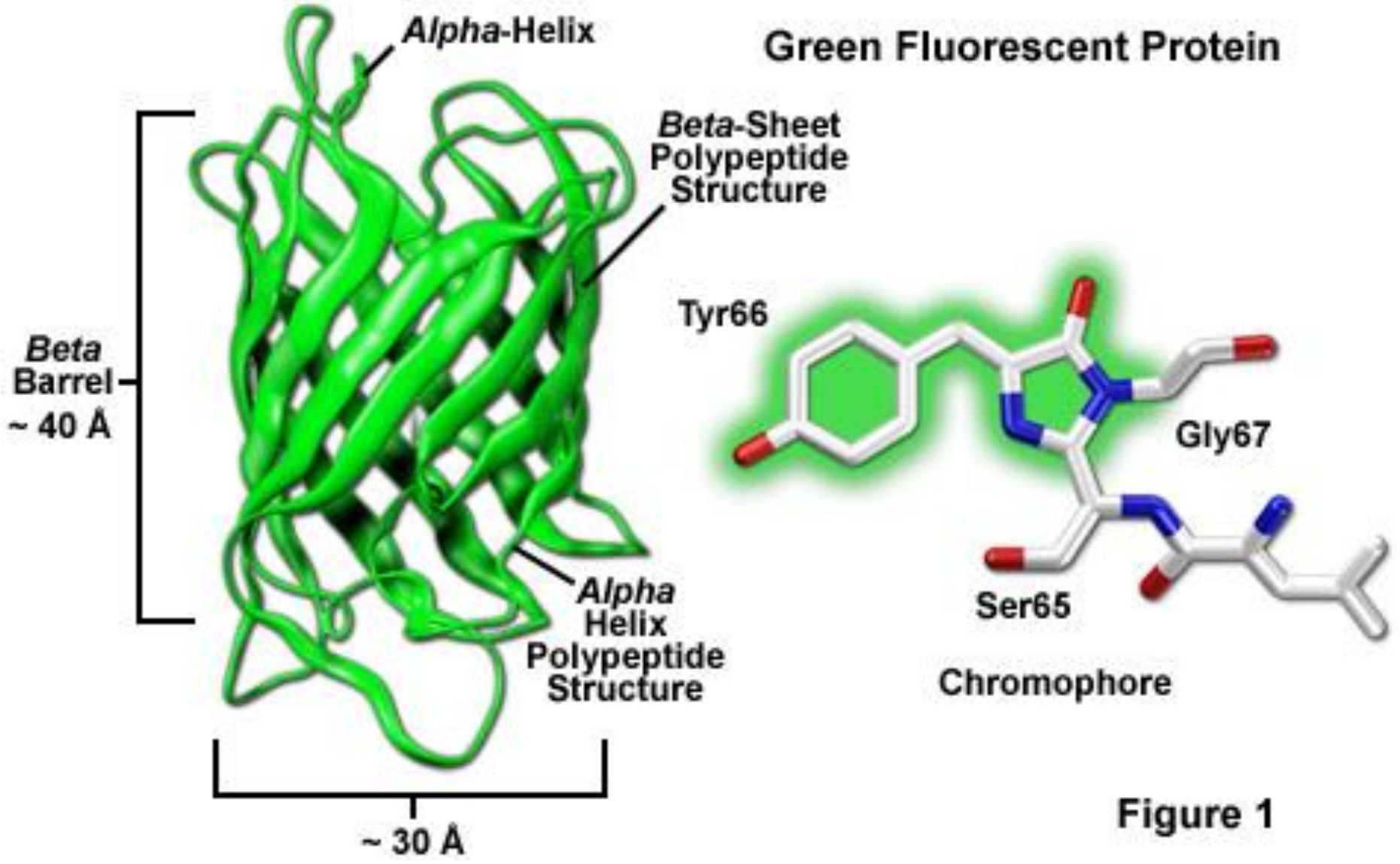

Figure 1

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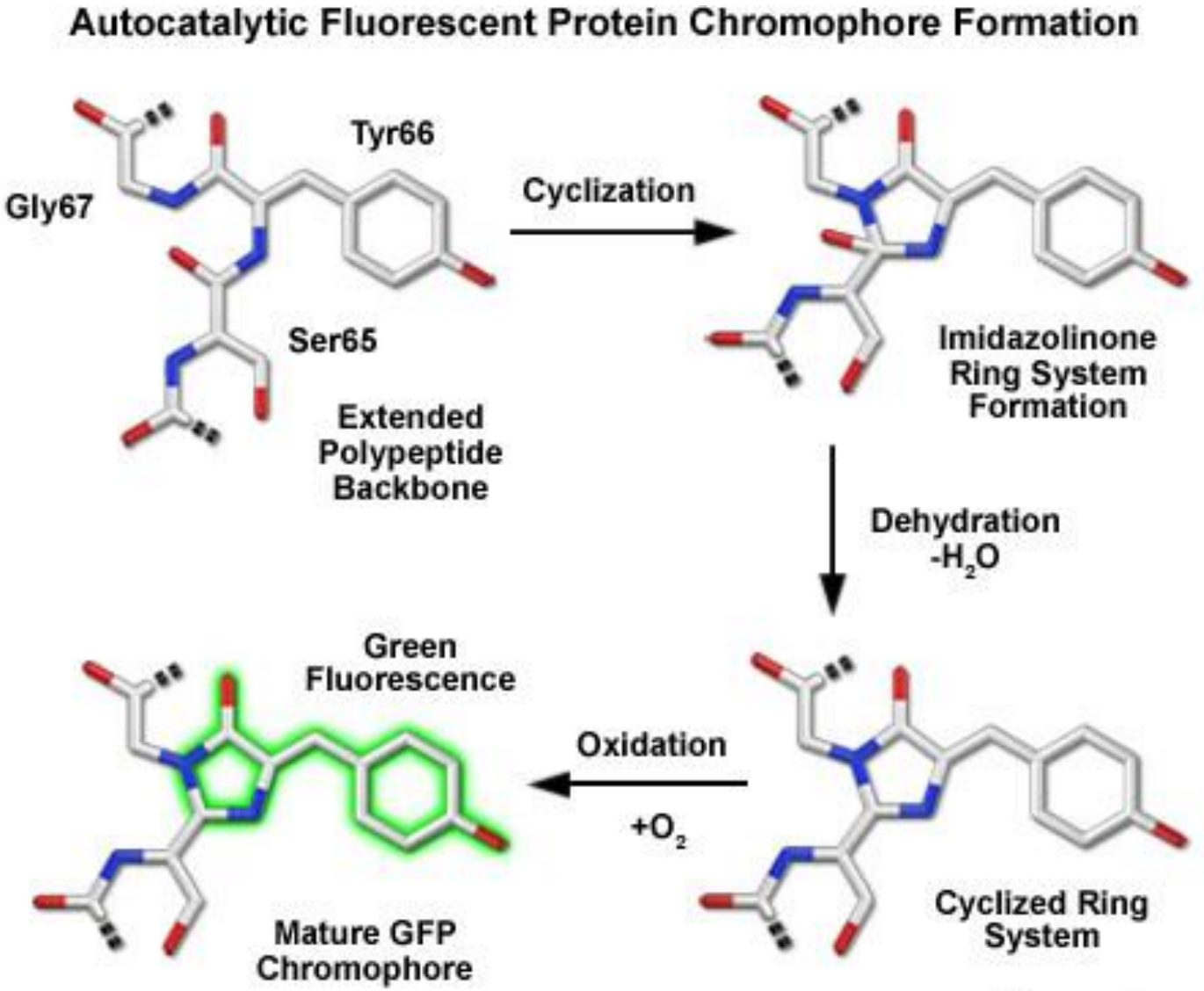

Figure 2

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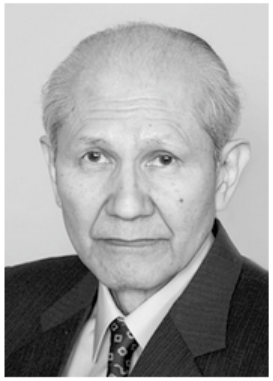

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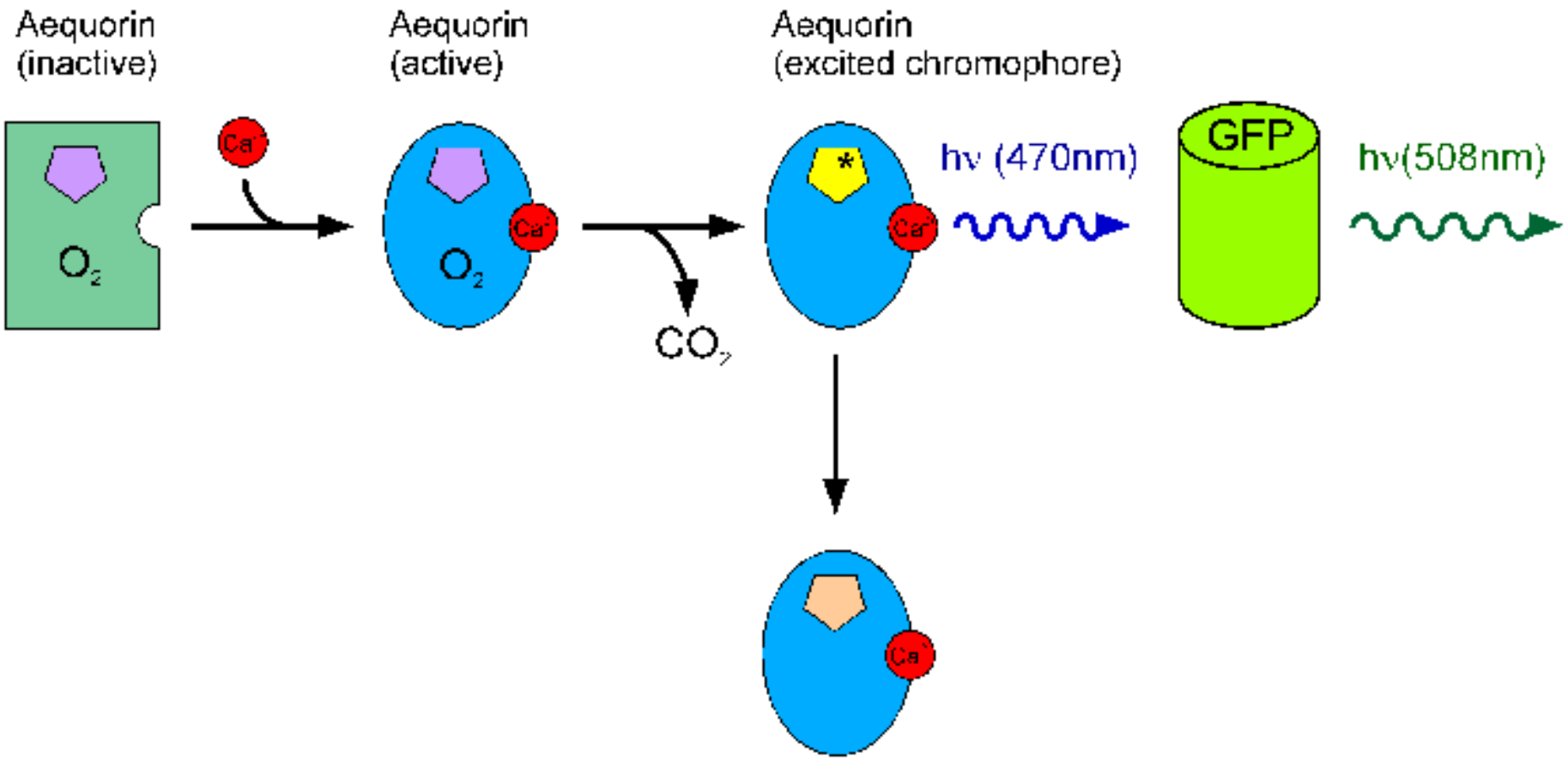
https://www.youtube.com/watch?v=cfg75ft7oOw

GFP COME "*REPORTER*"

- Emette fluorescenza → Può rendere visibili cellule in organismi trasparenti
- Non richiede substrati → Può permettere osservazioni *in vivo*
- Piccole dimensioni → Può essere fusa a proteine cellulari per l'analisi del traffico e localizzazione di proteine

GFP COME "*REPORTER*"



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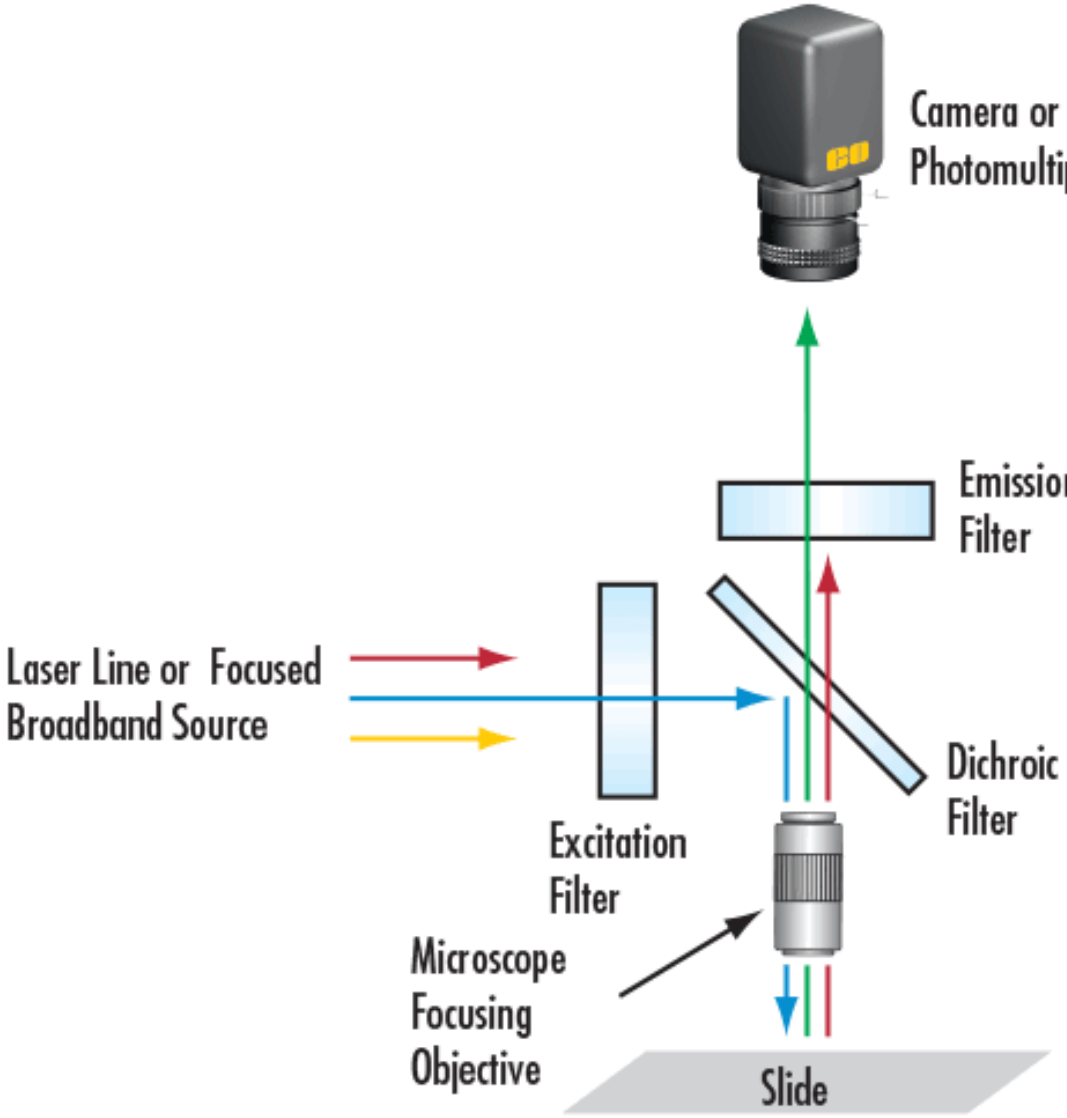
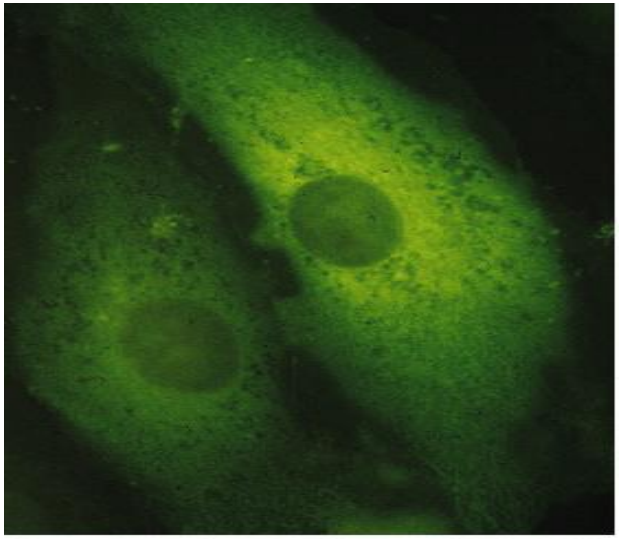
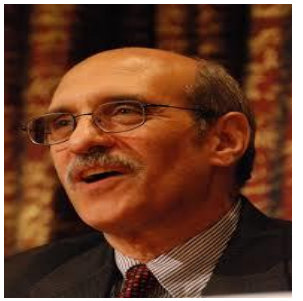




Green Fluorescent Protein



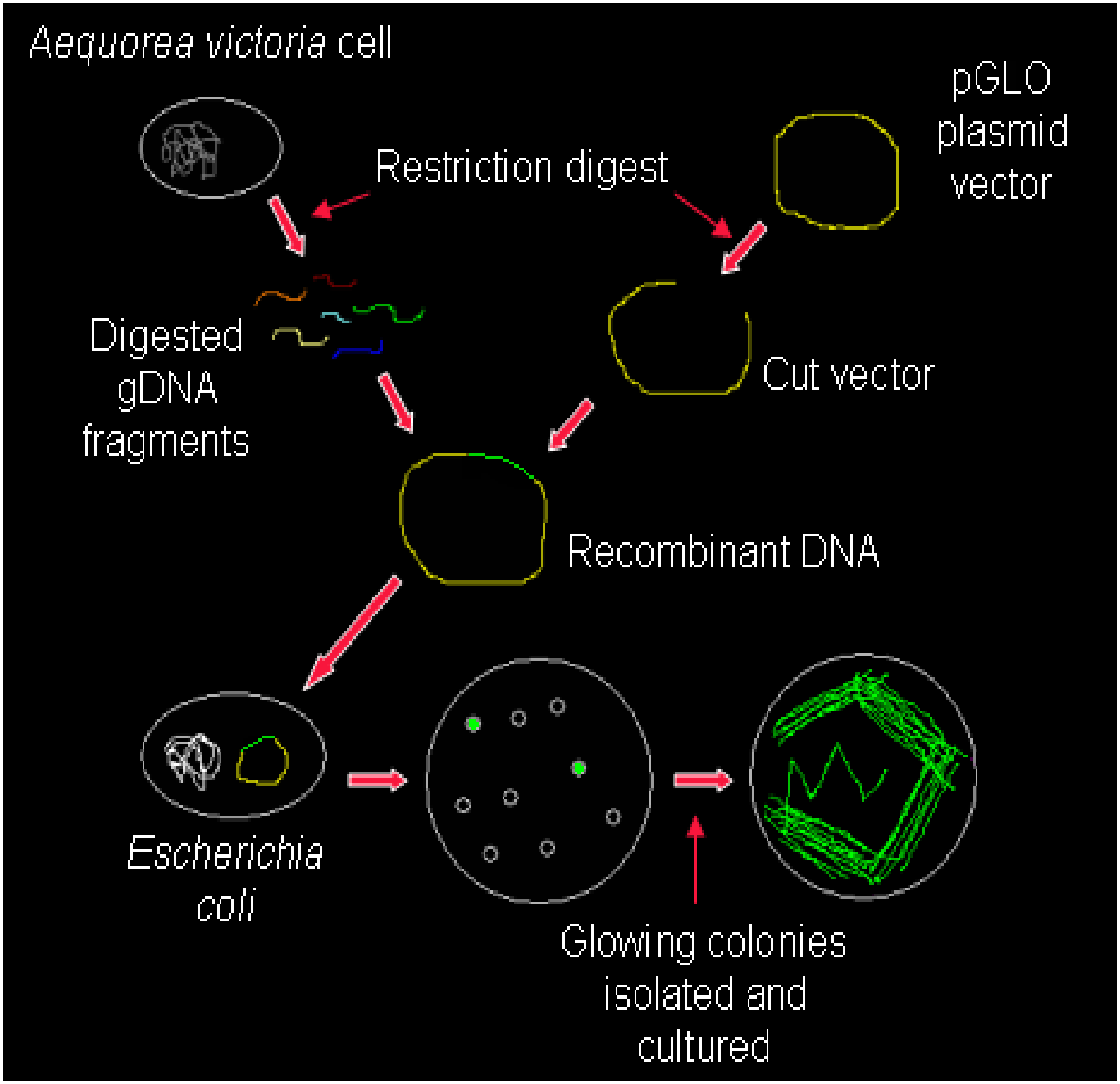
- Fu Douglas Prasher il primo a intuire la possibilità di usare la GFP come ***reporter*** in organismi trasparenti e questa intuizione aprì la strada alla straordinaria rivoluzione che ha portato la GFP a meritare il premio Nobel.

- M. Chalfie clonò il cDNA della GFP per utilizzarla come ***reporter*** (*E. coli* prima, *C. elegans* poi) per studiare il pattern di espressione di geni

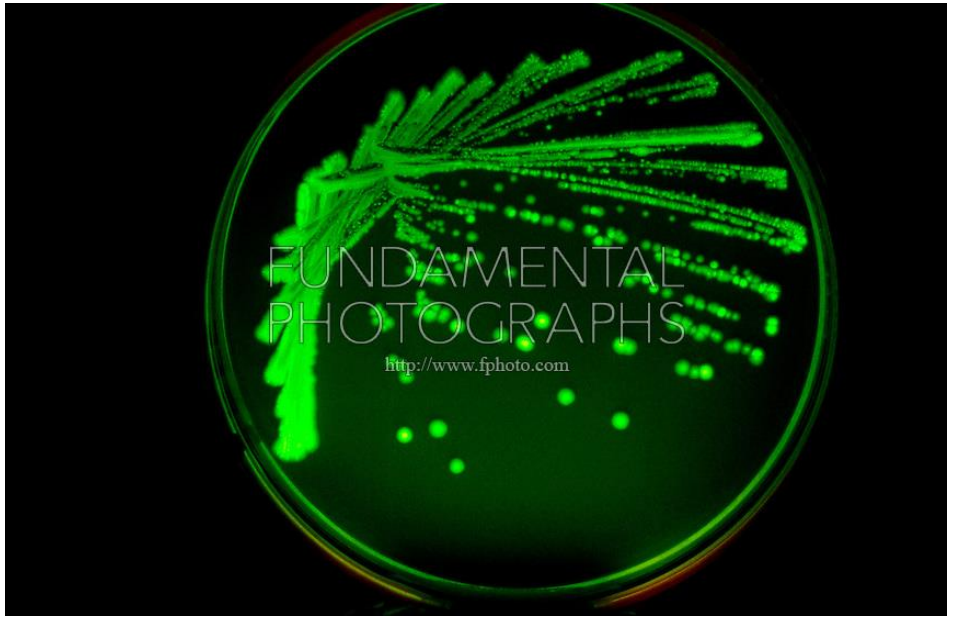
Green Fluorescent Protein

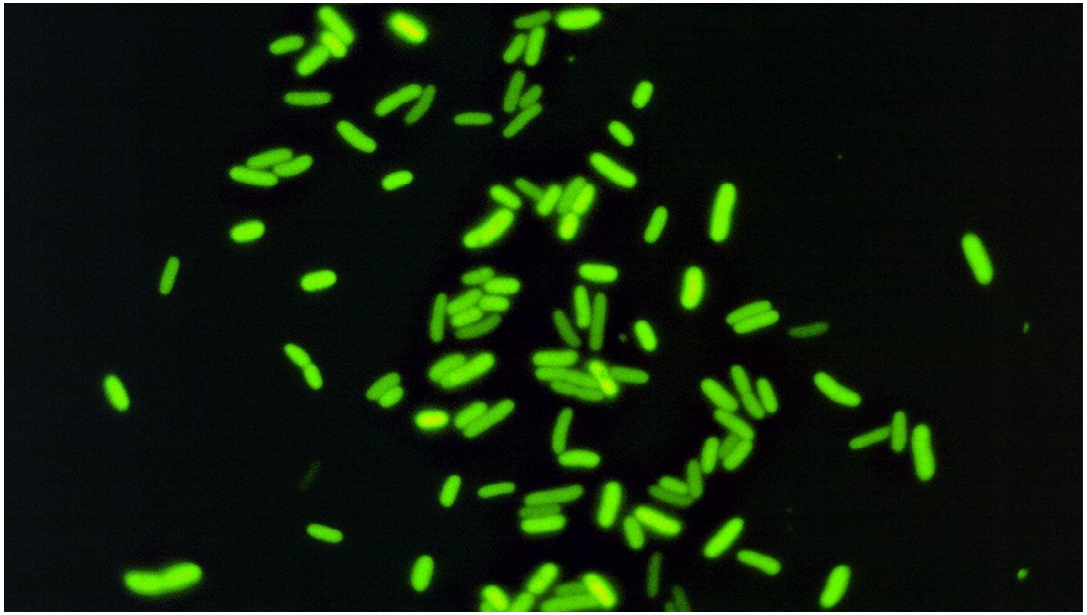
Nel 1994 la GFP è stata clonata...

Una studentessa del gruppo di Chalfie, Ghia Euskirchen,
riuscì a far esprimere il gene della GFP, che Chalfie aveva avuto da Prasher, in *E. coli*, cosicché diventasse verde quando il batterio veniva illuminato con luce blu.

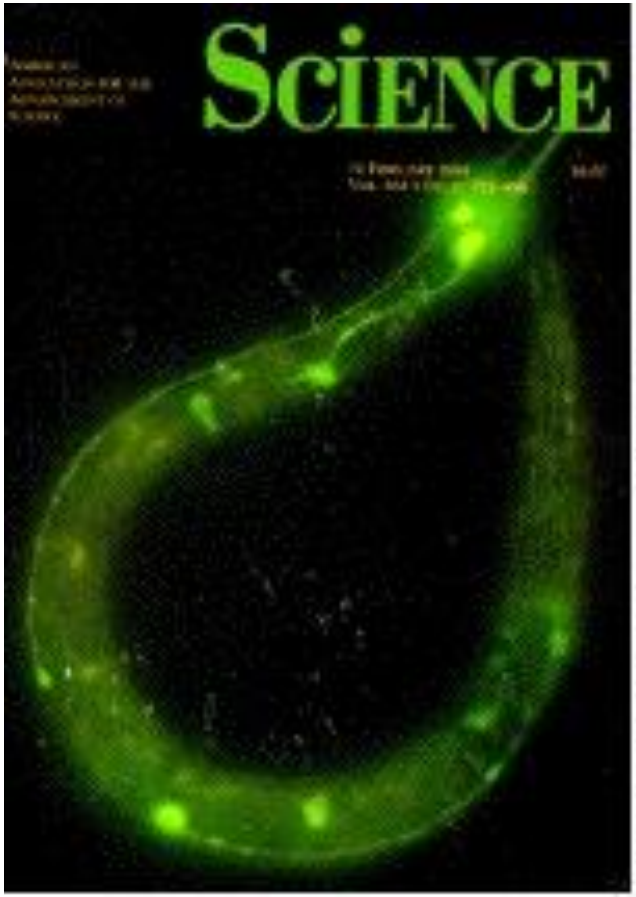


Escherichia coli





Caenorhabditis elegans



Green Fluorescent Protein as a Marker for Gene Expression

Martin Chalfie,* Yuan Tu, Ghia Euskirchen, William W. Ward, Douglas C. Prasher†

A complementary DNA for the *Aequorea victoria* green fluorescent protein (GFP) produces a fluorescent product when expressed in prokaryotic (*Escherichia coli*) or eukaryotic (*Caenorhabditis elegans*) cells. Because exogenous substrates and cofactors are not required for this fluorescence, GFP expression can be used to monitor gene expression and protein localization in living organisms.

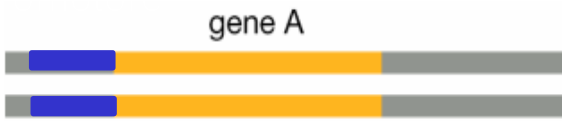
Light is produced by the bioluminescent jellyfish *Aequorea* victoria when calcium binds to the photoprotein aequorin (*1*). Although activation of aequorin in vitro or in heterologous cells produces blue light, the jellyfish produces green light. This light is the result of a second protein in *A. victoria* that derives its excitation energy from aequorin (*2*), the green fluorescent protein (GFP).

Purified GFP, a protein of 238 amino acids (*3*), absorbs blue light (maximally at 395 nm with a minor peak at 470 nm) and emits green light (peak emission at 509 nm with a shoulder at 540 nm) (*2*, *4*). This fluorescence is very stable, and virtually no

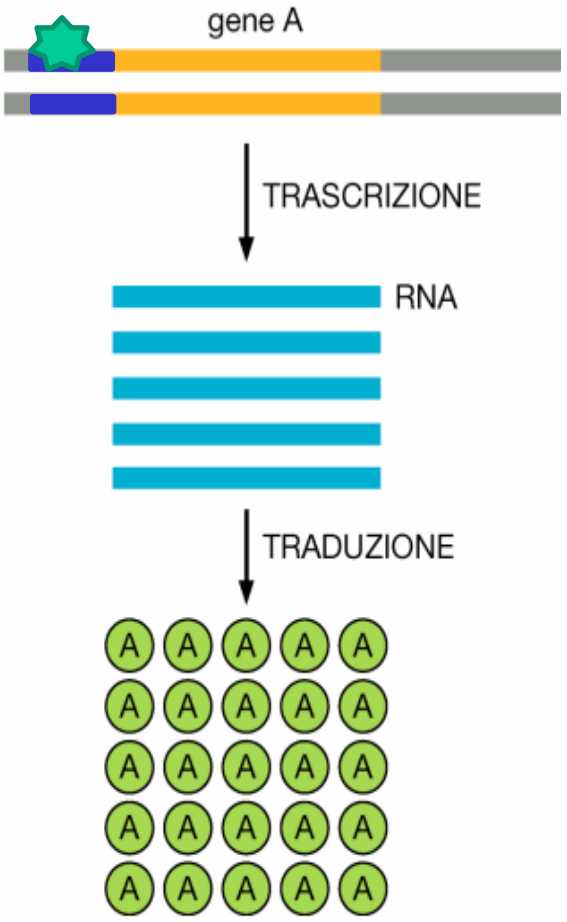
1) Studio di promotori per analisi dell'espressione genica

I geni possono essere espressi a livelli diversi in organi/tessuti diversi

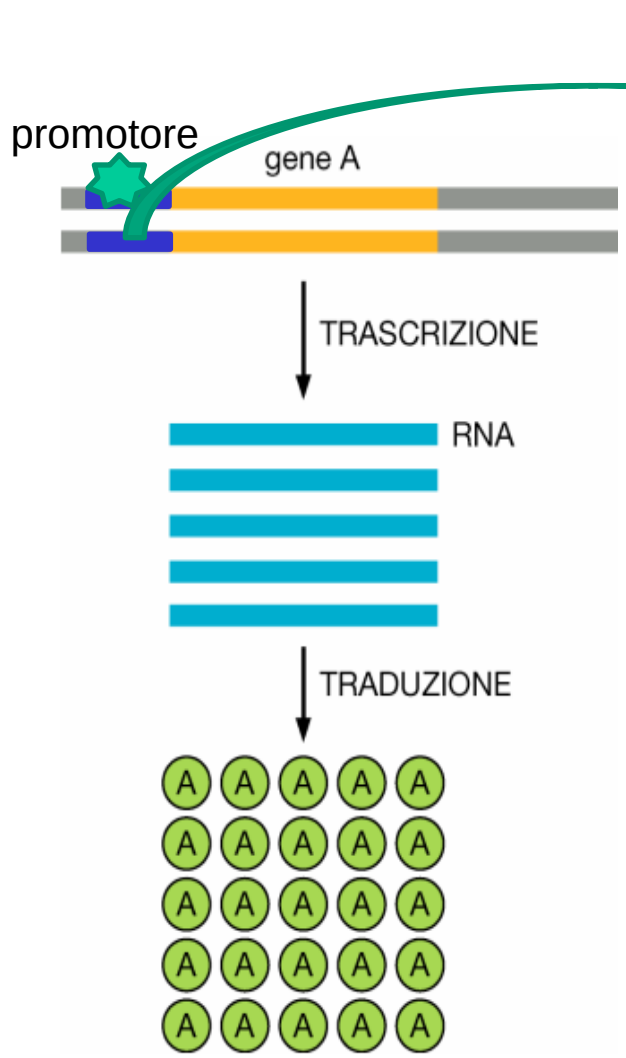
Cellula tessuto A



Cellula tessuto B

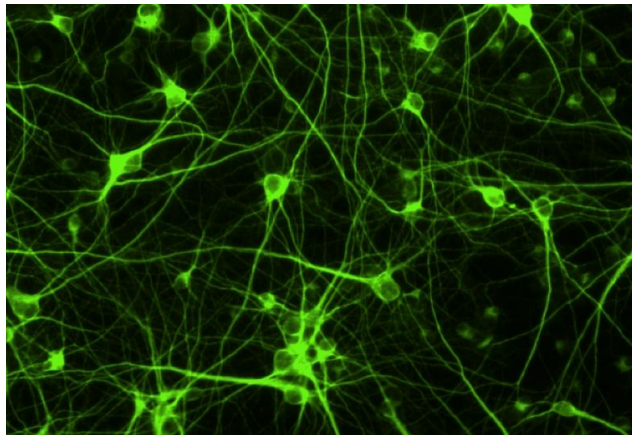


«istruire» una cellula o un animale con DNA della GFP ed un promotore

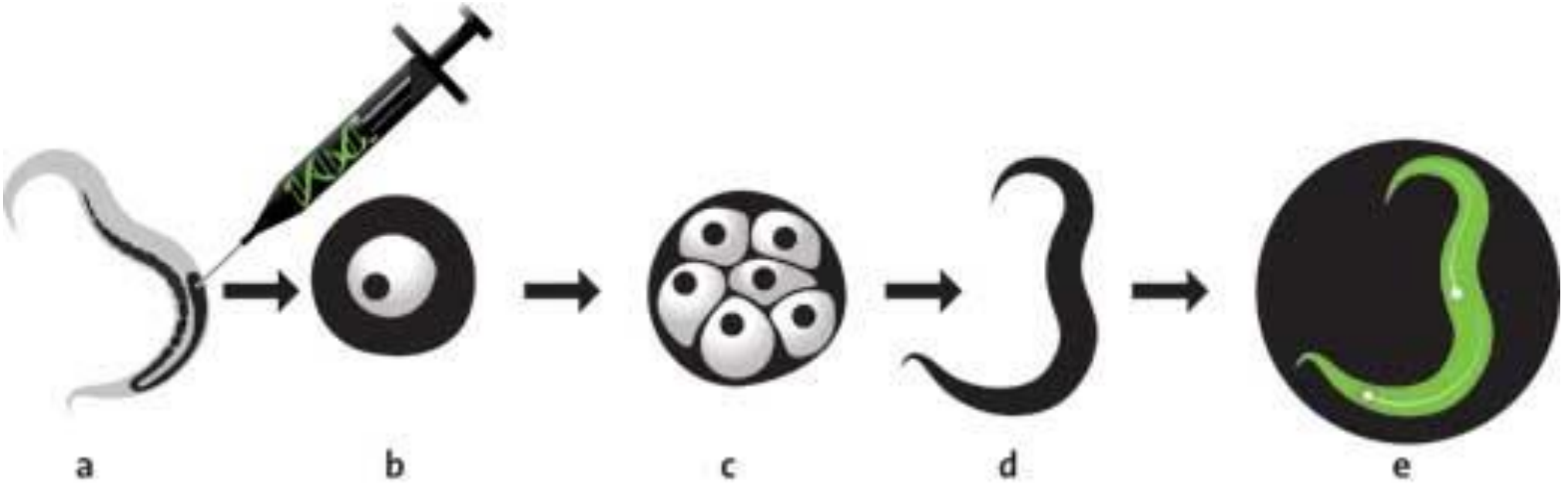


Promotore
gene A
gene GFP

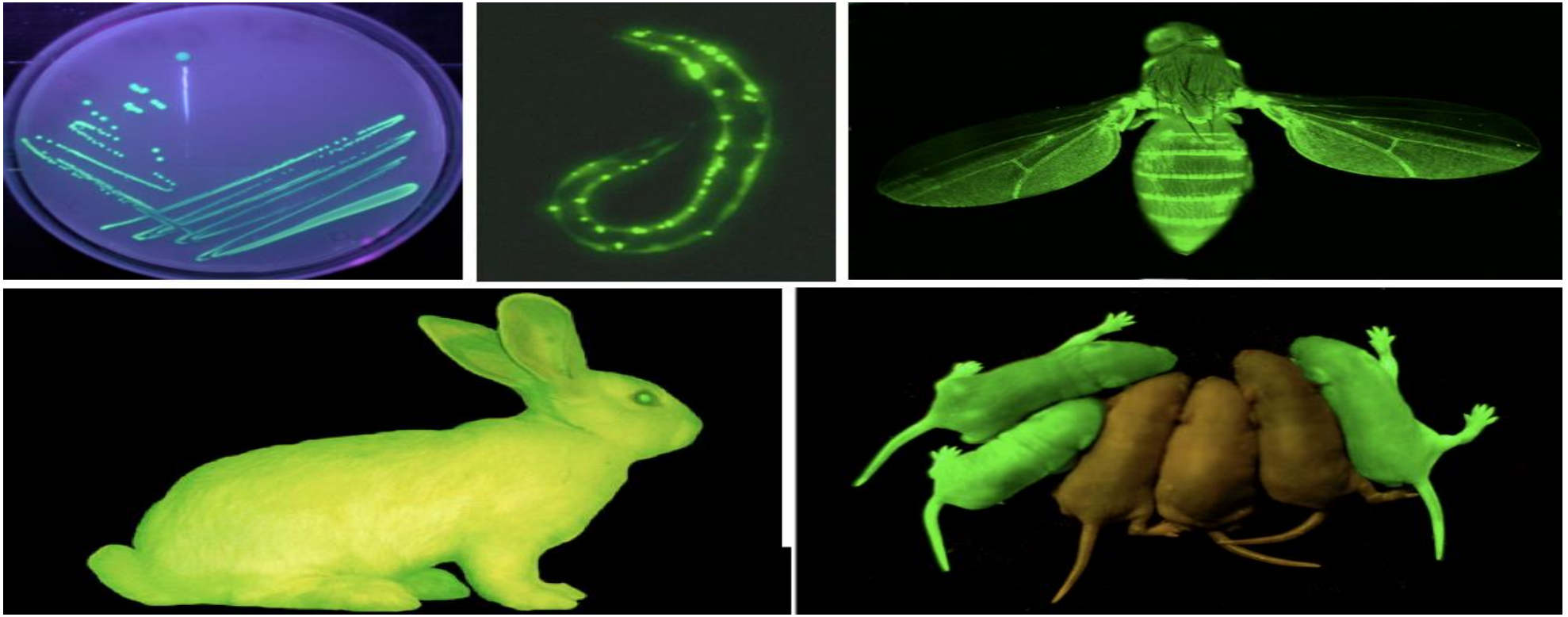
trasferimento
in cellule o animali



Caenorhabditis elegans



Green Fluorescent Protein



….e da allora, a scopi scientifici, industriali e commerciali, cellule, alghe, batteri vermi e perfino conigli, maiali o pesci sono stati resi fluorescenti.

Utilizzata come tracciante per studiare **l’espressione, la funzione** e il **destino** delle proteine.

8 October 2008

KUNGL. VETENSKAPSAKADEMIEN
THE ROYAL SWEDISH ACADEMY OF SCIENCES

The Nobel Prize in Chemistry 2008
Osamu Shimomura, Martin Chalfie, Roger Y. Tsien

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Osamu Shimomura - Facts

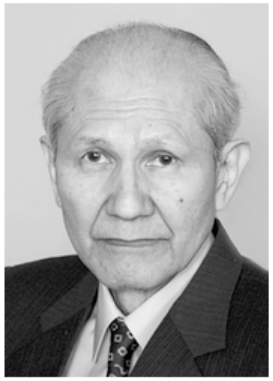

Photo: U. Montan

Osamu Shimomura

Born: 27 August 1928, Kyoto, Japan

Affiliation at the time of the award: Marine Biological Laboratory (MBL), Woods Hole, MA, USA, Boston University Medical School, Massachusetts, MA, USA

Prize motivation: "for the discovery and development of the green fluorescent protein, GFP"

Field: biochemistry

Prize share: 1/3

Work

Some organisms produce what has been named Green Fluorescent Protein (GFP), which emits a shimmering light. The formation of GFP is regulated by a gene that can be incorporated into the genomes of other organisms. Because GFP can be linked to other proteins thanks to genetic engineering, it has become an important tool for studying biological processes in cells. The first steps in achieving this were taken by Osamu Shimamura, who isolated GFP from the jellyfish Aequorea victoria in the 1960s and found that the protein glowed green when illuminated with ultraviolet light.

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The Nobel Prize in Chemistry 2008
Osamu Shimomura, Martin Chalfie, Roger Y. Tsien

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Martin Chalfie - Facts

Photo: U. Montan

Martin Chalfie

Born: 15 January 1947, Chicago, IL, USA

Affiliation at the time of the award: Columbia University, New York, NY, USA

Prize motivation: "for the discovery and development of the green fluorescent protein, GFP"

Field: biochemistry

Prize share: 1/3

Life

Martin Chalfie was born in Chicago. His parents worked in the garment industry, but they encouraged their three sons to pursue academic careers. Chalfie took an interest in the natural sciences, especially chemistry, and received a doctorate in biochemistry at Harvard. He did various short-term jobs before continuing studies for his doctorate. Later he conducted research in Cambridge, England, where he did his Nobel Prize-awarded work. Since 1982 he has served at Columbia University. He married Tulle Hazelrigg, and they have one daughter, Sarah.

Work

Some organisms produce what has been named Green Fluorescent Protein (GFP), which emits a shimmering light. The formation of GFP is regulated by a gene that can be incorporated into the genomes of other organisms. Because GFP can be linked to other proteins thanks to genetic engineering, it has become an important tool for studying biological processes in cells. Martin Chalfie began to use GFP for this purpose in 1988. He inserted the GFP gene into the ringworm C. elegans and succeeded in coloring six individual cells that could then be tracked.

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The Nobel Prize in Chemistry 2008
Osamu Shimomura, Martin Chalfie, Roger Y. Tsien

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Roger Y. Tsien - Facts

Photo: U. Montan

Roger Y. Tsien

Born: 1 February 1952, New York, NY, USA

Died: 24 August 2016, Eugene, OR, USA

Affiliation at the time of the award: University of California, San Diego, CA, USA, Howard Hughes Medical Institute

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8 October 2008

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The Nobel Prize in Chemistry 2008
Osamu Shimomura, Martin Chalfie, Roger Y. Tsien

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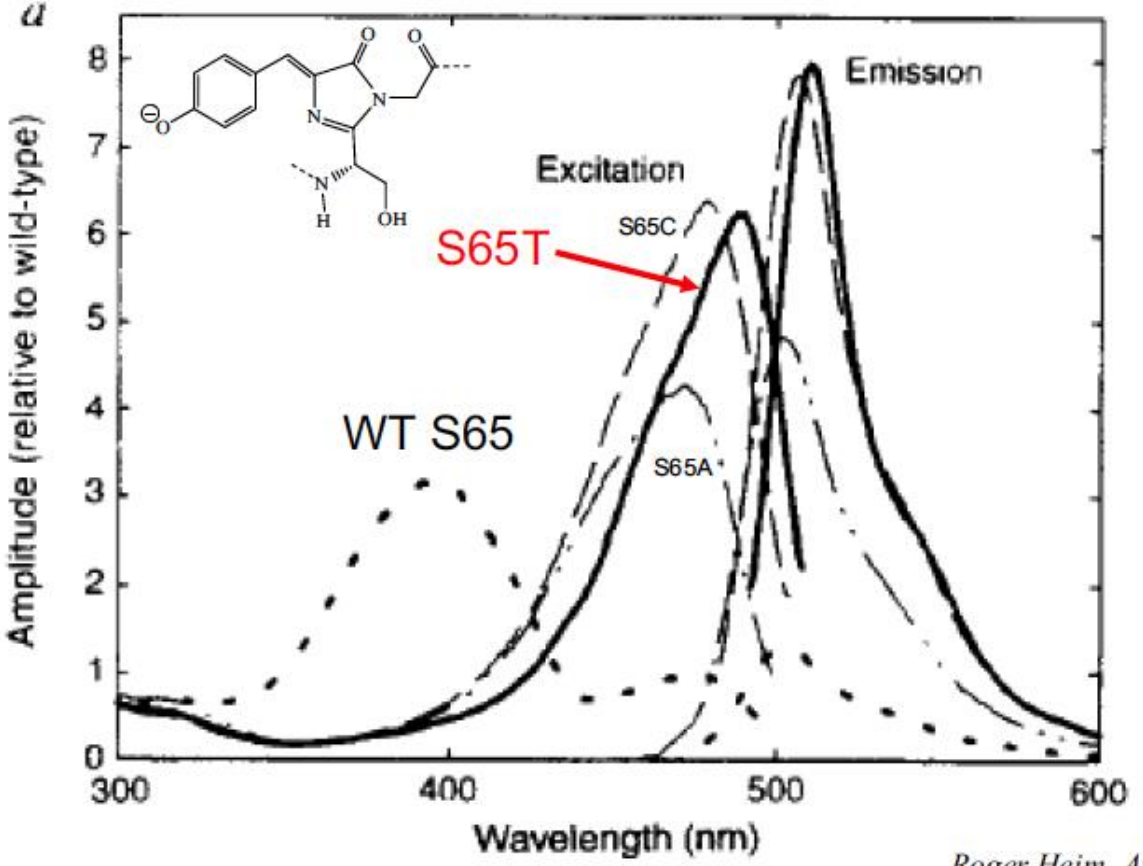
Mutations of Ser65 improve excitation spectra



Roger Heim, Andrew Cubitt

Roger Y. Tsien - Facts

Photo: U. Montan

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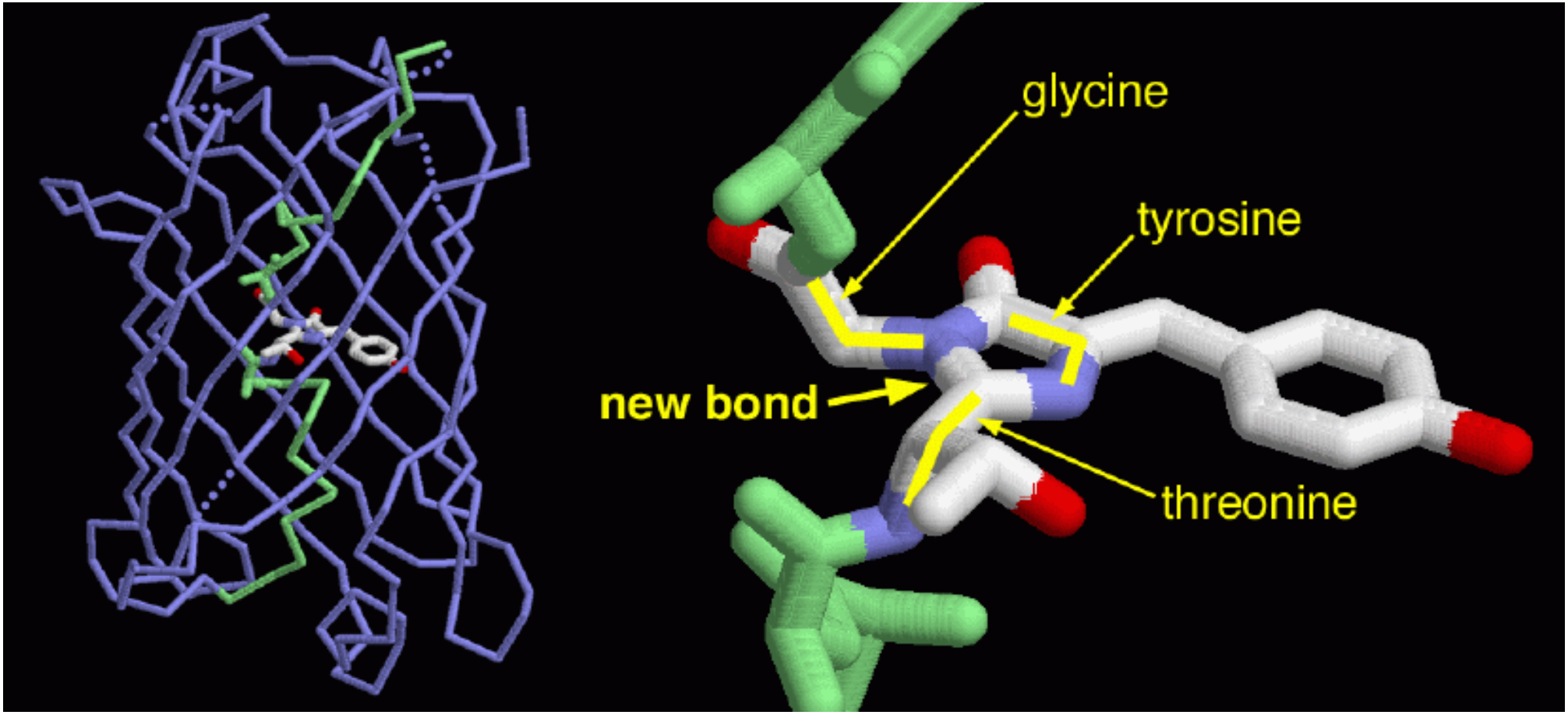
Il Cromoforo $\underline{\text{Thr}_{65}}$-$\text{Tyr}_{66}$-$\text{Gly}_{67}$:EGFP



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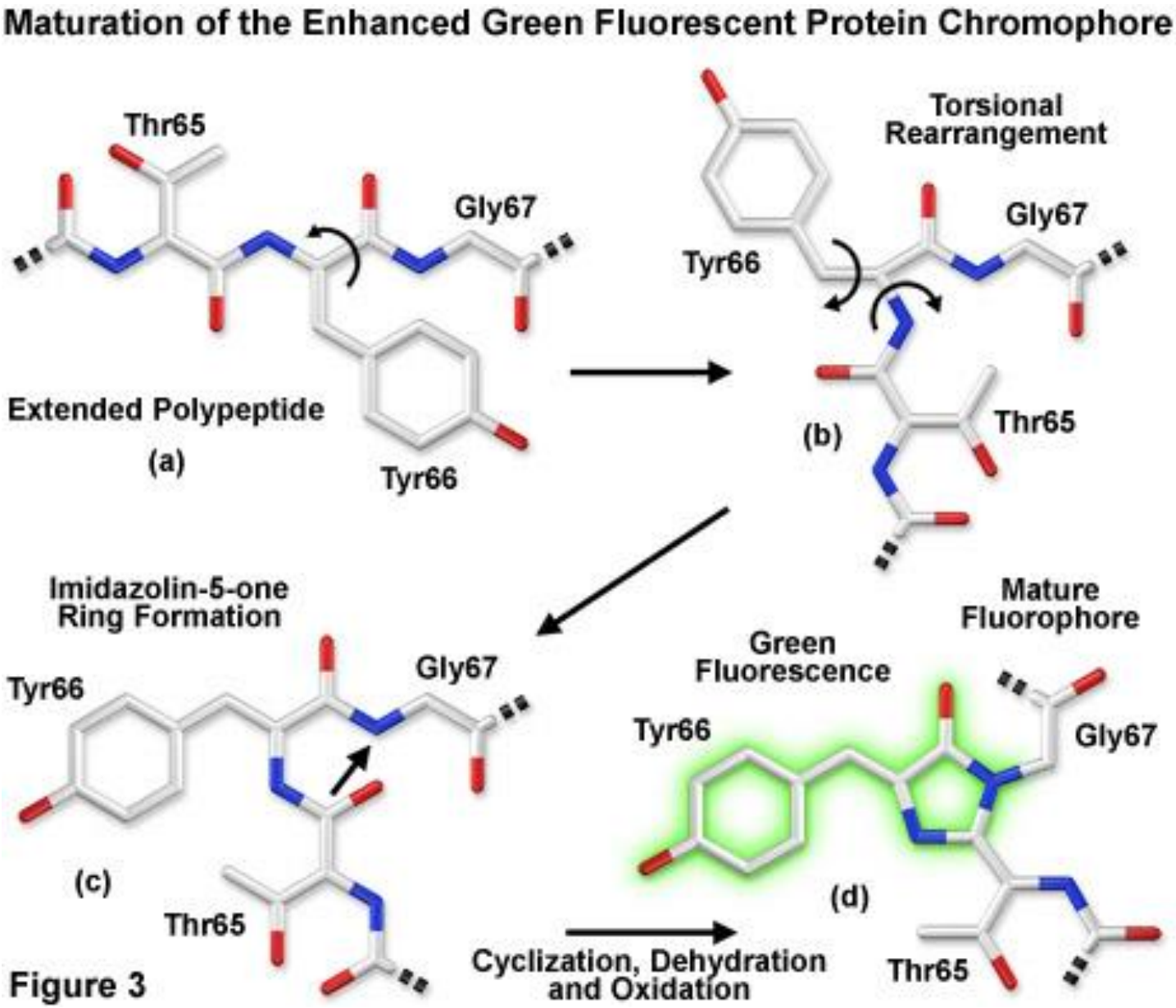
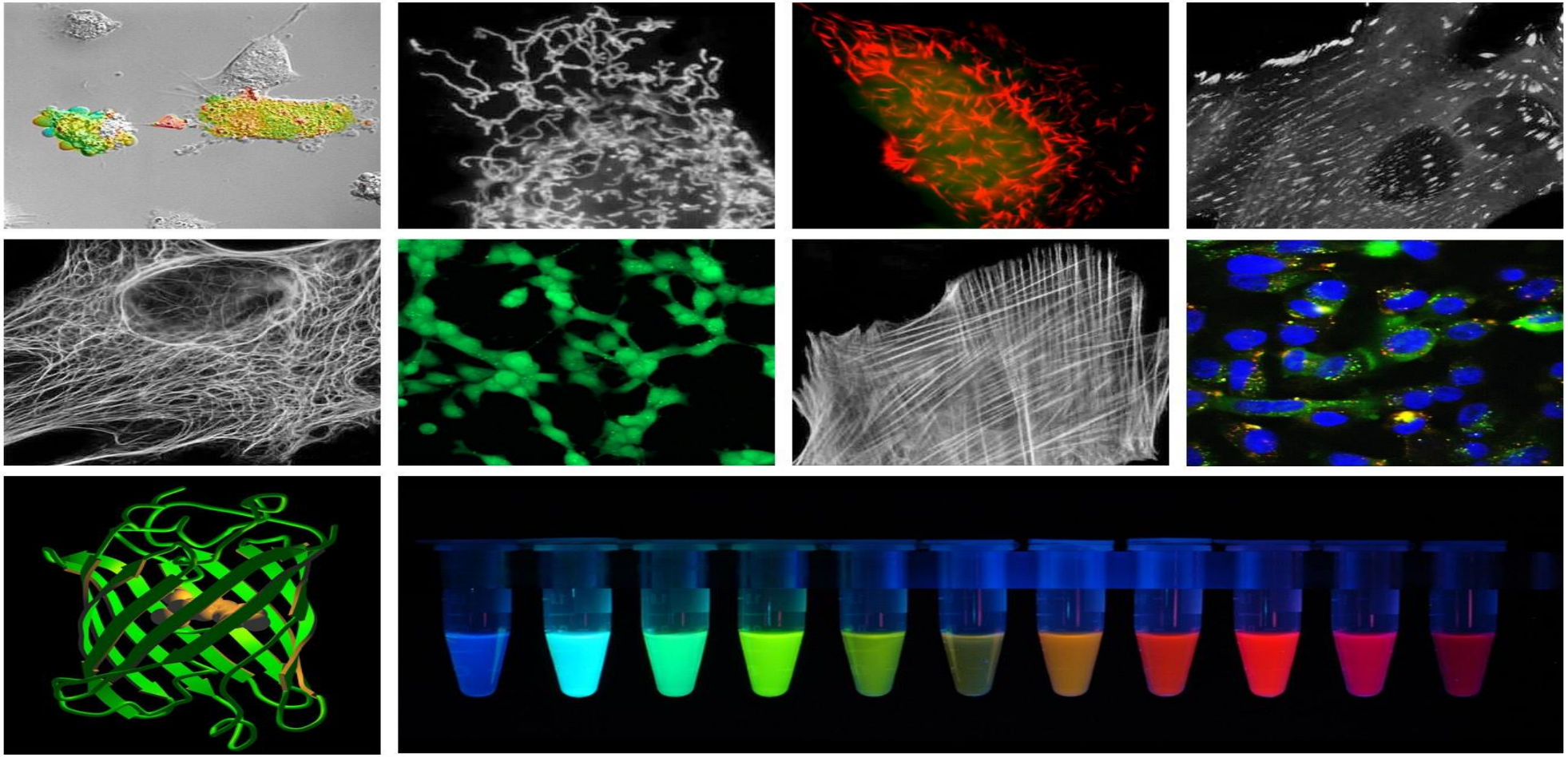


Enhanced Green, Cyan and Yellow Fluorescent Proteins

Roger Tsien mise a punto nuove tecniche, produsse molte proteine mutanti, che emettono fluorescenza più **rapidamente**, con **maggiore intensità** e anche di **diversi colori**



…di tutti i colori

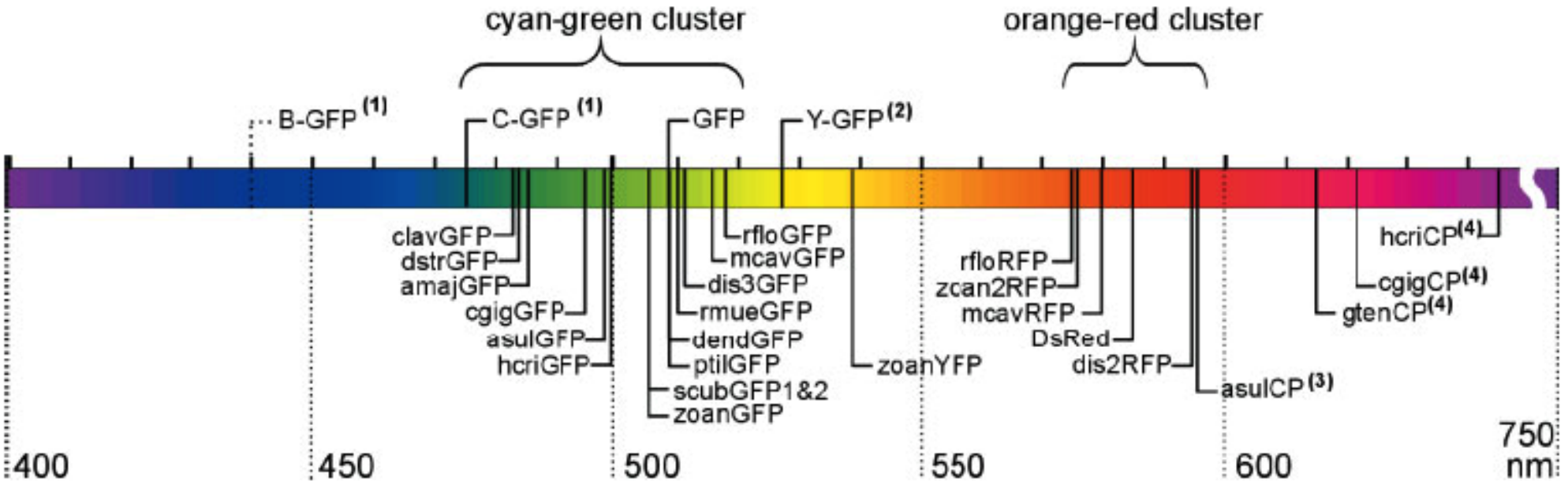
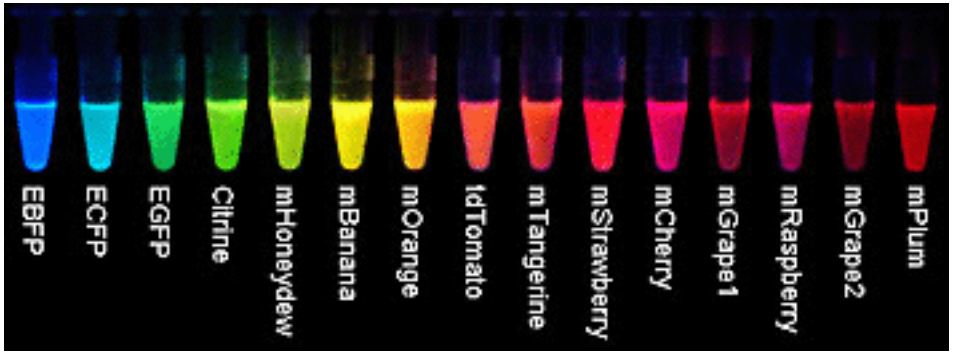


Figure 5. Spectral properties of variants of the GFP family (Matz et al., 2002).



8 October 2008

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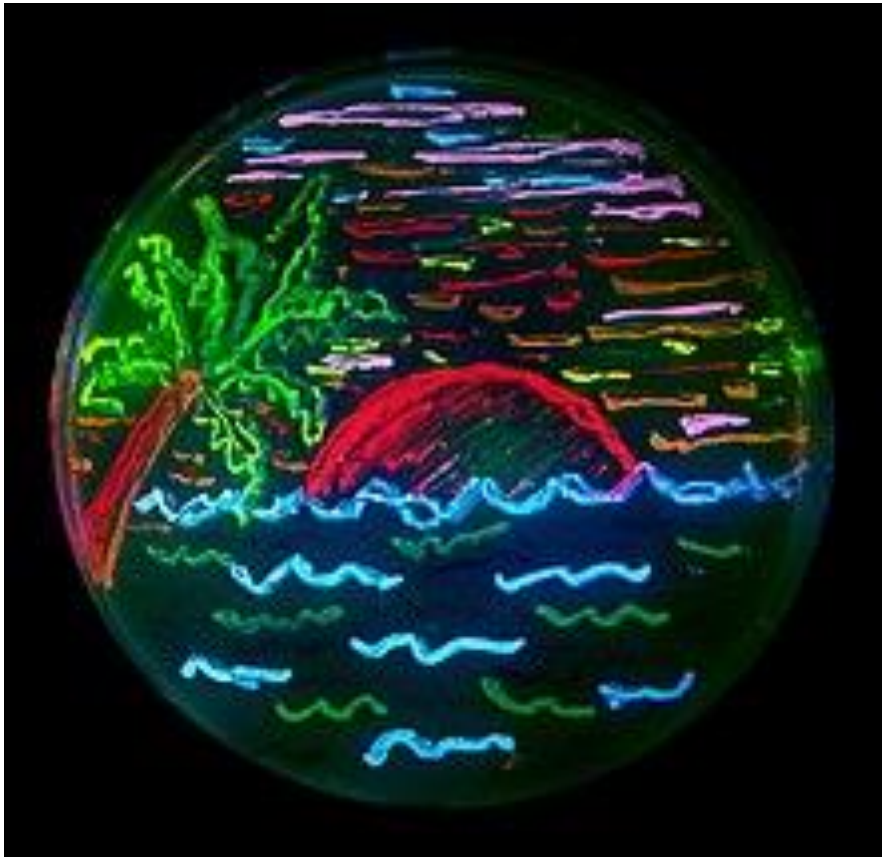

The Nobel Prize in Chemistry 2008
Osamu Shimomura, Martin Chalfie, Roger Y. Tsien

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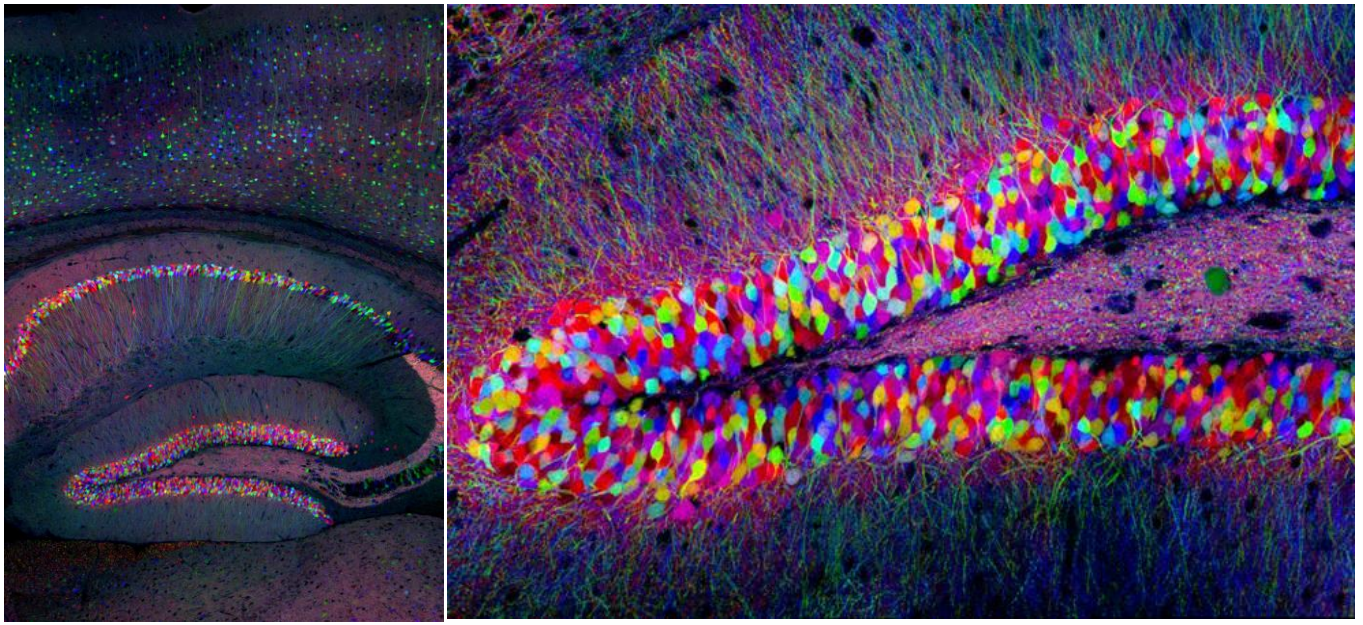
https://www.youtube.com/watch?v=qK9aYnkIr3w

“Brainbow”



Researchers at Harvard University in the USA have coloured the nerve cells in a mouse's brain so that it fluoresces in all the colours of the rainbow. The nerve cells produce different amounts of three GFP-like proteins that fluoresce yellow, cyan and red, mimicking the colours used in a printer. This enables researchers to see how individual nerve cells in the brain are woven together in a network. Photo: Livet et al (2007) Nature 450 56-63.

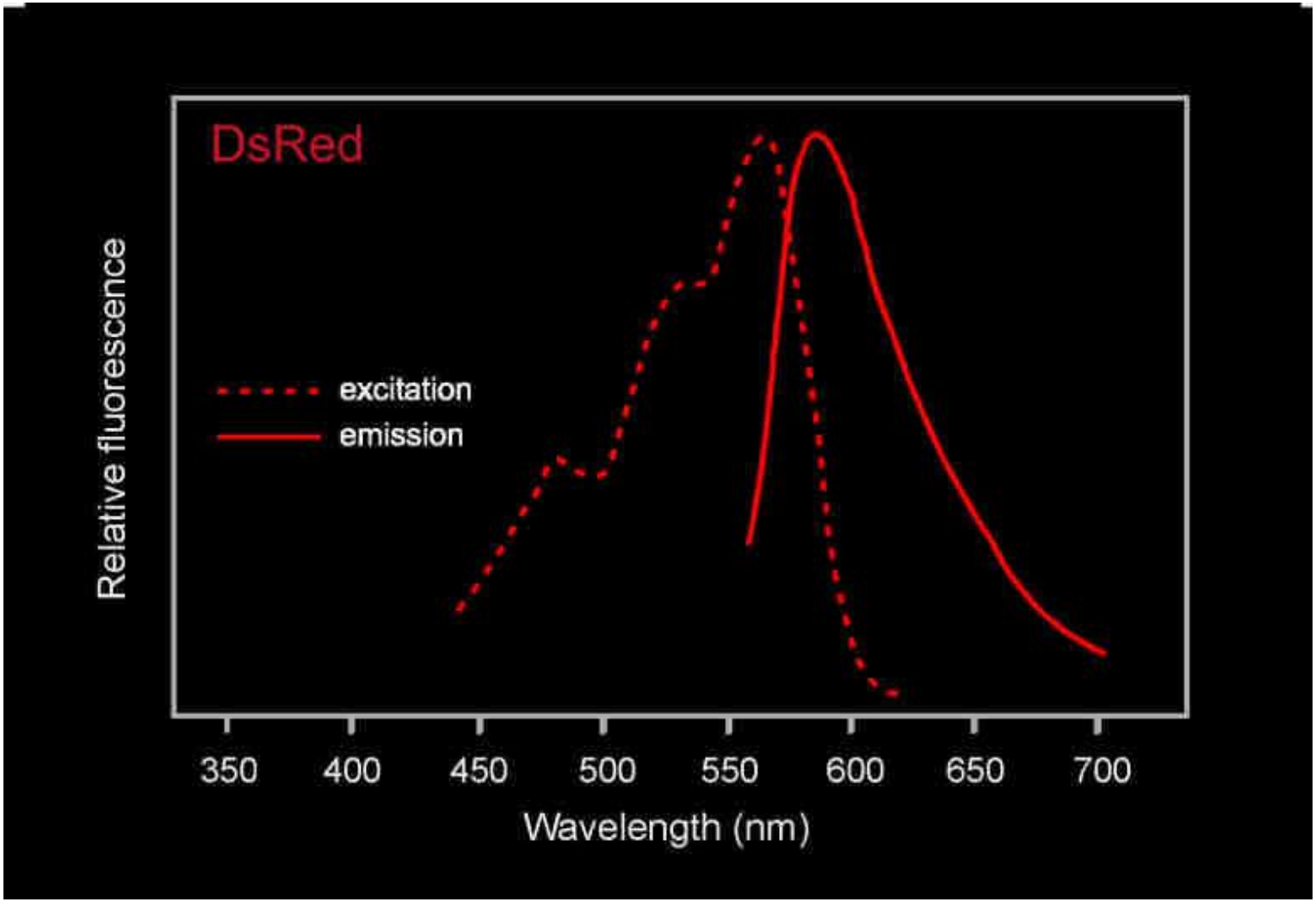
…e con l’aiuto dei coralli (Discosoma)

…e con l’aiuto dei coralli (Discosoma)

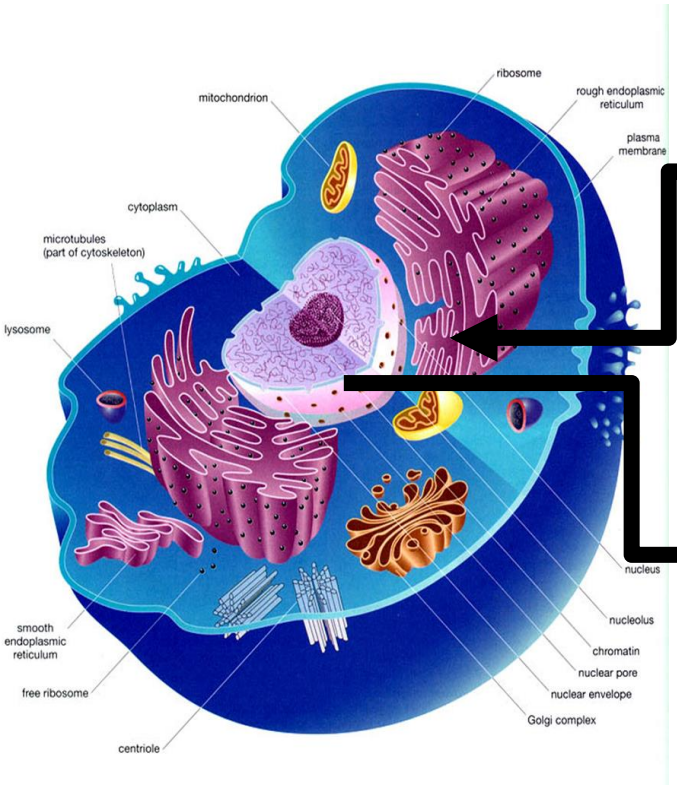
COME ELIMINARE I DISCOSOMA DAL PROPRIO ACQUARIO

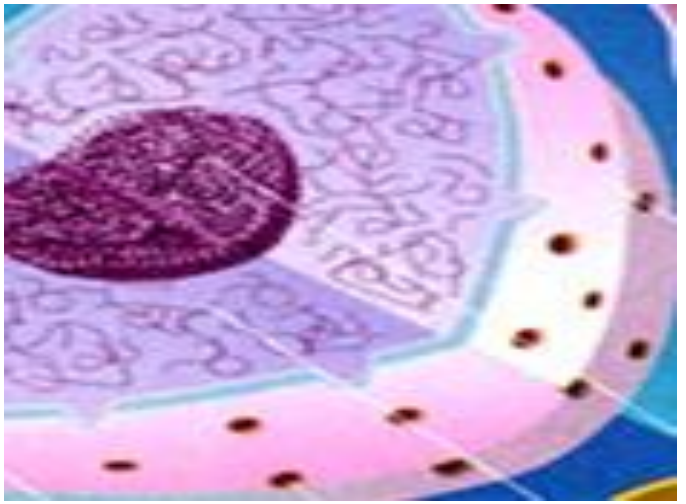
I **Discosoma** (o **Actinodiscus**), conosciuti anche come **Coralli Fungo**, in acquario possono essere tremendamente infestanti, e la cosa è dannatamente fastidiosa se il vostro acquario marino è dedicato ai coralli duri a polipo piccolo.

…e con l'aiuto dei coralli (Discosoma)



2) Uso della proteina GFP per lo studio del traffico e delle connessioni cellulari





Attraverso semplici tecniche di Biologia Molecolare si possono generare Proteine di Fusione

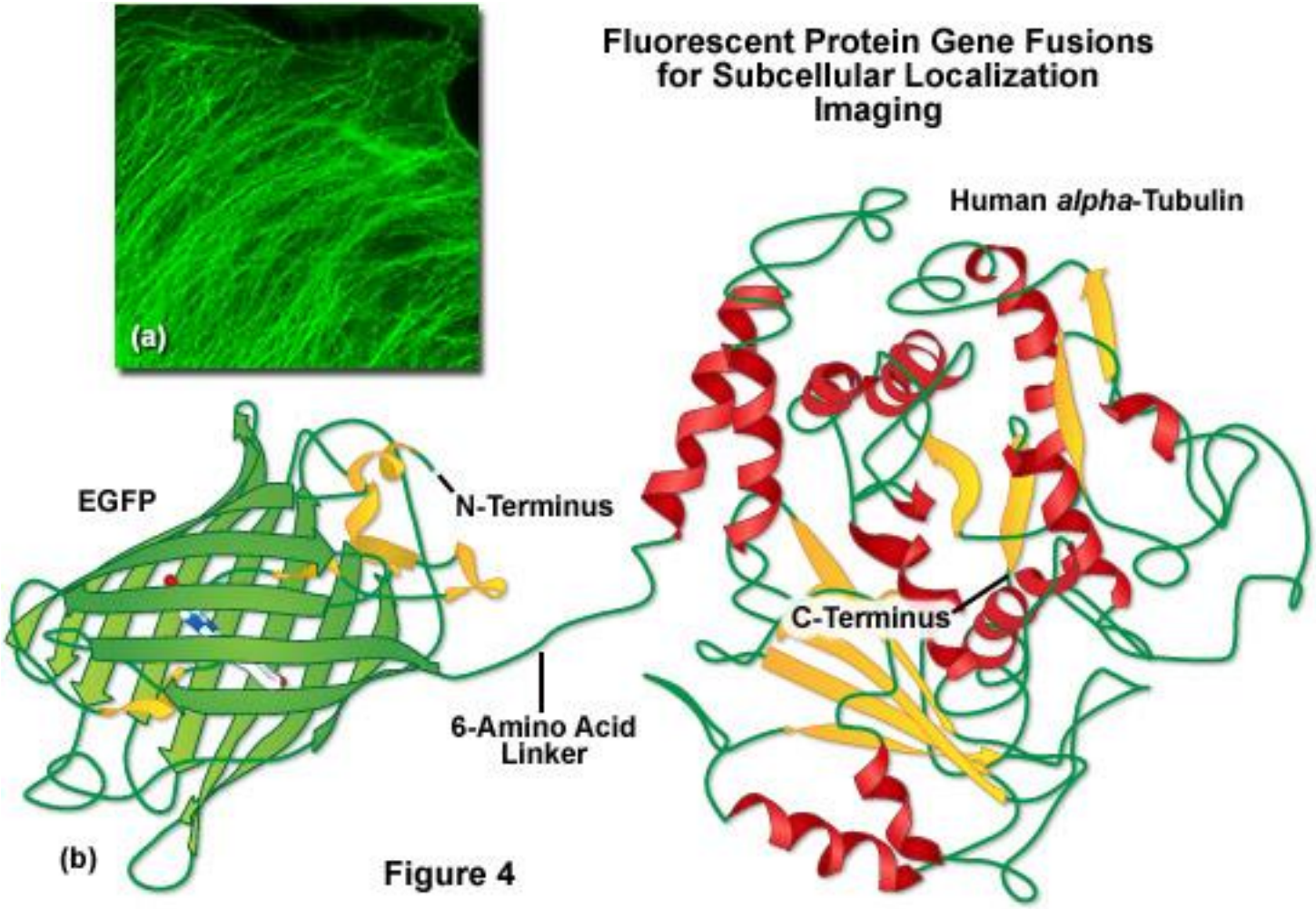
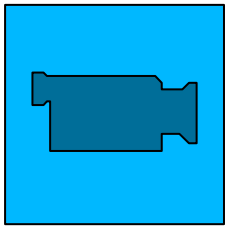


Figure 4

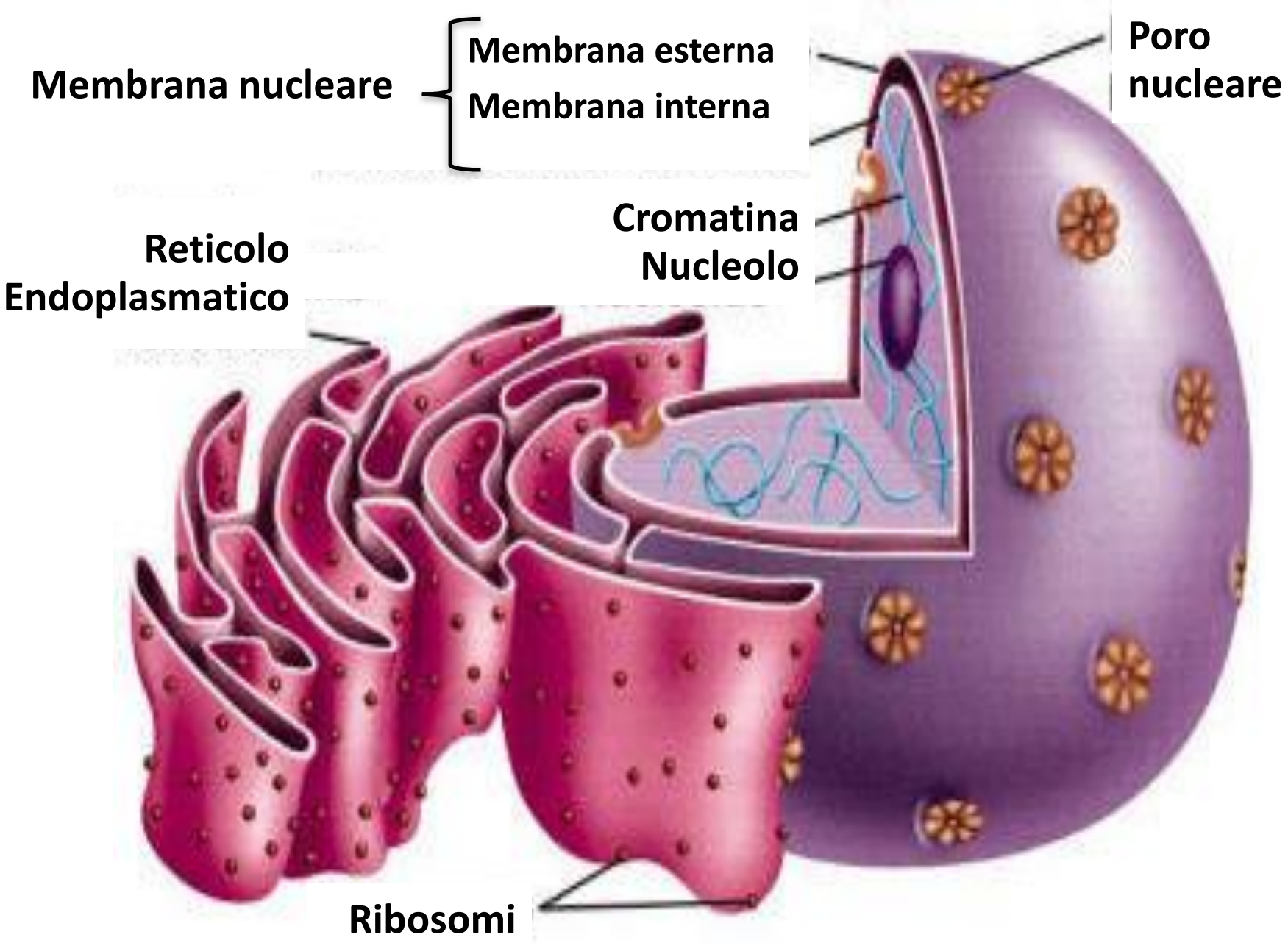
Studiare la localizzazione di proteine e il loro traffico «live»

https://www.youtube.com/watch?v=zyGVC2-WBcA

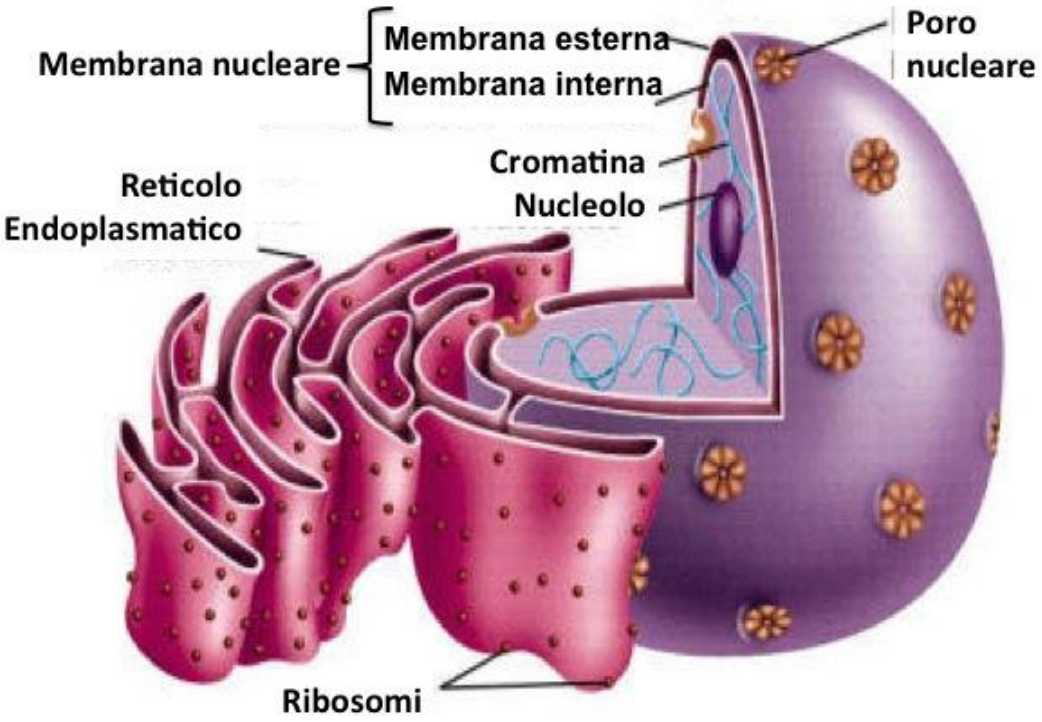


- **Esistono segnali di trasporto di proteine nel nucleo?**
- **Come sono fatti questi segnali?**
- **Come si dimostra l'esistenza di questi segnali?**

Struttura del nucleo



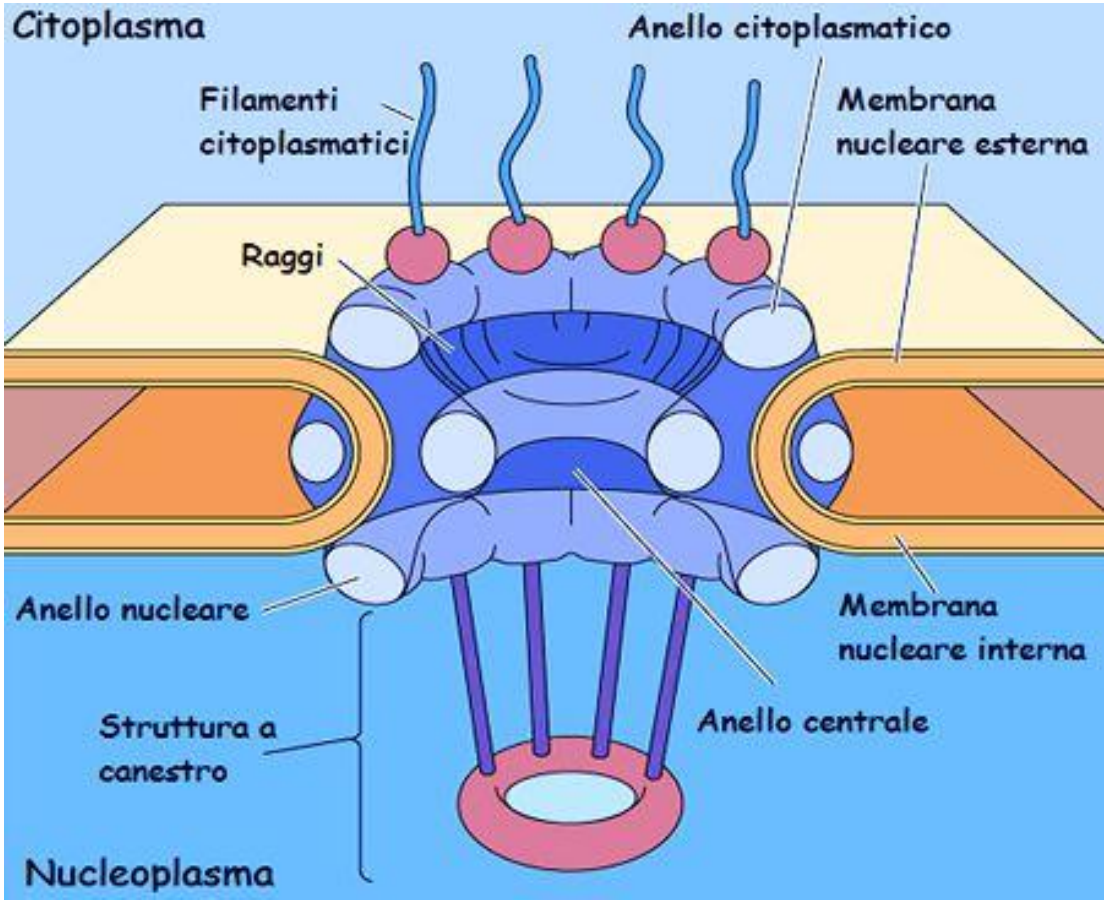
Struttura del nucleo

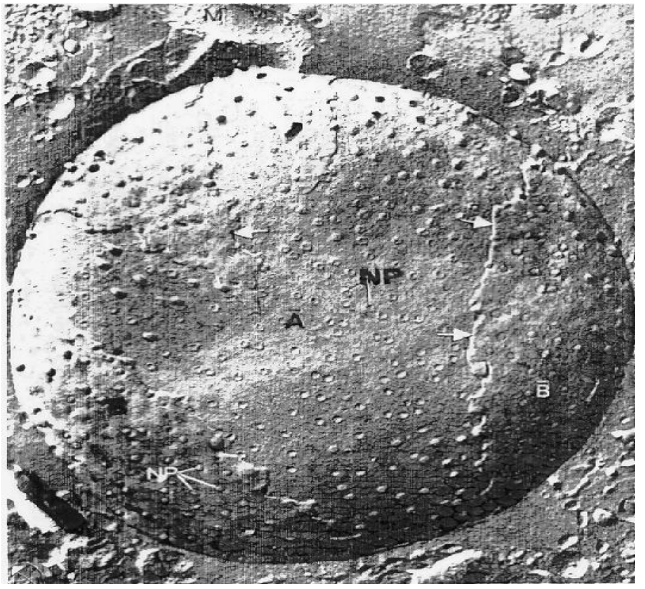


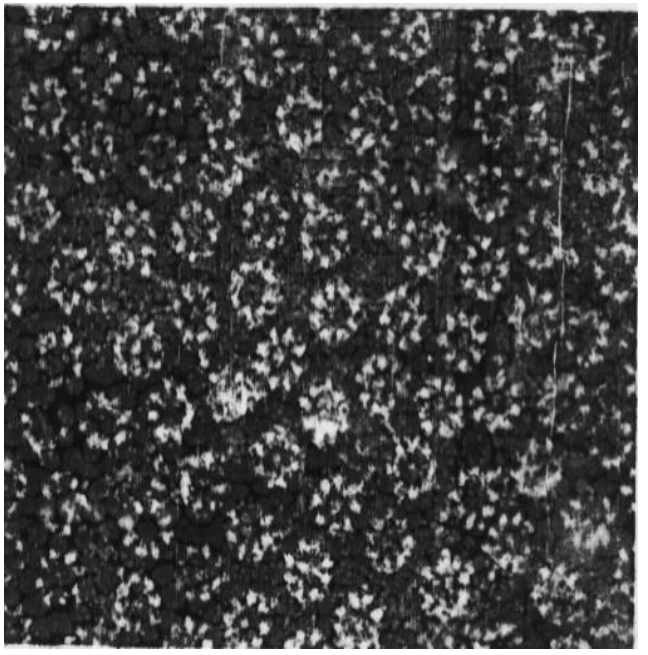
- All'interno dell'involucro nucleare vi è la **lamina nucleare**, una rete di filamenti con **funzione di supporto**
- Il nucleo è perforato da grosse strutture note come «**i complessi dei pori nucleari»**
- Costituiti da **più di 50** proteine diverse chiamate **nucleoporine** con simmetria ottagonale

I PORI NUCLEARI NON SONO DEI SEMPLICI BUCHI!

Struttura del poro nucleare

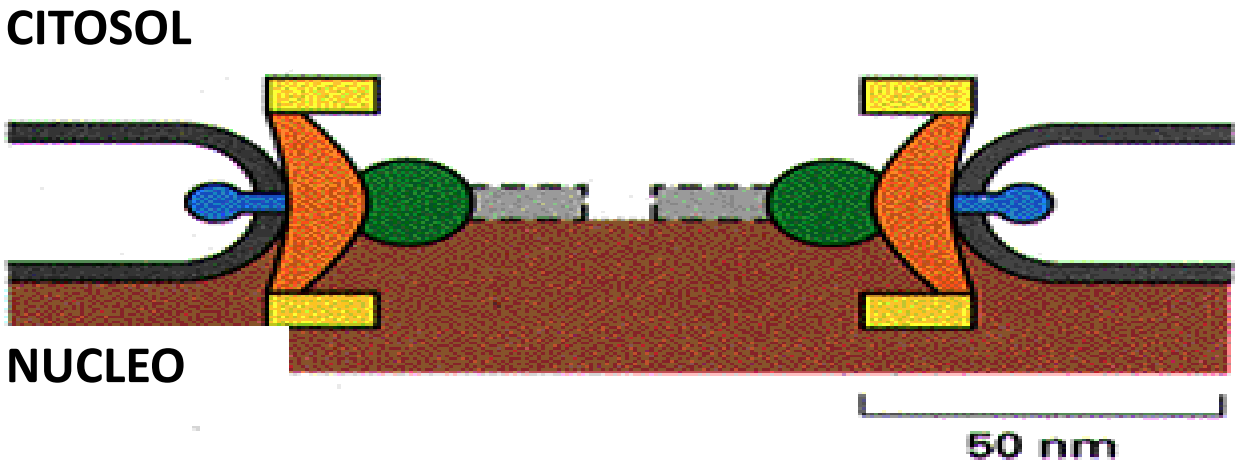






- Consentono il **"*passaggio selettivo di molecole*"**
- Possono essere **numerosi con struttura ordinata** ottagonale
- Ai pori sono associate strutture ad **entrambi i lati**

Alcune piccole molecole passano liberamente attraverso i pori



Dimensioni delle proteine che entrano nel nucleo per diffusione libera

< 9nm SI

Dimensioni delle proteine che entrano nel nucleo tramite **trasportatori**

> 9nm NO

Molte molecole entrano ed escono dal nucleo

COSA ESCE

rRNA, tRNA, mRNA, ribosomi, proteine

COSA ENTRA

Proteine: polimerasi, istoni, fattori di trascrizione,
Proteine ribosomiali, etc.
Nucleotidi, etc.

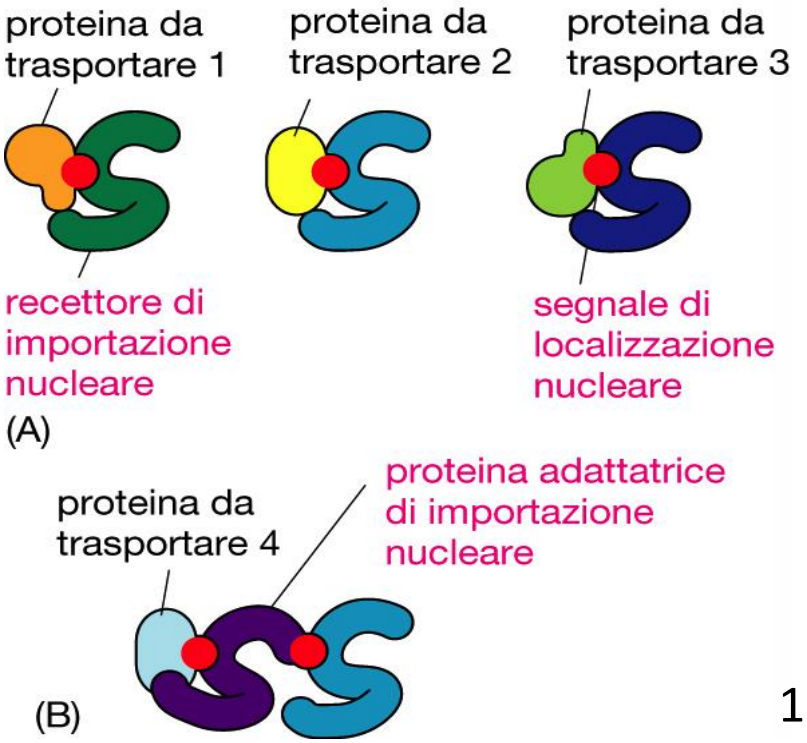
- **Esistono segnali di trasporto di proteine nel nucleo?**
- **Come sono fatti questi segnali?**
- **Come si dimostra l'esistenza di questi segnali?**

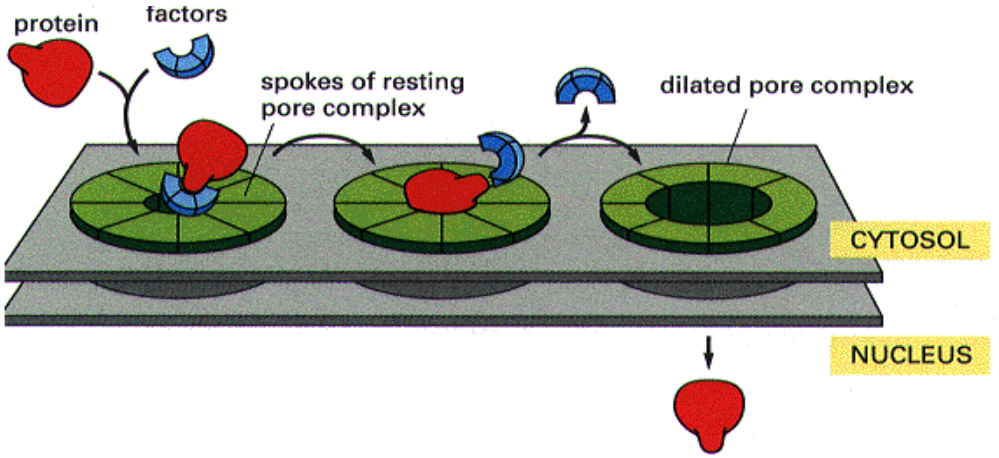
Le sequenze NLS

- **N**uclear **L**ocalization **S**ignals
- Sequenze ricche di a.a. basici **PKKKRKV**
- All'interno della proteina
- Frequentemente bipartite

PROTEINA	**SEQUENZA**
ANTIGENE T (SV40)	Pro-Lys-Lys-Lys-Arg-Lys-Val
PROTEINA 72kDa (adenovirus)	Pro-Lys-Lys-Lys-Lys-Lys-Arg
VP2/3 (polioma)	Gln-Lys-Lys-Lys-Arg-Lys-Leu
70kDa (drosophila)	Lys-Arg-Gly-Lys-Arg-Lys-Lys
c-myc (pollo)	Glu-Gln-Lys-Arg-Arg-Arg-Arg

Le sequenze sono riconosciute da recettori specifici





1. Proteine solubili (importine) **riconoscono** le sequenze
2. **Il complesso Importina- proteina** nucleare si lega alle proteine del Poro (**NUP**) (RIPETIZIONI FG)
3. Il **Poro si dilata** ed il complesso lo attraversa
4. **L'importina si dissocia** dalla proteina nucleare
5. L'importina **torna nel citosol**

Esempi:
- **Importine α e β,**
- **TNPO1**

Come si dimostra l'esistenza di questi segnali?

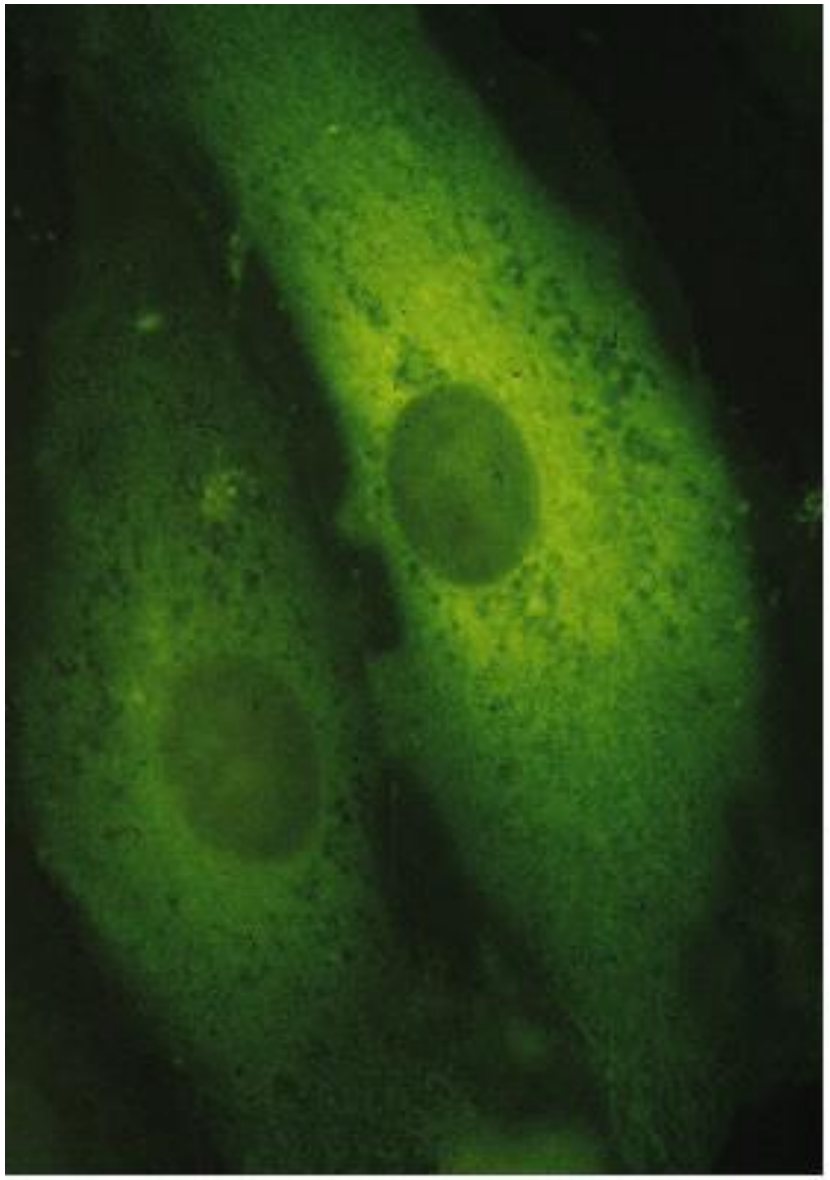
- 1. RIMOZIONE DELLA SEQUENZA
- 2. MUTAZIONE DELLA SEQUENZA
- 3. TRASFERIMENTO DELLA SEQUENZA SU UN'ALTRA PROTEINA

Come si dimostra l'esistenza di questi segnali?

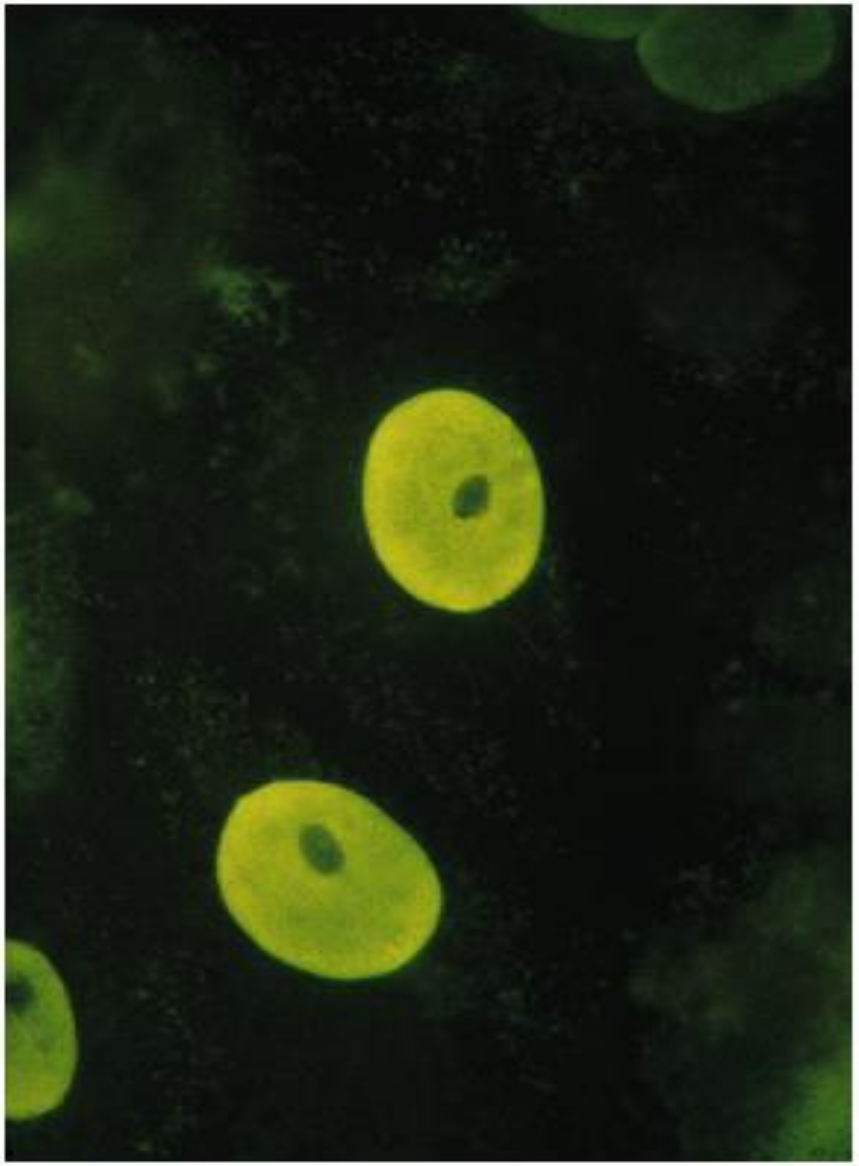
- 1. RIMOZIONE DELLA SEQUENZA
- 2. MUTAZIONE DELLA SEQUENZA
- 3. TRASFERIMENTO DELLA SEQUENZA NLS SU UN'ALTRA PROTEINA

EGFP!

LA PROTEINA REPORTER PRIVA DI NLS NON SI LOCALIZZA NEL NUCLEO



LA PROTEINA REPORTER CON NLS SI LOCALIZZA NEL NUCLEO



Clonaggio di un segnale di importo nucleare (NLS) in vettore pEGFP

- Reazione di Annealing
- Digestione enzimatica del vettore pEGFP

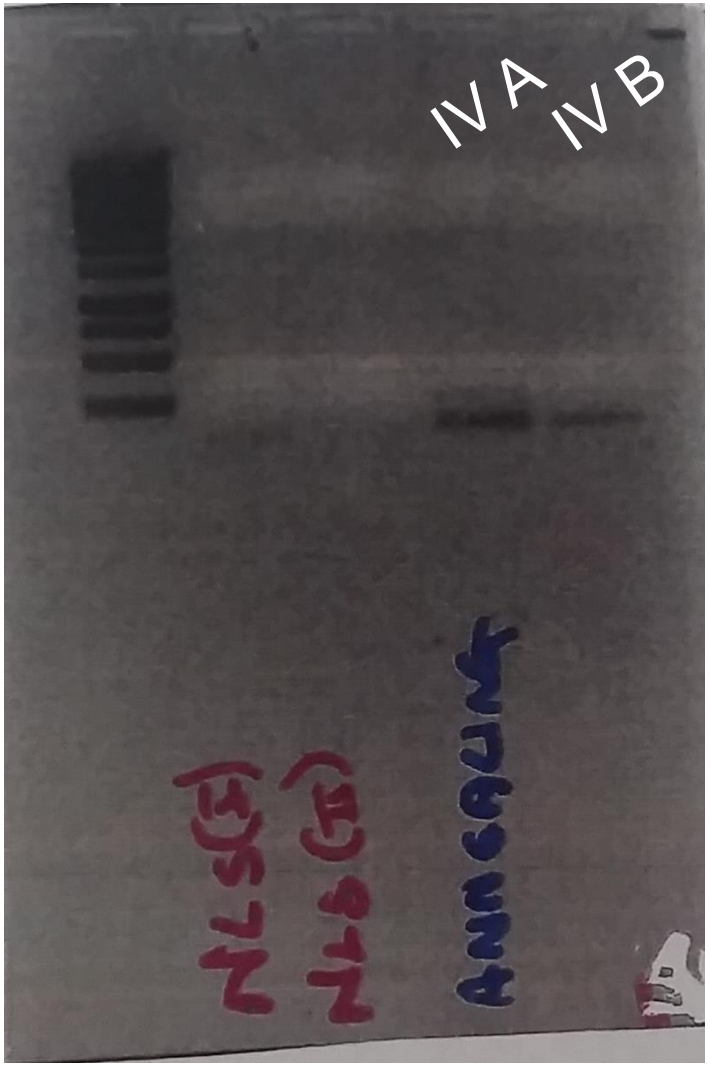
Preparazione e verifica dei plasmidi

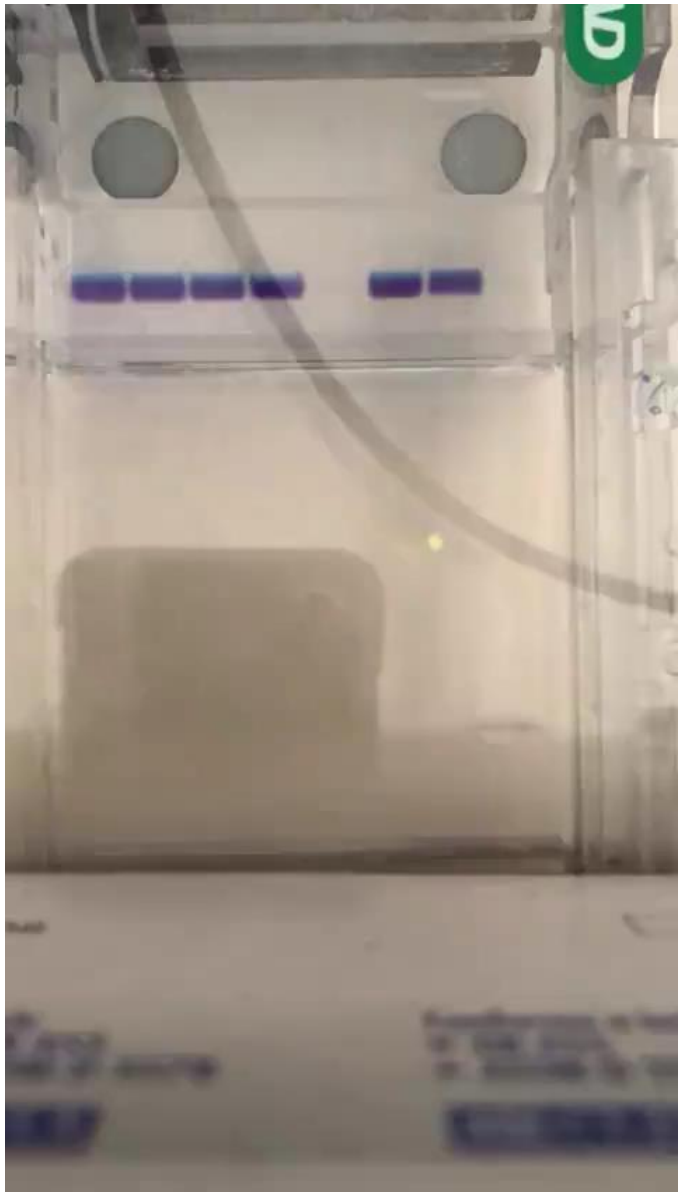
- Purificazione del vettore mediante elettroeluizine
- Reazione di Ligasi
- Trasformazione batterica e piastratura

Trasfezione di NLS-pEGFP in cellule HEK293

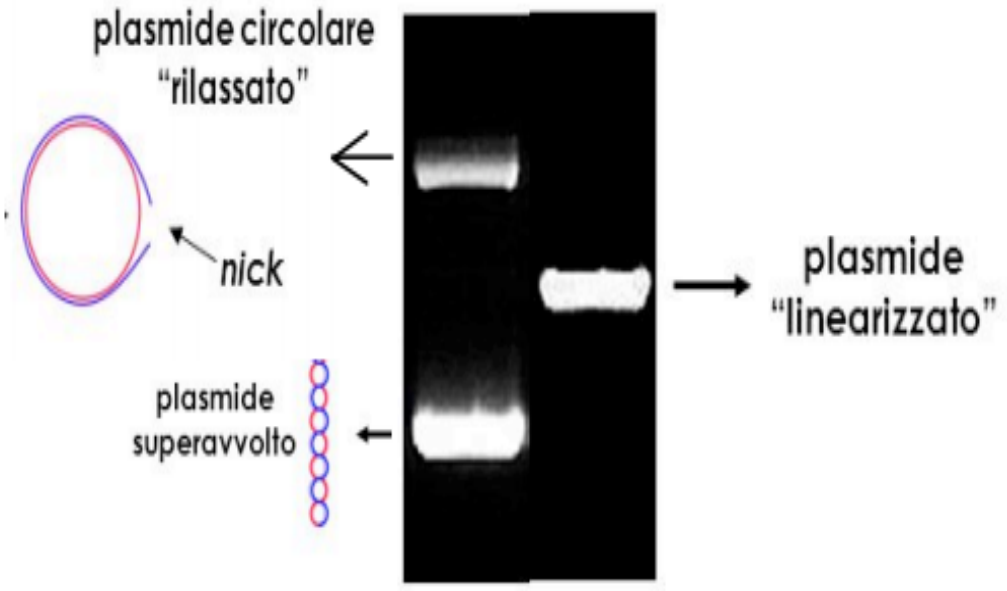
- Miniprep
- Verifica della **localizzazione** di NLS-pEGFP mediante IF

Reazione di Annealing



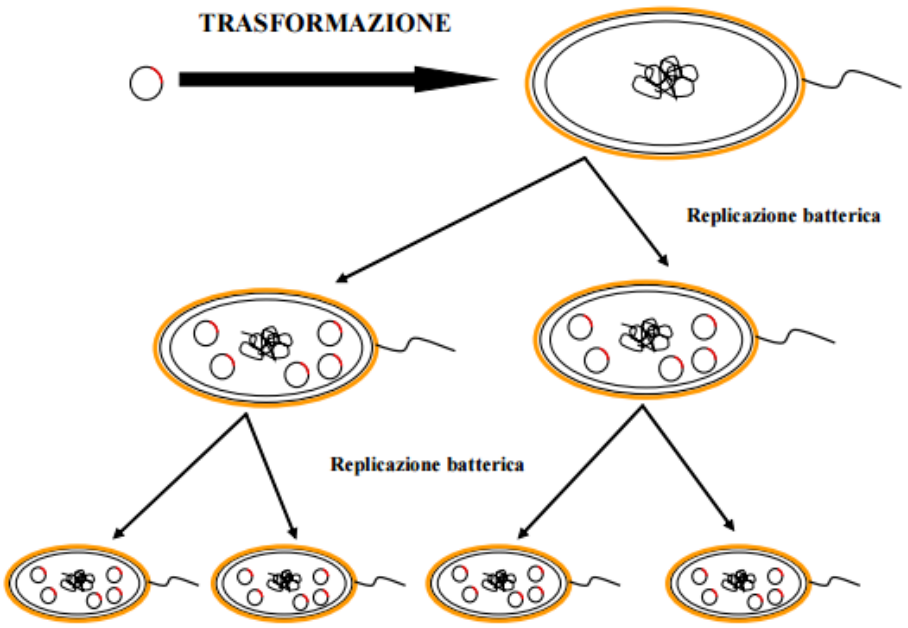


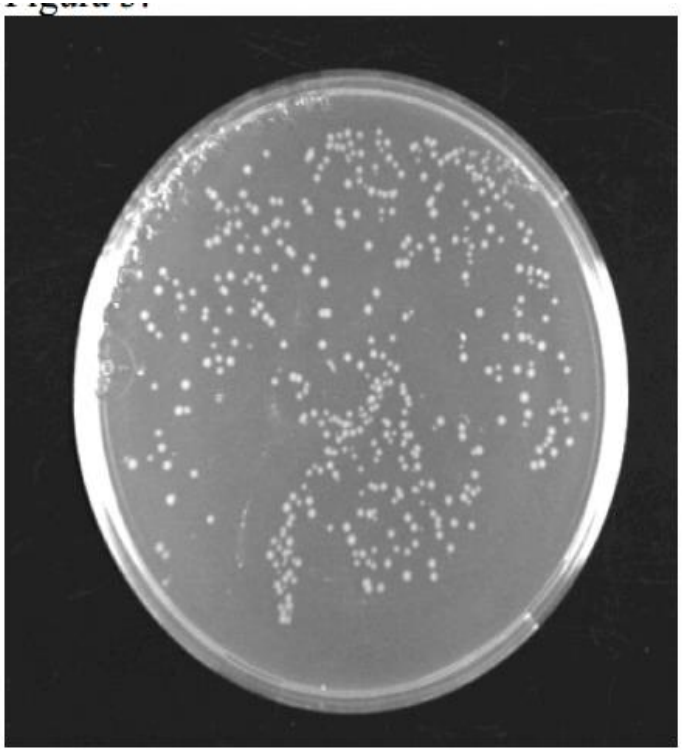
Digestione del vettore pEGFP



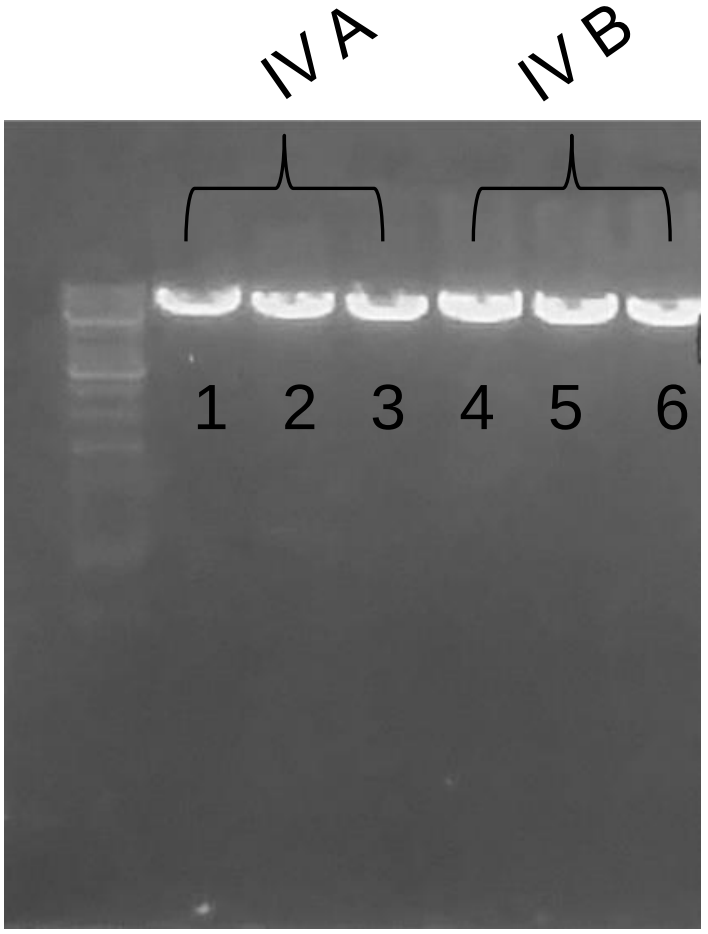
1: Vettore pEGFP NON digerito
2: Vettore pEGFP digerito

TRASFORMAZIONE BATTERICA



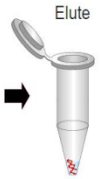


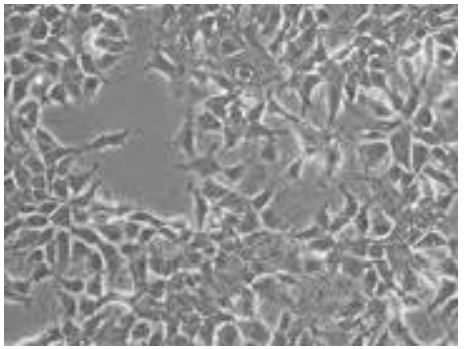
Miniprep



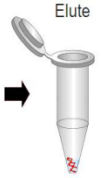
Trasfezione in cellule Hek293

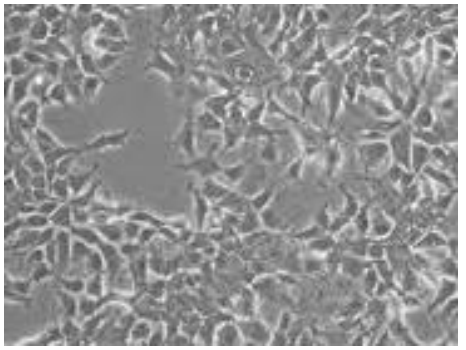
1) pEGFP



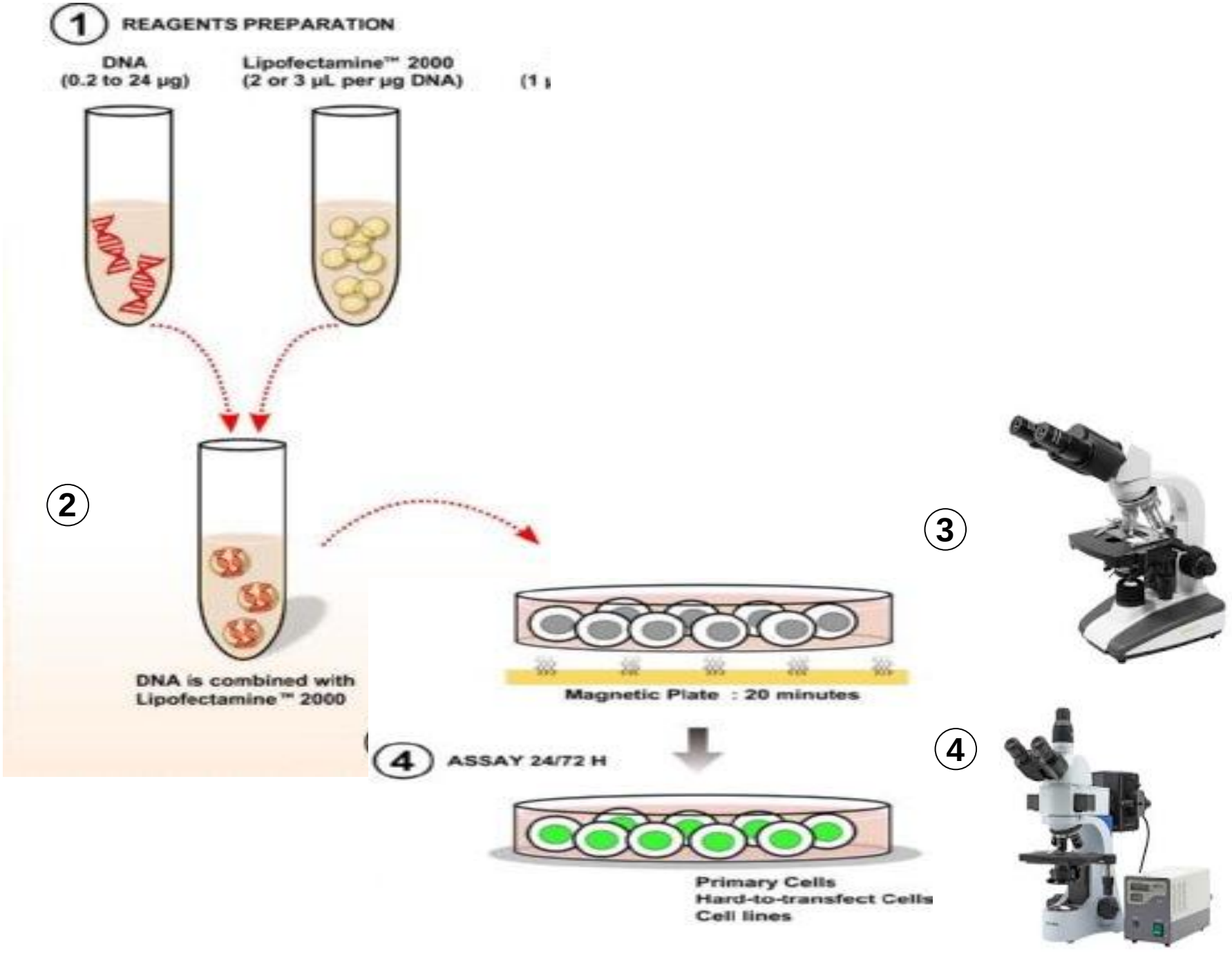


2) NLS-GFP



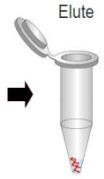


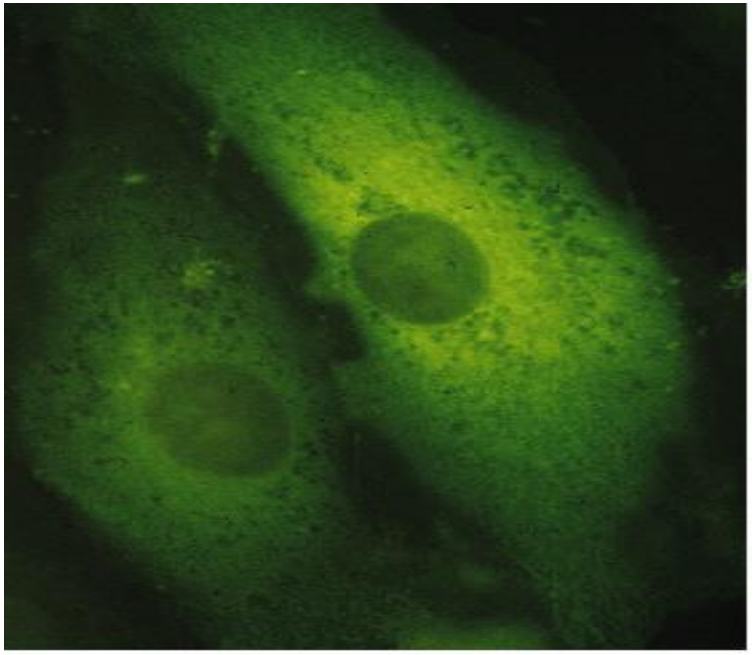
Trasfezione



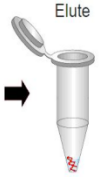
Risultati trasfezione in cellule Hek293 dopo 48h

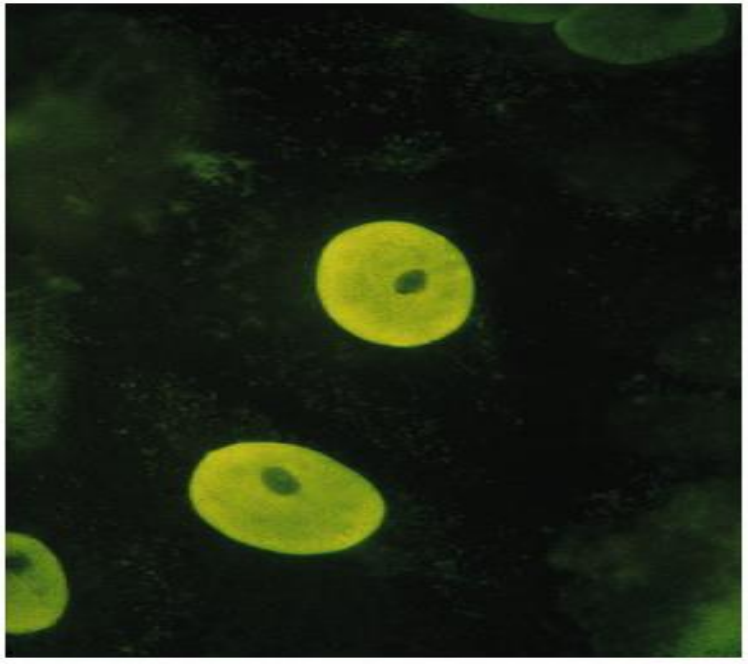
1) pEGFP





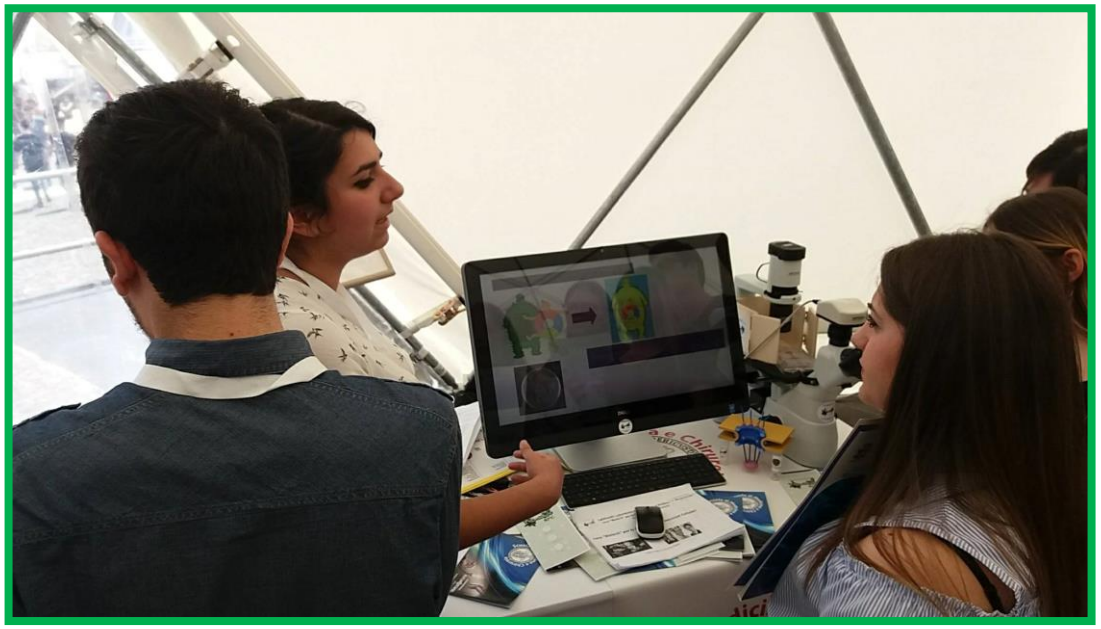
2) NLS-GFP



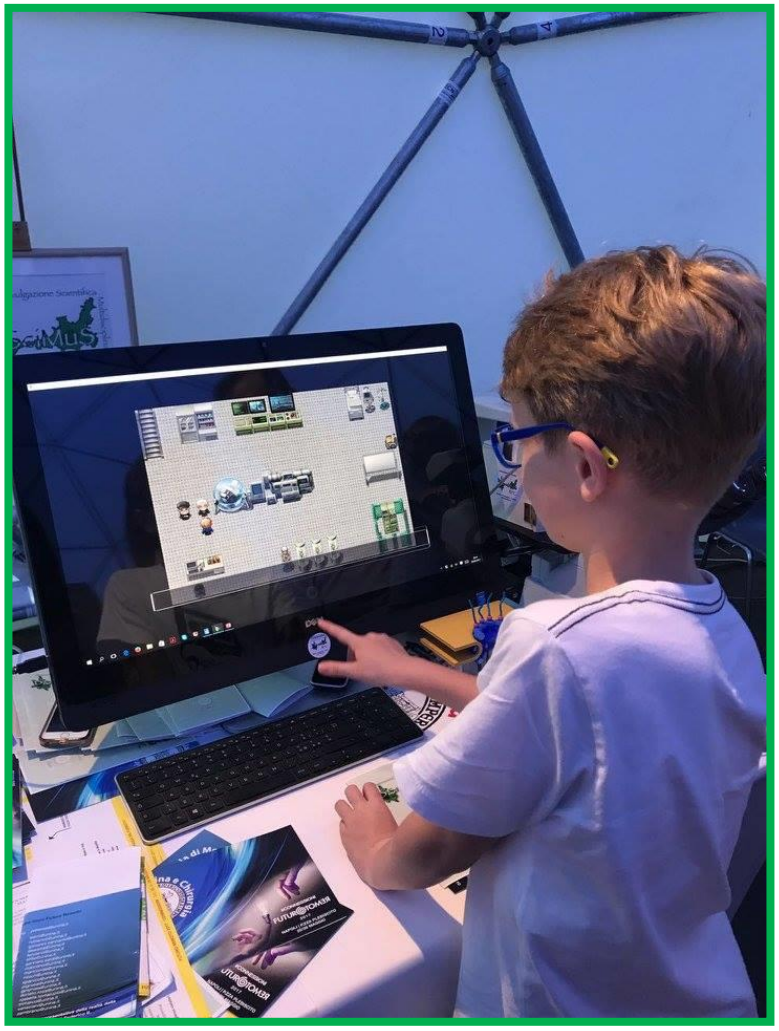


Esperimento effettuato per verificare la localizzazione cellulare di una sequenza NLS legata a GFP

La fase dimostrativa «attiva»



La fase dimostrativa «attiva»



La fase dimostrativa «attiva»

Il nostro progetto di laboratorio in Alternanza Scuola Lavoro:
"Uso della proteina GFP per lo studio delle connessioni cellulari"

Una cellula eucariotica è suddivisa in numerosi compartimenti funzionalmente distinti, racchiusi da membrane. Tra questi, l'involucro nucleare racchiude il DNA e definisce il compartimento nucleare. L'involucro nucleare consiste di due membrane concentriche, interna ed esterna separate da una spazio perinucleare e penetrate da complessi proteici detti "**pori nucleari**". Questi complessi sono canali acquosi che consentono un traffico bidirezionale tra il citosol e il nucleo. La selettività di questi processi di importazione ed esportazione richiede la presenza di specifici segnali di localizzazione che sono presenti sulle proteine: un segnale di localizzazione nucleare (**NLS**) e/o un segnale di esportazione nucleare (**NES**).
Nella nostra attività di laboratorio legata al percorso di **Alternanza Scuola Lavoro "LabBiomEt - Laboratorio di Biomedicina e BioEtica"**, applicando il metodo scientifico, abbiamo valutato la funzionalità di una putativa sequenza NLS attraverso il clonaggio in un vettore esprimente la proteina EGFP utilizzata come *reporter*.

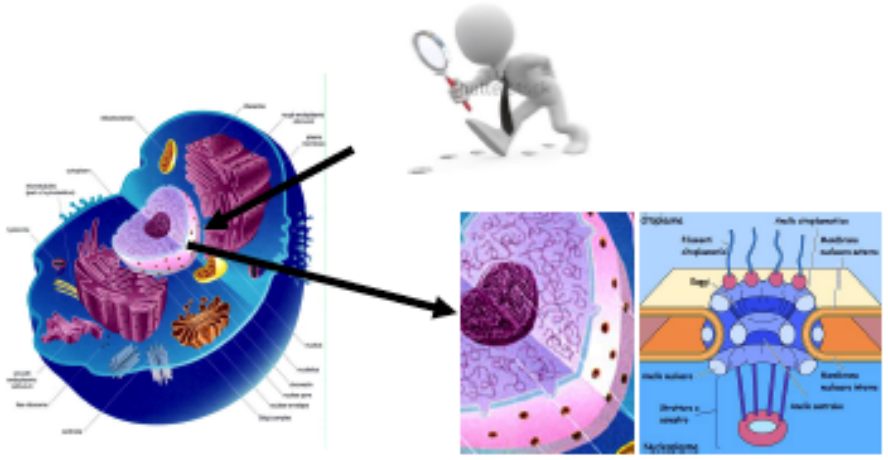


Fig. 5 Il nucleo e il complesso del poro

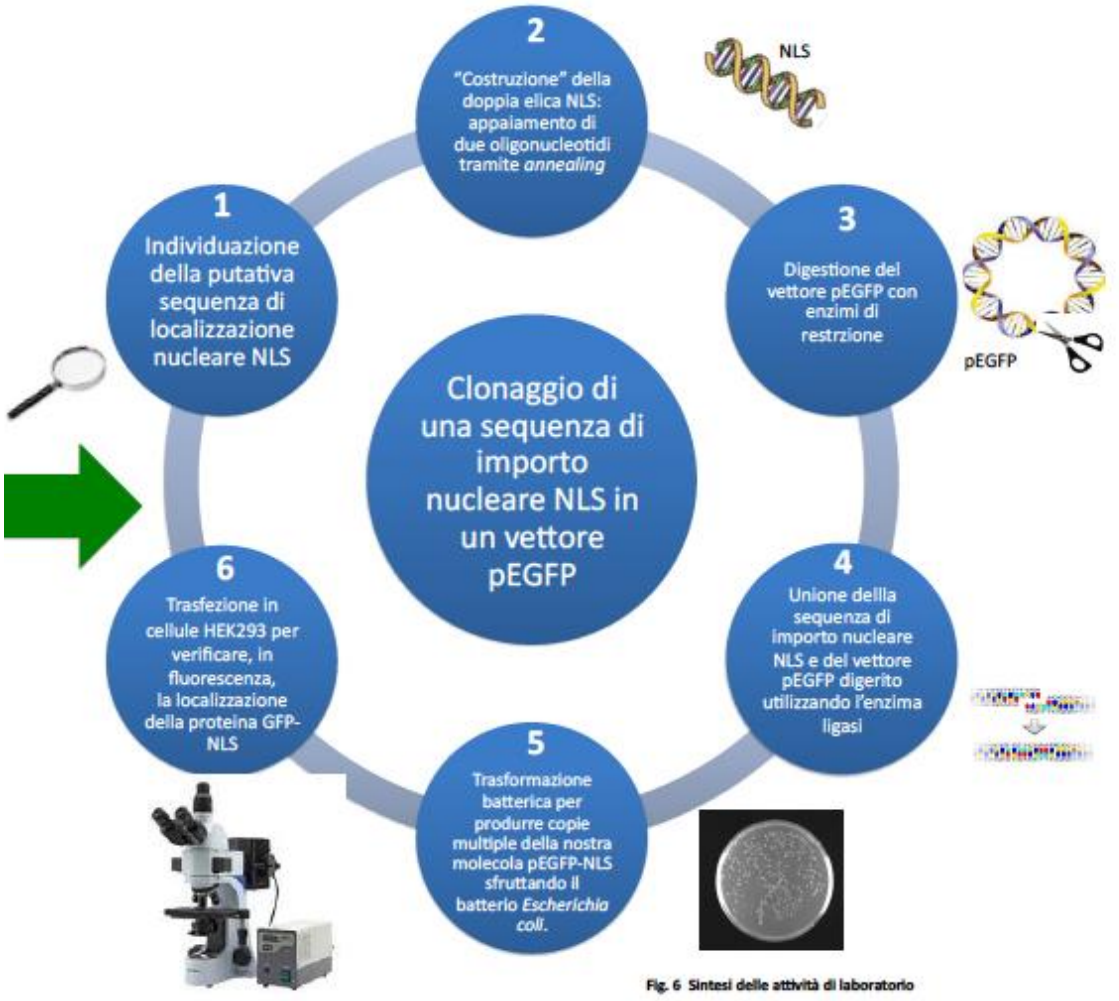


Fig. 6 Sintesi delle attività di laboratorio

La fase dimostrativa «attiva»

Conclusioni:

- Una volta prodotta la molecola di DNA ricombinante che contiene la sequenza nucleotidica NLS unita alla sequenza di EGFP, lo step successivo è stato quello di esprimere tale proteina EGFP+NLS in cellule eucariotiche HEK293 e, sfruttando le proprietà fluorescenti della proteina EGFP, ne abbiamo analizzato il compartimento di localizzazione attraverso un microscopio a fluorescenza.

- Come possiamo notare dalla Figura 5 la proteina EGFP-NLS si localizza perfettamente nel nucleo della cellula eucariotica se confrontata con un vettore contente solo EGFP senza sequenza NLS.

- Quindi dagli esperimenti eseguiti durante le nostre attività di laboratorio possiamo affermare che la proteina GFP non solo emette fluorescenza ma è stato un utilissimo strumento per lo studio delle connessioni cellulari "citosol-nucleo"

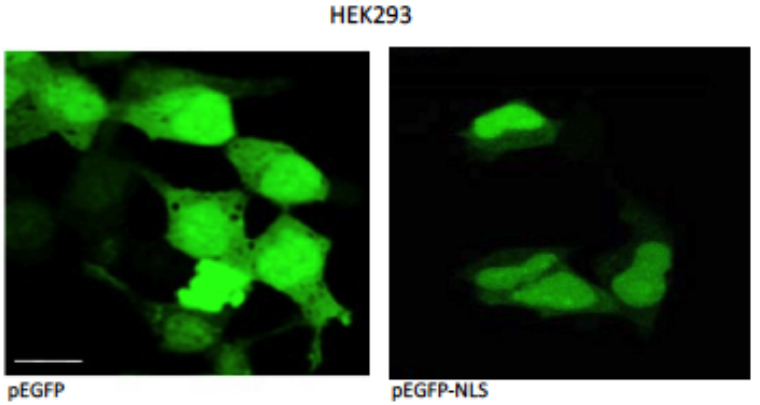
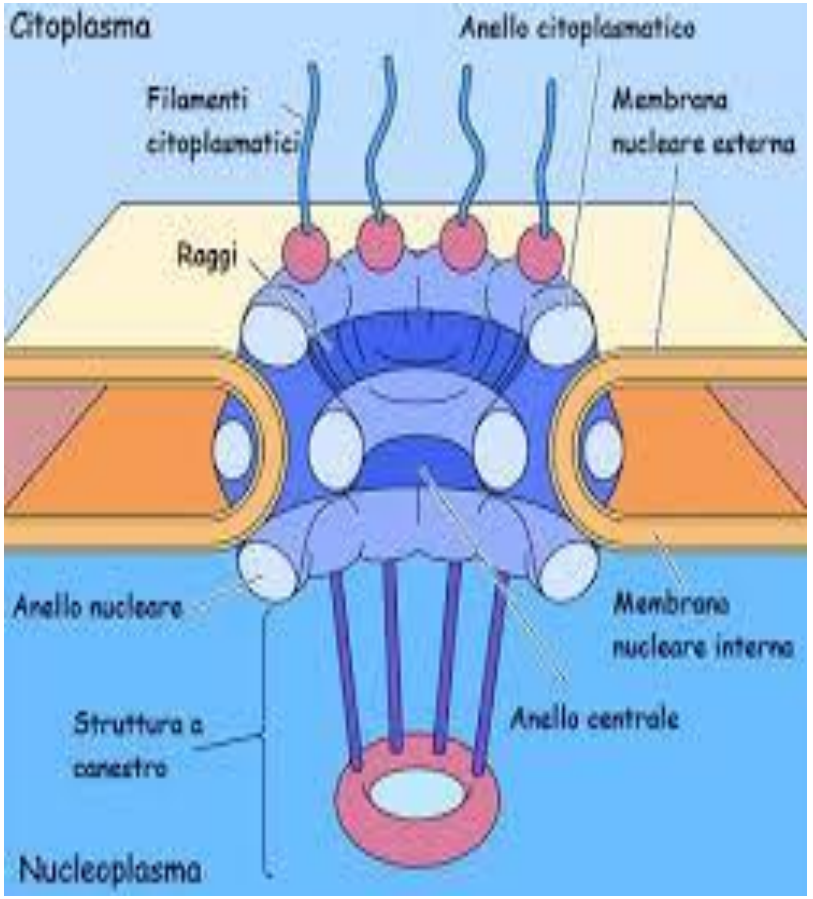


Fig. 7 Le cellule HEK293 trasfettate con pEGFP (controllo) e pEGFP-NLS sono state visualizzate al microscopio a fluorescenza.

La fase dimostrativa «attiva»



La fase dimostrativa «attiva»

La fase dimostrativa «attiva»

PLS *Virtual Summer School* per Studenti (PVS3) – 7 settembre 2021

GRAZIE PER L'ATTENZIONE!

Nicola Zambrano
zambrano@unina.it

https://www.youtube.com/watch?v=x5ox71qIa-0

https://www.youtube.com/watch?v=jKz07IpMwJo

https://www.youtube.com/watch?v=wxf4a4SX84A