evropský sociální fond v ČR
EVROPSKÁ UNIE
MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY
OP Vzdělávání pro konkurenceschopnost
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

Název: Biotechnology and Fluorescent protein

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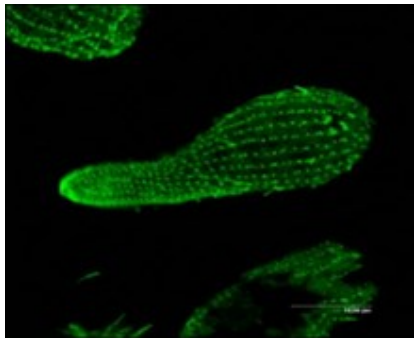
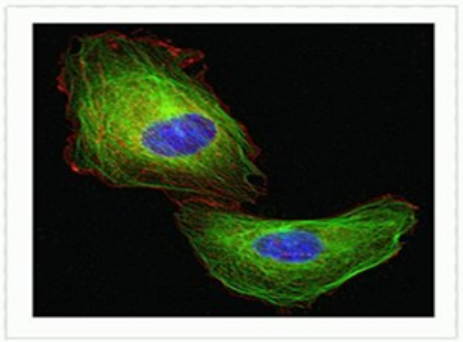
Reg.č.projektu: CZ.1.07/2.3.00/20.0148

Název projektu: Mezinárodní spolupráce v oblasti "in vivo" zobrazovacích technik

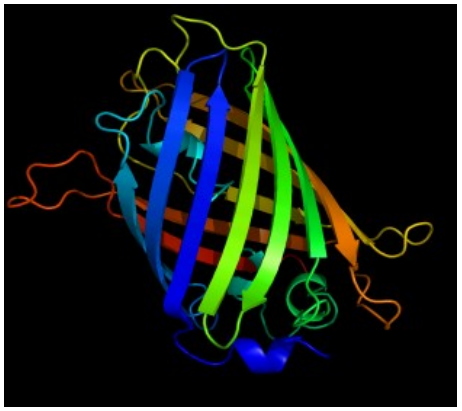


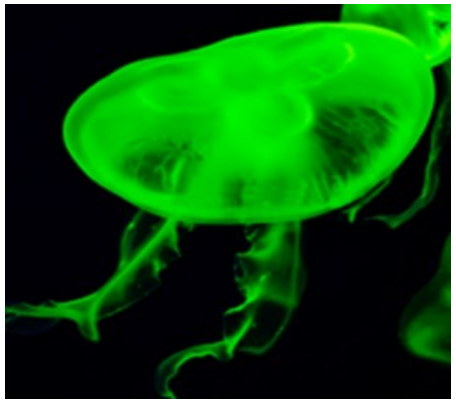
INTRODUCTION



GFP - Green fluorescent protein

- A protein composed of 238 amino acid residues (26.9 kDa)
- exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range
- GFP was isolated from the jellyfish *Aequorea victoria*.
- In cell and molecular biology, the GFP gene is frequently used as a reporter of expression
- The GFP gene has been introduced and expressed in many bacteria, yeast and other fungi, fish, plant and mammalian cells.

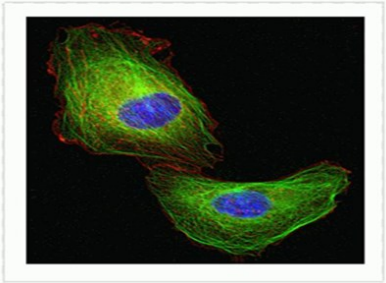


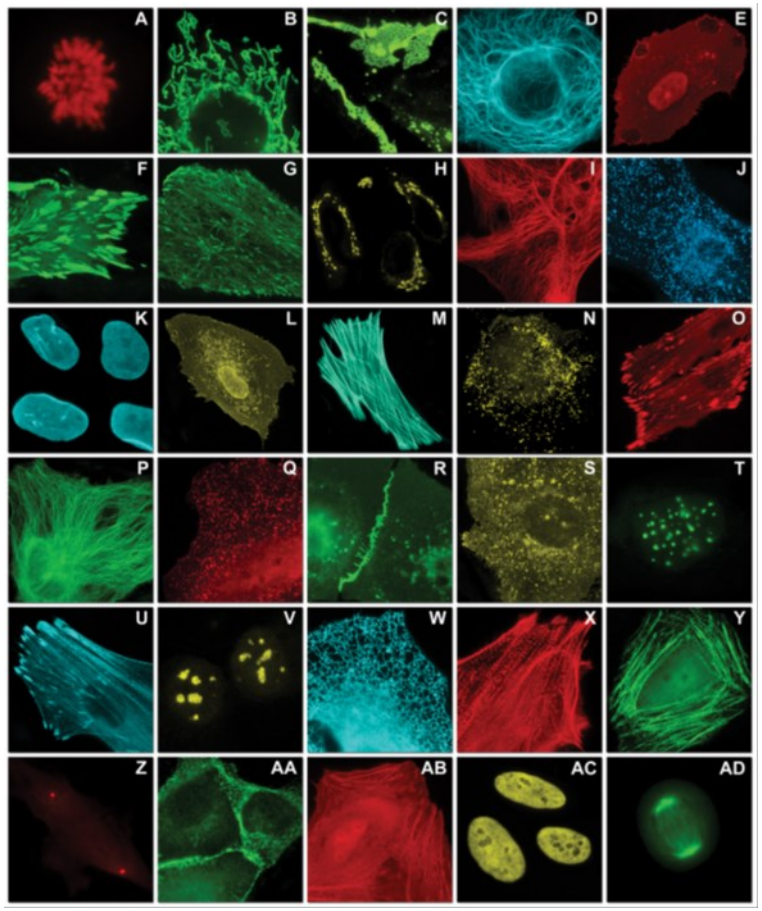


Green fluorescent protein (GFP)

- Nobel prize in Chemistry (2008):
  - for the discovery and development of the green fluorescent protein, GFP
    - Osamu Shimomura
    - Martin Chalfie
    - Roger Y. Tsien

- Now GFP is found in laboratories all over the world where it is used in every conceivable plant and animal





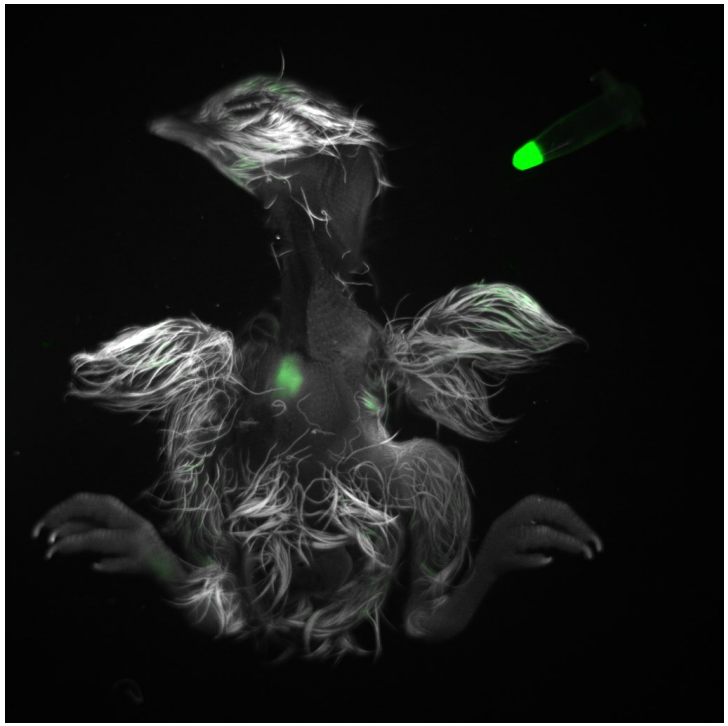
GFP Types

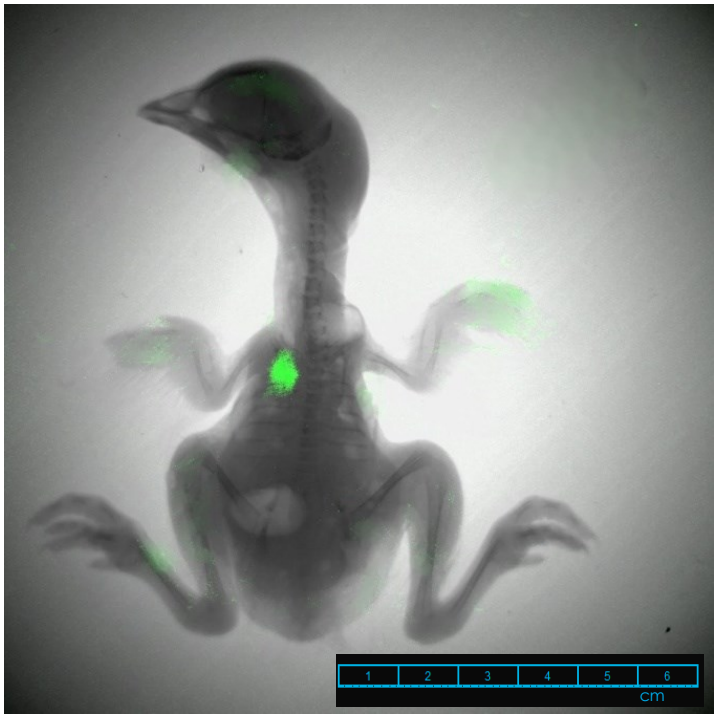
- Fluorescent proteins enable the creation of highly specific biosensors to monitor a wide range of intracellular phenomena.
- Mutagenesis of *A. victoria* GFP has resulted in fluorescent proteins that range in color from blue to yellow

Fluorescence Detection

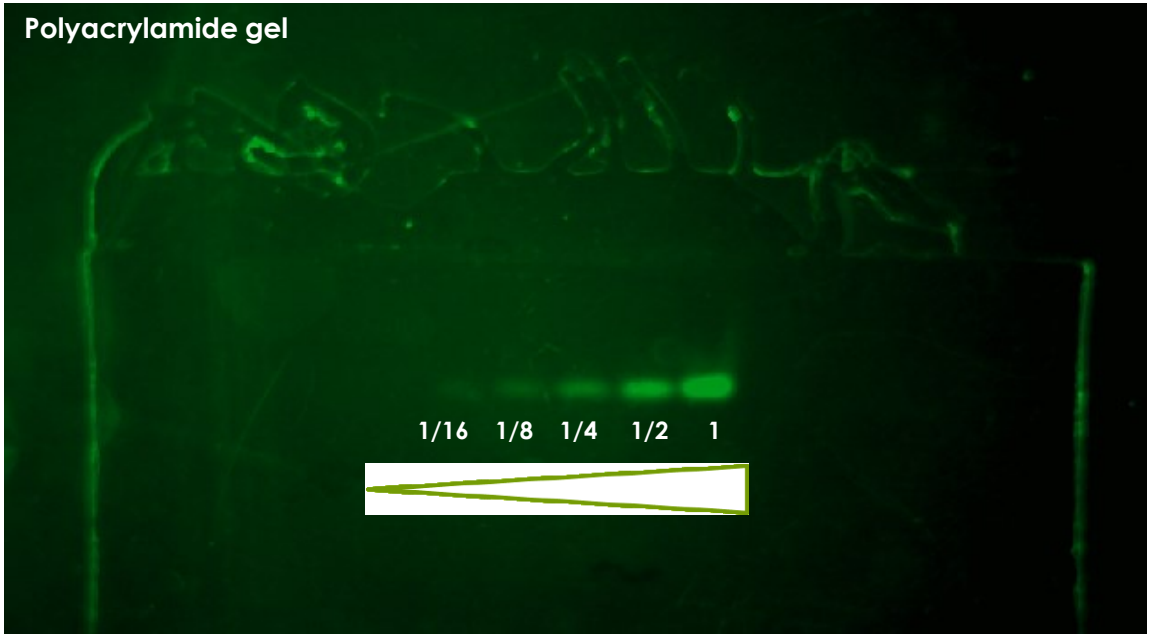
- Transluminator
- Fluoresence spectrophotometry
- In-vivo Xtreme
- Fluorescence microscopy

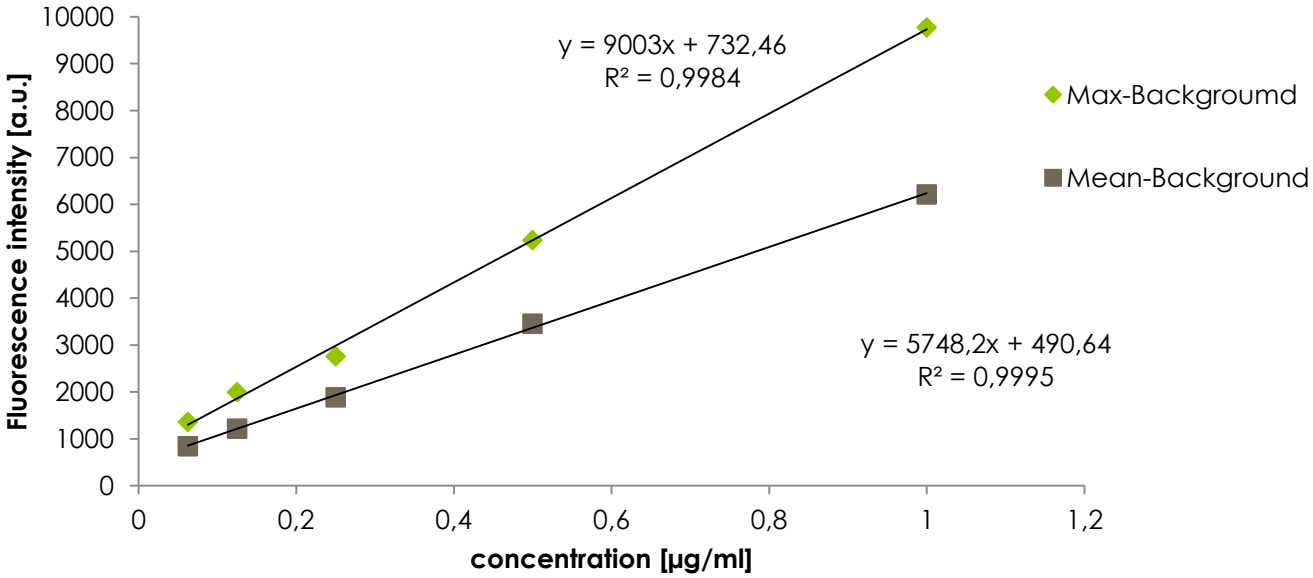
In-vivo Xtreme





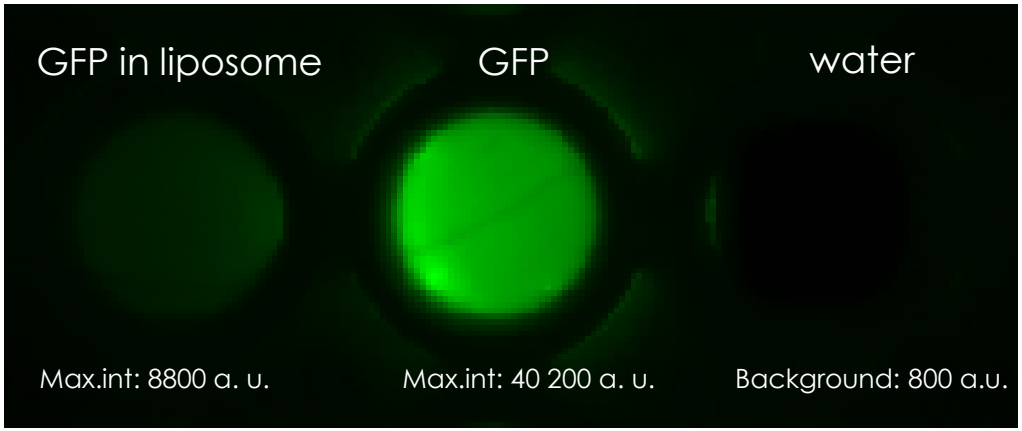
In-vivo Xtreme



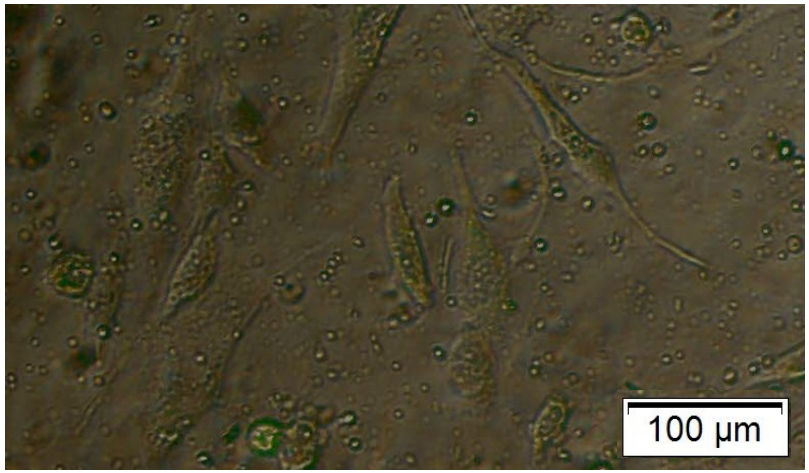


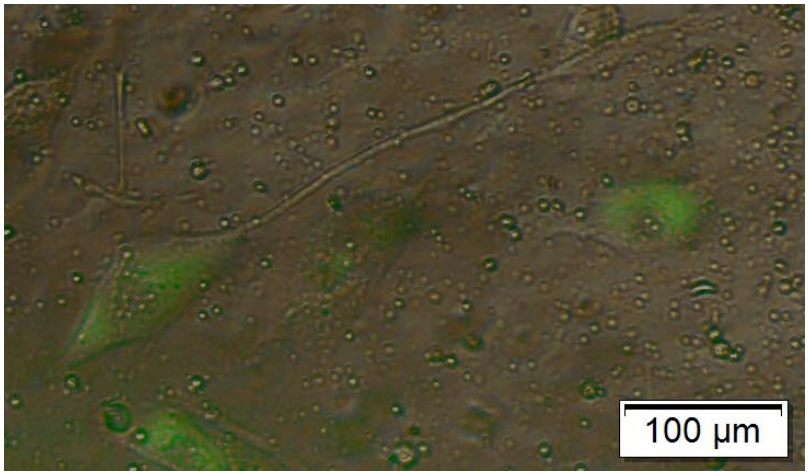
GFP encapsulated in liposome

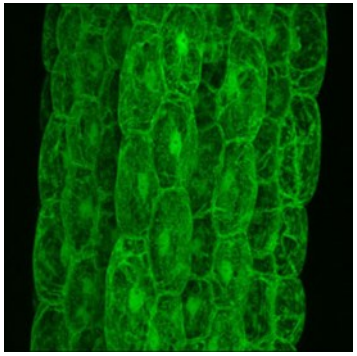
In-vivo Xtreme



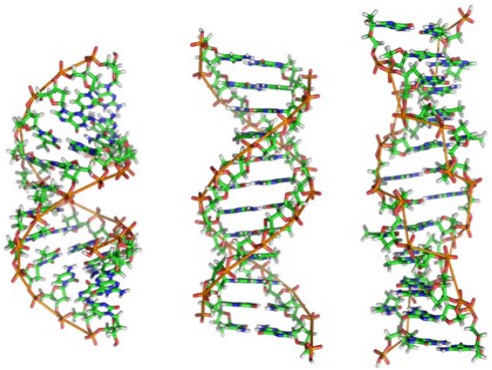
Fluorescence microscopy

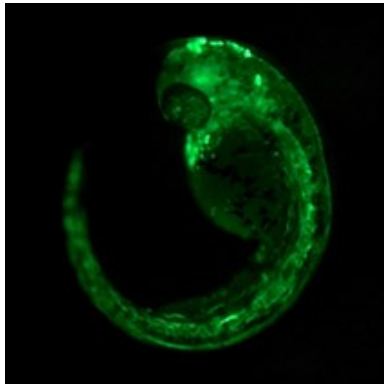






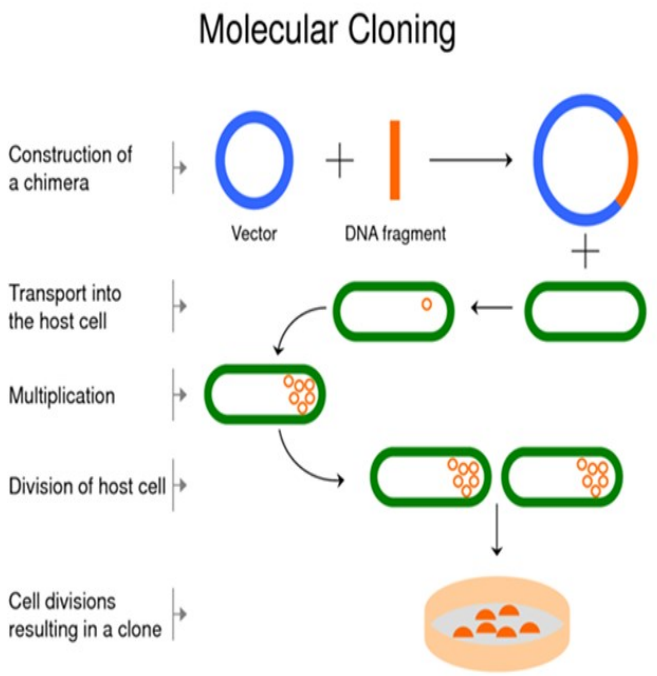
MOLECULAR CLONING

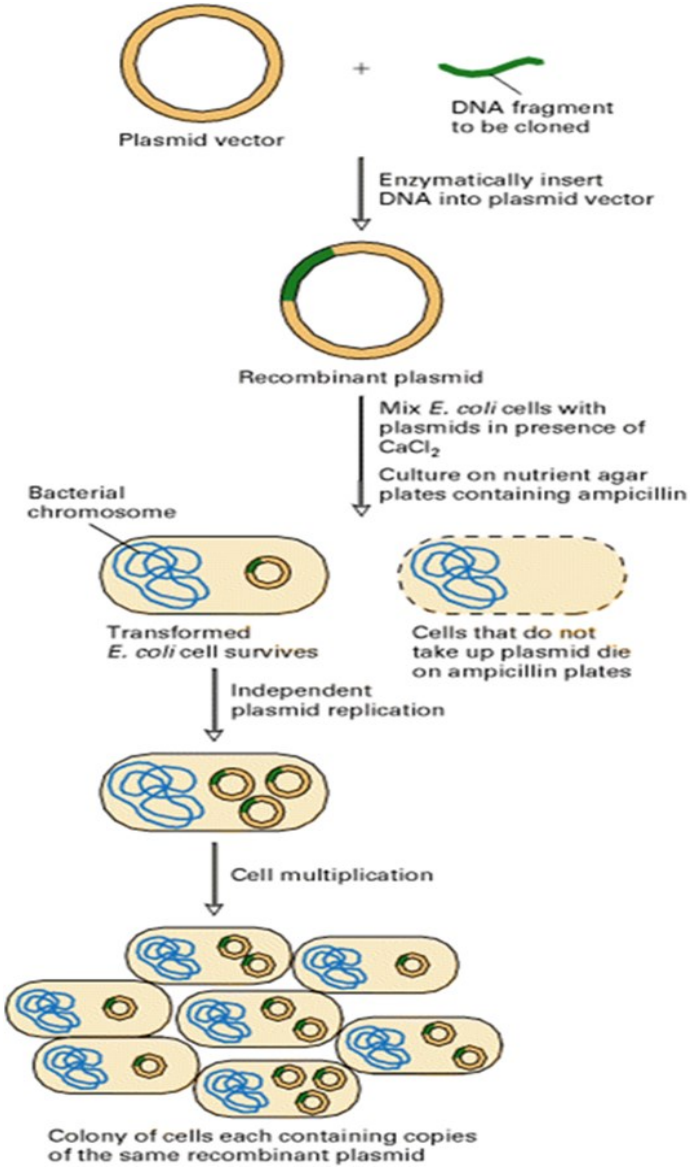




Molecular Cloning

- One the most basic techniques of molecular biology to study protein function is **cloning**
- The DNA fragment is inserted into a plasmid vector to create recombinant DNA molecules.
- The recombinant DNA is introduced into a host organism, *E. coli* bacteria
- The recombinant DNA molecules are replicated with the host DNA





The insertion of the DNA fragment of interest into the plasmid vector

↓

Mix *E.coli* cells with recombinant plasmid

↓

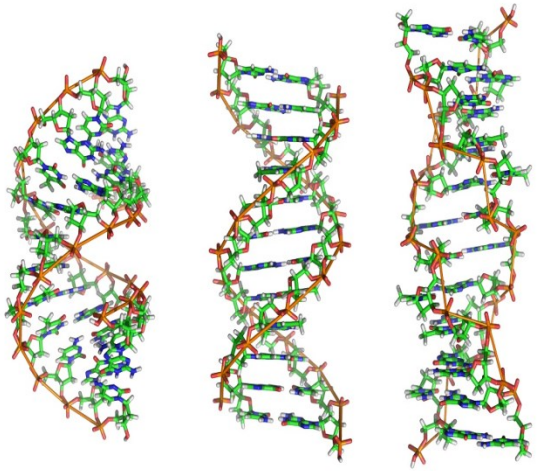
Cell Multiplication

↓

Colony cells containing copies of the recombinant plasmid

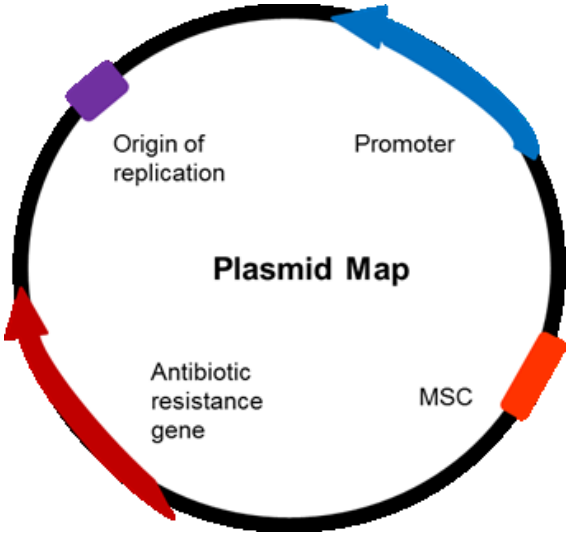
DNA fragment

- The genomic DNA or RNA is extracted from the organism of interest
- The DNA or RNA is purified using the standard method to remove contaminating molecules
- The specific gen of interest is amplified using PCR method and later proceeding with the molecular cloning



Plasmid Vector

- Plasmids have become an essential tool in molecular biology
- Plasmids are fragments of double-stranded DNA that can replicate independently of chromosomal DNA, and carry genes
- Their size is between 1,000-20,000 base pairs and they are stable long-term

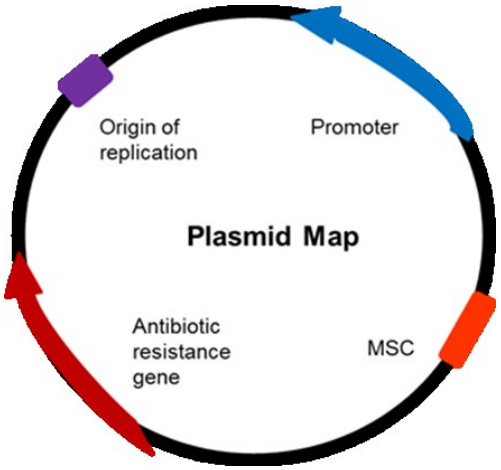


Vector component

- Origin of Replication (ORI)
- Antibiotic Resistance Gene
- Multiple Cloning Site (MCS)
- Promoter Region

Vector Components

- **Origin of Replication (ORI)** DNA sequence which allows initiation of replication within a plasmid
- **Antibiotic Resistance Gene**, allows for selection of plasmid-containing bacteria
- **Multiple Cloning Site (MCS)**, contains several restriction sites allowing for the easy insertion of DNA
- **Promoter Region**, drives transcription of the target gene



Host Organism

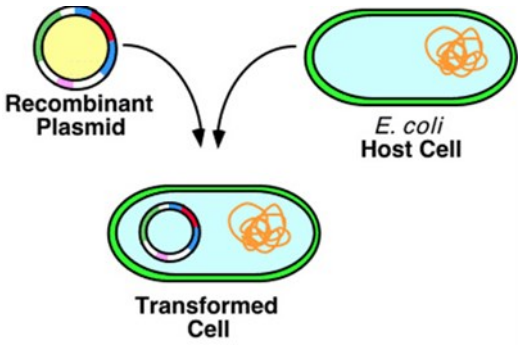
- The majority of molecular cloning experiments begin with a laboratory strain of the *E. coli* (Competent cells)
- *E. coli* has small genome size, it is about 4,400 genes
- *E. coli* grows rapidly at a rate of one generation per twenty minutes.
- This allows for preparation of log-phase cultures with overnight incubations

Competent Cells

GFP in pGLO plasmid

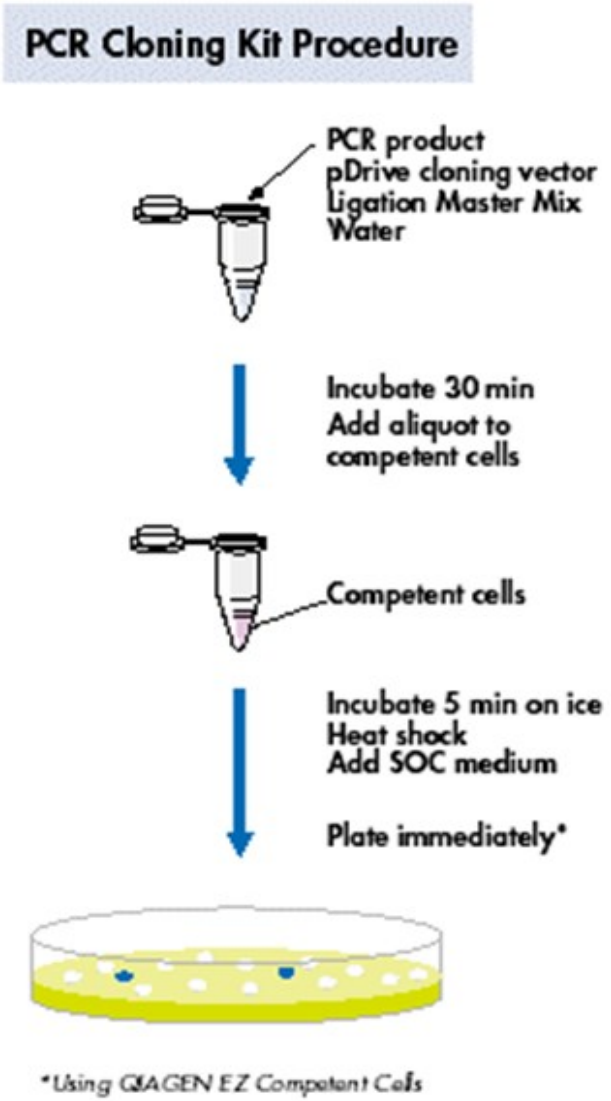
- The pGLO plasmid is used in Biotechnology
- The plasmid contains the green fluorescent protein (GFP) and the ampicillin resistance gene
- The GFP gene is expressed in the presence of arabinose, which makes the transgenic organism shows fluorescence under UV light
- GFP can be induced in bacterias which contains the pGLO plasmid and grows on arabinose and ampicilin plates

pGLO™ Bacterial Transformation Kit (Biorad)



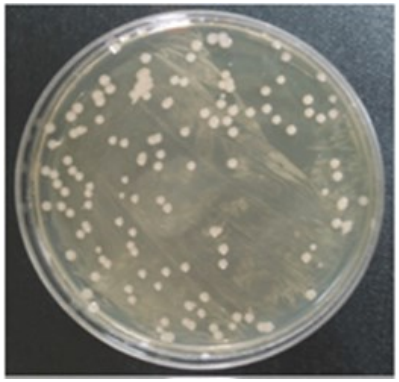
Cloning

- Add 2 µl of the pGLO plasmid into vial of chemical competent cells.
- Incubate on ice 30 minutes.
- Heat shock the cells for 45 seconds at 42°C.
- Transfer the tubes to ice for 3 minutes.
- Add 250 µl of SOC medium.
- Shake the tube horizontally (200 rpm) at 37°C for 90 minutes.
- Spread 50 µl and 200 µl from transformation on selective plate (ampicilin+arabinose)
- Incubate overnight at 37 °C.



Results of Cloning

pGLO Cloning

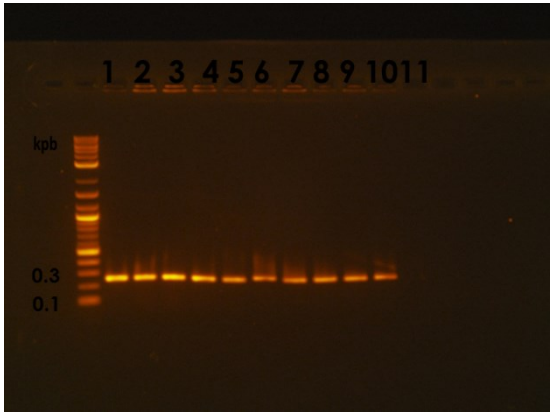


LB+ampicilin+arabinose

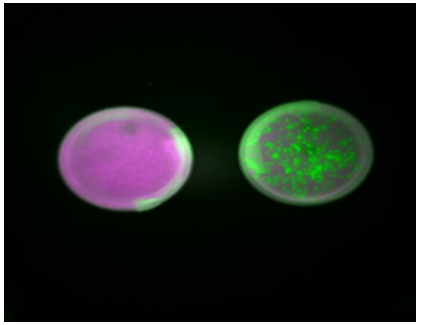
A

B

PCR Screening



In-vivo Xtreme



Analysis of Positive Clones

- Pick 10 colonies and make **PCR Screening**

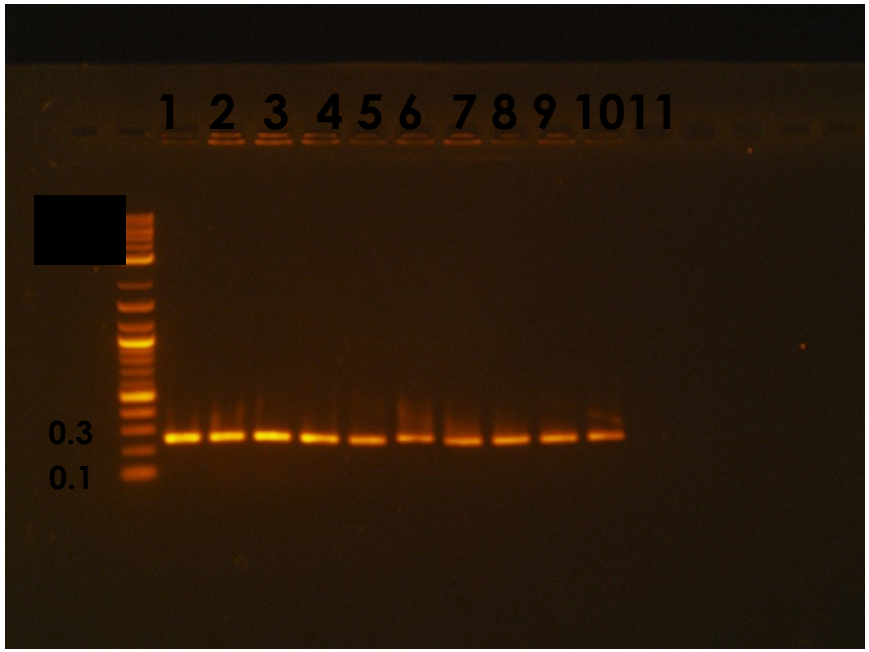
PCR (GFP Plasmid) 35x

4 min 95 °C
1 min 95 °C
30s 50 °C
30s 72 °C
10 min 72°C
10 min 72°C
10 min 10 °C

PCR product **272bp**

Electrophoresis
1% AgarGel
Ethidium Bromide 5 µl/100µl
TAE Buffer, 100v
60 min, UV

PCR
1-10. Colonies
11. Negative control

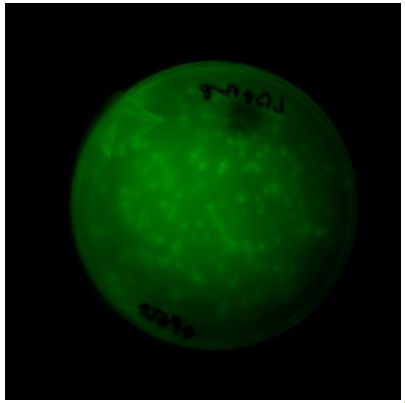


All colonies were positive transformants!!

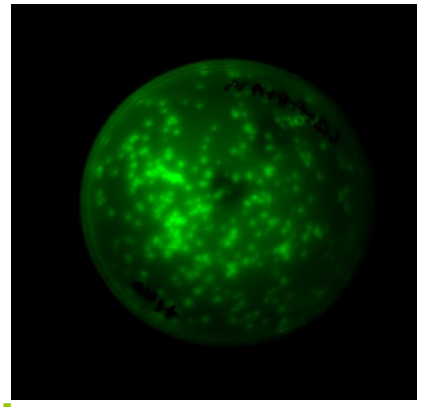
pGLO GFP - fluorescence detected

In-vivo Xtreme

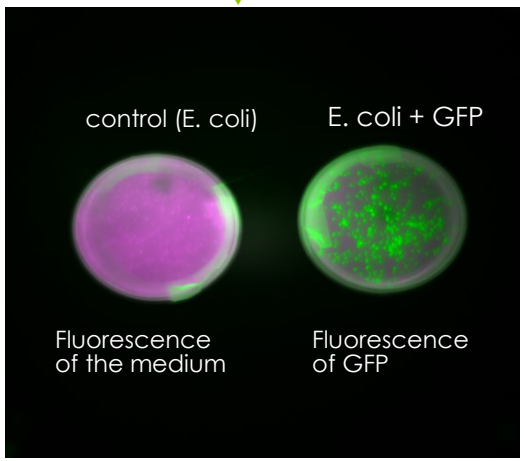
control (E. coli)



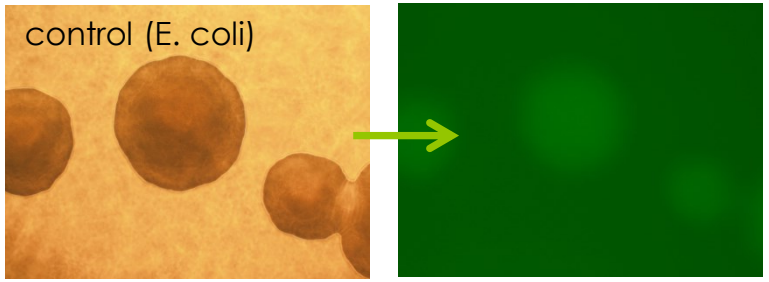
E. coli + GFP

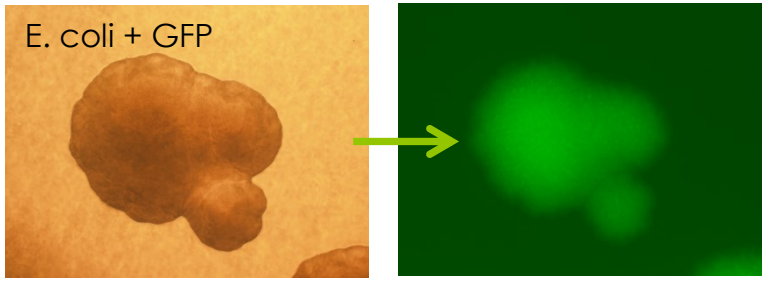


Unmixing software



Fluorescence microscopy

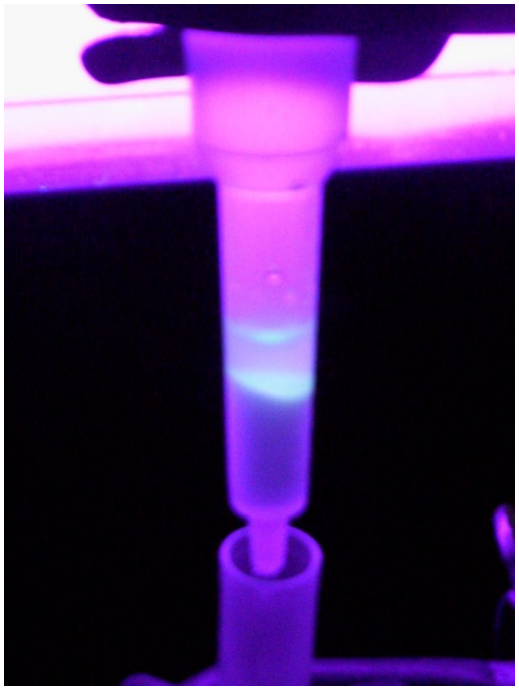




fluorescence

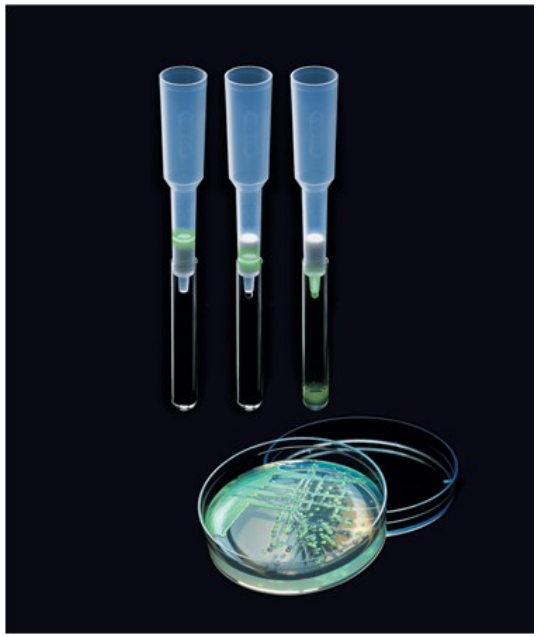
Bacterial Lysis and Protein Chromatography

- The positive transformants were grown in LB broth with 100 mg/liter ampicillin and 0.2% arabinose
- Shaking the culture overnight at 32 °C
- Enzymatic lysis of the bacterial cell wall with10 mg/ml of lysozyme and freezing at -80°C
- GFP was purified from the bacterial lysate using hydrophobic interaction chromatography (HIC) columns (Macro-Prep® Methyl HIC Column, Biorad).
- The protein elution was made with TE buffer



Chromatography Column using the UV light

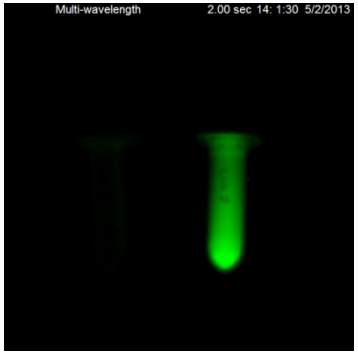
Results of GFP protein Isolation



Protein Chromatography

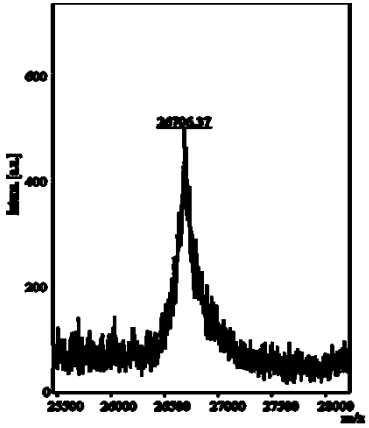
A

In-vivo Xtreme



B

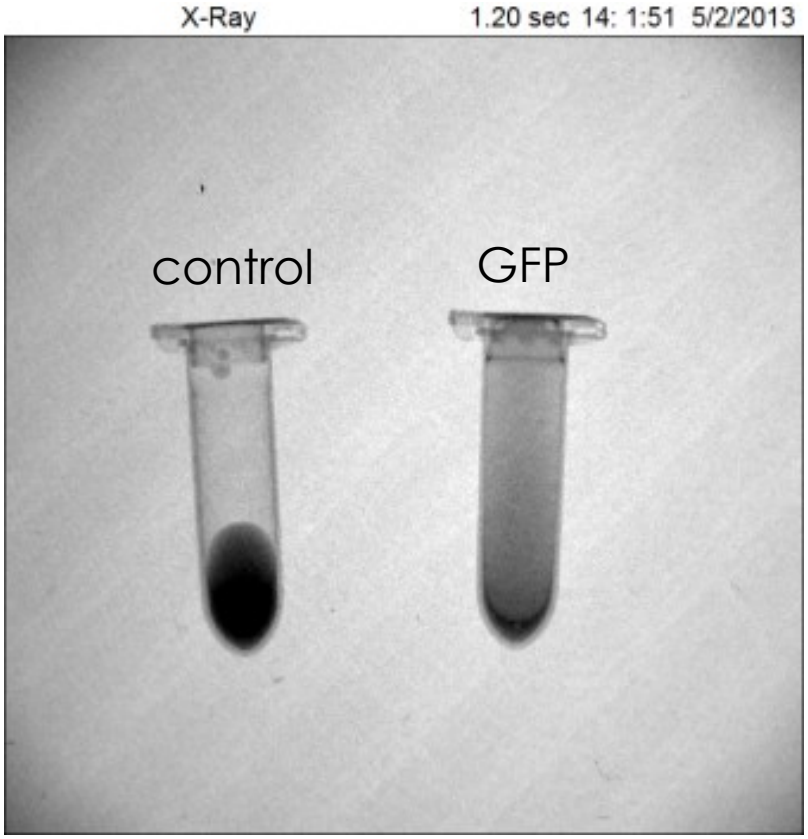
MALDI-TOF

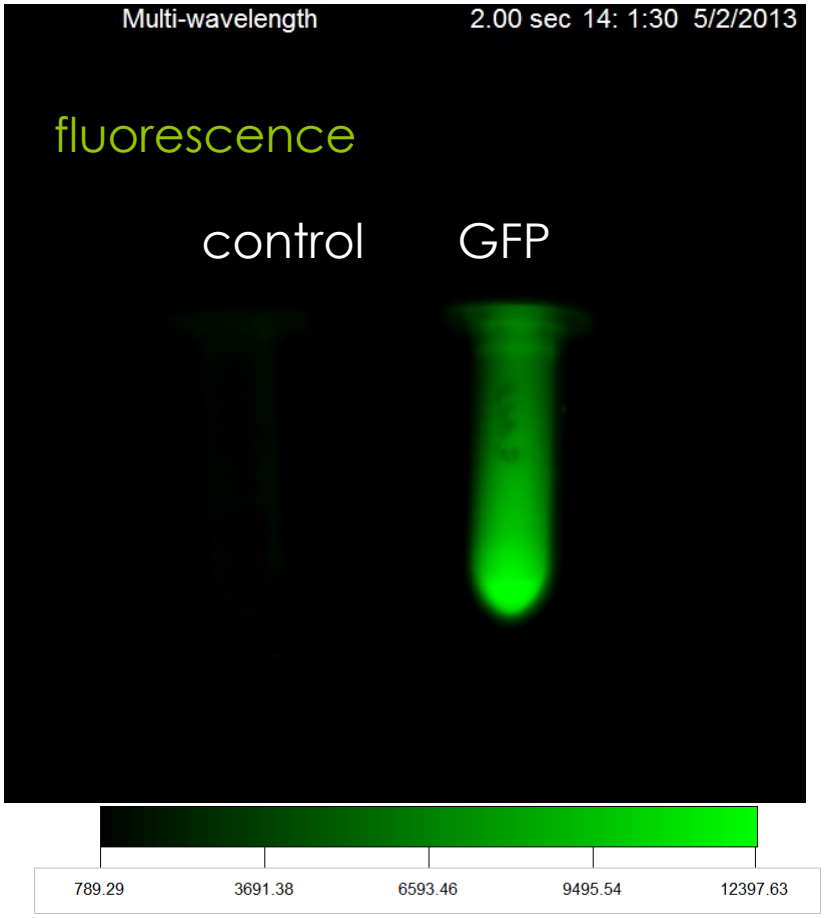


GFP protein

Fluorescence Detected

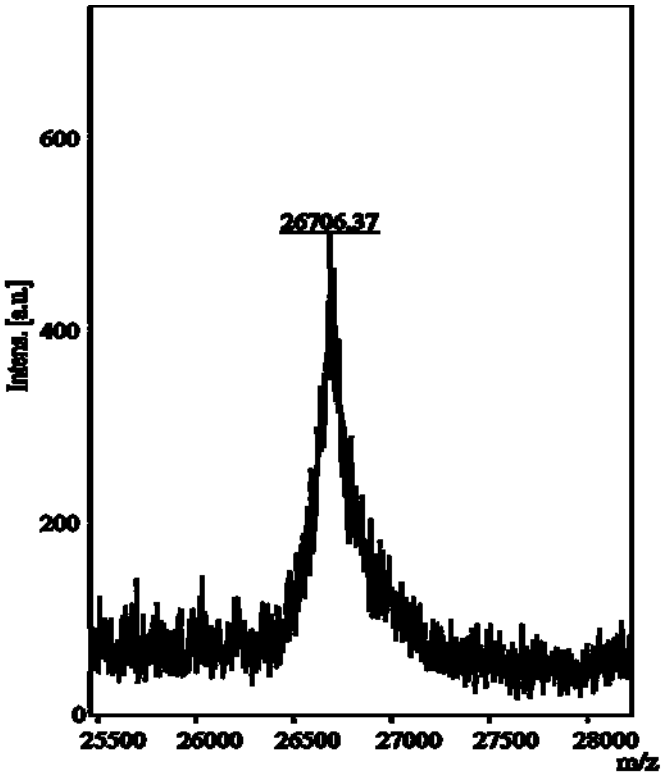
In-vivo Xtreme





GFP Espectra MALDI-TOF/TOF

- The matrix used in the MALDI method was a-cyano-4-hydroxycinnamic acid (CCA).
- The matrix was prepared in TA30. The MS spectra were acquired by averaging 20 sub spectra from a total of 500 shots of the laser
- The results in MALDI are correct, the expected size of the full GFP is 26700 Da



Spectra of Green Fluorescent Protein by MALDI-TOF

Acknowledgements

- **MSc. Miguel Angel Merlos Rodrigo**
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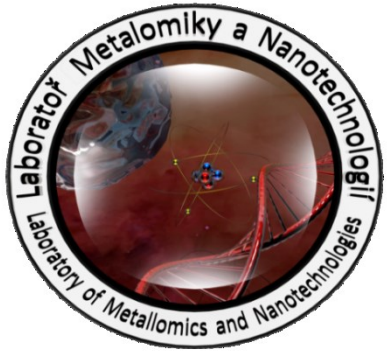
Děkuji za pozornost !



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