GMIT
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GALWAY-MAYO INSTITUTE OF TECHNOLOGY

Presented by:
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Yr 4 Medical science.
BSc. Medical Science Honours
Level 8.
G00296946

Background:

- GFP sourced from *Aequoria Victoria* jellyfish.
- EGPP has own chromophore.
- 11-B stranded sheets around α-helix.
- S65T and F64L mutations enhanced the folding and fluorescence of EGFP.
- Glutamine 222 and arginine 96.
- EGFP-pBAD plasmid expression system prevents protein toxicity.
- Expression system for optimal protein solubility.

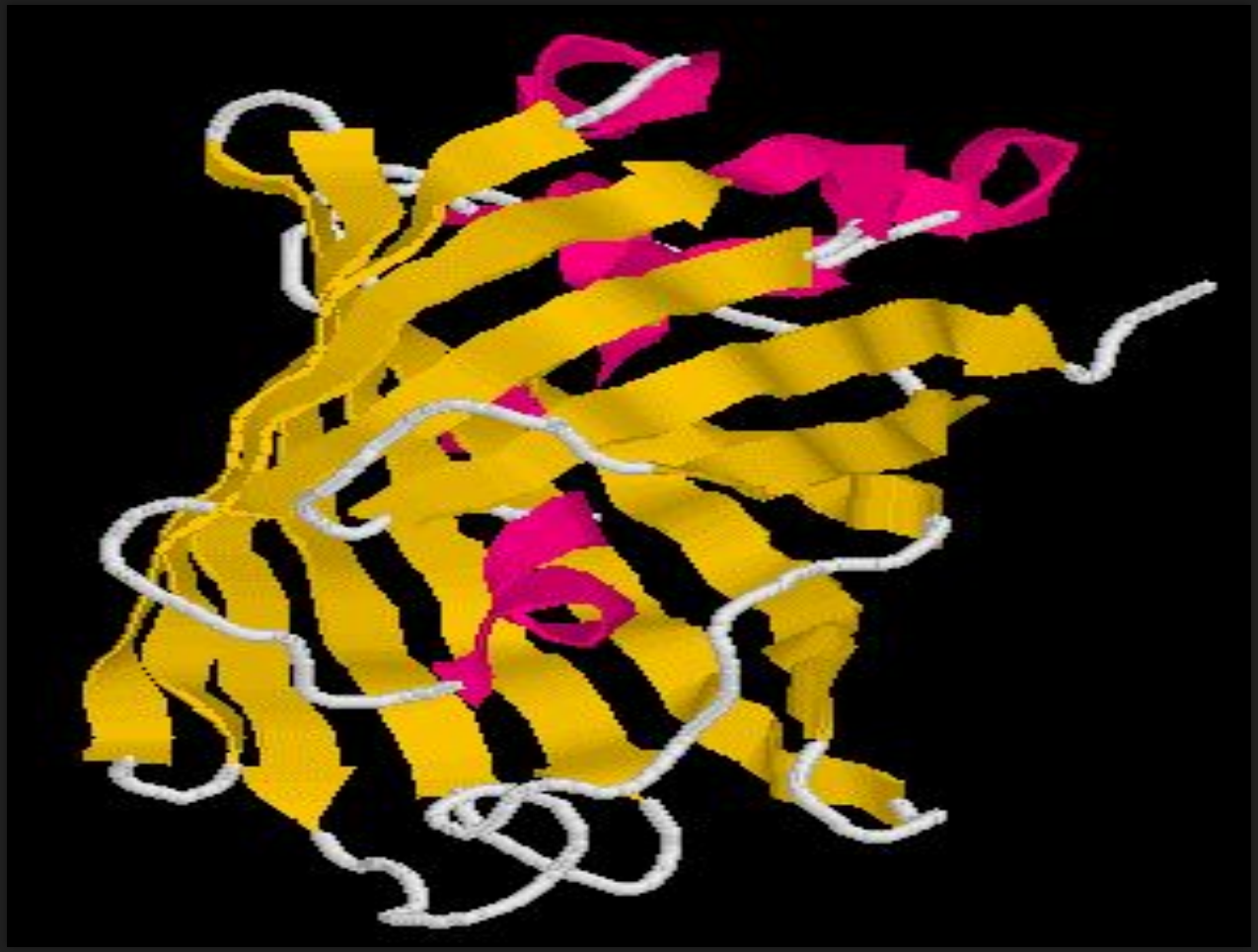


Fig. 1: Rasmol software generated Cartoon display EGFP structure from ‘2y0g’ protein databank imported protein reference.
The yellow strands are B-sheets. Alpha helices are coloured magenta.
The white strands are the remainder of the molecule.
Protein reference deposited by Royant and Noirclerc Savoye (2011).

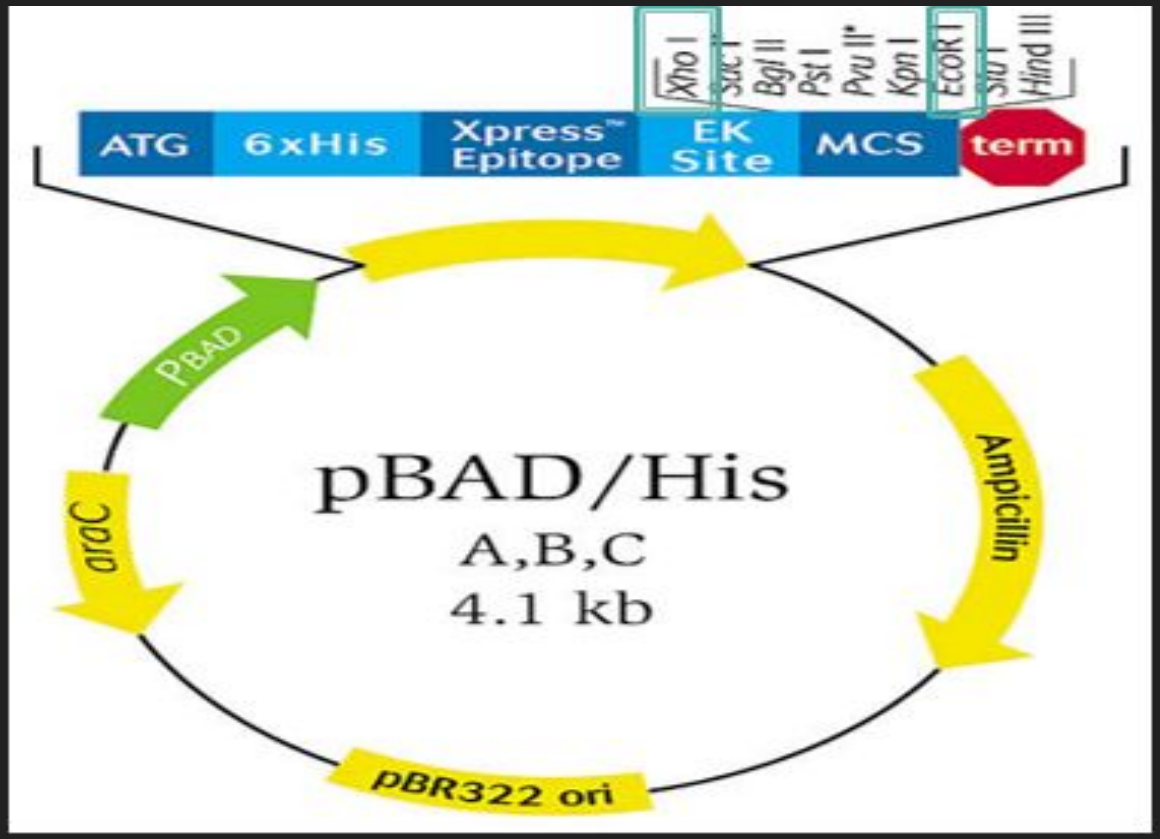


Fig. 2: Thermo scientific pBAD histidine tagged plasmid.

The ATG is the start codon of the DNA plasmid.

6xHis tag is located on the N-terminus of the plasmid near the start codon amino acid sequence.

The multiple cloning site is attached to the terminator to end transcription of the EGFP target gene.

EcoR1 restricts at 932bp. XhoI restricts at 205bp.

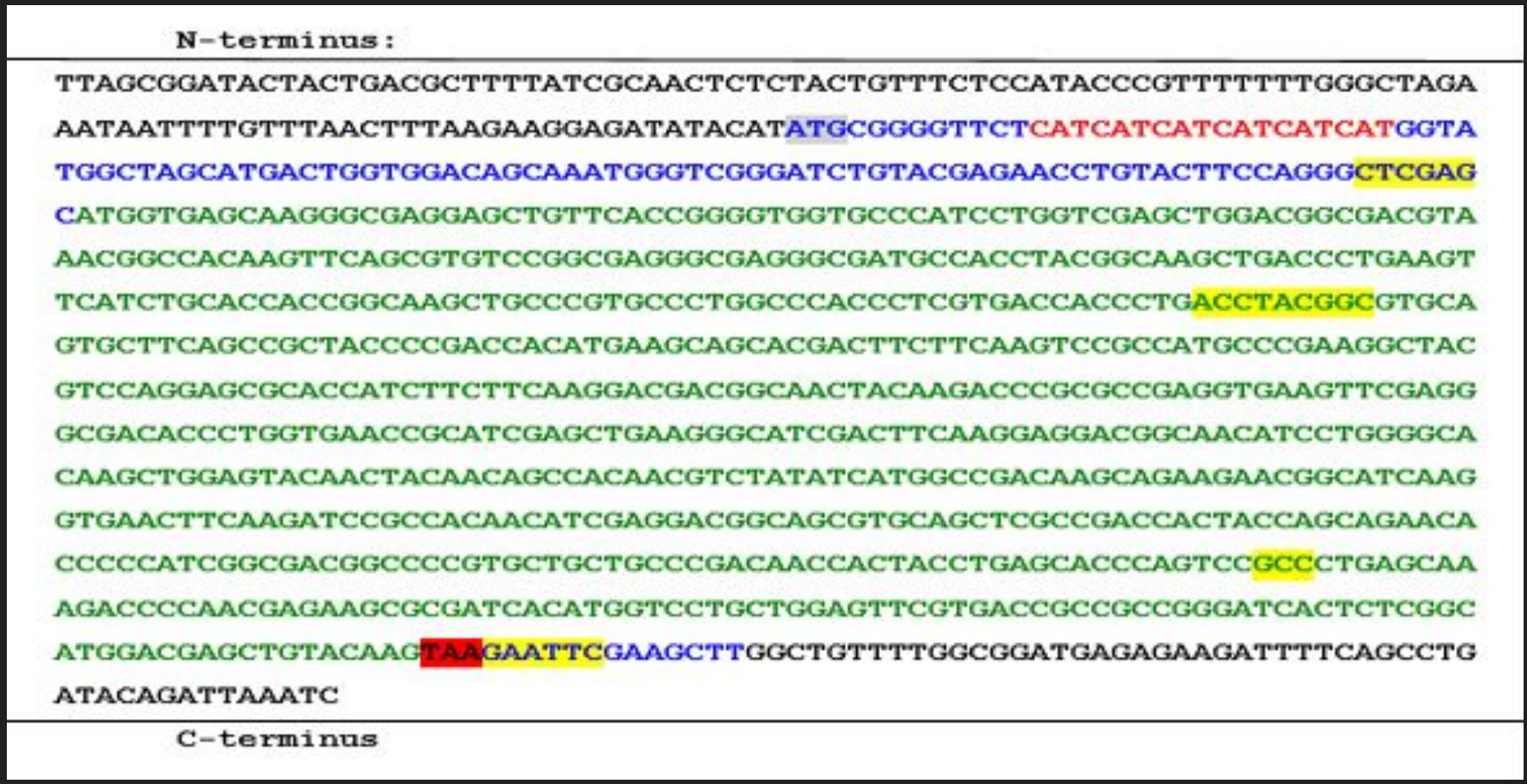


Fig. 3: EGFP-pBAD genetic sequence.

ATGGTGAGC - EGFP Sequence (717 Nucleotides - 239 Amino Acids)

ACCTACGGC - EGFP Chromophore (TYG); A206 = GCC

CATCATCAT - 6X Histidine Sequence

TCCGGACTC - Multiple Cloning and pBAD Components

ATG - Methionine Start Codon

TAA - Stop Codon

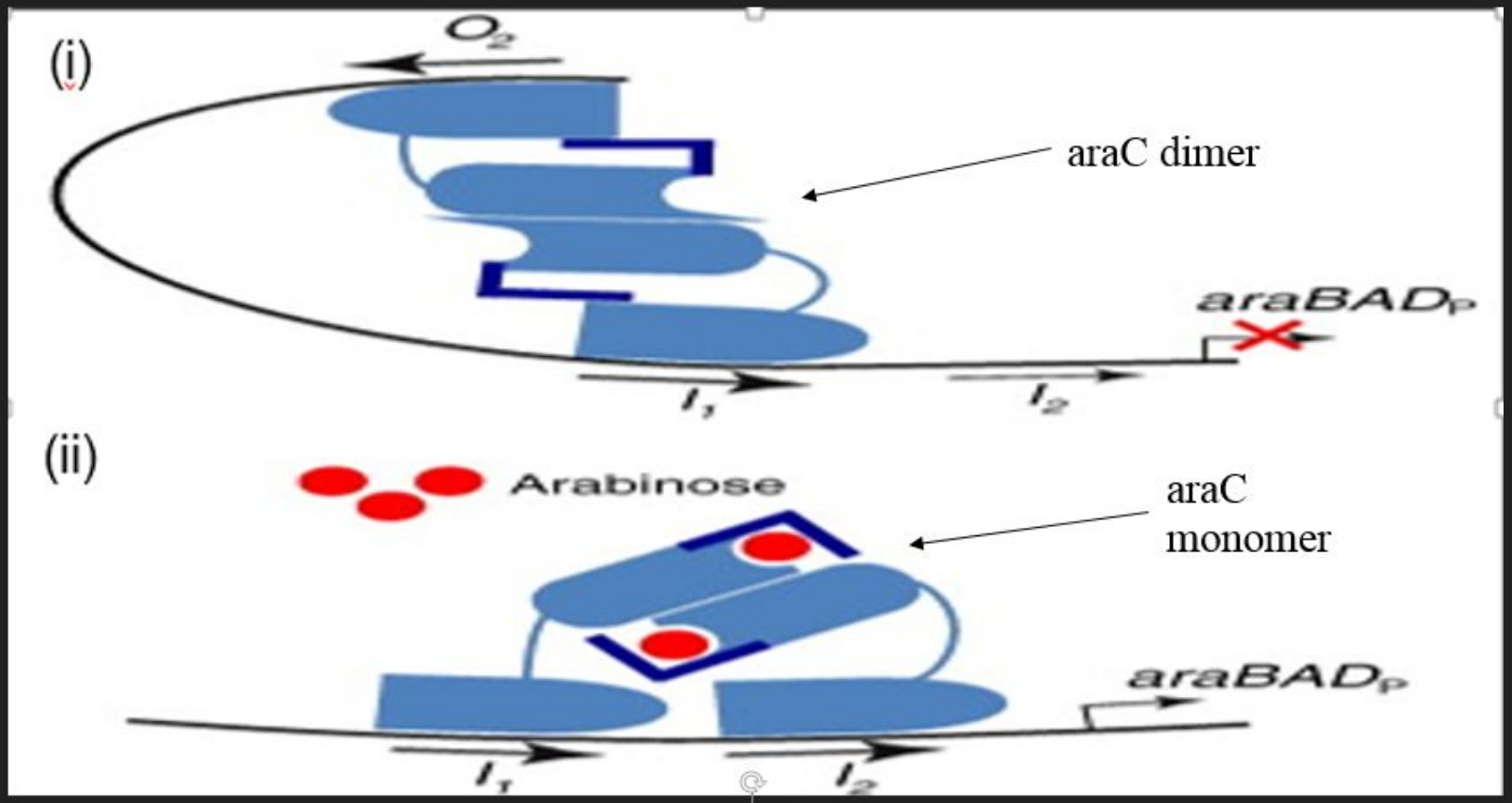


Fig. 4: AraBAD promoter repression and transcription

(i) Arabinose absence: AraC binds to operator and initiator to form 210bp DNA loop.

(ii) Arabinose presence structurally changes AraC component of the arabinose operon. AraC binds to the initiator sites and terminates DNA looping. RNA polymerase has access to araBAD promoter.

araBAD promoter activation causes transcription of the recombinant gene.

(Yang et al. 2011).

Aims:

- To optimise the expression of EGFP in Top10 *E. coli* strain.
- To optimise the purification of EGFP.
- To purify and polish monomeric EGFP for crystallisation.

Overall procedure:

Plasmid

- DH5α *E. coli* EGFP-pBAD plasmid purification
- restriction of plasmid DNA
- 1% agarose gel electrophoresis

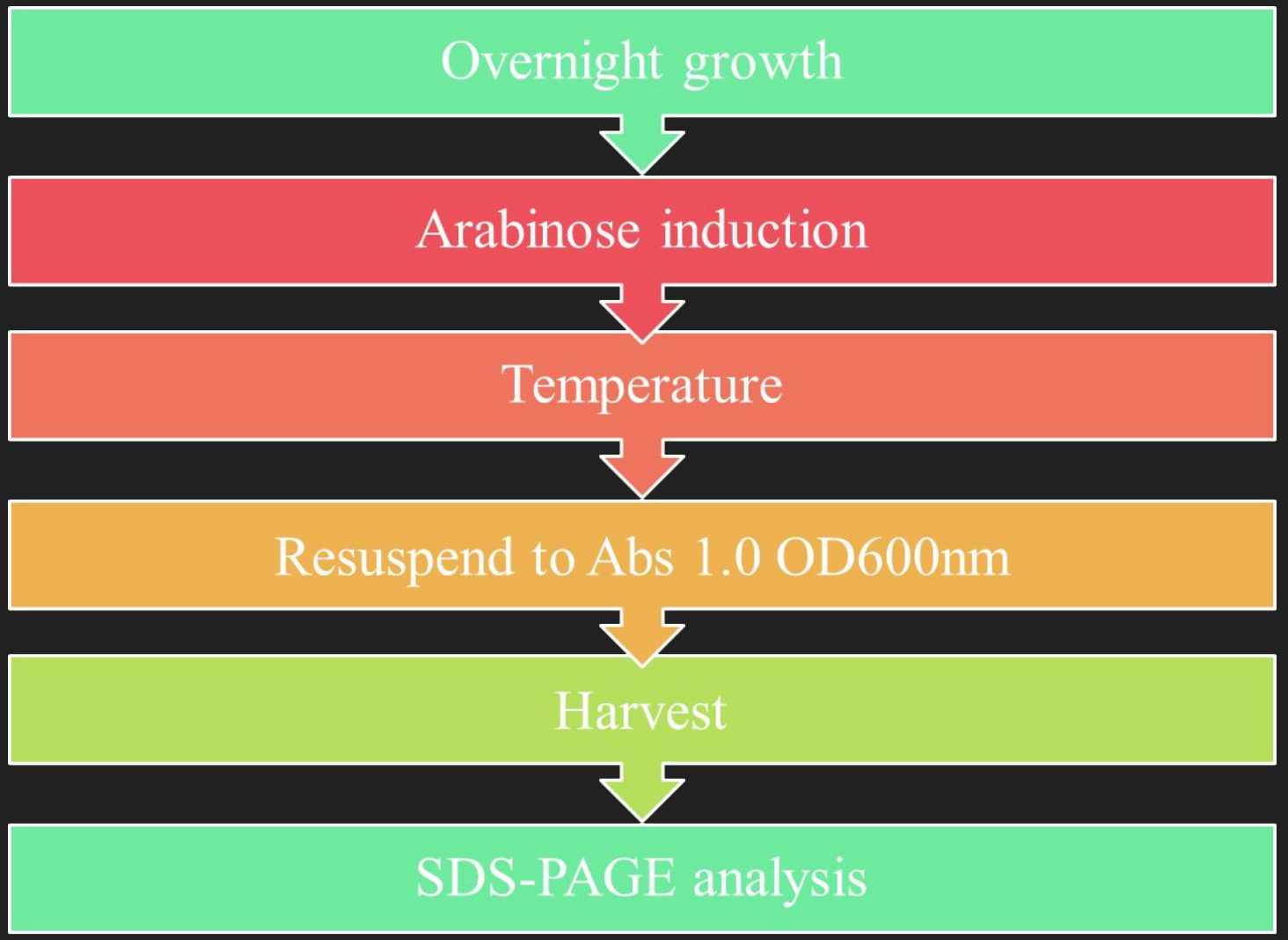
Transformation

- Chemically competent Top10 *E. coli*-CaCl2
- Heat shock transformation

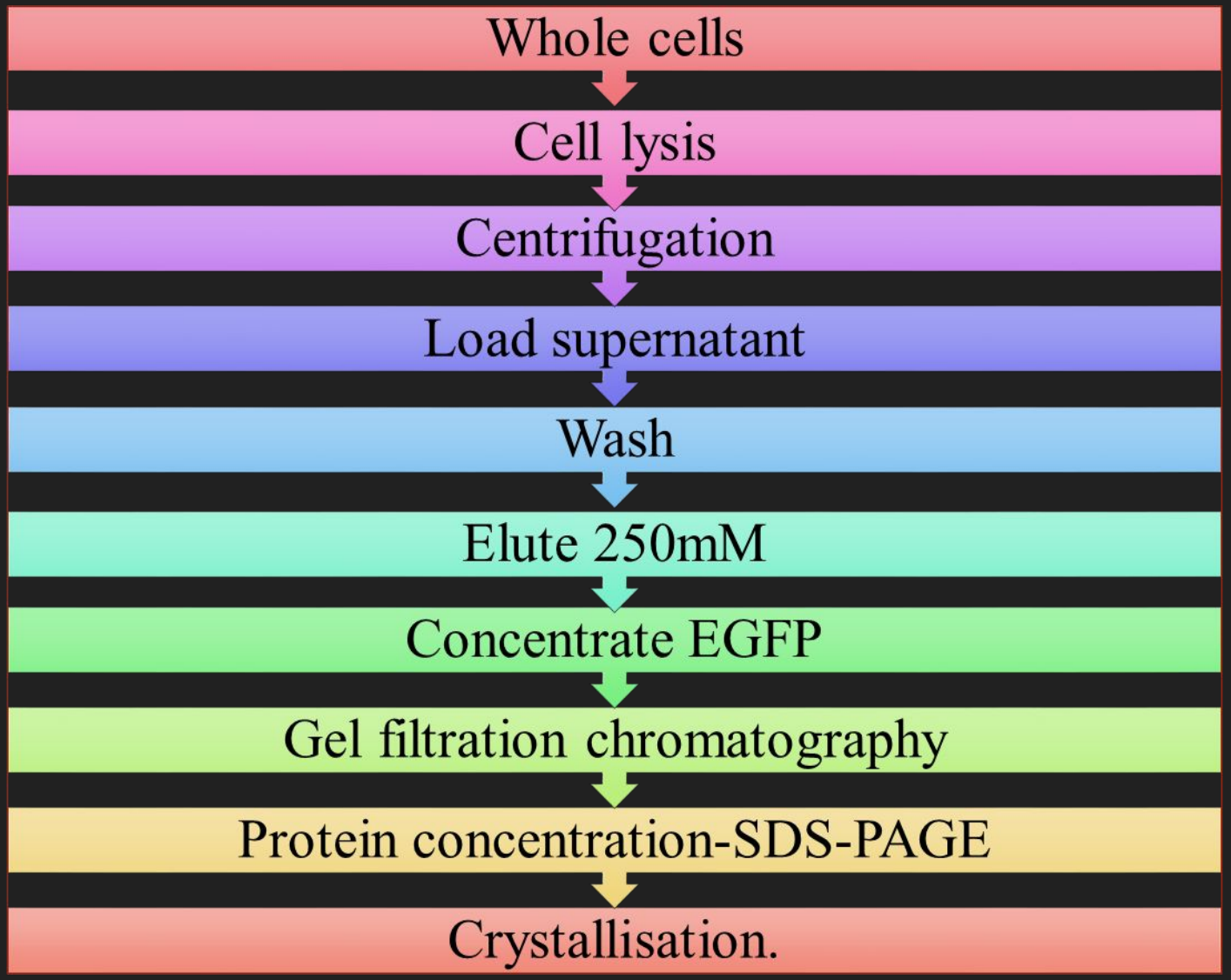
Main procedures:

- Expression
- Purification
- Polishing
- Crystallisation

Expression Optimisation



Purification optimisation, gel filtration chromatography and crystallisation



Results:

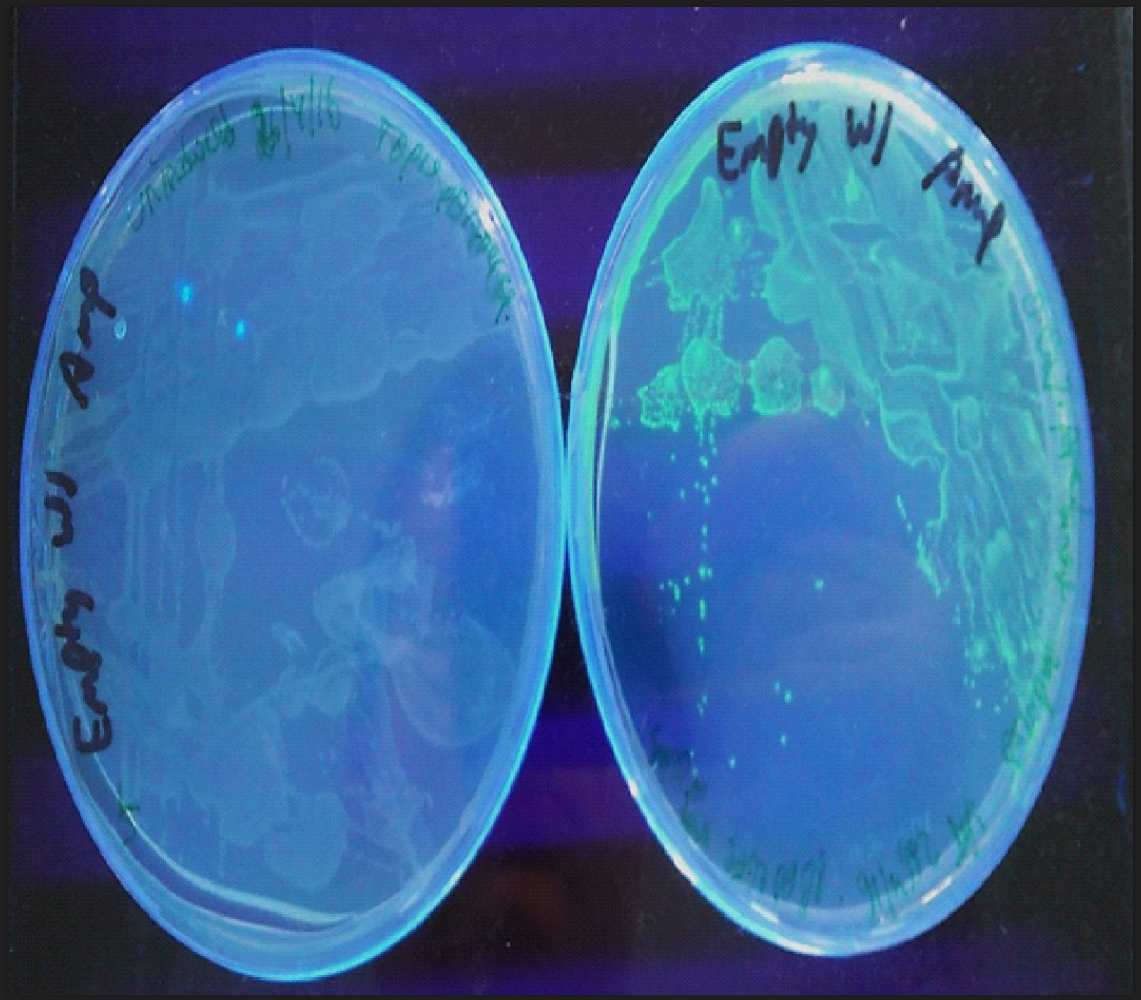


Plate 1: Uninduced vs Arabinose induced transformed Top10 *E. coli* cells.
Arabinose induced cells by 0.02% arabinose in the right LB Amp 100 agar plate.
Picture taken under UV-light.

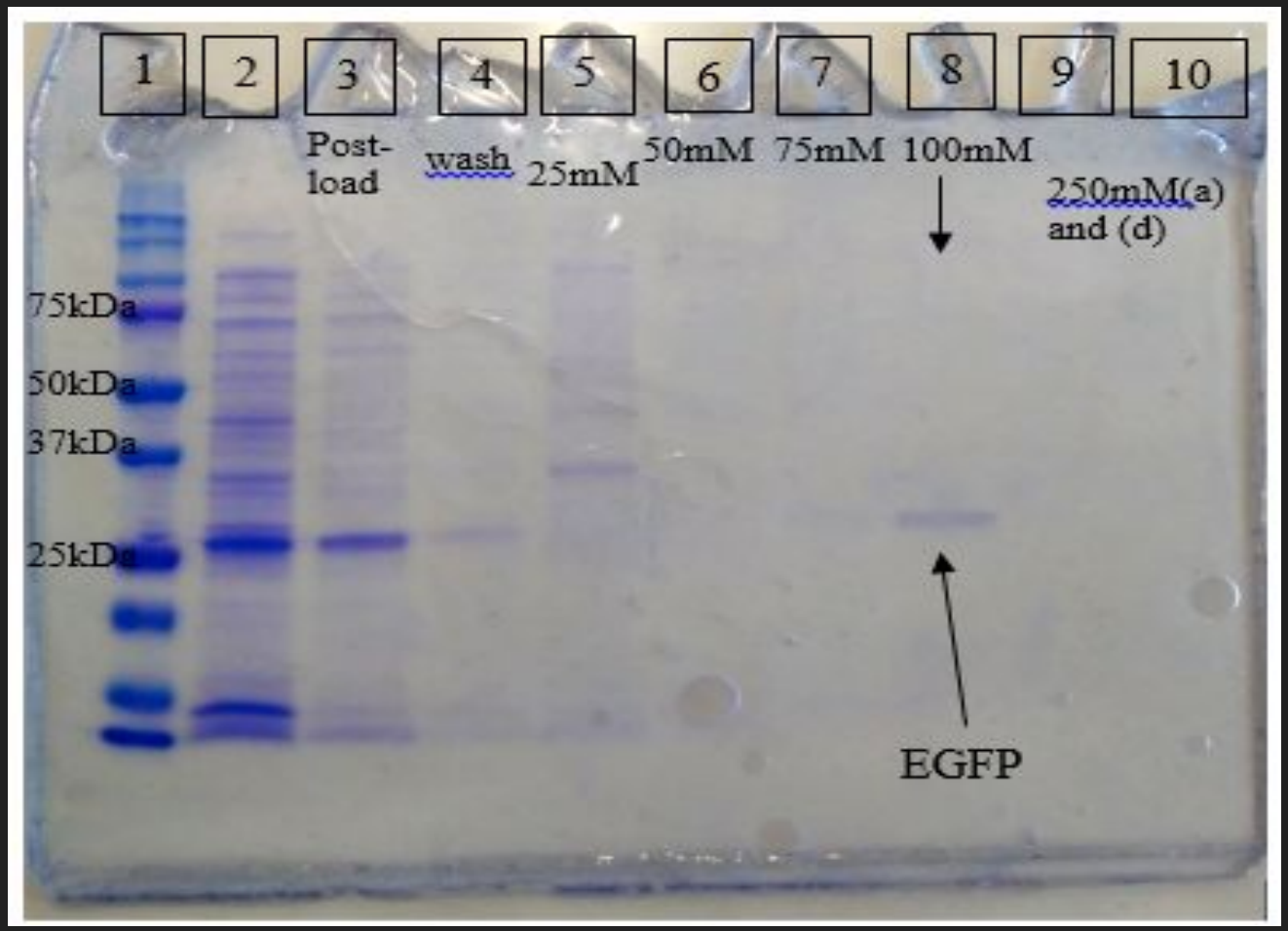


Plate 8: small scale nickel affinity chromatography purification gel.

Lanes 1-10: hyperladder, load, post-load, wash (lysis buffer only), 25mM, 50mM, 75mM, 100mM, 250mM (a), 250mM (d).

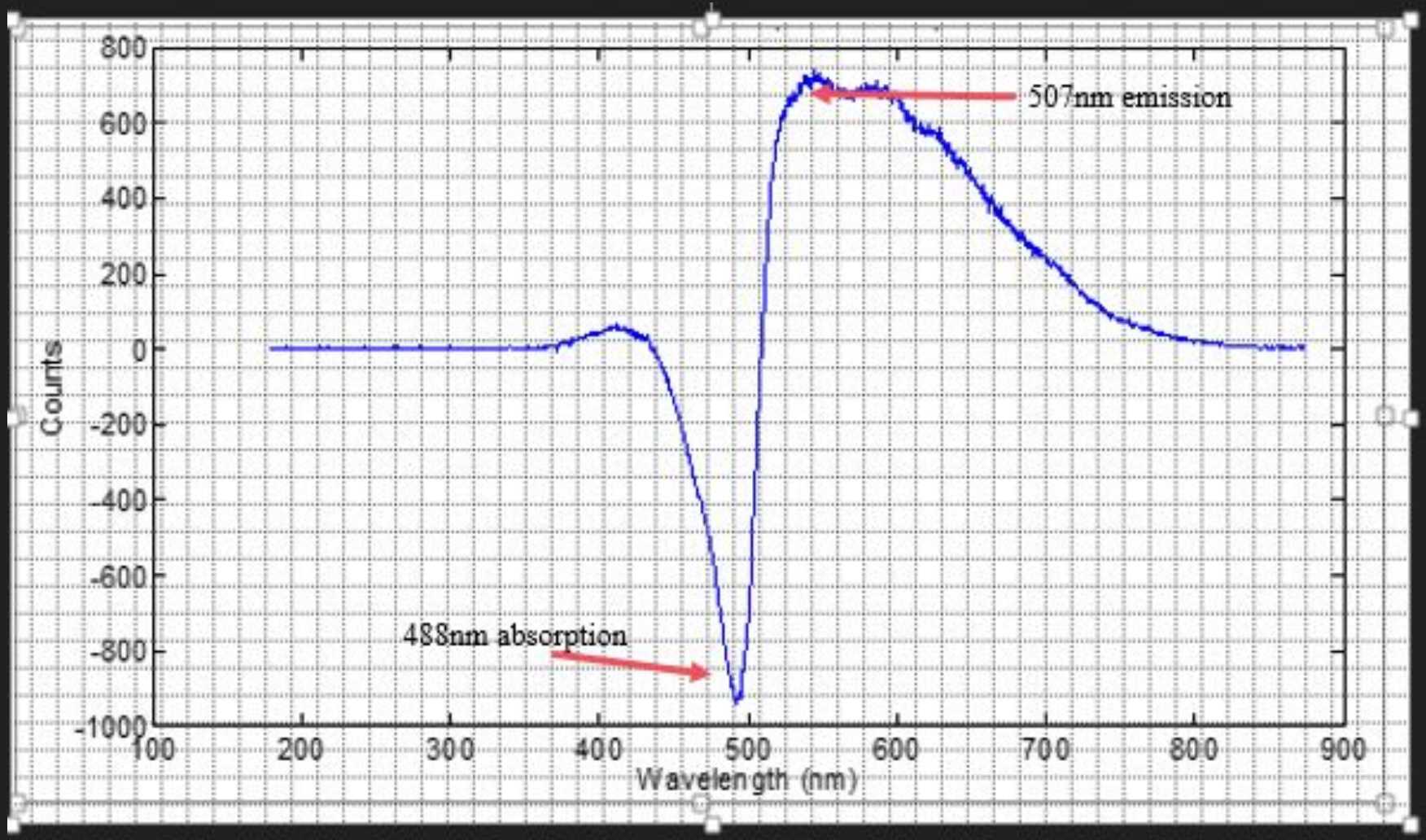


Fig. 6: 488nm absorption and 507nm emission of 250mM Imidazole nickel affinity chromatography elution fraction.

Wavelengths were obtained by software subtraction of background emission measurement from unsaturated emission measurement. EGFP was purified with optimised purification and spectrally analysed.

Applications:

- Monomeric EGFP is gold standard for other fluorescent protein variants.
- Gram negative lipid A and LPS detection.
- Toxicity biosensors to detect SDS and heavy metals.
- Delivery of EGF1 domain peptide to clots by nanoparticles.
- pH sensitive GFPs in synaptic vesicles to track neurotransmitter migration by pH change and fluorescence.
- Study of mitosis and malignancy in live animals.
- Fluorescent protein indicators to detect and measure redox potential, calcium, membrane potential.

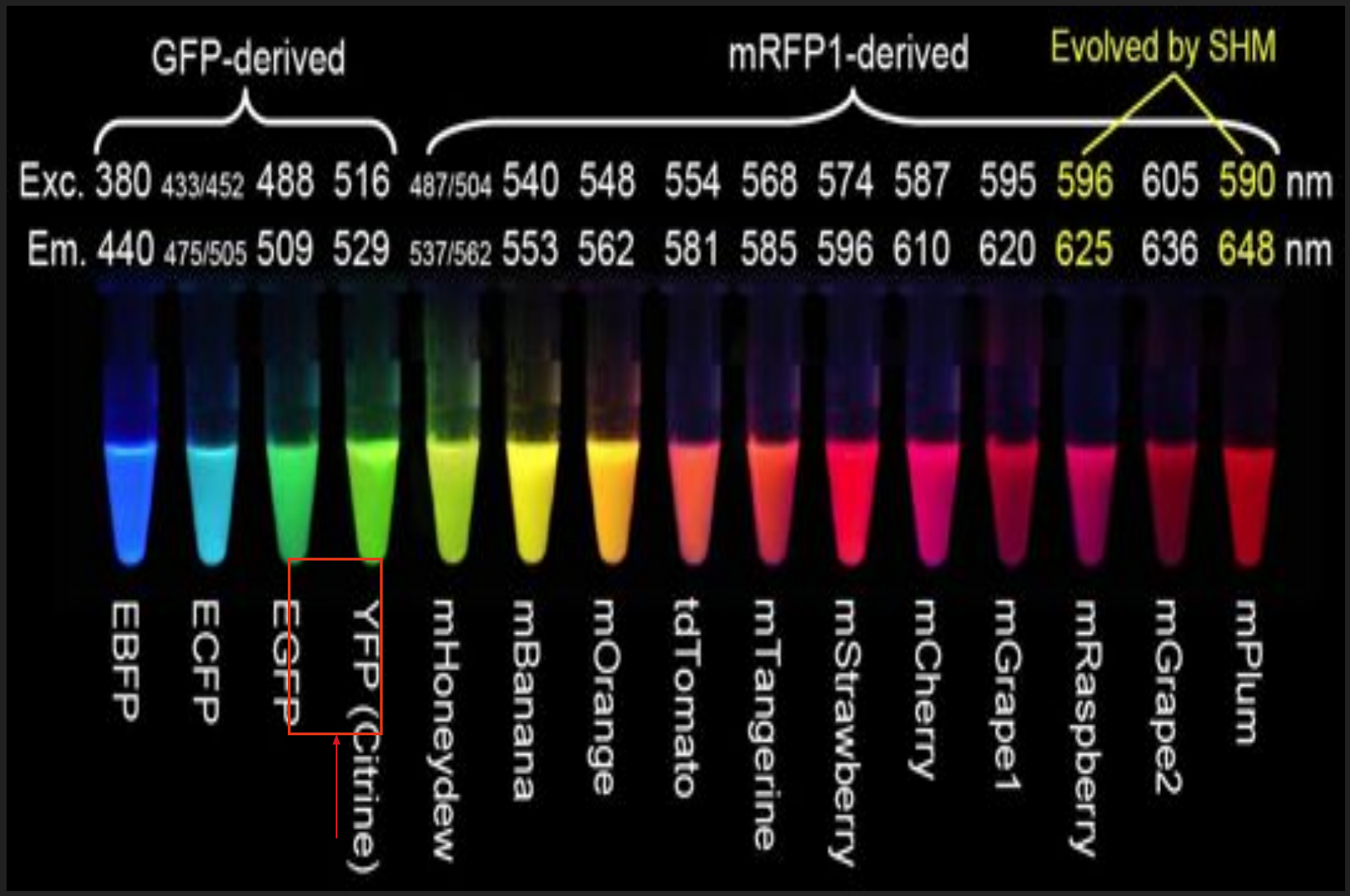


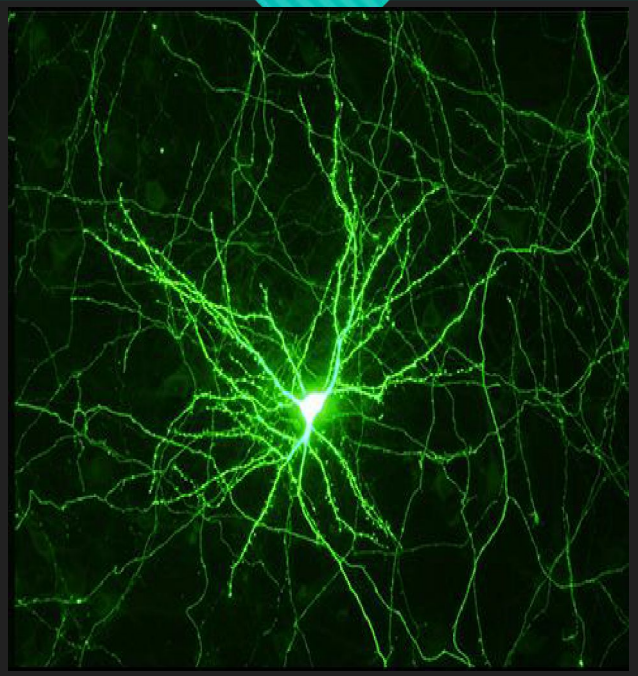
Fig. 7: Fluorescent protein types adapted from native GFP (Tsien, 2009).

Literature references:

- Bornhorst B., Falke J. (2000) "[16] Purification of Proteins Using Polyhistidine Affinity Tags", *Methods in Enzymology; 326: 245-254.*
- Donna E. Crone, Yao-Ming Huang, Derek J. Pitman, Christian Schenkelberg, Keith Fraser, Stephen Macari and Christopher Bystroff (2013). GFP-Based Biosensors, State of the Art in Biosensors - General Aspects, Dr. Toonika Rinken (Ed.), *InTech*.

- Price, W., Handelman, S., Everett, J., Tong, S., Bracic, A., Luff, J., Naumov, V., Acton, T., Manor, P., Xiao, R., Rost, B., Montelione, G., Hunt, J. (2011) "Large-scale experimental studies show unexpected amino acid effects on protein expression and solubility in vivo in *E. coli* ", *Microb Inform Exp*, 1(1), 6.
- Saraswat, M., Musante, L., Ravidá, A., Shortt, B., Byrne, B., Holthofer, H. (2013) "Preparative Purification of Recombinant Proteins: Current Status and Future Trends", *BioMed Research International*, 2013, 1-18.
- Tsien, R. (2009) "Constructing and Exploiting the Fluorescent Protein Paintbox (Nobel Lecture)", *Angewandte Chemie International Edition*, 48(31), 5612-5626.
- Yang, J., Tauschek, M., Robins-Browne, R. (2011) "Control of bacterial virulence by AraC-like regulators that respond to chemical signals", *Trends in Microbiology*, 19(3), 128-135.

Questions?



Thank You for making me glow green. I am not pigmented. I only produce EGFP. Native GFP fluoresces in the UV-spectrum a lot more. EGFP is spectacular in the visible-spectrum.

Expected Questions:

Addgene plasmid handbook: DH10B *E. coli*:
F- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ (ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ-

TOP10 (Invitrogen):
F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ (ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$

- Very similar to DH10B- Dr Damian Deckiert stated that Top10 cells are similar to DH10B but most likely rebranded as Top10 cells.

Copied text from *E. coli* genotypes.
http://openwetware.org/wiki/E._coli_genotypes#TOP10_.28Invitrogen.29

HeLa cells in genotoxicity studies most common human cell line
(cervical cancer-Henrietta Lachs- George Gey). Polio virus. Cloned in 1955.

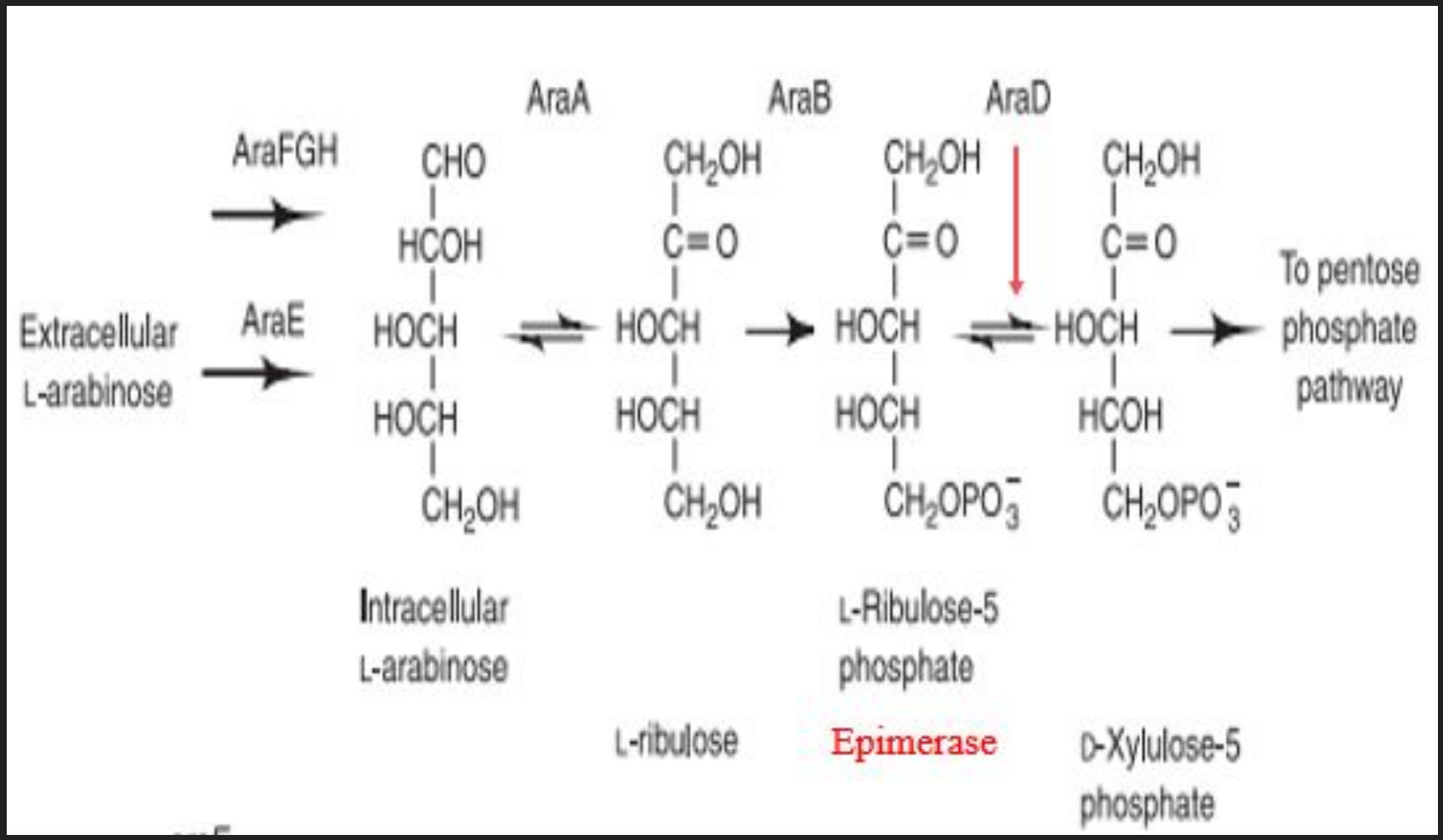


Fig. 8: Arabinose metabolism pathway from extracellular L-arabinose to pentose-phosphate pathway.

Schleif, R. (2010) "AraC protein, regulation of the l-arabinose operon inEscherichia coli, and the light switch mechanism of AraC action", *FEMS Microbiology Reviews*, 34(5), 779-796.