Analysis of green fluorescent protein bioluminescence in vivo and in vitro using a glow discharge

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Abstract. The discovery of fluorescent proteins has been a revolution in cell biology and related sciences because of their many applications, mainly emphasizing their use as cellular markers. The green fluorescent protein (GFP) is one of the most used as it requires no cofactors to generate fluorescence and retains this property into any organism when it is expressed by recombinant DNA techniques, which is a great advantage. In this work, we analyze the emission spectra of recombinant green fluorescent protein in vivo and in vitro exposed to a glow discharge plasma of nitrogen in order to relate electron temperature to fluorescence intensity.

1. Introduction
The green fluorescent protein (GFP) is produced by bioluminescent organisms such as the jellyfish Aequorea victoria, and the IndoPacific sea anemone relative Discosoma; this protein has two excitation maxima at 395 and 470 nm, and a single major emission peak at 509 nm which enable it to exhibit intrinsic fluorescence. The GFP has become the most important marker in cell and molecular biology and a very useful biosensor, since it is used as reporter gene, marker for tumor cells, fusion tag with cloned genes in cellular dynamics studies, or arsenic traces detector in water. In this work, the recombinant GFP was expressed by competent Escherichia coli cells and purified at room temperature employing hydrophobic interaction chromatography. Both systems, the transformed cells expressing GFP and the purified recombinant protein, were exposed into a glow discharge produced by a continuous gas flow at different pressures of N₂.

2. Background
The GFP has two excitation peaks, the first near to 395 nm, the second around 475 nm; and also two emission peaks, if excited at the 395 nm peak, the emission is at 508 nm, and on the contrary, if excited at 475 nm, then the broadcast will appear at 503 nm. When this occurs the protein captures more light outside the visible spectrum and the emission becomes visible green light [1-6]. GFP is conformed by a chain of 238 amino acid residues, forming 11 beta strands, organized in a cylinder that

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encloses an alpha helix. It contains a special chromophore (constituted by 3 consecutive amino acids: Ser, Tyr and Gly), a chemical group that absorbs and emits light. After synthesis of the protein, three amino acids go through a cyclization and spontaneous oxidation. The carbon atom in the serine’s carbonyl forms a covalent linkage to the N atom of the amino group of glycine, followed by removal of water and oxidation of C alfa link – C beta tyrosine to form a double bond. The structure obtained contains a system of conjugated double bonds which gives the property of fluorescence [1, 7-11].

Fluorescent proteins have many applications in science; one of the most common is their use as markers fused to polypeptides or indicators of intracellular chemical - physiological states, in all types of cells, cellular subcompartments and organisms from prokaryotes to mammals. If GFP is attached to other proteins in which the fluorescence depends on the environmental conditions, such compound molecule operates as a sensor in monitoring several cellular complex processes, such as intracellular temperature, the activity and signs displayed in cascades, pH changes, dynamics of second messengers, protein interactions and conformational changes [1,12].

3. Method
3.1. GFP expression and purification
Green fluorescent protein was expressed in Escherichia coli competent cells, HB101 K-12 strain. Individual colonies of 1.5 mm in diameter grown in LB solid medium 4%, were transformed with 10 µl of pGLO plasmid at a concentration of 0.08 µg/ml in 1 ml of transformation solution (50 mM CaCl2, pH 6.1) with 1 ml of LB liquid medium 4%. Subsequently, they were cultured on LB solid medium 4%, supplemented with ampicillin (0.1 g/L) and L - arabinose (5 g/L) at 37 °C for 24 h. The expression was monitored using a long wavelength UV lamp. After 24 h, the transformation efficiency was determined. Individual colonies expressing GFP were grown in tubes containing 2 ml of LB/amp/ara broth [ 4% LB, ampicillin (0.1 g/L) and L - arabinose (2 g/L)] with vigorous shaking of 200 rpm at 32° C for 24 h . They were then harvested by centrifugation at 13 300 rpm for 5 min and resuspended in 250 µl of Tris - EDTA buffer (TE; 10 mM Tris, 1 mM EDTA, pH 8.0). The cells were lysed by incubation with lysozyme followed by freezing for 24 h. After thawing, they were subjected to centrifugation at 13 300 rpm for 10 min to separate cell debris. GFP was purified at room temperature on columns of hydrophobic interaction chromatography (8 x 40 mm) pre-packed with methyl resins (Macro-Prep®) and pre-equilibrated with 2 ml of equilibration buffer (2M (NH4)2SO4/TE , pH 8.0). For each 250 µl of supernatant, there were used 250 µl of binding buffer (4M (NH4)2SO4/TE , pH 8.0) and 250 µl of wash buffer (1.3 M (NH4)2SO4/TE , pH 8.0), followed by 750 µl of TE as elution buffer. All reagents were obtained from BioRad.

3.2. Spectroscopies
Both systems, transformed cells expressing GFP and the purified recombinant protein in TE buffer, were exposed to a glow discharge produced by a continuous flow of N2. The experimental setup consisted of a cylindrical chamber with two circular aluminum plate electrodes placed at its center. Prior to discharge, it was held a vacuum of 10⁻³ Torr in order to remove impurities and/or residual gases. In the course of the experiment the pressure was 1 Torr for N2. To generate the plasma, a potential difference was applied to the electrodes, regulated by a power source which provided direct current (V = 238 V, I = 50 mA).

For analysis in vivo, the expressing GFP bacterial colonies on solid medium were mounted in a 1 cm path length quartz cell, using as controls the LB/amp/ara medium without bacteria and LB/amp bacteria. For analysis in vitro, 3.1 ml of GFP in TE buffer analysis were placed in a quartz cell of 1 cm path length, using TE buffer as control. Emission spectra were observed for both systems in the wavelength range 200 to 1100 nm, using an Ocean Optics spectrometer HR4000CG with 0.15 nm resolution.
4. Results
Green Fluorescent Protein was successfully expressed using the plasmid pGLO and HB101 K-12 strain of *Escherichia coli*. The efficiency of transformation was on the order of $10^3$ transformers per microgram of DNA. The phenotype of the bacterial colonies is consistent with the expected and reported in the literature. In the region of 500-600 nm, it was observed an emission band (in vivo and in vitro) in which the most intense line is N$_2^+$. Such band is not typical of a glow discharge of nitrogen at the experimental conditions, so it was attributable to the protein composition, culture medium and/or TE buffer. The fluorescence intensity at 509 nm is evidence that N$_2$ plasma radiates with sufficient energy to activate the GFP chromophore (figures 1, 2).

![Figure 1](image1.png)  
**Figure 1.** Analysis *in vivo*. Bacteria in culture medium exposed to a nitrogen plasma.

![Figure 2](image2.png)  
**Figure 2.** Analysis *in vitro*. Left: Control sample which contains TE buffer. Right: Sample of green fluorescent protein (GFP) dissolved in TE buffer.

The figure 3 shows the emission spectra of GFP *in vivo* (bacterial colonies on solid LB/amp/ara medium) and *in vitro* (TE buffer) in which emission lines and bands corresponds to a typical N$_2$ plasma generated by a glow discharge, also we can observe the presence of a band in the 500-600 nm range whose species is N$_2^+$, and a peak at 509 nm corresponding to the fluorescent emission of the GFP.

![Figure 3](image3.png)  
**Figure 3.** Emission spectra of GFP *in vivo* and *in vitro*. 
5. Conclusions
The fluorescence intensity of in vivo and in vitro systems did not exhibit measurable changes at exposition times into the glow discharge, this is indicative that GFP molecules do not suffer significant changes in their native structure under the experimental conditions.

The experiments showed the advantages of using a glow discharge as external source in order to stimulate the chromophore. We expect that the sample exposition to a more intense plasma will provide favorable conditions to analyze the protein fluorescence anisotropy.

Acknowledgments
The authors wish to thank Autonomous University of State of Mexico for financial support through project ID 3697/2014/CID.

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