Optical nanobiotechnologies for the intracellular study of protein functions

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In the post-genomic era, the main scientific issue in the field of molecular biology is the understanding of protein function in terms of dynamics, working environment, and interaction with other proteins, within living cells.

In the framework of NEST activities, a major goal is the development of novel optical nanobiotechnologies for the study of protein function. To achieve it, we have adopted a combined approach of biomolecular physics, biochemistry, and molecular biology, which mainly entails the use of intrinsic fluorescent biomolecules as probes, and high-resolution, high-sensitivity spectroscopy as detection method.

Development of new mutants of GFP with improved photophysical characteristics

One of the GFP mutants previously developed at NEST center, E^2GFP, was showed to possess photochromic characteristics advantageous for long-lasting imaging of protein trafficking within cells as well as application in bioelectronic data storage [1,2,3]. However, the strong dependence of E^2GFP fluorescence upon the presence of chloride ions appeared a major obstacle for its use in biological environments enriched in Cl^-. Indeed, at constant physiological pH of 7.4, E^2GFP fluorescence decreases of 5- to 10-fold (Fig. 1) with increasing chloride concentration with a Kd of about 22 mM as precisely measured by absorption spectroscopy (Fig. 2).

E^2GFP is basically a GFP optimized for transcription and folding with 2 additional mutations: S65T and T203Y. In order to dissect the single mutation contribution to the E^2GFP properties we expressed and purified by 2-step chromatography (affinity followed by anion exchange) the 4 mutants: wt (E^0GFP), S65T (EGFP or enhanced GFP clontech), T203Y (E'GFP) and S65T-T203Y (E^2GFP). We discovered that E'GFP can be reactivated likewise E^2GFP while E^0GFP and EGFP cannot. Moreover the new E'GFP mutant has a much stronger fluorescence resistance to chloride suggesting its preferential adoption for biological imaging applications requiring photochromism.

GFP mutants as biosensor of intracellular pH

Intracellular pH is an important modulator of cell function. For instance, many enzymes exhibit pH dependence in the physiological range such that their activities are affected by small variations in intracellular pH [4]. Therefore, monitoring of pH at cytoplasmic as well as subcellular localization may in some cases be a key factor to understand protein function.

New mutants of the green fluorescent protein that operate as pH indicators have been synthesized in the past; however, most of them suffer of major drawbacks, namely: 1) poor sensitivity in the physiological range of pH, 2) dependence of optical response on the degree of GFP transfection [5].

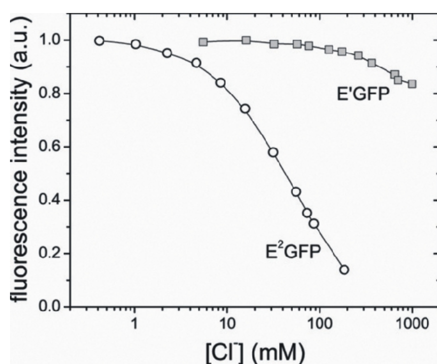

Fig. 1

Fig. 1
Comparison between fluorescence intensity of EGFP (F64L, S65T) and E^2GFP (F64L, S65T, T203Y) as function of chloride concentration at pH 7.4

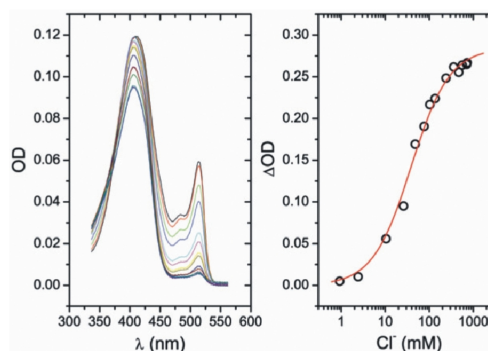

Fig. 2

Fig. 2
Left: absorption spectra of E^2GFP at different chloride concentration and at pH 7.4; right: optical density at 514 nm vs chloride concentration

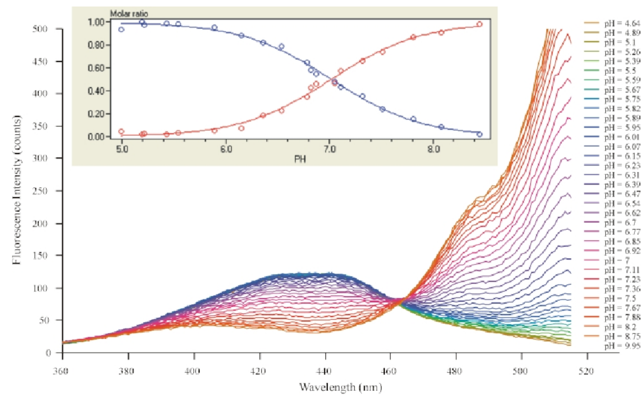

Fig. 3
Excitation spectra of E^{2}GFP at pH ranging between 4 and 9. Inset: molar fraction of protonated and deprotonated chromophore and pKa determination from absorption studies

In 2004, we developed a new GFP-based pH biosensor that is not limited by such problems. Indeed, the photophysical characterization of the GFP mutant E^{2}GFP, previously developed at NEST, showed that: 1) interconversion equilibrium between the protonated and the deprotonated states of the chromophore has a pK=7, and 2) excitation spectra taken a different pHs show an isosbestic (i.e. not changing) point around 458 nm (Fig. 3).

Therefore, the ratio of E^{2}GFP fluorescence excited at 488 nm and 458 nm (typical emission lines of a simple Ar-laser) is dependent on medium pH regardless of protein concentration, and display the maximum sensitivity around pH=7. Accordingly, we were able to create an absolute calibration curve of fluorescent ratio vs pH in living cells (CHO-A1 and HeLa) (Fig. 4), and subsequently to monitor physiological changes of intracellular pH upon external stimulation with high sensitivity.

Cellular trafficking of the HIV-1 transactivating protein (Tat)

The Tat protein of human immunodeficiency virus type 1 (HIV-1) is an 86- to 101-amino-acid regulatory protein that transactivates HIV-1 provirus expression.

Besides having transcriptional functions at the HIV promoter, the Tat polypeptide is also unique in that it is released by expressing cells and enters non-producing cells when present in the extracellular environment [6].

Tat released by infected cells is likely to exert autocrine and paracrine activities with possible important implications for HIV disease pathogenesis.

The capacity to cross cell membranes depends upon the integrity of the basic region of the protein, a 9-amino acid, arginine-rich sequence (protein transduction domain or PTD) that also corresponds to the nuclear localization signal and the TAR-binding domain of the protein [7].

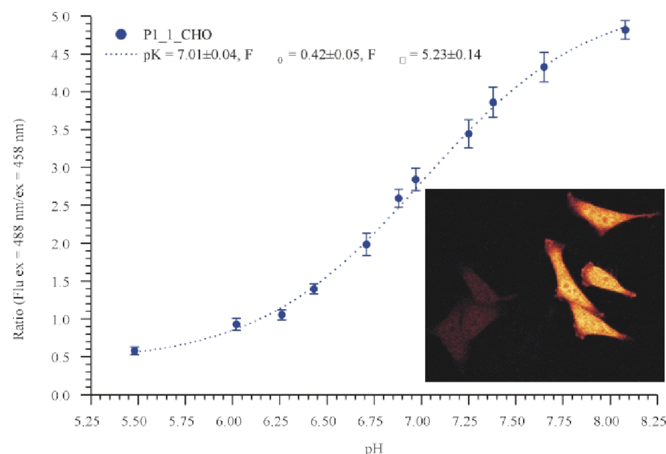

Fig. 4
Calibration curve of fluorescence emission ratio vs pH for E^{2}GFP in CHO-A1 cells; inset: HL3T1 cells expressing E^{2}GFP at pH=7.4

The ability of exogenous Tat to be taken up by cells has generated great interest owing to the potential therapeutic applications of this molecule, as it could be used as a vector for delivering heterogeneous proteins and drugs that would otherwise not have access to the intracellular environment [8]. In the framework of our previous studies on Tat intracellular trafficking and localization [9,10,11], our 2004 activity tried to elucidate the type of endocytic pathway utilized by Tat fusion proteins and determine the kinetics of this process within the cell, with the perspective of using Tat-mediated transduction for molecular therapy applications. Following our previous results on the internalization of Tat fused to green fluorescent protein and glutathione S-transferase (GST-Tat-eGFP) through the caveolar pathway [10], we have decided to exploit the properties of novel Tat recombinant proteins. The first one is a Tat-GFP fusion protein lacking the GST cargo, purified by heparin-affinity chromatography. The protein obtained is highly biologically active and monomeric as we revealed by a Luciferase Assay for transactivation and a size-exclusion chromatography, respectively.

We added Tat-GFP fusion protein to HL3T1 and CHO cultured cells and performed live-cell time-lapse analysis by confocal microscopy (Fig. 5).

This recombinant protein has shown the same internalization pathway as the GST-Tat-GFP revealing no artifacts due to the presence of the GST cargo. As in the case of GST-Tat-GFP fusion protein we were still not able to detect the nuclear accumulation of Tat-GFP. One possible explanation of GFP fluorescence loss is that an unfolding step is necessary for Tat fusion protein nuclear translocation. In order to overcome this problem we have

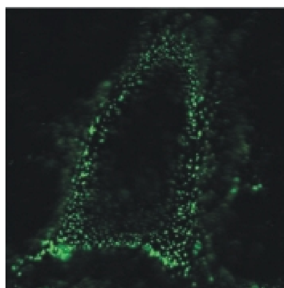

Fig. 5
Fluorescence micrograph of Tat-EGFP internalization in a living HeLa cell; fluorescent clusters represent caveolar organization of Tat-EGFP, similar to that observed for GST-Tat-GFP [10]

engineered a second new recombinant protein: a full-length Tat fused to a tetracysteine peptide (CCAACC) that can be labeled using a fluorescein containing two arsenoxides (FlAsH). Biarsenical fluorophores are fluorescent labeling reagent of proteins that are reactive to genetically engineered four close cysteines within a tetracysteine motif (Cys-Cys-Xaa-Xaa-Cys-Cys, where Xaa is a noncysteine amino acid) [12]. The reaction is entropically favored and biarsenical fluorophores react selectively to the tetracystein motif rather than other cysteines even in the living cells. When the labeling reagent binds to the tetracysteine motif, there is a strong increase of fluorescence emission. The syntesis of FlAsH was carried out by reaction of a mercury derivative of fluorescein with arsenic trichloride, in presence of palladium acetate as transmetallation catalyst (Fig. 6). Future activity will focus on the selective binding of FlAsH to Tat-tetracysteine and monitoring of its activity within the cell.

Fluorescence imaging of cyclin T1 kinetics in living cells

Cyclin T1 is the cyclin partner of the CDK9 kinase forming the pTEFb complex, a key factor in the regulation of HIV transcription. Transcriptional regulation in mammalian cells is a highly dynamic process, requiring temporal and spatial

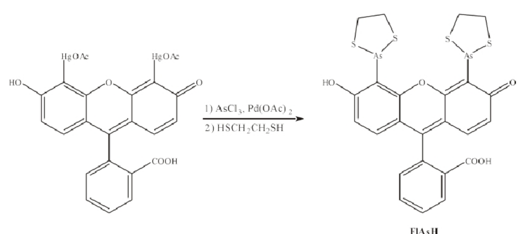

Fig. 6
Synthesis of FlAsH from fluorescein mercuric acetate.

coordination of functional protein complex assembly. Several of the factors participating in these events are found to reside in specific sub-nuclear compartments. In particular, cyclin T1 is recruited to promyelocytic leukaemia (PML) nuclear bodies thought direct interaction, as demonstrated by FRET experiments [13].
The kinetic properties of cyclin T1 in nucleus of living cells were determined by analysis of the fluorescence recovery curves obtained after photobleaching (FRAP) (Fig 7a-b).

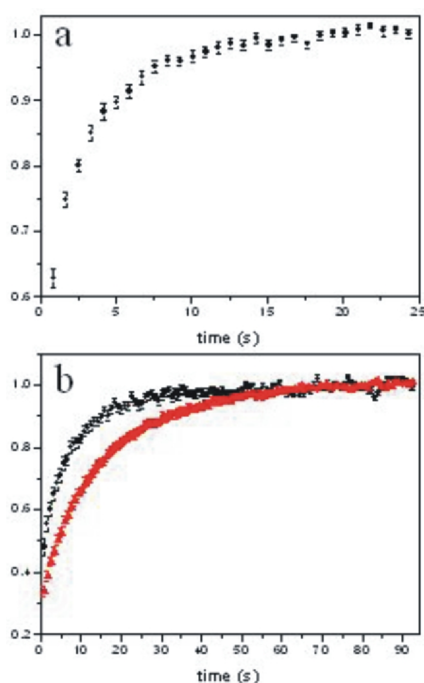

Fig. 7
FRAP of cyclin T1 within the nucleus. (a) Kinetics of recovery after bleaching of YFP-cyclin T1 within nucleoplasm. Recovery of the fluorescence signal reached a plateau after about 20 s. The time for half recovery was less than 1.2 s. (b) Kinetics of recovery after bleaching of YFP-cyclin T1 within PML nuclear bodies compartments. Recovery of fluorescence signal indicates when a protein-protein interaction occur: reassociation of cyclinT1 with PML was rapid in absence of a positive FRET signal (white curve); the presence of positive FRET signal between cyclin T1 and pml protein slower the reassociation kinetics of YFP-cyclin T1 (grey curve).

YFP-Cyclin T1 moves rapidly throughout the nucleoplasm; recovery was complete within 20 s, with a half time of about 1.15 s (Fig. 7a). The diffusion coefficient, D, value was 1.2 mm^2 s^{-1} which is consistent with a diffusion mechanism [14].
PML nuclear bodies represent stable, static sub-nuclear protein compartments; cyclin T1 might move rapidly in and out of PML nuclear bodies. The kinetics of cyclin T1 within the PML nuclear bodies reveals two different recovery curves (Fig. 7b). Interestingly, such different kinetics reflects the presence of direct interaction with PML (as assessed by FRET experiments in vivo). In absence of a positive FRET signal the reassociation of cyclin T1 with PML was rapid; fluorescence recovery was complete within 30 s, and the time for half recovery was less than 3 s. The D value was 0.54 mm^2 s^{-1} indicating that cyclin T1 continously and rapidly associates with the PML nuclear bodies. On the contrary, the presence of positive FRET signal between cyclin T1 and PML, slower the reassociation kinetics of cyclin T1: the time for half recovery shift from 3 s to 8.3 s with D value of about 0.17 mm^2 s^{-1}. These observations strongly support the model in which the presence of cyclin T1 in the nucleoplasm matrix is regulated and coordinated though direct interaction of the PML protein [13].

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