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Towards single-molecule observation of protein synthesis

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Sumario. El ribosoma es el motor molecular responsable de la síntesis de proteínas dentro de las células. El movimiento del ribosoma a lo largo del RNA mensajero (mRNA) para leer el código genético es asincrónico y ocurre a través de múltiples vías cinéticas. Consecuentemente, un estudio a nivel de una sola macromolécula es deseable a fin de poder desentrañar las complejas dinámicas involucradas. En esta comunicación, se presenta el desarrollo de una química de superficie de avanzada para fijar un ribosoma activo al cubre-objeto (coverslip) del microscopio y seguir la incorporación de aminoácidos mediante microscopía de fluorescencia. El ribosona es etiqueteado con un punto cuántico (QD) con el propósito de localizarlo en la superficie, mientras que un aminoácido específico (lisina) es marcado con Bodipy-FL. Este tinte fluorescente es lo suficientemente pequeño para acceder a los canales ribosomales, dejando así, intacta, la actividad ribosomal. De esta manera se posibilita observar la síntesis de proteínas a tiempo real a medida que los aminoácidos etiqueteados son incorporados en la cadena polipeptídica

Abstract. The ribosome is the molecular motor responsible for the protein synthesis within all cells. Ribosome motions along the messenger RNA (mRNA) to read the genetic code are asynchronous and occur along multiple kinetic paths. Consequently, a study at the single macromolecule level is desirable to unravel the complex dynamics involved. In this communication, we present the development of an advanced surface chemistry to attach an active ribosome to the microscope coverslip and follow the amino-acid incorporation by fluorescence microscopy. The ribosome is labeled with a quantum dot (QD) in order to localize it on the surface while a specific amino acid (lysine) is marked with Bodipy-FL. This fluorescent dye is small enough to enter the ribosomal channel thus leaving intact ribosomal activity. It should then be possible to observe the protein synthesis in real time as the labeled amino acids are incorporated into the polypeptide chain.

Key words. Effect of visible radiation in biological systems 87.50.W, Laser diodes, 42.55.Px

1 Introduction

Proteins are synthetized in all cells by the ribosome, a molecular motor that reads the genetic code carried by the messenger RNA (mRNA) and translates it into a chain of amino acids. The reading process takes place as the ribosome moves along the mRNA to translate successive codons, each codon of three nucleotides corresponding to one specific amino acid. The aminoacids are attached to transfer RNA (tRNA) that carry the

matching anticodon. The ribosome associates the codon and anticodon and catalyses peptide bond formation. The motions of the ribosome along the mRNA required to perform these functions are asynchronous and follow multiple kinetic paths, depending for example on when the proper aminoacid attached to its tRNA (aa-tRNA) will find its way into the ribosome, or whether secondary structures on mRNA will have to be melted by the ribosome to go on with the translation. Consequently, a study at the single molecule level is desirable to unravel the complex dynamics involved.

Cryo-electron microscopy and X-ray diffraction studies have given precious information about the structure of the ribosome and its factors [1-4]. Optical microscopy does not have the required resolution, but it provides us with a non invasive method to study the ribosome dynamics in real time. The resolution of an optical microscope, limited by diffraction, does not allow us to observe directly molecules that are 20nm or smaller, but specific labeling either by fluorescent markers, or using micron-size beads, makes it possible to detect single molecules. The typical time scale for the ribosome motion along the mRNA is of a few aminoacids incorporated per second, and it can even be slower in vitro. This rate is well suited to the tenth of milliseconds required to observe the fluorescence of a single molecule with a good signal to noise ratio.

In recent years, single molecule fluorescence microscopy has brought new information about the motions involved in the process of one aminoacid incorporation, in particular about conformational changes detected using Förster Resonance Energy Transfer (FRET)[5,6]. A few experiments have even been able to observe several elongation steps on a single ribosome attached to a surface either directly[7,8] or via one end of the mRNA[9]. In these experiments, ribosomal activity was monitored either via the motion of a micron size bead attached to the 3' end of the mRNA[7] or controlled using optical tweezers[9], or through the fluorescence of the resulting protein GFP after synthesis and folding[8].

2 Experimental setup

Our setup uses total internal reflection single-molecule fluorescence microscopy (TIRFM) to study kinetics of amino-acid incorporation inside the growing protein. The mRNA-ribosome complex is attached to a polyethylene glycol (PEG)-modified coverslip surface by a streptavidin-biotin linkage. The ribosome is labeled with a quantum dot (QD) to be localized on the surface while a specific amino acid (lysine) is marked with Bodipy FL, a fluorescent dye small enough to enter the ribosome canal without preventing its activity. The protein synthesis can then be observed in real time as the labeled amino acids are incorporated into the polypeptide chain (See Fig. 1). In spite of its relatively low photostability, we previously showed that we are able to observe single molecules of Bodipy-FL with exposure times small enough to allow monitoring of translation in vitro[10].

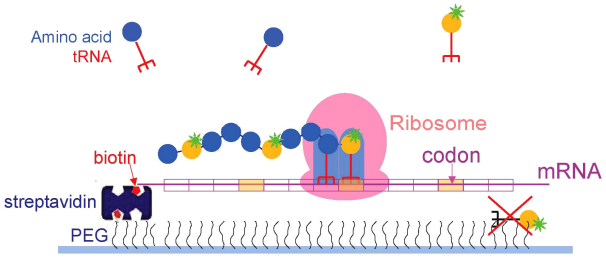

Figure 1. General principle of our experiment. A ribosome complexed with its mRNA and tRNA is attached to a PEG coated surface via a biotin-streptavidin linkage. Specific aminoacids (lysine, in yellow) are labeled with a fluorescent marker (Bodipy) making it possible to observe their incorporation into the protein by the ribosome. The PEG surface is further passivated to reduce non-specific binding of tRNA-lysine-Bodipy directly to the surface.

Excitation of the two fluorophores is performed using an objective based total internal reflection microscopy setup, based on an inverted Olympus IX70 microscope (see fig.2). The excitation laser is reflected at the interface between a microscope coverslip and the sample, so that only an evanescent wave is exciting the medium, over a distance of approximately 100nm above the surface. In that way, only the fluorophores staying very close to the surface for the whole observation time, i.e. attached to it, will be detected. The laser at 488nm can excite both the Bodipy molecules and the quantum dot, but we can use another laser at 638nm to excite the quantum dot only, so that we avoid photobleaching the bodipy prematurely. The two fluorescence spectra are well separated (the bodipy fluoresces between 510 and 600nm while our quantum dot emits around 655nm), so that we can detect them separately using different sets of beamplitters and filters. This can be performed either successively by switching beamsplitter and filter in the microscope or simultaneously using half the field of view of the camera for each fluorophore.

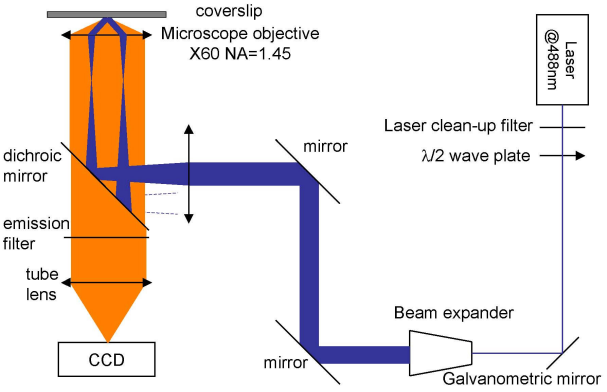

Figure 2: Schematics of our optical setup.

In figure 2 the Bodipy-FL molecules are excited by an evanescent wave from a diode-pumped frequency dou-

bled solid-state laser emitting at 488nm. The laser beam is expanded and focused in the back focal plane of a microscope objective (NA 1.45) to create a parallel beam at an angle of incidence larger than the limit angle between the microscope coverslip and the sample. The half wave plate rotates the polarization of the laser such that it ends up linearly polarized in the plane of incidence of the total internal reflection (TM polarization). The clean-up filter is centered at 488nm with a width of 10nm, to remove any higher wavelength that could end up in the fluorescence channel. The Bodipy fluorescence is collected by the large aperture microscope objective and isolated from the excitation laser thanks to a dichroic beamsplitter (HQ535/50) and an emission filter (Q505LP). It is detected by an electron multiplying charge coupled device (EMCCD) camera (C9100, Hamamatsu), which pixel resolution is 260 nm in binning 2x2. The galvanometric mirror controls the excitation time, synchronized with the camera acquisition, to avoid photobleaching the bodipy molecules prematurely. To excite the quantum dots (QD655) only, we replace the 488nm laser by a diode laser at 638nm, and switch to a different set of beamsplitter and filter (Q660LP and HQ665LP). When we wish to detect the two markers simultaneously, we can excite them both with the 488nm laser through a dichroic beamsplitter, and separate the two fluorescence spectra (using extra beamsplitters) on two separate areas of the CCD camera. The field of view is then reduced by half.

To monitor the motion of one ribosome over the whole translation process, we have to attach it to a surface, here the microscope coverslip, in such a way that it is still free enough to move and perform its function. This step is one of the main difficulties of this experiment. For that purpose we have used a polyethylene glycol (PEG)-modified glass coverslip surface where a fraction of the PEG have a biotin at their extremity, as described in ref. [11]. The surface is then covered with streptavidin, so that other biotinylated molecules can be attached to it. Several strategies have to be used to attach a biotin at the end of the mRNA: either through hybridation of the mRNA to a biotinylated single strand DNA, or by direct biotinylation of the mRNA (see figure 1). In order to observe protein synthesis in real time, we have designed a microfluidic system where we can electrically control the injection into our sample (see fig. 3).

In order to understand the influence of mRNA secondary structures on the ribosome activity we develop a complementary experiment in which we apply a control force at one end of the mRNA with an optical tweezer. For this purpose, a microsphere is attached to the end of the mRNA. It is then manipulated using the optical trap created near the coverslip by a YAG laser beam focused through the same high numerical aperture microscope objective than the one used for TIRFM. Ultimately, both methods can be applied simultaneously for studying the influence of controllable perturbations or friction on the translation kinetics (fig. 4).

3 Conclusions

We present a method, including advanced chemical, biochemical and optical techniques, to study in real time the translation at the single molecule level. Using a two-color fluorescence microscopy setup, it will be possible to co-localize a single ribosome and labeled amino-acids being incorporated to the growing protein.

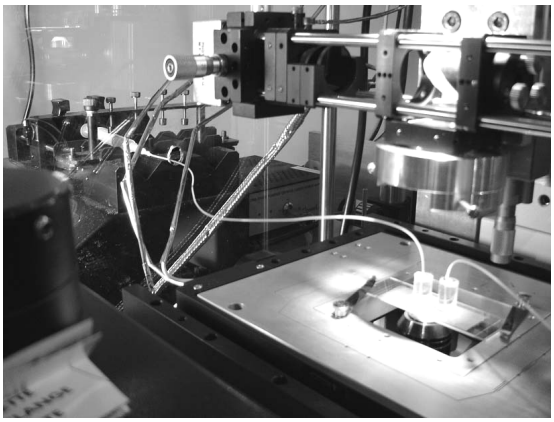

Figure 3: Picture of our microfluidic cell. The microscope coverslip is cleaned and chemically prepared and then taped to a microscope slide with small holes and PDMS blocks through which the reactants are injected for all the following steps of the surface chemistry and of the protein synthesis experiment.

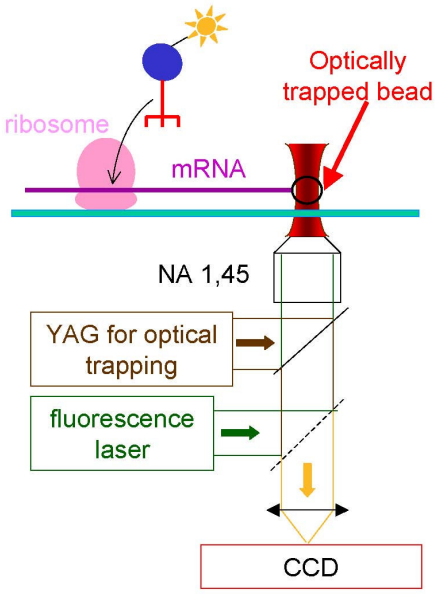

Figure 4: Design of a future experiment where we would combine fluorescence microscopy and optical tweezers to observe the aminoacid being incorporated in the protein while applying a force on the mRNA.

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