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Report on methodologies for the detection and manipulation of single molecules

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Deliverable Nature	
R = Report, P = Prototype, D = Demonstrator, O = Other	R
Dissemination Level	
PU = Public PP = Restricted to other programme participants (incl. the Commission Services) RE = Restricted to a group specified by the consortium (incl. the Commission Services) CO = Confidential, only for members of the consortium (incl. the Commission Services)	PU

A. Abstract / Executive Summary

During the project LENS developed innovative techniques for nanometre single molecule localization, fast single molecule manipulation, and combined manipulation and single molecule imaging,

1) Single molecule localization of single molecules in three dimensions: Live cells are three-dimensional environments where biological molecules move to find their targets and accomplish their functions. However, up to now, most single molecule investigations have been limited to bi-dimensional studies owing to the complexity of 3d-tracking techniques. We developed a novel method for three-dimensional localization of single nano-emitters based on automatic recognition of out-of-focus diffraction patterns. Our technique can be applied to track the movements of single molecules in living cells using a conventional epifluorescence microscope. We first demonstrated three-dimensional localization of fluorescent nanobeads over 4 microns depth with accuracy below 2 nm in vitro. Remarkably, we also established three-dimensional tracking of Quantum Dots, overcoming their anisotropic emission, by adopting a ligation strategy that allows rotational freedom of the emitter combined with proper pattern recognition. We localize commercially available Quantum Dots in living cells with accuracy better than 7 nm over 2 microns depth.

2) Single molecule tracking techniques applied to investigate the molecular basis of Alzheirmer's disease. This approach has allowed discovering new features normally not accessible with standard methods based on data averaging. This study has contributed to add new information on the biology of the toxic Abeta petide oligomers by describing their dynamic behaviour on the plasma membrane of living cells. Additional results on the interaction of Abeta oligomers with components of the plasma membrane have also provided proof of evidence for a mechanism of toxicity that was object of debate in the past. Although the interaction of Abeta oligomers with the cellular membrane is a well-known process, the manner through which they cause cell dysfunction can depend on several factors. The findings related to this deliverable have revealed a new potential mechanism of toxicity attributable to the loss of function of specific membrane components consequent to an alteration of their mobility caused by the binding to slowly diffusing Abeta oligomers. The dynamic behaviour and the membrane effects of toxic amyloid aggregates formed by proteins or peptides other than Abeta have also been object of investigation. Results similar to those obtained in the case of Abeta oligomers were found for amylin (involved in the development of type II diabetes), prion sup35 and beta2-microglobulin (associated with a familial form of systemic amyloidosis), supporting the hypothesis that amyloid diseases share similar mechanisms of toxicity.

3) High-speed optical tweezers. We developed a dual-trap force-clamp configuration that applies constant loads between a binding protein and an intermittently interacting biological polymer. the method has a measurement delay of only ~10 ms, allows detection of interactions as brief as ~100 ms and probes sub-nanometer conformational changes with a time resolution of tens of microseconds. We tested our method on molecular motors and dnA-binding proteins. We could apply constant loads to a single motor domain of myosin before its working stroke was initiated (0.2–1 ms), thus directly measuring its load dependence. We found that, depending on the applied load, myosin weakly interacted (<1 ms) with actin without production of movement, fully developed its working stroke or prematurely detached (<5 ms), thus reducing the working stroke size with load. our technique extends single-molecule force-clamp spectroscopy and opens new avenues for investigating the effects of forces on biological processes.

B. Deliverable Report

1 Introduction

1) Single-molecule techniques represent an innovative approach for biological investigations. Thanks to the possibility to study the behaviour of molecules *in singulo*, during the last two

decades it has been possible to enrich the current knowledge of many biological processes, complementing information derived from bulk experiments, and study the effects of the natural variability between molecules[1]. Single-molecule *in vitro* experiments revealed the machinery of motion by molecular motors and how these enzymes accomplish transportation by cooperative mechanisms[2-7]. On the other hand, the application of single molecule tracking to membrane proteins in living cells disclosed fundamental information about the dynamics and the interplay of different membrane components[8-10]. Essential features of sub-cellular structures and interactions between biomolecules have been revealed thanks to the implementation of single-molecule techniques inside living cells[8, 11]. More recently, the need for accurate molecular localization has been further pushed by the development of super-resolution microscopies based on localization, such as PALM and STORM[12, 13]. Nevertheless, most of these studies are based on two-dimensional imaging, whereas many intracellular processes are strongly influenced by the three-dimensional structure of the cellular environment[14]. Many techniques have been proposed in last years to obtain nanometer axial localization. On one hand, many approaches have been proposed, mainly based on the deformation of the point spread function or on multiplane imaging, which all require custom optical setups to be applied. On the other hand, axial localization within few nanometers was demonstrated through off-focus imaging, by the simple observation of the point spread function of probes imaged out of the focal plane. Here we describe a new method, based on this approach, through which axial localization, as well as x, y localization, is achieved for both fluorescent beads and Quantum Dots in living cells in a standard epifluorescence microscope. We developed an algorithm that automatically measure the three coordinates provided an appropriate calibration. We also describe a method to obtain a fine calibration for both beads and Quantum Dots and discuss our results. Through our algorithm, simultaneous localization of all three dimensions within 2 nm accuracy is achieved, over 4 microns range, for 200 nm fluorescent beads. On the other hand we demonstrate localization within 7 nm, over 2 microns range, for QDots in living cells.

2) It is well established that cytotoxic Abeta oligomers are the key factor that triggers the initial tissue and cell modifications eventually culminating with the development of Alzheimer's disease. Abeta 1-42 oligomers display a high degree of polymorphism, and several structurally different oligomers have been described. Most of the current research on the molecular mechanisms of Alzheimer's disease is however based on averaged results obtained using bulk methods. In this case, many important details can be missed and only the most prominent features are eventually taken into account. This consideration may explain at least part of the discrepancies arising from the several models that have been proposed during the last years. Within this context, we aimed at providing a better understanding of the pathogenesis of Alzheimer's disease by studying the dynamic features of this complex system at a single molecule level.

3) When a molecular bond is weak, the unbinding kinetics becomes rapid and application of single molecule techniques during the short lifetime of the molecular complex becomes challenging. Such molecular interactions include receptors with low- binding-affinity ligands, nonprocessive motors interacting with their tracks and nucleic acid–binding proteins interacting with nonspecific sequences during the target search. Different protein conformations usually have different binding strengths, some of them characterized by rapid unbinding kinetics. Molecular interactions on the millisecond time scale are very common, and single-molecule force spectroscopy of such short-lived molecular complexes requires sub-millisecond resolution and control of the applied load. We developed an optical tweezers technique for the detection of interactions and application of controlled forces on the sub-millisecond time scale.

2 Results

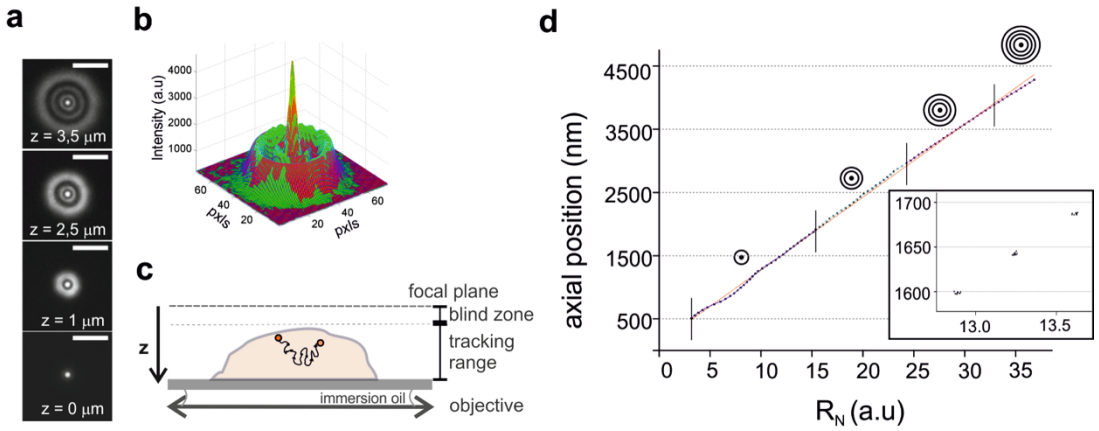

Figure 1 a) 200 nm fluorescent nanobeads embedded in 2% agarose film, approximately 40 μm thick. Nanobeads located at different depths inside the gel produce diffraction patterns of size dependent on the distance z from the focal plane. Scale bar = 3 μm. **b)** A custom algorithm automatically recognizes and analyses the diffraction pattern, by the calculation of a proper function (red surface plot) to fit experimental data (green surface plot). **c)** Sketch of the accessible tracking range. Proper probe defocusing can be obtained by adjusting the position of the focal plane above the sample. In this way, the entire volume of the cell is potentially accessible for tracking. **d)** Calibration curve obtained for fluorescent nanobeads. The cartoons represent the number of rings in different axial regions as indicated by vertical lines. Red curve is a linear fit to the data. Coloured curves are non-linear fits to the data in the different regions. Inset shows a zoom of a region of the calibration curve. At each axial position 10 images of the bead are acquired and analyzed through our algorithm to calculate R_N.

1) The intensity pattern produced by a sub-diffraction object positioned at the focal plane is representative of the PSF of the optical system, and it is well described by a bidimensional Gaussian. If the same object is imaged out of the focal plane its intensity pattern, that is still representative of the PSF of the optical system, can be represented by a diffraction pattern as shown in Fig.1a. In particular, it has been shown that the number of rings in the pattern and its radius are indicative of the distance of the object from the focal plane (Fig.1a)[15, 16]. Based on this property it is possible to produce a calibration curve that unambiguously relates this radius to the z-position of the fluorescent probe relative to the focal plane. First, 200 nm fluorescent beads were embedded in a 2% agarose gel and through a piezo stage the objective was moved in the z-direction by 50 nm steps. 10 images of the same bead were acquired, at each step, by an EMCCD camera and analyzed through a custom made Matlab code to measure the radius of the outermost ring. Our algorithm automatically identifies the number and the location of diffraction rings on a radial intensity profile calculated on a Region Of Interest containing the emitter. These parameters are set as initial values for the fitting function *f*. The function *f* is composed by a central Gaussian peak surrounded by N Gaussian circles. For each image acquired during the calibration procedure described above, the function *f* is fitted to the experimental data to find out the coordinates x_0, y_0 of the central peak of the diffraction pattern and the radius of the outermost ring R_N of the bead (Fig.1b). By plotting R_N as a function of its correspondent axial position a stepwise plot as that shown in fig. 1d is obtained. In the axial range between 0 and 0,5 μm the software is not able to provide any value for R_N, due to the absence of rings in the pattern.

By fitting the plot in fig. 1d with an appropriate nonlinear function a calibration function was obtained, that provides a precise prediction of the axial position of the bead for any given value of R_N. The absolute error to associate to the beads axial localization was calculated as the standard deviation of the axial position predicted by the calibration function between

points belonging to the same axial step in fig.1d (inset). X and y localization error were also evaluated as standard deviations. On average x, y and z localization within 2 nm was obtained over 4 microns range.

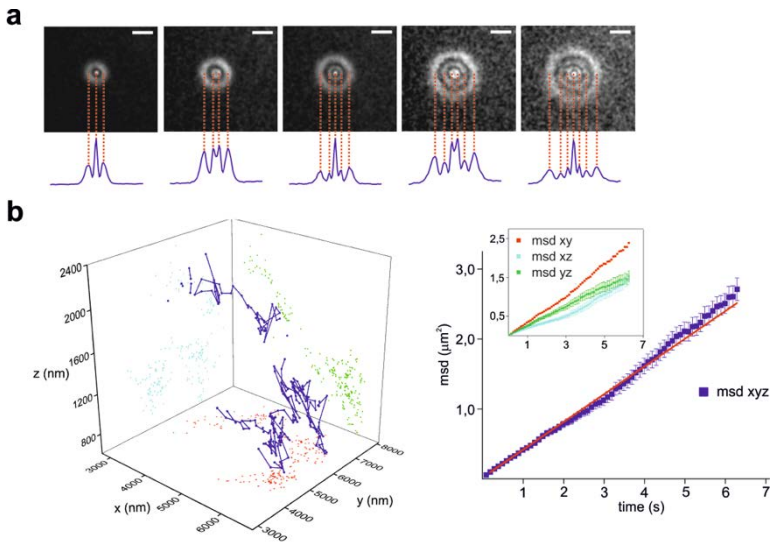

Figure 2 a) Diffraction patterns of an out-of-focus quantum dot (QD655-WGMA, wheat Germ Agglutinin-QD conjugated) targeted to the membrane of a fixed human neuroblastoma cell, at different depths. Profiles are radially isotropic thanks to flexible ligation strategy through PEG linker. Profiles present a central "hole" at specific axial positions. Our algorithm recognizes these cases and chooses a proper fitting function. Scale bar = 2 μm. **b)** Three-dimensional trajectory of GM1 in a living neuroblastoma cell (blue line). Gaps in the QD trajectory correspond to blinking events. **c)** msd analysis of GM1 trajectory displayed in panel a). Blue squares show three-dimensional msd. Red line represents weighted linear regression of data (slope 0.396±0.002 μm2/s). Inset shows msd of trajectory projections along the three planes xy,xz,yz. Error bars are standard errors**.**

We then performed an analogous calibration procedure to characterize our technique in terms of localization accuracy and imaging depth using QDs. Due to the need for a three-dimensional sample we used cells' membrane as a support for QDs. Such a sample has also the advantage of reproducing the typical conditions encountered when performing 3D-tracking experiments in living cells, both in terms of background fluorescence and mechanical stability of the QDs support. GM1 ganglioside on the membrane of fixed human neuroblastome cells was targeted with Wheat Germ Agglutinin conjugated with QDots 655. Images of QDots located at different axial positions were acquired at 150 ms exposure time and 300 EMgain by following the stepping procedure described previously for the case of fluorescent beads (Fig.3a). A calibration function was obtained, similar to that obtained for fluorescent nanobeads, but limited to 2 μm range, due to the much lower S/N ratio in living cell experiment conditions. On average x, y and z localization within 7 nm over 2 microns was obtained for QDots tracked on the membrane of living neuroblastoma cells. The technique was validated in living cells by precisely tracking of GM1 movements on the membrane of a neuroblastoma cell in three dimensions at 100 ms integration time over 23 seconds. As shown in Figure 2b, in this short time the particle could span a volume of about (3 x 4 x 1.4) μm (x,y,z). We could analyse GM1 movements in 3d (Fig. 2c) and separate them along the three spatial directions (fig. 2c inset).

2) The goal of this part of the deliverable was to provide a better understanding of the pathogenesis of Alzheimer's disease by studying the dynamic features of this complex system at a single molecule level. It consisted of three main objectives:

1. Monitor the surface mobility of single A-beta oligomers in living cells.
2. Challenge the controversial hypothesis that supports the specific binding of A-beta oligomers to lipid rafts.

3. Compare the membrane dynamics of different amyloid oligomers and their influence on plasma membrane components.

Overall, the experimental strategy for 2D surface SPT is based on labelling immunochemically the target species with antibodies coupled to small (10-30 nm) and extremely photostable fluorescent probes called 'quantum dots' (QDs). Real time recordings of single molecules moving on the plasma membrane of living cells are carried out using a high sensitivity camera. The accurate localization of the fluorescent particle is obtained by fitting its point spread function with a Gaussian function. The analysis of the trajectories allows calculating the mean square displacement and the diffusion coefficient of each particle. These parameters are used to fit the data to several models of diffusion, and distinguish between different types of motions (random walk, confined motion, directed motion, etc.).

By taking advantage of this approach, we have successfully addressed most of the objectives described above:

1. By using specific antibodies coupled to highly fluorescent probes, we have been able to track single Abeta oligomers on the plasma membrane of neuroblastoma cells, and found that their mobility is limited.
2. Simultaneous imaging of Abeta oligomers and lipid rafts labelled with cholera toxin (it binds specifically to GM1, a ganglioside characteristic of lipid rafts) has shown that Abeta oligomers can interact with and decrease the mobility of the raft components.
3. We ahev compared two types of Abeta oligomers that have been shown to possess similar size but distinct toxic properties, as a consequence of their biophysical and structural differences. We found that only the toxic oligomers appear to interact and alter the mobility of GM1.
4. Single particle tracking of amyloid aggregates composed by amylin, sup35 prion, and beta2-microglobulin display a confined mobility on the plasma membrane, comparable to the structurally similar Abeta oligomers. These aggregates were also found to reduce the mobility of GM1.

We have also collaborated to a study on lipid rafts mobility during cell polarization, and to another one on the interaction between the LDL receptor and IDOL, a protein involved in its degradation. These studies are related to the development of cancer and atherosclerosis, respectively.

3) We developed a technique named ultrafast force-clamp spectroscopy, that recently allowed us application of constant loads between a single intermittently interacting biological polymer and a binding protein with a delay in application of the load of only ~10 μs (78).

A sketch of the operational principle of the method is shown in Fig. 3a, in which A is the binding protein, and B is the polymer. A net constant force is applied to the bead-polymer-bead complex (dumbbell) through two feedback systems that clamp the force on the two beads to two different values. The dumbbell, thus, moves against viscous drag at constant velocity (v) when molecules A and B are not bound. The net force is alternated in direction, so that the dumbbell oscillates in a triangular wave fashion within a limited spatial interval (Fig. 3b). When A binds to B, the force Ftot is transferred to the surface-coupled molecule A; because the system was designed to maintain a constant force, the dumbbell movement stops, except for possible conformational changes of the molecules after binding.

The time taken to transfer the force from the viscous solution to molecule A and stop the dumbbell is the relaxation time of the molecular complex. When applied to the interaction between fast skeletal muscle myosin and actin using highly pretensioned actin filaments and 500-nm diameter beads, the transfer time was ~10 μs. For lactose repressor interactions with

a weakly tensioned DNA (~3 pN), the transfer time was ~100 μs (Fig. 3, c and d). This time is very short compared to the duration of typical protein interactions which, thus, occur under a real force-clamp configuration. Any conformational change occurring after the formation of the molecular bond is also performed under the same constant load (Fig. 3e). Using this system, we directly measured load- dependence of the amplitude of myosin working stroke.

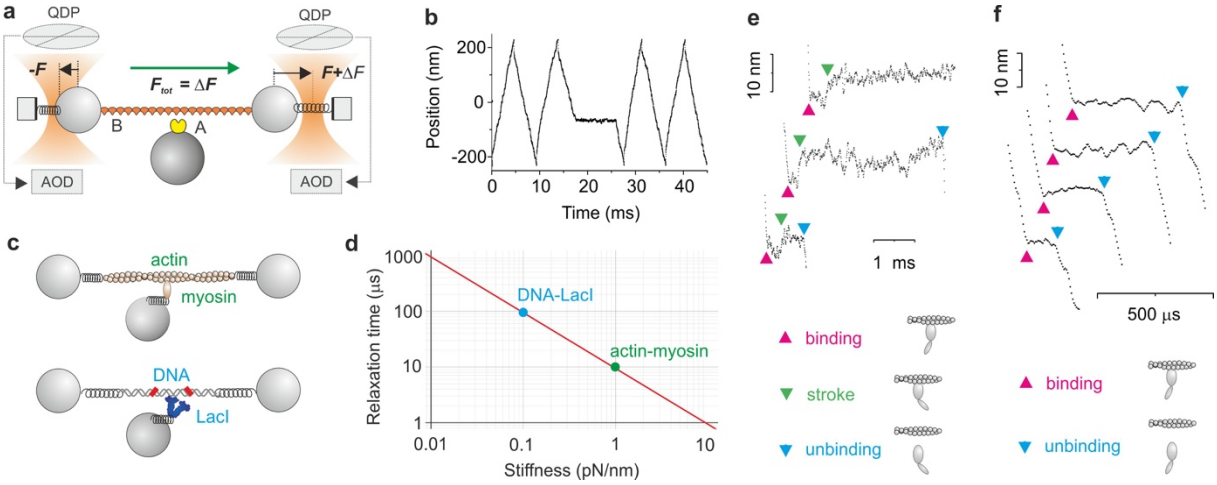

Figure 3 Ultrafast force-clamp spectroscopy. (a) Schematic of the operational principle of the method illustrating constant Ftot applied to molecule B through two feedback systems clamping the force on the left and on the right bead. The force is measured using quadrant detector photodiodes and kept constant by moving the traps via AODs. (b) Position of the dumbbell. The net force is switched to keep the dumbbell within a confined spatial interval (200 nm). The dumbbell stops when A binds to B. (c) Mechanical model for actin-myosin and DNA- LacI interaction. (d) Relaxation times calculated from the models in panel c using 500-nm diameter beads. (e) Actin-myosin interactions longer than 1 ms showed that the myosin working stroke is developed 0.2–1 ms after attachment. (Filled arrowheads pointing down) Myosin working stroke. (f) Submillisecond single actin-myosin interactions detected with ultrafast force-clamp spectroscopy. (Filled arrowheads pointing up) Actin-myosin binding. (Open arrowheads pointing down) Actin-myosin detachment.

Another advantage of this system is that interaction events are detected through variations in the dumbbell velocity, which display a high SNR. The variation of velocity upon binding grows with the applied force and decreases with the beads size. The dead time for the detection of actin-myosin binding was ~100 μs at ~5 pN, using 500-nm diameter beads, which allowed the detection of weak binding events (Fig. 3 f), and premature detachment of myosin from actin. On Lac repressor DNA, short-lived interactions, which are probably involved in a Lac repressor- facilitated diffusion mechanism, were detected.

3 Conclusions

Off-focus imaging is a very simple imaging technique and has the big advantage that does not rely on a custom built optical setup, but can be implemented in a conventional epifluorescence microscope equipped with laser sources for fluorescence excitation and an EMCCD camera for detection. Despite its simplicity, off-focus imaging has not been widely applied for single-molecule experiments in living cells so far, mainly because of the lack of a method that allows automatic recognition and analysis of off-focus intensity patterns over several microns depth. We developed a novel pattern-recognition method that overcomes such limitations. We name our technique Pattern-Recognition Out-of-Focus (PROOF) imaging. Using 200 nanometre fluorescent beads, our method allows three-dimensional localization accuracy within 2 nm over 4 microns range. Remarkably, and contrary to previous reports[17], we demonstrate off-focus imaging of Quantum Dots, by using a ligation strategy that allows rotational mobility of the probe and proper pattern recognition. We could

track commercially available QDots on the membrane of living cells over 2 microns range with a localization accuracy below 7 nm in three dimensions. We anticipate wide application of our technique, which can be applied in a conventional epifluorescence microscope, allows the use of commercially available functionalized QDs, and for which we provide a freely distributable analysis software.
The results obtained in this deliverable on the intimate behavior of toxic oligomers and their interaction with specific membrane components contribute to improve the understanding of the basic mechanisms of Alzheimer's disease and amyloid diseases in general.

Several optical manipulation experimental configurations have been developed in the past to investigate load dependence of short-lived molecular complexes, but each has limitations. To our knowledge, our ultrafast force-clamp spectroscopy is unique in combining 'instantaneous' application and clamping of the force, detection of molecular interactions as short as ~100 μs and the capability to probe sub-nanometer conformational changes occurring during the interaction on a time scale of a few tens of microseconds.

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