

Introduction to Single-Molecule Analysis and Computation: The Focus Project

Monica Mazzolini and Vincent Torre

The FP7 project *FOCUS* “Single-molecule activation and detection” belongs to the FET Proactive 7 Programme: “Molecular Scale Devices and Systems” and is aimed at investigating and developing molecular devices (MD) where single molecules become computing elements. X-ray diffraction and NMR spectroscopy provides information on the structure of molecules with an atomic resolution, but the generated data are averaged over millions of molecules. Single-molecule analysis, in contrast, investigates properties of individual molecules not averaged over a large ensemble of similar molecules. The great majority of these properties, and of the related measurements, refers to nonequilibrium conditions. In order to obtain classical thermodynamic quantities such as changes of the free energy ΔF , special methods of statistical mechanics, such as those introduced by Jarzinski [5, 6] can be used. Single molecule experiments provide a direct view of molecular events in action and offer the possibility to verify basic notions of chemical reactions such as Kramers theory and Eyring’s transition state theory. Indeed, it is possible to characterize very precisely Kramers diffusion coefficient and free-energy barrier by measuring the temperature and viscosity dependence of the transition path time for protein folding [2].

The first single-molecule analyses were those obtained with patch-clamp recordings [3] providing a direct measurement of the current flowing through a single ion channel. These recordings completely changed electrophysiology and our way to understand the operation of ion channels. Subsequently, single-molecule analysis was extended to probe the folding and unfolding dynamics of single

M. Mazzolini · V. Torre (✉)

Neuroscience Area, Scuola Internazionale Superiore di Studi Avanzati (SISSA),
via Bonomea, 265, 34136 Trieste, Italy
e-mail: torre@sissa.it

M. Mazzolini

CBM S.c.r.l. Basovizza, Area Science Park, 34139 Trieste, Italy

molecules by using force spectroscopy [4, 8, 11] either by optical tweezers (OT) [9] or by atomic force microscopy (AFM) [1]. In force spectroscopy, a molecule is mechanically stretched and its elastic response is measured in real time.

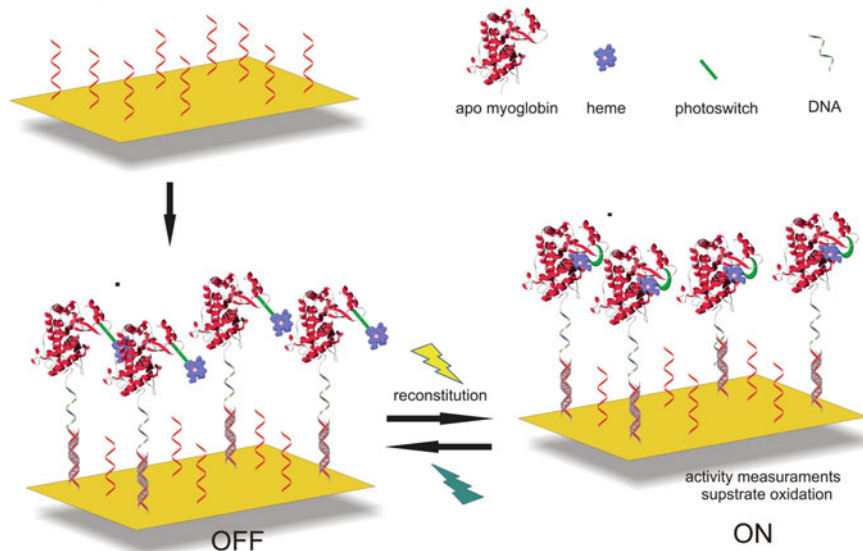
Single molecule force spectroscopy (SMFS) is an application of AFM and consists in the application of the force (F) required to unbind a ligand or to unfold a polymer while the distance (D) between the AFM cantilever tip and the sample is measured as the coordinate reaction, with pN and nm resolution. Force-distance (F-D) curves characterizing the stretching of a protein allow the identification of folded and unfolded regions giving insight in the secondary structure [4].

An OT is composed of a highly focused laser beam creating an optical trap in which samples and biological specimen [9] are analysed. This optical trap is equivalent to a restoring force i.e. a simple spring, which follows the well-known Hooke's law. Therefore by knowing the stiffness of the spring, the OT can be used to measure forces in the pN range. An OT is capable of manipulating nanometer and micron-sized dielectric particles with a very high precision in the nm range and is used to manipulate and study single molecules by interacting with a bead that has been attached to that molecule.

Fluorescence can be used to observe one molecule at a time by using photo-multiplier tubes, or avalanche photodiodes or special-purpose CCD cameras. Single molecule spectroscopy usually requires the attachment of a fluorescent probe to the molecule under investigation, which can be quantum dots (QD), gold nanoparticles or organic fluorophores [7, 13] Single molecule fluorescence resonance energy transfer (smFRET) [12] is used to measure distances at the 1–10 nm scale and is based on a single donor and acceptor FRET pair. Data collection with cameras will then produce movies of the specimen that must be processed to derive the single-molecule properties.

Single fluorophore can be chemically attached to proteins or DNA, and the dynamics of individual molecules can be tracked by monitoring the fluorescent probe. For instance, single molecule labelling has yielded a vast quantity of information on how molecular motors move along in cells and over microtubules. A typical fluorophore used in many biological applications is a QD, which is a semiconductor nanocrystal with excellent optical properties. Antibodies, streptavidin and DNA can be used to target QDs to specific proteins in cells and neurons.

The core of the FOCUS project is to address a long standing issue of programmable assembly, which Nature masters on a fine molecular level, but scientists have not yet achieved: Nature has developed highly sophisticated systems able to detect single photons and molecules and to perform reliable computations in a noisy environment dominated by thermal motion. Can we design and assemble molecular devices with similar properties?



*The diagram of the MD developed within the FOCUS project.
(Fruk L., FET-NEWS, 2013)*

This project wants to build a novel generation of biologically inspired MDs based on the developments of new photonic devices. These devices will use plasmon polariton and two-photon technology, enabling focused light spots with a diameter around 10 nm, i.e. *the size of a single protein*. FOCUS is also developing new light-sensitive molecules that are selectively activated by our new photonic devices. These new technological innovations will provide a way to control activation of single light-sensitive molecules and will allow the investigation of molecular computation in a biological environment and with an unprecedented resolution. A guiding idea beyond the FOCUS project is diffusion, which is seen as a basic component of biological computation.

FOCUS has formed a highly interdisciplinary consortium composed of nanotechnologists able to fabricate the new photonic devices—i.e. Enzo Di Fabrizio (IIT), Alpan Bek (METU) and Marco Lazzarino (CBM), chemists able to develop the photoswitches and assemble the MDs—i.e. Pau Gorostiza (IBEC) and Ljiljana Fruk (KIT) and biologists able to understand molecular mechanisms—i.e. Vincent Torre (SISSA) and Fabio Benfenati (IIT). The two companies RappOptoElectronic and NT-MDT Europe BV will transform the new tools and devices into marketable products.

The nanotechnological component of FOCUS has the objective to design and fabricate new photonic plasmonic devices able to provide a well-focused beam of light with a diameter of 10 nm, close to the size of a single molecule. In order to identify the best possible geometrical configuration capable of strong light focusing with low background signal, simulations on three different systems were performed. In particular, we have chosen (1) single nanowire antenna, (2) sub-

wavelength holes in metallic slab (3) hollow conical structure. The three devices were also fabricated by means of a combination of focus ion beam and electron beam lithography.

Chemistry has also an important role in FOCUS and its objective is to develop and validate procedures for affinity labelling enabling the attachment of a photoswitch to different proteins, and to evaluate their toxicity and functionality. Within the consortium, chemical and biological assays were implemented and validated to evaluate the conjugation of photoswitchable compounds to the kainate receptor GluK2, and four full photoswitchable compounds were designed, synthesized and assayed.

FOCUS aims also to elucidate how molecular reactions based on diffusion control phototransduction and in particular to understand the role of diffusion in signal amplification and gain control in rods and to determine how diffusion interconnects and concatenates molecules in phototransduction. Within the project, a recording set-up system has been developed to measure the photocurrent from rods using the suction electrode technique and have investigated differences in rods with different geometries, where diffusion is expected to play a major role. This system is also combined with an optical fibre illuminating rods in a configuration allowing the stimulation of few rhodopsins in a specific position and timing. These experiments are expected to solve long standing issues in phototransduction.

These novel probes provide also the opportunity to analyse the biological effects of the activation of a reduced number of ion channels, receptors and proteins possibly at a single molecule level. In this case, it is mandatory to have light beams with well defined wave-front profiles. In order to use light-sensitive molecules for single molecule analysis, it is important and mandatory to be able to have very restricted beams of light, exciting a single molecule or a confined and selected region. Usual light beams obtained with conventional optical components like optical fibres, have Gaussian profiles with characteristic side lobes, which do not allow the controlled excitation of a single light-sensitive molecule. Another result obtained within the FOCUS project is the observation that peculiar light exits from “apertureless” tapered optical fibres (TOFs), commercially available. What is more important is that by using TOFs fed by a laser beam it is possible to have confined spots of light with almost no side lobes, and we demonstrated that their use reveal new fundamental features of phototransduction in vertebrate rods that contain the rhodopsin, a molecule intrinsically light-sensitive at the basis of vision in the animal kingdom. A rod cell is subdivided in a synaptic region, an inner segment (IS) and an outer segment (OS) that contains a stack of lipid discs surrounded by a plasma membrane. In dark condition, cyclic nucleotide-gated (CNG) channels that are localized in the plasma membrane are open and sodium and calcium ions enter inside the cell but this current is abolished in presence of light; in fact when photons reach rhodopsin molecules that are densely packed on disc membranes—phototransduction starts [10]. Using an apertureless TOF, we deliver restricted illumination at various positions of the OS at the base, middle and tip. Our results show that by using apertureless TOFs novel properties of phototransduction are obtained demonstrating that there are differences along the

OS. These apertureless TOFs can also be used in optogenetics for the activation of light-sensitive ionic channels in specific regions of a neuron, such as distal dendrites and/or single spines. These TOFs allow the activation of a very limited number of light-sensitive receptors and proteins and by improving their performances a single light-sensitive molecule could be excited. These TOFs could become useful tools for single-molecule investigations.

During the first period of the project's life, the consortium also identified and validated experimental configurations aiming to the activation of subsets of receptors in the perspective to track the triggered diffusion processes and to reveal basic computational mechanisms involving the neuronal input processes. In the same period of time, the input-output relationships in molecular computation have been investigated. A theoretical study of the input-output (stimulus-response) relationships using both analytical methods from statistical physics and applied mathematics and numerical simulations was performed.

Within the consortium, scientists and engineers have optimized the integration of the AFM cantilevers with plasmonic antennas. We have produced PC-PA (photonic crystal-plasmonic antenna) directly on AFM cantilever by combining focused ion beam milling and induced electron growth. Partners have examined, tested and investigated the properties of PC-PA cantilevers using their modified commercial AFM.

The core of the FOCUS project is where the new photonic devices, the photoswitchable gates and the biological investigation of diffusion are integrated in the prototype of the new molecular computing device, schematically represented in the figure above. DNA-proteins that conjugate for attachment onto the surface and where the length of attachment controls the diffusion of anchored proteins have been developed. The activation of these proteins is controlled by light through use of azobenzene photoswitch, and the new photonic devices developed within the project. A set of DNA oligonucleotides was designed so that the length of the DNA can easily be readjusted if needed for future studies of diffusion and cofactor reconstitution. Regarding protein modification, several mutant myoglobin (Mb) proteins have been expressed and purification methods optimized. Mutants were designed as the native Mb did not have a suitable anchoring group for attachment of two different classes of molecules. In addition, new chemical procedures were developed for attachment of modified DNA strands onto tyrosine residues of the protein as the crystallographic data analysis has shown that the tyrosine residues are a suitable target for DNA attachment.

Another objective is the development of the capability to concentrate light to very small spots of light in the 10 nm range using two photon plasmonic engineering. In accordance with the two tasks of building a nanoparticle-based molecular switch and addressing the individual molecular switch, we have the objectives to fabricate a substrate with a dense array of metal nanoparticles and to set-up an instrument capable of performing nano-manipulation. At the same time, the project also has the objective to realize an optical characterization tool for testing plasmonic properties of the nanoparticle decorated substrates.

The methodologies developed within FOCUS will certainly have wide implications not only to study biological processes but also in the fields of photonics, single molecule manipulation and detection, chemical modification and programmable assembly.

References

1. Binning G, Quate CF, Gerber Ch (1986) *Phys Rev Lett* 56(9):930–935
2. Chung & Eaton (2013) Single-molecule fluorescence probes dynamics of barrier crossing. *Nature* 502:685–689
3. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* 391(2):85–100
4. Hoffmann T, Dougan L (2012) Single molecule force spectroscopy using polyproteins. *Chem Soc Rev* 41(14):4781–4796
5. Hummer G, Szabo A (2001) Free energy reconstruction from nonequilibrium single-molecule pulling experiments. *Proc Natl Acad Sci* 98(7):3658–3661
6. Hummer G, Szabo A (2010) Free energy profiles from single-molecule pulling experiments. *PNAS* 107(50):21441–22144
7. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S (2005) Quantum dots for live cells, in vivo imaging ad diagnostics. *Science* 307:538–544
8. Müller DJ, Wu N, Palczewski K (2008) Vertebrate membrane proteins: structure, function, and insights from biophysical approaches. *Pharmacol Rev* 60(1):43–78
9. Neuman KC, Block SM (2004) Optical trapping. *Rev Sci Instrum* 75(9):2787–2809
10. Pugh EN Jr, Lamb TD (2002) Phototransduction in vertebrate rods and cones: molecular mechanisms of amplification, recovery and light adaptation. In: *Handbook of biological physics*. Elsevier Amsterdam, pp 183–255
11. Rief M, Gautel M, Oesterhelt F, Fernandez JM, Gaub HE (1997) Reversible unfolding of individual titin immunoglobulin domains by AFM. *Science* 276(5315):1109–1112
12. Roy R, Hohng S, Ha T (2008) A practical guide to single-molecule FRET. *Nat Methods* 5(6):507–512
13. Zhang J, Fu Y, Conroy CV, Tang Z, Li G, Zhao RY, Wang G (2012) Fluorescence intensity and lifetime cell imaging with luminescent gold nanoclusters. *J Phys Chem C Nanomater Interfaces* 116(50):26561–26569