1.3.8 Advanced microscopy techniques in living cells

Progress in fluorescence imaging is enabling the study of biological events at unprecedented detail, thanks to novel microscopy techniques that provide imaging and mapping of several parameters in the environment down to the nanoscale resolution. In the NEST laboratory we have carried out both the development of novel fluorescent nanotools for biosensing and of novel techniques in order to study dynamics and interaction of several moieties on the membrane and in other internal organelles and vesicles of living cells. Some of these techniques may have interesting application in diagnostic based on ultrasensitive techniques.

An important area of activity at NEST regards the implementation and development of epifluorescence, confocal and TIRF microscopy techniques and their application to the study of bioevents (such as protein dynamics, oligomerization state, and interactions). This often requires the development of novel fluorescent indicators, which is done in our laboratory also by a chemical synthesis approach. On the other side, new image acquisition and analysis methods (e.g. based on fluorescence correlation spectroscopy, FCS, or on single particle tracking, SPT) can increase the amount of biological information that can be recovered compared to conventional strategies based on perturbation (e.g. FRAP). These methods are now routinely used in several research projects, as shortly described in the following.

The development of new fluorescent probes can in principle conjugate the extremely high sensitivity of fluorescence (down to single molecule) to its intrinsic sensitivity to any molecular factor capable to affect the long-living excited state. Molecular recognition events in living systems, or structural rearrangements in specific domains (e.g. cell membranes), are often related to transient changes of the physicochemical properties of their local (nm-scale) environment. Thus, environmentally-sensitive probes may help understanding subtle biological phenomena. By rational molecular design, we successfully developed a palette of intracellular fluorescent reporters, based on coumarin, butenolide, and styryl cores, endowed with excellent brightness and photostability, and provided with functional groups for conjugation to biomolecules [1-4]. As an example, for a new “molecular rotor”, a viscosity sensitive dye, we demonstrated strong absorption in the green region of the spectrum, very little solvatochromism, and strong emission sensitivity to local viscosity [4]. Actually the emission increase was paralleled by an increase in emission lifetime; owing to its concentration-independent nature, fluorescence lifetime is particularly suitable to image environmental properties, such as viscosity, at the intracellular level. Accordingly, we demonstrated that intracellular viscosity measurements could be efficiently carried out by lifetime imaging with our probe and phasor analysis, an efficient method for measuring lifetime-related properties (e.g., bionalyte concentration or local physicochemical features) in living cells (Fig. 1) [4]. Moreover, we analyzed the interactions between organic dyes and the possible scaffolds that can be functionalized; in particular, we studied surface enhanced Raman scattering (SERS) for fluorophores conjugated to metallic nanoparticles, and how the use of dendrimers (reproducible ramified polymers, which carry on their surface

functionalizable moieties) can increase the versatility of fluorescence-based approaches [5].

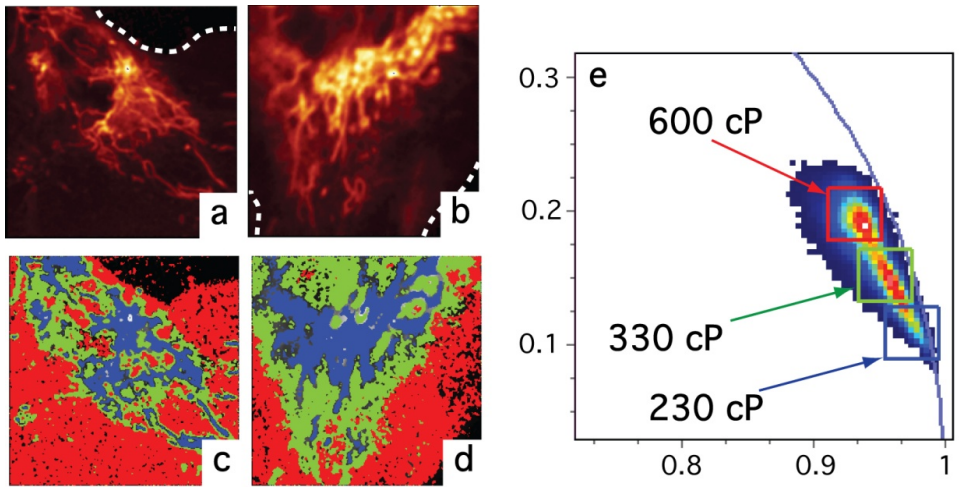

Figure 1 Cultured HUVEC cells treated with our styryl molecular rotor [4]. Panels a-b: fluorescence intensity images. Panels c,d: "phasor" (lifetime) images corresponding to a,b; the color code is: red for membrane, green for lysosomes, blue for mitochondria. Panel e: phasor plot for images c,d; the three different regions corresponding to membrane (red), lysosomes (green) and mitochondria (blue) are enclosed in colored squar

es and the related viscosities are added.

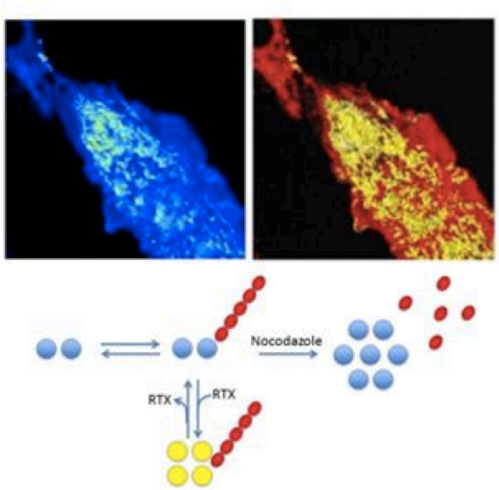

Figure 2. Intensity (left) and N&B map (right) of TRPV1 upon activation. Red pixels correspond to dimers, yellow pixels to functional tetramers. Schematic representation of TRPV1 oligomerization state under different conditions.

In one of the approach to study the dynamics of molecules in living cells, classical FCS and its recently developed variants have been introduced to study protein diffusion, aggregation state, and interactions in the context of a living cell sample and in presence of many molecules. For example, the Number&Brightness (N&B) fluctuation analysis has been used to quantitatively investigate the aggregation state of the transient receptor potential cation channel subfamily V member 1 (TRPV1), a non-selective membrane protein activated by heat and capsaicin and involved in nociception [6]. By N&B we demonstrated that TRPV1 is dimeric under physiological conditions whereas it mostly converts into a functional tetramer under ligand-binding conditions (Fig. 2). Moreover, fast spatiotemporal correlation spectroscopy allowed us to determine the lateral diffusion laws of a GFP-tagged transmembrane transferrin receptor in live Chinese hamster ovary cells, a well-known benchmark of membrane-skeleton–dependent transiently confined diffusion; this approach can be used also with dim and dense molecole and represents a powerful tool for the determination of

kinetic and thermodynamic parameters over very wide spatial and temporal scales. [7]

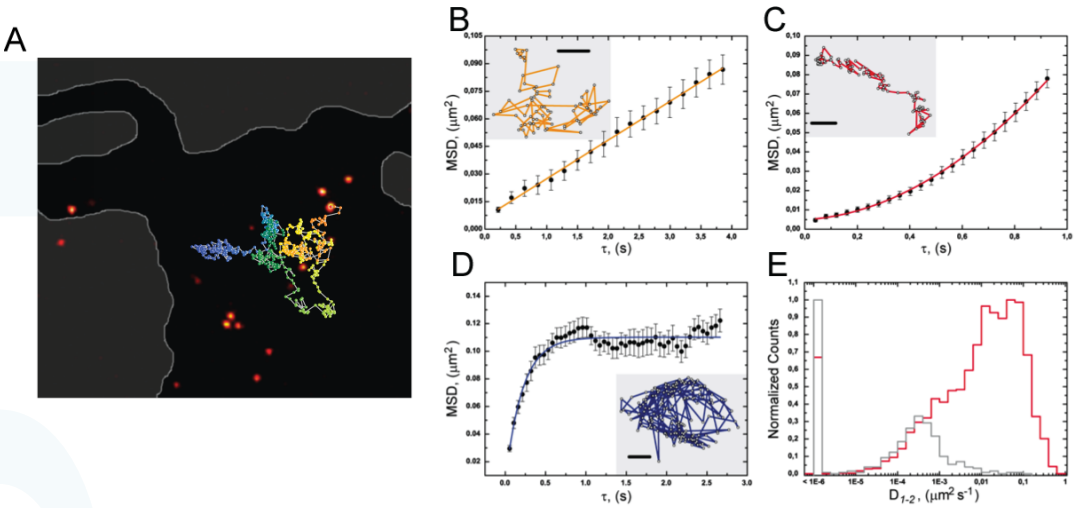

Figure 3. TrkA SM trajectories at the basal membrane of living cells. A) TIRF microimage showing single Qdot-labelled ACP-TrkA receptor molecules at the basal plasma membrane. The border of the basal membrane is depicted as a light-grey line and areas outside the cell are grayed. A typical trajectory recorded for TrkA molecules is superimposed to a spot of the image; the color code represents the trajectory time progression. B–E) MSD vs lag-time (t) plots for trajectories (insets) epitomizing three diffusive regimes associated to individual ACP-TrkA receptors: (B) Brownian (yellow curve), (C) drifted (red curve) and (D) confined (blue curve) regimes. Bar: 0.16 mm (1 pixel). (E) Global distribution of average diffusivities ($D_{1\text{-}2}$) associated to each single trajectory (red curve) and $D_{1\text{-}2}$ distribution of Qdot immobilized to the glass surface (gray curve). The bin "<1E-6" includes negative values for $D_{1\text{-}2}$, mostly arising from cases where the uncertainty is higher than the absolute value.

In a parallel approach, SPT in combination with TIRF microscopy have been used to address the study of tropomyosin receptor kinase A (TrkA) lateral diffusion in the membrane of living cells. TrkA is the high-affinity nerve growth factor (NGF) receptor, and cooperates with the low-affinity p75 neurotrophin co-receptor (p75NTR) to transduce NGF signals in neuronal cells. The dynamic interplay between NGF, TrkA and p75NTR is critically involved in several physio-pathological processes. The quantitative description of the early steps of NGF-induced TrkA and p75NTR separate and concerted responses at the cell plasma membrane is currently a hot topic studied by our laboratory. We have demonstrated that the insertion of the acyl carrier protein (ACP) tag at the extracellular domain of TrkA receptor allows for the specific labeling of the receptor pool exposed at the cell surface, when the construct is transfected in living cells [8]. The ACP tag has shown to not interfere with TrkA receptor function, both in terms of ligand responsiveness and downstream signalling. Eventually coupled to semiconductor quantum dots (Qdots) or to fluorophores, the ACP-TrkA construct has allowed recording heterogeneous diffusion patterns of receptor single molecules (SMs) at the cell plasma membrane, with a localization precision down to 10 nm and a temporal resolution in the ms time scale (Fig. 3) [8]. Software has been implemented and developed for almost automated analysis of the obtained SM trajectories. Algorithms currently available at NEST process trajectories obtained by one-color SMs temporal series and calculate: i) the most significant diffusion and confinement parameters,

starting from the mean square displacement (MSD) of trajectories or subtrajectories; ii) the fraction and dynamic changes of all monomers and/or homo-oligomers detected by measuring fluorescence intensities of the tracked objects [8, 9].

The heterogeneous movements of TrkA at the membrane have also been correlated to its function, in particular with the binding of different biologically relevant ligands: NGF, NGF R100E HSANV mutant, proNGF and NT-3 [9]. We have provided evidence that a close correlation exists between the initial receptor membrane dynamics triggered upon binding and the specific biological outcomes induced by different ligands for the same receptor. Indeed, in the absence of ligands, most of TrkA receptors are fast moving monomers ($D \approx 0.47$ $\mu m^2/s$); about 20% TrkA molecules are moving at least an order of magnitude slower and around 4% are almost immobile within regions of about 0.6 μm in diameter. Ligand binding results in increased slow and/or immobile populations over the fast one, slowing down of non-immobile trajectories and reduction of confinement areas, observations which are consistent with the formation of receptor dimeric and oligomeric states. Notably, the extent of TrkA lateral mobility modification is strictly ligand-dependent and each ligand promotes distinct trajectory patterns of TrkA receptors at the cell membrane (ligand "fingerprinting" effect, Figure 4). This ligand-signature of receptor dynamics results from a differential combination of receptor-binding affinity, intracellular effectors recruited in the signalling platforms and formation of signalling/recycling endosome precursors [9].

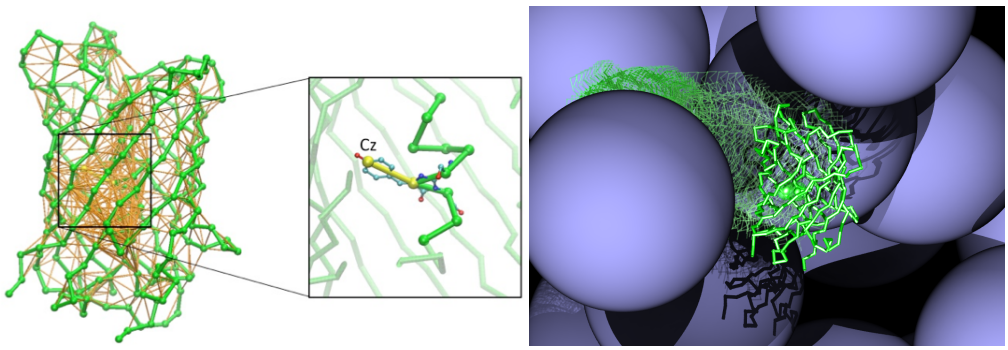

Figure 5. Left: CG Minimalist model for the GFP (green ball&sticks); an indicative representation of the local interactions topology (*T*) is reported as orange bonds. An expanded view of the chromophore surrounding is shown, with the atomistic structure of the chromophore superimposed. The chromophore beads are in yellow. Right: artistic representation of the diffusion of a single GFP molecule in a crowded environment.

Efforts were also dedicated to the simulation of the dynamics of proteins in crowded environments, such as cytoplasm and plasmamembrane. As a benchmark, the dynamic of GFP was addressed. After the development of a strategy for developing corse grained (CG) "minimalist" models for biopolymers, and its application to the theoretical study of RNA, DNA, and polypeptides [10], a minimalist model for the GFP was optimized by combining statistics-, physics- and structure-based information by means of a Genetic Algorithm, resulting in

accurate monomer internal fluctuations and homodimer binding modes [11]. The diffusive dynamics of GFP in concentrated environments was addressed by embedding the protein in a simplified cytoplasm-like solution consisting of spherical crowding agents. Thanks to the extreme simplifications of both the tracer and crowding agents and the rigorous procedure to evaluate the inter-molecular interactions, μs-long multiscale simulations of the GFP were easily accessible at different crowders concentrations and reproduced the *in vivo* measured diffusion coefficients (Fig. 5) [11].

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