1.3.27 New paradigms in nanoscale biophysics: looking at life (mis)regulation inside cells

Capturing life (mis)regulation at the nanoscale is a crucial challenge for present and future biophysics. At this scale, molecules are the main actors. To successfully tackle molecular behavior within living matter, two crucial requisites are typically needed: (i) nanometer spatial resolution, (ii) micro-to-millisecond temporal resolution. In this context, optical microscopy is a valuable methodological platform: by using fluorescence as readout, spatial and temporal details of molecular processes can be investigated directly within living matter.

We introduced/developed a number of methods, based on the statistical analysis of fluorescence fluctuations, in order to increase the quantitative information that can be extracted from time-resolved optical-microscopy experiments and provide further insight into dynamic molecular processes. In particular, new spatiotemporal variants of fluorescence correlation spectroscopy (FCS) were proposed which are able to resolve the dynamic behavior of molecules well below the nominal imaging resolution of the optical setup (e.g. the imaging derived Mean Squared Displacement analysis, or iMSD, is reported in Fig.1a-d) [1-5].

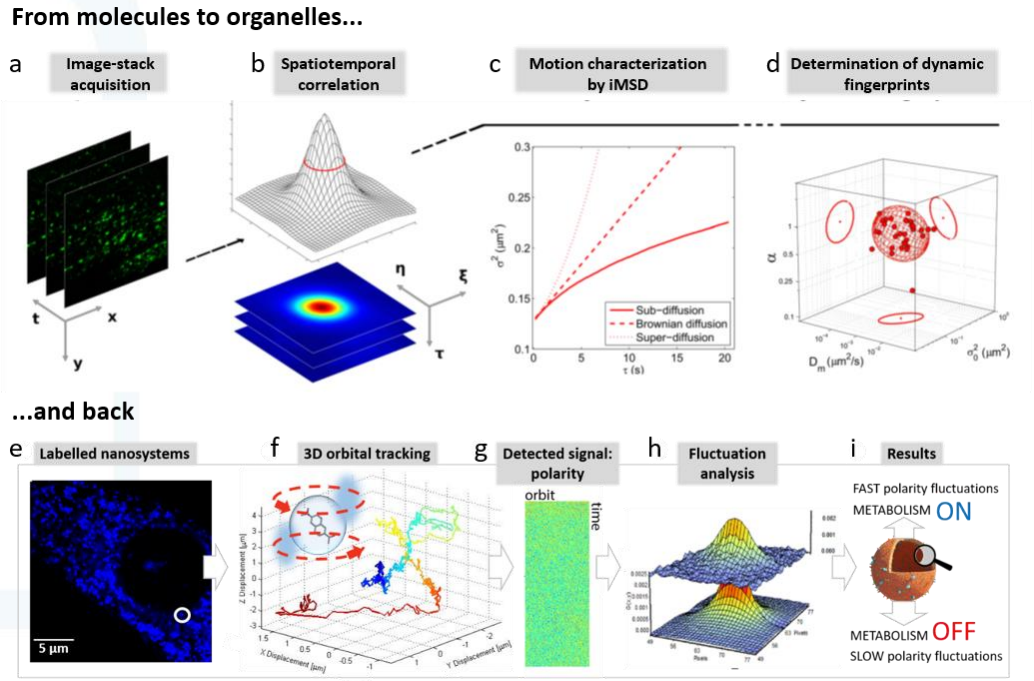

Figure 1. From molecules to organelles: Schematic representation of the iMSD-based dynamic fingerprint analysis. **a**) A stack of images of fluorescently-labelled intracellular structures is acquired by time-lapse confocal microscopy. **b**) Spatiotemporal correlation function is derived from image analysis by the iMSD algorithm (see Materials and Methods for equations). **c**) Gaussian fitting of correlation functions allows to extract the iMSD plot, which in turn depicts the average diffusion law of the structure of interest (exemplary cases are reported: super-diffusion, dotted red line; isotropic diffusion, dashed red line; sub-diffusion, solid red line). **d**) The short-range diffusion coefficient (D_m), the anomalous diffusion coefficient (α), and the y-axis intercept of the iMSD plot, indicating the average size of the diffusing structures. These three parameters are organized in a 3D plot, used to identify the 'dynamic fingerprint' of the diffusing structure. Adapted from Digiacomo et al. Scirep 2017 (Ref. 11). **From organelles to molecules:** Here, lysosome lumen is labelled by the polarity-sensitive 6-acetyl-2-dimethylaminonaphthalene (ACDAN) probe (**e**). 3D orbital tracking affords the trajectory of single organelles (**f**) while detecting luminal polarity (**g**). Fluctuation analysis finally yields the amplitude and timing of polarity fluctuations (**h**), which in turn are informative of lysosome metabolic status (**i**). Adapted from Ref. [13].

Methodological demonstrations were provided in a series of applications of relevant biological/biomedical interest, including: the spatiotemporal regulation of intracellular transport [6-9], the dynamics and oligomerization of membrane receptors and their ligands [10], the intracellular trafficking of drug-delivery vectors within subcellular nanostructures [11, 12].

For instance, the spatiotemporal dynamics of Transient Potential Vanilloid 1 (TRPV1) membrane receptors (implied in nociception and target of pain therapy) was addressed by combining for the first time the nanoscale resolution of Fluorescence Resonance Energy Transfer (FRET) with the iMSD analysis [10]. It was found that TRPV1 is split in three pools with distinct functional roles: i) TRPV1 trapped in caveolae, and implied in long-term receptor desensitization; ii) TRPV1 stabilized by microtubules, and organized in large supramolecular assemblies, and iii) TRPV1 a freely diffusing on the plasmamembrane and acting as a receptor reservoir [10].

In spite of these technological achievements, molecular details of life regulation are still destined to remain elusive within subcellular, membrane-enclosed, nanosystems such as vesicles, organelles, or even entire subcellular protrusions. At this scale, in fact, the molecular actors are part of a reference nanosystem that is endlessly changing position in space and time in the complex 3D cellular environment. This condition imposes a third requisite to be concomitantly met in the same experiment, large volume sampling to localize the target nanosystem. Unfortunately, no method has the capability to subtract the 3D evolution of the entire nanosystem while preserving the temporal resolution needed to probe molecular details on it. We tackled this bottleneck by sending the excitation beam in a periodic orbit around the nanostructure of interest, with the recorded signal (e.g. fluorescence or scattered light) used as feedback to localize the nanostructure position with unprecedented spatial and temporal resolution. At this point, state-of-the-art imaging/analytical approaches (e.g. fast spatiotemporal correlation spectroscopy) can be used along the orbit to push biophysics to an entirely new level: molecular analysis on a moving, nanoscopic, reference system. The potential of this strategy have been recently demonstrated in a paradigmatic application in which metabolism-dependent solvent polarity fluctuations were measured in the lumen of a 3D-trafficking lysosome (as reported in Fig. 1e-i) [13]. Overall, by such a toolbox of techniques we propose a paradigm shift in the way we address the natural physiopathology of living matter at the sub-cellular scale, where molecular information is still hidden behind a plethora of dynamic intracellular nanostructures. The direct involvement of these latter in the processing of drug-delivery vectors suggests them as a due target for a new era of *theranostic* strategies using engineered nanoarchitectures. Preliminary studies are actively carried out, also in collaboration with external biomedical research units and medical clinics. If successful, this research activity is expected to open new perspectives in biophysics and related fields, nanomedicine above all.

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