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Time-resolved biophysical approaches to nucleocytoplasmic transport

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ABSTRACT

Molecules are continuously shuttling across the nuclear envelope barrier that separates the nucleus from the cytoplasm. Instead of being just a barrier to diffusion, the nuclear envelope is rather a complex filter that provides eukaryotes with an elaborate spatiotemporal regulation of fundamental molecular processes, such as gene expression and protein translation. Given the highly dynamic nature of nucleocytoplasmic transport, during the past few decades large efforts were devoted to the development and application of time resolved, fluorescence-based, biophysical methods to capture the details of molecular motion across the nuclear envelope. These methods are here divided into three major *classes*, according to the differences in the way they report on the molecular process of nucleocytoplasmic transport. In detail, the first class encompasses those methods based on the perturbation of the fluorescence signal, also known as ensemble-averaging methods, which average the behavior of many molecules (across many pores). The second class comprises those methods based on the localization of *single* fluorescently-labelled molecules and tracking of their position in space and time, potentially across *single* pores. Finally, the third class encompasses methods based on the statistical analysis of spontaneous fluorescence fluctuations out of the equilibrium or stationary state of the system. In this case, the behavior of *single* molecules is probed in presence of many similarly-labelled molecules, without dwelling on any of them. Here these three classes, with their respective pros and cons as well as their main applications to nucleocytoplasmic shuttling will be briefly reviewed and discussed.

1. Introduction

In eukaryotic cells, the cytoplasm and the nucleus are spatially separated by a double membrane, the nuclear envelope (NE). Embedded in the NE are the nuclear pore complexes (NPCs), which allow the passage of ions and molecules across the NE and, at the same time, regulate the exchange of larger molecules, such as RNAs, proteins, or ribonucleoprotein (RNP) particles between nucleus and cytoplasm [1].

The overall shape of the pore is known since pioneering studies, among others, were conducted yeast by electron microscopy (EM) [2] and on *Dictyostelium discoideum* by cryo-electron tomography (cryo-ET) [3]: the pore is a channel-like structure of about 40–90 nm in length and 40–75 nm in width, showing an asymmetric structure with flexible protein filaments extending out from the pore (approximately 50 nm) into the cytoplasmic environment, and an open basket-like structure extending to about 75 nm into the nucleus. More recently, mainly thanks to the straightforward combination of EM and ET with mass spectrometry (MS) analysis, structural modelling, and X-ray crystallography, our knowledge about the finest structural details of the NPC is enormously increasing (for more details see Refs. 3–7). Collectively, the achievements brought by structural studies promise to open new perspectives for our understanding of the molecular mechanisms underlying NPC function in normal and altered conditions [8].

At the molecular level, the whole NPC consists of about 30 different polypeptides designated nucleoporins (Nups), with a very controlled stoichiometry, and a total mass of ~125 MDa [9–11]. Most of the Nups lack a fixed secondary structure but rather contain domains rich in phenylalanine-glycine (FG) repeats [12] which are very flexible. These FG-Nups are generally located within the central channel of the NPC, forming a selective barrier that inhibits the efficient translocation of large molecules (>40 kDa) unless they are chaperoned by transport receptors [13], such as Importin β (Impβ). Impβ, one of the major transport receptors, recognizes cargo molecules in the cytoplasm and forms a transport complex either directly or indirectly (i.e. through Importin α, Impα) [14]. Cargo-receptor complexes are able to interact with Nups at the cytoplasmic filaments or at the peripheries of the central pore [15]. From here, the cargo-receptor complex is transferred to the FG-repeat domain of nucleoporins in the center of the NPC (e.g. Nup153 [16]), where the FG-Nups offer a functional redundancy of binding sites for Impβ [17,18]. How nucleoporin-Impβ interaction drives NPC-passage is, however, not a trivial problem, and several models address this issue (for a detailed review refer to [19]). In spite of their variety, these models differ mainly in the physical arrangement and mobility (static vs. dynamic) of the FG-domains within the

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NPC. Whatever the organization of the FG-Nups in the NPC, the transport process is terminated by the intervention of Ran guanosine triphosphate (RanGTP), which dissociates Impβ from the FG-Nups at the level of the nuclear basket and causes the release of the cargo molecule into the nucleus. The newly formed Impβ-RanGTP complex is selectively transferred into the cytoplasm to initiate a new round of transport.

Given the highly dynamic nature of the overall process, a variety of time resolved biophysical strategies were applied to nucleocytoplasmic transport of molecules (summarized in Fig. 1). They can be roughly

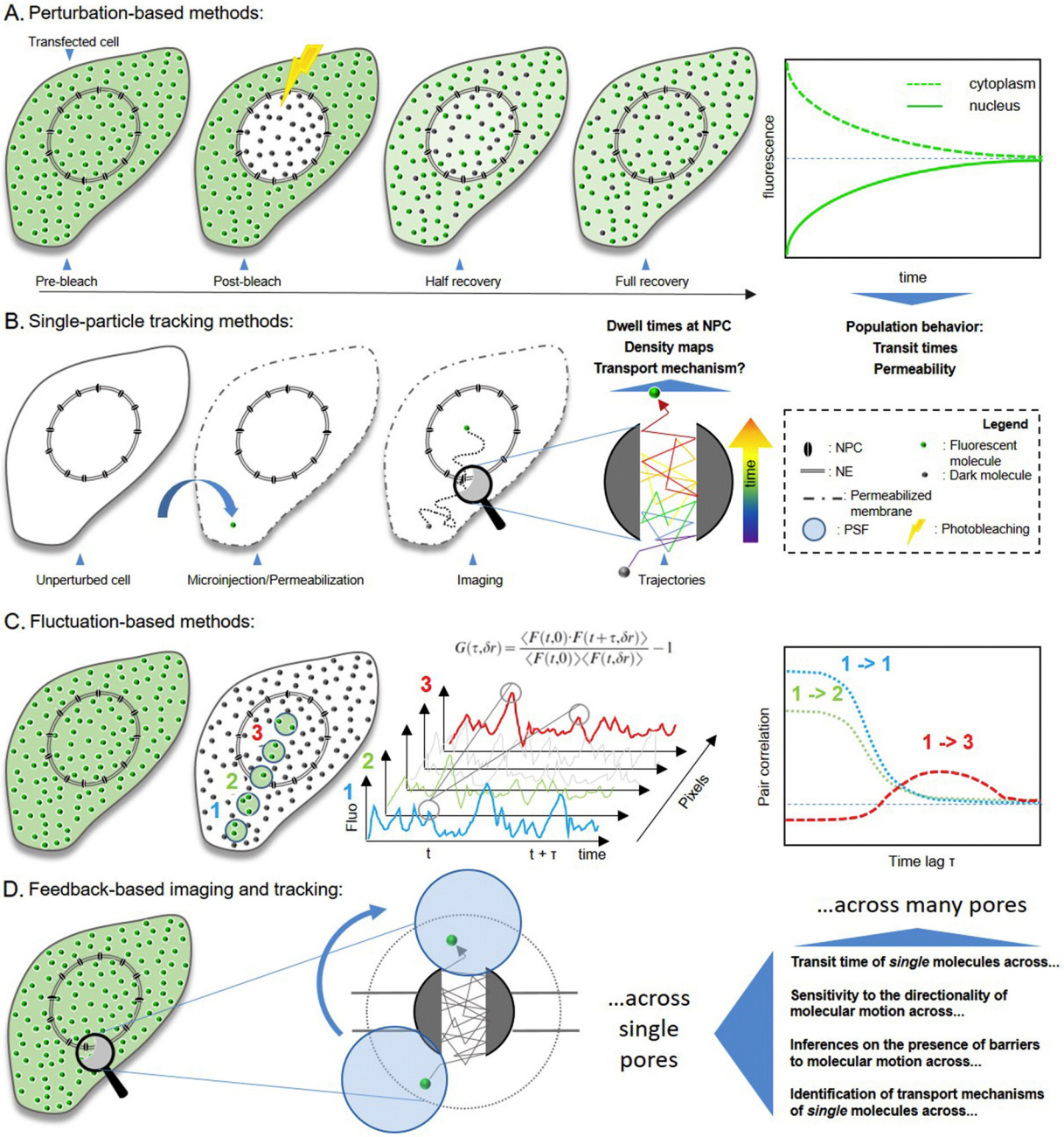

Fig. 1. Schematic representation of the major classes of time-resolved biophysical approaches to nucleocytoplasmic transport. A) Perturbation-based methods. A schematic representation of the FRAP method is reported, with the nucleus of a cell being phobleached to then follow the recovery of fluorescence due to the exchange of 'dark' and 'green' molecules across the NE. A typical plot of exponential fluorescence recovery in the nucleus (and concomitant decrease in the cytoplasm) is reported. From such a measurement, under proper modelling of the process under study, the dynamic behavior of a population of molecules can be extracted, in terms of characteristic time of fluorescence recovery, immobile/mobile fraction of molecules, etc. B) Localization-based techniques. Typically, the molecule of interest must be properly purified, labelled, and introduced into the sample by microinjection or permeabilization procedures. At this point, single-molecule imaging can be performed, provided that the label yields the required amount of photons to allow localization with the desired precision. Under optimal conditions, trajectories of single molecules transported across the pore can be described (as schematically represented here). From trajectories, residency times at the pore and/or density maps of single-molecule localizations can be extracted. C) Fluctuation-based techniques rely on the rapid acquisition of fluorescence signal fluctuations from a system (e.g. a transfected cell as in the example here) left at equilibrium or steady state (no large perturbation is introduced). Spatiotemporal analysis of such fluctuations (e.g. by the pair correlation function reported here) provide sensitivity to single molecules in presence of many similarly labelled molecules, large amount of information in a single measurement and compatibility with the use of relatively dim molecules (e.g. GFPs) in live, unperturbed cells. For instance, by the pCF algorithm, average transit times of single molecules across (many) pores can be measured and inferences about the nature of the pore as a barrier to molecular motion can be drawn. D) Feedback-based methods. In this case, the observation volume defined by the PSF is rapidly orbiting around the object to be tracked (the pore in this case), with a response time of few milliseconds and a location precision in the nanometer range. In other words, standard analytical tools (e.g. fluctuation analysis) can be brought onto the reference system of a *single* pore to follow the translocation of *single* molecules with great precision and a time resolution that is faster than the motion of the overall NPC.