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Visualizing single molecules interacting with nuclear pore complexes by narrow-field epifluorescence microscopy

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Abstract

The utility of single molecule fluorescence (SMF) for understanding biological reactions has been amply demonstrated by a diverse series of studies over the last decade. In large part, the molecules of interest have been limited to those within a small focal volume or near a surface to achieve the high sensitivity required for detecting the inherently weak signals arising from individual molecules. Consequently, the investigation of molecular behavior with high time and spatial resolution deep within cells using SMF has remained challenging. Recently, we demonstrated that narrow-field epifluorescence microscopy allows visualization of nucleocytoplasmic transport at the single cargo level. We describe here the methodological approach that yields 2 ms and ~15 nm resolution for a stationary particle. The spatial resolution for a mobile particle is inherently worse, and depends on how fast the particle is moving. The signal-to-noise ratio is sufficiently high to directly measure the time a single cargo molecule spends interacting with the nuclear pore complex. Particle tracking analysis revealed that cargo molecules randomly diffuse within the nuclear pore complex, exiting as a result of a single rate-limiting step. We expect that narrow-field epifluorescence microscopy will be useful for elucidating other binding and trafficking events within cells.

Keywords: Single molecule fluorescence; Nuclear transport; Particle tracking; Narrow-field epifluorescence microscopy; Cell imaging

1. Introduction

1.1. Visualizing single cargo molecules interacting with nuclear pore complexes

The method described here provides a means to determine fundamental mechanistic properties of the nuclear pore complex (NPC) by studying the dynamic behavior of single molecules interacting with NPCs in intact nuclear envelopes (NEs). Monitoring single particle behavior in the complex environment of an NPC is challenging for a number of reasons. First, high localization precision is necessary. NPCs are large (~60–120 MDa), octagonally rotationally symmetric structures comprised of at least 30 different nuclear pore proteins (Nups) [1–3]. The pore itself is ~90 nm in length and is ~50 nm wide at its narrowest point. Flexible filaments extend ~50 nm into the cytoplasm, and a filamentous open basket structure extends ~75 nm into the nucleoplasm (Fig. 1) [1,4]. High localization precision for cargos moving within these NPC structures (spanning ~200 nm) is most easily obtained from micrometer-scale images using particle tracking algorithms. It is certainly desirable to be able to track more than one cargo molecule simultaneously as they traffic through distinct NPCs within the same imaging field. Second, image acquisition must be rapid. Diffusing molecules move quickly, and thus, are inherently difficult to track in three dimensions. The transport event itself occurs in <10 ms [5,6]. Third, sensitive, high-quality instrumentation is necessary to maximize the signal-to-noise ratio (S/N). The signals arising from a single molecule are inherently weak, especially for the desired time and spatial resolution. And fourth, the imaging technique must allow deep cell penetration and must provide accurate positional informa-

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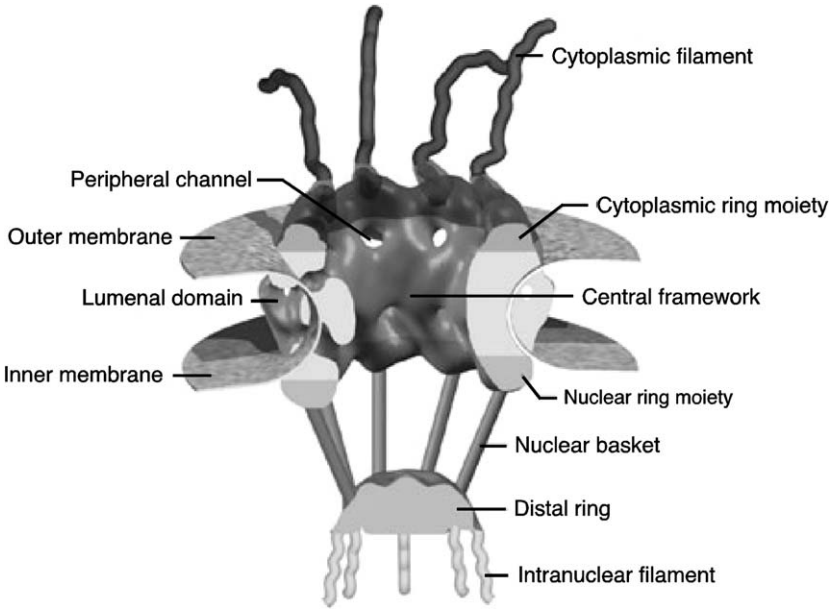

Fig. 1. The nuclear pore complex. Reproduced and modified with permission from the authors and from Nature Reviews (www.nature.com/reviews) Molecular Cell Biology [1] copyright (2003) Macmillan Magazines Ltd. The original figure was modeled and prepared by D. Stoffler using ViPEr, a Visual Programming Environment, that was developed by D. Stoffler and M. Sanner at The Scripps Research Institute, La Jolla, California, USA.

tion perpendicular to, rather than within, the plane of the NE. NEs are typically embedded deep within cells, and the desired spatial tracking dimension is perpendicular to the NE surface.

In this paper, we describe a fluorescence imaging approach that provides 2 ms and ~15 nm resolution on immobile particles and allows direct observation of molecules interacting with NPCs. The fundamental characteristics of cargo movement determined via this technique have provided an advanced molecular understanding of NPC function and transport dynamics [5].

1.2. Overview of nuclear transport

NPCs are permeable to molecules smaller than ~20–40 kDa (~4–5 nm diameter) without specific recognition (''signal-independent transport,'' or ''passive diffusion''). Larger molecules, up to ~25 MDa (~40 nm diameter), must form a complex with at least one transport receptor to transit through the NPC (''carrier-mediated, signal-dependent transport,'' or ''facilitated translocation'') [7–9]. Importins and exportins are soluble protein cofactors that recognize and bind to import and export cargos through nuclear localization sequences (NLSs) and nuclear export sequences (NESs), respectively. Import complexes (ICs), consisting of cargo and importin(s), are disassembled after transit through the NPC by Ran-GTP, the GTP-bound form of the G-protein Ran. In contrast, export complexes, consisting of cargo, exportin and Ran-GTP, disassemble upon Ran-GTP activation (leading to GTP hydrolysis) by Ran's cytoplasmically localized GTPase activating protein, RanGAP [8]. The Ran-GTP concentration gradient is established and maintained by cytoplasmic RanGAP and nucleoplasmic RanGEF, the chromosome-bound guanine-nucleoside exchange factor for Ran, which catalyzes GDP/GTP exchange (Fig. 2) [8]. Model cargos can be generated by attaching an NLS and/or an NES to a molecule of interest. Expression plasmids are available for transport cofactors (including Ran, RanGAP, and many importins and exportins), and thus, the role of these transport cofactors can be directly tested in *in vitro* assays with permeabilized cells.

An extensive network of thousands of phenylalanine–glycine (FG) repeat motifs located on almost half the Nups (FG-Nups) provides binding sites for importins and exportins and thereby provide the NPC interaction sites for cargo complexes [2]. These FG-Nups are distributed throughout the NPC structure on both the cytoplasmic filaments and nuclear basket, and in the pore itself [3,10,11]. The FG-repeat regions are highly unfolded (disordered) and highly flexible [12]. The structure, distribution and properties of the FG-Nup filaments within the NPC central pore (invisible in Fig. 1) have been intensely debated. It is clear that these filaments play critical roles in selectivity and permeability regulation.

1.3. Why single molecules?

There are at least four main advantages of single molecule experiments. First, reactions do not need to be synchronized. This avoids a main drawback of ensemble kinetic experiments where it is often difficult to obtain a homogeneous population that can be instantaneously triggered to begin the reaction of interest. Second, fluctuations and distributions of dynamical processes and kinetic parameters can be extracted from studies on single molecules that are simply lost by ensemble averaging. Thus,