Distinct, but not completely separate spatial transport routes in the nuclear pore complex

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The nuclear pore complex (NPC), which provides the permeable and selective transport path between the nucleus and cytoplasm of eukaryotic cells, allows both the passive diffusion of small molecules in a signal-independent manner and the transport receptor-facilitated translocation of cargo molecules in a signal-dependent manner. However, the spatial and functional relationships between these two transport pathways, which represent critical information for unraveling the fundamental nucleocytoplasmic transport mechanism, remain in dispute. The direct experimental examination of passive and facilitated transport with a high spatiotemporal resolution under real-time trafficking conditions in native NPCs is still difficult.

To address this issue and further define these transport mechanisms, we recently developed single-point edge-excitation sub-diffraction (SPEED) microscopy and a deconvolution algorithm to directly map both passive and facilitated transport routes in three dimensions (3D) in native NPCs. Our findings revealed that passive and facilitated transport occur through spatially distinct transport routes. Signal-independent small molecules exhibit a high probability of passively diffusing through an axial central viscous channel, while transport receptors and their cargo complexes preferentially travel through the periphery, around this central channel, after interacting with phenylalanine-glycine (FG) filaments.

Strikingly, these two distinct transport zones are not completely separate either spatially or functionally. Instead, their conformations are closely correlated and simultaneously regulated.

In this review, we will specifically highlight a detailed procedure for 3D mapping of passive and facilitated transport routes, demonstrate the correlation between these two distinct pathways, and finally, speculate regarding the regulation of the transport pathways driven by the conformational changes of FG filaments in NPCs.

NPC Structure and Function

In eukaryotic cells, the double lipid bilayer of the nuclear envelope (NE) separates the nucleus from the cytoplasm. Transcription in the nucleus and translation in the cytoplasm require eukaryotic cells to perform highly regulated, massive transport between the two segregated compartments. Although large ribonucleoprotein complexes were recently reported to be exported from the nucleus through nuclear membrane budding, the majority of the bidirectional trafficking of macromolecules between the nucleus and the cytoplasm is still believed to be mediated by thousands of nuclear pore complexes (NPCs) embedded in the NE. As revealed by electron microscopy, the architecture of the NPC consists of a central scaffold region with an approximate length of 40–90 nm and an inner diameter of 50 nm, multiple flexible fibrils extending approximately 50 nm into the cytoplasm and a basket structure that protrudes approximately 75 nm into the nucleus. The NPC is a large assembly composed of approximately 30 different proteins, each of which is present in multiples of eight copies, known...
as nucleoporins (Nups). Approximately one-third of these Nups possess a native unfolded structure with domains that are rich in phenylalanine-glycine (FG) repeats. These FG Nups form a selective permeability barrier in the NPC that allows two modes of transport to occur: the passive diffusion of small molecules (< 40 kDa) in a signal-independent manner and the facilitated translocation of cargo molecules (up to ~25–50 MDa) in a signal-dependent manner. The passive diffusion of small molecules through the FG barrier in the NPC suggests that the available spatial channels (or gaps) in the barrier are sufficiently large for small molecules to diffuse through them, without consuming any chemical energy. In contrast, in the facilitated translocation pathway for signal-dependent cargo molecules, these molecules either permeate the NPC with a low efficiency or are repelled because they observed common pathways and their cargo complexes achieve efficient translocation through the NPC by dissolving in the FG hydrogel network, rather than by collapsing the FG filaments toward their anchors. Finally, the forest model posits that two separate transport receptors collapse or dissolve in the FG hydrogel network, allowing the passive diffusion of small molecules to occur.}

### Spatial Transport Routes through the NPC

To maintain cellular viability and growth, the NPCs must mediate massive transport of various molecules between the cytoplasm and the nucleus. The sizes of in-transit molecules can be as small as half a nanometer or as large as several tens of nanometers. It has been estimated that a single NPC could mediate the transport of up to 28 MDa of materials between the cytoplasm and the nucleus per second. Given the physical confinement of the nuclear pores and the millimolar filamentous environment, especially in the central scaffold region of the NPC, the sizes of in-transit molecules can be as small as half a nanometer or as large as several tens of nanometers. From these studies further indicated that these two transport modes were functionally independent, without any competition occurring between them. However, at the same time, many other researchers argued against the above conclusions because they observed common pathways shared by passive and facilitated transport, not only spatially but also in terms of function.

### Table 1. Although no current model covers all of the known properties of nuclear transport, some models do make different, testable predictions for passive and active transport through the NPC

| Prediction Model | I. Whether a single central channel or multiple channels for passive diffusion of small molecules exist in the NPC. | II. Whether passive and facilitated transport exhibit spatially separate or shared pathways in the NPC. | III. Whether transport receptors and their cargo complexes collapse FG-repeat filaments or dissolve into the filamentous meshwork during their transport. |
|------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Polymer brush    | n/c                                             | n/c                                             | Collapse                                        |
| Selective phase/hydrogel | Multiple                                       | Joined                                           | Dissolve                                        |
| Oily Spaghetti/ROD | Single                                          | Separate                                        |                                                  |
| Forest           | A single central channel and eight peripheral channels | Joined                                           | n/c                                             |

n/c means not clear.
To address the long-lasting disputes described above and to distinguish among the proposed transport models, direct mapping of the routes of passive and facilitated transport in native NPCs is required. However, the size of the NPC, the fast, inherently 3D movements of molecules through the NPC, and the natively unfolded FG Nups that fill the nuclear pore constitute a challenge to current imaging techniques. First, the 200-nm long NPC, which is just below the diffraction limit of light microscopy, requires imaging approaches with a high spatial resolution, such as electron microscopy (EM) or super-resolution light microscopy. Nevertheless, while the spatial resolution of EM can be as high as several nanometers, the fixed or frozen samples of NPCs used in EM imaging prevent real-time tracking of molecules through the NPC. Alternatively, super-resolution light microscopy is usually capable of capturing dynamic, real-time images at a sub-diffraction-limit spatial resolution of 20–80 nm, but temporal resolution is sacrificed due to adapting minute-or even hour-long detection times, which is far above the millisecond nuclear transport time.54,59,60,63-65 Furthermore, the inherently 3D movements of in-transit molecules in the NPC require an imaging technique that is able to follow the molecules through the NPC in 3D, within their millisecond transport times. Unfortunately, the currently available 3D imaging methods generally cannot capture or distinguish such rapid diffusion in a sub-micrometer bio-cavity such as the NPC.7,9,75 Finally, the complexity of natively unfolded FG Nups in the NPC makes mimicking real nuclear transport challenging.70-75

SPEED microscopy. To meet the above requirements for the mapping of nuclear transport, an innovative imaging approach that combines single-particle tracking and a deconvolution algorithm has recently been developed in our laboratory.79-82 First, single-particle tracking of in-transit molecules through a single NPC is realized using single-point edge-excitation sub-diffraction (SPEED) microscopy. The first goal of SPEED microscopy is to illuminate a single NPC in the NE in permeabilized and living cells. In SPEED microscopy, a laser beam is shifted from the center of an objective to enhance the inclined diffraction-limited spot illumination volume at the focal plane of the objective at an angle of 45° from the perpendicular direction (Fig. 1A). Such an inclined illumination volume generates a point spread function (PSF) smaller than the average distance between the nearest neighboring nuclear pores in three dimensions.81 For example, if a 488-nm laser beam is used to illuminate a single GFP-labeled NPC in cells, the resultant illumination volume associated with a 260-nm PSF in three dimensions is sufficiently small to selectively excite a single NPC from thousands of NPCs on the NE with an average neighboring distance of 400–500 nm.79 Next, a second laser with a different wavelength following the same beam path as the 488-nm laser is used to conduct single-particle tracking of labeled in-transit molecules through this illuminated NPC. Noteworthy, the major difference between SPEED microscopy and a previous reported highly inclined and laminated optical sheet microscopy is the different illumination volume formed at the focal plane of objective. The former provides a diffraction-limit point excitation and the latter generates a plane illumination. The illumination of a single NPC in the NE provides the following advantages.79-81 (1) Single NPCs in cells can be...
directly localized with a 1–3 nm spatial localization precision, rather than localizing the partial or entire NE, as in previous measurements.\(^\text{56}\) (2) The diffraction limit illumination volume of SPEED microscopy enables a very high detection speed with a CCD camera. (3) A better spatial localization precision is generated for single moving molecules due to minimizing the partial or entire Gaussian width. As an example, we achieved a localization precision of 9 nm for immobile molecules and of 10 nm for moving molecules at 400 μm by collecting ~1,100 photons from four Alexa fluor 647-labeled Imp β1 molecules.\(^\text{57}\) (4) Accurate determination of the relative locations of single trajectories of single in-transit molecules and the centroid of the NPC results in determination of the real-time 2D spatial distribution of molecular locations in NPCs. (5) Single-pore imaging provides the essential conditions for further development of a deconvolution algorithm to realize 3D mapping of transport routes in the NPC.

Side view of nuclear transport via SPEED microscopy. To distinguish the import and export events occurring through the NPCs, we employed SPEED microscopy to illuminate single NPCs at the equator of the NE and to track single in-transit molecules through NPCs.\(^\text{70}\) After the centroids of single NPCs and the single-molecule trajectories of in-transit molecules through these NPCs were overlaid, a 2D plot of the spatial locations of in-transit molecules in the NPC was obtained. With knowledge of the starting and ending locations for each transport event, the transport direction (import or export), transport time (dwell time in the NPC) and transport efficiency (percentage of molecules successfully traversing the NPC) can be determined. The 2D spatial locations of in-transit molecules were further converted into 2D probability density maps after filtering using the Gaussian blur function. However, the obtained 2D probability density maps cannot be directly used to distinguish spatial transport routes. As shown in Figure 1B–D, the 2D maps generated for passive diffusion and facilitated transport revealed that their spatial locations display different distributions but also share some overlapping regions in the NPC. If the differences between the two transport routes are emphasized, a conclusion of distinct routes might be drawn, whereas if the overlap between the two distributions is focused upon, it would be inferred that the two transport pathways share space in the NPC. Therefore, whether passive and facilitated transport display spatially separate or shared transport routes remains to be determined.\(^\text{7,10}\) In our experiments, the 2D spatial locations of transport events in multiple NPCs were superimposed to obtain a sufficient number of locations to generate a 2D probability density map. Superposition was conducted using the centroid of each NPC as the reference point.\(^\text{7,10}\) As shown in Figure 2B, under a Cartesian coordinate system, the 3D locations \((r, x, y)\) of single molecules in an NPC are projected as their 2D spatial distribution \((r, x)\) after microscopy imaging. Under a cylinder coordinate system (Fig. 2C), the 3D information on spatial locations \((r, x, y)\) is also simplified based on the superposition of multiple NPCs. Specifically, the centroid of each NPC can be well defined as \((r = 0, x = 0)\), but independent of \(θ\) in the cylinder coordinate system. When multiple NPCs are overlaid based on the centroid of each NPC (the orientations of all of these NPCs were precisely defined in our experiments), the relative locations of single-molecule trajectories in \((r, x)\) are reliably localized, but not the \(θ\) coordinate. The distribution of single-molecule locations along the \(θ\) dimension is then averaged to make it uniform, and thus, \(θ\) becomes a constant for each single-molecule location, as \((r, x, constant)\). Therefore, the 3D coordinate \((r, x, 0)\) of the cylinder coordinate system is simplified as the 2D coordinate \((r, x)\).

Finally, a deconvolution algorithm was used to obtain the 3D spatial information on spatial transport routes in NPCs. Using the above deconvolution process, the 2D spatial distributions of the passive diffusion and transport receptor-facilitated transport pathways were converted into 3D probability density maps (Fig. 3).\(^\text{7,10}\) Recently, the distribution of single-molecule locations along the \(θ\) dimension in each NPC was experimentally confirmed to be approximately homogeneous.\(^\text{7,10}\)

3D mapping of spatial transport routes for passive diffusion vs. transport receptor-facilitated translocation in NPCs. Using the above deconvolution process, the 2D spatial distributions of the passive diffusion and transport receptor-facilitated transport pathways were converted into 3D probability density maps (Fig. 3).\(^\text{7,10}\) The 3D spatial probability density maps revealed the probable spatial dwelling locations for the targeted in-transit molecules in the NPCs, which actually reflected the real-time transport routes of these molecules through the NPCs. Clearly, small molecules exhibit a high probability of passively diffusing through an axial central path (Fig. 3A and B). Conversely, transport receptor-facilitated translocation largely avoids this central channel and preferentially occurs through the periphery around the central path (Fig. 3C–F). Fundamental similarities and obvious differences were observed when comparing the cargo-free and cargo-bound transport receptor Imp β1 (Fig. 3C–F). The efficient nuclear transport of large cargo complexes is realized through interactions between Imp β1 and the FG Nups, and thus, these cargo complexes have to follow the spatial path of Imp β1. Simultaneously, the altered binding affinity of Imp β1 to the FG Nups caused by cargo binding, the large
molecular size of cargo complex and the orientation of the cargo complex in the pore could result in the differences in the spatial transport routes for cargo-free and cargo-bound Imp β1. Moreover, the existence of distinct spatial transport routes for passive and facilitated transport has been repeatedly confirmed for the transport routes of other small molecules and transport receptors. Thus, the 3D imaging approach clearly provided strong evidence supporting a single central channel configuration and the existence of distinct spatial transport routes in the NPC.

Bottom view of nuclear transport via SPEED microscopy. In addition to obtaining a side view of nuclear transport, single NPCs can be illuminated from the bottom (see supporting information in ref. 79). The advantage of this approach is that it allows direct observation of the spatial routes of passive and facilitated transport in a cross-section of the NPC. Based on the Gaussian widths of the in-transit molecules that indicate their positions in the axial direction of nucleocytoplasmic transport, whether these transiting molecules are inside or outside the NPC were determined. The single-molecule trajectories of the events inside the NPC are then superimposed to obtain their spatial distribution in a cross-section of the NPC. The spatial distributions of small dextran molecules and Imp β1 clearly indicated their distinct spatial transport pathways in the NPC cross-section. The small molecules were concentrated in the central region, whereas Imp β1 preferentially remained in peripheral regions and seldom occupied the central channel. These results agreed very well with the cross-sectional view of passive and facilitated transport routes obtained from the deconvolution process (Fig. 3B and D) and supported the existence of a configuration involving distinct spatial transport routes for passive and facilitated transport in the NPC.
Distinct spatial routes have been identified for passive and facilitated transport in native NPCs, but whether they are spatially and functionally separated remains a critical question to be answered. First, it appears that there is no rigid line that spatially separates the two transport zones in the NPC, and instead, there is spatial overlap between them at their interface (Fig. 4). Furthermore, the overlapping region depends on the size of small passively diffusing molecules, as it was observed that smaller molecules passively permeate deeper into peripheral regions than larger ones (Fig. 4B). In peripheral regions, the locations where Imp β1 was observed to be concentrated suggested that there are many available binding sites provided by the FG Nups. Due to the weak hydrophobic interactions between most FG Nups, they could form filamentous or porous structures, and the small gaps (or holes) between filaments would then allow smaller molecules to passively diffuse around these structures. When the molecular size of small molecules was reduced from 29 kDa to 0.3 kDa, it was found that 0.3-kDa fluorescein molecules diffused approximately 2-fold farther into the peripheral filamentous regions than 29-kDa GFP molecules. Therefore, the distinct transport routes are not completely spatially separated. Moreover, it is reasonable to expect that smaller molecules, such as ions or water molecules, could diffuse farther, potentially extending across the entire peripheral region. On the other hand, the facilitated translocation of larger cargo complexes may occupy more space in the central channel simply due to the size of these complexes and, thus, cause more overlap between the two transport zones. Interestingly, experimental data indicate that it is unlikely that facilitated transport would completely occupy the central channel and block passive diffusion pathways in the center of NPCs under physiological conditions (unpublished data). It has been reported that very large cargo molecules require more transport receptors to assist their transit through a nuclear pore. Large numbers of transport receptors in a pore (termed a high pore occupancy) would induce conformational changes in FG filaments, which would subsequently result in alteration of the overall configuration of the transport routes in the NPC. Based on our preliminary data, we found that the FG filaments displayed different conformations under a low and high pore occupancy of Imp β1 in the NPC. Some FG filaments were forced away from the axial center and toward their anchors on the walls of the pores. Conversely, other filaments extended into the center, stimulated by the change. Recently reported in vitro measurements suggested similar changes. Such alterations of FG filaments have direct effects on the spatial geometry of the transport routes and subsequently affect the kinetics of both passive and facilitated transport. Moreover, the changes in these two pathways have been found to be closely correlated and nearly complementary to each other within nuclear pores. Therefore, distinct, but closely correlated transport routes for passive and facilitated transport exist in the NPC.
Furthermore, the inhomogeneous spatial distribution of Impβ{sub}1{superscript} suggests that the spatial distribution of FG filaments along the NPC is not homogenous. Hence, these FG filaments likely cluster to form distinct sub-regions along the NPC, which may provide unique micro-environments for specific biochemical interactions or multiple transport pathways for different cargo complexes, assisted by various transport receptors.

Conclusion and Perspective

Two core questions are closely related to the understanding of nuclear transport mechanisms. One is how passive and facilitated transport share the nuclear pore space, and the other concerns the structure of the selective barrier formed by the unstructured FG Nups in the NPC.

Regarding the former, our tests involving various molecules (small molecules with a molecular weight of 0.3–40 kDa; transport receptors Impβ{sub}1, transportin, NTF2 and Tap/p15 and cargo complexes) have revealed that passive and facilitated transport exhibit distinct spatial transport routes that are not, however, completely separate either spatially or functionally. It has been reported that the transport kinetics and spatial transport routes involved in the passive diffusion of small molecules can be altered at a high Impβ{sub}1 pore occupancy (Fig. 5). Moreover, the degree of spatial overlap and functional interference between these molecules must occur through the same axial central channel in NPCs.

Finally, the 3D probability density maps obtained for both passive diffusion and transport receptor-facilitated transport further indicate the spatial distribution of FG filaments in the NPC. Driven by its interaction with FG filaments, Impβ{sub}1 can efficiently migrate through the NPC, despite the fact that its size is far above the cut-off threshold for passive diffusion. Studies have indicated that Impβ{sub}1 can interact with almost all of the FG Nups within the NPC, suggesting that the spatial pattern of Impβ{sub}1 localization in the NPC also reveals the location of the FG filaments, with a higher Impβ{sub}1 density suggesting a higher density of FG filaments. Thus, the low probability of observing Impβ{sub}1 in the axial central channel suggests that there are no, or very few, FG filaments in the central region. As a result, the relatively less filamentous central conduit functions as the major diffusion path for the passive diffusion of signal-independent small molecules.

Moreover, the inhomogeneous spatial distribution of Impβ{sub}1 suggests that the spatial distribution of FG filaments along the NPC is not homogenous. Hence, these FG filaments likely cluster to form distinct sub-regions along the NPC, which may provide unique micro-environments for specific biochemical interactions or multiple transport pathways for different cargo complexes, assisted by various transport receptors.
pathways depends on (1) the molecular size of small, passively diffusing molecules and facilitated cargo complexes and (2) the regulatory effect of the studied transport receptors on the FG Nups. Although further tests are still needed to examine the spatial transport routes of very small or very large molecules in transit in native NPCs, our expectation is that very small, signal-independent molecules, such as ions and water molecules, might be capable of diffusing across the entire FG filamentous region. Meanwhile, the facilitated transport of very large cargo complexes may extend into the axial central channel or involve conformational changes to allow these complexes to squeeze through the compact selective barrier. Concerning the latter question, the configuration of FG filaments in the pore may ultimately determine the geometry of transport routes for passive and facilitated transport in NPCs. Both the conformation of FG Nups and the geometry of transport routes can be further regulated by the pore occupancy of transport receptors to ultimately meet the trafficking needs of cells. Novel experimental designs and new techniques are urgently needed to ultimately structure the selective barrier in the NPC.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.

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