Resolving molecular ageing processes of nuclear pore proteins using a microfluidic droplet assay

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The physiological permeability barrier for molecular traffic between the nucleus and the cytosol is filled with intrinsically disorder proteins (IDPs) and assembled by the nuclear pore complex (NPC). These highly enriched disordered nuclear proteins contain domains with high amounts of phenylalanine and glycine (FG-Nups). In order to understand the physico-chemical properties of such molecular "gatekeeper", we made use of a microfluidic device capable of controlled protein condensation, combined with fast and parallelised data acquisition. Our microfluidic device permits studying phase separation on the seconds time scale (due to diffusive mixing and laminar flow), coupled with rapid optical inspection of permeability barrier properties. This time resolution is challenging to achieve by conventional benchtop experiments such as coverslip assays. Our experiments show a rapid aging of FG-Nups into different material states (liquid, gel, solid etc.) within minutes under physiologically relevant concentrations. Already early droplets show typical properties of a liquid state and resemble a barrier and cargo delivery properties found for physiological nuclear transport. This includes formation of a natural barrier for cargoes larger than ~4 nm, unless accompanied by nuclear transport receptors (NTRs). For a better understanding of the evolution of supramolecular structures as well as the mechanical properties of FG-rich droplets, we combine our microfluidic system together with coherent anti-Stokes Raman spectroscopy (CARS) and particle tracking microrheology (PTM). This interdisciplinary approach provides a coherent picture to explain how the balance between homo- and heterotypic interactions in FG/NTR mixtures modulates the phase behavior and how this relates to the permeability barrier function of the condensed liquid state. The microfluidic platform described above can work as a general tool to study LLPS of phase separating proteins, particularly those that undergo rapid maturation to gel or amyloid like states, such as e.g. FUS, Tau and alpha-synuclein or other proteins associated to neurodegeneration.

Nuclear Pore Complex

The metazoan NPC is a ~120 MDa complex built from multiple copies of ~30 different nucleoporins (Nups). The complex forms a barrel- or ring-like structure with a core that is ~110 nm wide and ~70 nm thick and that traverses the nuclear envelope (Figure 1 and 2) [2]. Multiple copies of 9 different Nups constitute the NPC permeability barrier (Figure 1). These Nups have large domains rich in phenylalanine (F) glycine (G), or FG, repeats such as FxFG, GLFG, and FG motifs (in what follows collectively abbreviated as FG-Nups). In contrast to the complex's scaffold proteins, FG-Nups are primarily disordered [3,4]. Interestingly, the central core populates mM amount of FG-Nups that fill the inner channel.

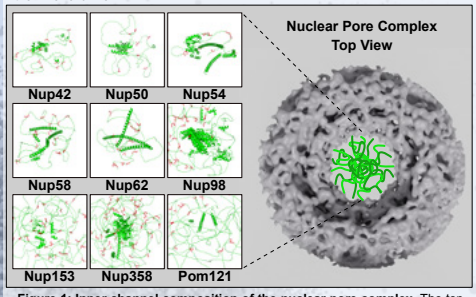

Figure 1: Inner channel composition of the nuclear pore complex. The top view on the nuclear pore complex shows a typical ring-like structure (Right panel) where the inner channel is densely filled Nups rich in F and G domains (Left panel, AlphaFold structures with backbone in green and FG-repeats in red).

The permeability barrier in the NPC is unique in that it behaves as a passive barrier with a "soft" cutoff size of ~4 nm for inert cargo [5,6]; however, with the use of special adapter proteins called nuclear transport receptors (NTRs), cargos as large as ~36 nm can pass through (Figure 2 and 3) [7]. In addition, NTRs can transit the cargo incredibly fast (~5 ms with e.g., Importin NTRs [8.9]) compared to the cargo alone. Due to the inability to visualize the molecular architecture of the NPC barrier, the nature of passive selectivity and facilitated transport is the subject of significant debate.

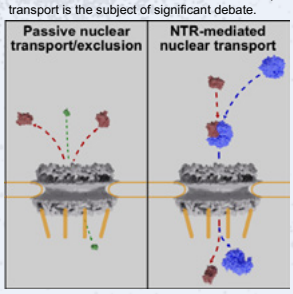

Figure 2: Schematic illustration of nuclear transport. (Left panel) Molecules with a diameter below 4 nm (green) can passively diffuse through the nuclear pore (grey). Molecules larger than 4 nm (red) are getting passively excluded. (Right panel) Only the binding of nuclear-transport receptors (NTRs) (blue) to larger cargos facilitates the transport through the pore.

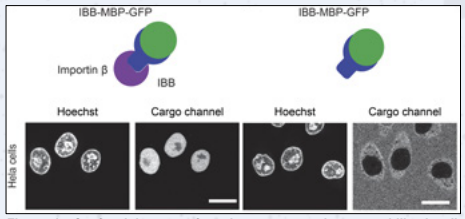

Figure 3: Confocal images of nuclear transport in permeabilised cell assays. (Left panel) Cargo construct IBB-MBP-GFP was added together with Importin-β to permebilised cells. The protein complex can actively fascilitated to the nucleus. (Right panel)Whereas the same cargo gets excluded from the nucleus when Importin-β is absent. Scale bars are 50 µm. Adapted from [1].

Microfluidic Design

Previous investigations indicate that FG-Nups readily phase separate, aggregate and mature to more solid species. Different studies capture FG-Nups at different phases and states in time. A promising approach to study the permeability barrier is to develop *in vitro* reconstitution models of FG-Nups that capture key features of the functional NPC such as selective transport and affinities to nuclear transport receptors (NTRs). Due to the transient nature of the LLPS state for FG-Nups *in vitro*, kinetical and mechanistic experiments including manual sample and bulk liquid handling are challenging to assess. Therefore, we developed a microfluidic application allowing reproducible formation of FG-Nup liquid droplets under laminar flow and thus enables the study of phase separation on the microsecond time scale. Furthermore, after diffusive mixing and droplet formation in the first mixer (MI in Figure 4 A and B) an additional mixer (MII in Figure 4 A and B) immerses the freshly formed FG-Nup droplet in cargo proteins. Thus, various protein interactions in the presence or absence of NTRs or other Nups can be screened. The efficient accessibility of cargo for fully quantitative kinetic and mechanistic measurements are crucial and has been improved over generations of microfluidic designs (Figure 4) [1]. Thus, our microfluidic approach provides a biophysical tool to investigate very transient molecular species quantitaively in a time resolve manner.

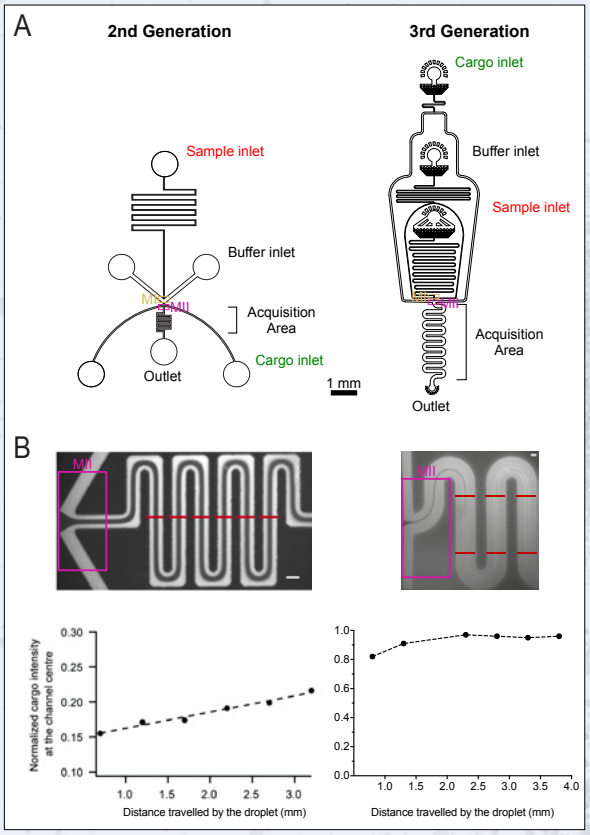

Figure 4: Development of our microfluidic chip design and hence following improvements in data acquisition. (A) Scheme on the left is the microfluidic device adapted from [1]. Design on the right shows updated design with further improvements on the microfluidic chip. Major changes were the combination of inlets and the adjustment of microfluidic resistance in order to allow controlled liquid handling with negative pressure applied at the outlet. (B) Changes in channel dimensions, resistance and thus flow ratios and speed provide a further improvement and rapid delivery of cargo molecules throughout the channel. As can be seen from the normalized cargo intensity plots from [1] (left) and our most recent design (right), we are able to equally distribute cargo molecules. Scale bars are 50 µm.

Kinetic Measurements

Microfluidic experiments showed that FG-Nups could adopt a purely liquid state that already exhibited permeability barrier like properties [1]. Those FG-Nup droplets displayed all properties of a liquid state, including coalescence, deformability, and fast fluorescence recovery in FRAP experiments. As can be seen in Figure 4 and 5, droplets are formed through rapid dilution of denaturant followed by a second mixing region, where their permeability properties are being tested. However, droplets flow at different speeds due to size and individual position in the flow channel. Therefore, we apply a tracking algorithm allowing the characterization and quantification of individual droplets with different persistence (Figure 5 B). As shown in Figure 5 A and B, in the second mixing region an IBB-MBP-GFP fusion construct was perfused over the FG-Nup droplets (Nup98). In addition, the cargo supply also contained varying concentrations of the NTR Importin-β. Physiologically, IBB (**Importin-β b**inding domain) is a domain from Importin-α, that can bind to Importin-β once Importin-α is bound to a nuclear localization signal (NLS). Based on the microfluidic quantification (Figure 5 C) it can be observed that the co-partitioning kinetics of IBB-MBP-GFP into Nup98 droplets occurs in an Importin-β concentration dependent manner, which resembles physiological nuclear import. The more Importin-β is present the more IBB-MBP-GFP can be imported. Further experiments in [1] also validate that those droplets also show passive exclusion properties, and that even large viral capsids can penetrate the liquid droplets rapidly. This establishes that the liquid state recapitulates major features of the intact NPC machinery and provides an important model system that warrant further investigation.

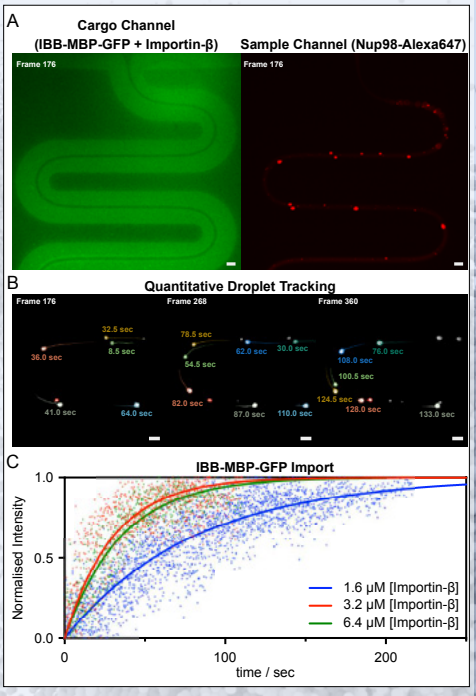

Figure 5: Microfluidic quantification of cargo import kinetics into FG-Nup droplets in an NTR dependent manner. A) Fluorescence intensity of cargo and Nup98 was acquired. Hence, the droplet signal (red) was converted to a mask and applied on the cargo signal (green) to obtain the cargo influx inside droplets. B) After background subtraction, droplets were tracked individually using the ImageJ plugin Trackmate over a set of frames [10]. C) The increasing cargo intensity for each individual droplet was quantified and normalised. The individual fittings line in the kinetic trace picture shows the fit performed on traces using a single rising exponential. The scale bars are 50 µm.

Mechanistic Analysis

Combining the controlled droplet formation and tracking with microrheology measurements allow for monitoring the regimes of composition bifurcation and droplet coarsening, but also the quantification of transient mechanical properties. Therefore, tracking droplet enclosed polystyrene beads (blue circles, Figure 6 B) allow the investigation of mechanistical properties of the FG-Nup liquid state and the maturing into hydrogel-like materials. In addition, applying coherent anti-Stokes Raman spectroscopy (CARS) on the microfluidic chip (Figure 6 C) can provide structural protein information. This technique probes the intrinsic motion of chemically bonded nuclei (e.g. amide C=O stretching in proteins) and provides a direct, label-free readout of each group. In the case of proteins, secondary structure and local environment causes specific changes in the Amide I (1600 – 1700 cm^{-1}) and Amide III (1200-1300 cm^{-1}) vibrational bands due changes in hydrogen bond configurations and allow for quantification of α-helix, β-sheet or disordered coil structures due to spectral decomposition [11,12]. Together with the controlled addition of specific NTRs as cargo at various stages of FG-Nup LLPS for different FG-Nups and/or mixtures this platform is excellently suited for gaining fundamental insight in the dynamics associated with the coupling between LLPS of IDP's with "sticky" domains and the occurrence of transient elastic effects due to non-covalent network formation.

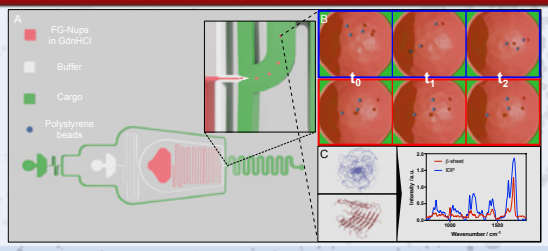

Figure 6: Microfluidic droplet formation combined with microrheology and coherent anti-Stokes Raman spectroscopy (CARS) measurements. A) The microfluidic chip is loaded with FG-Nups solved in GdnHCl (red) together with buffer (white). Due to fast dilution of GdnHCL in the first mixer and further dilution with cargo solution (green), the FG-Nups show rapid droplet formation. B) Mechanical properties and coarsening can be further studied by microrheology of polystyrene beads (blue circles at different time points for liquid-like (blue boxes) and stiff (red boxes) droplets) or C) coherent anti-Stokes Raman spectroscopy (CARS) directly on chip. This provides structural protein information (IR spectra for disordered (blue curve) and beta-sheet (red curve) structures) and allows to quantify secondary structure content.

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Acknowledgment

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