Chapter 1

General introduction
Chapter 1

The Nuclear Pore Complex

The central dogma in biology, describing the information flow in biological systems, states that DNA is transcribed into RNA, followed by translation of RNA to protein. Subsequently, proteins are folded and targeted to specific compartments to perform their function(s). Eukaryotic cells have a nucleus containing the DNA, while the ribosomes that synthesize proteins from RNA are located in the cytoplasm. Thus, transcription and translation are separated and located in different compartments, which allows for more control for regulation. However, it also requires efficient and regulated transport in and out of the nucleus. The Nuclear Pore Complex (NPC) is the main gateway for this traffic. This protein complex is embedded in the nuclear envelope (NE), spanning both its inner and outer nuclear membrane. NPCs are conserved in eukaryotes from yeast to humans, although the size and molecular mass are quite different between species (61): the NPC is 66 MDa in *Saccharomyces cerevisiae* (158) and 125 MDa in vertebrate (148). The amount of NPCs per nucleus varies greatly between species, cell type and cell cycle stage (61). In yeast there are approximately 50-200 NPCs on average, or 12 NPCs/µm² NE (193). Nevertheless, several basic principles described below are conserved between species and knowledge obtained in one organism can mostly be applied to other organisms as well.

Structure of NPC

*Core structure*

NPCs are big 50-125 MDa proteinacious complexes. The overall structure of the NPC has been elucidated using various techniques. Electron microscopy (EM) showed structures in the nuclear envelope (4, 21) with eight-fold symmetry (52, 53, 194). Later studies showed a donut-shaped structure with an additional two-fold symmetry in the plane of the membrane (66). Developments of new techniques, e.g. cryo-electron tomography (cryo-ET), resulted in further improvement of the resolution of the determined structures (11, 114). Next to this core donut-shaped scaffold structure there is a basket-like structure attached to the scaffold on the nuclear side, also called the nuclear basket, and on the other side filaments protrude into the cytoplasm, the cytoplasmic filaments (59, 76, 154).

The protein composition of NPCs is relatively simple; only ~30 proteins
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...nucleoporins or nups), present in multiple copies, form the complex (156). These nups can be divided in three classes. The first class exists of three nups that contain transmembrane domains and anchor the complex to the NE. About half of the nucleoporins form the second class. They have a structural function and form the scaffold of the complex. The third class comprises the partially unstructured phenylalanine-glycine (FG) nups. These nucleoporins contain intrinsically disordered domains with multiple FG-repeats that occupy the center of the pore. Via a structured domain they are anchored to the scaffold of the NPC. The unfolded FG-domains play an important role in the function of the NPC, as they form the permeability barrier and facilitate transport through the NPC. The function of the NPC will be discussed in more detail later in this chapter.

A more detailed view of the structure of the yeast NPC was presented in a 3D probability map of mass densities that was obtained using a combined computational, biochemical and cell biological approach (Figure 1.1A) (6, 7). The cytoplasmic filaments and the nuclear basket were omitted in this study. This map shows that the NPC is build from 8 spokes. In addition to the eight-fold symmetry, the cytoplasmic halve of the complex is almost identical to the nucleoplasmic halve. The outer and inner rings form the scaffold of the NPC. The membrane rings anchor the complex to the NE. The structured parts of the FG nucleoporins together with the linker nups form the inner side of the pore. After this model of the yeast NPC, the structure of the human NPC...
was elucidated in more detail by combining the cryo-EM and ET approaches with single-particle electron microscopy, and crosslinking mass spectrometry (Figure 1.1B) (18). This showed some clear differences between the yeast and human NPC in their structure, most prominently the two double rings of y-shaped complexes in human NPCs, while there are only two single rings in yeast, which might explain (part of) the size difference between the NPCs of these species.

Further details of the localization of different nups was obtained using more recently developed techniques like super-resolution light microscopy (107, 177). Similar techniques were also used to count the copy number of the different nups in the yeast NPC (121), of which the results agree mostly with the previously published copy numbers (7, 156), with the main differences seen for Nic96 and Nsp1 that show only 16 copies instead of the 32 published earlier. The evolution of these newer techniques will probably soon result in a more complete and accurate structure.

**FG-nups**

The center of the pore is filled with the intrinsically-disordered domains of the FG-nups. In yeast there are 13 FG-nups identified, which are all largely unfolded and contain multiple FG repeats. Different types can be distinguished, with different FG-repeats and inter-repeat sequences. Nups containing GLFG-repeats are cohesive, meaning they can interact with other GLFG-repeats, while FxFG-nups do not bind tightly to each other (138). Also the residues flanking the FG-repeats have different properties in the various FG-nups, resulting in more extended or collapsed conformations (197). Together these unfolded domains are responsible for the permeability barrier of the NPC while at the same time allowing the fast transport of cargo-transport factor complexes.

Several models of how the selective barrier works have been proposed, which can be divided into two main groups. The first group assumes that the FG-nups in the pore behave as a polymer brush, thereby entropically excluding molecules because of crowding (104, 113, 157). The energy obtained by binding of transport factors to the FG-repeats then overcomes this entropic barrier. The ‘reduction of dimensionality’ model is an extension of the entropic gate models and proposes that the FG-repeats form a layer aligning the scaffold of the NPC. This enables a faster two-dimensional diffusion of transport factors over this layer, instead of a 3D motion through the whole center of the pore, while the rest of the FG-domains exclude other molecules (142).
The second group of models is based on the assumption that the FG-nups interact with each other, thereby forming a selective phase or hydrogel (151, 153). Transport is facilitated by binding of transport factors to the FG-repeats, replacing the FG-FG interactions. Other molecules cannot diffuse through the pore because the FG-nups form a meshwork with only small holes in between. Others proposed a combination of these models, where the more cohesive nups form a selective phase, while more extended nups act as a polymer brush (138, 197). Recent experiments also point in this direction (46), showing that the truth will probably combine aspects of both models.

One way to get more insight in the combined behavior of the disordered proteins is to use computer simulations. Single nups or a group of peptides can be modeled using atomistic models (35, 94, 122, 197), but this molecular dynamics is not feasible for the collective FG-domains in the NPC because too much calculation time would be needed. Different computational models have been put forward, from more coarse-grained where the FG-domains are divided in segments of multiple residues (123, 128, 129, 134, 135), to models that account for the amino-acid sequence of the nups (56, 179). These models, in combination with in vivo experiments, are useful tools to study the properties and roles of FG-nups and be a valuable source of information on the mechanism of transport through the NPC.

**Functions of NPC**

*Cellular functions*

The main function of the NE is to separate transcription and translation, therefore NPCs should be able to prevent diffusion of the biological molecules that are essential for those processes. Indeed, the NPCs effectively block the passive crossing of large structures, like the chromatin, mRNPs and ribosomes. Next to this main task, an important function of the NPC is to facilitate the creation and maintenance of gradients across the nuclear envelope in a fast and regulated way, to be able to respond quickly to changing conditions. To achieve this, it is important that there is a large difference between the rates of active transport and the leak of molecules. This poses the biggest challenge for the NPC: being effectively ‘open’ to facilitate the efficient and fast transport of a wide variety of cargos, including large complexes like mRNPs, but at the same time being ‘closed’ to prevent leak of non-cargo. These dual properties make the NPC an important object to study, for their biological function as
well as for applications in biomimetic materials and pores (23, 77, 91).

Next to the function in transport, research in recent years has shown that NPCs also play a role in other cellular processes (174). The nuclear basket of the NPC extends into the nucleus, and therefore seems the most likely candidate for interactions with the chromatin, but also linker nups in the scaffold of the NPC and FG-nups have been described to play a role in gene transcription regulation (reviewed in (145)). It has been shown that highly active genes and a selection of inducible genes localize to the NPCs when they are activated (36). In addition to this role in transcription of genes, the constituents of the nuclear basket also are involved in mRNA validation by retaining improperly processed mRNAs and recognizing malformed mRNP (40). Also in double strand break repair of DNA the NPCs are involved in recruiting the damaged DNA to the periphery of the nucleus (19, 178). This all shows that NPCs are not just transport machines, but they play an extended role in the biology of eukaryotic cells.

**NPC structure and function during ageing**

Recently, it was found that some nups are among the most long-lived proteins in cells (32). Especially the scaffold nups are not turned-over, while FG-nups are generally quickly exchanged. Also the different sub-complexes have a different lifetime (32, 159, 185). This can result in loss of stoichiometry of the NPC complexes. In addition, because of the low turn-over, damage of the proteins can accumulate in the NPC, especially in post-mitotic cells that don’t divide. Also in dividing cells the damage can accumulate, especially if cells divide asymmetrically, like *S. cerevisiae*.

Yeast is a good model to perform ageing research, because its lifespan is only about 3 days, in which a mother cell has done approximately 30 divisions. In principle, it is easy to obtain high quantities of aged cells, as yeast is easy to culture. However, following or purifying old cells is a real challenge: when a mother cell reaches age 30, it is outnumbered by ~10^9 younger cells. Therefore methods to enrich for old mother cells were developed. Mother cells can be labeled by a tag on the cell wall, which is retained by the mother, whereas daughters receive newly synthesized cell walls and are thus not tagged. After several divisions the mother cells can be purified by the tag. Another approach is to trap mother cells in a microfluidic chip, which has the additional benefit that it allows following a cell during its lifespan.

When a yeast cell divides, the daughter cell is rejuvenated, even when the
mother cell is becoming older. For NPCs it was shown that daughter cells born from young cells are enriched in old nups (86), and that a soluble pool of nup Nsp1 is involved in the inheritance (111, 115). However, in older yeast cells extrachromosomal rDNA circles (ERCs) that are damaging for the cell are found, and these ERCs bind to NPCs. Mothers retain these NPCs. So, when a cell gets older, more of the NPCs are retained (34, 165), increasing the fraction of old NPCs in ageing cells. All this could lead to loss of functions of the NPC, causing or accelerating ageing. A first demonstration of the effects of mutations of the NPC on ageing showed that yeast strains with altered NPCs, where FG-domains of different nups were removed, have a decreased or increased replicative lifespan (RLS), depending on which FG-domain was deleted (106). In addition older cells display a lower level of accumulation of NLS-cargo. This points towards a role of NPCs in ageing, but the exact changes in NPC structure and function are still unclear.

**The role of the NPC in separating the nuclear and cytoplasmic proteome**

*Regulating protein localization*

To localize a protein to a desired location and maintain it there, or to exclude it from certain places, cells can use different approaches. One way is to trap the protein to a less mobile binding partner, thereby preventing the movement of the protein of interest. For example histones are bound to DNA, which retains them in the nucleus. Another strategy is to block the path of the protein to the undesired location, based on size or other biophysical properties. Ribosomes are excluded from the nucleus because they are too big to diffuse through the NPC. A last method that can be used is active transport, both towards the destination or pumping away from/out of the unwanted location. This can be seen when a transcription factor is actively transported into or out of the nucleus in response to a signal. The NPC also uses all three strategies in regulating the homeostasis of biomolecules in the nucleus and cytoplasm. Trapping at a binding partner can be done at the nups that are asymmetrically localized, providing specific binding sites on either side of the NE. Blocking passive diffusion and using active transport are both done by the collective behavior of intrinsically-disordered FG-domains that fill the center of the pore. How the FG-nups form the permeability barrier and facilitate active transport is discussed in more detail below.
**Passive diffusion, or permeability of the NPC - soluble cargo**

Permeability of the NPC is an old topic. Already in the 1960s the first measurements were done using injections with gold particles. There is a wealth of data from diverse eukaryotes, describing nuclear entry of fluorescently-labelled proteins, dextrans or gold particles in isolated nuclei or detergent-permeabilised cells or after micro-injection (this data is discussed in more detail in chapter 2 and is summarized in Table 2.1). In higher eukaryotes, the data consistently show that probes bigger than 40-60 kDa are excluded on the minute timescale. *In vivo* experiments using live cells expressing reporter proteins are done at much longer timescales and show that bigger proteins can enter the nucleus on an hour’s timescale. In particular multimeric GFP, consisting of 3, 4 or even 6 GFP domains fused together, are able to enter the nucleus (12, 25, 164, 190). A complication with the mammalian *in vivo* measurement is that on these longer timescales entry into the nucleus may have happened during mitosis when the nuclear envelope is broken down.

*In vivo* studies in *S. cerevisiae* are easier to interpret as the nucleus does not break down during mitosis. Several assays have been used. Influx of GFP fused to a nuclear export signal (NES) was measured after inhibiting export by incubating the cells on ice for 1 hour (167, 168). Because at 0°C active export is more affected than passive diffusion, proteins that are small enough to pass the NPC should equilibrate between the cytoplasm and the nucleus. On a timescale of an hour all reporters bigger than 66 kDa were excluded from the nucleus. However, it is questionable whether measurements at 0°C are physiologically relevant. At 30°C there is continuous import and export, and the collective traffic through the NPC may affect leak. Moreover, the physicochemical properties of the FG-nup network is likely different at 0 versus 30°C. In comparison, FRAP-based assays in living cells yield import, influx and efflux rates under steady state conditions. Efflux of GFP-cNLS-GFP has a rate constant of 0.02 s⁻¹ (118), meaning that ten-thousands of molecules in this size range would distribute between the cytosol and nucleus in minutes. So, in live cells at 30°C, molecules in this size range (~56 kDa) are indeed not excluded from the nucleus. In fact, a soluble 3xGFP reporter (~81 kDa) is equally distributed over the nucleus and cytoplasm after 1 hour of expression (54). In a different assay to determine the permeability of the NPC in yeast, expression of a 70 kDa transcription factor was used, and although only relative activities were reported, a background activity was observed.
indicating that the 70 kDa transcription factor does enter the nucleus (138). Thus, for baker’s yeast, there is \textit{in vivo} data to show that proteins larger than 60 kDa can equilibrate via diffusion between the cytoplasm and nucleoplasm when looking at minutes to hour’s timescales.

\textbf{Passive diffusion, or permeability of the NPC - membrane proteins}

Compared to soluble proteins, passive entry of membrane proteins into the nucleus is less studied. Because the membranes are continuous, membrane proteins can diffuse from the ER to the ONM and then pass the NPC at the pore membrane to reach the INM. The soluble domain may pass through the lateral channels in the scaffold of the NPC (66, 114), and it is unclear whether FG-nups are involved in forming a barrier for passive entry. Based on the small size of lateral channels the size of the soluble pore-facing (or extralumenal) domain will restrict the diffusion of membrane proteins. In mammalian cells, proteins that use a diffusion-retention mechanism to localize to the inner nuclear membrane fail to accumulate at the INM when the cytoplasmic domain is larger than \textasciitilde60 kDa (133, 172, 187, 195). Also in yeast the size of the extralumenal domain is important for diffusion over the NPC, as fusing a large globular domain to natively INM resident Doa10 prevents the protein from travelling to the INM (33). This shows that indeed the path through the NPC for membrane proteins seems more restrictive in size then for soluble proteins, consistent with the lateral channels being less spacious than the central channel. The effect of FG-nups on the passive entry of membrane proteins has not been studied.

\textbf{Active transport through the NPC - soluble cargo}

A broad range of cargos is selectively translocated over the NE. RNA transcripts have to be exported from the nucleus to reach ribosomes for translation. In addition, many different proteins have to be transported into the nucleus, for example histones, transcription factors and polymerases. The transport of cargos over the NPC is facilitated by binding to transport factors or karyopherins (kaps), which interact with the FG-repeats in the center of the pore. In yeast there are 14 different kaps known that are involved in nucleocytoplasmic trafficking (45). These kaps are called importins when they transport cargo from the cytoplasm into the nucleus, while exportins mediate nuclear export. Cargo molecules contain nuclear transport signals, which are either recognized by an importin or exportin directly or via an adapter molecule. The signal for molecules that should be imported into the nucleus is
called a nuclear localization signal (NLS). There are several different types of NLSs that each interact with specific importins in yeast, although there is some redundancy in the specificity (45). Some importins, like the yeast Kap95, bind the cargo via an adaptor protein, in this case Kap60, that recognizes the NLS in the cargo molecule, and then travel as a trimeric complex through the NPC (5). Export from the nucleus is mediated by exportins. A versatile example is the exportin Xpo1 (homolog of human Crm1), which exports proteins containing a leucine-rich nuclear export signal (NES) and can export RNAs and ribonucleoproteins (50).

The directionality of transport over the NPC and the energy source that is required for the formation of gradients and fast signaling responses come for most cargos from the RanGDP/GTP gradient (125). In the nucleus a high concentration of RanGTP is present, which is maintained by a guanine-nucleotide exchange factor RanGEF that exchanges the Ran-bound GDP nucleotide for GTP (3, 79, 132). On the cytoplasmic side of the NPC a Ran-specific GTPase activating protein RanGAP1 hydrolysates RanGTP to RanGDP, assisted by RanBP1 and RanBP2, which creates a higher concentration of RanGDP on the cytoplasmic side (14, 89). The factors maintaining this gradient are bound to nuclear or cytoplasmic constituents (chromatin and cytoplasmic filaments of the NPC, respectively), thereby staying at the desired side of the pore (44, 68, 132). In an import cycle, the cargo with NLS is bound by a kap in the cytoplasm, which facilitates the diffusion through the pore. Arriving on the nuclear side of the NPC the kap-cargo complex is dissociated by binding of RanGTP to the kap. This releases the cargo in the nucleus. Exportins form a trimeric complex with RanGTP and cargo in the nucleus, and the cargo is released on the cytoplasmic side because RanGTP is hydrolysed to RanGDP. NTF2 is the transport factor that transports RanGDP back to the nucleus, where it is converted again by RanGEF to RanGTP, hereby completing the cycle (131, 152, 170). Although the RanGDP/GTP gradient is required for most cargo transport, part of the mRNA export requires Dbp5 and Gle1 and is independent of RanGTP gradient (8, 192).

Active transport through the NPC - membrane proteins

The above-mentioned mechanism describes the active transport of soluble cargo over the NPC, but also membrane proteins have to be transported via the NPC. Several mechanisms have been proposed for the sorting of membrane proteins to the inner membrane of the nuclear envelope (10, 20,
It is known that many membrane proteins accumulate at the INM by retention in the INM (67, 172). In cells with closed mitosis, or post-mitotic cells, this retention can only happen after passing through the NPCs. However, in metazoans with open mitosis, the NE disassembles before chromosome segregation and afterwards it is reassembled around each segregated mass of chromatin. Here, no NPC-passage is needed, as recruitment of INM-proteins to the chromatin can happen during the reformation of the NE. Other signals present in these proteins are required to retain or retrieve the protein in the ER/NE-membrane network, but these are not sufficient to target the protein specifically to the INM (187). It is possible that some of these proteins use a piggyback mechanism, where the membrane protein binds to a soluble protein that contains a NLS to be imported (54). For the mammalian protein Sun2 and UNC-84, another Sun-protein from *C. elegans*, a combination of several mechanisms was shown to target the protein to the INM (181, 187). They contain a NLS that contributes to the INM localization, but this NLS is not required for accumulation. Lastly, a role of FG-repeats in INM proteins, where the FG-repeats act as an intrinsic transport factor, has been proposed (203). This mechanism might actually also be used in soluble proteins, as in both the soluble as membrane-bound nuclear proteome FG-repeats are enriched (202), but further evidence is needed to get more insight into these modes of transport to the nucleus.

In *S. cerevisiae* there are two membrane proteins, Src1/Heh1 and Heh2, whose localization in the nucleus depends on an active transport mechanism, involving transport factors, FG-nups and the RanGDP/RanGTP gradient, similar to the active transport mechanism of soluble proteins (88, 120). In addition to a (strong) NLS, the sorting signal in these proteins consists additionally of a long, intrinsically-disordered (ID) linker, and the combined NLS-L is sufficient and required for INM targeting (120). Retention in the INM also contributes to the localization of these proteins, but it is not required for this mode of transport.

**Experimental approaches to studying the functions of the NPC**

In the previous subsections the structure and functions of the NPC that are known up till now have been described, but clearly not everything is yet fully understood. To get more knowledge about the NPC and its selectivity, we use
S. cerevisiae, or baker’s yeast, as a model organism. It is a unicellular eukaryote, but still quite closely related to humans, as they are both members of the opisthokonts, a group of eukaryotes with a common ancestor that share characteristics. Yeast is a proven model to understand human biology, as is evidenced by the recent Nobel prizes in medicine of 2009 and 2013, where studies in yeast were a large part of and essential for the discoveries.

In early years several types of *in vitro* experiments have been performed, with for example isolated nuclei and purified proteins. This yielded much knowledge about the NPC, as the controlled environment allows to focus on specific components and helps to disentangle different processes. However, with progressing insight it has become clear that these *in vitro* results are not always transferable to what happens *in vivo*. For example, for the transport through the NPC it is not fully understood how cell cycle stage, karyopherin concentration, the continuous transport of all other cargo and other cellular parameters like crowding affect the kinetics, which makes it difficult to mimic these properties *in vitro*.

Although working *in vivo* will give the most complete answers, it also causes complications because of the complexity of cells, especially for targeting and sorting studies. Many different signals can influence the localization of proteins, making it challenging to disentangle the function of separate domains. Also by mislocalizing a native protein you might affect other cellular processes or even kill cells, making interpretation difficult. To decrease the complexity, artificial reporter proteins can be used. By using reporter proteins with the domain of interest fused to a tag that enables localization, in combination with artificial, non-native or non-functional domains, a clearer answer can be obtained. In addition it is easier to make mutations, making it possible to study the function of specific properties, sequences or structures. Therefore reporter proteins are a useful tool to study nucleocytoplasmic transport.

To study cells during ageing, single-cell approaches are preferred over batch-ageing experiments, because you can follow individual cells during the whole lifespan and know their exact age. Microdissection, where you manually remove the daughter cell after each division (127), has been used for a long time, but it is very laborious and time-consuming, and therefore not suitable for more high-throughput measurements. Moreover, the only read out from these experiments is a measure of lifespan. In recent years several microfluidic devices were developed that can be used at a microscope to follow the mother
cell in its whole lifespan (31, 41, 101, 196, 200). They are all based on trapping the mother cell, while flushing out the daughter cells by the flow of the medium through the chip. In all but one design (196) the trapping makes use of the fact that mother cells are bigger than daughter cells at the moment of division. These chips enable us to follow the lifespan of a mother cell and count the divisions, but it is also possible to study the localization or abundance of a protein throughout the complete lifespan, all on a single cell level. This opens up opportunities to study the mechanism of ageing in yeast, and more specifically for us the role and behavior of NPCs in ageing.

As mentioned before, another useful tool that can be used to understand biology is ‘in silico’ computational modeling. Although similar drawbacks apply as for in vitro experiments, it is a great approach to obtain molecular understanding of for example the selective barrier that is formed by the unfolded FG-nups. It is time-consuming to make mutants in vivo, but in a model this can easily be done, and predictions can be made on the effects of these mutations. With careful correlation of computational and in vivo data, models can provide valuable information and new insights to be tested.

Outline

In this thesis several studies on the role of the NPC in regulating the nuclear and cytoplasmic contents in young cells and during ageing in yeast are described.

The permeability barrier is the main topic of chapter 2. Here, we ask how permeable the pores are for soluble and membrane proteins and what defines the permeability barrier. We describe a systematic analysis of the effect of protein size on the diffusion through the NPC, using both soluble and membrane protein reporters. A new assay was developed to visualize the ability of membrane proteins to pass the NPC. In addition, the role of different FG-nups on the permeability barrier was studied, using FGΔ-mutant strains. These results were compared with a coarse-grained molecular dynamics model of the intrinsically-disordered domains of the FG-nups in the pore, which shows a clear correlation between the leakiness of the NPC with the average FG-mass density in the center of the pore. In the appendix we elaborate on the mistargetting observed for some membrane reporters with a FKBP-domain and the effect of rapamycin on this, showing that this localization does not influence the assay of NPC permeability for membrane proteins.

The third chapter is focused on the intrinsically-disordered linker that
is part of the INM sorting signal of *S. cerevisiae* protein Heh2. To get more insight in the transport pathway through the NPC for membrane proteins we ask if flexibility, which is intrinsic to ID regions, is required for the transport mechanism. We changed the biophysical properties of the linker and measured the effect on INM accumulation. Reporters with polyproline linkers of up to 149 consecutive prolines were used and these artificial proteins expressed and localized to the INM. However, it was also clear that flexibility and a large Stokes radius, representing a more extended conformation, are essential for localization at the inner membrane. This suggests the linker may need to bend to move through the NPC.

Chapter 4 describes the current state of a project to assess the composition and function of NPCs in ageing cells on a single cell level. It describes the development of a method using microfluidic devices that can be applied to follow the abundance and stoichiometry of nups at the NPC throughout the lifespan. Furthermore, it can be used to study how well the NPCs function in aged cells, by following the localization of several reporter proteins during ageing. In chapter 4, I describe the assays, design and issues related to the method, using preliminary data describing the age-related changes in stoichiometry of nups at the NPC and the age-dependent import of GFP-tcNLS and export of GFP-NES.

The major findings of chapters 2, 3 and 4 are discussed in Chapter 5.