

## Contribution of Electron Microscopy to the Study of the Nuclear Pore Complex Structure, Composition, and Function

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In interphase eukaryotic cells, the nucleus is separated from the cytoplasm by a double nuclear membrane system called the nuclear envelope. The nuclear envelope enables the two cell compartments to have their distinct composition, and separates the genetic machinery from protein synthesis. Molecular exchange between the nucleus and the cytoplasm, however, is essential for keeping eukaryotic cells alive, and is involved in the regulation of many cellular functions, including gene expression. Thus, eukaryotic cells have evolved an elaborate system for maintaining constant communication between the nucleus and the cytoplasm. A major component of this system is the nuclear pore complex, a large and extremely elaborate protein structure embedded in the nuclear envelope which mediates bidirectional nucleocytoplasmic trafficking of macromolecules. The nuclear pore complex has a molecular mass of 125 megadaltons, and is composed of 30 different proteins, arranged in an octagonal structure that is 120 nm in diameter. The structure of the nuclear pore complex has been extensively investigated using a variety of electron microscopy techniques, including classical electron microscopy techniques such as embedding and thin-section, as well as state-of-the-art electron microscopic technologies such as cryo-tomography. In addition, immuno-gold electron microscopy has been used to reveal the molecular composition of the nuclear pore complex, and to dissect nuclear transport into distinct steps. This chapter reviews the contribution of several electron microscopy techniques to the study of the nuclear pore complex structure, composition and function.

**Keywords** nuclear import; nuclear pore complex; electron microscopy; *Xenopus* oocyte

### 1. Introduction

The separation of the genetic machinery from protein synthesis by the double nuclear membrane makes nucleocytoplasmic transport a major and essential cellular activity. Most of the RNA synthesized in the nucleus is exported to the cytoplasm, where it is used for protein synthesis. Proteins required for nuclear function are synthesized in the cytoplasm and imported into the nucleus. Molecular trafficking between the nucleus and the cytoplasm occurs through specialized elaborate protein channels embedded in the double-membrane nuclear envelope, called nuclear pore complexes (NPCs). Transport through the NPC is not only essential for maintaining cell metabolism, but is also extremely important for the regulation of gene expression. Nuclear import of large nucleic acids is also the basic mechanism by which therapeutic genes can be specifically delivered to organs harboring a disease (i.e., in gene therapy). In addition, many viruses depend on nucleocytoplasmic transport to replicate and spread infection.

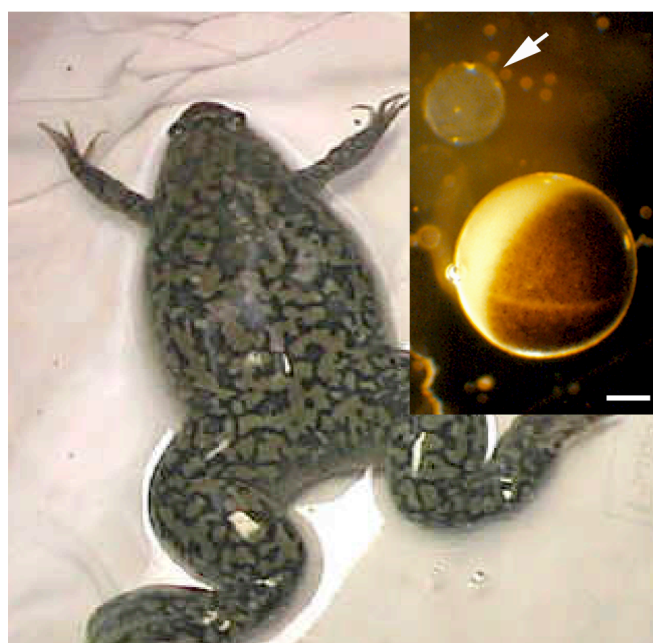
As the NPC is the major player in nucleocytoplasmic transport, extensive studies have been carried out to elucidate its structure, composition and function. Several of these studies have used microscopy techniques. The aim of this chapter is to illustrate the contribution of electron microscopy to the study of the NPC structure, molecular composition and function, with an emphasis on explaining the different electron microscopy techniques.

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## 2. *Xenopus laevis* oocyte as a model system to study NPC

With the exception of some structural studies performed with yeast NPC [1, 2] and with intact *Dictyostelium* nuclei [3], structural studies of the NPC have been performed using nuclear envelopes or nuclei isolated from amphibian oocytes. In particular, the large nucleus of *Xenopus laevis* oocyte (Fig. 1) has become a favorite system of study for scientists in the field of nuclear envelope and NPC because each oocyte has a very large nucleus (about 0.4 mm in diameter) that is easy to isolate and manipulate manually. The *Xenopus* oocyte nuclear envelope contains a high density of NPCs: 50 NPC/ $\mu\text{m}^2$ . This is extremely large compared for example with nuclei from tissue culture cells, which contain only 3-5 NPC/ $\mu\text{m}^2$  [4]. In addition, *Xenopus* nuclear envelopes yield very well preserved NPCs. Injection of *Xenopus* oocytes with transport substrate in their cytoplasm or nuclei is also relatively easy; thus, *Xenopus* oocytes are also a popular system to study nucleocytoplasmic transport [5].



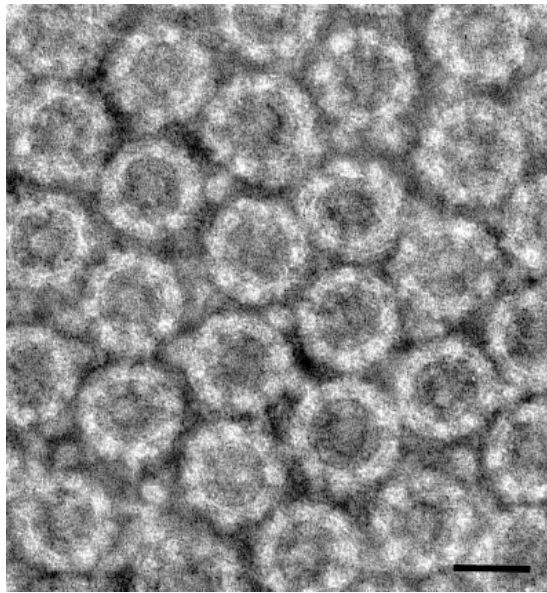
**Fig. 1** Female *Xenopus laevis* frog. The inset in the top right shows an oocyte with its pigmented and unpigmented hemispheres, and an isolated nucleus (indicated by the white arrow). Scale bar = 0.2 mm.

## 3. Conventional electron microscopy of the *Xenopus* NPC

The NPC is one of the largest protein complexes in the cell, with a total mass of 125 megadaltons in vertebrates [6] - about 30 times larger than a ribosome. With dimensions of 120 nm, the detailed structure of the NPC can only be visualized using electron microscopes. Much of our current understanding of the NPC structure comes from electron microscopy studies of *Xenopus* oocyte NPCs using a variety of electron microscopy techniques and sample preparations.

As with any other biological sample, NPCs do not scatter electrons, and therefore, do not yield well-contrasted images in the electron microscope without the use of staining. Thus, negative stain electron microscopy has been used to visualize the NPC. For this technique, a thin biological sample is placed on a carbon-coated electron microscopy grid. After allowing the sample to settle on the grid, a drop of a solution of heavy-metal salts, commonly derived from uranium or molybdenum (for example uranyl acetate or ammonium molybdate), is added to the grid and blotted dry after few minutes. The heavy-

metal salt covers the carbon film everywhere on the grid except where it has been excluded by the specimen, hence the name *negative stain*. When visualized with an electron microscope, the electrons are deflected by the heavy-metal salt but not by the specimen, thus creating a contrasted image where the sample appears grey or white against a black background. When isolated, *Xenopus* nuclei are placed and spread on a carbon-coated electron microscopy grid, the thin nuclear envelope detaches easily from the nuclear content and absorbs to the grid. As illustrated in Fig. 2, after negative staining of a *Xenopus* nuclear envelope and visualization of the specimen with a transmission electron microscope (TEM), a face-on view of the NPCs is obtained. In this view, the nuclear membrane appears black and the NPCs appear as round particles with diameters of 120 nm and an octagonal symmetry.

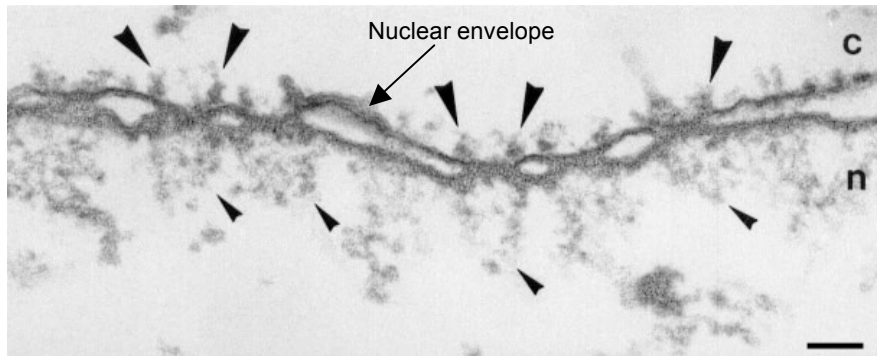


**Fig. 2** Electron micrograph of negatively stained intact NPCs from *Xenopus* oocyte nuclei. These NPCs are embedded in the nuclear membranes and have been negatively stained with a solution of 1% uranyl acetate. Scale bar = 100 nm.

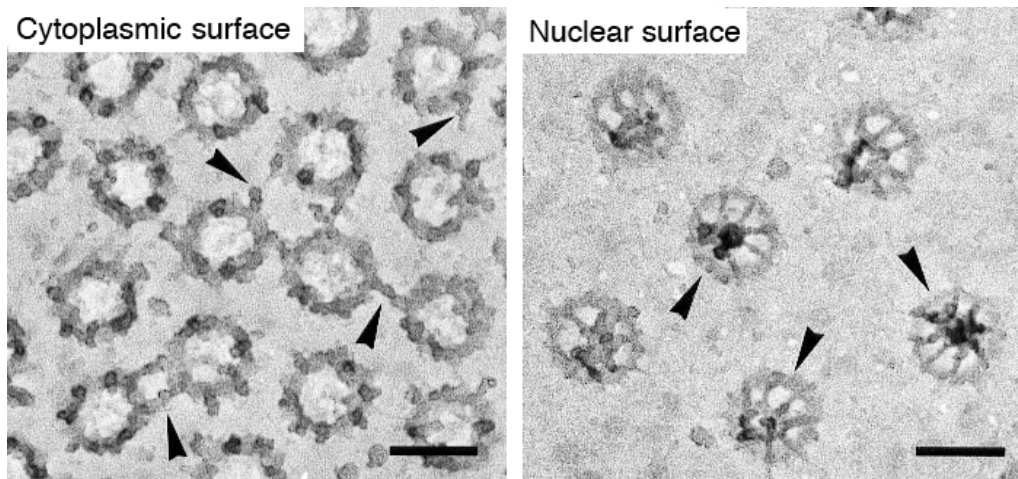
Embedding and thin-section electron microscopy is another technique that has been used extensively to visualize the NPC. For this technique, the specimen is fixed, dehydrated (by placing the specimen in solutions of increasing ethanol concentration), and permeated with an epoxy resin that polymerizes to form a solid block of plastic with the specimen embedded in it. The plastic block is cut into extremely thin sections (50-70 nm thick) with a diamond knife on a microtome. The thin sections are then placed on an electron microscopy grid and stained with a solution of heavy-metal salt (for example, uranyl acetate) to create contrast. When an isolated *Xenopus* nucleus is prepared by this technique and visualized with a TEM, the nuclear envelope and NPCs are revealed in cross-sections or side views (Fig. 3). These views show the typical structure of the nuclear envelope with the two parallel membranes, and the NPCs as electron-dense structures embedded in the spaces where the inner and outer nuclear membranes join together.

Although the NPC appears highly symmetrical in face-on views (Fig. 2), in cross-section views, the NPC appears highly asymmetric with distinct filaments associated with both the cytoplasmic and nuclear periphery (Fig. 3). The existence of these filamentous structures has been documented using more elaborate electron microscopy methods and instrumentations that directly produce images of the surface of a specimen. These include (i) TEM of samples that have been quick frozen, freeze-dried and metal shadowed [7]; and (ii) high resolution scanning electron microscopy of *Xenopus* nuclear envelopes [8]. As documented in Fig. 4, electron microscopy views of the cytoplasmic surface of the NPC reveal the pres-

ence of eight short filaments attached to a 110 nm outer diameter ring. The nuclear surface of the NPC, however, reveals a fine basket-shaped structure, which is formed by eight filaments that are fused to a distal ring (Fig. 4).



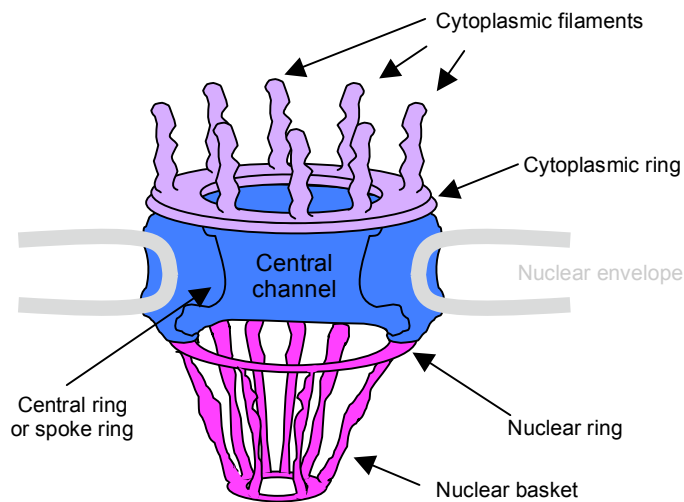
**Fig. 3** Electron micrograph of a cross-section of an Epon-embedded *Xenopus* oocyte nucleus. In this view, filaments are revealed attached to both the cytoplasmic (large arrowheads) and nuclear (small arrowheads) side of the NPC. The cytoplasmic and nuclear side of the nuclear envelope are indicated by *c* and *n* respectively. Scale bar = 100 nm.



**Fig. 4** Electron micrographs of the cytoplasmic and nuclear surface of intact *Xenopus* oocyte nuclear envelopes that were quick-frozen, freeze-dried, and metal shadowed, and visualized on a TEM. In the cytoplasmic face, the arrowheads point to filaments protruding from the cytoplasmic rings of the NPC. In the nuclear face, the arrowheads point to the nuclear baskets. Scale bar = 100 nm.

The large number of electron microscopy studies on *Xenopus* oocytes have identified five major structural components of the NPC as illustrated in Fig. 5: i) a massive membrane embedded central ring, called the NPC central framework, the spoke ring, or the spoke complex; ii) a cytoplasmic ring that sits on the cytoplasmic side of the central ring; iii) a nuclear ring attached to the nuclear side of the central ring; iv) eight cytoplasmic filaments attached to the cytoplasmic ring; and v) a nuclear basket attached to the nuclear ring. The central ring has an outer diameter of 125 nm and an inner diameter of 50 nm. Molecular transport occurs through the 50 nm central channel of the central ring.

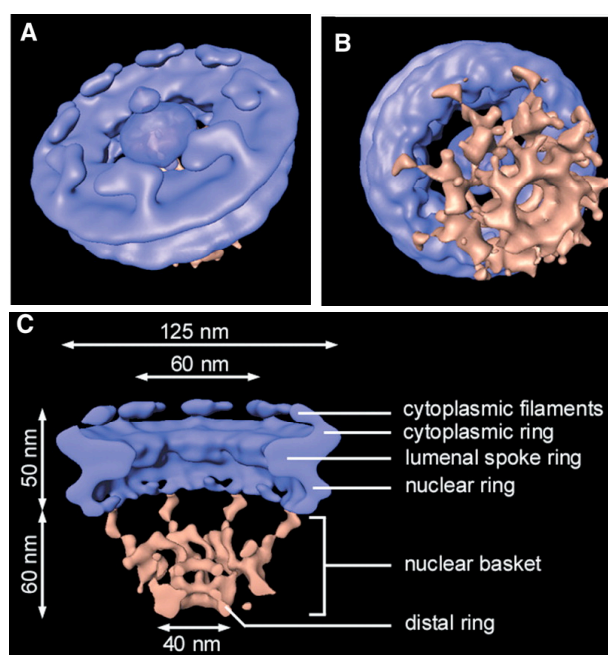
Early structural analysis of the NPC documented the presence of a massive particle in the central channel, called the central plug. Because this central particle is of variable appearance within a given NPC population, it was a subject of much debate. Some scientists asserted that it was an integral component of the NPC, while others maintained that it was material in transit. Recent structural analysis (see below) supports the latter conclusion; however, it is surmised that the central channel is not completely empty, but instead contains a high concentration of polypeptide chains of NPC proteins that reside in the central ring and extend into the central channel. These polypeptide chains are rich in phenylalanine-glycine (FG) sequence repeats, and seem to be highly dynamic, allowing the diffusion of small molecules (< 9 nm) and accommodating carrier-mediated transport of macromolecules that are up to 39 nm in diameter [9].



**Fig. 5** Schematic diagram of the major components of the NPC, based on electron microscopy images.

#### 4. Contribution of cryo-electron tomography to the structure of the NPC

To reveal more details of the fine structure of the NPC, several electron microscopy tomography studies have been conducted [3, 10-12]. In these studies, the three-dimensional (3D) structure (tomogram) of the specimen is reconstructed from a set of different views of the specimen, which are obtained while tilting the specimen around a single axis in the electron microscope. These tomography studies were first performed on NPCs that were negatively stained [10]. More recently, cryo-electron tomography has been used to preserve the NPCs in an almost physiological, fully hydrated state, without the distortions and possible artefacts from stains or fixatives used in traditional electron microscopy. In cryo-electron microscopy, the specimen is spread on an electron microscopy grid, and the grid is rapidly plunged into a liquid cryogen, such as propane or ethane. This produces a high speed of freezing and makes the water contained in the sample to pass from liquid to a vitreous state. Electron microscopes with special sample holders that keep the specimen hydrated and at liquid nitrogen temperature (-196 °C) are used to visualize frozen-hydrated samples. Cryo-electron tomography of *Xenopus* NPCs [11, 12], and more recently of *Dictyostelium* NPCs [3], have now yielded a 3D reconstruction of the NPC with a resolution of 8 nm (Fig. 6). In these tomograms, the structure of the central ring, cytoplasmic filaments, and nuclear basket has been revealed in detail. In addition, the controversial central plug is very variable and occupies different positions within the central channel, an indication that the controversial central plug represents material in transit. These tomographic reconstructions have also documented that the NPC is a highly dynamic structure that deforms to accommodate the transport of macromolecules.

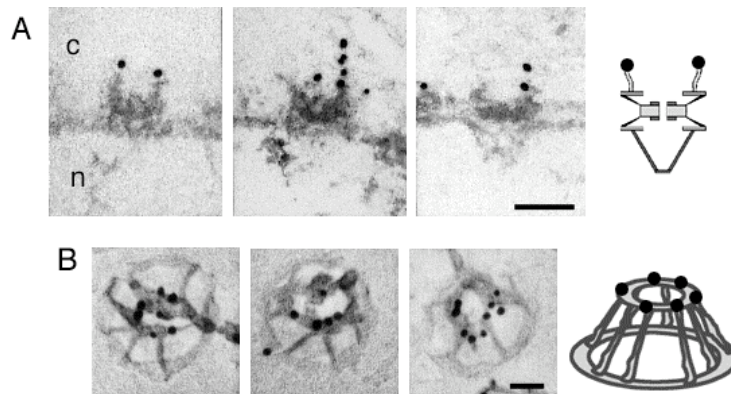


**Fig. 6** Surface renderings of the electron tomogram of the *Dictyostelium* NPC. (A) Cytoplasmic surface. (B) Nuclear surface. (C) Cut-away view. From Beck et al. (*Science*, 306:1387-90, 2004; reference [3]). Reprinted with permission from The American Association for the Advance of Science.

### 5. Contribution of immuno-gold electron microscopy to the mapping of proteins within the NPC structure

Proteomic analysis of the NPC has indicated that it is composed of 30 different proteins, called nucleoporins [13, 14]. Multiple copies of these nucleoporins assemble in the NPC to give a total mass of 125 megadaltons [6]. Immuno-gold electron microscopy has been used to determine the detailed localization of some of these nucleoporins within the structure of the NPC. For this technique, antibodies conjugated with small gold particles (which are electron dense) are used to localize specific proteins. The immuno-labeling may be done with the primary antibody directly conjugated to gold particles, or by an indirect method that uses an unlabeled primary antibody and a gold-labeled secondary antibody. In the first method, the distance between the gold particle and the protein to be localized is smaller than with the second method, thus allowing for a more precise localization of the protein. The usual procedure for immuno-gold electron microscopy, termed post-embedding labeling, consists of first fixing, embedding, and thin-sectioning the sample (as described in Section 3), and then performing the immuno-staining on the sections mounted on electron microscopy grids. A disadvantage of this method is that the embedding resin that preserves the antigenicity of the sample gives a limited preservation of the sample structure; in particular, membranes are not well preserved. In addition, the antigenicity of many molecular components might be destroyed during fixation and sample processing. Thus, for the localization of nucleoporins within the detailed structure of the NPC, it is preferable to perform the immuno-labeling before embedding of the sample into resin and thin sectioning (a technique called *pre-embedding labeling*). Using this procedure, several nucleoporins have been localized in *Xenopus*, mammalian and yeast NPCs [2, 15-18].

For pre-embedding immuno-gold labeling of *Xenopus* oocyte nucleoporins, the nuclei are manually isolated and incubated with the primary antibody conjugated with gold particles. After several washings to eliminate unbound antibody, the nuclei are then processed for conventional embedding and thin-section electron microscopy. Often the isolated nuclei are permeabilized with the detergent Triton X-100 before incubation with the antibody, in order to allow the antibody to access both the cytoplasmic and nuclear side of the NPC. Figure 7A illustrates the localization of the nucleoporin Nup214 using this methodology. Immuno-gold labeling of *Xenopus* nucleoporins can also be performed in conjunction with quick-freezing, freeze-drying and metal shadowing (Fig. 7B).



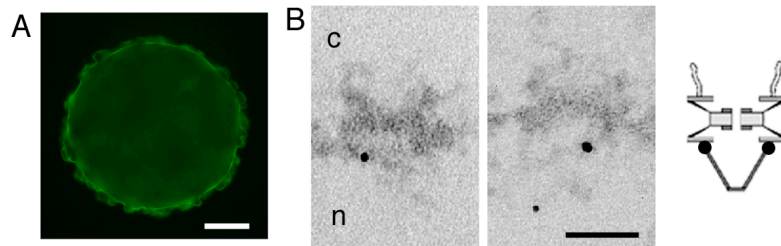
**Fig. 7** Immuno-gold localization of nucleoporins. (A) Localization of the nucleoporin Nup214 in cross-sections of *Xenopus* NPCs. Manually isolated *Xenopus* oocyte nuclei were permeabilized with Triton X-100, immuno-labeled with an anti-Nup214 directly conjugated to 8-nm gold particles, and prepared for electron microscopy by embedding and thin-sectioning. The cytoplasmic and nuclear side of the NPC are indicated by *c* and *n* respectively. (B) Localization of the nucleoporin Nup153 at the NPC nuclear basket. Manually isolated *Xenopus* oocyte nuclei were permeabilized with Triton X-100, immuno-labeled with an anti-Nup153 antibody directly conjugated to 8-nm gold particles, and prepared for electron microscopy by quick freezing, freeze-drying, and metal shadowing. See reference [15] for a more detailed protocol. Scale bars = (A) 100 nm and (B) 50 nm. On the right are schematic diagrams of the NPC, with black dots indicating the localization of Nup214 and Nup153.

To overcome the limitation of unavailable anti-nucleoporin antibodies, I have developed an experimental approach to localize nucleoporins (Fig. 8) [19]. This approach involves the microinjection of *in vitro* synthesized mRNA from a vector encoding an epitope-tagged nucleoporin (for example, the nucleoporin of interest could be tagged with GFP) into the cytoplasm of *Xenopus* oocytes. After the protein is expressed (usually within 24 hours), the nuclei are then isolated and immuno-gold labeled using an anti-tag antibody. This new approach to localize nucleoporins within the structure of the NPC overcomes limitations of standard immuno-gold labeling resulting from the high cross-reactivity of many anti-nucleoporin antibodies, caused by the presence of highly antigenic repetitive sequence motifs within many nucleoporins. Thus, this new approach allows for a greater specificity and resolution that has not been possible with previous methods. Moreover, this procedure is much easier and faster to perform than the production of direct antibodies. For example, when using an injection of mRNA encoding for GFP-tagged nucleoporins, the expression of the heterologous protein is easily checked by direct observation of the isolated nuclei under a fluorescent microscope (Fig. 8A).

## 6. Contribution of electron microscopy to the function of the NPC

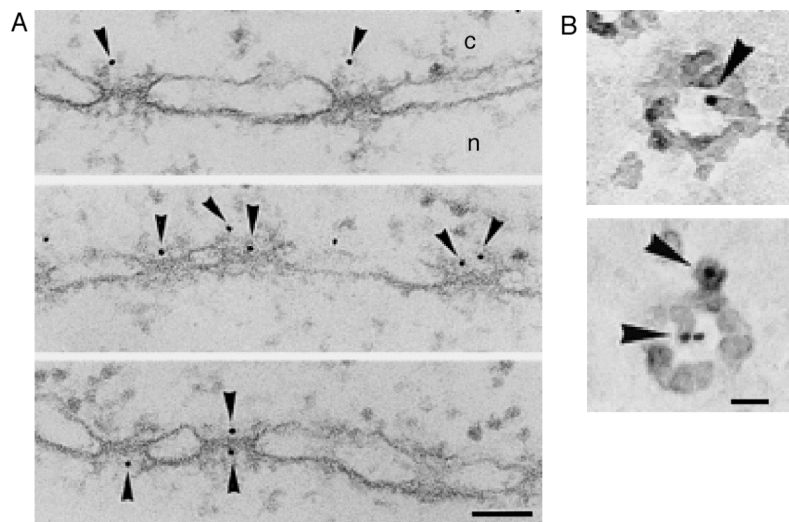
Nucleocytoplasmic transport has also been studied using electron microscopy, and microinjection of the *Xenopus* oocyte has been the system of choice for these studies also (review in reference [5]). For this system, the oocytes are injected with gold particles which are coated with nuclear transport substrates (protein or RNA). After preparation of the oocytes by embedding and thin-section electron microscopy,

the fates of the gold particles are then determined by TEM. In pioneering studies, Feldherr and co-workers used this system, and demonstrated that nuclear transport occurs through the NPCs [20]. Moreover, in performing double injection experiments (20 nm-gold particles coated with t-RNA and injected into the nucleus, and 120 nm-gold particles coated with a nuclear protein and injected into the cytoplasm), they also demonstrated that individual NPCs function in both directions (i.e., for both nuclear import and nuclear export) [21].



**Fig. 8** Immunogold localization of the nucleoporin Nup98 by expression of GFP-tagged Nup98 in *Xenopus* oocytes. After confirming by fluorescence microscopy (A) that *Xenopus* nuclei, isolated from oocytes that were injected with an mRNA coding for GFP-Nup98, were fluorescent, the nuclei were immuno-labeled with an anti-GFP antibody directly conjugated to 8-nm gold particles and prepared for electron microscopy by embedding and thin-section electron microscopy (B) according to the protocol described in [19]. In (B), the cytoplasmic and nuclear side of the NPC are indicated by *c* and *n* respectively. Scale bars = (A) 1  $\mu$ m and (B) 100 nm. On the right is a schematic diagram of the NPC with black dots indicating the localization of Nup98.

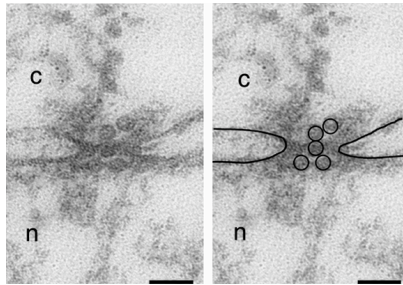
As illustrated in Fig. 9, the *Xenopus* oocyte nuclear import assay, in combination with electron microscopy, allows for the visualization of nuclear transport of single gold particles (coated with transport substrate) through individual NPCs at the level of distinct NPC components.



**Fig. 9** Electron microscopy visualization of nuclear import of gold particles coated with nucleoplasmin (a nuclear protein). (A) *Xenopus* oocytes were microinjected in their cytoplasm with 8-nm gold particles coated with nucleoplasmin. After incubation at room temperature for 10 minutes (top micrograph), 20 minutes (middle micrograph), and 50 minutes (bottom micrograph), the oocytes were fixed and processed for embedding and thin-section electron microscopy as described in [5]. (B) Electron micrographs of the cytoplasmic surface of NPCs from the *Xenopus* oocyte that were microinjected in their cytoplasm with 8-nm gold particles coated with nucleoplasmin, incubated at room temperature for 10 minutes, quick frozen, freeze-dried, metal shadowed, and visualized on a TEM. Both microscopy techniques show that at early time points (10 minutes), gold particles are seen associated with the NPC cytoplasmic filaments. In (A), the cytoplasmic and nuclear side of the NPC are indicated by *c* and *n* respectively. Scale bars = (A) 100 nm and (B) 50 nm.

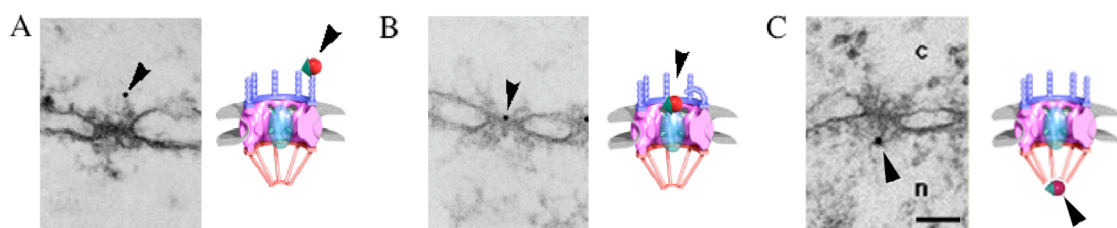


Using microinjection of *Xenopus* oocytes with gold particles of various sizes that were coated with nuclear transport substrate, Feldherr and his co-workers also demonstrated that there is a limit size for molecular transported through the NPC [21]. More recently, Panté and Kann [9] used the same approach, but with well-characterized gold particles in which the exact size of the protein coat (defined not only by the nuclear proteins but also by the soluble nuclear import receptors importin  $\alpha$  and importin  $\beta$ ) was known. They determined that the functional diameter of the NPC is 39 nm - a finding supported by the nuclear import of capsids of the hepatitis B virus, which have diameters of 36 nm and are seen crossing the NPC intact (Fig. 10).



**Fig. 10** Electron microscopy visualization of the nuclear import of hepatitis B virus (HBV) capsids. For these experiments, *Xenopus* oocytes were injected with phosphorylated recombinant HBV capsids, and prepared for electron microscopy by embedding and thin sectioning. On the right is the same micrograph as on the left but with the nuclear membranes and the HBV capsids outlined. The cytoplasmic and nuclear sides of the NPC are indicated by *c* and *n*, respectively. Scale bars = 100 nm.

The *Xenopus* oocyte system, in combination with electron microscopy, has also been used to characterize the structural events of nuclear import; in particular, specific transport intermediates arrested at the NPC has been visualized [22-24] (Fig. 11). For these studies, inhibitors of nuclear transport have been used to arrest the gold particles (coated with nuclear transport substrates) at the NPC. As illustrated in Fig. 11A, when *Xenopus* oocytes are injected with lectin wheat germ agglutinin (an inhibitor of nuclear transport) before injection of gold particles coated with the nuclear protein nucleoplasmin, the gold particles are arrested at the terminal end of the cytoplasmic filaments of the NPC. Inhibition of nuclear import by chilling, however, results in the gold particles being arrested at the cytoplasmic entrance of the NPC central channel (Fig. 11B). A third nuclear import intermediate is also depicted at the nuclear side of the NPC when *Xenopus* oocytes are co-injected with a mutant form of the nuclear transport receptor importin  $\beta$ , which does not bind to the small GTPase Ran [23] (Fig. 11C). This importin  $\beta$  mutant fails to deliver the gold particles into the nucleus, which indicates that the Ran binding site in importin  $\beta$  is essential for the last step of the nuclear import of proteins [23]. Thus, these studies have revealed the distinct molecular steps in the mechanism of the nuclear import of proteins.



**Fig. 11** NPC cross-sections revealing three different transport intermediates arrested at the NPC. For these experiments 8-nm gold particles coated with the nuclear protein nucleoplasmin (gold-NP) were injected into the cytoplasm of *Xenopus* oocytes and the oocytes were processed for embedding and thin section electron microscopy one hour after injection. In (A) the lectin wheat germ agglutinin, which inhibits nuclear transport, was injected before gold-NP. In (B) pre-cooled oocytes were kept on an ice bath during injection with gold-NP, and incubated for one hour at 4°C after injection. In (C) gold-NP was co-injected together with a mutant form of importin  $\beta$  lacking the first 44 amino acid residues ( $\Delta$ N44-imp  $\beta$ ), which does not bind Ran [23]. Arrowheads point to gold-NP arrested at the NPC. In (C), the cytoplasmic and nuclear side of the NPC are indicated by *c* and *n* respectively. On the right of each panel are schematic diagrams of the NPC indicating the distinct arrested intermediates (arrowheads) visualized by electron microscopy. Scale bar = 100 nm.

## 7. Summary

Electron microscopy has been widely used for the study of the NPC structure, molecular composition, and function. These studies have significantly contributed to advances in the field of nuclear transport. The use of conventional electron microscopy has revealed NPC structural components (such as the cytoplasmic filaments and nuclear baskets) and the use of cryo-tomography has revealed the fine structure of the NPC with a resolution of 8 nm. In addition, immuno-gold labeling has helped to map nucleoporins within the NPC structure, and nuclear import assays with microinjected *Xenopus* oocytes have revealed the distinct molecular steps of the mechanism of nuclear import.

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