Scanning Force Microscopy of Nuclear Pore Complexes

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Abstract

Within the scope of this thesis an interdisciplinary approach has been pursued for the investigation of native biological membranes using Scanning Force Microscopy (SFM) in liquid environments. Physical examination of fragile biological samples at nanometer resolution required the optimization of imaging conditions through careful inspection of the forces exerted by the sensor. Highest resolution images were obtained using the amplitude modulation (tapping) mode of operation whereby lateral shear forces caused by the scanning tip could be significantly reduced. Typically, average normal forces exerted on the samples were smaller than 0.2 nN.

Even greater force sensitivities were obtainable by employing an electronic positive feedback loop (resonance or Q-control) which permitted the maintenance of force sensor oscillations against viscous damping forces, thus effectively increasing the quality factor of the resonator. However, especially when working on native biological samples, enhanced force resolution made the system more susceptible to disturbances arising from contaminations and loose material. Therefore, Q-control was not employed for imaging applications.

Nuclear Pore Complexes (NPCs) are large protein structures responsible for transport of large macromolecules across the nuclear envelope which separates the nucleus from the cellular plasma. The cytoplasmic topology of the NPCs was found to be strongly influenced by various exogenous and endogenous biochemical agents affecting transport through the NPC.

Mutant constructs of the importin ß (Impß) transport receptor were observed to modify the vertical aspect of the NPCs, i.e. pore depth, in a manner that was strongly correlated with their binding affinity to the NPC. Impß mutants which irreversibly bind to the NPC induced an accumulation of protein material over the mouth of the pore. On the other hand, an Impß mutant with reduced binding affinity to the NPC was observed to dilate pore diameter by approximately 7%.

EGTA and WGA, two well-known inhibitors of nucleo-cytoplasmic transport, caused distinct changes in NPC morphology. While 95% of the NPCs exposed to EGTA exhibited a plug-like structure which protruded from the interior of the pore, the effect of WGA was to reduce pore depth by 50%.

Amphipathic alcohols (1,2- and 1,4-cyclohexanediol and 1,6-hexanediol) were observed to reversibly increase pore diameter to an extent comparable to that observed in response to Impß mutants exhibiting reduced NPC binding.
Using SFM we were therefore able to correlate topological changes in the NPCs to previously described modifications of transport properties attributed to these agents. Our results may be interpreted in terms of the molecular mechanisms governing nucleo-cytoplasmic transport. We propose that the topological changes induced by amphipathic alcohols and Impβ mutants with disrupted NPC binding affinity may be explained by the molecular rearrangement of the constituents of the NPC which are involved in nucleo-cytoplasmic transport. Thus the increase in NPC lateral aspect may be directly related to the previously observed increase of non-selective transport associated with these agents.
Zusammenfassung

Im Rahmen dieser interdisziplinären Dissertation wurden natürliche biologische Membranen in Flüssigkeiten mit Hilfe eines Rasterkraft-Mikroskops untersucht. Um diese empfindlichen Proben auf der Längenskala von wenigen Nanometern physikalisch charakterisieren zu können mussten die Abbildungs-Parameter und die Umgebungsbedingungen sorgfältig optimiert werden. Dies geschah durch detaillierte Untersuchung der vom Sensor ausgeübten Kräfte. Abbildungen mit höchster Auflösung wurden mit dem Amplitudenmodulations- (Tapping-) Modus erzielt. Dabei konnten laterale Scherkräfte, verursacht durch die sich bewegende Spitze, stark reduziert werden. Im Mittel waren die Proben Normalkräften von typischerweise weniger als 0.5 nN ausgesetzt.


Die Kernhülle grenzt den Zellkern vom umgebenden Cytoplasma ab. Kernporen sind grosse Proteinstrukturen, die für den Transport von Makromolekülen durch die Kernhülle verantwortlich sind. Mit Hilfe der Rasterkraft-Mikroskopie konnten wir zeigen, dass biochemische Substanzen die den Transport durch die Kernporen beeinflussen die räumliche Struktur der Kernporen verändern.


EGTA und WGA sind Substanzen die den Transport durch die Kernporen blockieren. Beide verursachten klar unterschiedliche Veränderungen in der Struktur der Kernporen. EGTA bewirkte, dass 95% der Poren durch zapfenähnliche Strukturen aus dem Innern der Pore verschlossen wurden, während sich bei der Behandlung mit
WGA die Porentiefe um 50% reduzierte.

Amphipatische Alkohole (1,2- and 1,4-Cyclohexandiol und 1,6-Hexandiol) verursachten eine reversible Zunahme des Porendurchmessers in einem Ausmass vergleichbar zu der Behandlung mit Impβ-Mutanten mit reduzierter Poren-Bindung.

Chapter 1

Introduction

The atomic force microscope [1] offers the possibility to investigate interactions between single atoms or molecules on virtually any surface. Such a high force sensitivity is achieved through the use of microfabricated spring-like sensors outfitted with a sharpened tip in conjunction with methods to measure spring displacements which are theoretically smaller than typical interatomic distances in solids. To construct an apparatus for the investigation of very small objects, i.e. a microscope, the force sensor is combined with piezoelectric materials which allow the positioning of the tip in three dimensions with sub-nanometer accuracy. By measuring the total interaction between the moving tip and the sample an image of surface topography is generated. This method is thus commonly referred to as Scanning Force Microscopy (SFM).

Originally derived from the Scanning Tunneling Microscope (STM) [2], SFM has evolved into a valuable technique used in many different disciplines. SFM is just one member of the Scanning Probe Microscopy (SPM) family which combines the use of a small movable probe with the simultaneous measurement of some physical or chemical surface property sensitive to the proximity of the probe. Such surface properties might include electrical conduction [3], electrostatic potentials, transmitted or reflected light, or the physical or chemical composition of the surface [4]. SFM has proven to be extremely versatile in that various modes of operation and schemes for the detection of force signals have been developed [5]. Moreover, operation may take place in distinct environments including low temperatures [6], vacuum, various gases and liquids [7]. Furthermore, many different types of materials and systems have been investigated using SFM, ranging from monoatomically flat silicon surfaces where true atomic resolution may be achieved [8], to intact cells where the resolution is limited by the elasticity and mobility of the sample [9]. Finally, interaction forces may be chemical, electrostatic, or magnetic etc. in nature depending on the properties of tip and sample [8, 10]. The smallest forces that can be measured using commercial instruments and sensors are on the order of some tens of pN.

In biological sciences SFM has evolved to a level not only complementary to optical and electron microscopy, but has in many respects exceeded their capabili-
1. Introduction

ties. One of the greatest advantages of SFM for biological research is that it can be operated under conditions closely approximating the natural environment of cells. Furthermore, this can be accomplished with a minimum of sample preparation comparable to optical microscopy. Thus, biomolecular interactions can be examined in aqueous environments and changes in (e.g.) protein conformation followed in real time. [11, 12, 13, 14]. At the same time, SFM has the potential for molecular or macromolecular resolution comparable to electron microscopy [15, 16]. Furthermore, since the sensor tip directly probes the structures of interest no additional staining procedures are required to visualize proteins, lipids, DNA or other biomolecules. In addition, apart from structural information SFM also offers the possibility to study biochemical processes with single molecules, for example, protein unfolding [17], receptor-ligand binding [18], and the energetics of bond formations between complementary DNA strands [19].

Among the first cellular membranes to be investigated with SFM was the nuclear envelope [20], the boundary between the nucleus and the cellular plasma. Particular attention has been devoted to the Nuclear Pore Complex (NPC), a large protein compound traversing the nuclear envelope and acting as a macromolecular conduit between the nuclear and cytoplasmic compartments. In recent years much attention has been paid to changes in NPC native structure [21] in response to $\text{Ca}^{2+}$ [13, 14, 22, 23] and ATP [24, 25].

The NPC represents the only pathway for bidirectional exchange of proteins, mRNAs and other macromolecules between the nucleus and the cytoplasm. While small molecules and ions may passively diffuse through the NPC, the transport of larger macromolecules is an energy consuming process mediated by specific transport receptors [26]. Specificity of the translocation process arises from the fact that only macromolecules tagged with the appropriate signal are recognized by the transport mediators. Therefore, the NPC forms a virtually impenetrable barrier for other molecules.

Regulated macromolecular transport between the nucleus and cytoplasm is of importance for such fundamental cellular processes as gene replication and protein synthesis. The functional properties of nucleo-cytoplasmic transport have primarily been investigated using biochemical methodologies [26, 27]. X-ray crystallography has been used to examine interactions between transport mediators and components of the NPC at molecular resolution [28]. Electron micrographic studies have imported information concerning mediator-NPC interactions along the translocation pathway [29, 30, 31]. However, to date no SFM studies of these physiologically relevant transport processes exist.

Through the use of SFM characteristic modifications in NPC topology could be discerned which were attributed to specific interactions between the NPC and transport modifying agents. My thesis focused on morphological alterations of the NPC in response to transport mediators, in particular importin $\beta$ (Imp$\beta$), one of the best understood nuclear import receptors [26]. It was shown that various Imp$\beta$
constructs modulated the vertical aspect (i.e. pore depth) of NPCs in agreement with their predicted NPC binding affinity. Interestingly, Impβ mutants with strongly decreased binding affinity were observed to increase the lateral aspect (i.e. diameter) of the NPCs.

In addition to transport receptors, various other biochemical agents which are known to alter transport capacity also modify NPC topology. Transport block by WGA was associated with changes in the vertical aspect of the NPCs. On the other hand, enhancement of non-selective transport through the application of amphipathic alcohols such as 1,2-cyclohexanediol dilated the NPC diameter.

Using SFM we were thus able to correlate structural changes in the NPC to previously described functional modifications of transport properties [27, 26, 32]. The results are interpreted in terms of the molecular interactions between distinct amino acid sequences within the NPC (FG-repeats) and transport receptors. These interactions are essential for proper nucleo-cytoplasmic transport.

The topological properties and modifications of NPCs are of interest in view of their biochemical and physiological consequences. While most experimental observations (modified pore depth and diameter) are in tune with expectations based on transport properties, some extreme NPC features remain unexplained. This indicates that the physical examination of biological systems can lead to new insights which are not accessible to functional characterization.

Imaging at the nanometer scale requires sophisticated sample preparations irrespective of the microscopic technique employed for experimentation. Correspondingly, conducting experiments in native environments demands that experimental conditions be carefully optimized to obtain stable, high-resolution SFM operation, while at the same time preserving the natural structure of the biomolecules. In this context the understanding and characterization of interactions between biological surfaces, sample support and the force sensor are particularly emphasized.
The operation principle of SFM is based on measurements of distance-dependent forces between a sharp probe and the sample surface. Since at the same time the tip is raster-scanned above the sample, a two-dimensional representation of tip-sample interactions is obtained. The way these interactions are transformed into a measurable signal depends on the type of sensor used and the mode of operation (section 2.2).

Since the measured signal corresponds to the sum of all forces exerted on the tip it reflects the topography as well as any other sample property to which the tip is sensitive; among these are elasticity, surface charge distribution, surface chemistry, and even magnetism, etc., if the tip is modified accordingly (section 2.1).

2.1 Tip-sample forces

As schematically illustrated in Fig. 2.1, the different forces acting on the probe tip may extend over short or long ranges, and may be attractive or repulsive (with respect to the sample). In this context, short range force fields are usually restricted to not more than a few atomic distances, while long range interactions may extend over several tens of nanometers.

2.1.1 Molecular interactions

Interaction potentials \( w(r) \) of the form \( w = -C/r^n \) (where \( C > 0 \) is a constant with units \( \text{Jm}^n \), and \( n > 0 \)) describe attractive interactions between two molecules separated by a distance \( r \) [33]. The force \( F(r) \) derived from this interaction potential is given by

\[
F(r) = -dw(r)/dr = -nC/r^{n+1}.
\]
2.1. Tip-sample forces

The total interaction energy of one molecule with all the other molecules in the system is given by

\[ w_{\text{total}} = \int_{r_0}^{L} w(r) \rho 4\pi r^2 dr = -4\pi C \rho \int_{r_0}^{L} r^{2-n} = \frac{-4\pi C \rho}{(n-3)r_0^{n-3}} \left[ 1 - \left( \frac{r_0}{L} \right)^{n-3} \right] \]  \hspace{1cm} (2.2)

where \( L \) is the system size, and \( r_0 \) and \( \rho \) are the diameter and density of molecules, respectively. For \( n < 3 \) the total interaction energy increases with increasing system size \( L \), meaning that contributions from more distant objects become more and more important (for a given density \( \rho \)), and the interaction is termed long range. On the other hand, for \( n > 3 \) the total interaction energy does not depend on the system size for \( L \gg r_0 \). Therefore, contributions from more distant objects vanish and the effective range of the interaction is finite (short range). The same analysis applies also to repulsive interaction potentials of the form \( w = C/r^m \) (\( C > 0, m > 0 \)).

The Lennard-Jones potential is commonly used to describe the total interaction between uncharged molecules

\[ w_{LJ}(r) = \frac{A}{r^{12}} - \frac{B}{r^6} \]  \hspace{1cm} (2.3)

with \( A \) and \( B \) the appropriate constants with units Jm\(^{12}\) and Jm\(^{6}\), respectively. The interaction potential consist of a repulsive \( r^{-12} \) term which originates from the Pauli exclusion principle. The steep slope of this term emphasizes the short range and impenetrable nature of the atomic electron orbitals. The attractive \( r^{-6} \) term is derived from the van der Waals-interaction and summarizes contributions of attractive electrostatic and electrodynamic dipole-dipole forces; firstly, pairs of molecules which have a permanent dipole tend to align to minimize their interaction energy. Secondly, polar molecules induce dipole moments in (non-polar) atoms or molecules. Thirdly, even non-polar atoms or molecules are subject to fluctuating dipole moments arising from the instantaneous polarization of the electron cloud.
2. Scanning Force Microscopy principles for soft samples

with respect to the positively charged nucleus. Thus nearby molecules are polarized in a way similar to the situation indicated above.

Charged atoms or molecules interact via long range Coulomb forces. The corresponding potential for two charges \( Q_1 \) and \( Q_2 \) is given by

\[
w(r) = \frac{Q_1 Q_2}{4\pi\varepsilon\varepsilon_0 r}
\]

where \( \varepsilon \) and \( \varepsilon_0 \) are the dielectric constant and the vacuum permittivity, respectively. This type of interaction may lead to bonds between different ionic species.

At short distances between tip and sample chemical interactions such as the formation of covalent bonds may occur. Further, particularly in aqueous solutions hydrogen bonds are important, i.e. bonds between two electronegative atoms mediated by a positively polarized hydrogen which is covalently bound to one of the atoms.

2.1.2 Interactions between surfaces

The molecular pair interactions described above have to be integrated over all the atoms and molecules in the system to obtain the total force acting between tip and sample. By doing so, one also has to take into account that neighboring atoms as well as the surrounding medium may influence the interaction between any pair of atoms. After integration the resulting force law may thus be formally rather different from the underlying molecular force law, or may even reverse sign under certain circumstances.

van der Waals forces. For a sphere with radius \( R \) in close proximity \( (d \ll R) \) to a large flat slab of material the integration of the \( -A/r^6 \) potential yields [33] (after having taken the derivative with regard to \( d \) to obtain the corresponding force law)

\[
F_{vdW}(d) = -\frac{H_a R}{6d^2}.
\]

The Hamaker constant is formally given by \( H_a = \pi^2 A \rho_1 \rho_2 \) (where \( \rho_1 \) and \( \rho_2 \) are the densities in the two bodies) but generally has to be determined using a continuum approach ignoring the molecular structure in order to circumvent the problems associated with the mutual influence of interacting molecules. Typical values for \( H_a \) are in the range of \( 10^{-19} \text{J} \) to \( 10^{-20} \text{J} \), experimentally verified for e.g. silicon oxide \((0.83 \times 10^{-20} \text{J})\) and hydrocarbon surfaces \((1.10 \times 10^{-20} \text{J})\) interacting in water, respectively. For a spherical tip of radius 20 nm and a silicon oxide surface separated by \( d = 1 \text{nm} \) in water the attractive force thus amounts to 27 pN. In air \( H_a \) and thus the interaction forces are approximately one order of magnitude greater.
2.1. Tip-sample forces

**Electrostatic forces.** In air or vacuum the influence of electrostatic forces is calculated by solving the Laplace equation with the appropriate boundary conditions determined by the charge distribution (or potential). In liquids the force laws are altered by the presence of mobile ions. Furthermore, the surfaces may actually get charged because surface ions are dissolved.

To determine the force between objects which carry surface charges in the presence of aqueous electrolytes the Poisson-Boltzmann equation has to be solved. It is found that mobile counterions tend to screen the surface charge such that the electrostatic potential decays exponentially away from the surface (for small surface potentials). The expression *diffuse electric double layer* describes the ionic atmosphere created by counterions near the surface charges. The range over which the potential is reduced to $1/e$ times the surface potential is given by the Debye length $1/\kappa$

$$1/\kappa = \left( \frac{e^2}{\varepsilon \varepsilon_0 k_B T} \sum_i \rho_i z_i^2 \right)^{-1/2}.$$  

(2.6)

The sum in the above equation extends over all ionic species, where $\rho_i$ is the bulk ion density and $z_i$ the ionic valency. The double layer repulsion force between two equally charged spheres separated by a distance $d$ larger than the Debye-length is approximated by [33]

$$F_{el}(d) = \frac{2\pi R \sigma^2 e^{-\kappa d}}{\varepsilon \varepsilon_0} \frac{e^{-\kappa d}}{\kappa}$$  

(2.7)

for small surface charge densities $\sigma$. Table 2.1 shows how the Debye length and double layer forces depend on ionic concentrations for a two spheres with surface charge $\sigma = 0.018 \text{ C/m}^2$ which corresponds to about one electronic charge per $3 \times 3 \text{ nm}^2$. It is assumed that only monovalent salts such as NaCl or KCl are present. While at low ionic concentrations the Debye length is large and thus interaction forces are appreciable far away from the surface, at high concentrations the repulsive force rises steeply at very small separations but decays quickly at larger distances.

<table>
<thead>
<tr>
<th>concentration (M)</th>
<th>$1/\kappa$ (nm)</th>
<th>$F_{el}$ (pN)</th>
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<tr>
<td></td>
<td></td>
<td>$d=3 \text{ nm}$</td>
</tr>
<tr>
<td>0.01</td>
<td>3</td>
<td>35</td>
</tr>
<tr>
<td>0.1</td>
<td>0.96</td>
<td>3</td>
</tr>
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Table 2.1: Debye length and double layer repulsion forces for two spheres with radii 20 nm in NaCl or KCl solutions. For distances $d < 1/\kappa$ the values for $F_{el}$ are underestimated by eq. 2.7. A better approximation for small separations involving higher order interaction terms, but excluding effects associated with the discrete nature of the atoms and ions involved, is given in parentheses [33].
2. Scanning Force Microscopy principles for soft samples

Elastic deformation forces. In a continuum approach (Hertz model) the elastic properties of tip and sample give rise to repulsive interactions. For sufficiently soft samples the elastic properties of the tip material can be ignored (Young’s modulus for a silicon tip $E_T = 179$ GPa). The sample indentation is thus related to the interaction force by

$$F_{\text{Hertz}}(d) = \frac{8\sqrt{2R}}{3\pi} \left( \frac{1 - \nu_S^2}{\pi E_S} \right)^{-1} |d|^{3/2}$$

(2.8)

where $R$ is the tip radius, and $\nu_S$ and $E_S$ are the Poisson’s coefficient and Young’s modulus of the sample, respectively. Since during indentation $d < 0$ the absolute value of the tip-sample distance is used. The Young’s moduli and Poisson’s coefficients of polymers or cell membranes are estimated to be on the order of $E_S = 0.1$ GPa and $\nu_S = 0.3$, respectively [34]. Assuming a sample indentation (deformation) of $d = -0.3 \text{ nm}$ and a tip with radius $R = 20 \text{ nm}$ the Hertz repulsion exceeds 300 pN even on these soft samples. Clearly, the biggest problem associated with this model is that the contact area between tip and sample is in the nanometer range while general information about mechanical sample properties are only available in macroscopic dimensions.

Hydrophobic and hydrophilic interactions. Hydrophobic surfaces strongly attract each other in aqueous environments over large distances, and large adhesion forces between these surfaces can occur. This effect arises because water molecules interact rather strongly via hydrogen bonds. Water molecules at a hydrophobic (or non-polar) surface have to reorient to be able to participate in hydrogen bonds. This, however, is associated with an entropic penalty since the water is forced to take on a more ordered structure. Therefore, as two hydrophobic surfaces come together the entropically unfavorable arrangement of water molecules is avoided.

Hydrophilic molecules, on the other hand, repel each other. This is because water molecules bind to ionic and hydrogen bonding groups on their surface. In other words, hydrophilic objects do not associate because they prefer to be dispersed in the surrounding aqueous medium.

Capillary and adhesion forces. These interactions are related to changes in free energy (the surface energy or surface tension) when the surface of an object or medium is increased or decreased. Two surfaces will associate with each other in a medium if the energy gained in bringing the two surfaces into contact is larger than the energy needed to separate them from the surrounding medium, or the energy spent in bringing the surfaces into contact is smaller than the energy expended in exposing them to the medium. The association of hydrophobic (or not ideally hydrophilic) surfaces in water may be an example for adhesive interactions.

Capillary forces are very important for SFM operation in air. When exposed to ambient conditions a thin layer of water is present on virtually all surfaces, espe-
cially on biological samples. This film of water generally dominates the attractive interaction between tip and sample in air, and may be as large as some tens of nN (Fig. 2.8).

Dissipative interactions. Energy is dissipated e.g. by inelastic sample deformations or when interrupting adhesive and capillary interactions by retracting the tip from the sample [35, 36]. The work of adhesion can be measured by the area under the force-separation curve associated with the hysteretic motion of the tip (Fig. 2.11).

Sample viscoelasticity and hydrodynamic damping lead to velocity dependent, frictional force terms in the tip equation of motion [37, 34]. Apart from dissipative interactions, when working in fluids and with soft materials the additional mass set in motion by the tip and the force sensor has to be taken into consideration [38].

In the context of SFM operation in liquid environments the discrete nature of the solvent molecules has to be taken into account at small tip-sample distances. When approaching the tip interfacial fluid layers between tip and surface may be compressed and may exhibit an increased viscosity since the fluid molecules need to be displaced from between the tip and sample.

2.2 SFM system characterization and modes of operation

2.2.1 SFM setup

For the experiments presented in this thesis the interaction between tip and sample is measured by monitoring the deflection of a light beam in response to the mechanical bending of a flexible force sensor. The sensor is a made out of a microfabricated thin long sheet of silicon or silicon nitride (thickness typically 400 nm to 2 μm) referred to as the cantilever. One end of the cantilever is generally fitted with a sharp tip of the same material while the other end is clamped to a bulk solid. The cantilever thus acts like a spring which is bent by forces acting on the tip.

A laser beam focused on the cantilever’s backside (i.e. opposite the side where the tip is located) is reflected back into a segmented photodiode. Bending of the cantilever results in a deflection of the laser beam, causing a change of the photocurrent induced in neighboring photodiode segments (Fig. 2.2).

A differential amplifier connected to the segments of the photodiode thus generates a voltage which is related to the vertical displacement of the force sensing tip. More precisely, what is measured is actually the angle $\theta$ of the cantilever deflection. Because the cantilever is typically four orders of magnitude longer (length $L \sim 100 \, \mu m$) than the vertical displacement of the tip ($|z - z_0| \sim 10 \, nm$, where $z_0$ is the tips resting position), $\theta$ is equal to the slope along the main axis of the
2. Scanning Force Microscopy principles for soft samples

Figure 2.2: Schematic representation of the SFM setup for operation in liquids. A similar cantilever holder is used for operation in air. The outputs from the photodiode segments A to D are combined as follows to measure the vertical deflection of the tip: 

\[(z - z_0) \propto \frac{(A+B-C-D)}{(A+B+C+D)}.\]

cantilever. Considering only the lowest order flexural mode of the cantilever beam the relationship between the tip displacement and \( \theta \) is expressed by

\[(z - z_0) = \frac{2}{3} L\theta.\] (2.9)

Thus, the output from the differential amplifier is directly proportional to the vertical tip displacement and may be calibrated in a simple manner by slowly pushing the tip into a hard surface. The smallest displacement that can be detected using the reflected beam method is on the order of 4 \(10^{-4}\) \(\text{Å}\) (at a signal to noise ratio of 1) with small cantilevers [39]. In practice the sensitivity is limited by the thermal motion of the cantilever (see section 2.2.4). For higher order flexural modes the ratio between \((z - z_0)\) and the slope along the cantilever axis is smaller than 2/3, i.e. the tip displacement is overestimated. Therefore, if interested in the high frequency response of the cantilever (e.g. when measuring strongly nonlinear tip-sample interaction forces, section 2.2.5) the full frequency spectrum in conjunction with a frequency dependent calibration of the tip displacement is required.

Torsional bending of the cantilever may be measured using two additional photodiode segments which record laser deflections in a direction orthogonal to that associated with vertical tip displacements. With this implementation friction forces arising when the tip moves across the sample can be quantified (Fig. 2.2).

2.2.2 Scanning, topography feedback and the environment

By applying voltages to appropriately positioned electrodes on the surface of a piezoceramic material the tip is moved relative to the position of the sample (Fig. 2.2 and 2.5). The piezo tube (cylinder) used in our system allows lateral and vertical
displacements of about \(2 \text{Å}/\text{mV}\) (total range \(80 \mu\text{m}\)) and \(0.15 \text{Å}/\text{mV}\) (total range \(6.5 \mu\text{m}\)), respectively. To obtain an image of the sample, the cantilever signal is recorded while the tip is raster-scanned laterally across the surface. The distance dependence of force interactions between tip and sample may be measured by moving the tip vertically to the sample surface.

In the most common modes of operation, while scanning the tip laterally, an electronic feedback circuit is utilized to keep the measured tip-sample interaction signal at a constant setpoint value. To this end the vertical position of the tip relative to the sample is continuously adjusted. This is achieved by applying to the piezo a signal which is proportional to the difference between the momentary cantilever signal and the desired constant setpoint voltage (see Fig. 2.5). For a homogeneous sample (i.e. with identical properties everywhere) and an ideal feedback mechanism the vertical piezo displacement would thus correspond to the actual sample topography. While in reality non-topographical properties will always contribute to the resulting image of the sample, their influence can generally be minimized by keeping the force between tip and sample as small as possible (section 2.4) by optimizing the electronic feedback parameters (PID-controller), and by using small scan velocities.

As illustrated in Fig. 2.2 the SFM can also be operated in liquid environments. To this end a transparent window is inserted in the cantilever holder to provide an optical path for the laser beam and to protect piezo and electronics from coming in contact with the liquid. The sample may be placed in a small drop of fluid (~200 µl) which is confined between sample and cantilever holder by capillary forces. The cantilever and the lower part of the holder must be immersed completely in liquids for two reasons: first, capillary forces would strongly disturb the cantilever motion, and secondly, free liquid surfaces and interfaces would diffract the laser beam randomly.

### 2.2.3 Contact mode

In contact mode operation the vertical tip displacement (i.e. the low frequency or DC component of the cantilever signal) is kept constant by the electronic feedback circuit. The total force between tip and sample is thus controlled by adjusting the setpoint value.

The cantilever can be modeled by a massless spring attached to a point mass \(m\), i.e. a one-dimensional harmonic oscillator, obeying the usual linear force law (restoring force)\(^{[40]}\)

\[
F_C = -k(z - z_0) \tag{2.10}
\]

where \(k\) is the spring constant (also termed force constant), \(z_0\) is the tip’s resting position, and \(z\) is the tip’s momentary position. This model completely neglects higher flexural modes associated with the continuous nature of the cantilever beam (section 2.2.5), though the force constant may be determined from material properties \((k = 3EI/L^3)\) where \(E\) is the Young’s modulus, \(I\) the area moment of inertia
2. Scanning Force Microscopy principles for soft samples

and $L$ the cantilever length).

To minimize the interaction forces and to increase the sensitivity cantilevers with small force constants must be used. This is generally achieved by making the cantilevers longer and thinner. From eq. 2.9, on the other hand, it is inferred that the optical deflection method is more sensitive for short cantilevers since the laser beam displacement is proportional to the deflection angle. In addition, short cantilevers are less sensitive to hydrodynamic friction forces, have a higher resonance frequency and thus allow faster scanning.

2.2.4 Tapping mode

This mode is also known as intermittent contact or amplitude modulation mode [41, 42]. In contrast to contact mode the cantilever is oscillated vertically at or near its resonance frequency by applying a sinusoidal voltage to the piezo (Fig. 2.5). In addition, it is not the low frequency or DC-signal from the cantilever that is recorded, but usually the root-mean-square (RMS) signal at higher frequencies.

In this mode the tip only intermittently "touches" the sample, and the oscillation amplitude is reduced by conservative and dissipative interactions with the surface; the amplitude may actually also increase if the oscillation frequency is lower than the resonance frequency and if there is a net-attractive interaction between tip and sample. While scanning it is thus the preassigned oscillation amplitude $A$ that is used as the feedback signal while scanning. The amplitude setpoint is usually described by the ratio $A/A_{\text{free}}$. For net repulsive interactions the forces exerted on the sample are minimized by keeping the amplitude reduction as small as possible (using a high amplitude setpoint).

The tip equation of motion in the harmonic oscillator approximation is given by

$$m\ddot{z}(t) = -k[z(t) - z_0(t)] - \gamma \dot{z}(t) + kA_0e^{i\omega t} + F_{ts}(z(t))$$

where $\gamma$ is the constant for intrinsic and viscous damping, $A_0$ is the piezo excitation amplitude, $\omega = 2\pi f$ is the excitation frequency and $F_{ts}(z)$ is the (nonlinear) tip-sample interaction force inferred from section 2.1. The cantilever oscillation is generally characterized by the eigenfrequency of the undamped harmonic oscillator $\omega_0 = \sqrt{k/m}$ and the quality factor $Q = m\omega_0/\gamma$. Typical free oscillation amplitudes ($F_{ts} = 0$) are $A(\omega_0) = QA_0 = 10 \, \text{nm}$. The quality factor is on the order of $10^2$ in air, while in liquids $Q \sim 3 – 10$. The lateral displacement of the tip associated with the cantilever oscillation amplitude is on the order of $10^{-4}$ nm and can thus be safely neglected.

In the case of small oscillation amplitudes the tip-sample force may be approximated by the first order expansion $F_{ts}(z) \approx F_{ts}(z_0) + (z - z_0)F'_{ts}(z_0)$. Inserted in eq. 2.11, the force constant $k$ can thus be replaced by the effective force constant $k_{\text{eff}} = k - F'_{ts}(z_0)$ and the effective eigenfrequency will be $\omega_{0,\text{eff}} = \sqrt{k_{\text{eff}}/m}$. This
2.2. SFM system characterization and modes of operation

![Frequency (sweep) spectra (RMS amplitude) in air and water as indicated. The piezo drive was operated at 200 mV(RMS) for the measurement denoted by \( A_{\text{piezo}} \). Amplitudes large enough for tapping mode operation are obtained in the range of 1 – 10 kHz. The piezo drive is turned off to measure the thermal spectra (amplitude data is 50 times expanded). A higher flexural cantilever mode is observed at about 45 kHz for the thermal spectrum in liquid.](image)

Figure 2.3: Frequency (sweep) spectra (RMS amplitude) in air and water as indicated. The piezo drive was operated at 200 mV(RMS) for the measurement denoted by \( A_{\text{piezo}} \). Amplitudes large enough for tapping mode operation are obtained in the range of 1 – 10 kHz. The piezo drive is turned off to measure the thermal spectra (amplitude data is 50 times expanded). A higher flexural cantilever mode is observed at about 45 kHz for the thermal spectrum in liquid.

Illustrates that the cantilever effectively "hardens" when subject to repulsive forces (more precisely, when \( F_{ts}(z) < 0 \)) and is "softened" by attractive forces (\( F_{ts}(z) > 0 \)).

In the presence of forces which are linear as a function of tip-sample distance and if damping is constant (at least within the range of the tip oscillation amplitude) the steady state cantilever motion is sinusoidal

\[
z(t) = z_0 + A(\omega)e^{i(\omega t - \phi)}
\]

while the oscillation amplitude as a function of excitation frequency is given by [5]

\[
A(\omega) = \frac{A_0\omega_0^2}{\left[(\omega_{0,\text{eff}}^2 - \omega^2)^2 + (\omega\omega_0/Q)^2\right]^{1/2}}
\]

and the phase lag of the oscillation compared to the excitation force is determined by

\[
\tan(\phi(\omega)) = \frac{\omega\omega_0}{Q \omega_{0,\text{eff}}^2 - \omega^2}.
\]

\( A(\omega) \) describes a Lorentzian shaped curve, with a maximum at the resonance frequency \( \omega_{\text{res}} < \omega_0 \). The phase \( \phi \) is equal to \( \pi/2 \) at \( \omega = \omega_{0,\text{eff}} \). Conservative as well as dissipative interactions affect the cantilever resonance frequency (the resonance moves in frequency space) and thus exert an influence on the oscillation amplitude measured at constant frequency. \( F_{ts}(z) \) as well as the dissipated energy may be
measured independently by tracking the resonance frequency and by simultaneously compensating for dissipative losses [43, 44, 45].

In Fig. 2.3 the measured amplitude spectrum $A(f = \omega/2\pi)$ is shown for the same cantilever positioned at a distance of about 10 \( \mu \text{m} \) above the sample, i.e. the tip is not interacting with the sample. Two of the curves correspond to the thermal noise spectrum measured in air and water, respectively, as indicated. In these measurements the cantilever is thus not excited by the piezo. From the equipartition theorem the thermal noise fluctuation amplitude may be estimated assuming that the cantilever behaves like a simple harmonic oscillator with small damping, and by inserting the force constant provided by the manufacturer ($k = 0.16 \text{ N/m}$)

$$\frac{1}{2} k \langle z^2 \rangle = \frac{1}{2} k_B T \Rightarrow A_{\text{th}} = 1.6 \text{ Å}. \quad (2.15)$$

This translates into a force sensitivity on the order of 25 pN. Clearly, the resonance of the cantilever in water is much broader and at a much lower frequency than in air. This is due to hydrodynamic damping and because additional (liquid) mass is set in motion by the oscillating cantilever. Further, the second flexural mode is manifested at a frequency $f \approx 45 \text{ kHz}$ for the cantilever in water.

The black curve corresponds to the frequency sweep spectrum in water of the cantilever excited by the piezo (at frequencies ranging from 2 to 14 kHz, as indicated in Fig. 2.3). The oscillating piezo vibrates not only the cantilever but induces acoustic waves in the liquid. Thus a complicated frequency spectrum with appreciable amplitudes in the range of 1–10 kHz arises which is due to resonances of the combined system of cantilever, liquid volume, cantilever holder and drive piezo. Oscillations at higher frequencies are strongly damped, and because of the insufficient signal to noise ratio are not useful for tapping mode operation. Therefore, cantilevers have to be used with resonance frequencies below 10 kHz in water. Stable tapping mode operation over several hours is achieved at drive frequencies of typically 8 kHz.

### 2.2.5 Tapping mode: anharmonic contributions

In the derivation of eq. 2.12 and 2.13 for the cantilever motion $z(t)$ the tip-cantilever system was modeled by a single-mode harmonic oscillator, thus ignoring higher flexural modes of the cantilever beam and nonlinear tip-sample interactions. The tip motion thus turned out to be strictly sinusoidal with a frequency determined by the excitation force. In the following it is demonstrated experimentally that the influence of flexural cantilever modes is indeed small even if the SFM is operated using tapping mode in liquids [36]. The presence of anharmonic oscillations, on the other hand, demonstrate that tip-sample interaction forces are highly nonlinear.

In Fig. 2.4 the frequency spectrum of a cantilever ($k = 0.16 \text{ N/m}$) interacting with the surface of a biological membrane (nuclear envelope) in water is compared to spectra from noninteracting cantilevers. The upper curve corresponds to the thermal spectrum obtained 10 \( \mu \text{m} \) above the sample showing the resonances of the
2.2. SFM system characterization and modes of operation

Figure 2.4: Frequency spectra in water above a nuclear envelope sample. Thermal spectrum (upper curve, 10 μm above the sample, amplitude data 100 times expanded), and spectra obtained with drive frequency set at 8 kHz corresponding to the way tapping mode is operated (middle curve: 10 μm above the sample, and lower curve: setpoint 80%. The latter is offset to lower values for clarity). Higher harmonics illustrate the nonlinearity of the tip-sample interaction. Apparently, no higher harmonics are generated at the second flexural cantilever mode (~ 45 kHz) indicating that the simple harmonic oscillator model is valid.

Comparison of the spectra where the cantilever is excited at 8 kHz with the thermal spectrum shows that the tapping mode signal does not show any significant contribution reminiscent of the second flexural resonance mode located around 45 kHz. Therefore, despite its simplicity the single-mode harmonic oscillator model is well suited to describe the tip motion in tapping mode. The amplitude signal measured by the optical deflection detection method (the RMS signal) is therefore well approximated by the quasistatic calibration method which involves measurement of the DC-deflection when approaching a hard sample surface.

The spectrum corresponding to the tapping tip (lower curve) shows contributions from higher harmonic vibrations arising from nonlinear interactions. From this spectrum certain characteristics of the tip-sample interaction such as the maximum force and the contact time may be extracted [46]. The harmonic distortions turn
out to be rather small in the experiments presented here because the tip interacts
with a soft sample. The relative amplitude of e.g. the second harmonic contribution
is smaller than 2.5% (note the logarithmic scale).

2.2.6 Quality factors

The quality factor $Q$ of the cantilever oscillator has a strong influence on how sensi-
tively forces can be measured. This is understood by noting that conservative
interactions mostly affect the location of the resonance frequency. Shifts in res-
onance frequency may experimentally be measured by the accompanying shift in
phase (see also Fig. 2.8)

$$\Delta \phi = \frac{2Q(\omega_0 - \omega_{0,\text{eff}})}{\omega_{0,\text{eff}}}. \quad (2.16)$$

Clearly, a higher $Q$ value will help to increase the signal to noise ratio. Equivalently,
the higher the quality factor the larger are the changes in oscillation amplitude in
response to resonance frequency shifts. This is immediately clear since the sharper
the resonance peak (eq. 2.22 and Fig. 2.6) the larger the slope of $A(\omega)$.

High quality factors may be obtained by using cantilevers with a high force con-
stant since $Q = m\omega_0/\gamma = k/\gamma \omega_0$. The drawback here is that the relative change in
resonance frequency in response to a linear force (force derivative $F'(z_0)$) is inversely
proportional to $k$

$$\frac{\omega_{0,\text{eff}} - \omega_0}{\omega_0} = \frac{F'(z_0)}{2k}. \quad (2.17)$$

This demonstrates the need for small (high $Q$ because of reduced hydrodynamic
drag), soft (low $k$) cantilevers with high resonance frequencies $\omega_0$ [47]. Another
disadvantage of high-$k$ cantilevers is that they cannot be operated in liquids using
the tapping mode setup described above (except for very high excitation amplitudes)
because their resonances are suppressed. Soft (low $k$) cantilevers are therefore needed
with resonance frequencies in the range of the system resonances (Fig. 2.3).

This problem may be circumvented by using an excitation mechanism which
directly drives the cantilever, but not the liquid holder. Such a system has been
developed during this thesis by employing a small magnetic coil positioned around
the cantilever holder which exerts a sinusoidal driving force on a magnetic particle
mounted on the backside of the cantilever [48, 49]. For imaging applications high
quality results can be obtained using either cantilever driving mechanism. For quan-
titative measurements of conservative and dissipative forces in liquids, however, the
magnetic drive is expected to be the method of choice.

2.2.7 Resonance control

Soft cantilevers for tapping mode in liquids are usually comparatively large and thus
strongly subject to hydrodynamic drag forces. This results in a strong damping of
2.2. SFM system characterization and modes of operation

Figure 2.5: Schematic circuit diagram describing Q-control, height feedback and scan controls. For height feedback, the photodiode signal (here the RMS amplitude) is compared to the setpoint voltage, and the difference is used to adjust the vertical tip position. The scan controls are used for lateral positioning of the tip. For Q-control operation, the (unaveraged) photodiode signal is passed through a variable gain amplifier and a variable phase shifter, and added to the conventional AC drive voltage source (denoted by ~). For Q-control as well as conventional tapping mode the excitation signal is supplied to the piezo to induce vertical oscillations.

The cantilever oscillation, which is equivalent to a low quality factor \((Q \sim 3 - 10)\) and consequently a low force sensitivity. The combined system of cantilever, liquid and cantilever holder can be regarded as the actual tapping mode resonator which is well approximated by the damped harmonic oscillator model (as shown above), but which exhibits a complicated frequency spectrum as shown in Fig. 2.3.

The quality factor of this resonator can be modified using a gain-controlled feedback loop which affects the external drive voltage applied to the excitation (drive) piezo. This method is called resonance control or Q-control because the effective quality factor of the resonator can be increased or decreased by adjusting electronic parameters [50, 51, 43, 52, 53]. As illustrated in Fig. 2.5 the signal from the photodiode is amplified (gain \(g\)), phase shifted (phase \(\alpha\)), and then fed back to excite the cantilever. The cantilever equation of motion has to be supplemented with a force term describing the Q-control feedback (second term on the right)

\[
mz(t) + k[z(t) - z_0(t)] + \gamma z(t) - F_{ts}(z(t)) = kA_0e^{iut} + gz(t + \alpha/\omega). \tag{2.18}
\]

In other words, what Q-control effectively does is to supply a drive signal to the normal excitation voltage which is proportional to the phase shifted cantilever motion.

In order to increase the effective quality factor of the oscillatory system the phase shift is chosen such as to produce a signal proportional but opposite to the velocity dependent damping term. This is achieved for any frequency \(\omega\) by setting \(\alpha = \pi/2\) since

\[
gz(t + \alpha/\omega) = gA(\omega)e^{i(\omega t - \phi + \alpha)} = \frac{g}{\omega} \dot{z}(t). \tag{2.19}
\]
Thus an effective damping factor $\gamma_{\text{eff}} = \gamma - g/\omega$ which is smaller than the original damping factor can be introduced and the system response is now determined by the effective quality factor

$$Q_{\text{eff}} = \frac{m\omega_0}{\gamma_{\text{eff}}} > Q.$$  (2.20)

When tuned properly the resonator thus becomes self-exciting. In fact, the conventional excitation source is only needed to stabilize the self-exciting oscillation. Note that $\gamma_{\text{eff}}$ and $Q_{\text{eff}}$ are externally controlled parameters. The "mechanical" quality factor of the cantilever is unaffected.

Using Q-control the complicated cantilever (more precisely, resonator) frequency spectrum in liquids can be reduced to a single sharp peak (Fig. 2.6).

What is the origin of this behavior? For a harmonic oscillator operated near resonance where $\phi \approx \pi/2$ (eq. 2.19) the Q-control drive term can be rewritten

$$gz(t + \alpha/\omega) = gA(\omega)e^{i(\omega t - \phi + \alpha)} = gA(\omega)e^{i\omega t}.$$  (2.21)

This is to demonstrate that if the system is driven at resonance the Q-control drive term is in phase with the conventional drive signal ($A_0k e^{i\omega t}$), i.e. the cantilever is excited with the same force as would be applied in normal tapping mode operation. In more general terms, if $-\phi + \alpha = 0$ the tapping mode excitation is recovered. However, if the above phase condition is not fulfilled then the Q-control and the conventional drive signal interfere destructively, and the oscillation amplitude is reduced (in other words, damping increases). This is the case if the resonator is excited at frequencies other than those satisfying the phase condition, thus producing the sharp resonance peak in the spectrum. Since all resonance peaks obtained with the conventional drive mechanism exhibit a phase dependence as described in eq. 2.14 (but generally shifted from the theoretical value $\phi \approx \pi/2$ because of the nonlinearity of the resonator) essentially any one of them can be singled out and enhanced using Q-control by choosing the appropriate setting for $\alpha$. In this regard Q-control can be considered as a phase-selective band pass filter.

Equivalently, when tip-sample interactions become significant phase $\phi$ and cantilever amplitude $A$ change due to conservative and dissipative [54] interactions. Since both phase shift and amplitude reduction affect the Q-control signal, the total cantilever excitation force decreases simultaneously to the decrease in oscillation amplitude (Fig. 2.9).

The relative magnitude of the signal in the Q-control loop compared to the contribution from the conventional excitation source determines the amplitude and the effective quality factor of the system. Quality factors up to 1000 can be reached with this method as determined by fitting of the Lorentzian $A(\omega)$ (eq. 2.13) or using the expression

$$Q = \omega_0/\Delta \omega.$$  (2.22)

where $\Delta \omega$ is the full width at half maximum of the oscillation energy ($\sim A^2(\omega)$) as a function of frequency. However, lower effective quality factors of about 200-300
2.3. Force resolution

2.3.1 Force measurements and sample properties

The distance dependence of tip-sample interaction forces is measured by moving the cantilever vertically to the sample surface. For an oscillating cantilever, the amplitude as well as the low frequency (DC) signal can be recorded simultaneously.
Figure 2.7: Amplitude and deflection measured simultaneously as a function of vertical piezo displacement on a hard, medium hard, and soft sample in liquids (panels from left to right). The sample is located where changes in amplitude and deflection become appreciable. A full approach/retraction cycle was recorded as indicated by arrows pointing to left/right, respectively. Red straight lines indicate unit slope. Note the increased range of piezo displacement for the measurements on the unfixed membrane, indicating that the tip is displacing freely floating or unbound membrane patches over large ranges (piezo displacements > 80 nm). The deflection measurement may be translated into average forces by multiplying the data with $k = 0.16 \text{N/m}$.

and are generally displayed as a function of vertical piezo displacement. The DC-signal corresponds to the average deflection of the cantilever (as may be inferred from the Fourier expansion of the periodic tip oscillation signal) and is thus related to the average force exerted on the sample. Note that the average tip-sample distance $z_0$ is equivalent to the piezo displacement only as long as the tip is not deflected by interactions with the sample. To plot the amplitude, or deflection, as a function of the true tip-sample distance the abscissa has to be redefined from the deflection data the straight line (with unity slope) corresponding to hard contact between tip and sample. The origin on the abscissa (cantilever displacement axis) is arbitrarily chosen since there is no absolute measure for the tip-sample distance.

In Fig. 2.7 examples are shown of force measurements in liquids on the surface of a plastic petri dish, a chemically fixed nuclear envelope, and an unfixed nuclear envelope (for further information about nuclear envelopes and chemical fixation see section 4.1). The full approach-retraction cycle is measured and displayed as indicated. The oscillation amplitude decays rapidly and the cantilever ($k = 0.16 \text{N/m}$)
is strongly deflected as a function of cantilever displacement while making contact with a rather hard plastic surface. By contrast, on the fixed membrane the amplitude is reduced much more slowly and the tip is much less deflected. Clearly, the softer sample is elastically deformed under the influence of the tip, and sample viscosity plays a role in the damping of the oscillation amplitude. These effects are negligible when the sample is much harder than the cantilever, and a hard-wall type of interaction can be assumed. Under these circumstances the slope of the DC-deflection and the slope of the amplitude reduction (as indicated in Fig. 2.7) are expected to be unity (i.e. the amplitude is reduced by 1 nm and the tip is deflected by 1 nm when the cantilever is approached to the surface by 1 nm); force-distance measurements on hard substrates can thus be conveniently employed to calibrate the photodiode signal in terms of tip displacement.

A fundamentally different situation is sometimes encountered in preparations of unfixed nuclear envelopes. The cantilever amplitude is hardly reduced while the time averaged deflection is already appreciable and even may exceed the tapping amplitude. This is attributed to floating or sponge-like membrane patches that are compressed and set in motion by the oscillating tip. The large DC-deflection is presumably due to the membrane patch being in contact not only with the tip but with the entire cantilever, and thus exerts forces along the full length of the cantilever. Imaging using tapping mode is prevented in such a situation because the slope of the amplitude reduction (i.e. the sensitivity $dA/dz$, see section 2.3.3) is too small for stable operation of the height feedback. For contact mode, even though the slope of the DC-deflection is appreciable only features on the scale of microns can be resolved because the entire cantilever rather than the tip is ploughed across the sample.

### 2.3.2 Measurements of attractive and repulsive forces

In Fig. 2.8 typical force measurements are shown obtained with tapping mode in air using a cantilever with $k = 20$ N/m ($f_{res} = 284$ kHz) and a mica sample. The complicated amplitude-distance behavior is due to the nonlinear forces between tip and sample. Though qualitatively well understood in terms of attractive and repulsive interactions as presented in section 2.1.2 detailed modeling is generally prevented since tip shape and tip/sample properties (surface charges, local elastic moduli, etc.) are not known in full detail [56].

At small amplitude reductions (displacement $> 15$ nm) the net interaction is attractive (mainly van der Waals) as can be determined from the simultaneous phase measurement presented below ($\phi > \pi/2$, see also eq. 2.14). The amplitude decays rather linearly though with a slope smaller than one, which may indicate the presence of static surface charges influencing the tip at large ranges. Approaching nearer to the sample a step-like transition occurs in amplitude and phase. At this point the tip enters an oscillatory state of higher amplitude. Since $\phi < \pi/2$ in this regime
Figure 2.8: Force measurement (approach and retraction) on mica in dry air using a cantilever with a high force constant. The red straight line corresponds to unit amplitude slope. Due to nonlinear tip-sample forces the cantilever oscillation switches abruptly from a low amplitude to a high amplitude state at a small tip-sample distance. The phase measurement is very sensitive to the slope of the interaction force. Phases $\phi > \pi/2$ indicate net attractive, $\phi < \pi/2$ net repulsive tip-sample interactions. The amplitude drops to zero almost completely before the cantilever is statically deflected, which is indicative of the high quality factor of the oscillator.

The net tip-sample force is repulsive, and the amplitude decays linearly as expected for a tip tapping on a very hard sample (Hertz interactions, exchange repulsion). When retracting the tip a qualitatively similar behavior is observed, though the onset of the transition between repulsive and attractive oscillatory states occurs at higher amplitudes. The hysteresis between the approach and retraction curves is a measure of the energy dissipated in adhesive contacts. Note that the discrete jumps in the amplitude curves are not related to the snap-in/out effect discussed in section 2.3.4, but are the result of nonlinear tip-sample interactions, and, as such, depend on sample properties and cantilever amplitude.
2.3. Force resolution

2.3.3 Increased sensitivity with resonance control

The sensitivity of the SFM is set by the smallest change in tip-sample interaction force that can be detected, i.e. the smallest detectable change in the magnitude of the oscillation amplitude (tapping mode) or DC-deflection (contact mode). Since a change in vertical tip-sample distance is related to a change in force, this sensitivity directly translates into how accurately the system feedback manages to follow sample topography. In amplitude modulation mode the sensitivity is thus defined as the slope of the amplitude change (usually the amplitude reduction) as a function of tip-sample distance $S = dA/dz$.

As discussed in section 2.2.6 the force sensitivity is directly proportional to the quality factor $Q$. Experimentally, by increasing the effective $Q$-value using the resonance control setup (section 2.2.7) an increased amplitude slope compared to conventional drive operation is obtained (Fig. 2.9). The apparent increase in $dA/dz$ is a result of the simultaneous decrease in excitation force applied to the cantilever (Fig. 2.9, lower panel). This is achieved by feeding back to the drive piezo a phase shifted tip oscillation signal which drives the system out of resonance when tip-sample interactions are turned on.

Interestingly, using Q-control the amplitude slope $dA/dz$ reaches the optimum unit value indicating that the sample is not deformed by the tip (see also section 2.3.1) [57]. The much shallower amplitude reduction obtained with conventional

![Figure 2.9: Comparison of Q-control and conventional tapping mode in terms of amplitude sensitivity, average forces and excitation force. The red straight line indicates unit amplitude slope. The measurements were performed on a fixed nuclear envelope.](image)
Figure 2.10: Average force (DC-deflection) exerted by the tip plotted as a function of the normalized amplitude for Q-control and conventional tapping mode. This plot has been constructed from the same data as in Fig. 2.9. The normalized amplitude corresponds to the setpoint used for height feedback operation.

Drive mode, however, is attributed to two effects: 1) the sample is actually indented by the tip during the downward path of the cantilever oscillation cycle, and 2) the cantilever performs a pivoting motion with the point of the tip resting on the sample (and serving as the pivot). This is supported by the observation that the amplitude never reaches zero, even if the tip is fully in contact with the sample.

The DC-deflection of the tip, however, is independent of how the cantilever is vibrated, and corresponds to the average vertical force exerted on the sample. Since the amplitude drops faster for Q-control, average forces related to a given amplitude setpoint are smaller (Fig. 2.9). This is illustrated in Fig. 2.10 where the average force data is plotted against the normalized amplitude for Q-control and conventional tapping mode, respectively.

Using Q-control smaller forces can be measured, larger force contrast can be obtained (e.g. with measurements of the phase φ), and tip-sample interactions are minimized. The result is that soft samples can be measured with less deformation. However, the problem remains that on native biological specimens imaging is often disturbed by loosely attached particles or contaminants. Q-control is obviously more sensitive to these influences as well, resulting in noisier images. To obtain images with a signal to noise ratio comparable to conventional tapping mode often low \( A/A_{free} \) setpoints have to be used, thereby negating the advantage of using smaller average forces. Indeed, on biological membranes such as nuclear envelopes conventional tapping mode may generally be operated at higher setpoints and thus at average forces which are again comparable to Q-control operation (Fig. 2.10).
Therefore, at typical tapping mode setpoints of about 90% to 95% of the free amplitude average forces are significantly lower than 0.2 nN.

Resonance control may thus be the method of choice for force measurements and for imaging applications on extremely soft samples which could not be investigated otherwise and where the lateral resolution is not expected to be better than 30 nm.

### 2.3.4 Operation in air vs. liquids

Most surfaces exposed to ambient conditions (air) are covered by a thin layer of water with a thickness of a few nanometers depending on the surface chemistry and relative humidity. These conditions are especially pronounced for biological samples and as we will see later may lead to scanning conditions that could compromise sample integrity and resolution. Water films exert strong capillary forces on the tip which can lead to instabilities for cantilevers with small force constants $k$. This effect is characterized by the *snap-in* or *snap-to-contact* indicated in Fig. 2.11 at short distances when the tip is vertically approached to the sample surface. When the cantilever is retracted a liquid neck forms between tip and sample, and the tip is released only when the cantilever restoring force becomes large enough (*snap-out*) to overcome the adhesive forces. The tip thus describes a hysteretic motion upon approach and retraction, and the area between the two curves can be used to

![Figure 2.11: Force measurement on mica in ambient environment using a cantilever with $k = 0.6 \text{N/m}$. The capillary forces exerted on the tip may be measured by the restoring force acting at the snap-out point, $F_{cap} = -27 \text{nN.}$](image-url)
measure the adhesion energy between tip and sample. Large attractive and adhesive forces of this type may also occur between hydrophobic sample surfaces and the tip or contaminants on the tip. Even though covered by a rather hydrophilic oxide layer clean tips are easily contaminated by hydrophobic impurities.

The resolution that can be obtained under these circumstances is drastically reduced because the interaction between tip and surface is dominated by the large attractive force which may distort soft samples. Furthermore, height (vertical) feedback operation becomes unstable. This effect can be avoided if tip and sample surface are completely immersed in aqueous environments. Fortunately, the native structure of biological samples is much better preserved in liquid environments as well.

In general, cantilever instabilities occur if the tip is moved in an attractive potential where the force gradient becomes larger than the force constant of the cantilever. In such cases two tip-sample separations $z_{1,2}$ exist, where $F_{ls}(z_{1,2}) = k$ and $F_{ls}(z) > k$ for $z_1 < z < z_2$. The tip is either driven towards the sample upon approaching $z_2$ or driven away from it upon retraction from $z_1$, respectively, explaining the expression snap-in and snap-out. This effect may be understood by looking at the total energy in the cantilever-sample system given by $E(z) = W(z) + k(z - z_0)^2/2$ where $W(z)$ is the tip-sample interaction potential (with an attractive and a repulsive term) and the second term describes the cantilever potential energy. At sufficiently small separations and small $k$ two local minima as a function of $z$ exist. By approaching (or retracting) the cantilever the minima change in depth relative to each other, and under the influence of $k_B T$ the tip eventually jumps from the more shallow to the deeper minimum. To prevent the snap-in/snap-out effect cantilevers with high force constants are used (section Fig. 2.3.2).

### 2.4 Image resolution

#### 2.4.1 Tip shape

The bulk of the cantilever tip generally experiences forces of different magnitude and orientation than the foremost tip asperity (Fig. 2.1). In a simplified picture the tip and sample molecules are assumed to exhibit a short range hard wall repulsion instead of e.g. the exchange term $\frac{A}{r^2}$ in eq. 2.3. The sample topography is thus more conveniently defined by an "infinitely" strong repulsion, and elastic, electrostatic etc. material properties can be neglected.

The lateral resolution in an image is thus solely determined by the geometrical shape of the tip. In mathematical terms, the image generated by SFM is a dilation of the sample topography with a kernel determined by the tip shape. This effect is often wrongly referred to as the convolution of tip and sample morphology. Fig. 2.12 shows an example of a line scan profile obtained on a nuclear pore complex (control sample) using an oxide sharpened silicon nitride tip. The tip shape is estimated
Figure 2.12: Profile of a nuclear pore complex. In the lower panel, horizontal and vertical axes are to scale, while the vertical axis is expanded in the upper panel. The estimated tip shape is shown schematically. Red solid line: sphere with radius 3 nm and cone with half angle 20°; blue dashed line: parabola with radius of curvature 3 nm.

from the deep local miniumum. The blue dashed curve corresponds to a parabola $z = \frac{x^2}{2r}$ with radius of curvature $r = 3$ nm, while the red solid curve is to model a tip composed of a cone with opening half angle of 20° and a spherical end with radius $r = 3$ nm. Smaller structures in the profile curve may indicate that the tip has additional minute asperities. In other words, the tip is rough on the nanometer scale, which increases the apparent resolution.

Clearly, since cantilever tips are far from resembling point-like particles the lateral resolution is affected by sample (or tip) tilt and sample roughness. For example, a gold particle with diameter of 5 nm is easily resolved on a atomically flat mica surface, while if deposited on a biological membrane as shown above chances are small that it could be detected unambiguously. It is thus relevant to note that a "typical" protein molecule consisting of 300 amino acid residues is estimated to have a diameter of about 4 to 5 nm [58].

Images of steep features such as edges on a calibration grid may actually reproduce the tip edge rather than the sample surface. To resolve this issue tips with a high aspect ratio are needed, i.e. with cylindrical shape rather than a cone. In the framework of this thesis conventional SFM tips have been modified with Carbon Nanotubes (NT) which can be regarded to consist of one or more graphene sheets rolled-up into a cylinder (and are thus termed single or multi-walled) and end caps which resemble halves of bucky balls. Apart from their interesting metallic or semiconducting properties which are determined by the chirality of the nanotube [59], NT’s have a high tensile strength, but may buckle sideways [60]. Buckling occurs
2. Scanning Force Microscopy principles for soft samples

2.4.2 Contact vs. tapping mode

In imaging applications not only force sensitivity and connected issues like lateral and vertical resolution is of concern, but also the speed at which images can be
2.4. Image resolution

Figure 2.14: Comparison of contact and tapping mode images on the same part of a nuclear envelope. The lateral resolution is reduced for contact mode because of shear forces even at the smallest possible deflection setpoint. In fact, the setpoint had to be manually adjusted during this scan to compensate for DC-drifts which are evidenced by the streaking in the upper quarter of the contact mode image.

taken. Apart from the issue that biological samples degrade quickly with time, thermal drift poses a severe problem at slow scan rates. Clearly, a major advantage of contact (DC) mode is that the full system bandwidth is available to correct for changes in topography using the height feedback, while in amplitude modulation mode the bandwidth is restricted by the tapping frequency and the quality factor.

A major disadvantage of contact mode, however, is that DC measurements are generally more prone to drift. Here drift may arise from thermal inequilibrium of the imaging environment, photodetector and electronics. Thus often the deflection setpoint has to be adjusted even during a single image frame in order to prevent the tip from losing contact with the sample or exerting forces larger than desired (Fig. 2.14). Drift in measurements of the cantilever RMS amplitude, on the other hand, is almost exclusively due to drift in resonance frequency, and turns out to be relatively insignificant in terms of normal tapping mode operation.

From Fig. 2.9 it is inferred that average normal forces are similar in both tapping and contact mode operation in liquids (~0.2 nN), i.e. the amplitude setpoint in tapping mode corresponds to the onset of the DC-deflection and thus coincides with the point where contact mode would be operational. The main advantage of tapping mode, however, is that friction forces due to lateral motion are minimized because the tip only intermittently touches the sample during the lower part of the oscillation cycle. In contrast, the tip is in full contact with the sample for the DC-mode, at comparable average forces, and thus produces larger lateral forces. This effect translates to a clearly reduced lateral resolution particularly on soft samples as presented in Fig. 2.14.
Chapter 3

Nuclear transport

3.1 Introduction to cell organization

The cell can be considered the smallest autonomously functioning unit of life since it contains all the basic biochemical machinery to fulfill life’s requirements; for example, the production and secretion of substances (e.g. hormones), the detection of external stimuli (sound, light etc.), the production of mechanical force (or displacement), the transduction of signals (e.g. electrical signals) and reproduction (germ cells), to name a few.

Here we will be exclusively concerned with eucaryotic cells, which, in contrast to procaryotes (e.g. bacteria), represent a greater level of complexity through subcellular compartmentalization. In its simplest interpretation the eucaryotic cell can be envisioned as being composed of three principle compartments. These are the cytoplasmic, nuclear and the reticular membranous compartments (Fig. 3.1). The cytoplasmic compartment is the largest compartment, houses the constituents of the cell and forms the outer barrier of the cell known as the plasma membrane. The nuclear compartment contains the genetic information of the cell and is confined within the cytoplasmic compartment. Functionally separating the nuclear and cytoplasmic compartment is the nuclear envelope which is an extension of the endoplasmic reticulum and in combination they form the reticular membranous compartment. The nuclear pore complex connects the cytoplasmic with nucleoplasmic compartments by spanning the lumen of the nuclear envelope and thereby serves as a nexus for the three cellular compartments.

In the following, some of the structures observable inside a cell are described. The plasma membrane, a 5 nm thick bilayer of phospholipids, forms the outer boundary of the cell. Embedded in it are membrane proteins dedicated to the exchange of ions and molecules with the extracellular environment. A filamentous protein network, the cytoskeleton, provides stability and structure to the cell. The cytoplasm is a concentrated aqueous solution containing proteins, ions, nucleic acids, other small and large molecules, and certain subcellular organelles such as mitochondria
which consume oxygen and make available to the cell chemical energy in the form of adenosine triphosphates (ATP). The endoplasmatic reticulum consists of sheets, sacs and tubes of lipid membranes, and is involved in the synthesis and transport of lipids and, in association with protein-RNA complexes called ribosomes, also in the synthesis of membrane proteins. Ribosomes are also found floating freely in the cytoplasm where they are involved in the synthesis of soluble proteins.

By compartmentalization the eucaryotic cell has achieved a division of labour which opened the way for tissue diversity. On the other hand, compartmentalization of cellular processes demands efficient and selective exchange of the regulatory factors (proteins, nucleic acids, etc.) across the organellar boundaries. In the following we will focus in more detail on some of the most important cellular processes in the interplay between the nucleus and the cytoplasm.

3.1.1 The nucleus

The nuclear envelope functionally delimits the nuclear from cytoplasmic compartments of the cell. It consists of two concentric lipid bilayers separated by the perinuclear space (Fig. 3.1). The outer membrane is continuous with the endoplasmatic reticulum (ER) such that the perinuclear space is directly connected to the ER lumen. The nucleus hosts the genetic information of the cell in form of nucleic acids, i.e. DNA. It is dedicated to support interactions between nucleic acids (DNA, RNA) by means of specialized enzymes (proteins). The transfer of genetic information from DNA to mRNA (messenger-RNA) is called transcription. mRNA can be viewed as a mobile carrier of the code, thus will eventually leave the nucleus to convey the information to the cytoplasm.

3.1.2 The cytoplasm

Translation of the genetic code into proteins, i.e. protein synthesis, and activation of the proteins is controlled in the cytoplasm. Thus, the cytoplasm can be envisioned as the realm of protein chemistry. Proteins are constructed according to the mRNA template. In general, three sequential bases of the mRNA chain code for one single amino acid. Start and stop codons are implemented in the mRNA reading code to ensure the correct operating sequence.

Protein synthesis takes place on the ribosomes which are large macromolecular assemblies of various proteins and RNA molecules. At a certain stage, ribosomal proteins have to be imported into the nucleus themselves, where they assemble with rRNA (ribosomal-RNA), and are consequently exported again to the cytoplasm. Further, all the proteins and enzymes involved in transcription (gene regulatory proteins, DNA- and RNA-polymerases, etc.) are expressed in the cytoplasm, but function in the nucleus and thus also have to cross the nuclear envelope.
3. Nuclear transport

3.1.3 Communication between the nucleus and the cytoplasm – the nuclear pore complex

The need for export of mRNA, tRNA (transfer-RNA), etc., from the nucleus and import of enzymes, RNA polymerases, etc., into the nucleus requires efficient and selective bidirectional translocation of molecules across the nuclear envelope. The only available pathway for the exchange of macromolecules between the nucleoplasm and the cytosol is via the Nuclear Pore Complex (NPC) (Fig. 3.1). The higher the transcriptional and translational activity of the cell, the more NPCs are available for macromolecular communication. Each NPC is thought to sustain macromolecular traffic in both directions simultaneously. As of yet there is no indication that some of the NPCs should be responsible for import while others function in export only (or vice versa).

3.2 Structure of the NPC

The NPC is a large protein compound spanning the nuclear envelope, with an estimated mass of about 125MDa in vertebrates. It consists of about 50 to 70 different proteins (nucleoporins) cylindrically arranged to form a pore or channel connecting nucleoplasmic and cytoplasmic compartments. The pore walls are constructed from 8 columnar domains sandwiched between a nuclear and a cytoplasmic ring. Viewed along the axis of the pore the NPC exhibits an octagonal symmetry [62]. The main body of the NPC forms a cylinder with a mean diameter of about 83 nm and a height of about 40 nm [13]. In addition, flexible fibrils protrude from the rings into the nucleoplasmic and cytoplasmic compartment, respectively. While cytoplasmic fibrils appear to extend straight from the NPC, the nuclear fibrils converge to form a cage-like structure known as the nuclear basket (Fig. 3.1).

Many nucleoporins are present in multiple copies in accordance with the highly ordered, eight-fold symmetry of the NPC. In addition, multiple copies of the same protein may exist along the axial direction of the pore. Thus, feasibly thousands, or more, individual proteins make up the elaborate structure of a single NPC. Many of these nucleoporins have been identified using genetic and biochemical methods, and their positions in the NPC have been localized using gold-labeled antibodies (immunogold labeling) in conjunction with electron microscopy (reviewed in [63]).

Despite these progresses, little is known about the detailed molecular arrangement of nucleoporins in the central pore of the NPC. Many nucleoporins contain characteristic stretches rich in phenylalanine (F) and glycine (G) residues. More specifically, motifs of the form FxFG (where x is usually a small polar or nonpolar residue) or GLFG (L=leucine) are present in high concentrations in the central channel, the cytoplasmic filaments, and the nuclear basket. From here on, we will refer to these motifs simply as FG-repeats. FG-repeats are interspersed by hydrophilic spacers consisting of about 15 polar or charged residues. In contrast, since F, G, and L are
3.3 Transport pathways

In this section, two different mechanisms of how material (cargo) is transported across the nuclear envelope are elaborated upon. These mechanisms are distinguished by whether a molecule is able to pass across the NPC on its own, or if
translocation through the pore has to be supported by additional mediators (adapter molecules and transport receptors). Figure 3.2 shows a schematic representation of passive and mediated translocation through the NPC. The issue of transport is intimately connected to questions about selectivity and the detailed molecular mechanisms governing the actual translocation process through pore. The definition of transport pathways goes beyond the simple differentiation between non-mediated and mediated transport. In the latter, pathways are distinguished by the types of mediators and the kinds of interactions involved between cargo, mediators, and NPCs. As an example, the classical import pathway via importin β (Impβ) will be presented in detail. Although many of the concepts of nuclear transport will be discussed in terms of the import of substrates into the nucleus, they can also be applied in an analogous manner also to nuclear export pathways. Mediated nuclear transport has recently been reviewed by Görlich and Kutay [26].

3.3.1 Passive diffusion

Small molecules, such as hydrated inorganic ions and water-soluble macromolecules with mass up to ~ 40kDa (the exclusion limit) are thought to passively diffuse through the central channel of the NPC. The diffusion rate of these ions or molecules through the NPC is comparable to that of freely diffusing particles of the same size. From measurements of diffusion rates of small molecules across the nuclear envelope it is suggested that the NPC contains a pathway for free diffusion equivalent to a water-filled cylindrical channel of about 9nm in diameter and 15nm in length [58, 65]. This simple model stresses the fact that for small molecules transport through the NPC may be achieved by passive diffusion due to a gradient of chemical activity and is thus not connected to any kind of active transport mechanism such as shuttling by a carrier or gating by the NPC itself. On the other hand, as discussed in detail in the following sections, molecules of any size may participate in a facilitated translocation mechanism and thus potentially speed up their passage through the NPC above diffusion levels provided that they are fitted with an appropriate targeting signal (see below). This is obviously especially important for molecules with sizes (masses, diameters) close to or larger than the exclusion limit.

3.3.2 Mediated transport

It has been shown that gold particles of diameter up to 20 nm are taken up into the nucleus via the NPC if the gold surface is made receptive to transport in the appropriate manner [66]. This finding clearly contrasts with the passive diffusion model with a rigid channel of 9 nm diameter. Inert objects like gold particles, proteins, or other large macromolecules lacking the appropriate modification (signals) are excluded from the nucleus. Thus a second mechanism of transport has been evoked for import (or export) of large objects, which depends on the specific interaction of
the modified surface (as in the example of the gold particles) with the NPC directly or via mediating factors in the cytosol (or nucleoplasm, respectively).

Mediated import can be conceptually classified according to the cytosolic and nucleoplasmic components involved in the process (see Fig. 3.2 (right)). In general, the material to be imported (cargo, substrate) has to carry a specific signal, without which, it is not recognized by the import mediators. The Nuclear Localization Signal (NLS) thus targets import substrates to the nucleus, while export requires a corresponding Nuclear Export Signal (NES). These signals thus occur in different flavors, of which the NLS is just one. Since NLS was the earliest characterized signal, transport pathways related to it are termed classical. As far as the NLS is concerned, it generally consist of short sequences of basic amino acids such as lysine and arginine.

The soluble transport receptor protein recognizes and binds cargo carrying the appropriate signal, and targets the complex to the NPC. Some transport receptors need additional adaptor proteins which actually recognize the NLS-cargo. In this case the trimeric cargo-adaptor-receptor complex docks to the NPC, and is consequently translocated through the pore. Import and export can occur through the same NPC, and several cargo complexes can be accommodated simultaneously in the pore.

Translocation involves specific interactions between the transport receptor and the nucleoporins. During translocation the receptor is complexed with the cargo molecule. After passage through the pore, the di- or trimeric complex presumably dissociates as to release the cargo into the nucleoplasm. Nuclear import is thus terminated. Commonly, the dissociation of the transport complex is stimulated by interaction with yet another protein, RanGTP. Binding of receptor and RanGTP occurs at the nucleoplasmic side of the NPC, i.e. at the nuclear basket.

In contrast to the passive diffusion of small molecules, signal-mediated transport can occur against a gradient of cargo activity. To overcome the corresponding gradient in chemical potential, energy has to be supplied to the transport process. Below we will see that energy is consumed in the creation of a gradient in high-energy phosphates which drive the transport process. In general, in the cell chemical free energy is provided by the hydrolysis of nucleoside triphosphates to a diphosphate, e.g.

$$\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{P}_1 \quad (\Delta G^0 \approx -7 \text{ kcal/mol})$$

where $\text{P}_1$ is the cleaved off inorganic phosphate and the value for $\Delta G^0$ is for standard conditions ($25^\circ\text{C}$, pH 7.0, all concentrations 1 M). ATP is probably the most common source of chemical energy in biological systems. For certain modes of transport through the NPC, however, hydrolysis of ATP seems to play only a minor role. It has been shown that GTP hydrolysis in the cytosol via the GTPase activation of Ran is required for continuous translocation to proceed [67]. In sections 3.4 and 3.5 the function and stoichiometry of the RanGTP-system are presented in more detail.

At this stage, a definition of the term "transport pathway" may be necessary.
3. Nuclear transport

Figure 3.2: Passive diffusion of small particles (left) and signal-mediated facilitated transport of a larger cargo molecule (right). For demonstration purposes, we focus on facilitated transport via the importin α/β pathway, and show only the most essential components involved. Cargo exhibiting a NLS (nuclear localization sequence) is recognized by the adaptor protein importin α, and translocation is mediated by the transport receptor importin β. Cargo is released after interaction of the complex with RanGTP, which results in an allosteric conformational change in Impβ structure that interferes with its association with both cargo and the NPC.

The most important characteristic of a given transport pathway is obviously the transport receptor itself (example: Impβ) since it is responsible for the actual interaction with the NPC. In a broader sense transport pathways are also defined by a unique combination of the following properties:

- Transport receptors (Impβ, transportin)
- Transport direction (import, export)
- Type of cargo (NLS-containing)
- Adaptor molecules utilized (Impα)
- Docking sites and specific interactions of the transport receptor with the NPC (or passive diffusion)
- Mode of cargo release (RanGTP binding to the transport receptor)
3.4 The RanGTP gradient

3.4.1 The RanGTP/GDP cycle

Ran is a small protein that acts as a GTPase, i.e. it catalyzes the hydrolysis of GTP to GDP. Many different transport pathways interact in one way or another with Ran. In fact, Ran influences, controls and drives many of the nuclear transport processes.

Ran can complex with either GTP or GDP since the intrinsic catalytic activity of Ran is relatively low. The hydrolysis of GTP is greatly accentuated by the presence of a GTPase-Activating Protein, or GAP. This role is performed by RanGAP1, which is abundant in the cytoplasm, but not in the nucleus. Thus in the cytoplasm Ran is encountered predominantly in a GDP-bound state.

The Guanine nucleotide Exchange Factor (GEF) RCC1 is located inside the nucleus. As its name implies it catalyses the exchange of Ran-bound GDP for GTP without altering the phosphorylation state. Since GTP normally occurs in a much higher concentration than GDP, Ran is expected to be loaded preferentially with the former in the nucleus. Thus a high concentration of RanGTP is maintained in the nucleus, while in the cytoplasm Ran is predominantly bound to GDP. In other words, a distinct gradient in the RanGTP concentration is generated across the nuclear envelope.

Many transport receptors proteins interact with Ran. Import receptors load cargo in the absence of RanGTP and therefore this process is restricted to the cytoplasm. In an analogous manner a high concentration of RanGTP in the nucleoplasm prevents complexing of import receptor and cargo. Through such a mechanism, the

- Translocation kinetics (diffusion rate)
- Energy consumption (ATP or GTP hydrolysis)
- Recycling and reloading of the transport mediators

In conclusion, signal-mediated transport can be characterized as a facilitated translocation against a concentration gradient which depends on transport mediators and energy supply. Cargo binds to transport receptors via specific signal sequences. The entry of the cargo complex into the NPC is based on specific interactions between transport receptors and the nucleoporins. Large molecules which don’t have the appropriate signal are excluded from the nucleus. In this respect the NPC can be envisioned as a gatekeeper which imposes selectivity by only allowing cargo to pass if complexed with the appropriate receptors. In other words, selectivity for import substrates is delegated to the receptor and adaptor machinery. This strategy thus allows nuclear transport to work with a higher level of diversification and flexibility.
3. Nuclear transport

import receptor is forced to release its substrate in the nucleus and re-export of the cargo to the cytoplasm is prevented (Fig. 3.2). The same scheme applies to many export receptors, except with opposite sensitivities for RanGTP and RanGDP: cargo is bound in RanGTP rich environment, i.e. in the nucleus, and released in the cytoplasm where RanGTP hydrolysis occurs. The RanGTP gradient thus regulates the affinity between cargo and receptors, distinguishes nuclear and cytoplasmic compartments, and helps control one of the fundamental properties of nuclear transport pathways: that of directionality [26].

Import termination in the nucleus occurs in response to an association with RanGTP in the vicinity of the nuclear basket, following which the receptors are recycled back to the cytoplasm in a complex with RanGTP (Fig. 3.3). In the cytoplasm, a phosphate group of RanGTP is cleaved off (hydrolysed) and the complex dissociates. The import receptors do not bind to RanGDP in contrast to their large binding affinity to RanGTP, and thus are in a state where they can be reloaded with cargo for a new round of transport.

Ran is constantly depleted from the nucleus. RanGTP is continuously exported from the nucleus in association with export receptors or with recycled import receptors. The hydrolysis to RanGDP is mainly localized in the cytoplasm, while Ran’s replenishment with GTP takes place in the nucleus. Therefore, in order for the RanGTP gradient to be maintained, RanGDP in turn has to be shuttled back to the nucleoplasm. Passage through the NPC is achieved by cooperative binding of RanGDP and a transport receptor called Nuclear Transport Factor 2 (NTF2) to the pore, and subsequent translocation (Fig. 3.3). The question arises how NTF2 is in turn recycled back to the cytoplasm. Several strategies may be evoked: 1) NTF2 is recycled by another energy consuming export mechanism. 2) NTF2 is continuously synthesized in the cytoplasm and bound or degraded in the nucleus. 3) NTF2 may be able to diffuse back to the cytoplasm when dissociated from RanGDP, which may indicate that the NTF2 pathway through the NPC is inherently asymmetric assuming that NTF2 should not be allowed to move freely from the cytoplasm to the nucleoplasm. The first strategy may be regarded as an intermediate step in the hierarchy of a complex system of transport receptors shuttling back and forth through the NPC. At some stage, however, some sort of asymmetry as proposed in 2) or 3) may have to be introduced in the transport system if the cascade of transport mediators (and their energy consumption) is not to be continued infinitely.

3.4.2 Energetics

The processes involved in GTP hydrolysis and dissociation of Ran and transport receptor require interactions with additional specific ligands [26]. The mechanisms describing how free energy is coupled into the transport system thus vary between different pathways. Here, neglecting specific details, the association between transport receptor and RanGTP is taken to indicate that free energy is supplied to the
transport mediators and their interaction partners (nucleoporins, adaptor molecules) as opposed to any components of the NPC moving actively over large ranges (tens of nanometer).

However, the actual energy consuming process of the cell is the maintenance of the RanGTP gradient across the nuclear envelope. The RanGEF factors inside the nucleus replenish the transport system’s supply of GTP. RanGTP inside the nucleus terminates import and drives export processes by binding the import and export receptors, respectively. The energy stored in GTP is released in the cytoplasm upon hydrolysis of RanGTP.

It has been shown that certain transport receptors in the cytoplasm present in superior numbers compared to the number of NPCs in the nuclear envelope can mediate a single round of transport each even in the absence of Ran or nucleotide hydrolysis [67]. This indicates that docking of receptors to the NPC is independent of Ran and GTP hydrolysis. More importantly, transport receptors are able to pass through the NPC without the input of energy. This demonstrates that processes involving energy consumption and the actual translocation through the pore are in principle functionally independent.

3.5 The importin α/β pathway

This section presents a more detailed description of the general transport strategies discussed above. The Impα/β transport receptor complex represents the cell’s main nuclear import pathway. The detailed analysis of Impβ protein sequence and its interactions with Impα, nucleoporins, and Ran is provided to give an introduction to the molecular mechanisms associated with nuclear transport. Properties of Impβ constructs are discussed in detail by Kutay et al. [27]

3.5.1 The cargo complex

Impα/β is one of the best characterized import pathways. It is responsible for the import of substrates carrying the classical NLS sequence. Impα functions as the adaptor molecule and in this capacity recognizes and binds NLS-cargo in a specific manner. Furthermore Impα’s association with Impβ is mediated via its “Importin β-Binding” (IBB) binding domain. In this respect the IBB domain of Impα may be considered analogous to the NLS. The association of Impβ and Impα is stabilized by interaction with NLS cargo.

Impβ is the actual transport receptor which binds the Impα-cargo complex and mediates translocation through the NPC. We next focus on the molecular basis of the interaction between Impβ and the other components. Impβ (from Xenopus laevis) consists of 876 amino acids and has a molecular mass of about 97 kDa. Its linear amino acid sequence is arranged into 19 HEAT motifs stacked in parallel. Each HEAT repeat consists of a pair of antiparallel α-helices (termed A and B).
3. Nuclear transport

Figure 3.3: NLS-containing substrates are imported via the Impα/Impβ-pathway. The imported cargo complex dissociates in the nucleus upon interaction with RanGTP. Subsequently the transport mediators are recycled in association with RanGTP. Hydrolysis of GTP (denoted by *) takes place in the cytoplasm. RanGDP is imported into the nucleus, where GDP is exchanged for GTP (nucleotide exchange denoted by Δ). Thus the RanGTP gradient across the nuclear envelope is maintained.

Figure 3.4: Schematic of the binding domains (indicated by double sided arrows) in Impβ for Impα (IBB), RanGTP, and the NPC. The binding domain for BIB-containing proteins is omitted for clarity since it partially overlaps with the site for IBB binding. Numbers indicate approximate putative positions of the corresponding amino acid residues.
The motifs are arranged such that the A-helices form the outer (convex face) and the B-helices are on the inner (concave) face of the protein, giving it a croissant-like conformation.

Its three dimensional structure strongly determines the manner in which Impβ associates with other molecules. The IBB-recognition site, i.e. the site where Impα is bound, is located on the concave face of Impβ and encompasses the B-helices of HEAT repeats 7 to 19 [68]. The concave (inner) face of Impβ expresses acidic residues which are complementary to the basic residue stretches of the IBB domain. In terms of the amino acid sequence residues 331 through 876 contain the binding sites for IBB (see Fig. 3.4). Carboxyl-terminal (C-terminal) truncations of Impβ thus functionally abolish Impα binding [27]. Impβ bound to the IBB domain assumes a rather globular appearance with a diameter of about 8.5 nm.

Impβ also sustains other pathways for nuclear import. For example, cargo such as various ribosomal proteins bind directly to Impβ without the need of an adaptor molecule [69]. Ribosomal proteins possess a BIB-domain (Beta-like Import receptor Binding domain) which can be regarded as a more complex nuclear import sequence. The binding site on Impβ for the BIB domain is located at residues 286 through 462 (Fig. 3.4).

### 3.5.2 Interactions between Impβ and the NPC

The complex Impβ-(IBB)-Impα-(NLS)-cargo (or Impβ-(BIB)-cargo) is translocated through the NPC as an unit. However, it is only Impβ which interacts with the nucleoporins and thus mediates passage. Impβ and other transport receptors are thought to specifically interact with nucleoporins containing FxFG-repeats (see sections 3.2 and 3.6). These nucleoporins are found on the cytoplasmic filaments, the nuclear basket, as well as in the central pore itself. The hydrophobic FG-sequences are interspersed by hydrophilic linkers of a few tens of residues in length.

Although several sites distributed along the outer (convex) face of Impβ are involved in nucleoporin binding, residues 1 to 618 of Impβ are necessary for full binding affinity [27] (Fig. 3.4). However, appreciable NPC binding can also be obtained for C-terminal truncations down to residue 364 (e.g. Impβ as in 1-364), as well as short N- (amino-) terminal truncations (e.g. Impβ 45-876). Crystallographic data indicate that the interactions between Impβ and nucleoporins mainly involve hydrophobic interactions on behalf of both binding partners. More specifically, phenylalanine residues (F) of the FxFG-sequence strongly interact with hydrophobic pockets on the convex face of Impβ [28]. One such pocket contains the isoleucine (I) residue at position 178 (I178). By replacing the nonpolar isoleucine for a negatively charged aspartic acid (D) residue (e.g. as in the Impβ 1-876 I178D mutant), the binding affinity between Impβ and the NPC drastically decreases. This is expected since the newly introduced charge makes the site less hydrophobic.

Since Impβ simultaneously binds to FG-repeats and cargo these binding sites
are inferred to be structurally separated (Fig. 3.4). This is accomplished because nucleoporin and IBB binding occur on the convex and concave face of Impβ, respectively, even though there is an overlap of the residue sequences containing the binding sites (1-618 and 331-876, respectively, Fig. 3.4).

3.5.3 Release of cargo and recycling of transport receptors

Impβ has a very high affinity for GTP-bound Ran. The Ran binding domain of Impβ is contained within the first 364 residues (1-364) (Fig. 3.4)[27]. The affinity for RanGTP is drastically reduced if only a few residues at the N-terminal end of Impβ are deleted, and is completely lost for Impβ fragments which don’t have the first 44 residues (e.g. as in Impβ 45-876).

RanGTP dissociates Impβ from Impα [70], and in this way terminates translocation of the import cargo complex and delivers the cargo into the nucleus. Crystallographic data show that the Ran binding site on Impβ involves the B-helices of HEAT repeats 1 to 3 and 7 to 8, and is thus located on the acidic concave (inner) face of Impβ [71]. Thus the RanGTP binding domain only slightly overlaps with the IBB (Impα) binding domain in terms of the amino acid sequence (Fig. 3.4). The dissociation of Impβ and Impα produced by RanGTP therefore seems to be related to a conformational change in Impβ rather than competition for the same binding site (see Fig. 3.2).

N-terminally truncated Impβ fragments without the first 44 amino acids (e.g. Impβ 45-876) are unable to dissociate from one or several sites at the NPC. This indicates that binding of RanGTP is needed to release Impβ from nucleoporins located near the nucleoplasm (i.e. the nuclear basket) which seem to act as the terminal binding sites during translocation. In addition, N-terminally truncated proteins are dominant-negative blockers of nucleo-cytoplasmic transport [27], meaning that transport via the Impβ pathway and at least several other import and export pathways are inhibited if the NPCs are exposed to these constructs. This indicates that the aforementioned irreversible binding of the truncated Impβ mutants is located at sites which are also shared by other import and export receptors shuttling through the pore.

After release of cargo, Impα and Impβ are reexported to the cytoplasm (Fig. 3.3). Impβ is exported directly while bound to RanGTP. Since RanGTP is able to dissociate Impβ from several sites on the NPC, this might indicate that the export pathway for Impβ differs from its import pathway. Once in the cytoplasm, RanGTP is dissociated from Impβ and GTP is hydrolysed with the aid of RanGAP1 (section 3.4.1). Impα, on the other hand, exits the nucleus via the exportin CAS pathway (Fig. 3.3)[26]. CAS binds preferentially to Impα which is dissociated from NLS-cargo, preventing reexport of the cargo. As with other export pathways, CAS binds its (export) cargo only if associated with RanGTP, and this trimeric complex is exported as an unit. In the cytoplasm the complex is dissociated and GTP is
3.6. Molecular mechanism of translocation

hydrolyzed. Therefore, once recycled to the cytoplasm Impα and Impβ are again ready to convey a new round of import.

3.5.4 Summary

In conclusion, the import of a substrate via the Impα/Impβ pathway involves hydrolysis of two RanGTP molecules [72] that are exported from the nucleus when Impα and Impβ are recycled for a new round of transport. Here, concatenated processes such as the recycling of NTF2 and CAS are not considered. Assuming that the change in free energy provided by the hydrolysis of GTP is approximately equal to that of ATP under standard conditions and concentrations (25°C, pH 7.0, all concentrations are 1 M), the above reaction would yield \( \Delta G^0 \approx -14 \text{ kcal/mol} \). This corresponds to an energy consumption of about \( 24 k_BT \) (\( T = 298 \text{ K} \)) per substrate. Assuming that the efficiency of the transport process is about 30%, the hydrolysis of two GTP molecules should provide sufficient energy to overcome the difference in chemical potential associated with a substrate which is 1000 times more concentrated inside the nucleus than in the cytoplasm \( \Delta \mu = k_BT \ln \frac{[s]_N}{[s]_C} \approx 7 k_BT \), where \([s]_N\) and \([s]_C\) are the substrate concentrations in the nucleus and the cytoplasm, respectively). This analysis is to demonstrate why signal-mediated translocation is also known as facilitated diffusion. However, in this context concentration gradients of RanGTP and other mediators are neglected.

3.6 Molecular mechanism of translocation

This section is to illustrate how translocation properties may be connected with molecular interactions in the NPC. The NPCs efficiently exclude non-targeted (inert) substrates even against a large concentration gradient. Molecular models for translocation have to account for the observed selectivity, the direction of transit associated with a given transport receptor, the rate at which cargo is transported, and that single import events can occur in the absence of GTP. [32, 63, 64, 73, 74, 75]

Several lines of evidence indicate that translocation of transport receptors through the NPC involves interactions with FG-repeats (section 3.2): immunogold localization studies show that FG-containing nucleoporins line the entire transport pathway from the cytoplasmic filaments, through the central pore, to the nuclear basket [63, 31, 29]; many of these nucleoporins have been shown to bind directly to transport receptors [28, 76, 75]; binding of receptors and FG-repeats has been elucidated at the molecular level using X-ray crystallography [28, 77].

Moreover, transport through the NPC is modified by treatments which directly or indirectly affect the FG-repeats. WGA is a carbohydrate-binding protein (lectin) which binds and crosslinks specifically O-linked N-acetylglucosamines (sugars) on hydrophilic protein stretches located between FG-repeats. Signal-mediated nuclear
transport is inhibited by exposing the NPCs to WGA [30], by depleting WGA-binding nucleoporins [78], or by antibodies targeted specifically to FG-repeats [79].

Single translocation events through the NPC can occur also in the absence of Ran or nucleotide hydrolysis [67]. Furthermore, translocation rates through the NPC on the order of $10^3$ per second have been observed by estimating the influx of fluorescently labeled transportin (an import receptor which is also called importin β) present in high concentrations in the cytosol [64]. Translocation rates for smaller concentration gradients (1 μM) across the nuclear envelope turned out to be only a factor of 6 smaller compared to the diffusive flux through a buffer filled channel 40 nm in diameter and 40 nm in length. This indicates that selective transport through the NPC may not be appreciably slower than passive diffusion.

Several models try to explain these observations. According to the Brownian affinity gate model [31, 74] FG-containing flexible filamentous proteins on both sides of the NPC could, on the one hand, provide high affinity binding sites for transport receptors, while at the same time preventing the access of inert macromolecules disturbing the energetically favored arrangement of the interacting filaments (entropic exclusion of unwanted molecules). In addition, the Brownian motion of the filaments may be involved in concentrating the transport complexes at the entrance to the central pore.

A similar model focuses on the FG-containing nucleoporins located in the central channel. Mutually interacting FG-repeats may create a foam of network or gel which could provide an energetically favorable phase for molecules with the appropriate surface chemistry. While solvated molecules generally exhibit a rather hydrophilic surface, it is known that transport receptors such as Impβ have a partially hydrophobic surface allowing them to interact with FG-repeats, i.e. with the NPC, via hydrophobic interactions (section 3.5.2,[28]). The selective phase model [64, 32] thus suggests that the hydrophobic environment created by mutually interacting FG-repeats acts as a permeability barrier for normal hydrophilic molecules, while transport receptors are allowed to partition into this hydrophobic phase. Even though the transport receptors cannot be expected to wrap around the entire cargo molecule as to veil its hydrophilic surface, it is speculated that the entropic penalty associated with introducing cargo into the hydrophobic phase is more than compensated for by the gain in free energy provided by the receptor-nucleoporin interaction.

Within the context of these models the concept of a highly selective transport process does not seem to contradict the demand for high translocation rates. Note that the selective phase model does not impose any preferential direction for transport. The idea that the admission of transport receptors to the NPC is energetically favorable explains why receptors can translocate even in the absence of exogenous input of energy (GTP hydrolysis) or Ran (section 3.4.2) [67].

Many import receptors efficiently bind RanGTP and are thus dissociated from the cargo molecule. This may also indicate that by interaction with RanGTP the import receptors are released from the energetically favored environment in the
NPCs. Moreover, rather than being coupled directly to the transport mediators, energy is in a first stage consumed by processes maintaining the RanGTP gradient across the nuclear envelope (section 3.4.2).

As discussed before, the RanGTP gradient also establishes compartmental identity and in doing so imposes directed transport of cargo molecules. There may also be a need for internal asymmetries in the NPC structure to provide directed or rectified transport of certain mediators (section 3.4). Indeed, it has been suggested that different FG-containing nucleoporins located along the translocation pathway may provide docking sites of increasing affinity (the affinity gradient model) [75]. Extensive localization studies in yeast NPCs, however, indicate that the distribution of nucleoporins is rather symmetric with respect to the midplane of the nuclear envelope, and that only few nucleoporins are exclusively located on either the nucleoplasmic or cytoplasmic side of the NPC [31]. Furthermore, higher affinity interactions at more distal parts of the NPC also signify longer interaction periods. Indeed, estimations of off-rates [75] seem to be incompatible with the experimentally observed transport rates.

During translocation through the NPC cargo molecules are transported over a distance of approximately 70 nm. In the current models of translocation the components of the NPC are not assumed to perform any active, energy consuming mechanical displacement of this magnitude. An exception to this may be the opening and closing movements observed in the nuclear basket [22]. Therefore, a mechanical ratcheting model of translocation may not be at the onset feasible.

In conclusion, current models describing the molecular basis of translocation properties assume that hydrophobic FG-containing nucleoporins provide the physical and chemical environment appropriate for spontaneous, selective translocation. Even though very specific interactions between transport receptors and FG-nucleoporins occur, their mutual affinity seems to be weak enough to account for the high translocation rates measured experimentally. Taking into consideration that hydrophobic FG-repeats are interspersed by polar and charged residues stretches, the central pore region seems to provide a rather complicated environment with partially hydrophobic as well as hydrophilic characteristics.
Chapter 4

SFM on nuclear envelopes

4.1 Experimental methods

4.1.1 Preparation of nuclei from Xenopus oocytes

Oocytes (immature, developing, unfertilized eggs) of Xenopus laevis are desirable experimental systems for several reasons. Primarily, oocytes represent intact, functioning cells that are easily manipulated under low power optics (Fig. 4.1). Furthermore, oocytes are able to live autonomously for several days in standard buffer solutions without the need for an elaborate cell culture setup. Finally, oocytes are biosynthetically active and possess the biosynthetic machinery to be used in the later development of the embryo. This machinery can be used to the experimenter’s advantage stimulate production of a desired protein by insertion of foreign DNA into the nucleus. The elevated biosynthetic rate of oocytes is also a fortuitous advantage to the investigator studying NPCs since they are present at very high densities (50/μm², Fig. 4.2).

In preparation for experiments oocytes are surgically removed from the ovaries of female frogs. It can thus be ensured that most of them are not undergoing meiosis, i.e. the oocytes contain a nucleus. Oocytes can be stored for several days at 18°C in Modified Barth’s Solutions (MBS, see Appendix B for details).

To extract the nucleus, oocytes are opened along the equator of the cell (defined by the separation of the animal and the vegetal pole, Fig. 4.1) using sharp forceps or needles. The nucleus is located near the animal pole and is surrounded mostly by yolk composed of lipids and proteins. Shortly after coming into contact with extracellular buffer, the yolk starts to disintegrate and the clean nucleus is expelled. This process may be accelerated by the manual removal of yolk. The nucleus is then immediately transferred into Mock Intracellular Buffer (MIB), or, as is often the case for experiments utilizing Impβ proteins, into Low Salt Buffer (LSB).

Nuclei are now ready for incubation in experimental conditions (section 5.1.1) and/or deposition onto a solid substrate. The latter is necessary to obtain a me-
4.1. Experimental methods

Figure 4.1: a) The animal pole of a *Xenopus laevis* oocyte (right) is pigmented and thus appears dark, while the vegetal pole is unpigmented and bright. The diameter of the oocyte is about 1 mm. To the left a nucleus is shown that has been removed from a different oocyte (diameter 0.3 mm). b) Picture obtained with the optical microscope of a BioScope AFM showing parts of an intact nucleus deposited on a plastic petri dish, and a triangular silicon nitride SFM cantilever engaged in scanning operation.

Mechanically stable surface for SFM investigations (Fig. 4.1).

4.1.2 Fixation

It is often necessary to chemically fix biological specimens, particularly native membranes or large biomolecular compounds, for high resolution SFM imaging. Fixation is a well known method for immobilizing, stabilizing and preserving biological samples for optical and electron microscopy. Current methods involve treatment with reactive aldehydes, particularly formaldehyde and glutaraldehyde, which form covalent bonds with the free amino groups of proteins and thereby cross-link adjacent proteins. The structure of proteins is generally not significantly distorted by fixation. In fact, in response to light fixation some enzymatic activity may even persist. In addition, biochemical reactions can be stopped within a few seconds after introduction of the fixatives. Thus reversible or transient interactions between proteins can be effectively frozen in time. This allowed the accurate control of the time interval nuclear envelopes were exposed to experimental conditions.

Nuclei were fixed by adding formaldehyde and glutaraldehyde to the experimental buffer solution to a final concentration of 2% and 1%, respectively. Specimens were typically fixed for a few hours to overnight. In this way, highly reproducible results from virtually 100% of the preparations could be obtained (but not to forget the importance of technical expertise, of course).
4. SFM on nuclear envelopes

4.1.3 Sample preparation for SFM

Specimens need to be well adhered to the supporting substrate for SFM investigation (Fig. 4.1). Floating membranes or particles would not only move with the SFM tip, but eventually contaminate it and prevent high resolution imaging. To this end the experimental conditions have to be optimized in several respects.

First, the surface chemistry of the substrate has to be carefully chosen as to provide a large adhesion force attracting the sample. Nuclear envelopes were found to adhere better to plastic petri dishes than to glass coverslips. This effect is attributed to the presence of greater surface charges (and dipoles) residing on the plastic. Positive surface charges can be introduced by coating the substrate with poly-L-lysine solution for some ten minutes before deposition of the specimen. Clearly, contamination of the surface drastically reduces the affinity between sample and substrate. It is thus important that intermediate steps in the preparation are not to take place in the region where the sample is to be deposited later.

Secondly, electrostatic forces play a dominant role in structure and function of biological systems. Sample-substrate adhesion, the interaction between the SFM tip and the sample, as well as the integrity of the sample itself are strongly influenced by the ionic activity of the surrounding medium [80, 81]. Unfortunately, often the requirements for the functional and structural integrity of the sample are diametrically opposed to those needed for stable measurements. An example illustrating this dilemma is presented in Appendix D. In experiments involving nuclear envelopes chemical fixation helped to relieve most of the requirements concerning ionic activity. Envelopes fixed in LSB could be imaged rather well in MIB, LSB or water. They adhered rather well to the substrate even if several membrane layers were folded on top of each other (e.g. as for a flattened out intact nucleus). At low ionic concentrations the Debye length describing the range of repulsive forces between two charged surfaces decays very slowly as a function of distance (section 2.1.2). During imaging the sensor operating at a constant tip-sample interaction force would therefore be positioned at a larger distance from the sample than in the case of high salt concentrations. On one hand, the advantage of low ionic concentrations is that tip-sample interactions are minimized and that the tip is less likely to pick up contaminations from the sample. On the other hand, lateral resolution is in principle decreased because of the larger tip-sample distance. However, the latter effect is expected to be negligible for samples such as nuclear envelopes because the typical lateral and vertical dimensions of surface structures are much larger than the Debye length.

Third, the technique used to deposit the sample has to be adjusted to the experimental conditions. As outlined below the capillary forces of a receding drop of buffer solution can be used to bring the sample in close contact to the substrate. Complete drying of the sample is undesirable and often avoidable for stable deposition of the specimen on the support. In any case, after rehydration NPCs imaged with SFM exhibit the expected morphological dimensions even if the liquid was removed for
short periods during preparation. This finding applies to fixed as well as unfixed nuclear envelopes. In contrast, proteins denature and collapse in specimens which are dehydrated for long periods. This is illustrated in Fig. A.3 for a nuclear envelope (cytoplasmic side) that was imaged in air using SFM. The height of the NPCs is reduced by a factor of 2-3 compared to hydrated samples.

The procedure for deposition of nuclei on a solid substrate depends on the surfaces to be imaged and whether the specimen is fixed or unfixed. Fixed envelopes are more amenable to handling since they are more robust. In order to image the cytoplasmic (outer) nuclear membrane the nucleus is allowed to settle down onto the substrate in a drop of buffer solution. The nucleus is then flattened out by slowly and carefully removing the buffer solution by the siphoning action of a cotton string. In this manner the receding liquid meniscus draws the nuclear envelope outward onto the substrate. To expose the nucleoplasmic (inner) surface of the nuclear envelope the nucleus, once attached to the substrate, is opened and the envelope stretched out using sharp forceps. The trick here is to get the envelope sticking to the substrate and not to the tweezer tips.

The inner membrane of unfixed samples is similarly prepared. To be able to image the cytoplasmic surface, however, the unfixed nucleus has to be first opened and the chromatin removed, otherwise the membrane would not adhere strongly enough to the support. After removal of the chromatin the nuclear envelope patch has to be carefully deposited onto the substrate such as not to confuse its orientation.

4.2 SFM investigation of nuclear envelopes

Imaging of nuclear envelope samples was performed with a BioScope AFM (Digital Instruments, Veeco Metrology Group, Santa Barbara, CA) using silicon nitride cantilevers (spring constant 0.16 N/m) with oxide sharpened tips (Olympus Optical Co., Ltd., Japan). Scanning was conducted in buffer solutions or water using tapping mode at a tip oscillation frequency of typically 8 kHz (section 2.2.4). Scan speed was adjusted to about 2 μm/s. Best results were obtained when restricting scanning to flat and optically clean regions of the envelope such as at the periphery of the intact nucleus.

4.2.1 Cytoplasmic membrane

The predominant structures of the outer surface of the nuclear envelope are the Nuclear Pore Complexes (NPCs). With a density of about 50/μm² they occupy most of the surface area (Fig. 4.2), while the outer lipid bilayer of the nuclear envelope is discernible between the pores. Using SFM the octagonal structural symmetry of the NPCs can be resolved (Fig. 4.3). The cytoplasmic filaments often observed in cross sectional electron micrographs (where the entire cell is fixed and stained) are either
too flexible to be imaged with SFM, or have altogether collapsed onto the envelope during sample preparation.

Images of the outer nuclear membrane often show remarkably clean surfaces of micrometer dimensions. Potentially, artifacts or contaminants might arise from disrupted membranes, attached endoplasmatic reticulum (ER), yolk, or cellular proteins. Unfixed samples often exhibit loosely bound particles or damaged membranes (see appendix A).

Figure 4.2: a) Large range SFM image of the cytoplasmic face of a fixed nuclear envelope. b) Image of an unfixed nuclear envelope. No obvious structural change is introduced by chemical fixation of the envelopes. Scale bars represent 500 nm in both cases.

Figure 4.3: Contour plot of a fixed NPC demonstrating the octagonal symmetry of the protein body (scale bar 50 nm). Two subdomains at the lower left of the protein body are hidden underneath material of unknown origin, and thus appear to condense into one big structure. Contamination with foreign material is commonly observed with preparations of native membranes.
4.2.2 Nucleoplasmic membrane

It proved considerably more difficult to routinely obtain high quality images of the nuclear face of NPCs, the nuclear baskets. The reason is that the inner nuclear membrane is supported by a cytoskeletal meshwork of filamentous proteins known as the nuclear lamina. This meshwork interconnects the NPCs with the nuclear membrane, and is thought to interact with chromatin as well. Occasionally, it was possible to image the nuclear lamina in preparations of unfixed nuclear envelopes (Fig. A.2 in appendix A). Oddly, while chemical fixation generally improves the resolution of macromolecular structures, it may prevent the lamina and chromatin from detaching from the nuclear envelope during sample preparation. Indeed, in most of the experiments on fixed inner membranes it was not possible to unambiguously identify the nuclear baskets. However, NPCs have been observed on unfixed samples (Fig. 4.4).

Figure 4.4: Images of the nucleoplasmic face of an unfixed nuclear envelope (scale bars 200 nm (left) and 100 nm (middle), respectively). On the right a radial profile across a single NPC is shown. Diameter $d$ and depth of the depression $\Delta z$ are measured as indicated. It cannot be ruled out that the observed ring-like structures correspond to the main bodies of the NPCs rather than the nuclear baskets.
Chapter 5

NPCs exposed to transport modifying agents

In this chapter, SFM analyses of changes in NPC morphology induced by various endogenous and exogenous agents are presented. Our motivation was to determine whether structural changes can be correlated with modifications of nuclear transport properties previously observed in biochemical studies. On one hand, we will be concerned with mutant constructs of the importin β transport receptor. Modifications in NPC topology can provide insight as to the properties of the transport receptors themselves and the nature of their interactions with the NPC. On the other hand, structural properties of the NPCs are characterized in response to various exogenous agents which are known to influence the NPC’s transport capacity.

Figure 5.1 gives a summary of the variety of NPC topological conformations that can be discerned using SFM, and which are discussed in detail in the following sections. Experimental conditions which have previously been characterized in functional terms are observed to be correlated with distinct structural changes. This indicates how the physical characterization of nuclear envelopes can contribute to the understanding of molecular processes occurring at the level of single NPCs.

Functional and structural observations associated with the exposure of nuclear envelopes to transport modifying agents are summarized in Fig. 5.2. Apparently, modified translocation and interaction properties can be directly related to changes in lateral and vertical aspect of the NPCs. These results and interpretations are discussed in detail in the following sections.

This chapter is organized as follows: after an introduction to experimental methods SFM measurements are presented whereby the discussion is guided according to whether nuclear transport is preserved, inhibited, or enhanced above normal levels under the corresponding experimental conditions. The chapter is concluded with a speculative explanation of the molecular basis of topological changes induced in the NPCs which might be relevant also for the understanding of functional transport properties.
5.1 Experiments

5.1.1 Experimental methods

Nuclear envelopes were prepared as described in section 4.1. Immediately after dissection of the oocytes, the nuclei were transferred to low salt buffer. This ensured that no endogenous substances (transport receptors, cargo, other cytosolic factors) that could potentially complicate the analysis were present. Subsequently, nuclei were exposed to the appropriate experimental conditions for two minutes (unless stated otherwise) at 4 °C.

To this end, nuclei were incubated with Impβ constructs at a concentration of 1 μM, alcohols at 2% by weight, WGA at 10 mM, or EGTA at 10 mM in low salt buffer. Subsequently, nuclei were fixed in 2% formaldehyde and 1% glutaraldehyde. Throughout the preparation, incubation volumes were kept small (< 100 μl in small transparent test tubes) thus allowing economical use of proteins and other chemicals, accurate control of the final concentrations, fast exchange of solutions, and the fast introduction of fixatives.

Impβ and mutant Impβ constructs were generated, expressed and purified in the...
5. NPCs exposed to transport modifying agents

As discussed in section 3.6 binding of Impβ to the NPC is independent of Ran or energy. Therefore, even though in our experiments nuclei are incubated in the absence of Ran, ATP, GTP and other cytosolic factors, interactions of Impβ with the NPC should not be affected. However, continuous transport through the pores is clearly not sustained.

Following deposition onto a solid substrate, the cytoplasmic side of the nuclear envelopes was imaged with SFM in water or LSB.

5.1.2 Data and statistical analysis

The dimensions of single NPCs were analyzed using radial cross sectional profiles which connect opposite maxima on the pore rim (see e.g. Fig. 5.4). The lateral distance between these two points corresponds to the diameter \( d \) of the pore. The radial profile cuts through the central pore region which either forms a depression as in Fig. 5.4 or may exhibit a bulge rising above the pore rim as in Fig. 5.9 (lower). A third point can thus be defined which characterizes the central pore region. In the cases of a depression or a bulge these correspond to the minimum and maximum of the central pore region, respectively. The vertical difference between this third point and the average of the maxima on the rim is denoted as \( \Delta z \). By convention, \( \Delta z < 0 \) corresponds to a pore exhibiting a depression, and a bulge yields \( \Delta z > 0 \).

Radial profiles are generally taken along the fast scan direction of the SFM in order to minimize artifacts induced by thermal drift. Diameter \( d \) and pore characteristic \( \Delta z \) are subsequently determined using a semi-automated process programmed in Matlab. Where applicable (Figs. 5.11, 5.16, 5.19) the mean values of \( d \) and \( \Delta z \) are represented by symbols. Error bars are determined by the standard error of the mean (SEM).

Where applicable, statistical tests have been performed using the two-sided Student’s-t distribution. Numerically determined probabilities describing whether two sets of measurements are statistically independent are given in appendix C for selected experimental conditions. Here statistical sample sizes (number of profiles) and numerical values for mean diameter and depth measurements also may be found for each of the experimental conditions described below.

5.2 Conditions supporting transport

This section is mainly concerned with Impβ constructs which in their capacity support nuclear import of cargo molecules to either a full or a reduced extent. Common to all constructs presented in this section is that they fully translocate through the NPC. The Impβ 45-462 mutant, a dominant-negative blocker of nuclear transport, is discussed in section 5.3.1. Table 5.3 gives an summary of the binding properties of the Impβ constructs used in experiments.
Figure 5.2: Summary of experimental observations associated with the exposure of NPCs to Impβ mutants, amphipathic alcohols, WGA, and EGTA. The first panel describes whether the indicated Impβ mutants are able to translocate through the NPC and how alcohols, WGA and EGTA interfere with the translocation process (data compiled from [27,32,77,81,83]). The size of the bars is to indicate how strongly translocation is enhanced and inhibited, respectively, with control describing normal translocation properties. Binding affinities to the NPC (second panel) for Impβ mutants are compiled from [27,81]. The molecular interactions between alcohols and NPCs have not yet been characterized experimentally. However, the interaction is expected to be rather weak. The volume of a protein (third panel) may be determined from its molecular mass (a typical globular protein with 300 amino acids has a mass of about 33kDa and a diameter of about 4.3nm [58]). Alcohols and EGTA have a comparably small mass. The fourth and fifth panels summarize SFM measurements of the vertical (pore depth) and lateral (diameter) dimensions of the NPCs. These measurements are described in detail in the following sections. Here, depth and diameter values are plotted as deviations from control values (experimental condition minus control).
5. NPCs exposed to transport modifying agents

\[\begin{array}{cccc}
\text{RanGTP} & \text{BIB} & \text{IBB (imp} & \text{NPC}
\hline
1-876 & + & + & +
1-462 & + & + & - & +
1-409 & + & - & - & +
45-462 & - & + & - & +
1-876 & + & + & + & -
l178 D & + & + & + & -
45-462 & - & + & - & -
l178 D & - & + & - & -
\end{array}\]

Figure 5.3: Table illustrating the properties of various genetically altered Impβ constructs used in experiments (adopted from [27]). "+" and "-" indicate supported and reduced interactions, respectively, with the indicated proteins or protein domains.

5.2.1 Controls

Control experiments designed to rule out nonspecific effects introduced during sample preparation and scanning generally accompanied our different experimental conditions. Our controls thus represent nuclei from the same batch of oocytes prepared in the same manner as in the experimental conditions, except that the experimental incubation is omitted and replaced by incubation in standard buffer for an identical period of time. The cytoplasmic side of control envelopes imaged by SFM exhibits NPCs with a clearly visible open channel in the center of the pore. The octagonal symmetry of the pore protein walls is often clearly resolved (Fig. 5.4, see also Fig. 4.3).

For controls, the mean NPC diameter measured from opposite maxima of the pore rim is \(d = 84.9 \pm 0.5\) nm. The vertical difference between the top of the pore rim and the minimum at the central channel is \(\Delta z = -21.1 \pm 0.4\) nm on average (see Fig. 5.4 for details). These values are in good agreement with previously reported experiments on untreated nuclear envelopes [23, 14]. The average depth of control pores is thus clearly much shallower than would be expected if resolution were limited by the finite radius and aspect ratio of the tip (Fig. 2.12). On one hand this confirms that SFM is a powerful method to resolve macromolecular structures located even deep in the pore. On the other hand, it remains unclear how to distinguish surface artifacts introduced during sample preparation (contamination, membrane folds, sample tilt) from authentic NPC structure. Thus, simultaneously prepared control samples provide the only means to discern the contribution of experimental conditions on NPC topology.
5.2. Conditions supporting transport

Figure 5.4: Images of control samples: large range image (left, scale bar 200 nm), three dimensional representation of a single NPC (middle), and a radial profile across the same NPC illustrating pore depth ($\Delta z$) and diameter ($d$) measurements (right). The same conventions also apply to the images obtained with other experimental conditions.

5.2.2 Imp$\beta$ 1-876 (full-length)

Imp$\beta$ binds to the NPC via specific interactions (section 3.5.2). The corresponding binding sites are distributed along the translocation path through the NPC. Translocation of import receptor molecules have been estimated to occur at rates on the order of $10^3 s^{-1}$ (section 3.6). In order to sustain translocation rates of this magnitude the binding between transport receptors and nucleoporins has to be transient.

Samples treated with full-length Imp$\beta$ exhibit NPCs of control-like characteristics. The central pore as well as the NPC structure are clearly visible. However, additional material can be resolved which presumably corresponds to the binding of Imp$\beta$ to the surface and central channel region of the NPC (Fig. 5.5).

The mean depth of the pore channel is decreased by approximately 5 nm or 25%, i.e. the pore is shallower ($\Delta z = -15.0 \pm 1.1$ nm, Fig. 5.8) relative to controls, while the diameter is unchanged ($d = 85.0 \pm 1.1$ nm). This is taken to indicate that on average many pores are occupied by transport receptors. Although no additional Imp$\alpha$ or cargo was supplied during sample preparation, small amounts of endogenous adaptor or cargo molecules might still be present, allowing a small fraction of the full-length Imp$\beta$ to be complexed with Imp$\alpha$ and cargo. Binding of these complexes to the NPC could therefore also contribute to the observed accumulation of material on the NPC and the significant decrease in pore channel depth.

5.2.3 Imp$\beta$ 1-462

In this mutant, 414 amino acids of the carboxyl or C-terminus have been truncated from the wild-type molecule. Thus, in contrast to full-length Imp$\beta$, this construct is
5. NPCs exposed to transport modifying agents

Figure 5.5: Binding of Impβ 1-876 (full-length) to the NPCs.

not able to form a complex with adaptor molecules exhibiting the IBB-domain, and import of NLS-carrying cargo is not supported (section 3.5.1). However, this construct still retains binding to the NPC, although binding affinity is slightly reduced (section 3.5.2). Indeed, on average the pore appears deeper ($\Delta z = -18.9 \pm 1.3 \text{ nm}$) compared to full-length Impβ. The structure of the pore walls, e.g. pore diameter, is unaffected ($d = 83.2 \pm 1.5 \text{ nm}$, Fig. 5.8).

5.2.4 Impβ 1-409

While pore diameter is also unchanged by this short fragment ($d = 85.0 \pm 0.9 \text{ nm}$), the minimum at the location of the central channel is deeper ($\Delta z = -27.3 \pm 1.2 \text{ nm}$) than with mutants retaining more of their carboxyl terminus (Fig. 5.8). This can be tentatively explained by an even lesser NPC binding affinity, translating into a shorter lived NPC-mutant interaction (cf. 3.5.2). Additionally, the large C-terminal truncation eliminates interactions not only with Impα, but also with substrates/cargo which have a BIB-domain (e.g. ribosomal proteins). These cargo molecules can bind to Impβ-like transport receptors directly, without the need of an adaptor molecule like Impα (cf. 3.5.1). For the 1-409 fragment the pores appear even deeper than in control samples. This might indicate that binding at the NPC of endogenous transport receptors and cargo (which are possibly still present in small amounts in our preparation) is competed by binding of the 1-409 fragment. In any case, a deeper pore i.e. less material inside the channel is expected for the 1-409 fragment because it is unable to interact with cargo, has a smaller molecular volume, and has a decreased binding affinity to the NPC.

5.2.5 Impβ 45-462 I178D

This construct is deficient in Ran- as well as Impα binding. In addition, the nonpolar isoleucine (I) side chain at position 178 is replaced by an aspartic acid (D) residue.
5.2. Conditions supporting transport

Figure 5.6: Binding of Impβ 45-462 I178D to the NPC is weak, but dilates pore diameter.

This mutation is known to strongly diminish the affinity between Impβ and the NPC (a more detailed discussion of the Impβ -NPC interaction is presented in section 3.5.2). Indeed, the single point-mutation in this fragment yields a completely different pore morphology: the central channel is not occluded, and exhibits a depth comparable to control NPCs ($\Delta z = -22.4 \pm 1.2$ nm, Fig. 5.19). This finding clearly demonstrates the specificity of the accumulation effect observed with the unmutated 45-462 protein (section 5.3.1).

Importantly, the I178D mutant is not a dominant-negative blocker of signal-mediated transport even though its affinity for Ran is completely abolished. It is able to fully translocate through the NPC, and does not bind irreversibly to putative terminal sites of translocation [82]. A reduction in binding affinity thus dominates the properties of this mutant despite having its Ran-binding domain ablated.

Even though pore depth is comparable to control values, the mean NPC diameter is dilated ($d = 90.8 \pm 1.1$ nm, Fig. 5.19) compared to controls and experiments with other Impβ mutants. The increase in diameter by about 7% is statistically significant (see appendix C). We attribute this effect to the rearrangement of nucleoporins induced by the introduction of the hydrophilic I178D mutation. A more detailed discussion of this interpretation is presented in section 5.5.

5.2.6 Impβ 1-876 I178D

NPCs exposed to this isoleucine mutant are virtually indistinguishable from NPCs incubated with the truncated (45-462) construct containing the same mutation. Both mean channel depth and pore diameter measurements coincide with the values corresponding to the 45-462 I178D protein ($\Delta z = -23.4 \pm 1.1$ nm and $d = 90.1 \pm 0.9$ nm, Fig. 5.19). Specifically, the pore diameter appears widened. This is taken to indicate that the interaction site involving the amino acid residue at position 178
5. NPCs exposed to transport modifying agents

in Impβ dominates the affinity between Impβ and the nucleoporins (section 3.5.2).

Remarkably, the translocation rate of this mutant was shown to be even larger than for wild-type Impβ [82]. This is attributed to the decreased binding affinity to the NPC which seemingly enables faster diffusion through the pore. Evolution may have selected against this version of Impβ because weakened binding may go along with a reduction in the specificity of the translocation process.

5.2.7 Discussion: supported transport

This section summarizes the effects of different transport permissive Impβ fragments on NPC topology. The structural analysis of NPCs exposed to the different mutants is used to characterize the docking and translocation steps of this import pathway (Fig. 5.7). In Fig. 5.8 average pore depth and diameter are shown for NPCs exposed for 2 minutes to 1 μM solutions of the various mutants. For comparison, the values corresponding to control conditions and incubations in the dominant-negative transport blocker Impβ 45-462 are given in the same plots.

Binding strength to the NPC appears to influence central channel depth. Pore depth increases from approximately $-28\text{ nm}$ to $-15\text{ nm}$ in the sequence 1-409 < 1-876 I178D < 1-462 < 1-876. The underlying trend is in rough agreement with the binding affinity to the NPC attributed to each mutant; more tightly bound proteins produced shallower pores. Obviously, the finding that the data points lie on a straight line is just for demonstrative purposes and has to be taken with a grain of salt. For example, the isoleucine construct (Impβ 45-462 I178D) would certainly be expected to have the lowest binding strength to the NPC of all tested mutants.

Changes in central pore depth seem to correlate also with the volume of the construct. The C-terminal truncated Impβ fragments (1-409 and 1-462) are not able to chaperon NLS-containing cargo via Impα through the NPC. In addition, Impβ 1-409 is also deficient in binding cargo containing a BIB sequence (section 3.5.1). The increasing pore depth thus not only follows a trend corresponding to increasing affinity to adaptor molecules and import substrates, but also to increasing size of the transport receptor itself. Pore depth is therefore also related to the expected volume of translocated material, even in the absence of endogenous import substrates. In this respect, the seemingly inconsistent depth characteristic of the full-

![Figure 5.7: Profiles of NPCs treated with Impβ mutants which sustain translocation through the pore (except for 45-462).](image)
5.2. Conditions supporting transport

Figure 5.8: Pore depth decreases with increasing affinity of Impβ fragments to the NPC, while pore diameter is influenced mainly by the characteristic of the receptor-nucleoporin interaction. Labels on the horizontal axis correspond to the experimental conditions in Fig. 5.7. All fragments except 45-462 support transport through the NPC.

length isoleucine mutant (Impβ 1-876 I178D) can be reconciled within the context of the other mutants. Pore depth is a function of the combined effects of binding affinity and material volume.

Despite greatly influencing NPC vertical aspect, N- and C-terminal truncations have little effect on NPC diameter. The radial dimension of NPCs exposed to truncated, but otherwise unmutated Impβ fragments (thus excluding I178D) is independent of size and binding affinity of the transport receptors. However, NPC diameter is influenced by the characteristics of the interactions between nucleoporins and protein mutants. This is illustrated here for the case of Impβ 1-876 I178D, in which NPC diameter is significantly increased. A more detailed discussion of this effect will be presented in sections 5.4.2 and 5.5.
5. NPCs exposed to transport modifying agents

5.3 Blocked transport

5.3.1 Impβ 45-462

The C-terminal truncation of this Impβ fragment prevents binding of Impα and associated cargo molecules. In addition, the amino or N-terminus truncation of the first 44 amino acids completely abolishes binding to Ran and hence severely affects various properties of nuclear transport:

- Impβ is not detached from the terminal binding sites at the nuclear basket.
- The import complex is not dissociated and cargo is not released into the nucleoplasm.
- Impβ 45-462 suppresses not only the import of NLS-containing substrates via the Impo-Impβ pathway but abolishes all active transport through the NPC [27].

This Impβ fragment is thus categorized a dominant-negative inhibitor of nucleocytoplasmic transport. This remarkable finding could indicate that although different import and export pathways use different routes through the NPC, the binding of this mutant presents a major bottleneck in nuclear transport by irreversibly occupying (or occluding) binding sites on the NPC. Another interpretation might be that the NPC plays an active role in regulation of nuclear transport. In other words that the terminal binding site is “still” occupied by the Impβ mutant is communicated to other domains of the NPC preventing other transport receptors from taking up their route [27]. This could be achieved by some sort of conformational changes translated to distant parts of the NPC.

SFM analysis of nuclear envelopes may help to shed light as to the mechanism of block attributed to the 45-462 fragment. Incubation for the same amount of time as for the other mutants results in a totally different NPC morphology (e.g. Fig. 5.9 (lower)). The central depression almost completely disappears, and is replaced instead by a bulge apparently filling the pore and even rising above the rim of the NPC.

The bulge is generally located centrally above of the actual pore, and the NPC walls can be discerned as a ring-shaped shoulder framing the protruding material. Thus the diameter of the NPC is still well defined, though with increasing accumulation of material the error in the measurement becomes larger. On a scale of a few nanometers, the structure of the bulge appears rather irregular. Often a small depression or dimple could be discerned at the top of the bulge near the central axis of the pore (Fig. 5.9 (lower)).

To gain further insight as to the origin of these morphological changes we performed time series of incubation periods in the 45-462 mutant ranging from 10 seconds to 20 minutes. At short periods, the NPCs are indistinguishable from those
5.3. Blocked transport

Figure 5.9: Nuclear envelopes incubated in Impβ 45-462 for 15 s (upper) and 5 min (lower images). Material starts to accumulate inside the pore, giving rise to a protruding bulge at longer incubation times. The accumulated material is generally located centrally above the pore, leaving the pore rim clearly resolvable (see profiles). The accumulation of protein may involve binding to the cytoplasmic filaments, giving rise to a rather irregular bulge shape with a dimple near the central pore axis (red arrow).

in control samples. However, after only brief exposure (~15 s) to the mutant, the topology of the NPC is changed as is clearly observed in the profile taken across the diameter of a NPC (Fig. 5.9 (upper) and 5.10). The central depression of the pore becomes increasingly shallower with longer incubation times with the mutant, until after about one minute of exposure the pore is completely obstructed and the bulge starts to emerge above the rim of the pore. In Fig. 5.11 this time dependent change in pore morphology is displayed by showing the average depth of the central pore ($\Delta z < 0$), or the rise of the bulge above the pore rim ($\Delta z > 0$), respectively. Clearly, the average height of the material above the NPC rim saturates at exposure times longer than one minute.
5. NPCs exposed to transport modifying agents

Figure 5.10: Time evolution of binding of Impβ 45-462 to the NPC.

Figure 5.11: Pore depth ($\Delta z$) shows the time dependent accumulation of Impβ 45-462. Pore diameter ($d$) does not seem to follow any trend related to the binding of protein. The large spread in diameter data at longer intervals is attributed to the increasing measurement uncertainty associated with protein accumulation. Control values are shown on the left for comparison.
5.3. Blocked transport

The NPC diameter was found to be unaltered by exposure to the 45-462 mutant protein; mean values range from between 80 to 90 nm (Fig. 5.11). The rather large variation in the data is attributed to the increasing accumulation of protein material which sometimes renders accurate measurements difficult. However, particularly at short incubation intervals pore diameter does not deviate significantly from control values. This result might indicate that binding of 45-462 protein is presumably not accompanied by changes in NPC conformation.

In conclusion, the observed drastic change in NPC morphology is attributed to the accumulation bound Impβ 45-462, rather than conformational changes in NPC structure. The rapid increase in NPC vertical aspect might be explained by the binding sites of the NPC being quickly occupied. The rise of material centrally above the pore and the irregularly shaped bulge structure might also indicate that the mutant proteins bind strongly to the filaments protruding towards the cytoplasm. This would be an indirect confirmation that the filaments are actually still functional when the nuclei are isolated during sample preparation. Several tens or hundreds of binding sites at the NPC and the filaments may be necessary to account for the observed amount of accumulated material. Indeed, modeling the pore volume available for binding and accumulation of protein as a cylinder 40 nm in diameter and 40 nm in length, on the order of 150 empty Impβ molecules (molecular diameter 8.5 nm, section 3.5.1) may be accommodated. This is one order of magnitude lower than the estimated number of FG-repeats in the same volume (section 3.2). At longer incubation times, all binding sites seem to be saturated with Impβ mutant proteins.

Interestingly, even though this mutant is known to rapidly block receptor-mediated transport because of its inability to be released from the nuclear basket, binding to the cytoplasmic face of the NPC continues as is evidenced by the continual accumulation of bound material beyond 15 seconds. This mechanism of block therefore does not seem to interfere with the initial steps of the translocation process. This could again be taken to indicate a passive role for the NPC, namely as the supplier of docking sites relevant for transport.

The affinity of the 45-462 protein to the NPC is greatly enhanced compared to other Impβ mutants, leading to the observed accumulation. It is, however, low enough for translocation of the receptor to occur at least to the site at the nuclear basket where interaction with RanGTP should normally take place. This indicates that the observed accumulation is not simply due to irreversible binding of the 45-462 mutant to sites on the cytoplasmic face of NPC.

Furthermore, it is important to note that none of the Impβ mutants with intact N-terminus showed the distinct change in pore topology as is shown here. This effect can therefore be attributed specifically to the inability of this fragment to bind Ran.
5. NPCs exposed to transport modifying agents

5.3.2 Wheat germ agglutinin (WGA)

Lectins are a class of proteins which bind specifically to sugar moieties on the surface of proteins. WGA is one such lectin exhibiting specificity for N-acetyl-glucosamines (GlcNAc) containing glycoproteins. Many of the nucleoporins that interact with transport complexes similarly express GlcNAc on their surfaces [78]. Thus, WGA is thought to impede facilitated signal-mediated transport through the pore by binding or cross-linking nucleoporins in the central pore region, thereby denying transport receptors access to specific binding sites at the NPC. Another interpretation is that WGA interferes with the partitioning of cargo into a selective phase existing within the lumen of the NPC. Small particles are still able to diffuse passively through the pore.

The diameter of NPCs exposed to WGA is similar to the control value ($d = 84.1 \pm 1.3$ nm, Fig. 5.16). However, although still clearly visible the average depth of the central pore depression is significantly reduced by about 50% ($\Delta z = -10.4 \pm 1.2$ nm). Furthermore, some pores exhibit additional structures located in the central pore region (Fig. 5.12). On the one hand, this could indicate that WGA physically obstructs the channel by linking nucleoporins across the pore and forming an impenetrable mesh. On the other hand, some of these structures may actually correspond to cargo trapped during transit.

5.3.3 EGTA

The nuclear envelope and adjoining endoplasmatic reticulum form a subcellular compartment involved in calcium signaling. Calcium release from these compartments regulates a wide variety of cellular processes including the activation/inactivation of enzymes, the gating of ion channels and nuclear permeability.

It has been shown that intermediate size molecules ($\sim 10$ kDa) which normally
diffuse passively through the NPC, are excluded from the nucleus when Ca\(^{2+}\) is depleted from the perinuclear space [83]. On the other hand, as is the case for WGA treatment, inorganic ions and very small particles are still able to passively diffuse through the NPC. Active signal-mediated transport might not be inhibited under low Ca\(^{2+}\) conditions in certain in vivo experiments [84, 83], cf. [85]. It thus appears that calcium depletion represents a separate mechanism of transport block.

Depletion of Ca\(^{2+}\) from the perinuclear space can be achieved by complexing Ca\(^{2+}\) which continuously leaks from the perinuclear space with calcium chelators in the cytoplasm. To this end we incubated nuclear envelopes in EGTA (Ethylene glycol-bis(2-aminoethyl)-tetraacetic acid) for 10 min.

Depletion of Ca\(^{2+}\) from the perinuclear space results in a very distinct alteration of NPC morphology. Under these conditions a structure appears in the center of the pore known as the central "plug" or "translocater", which is clearly distinguishable from the accumulation of material caused by Imp/3 45-462 because of its more regular structure and clear separation from the inner circumference of the NPC (Fig. 5.13, [13, 14, 22, 23]).

The naming of this structure arose from the notion that it could either represent cargo caught in transit through the NPC [13], or an actively moving component of the NPC involved in regulation of transport through the pore [23]. The question as to the origin of the "plug" has been investigated with SFM of unfixed nuclear envelopes which were sequentially first depleted then replenished of perinuclear Ca\(^{2+}\) while concomitantly taking images of the NPCs [14]. The results showed that the protrusion of the plug towards the cytoplasmic side could be reversed by adding back free Ca\(^{2+}\) to the bathing solution. This finding clearly favors the interpretation that the "translocater" is an integral part of the NPC itself.

Our experiments examining Ca\(^{2+}\) store depletion agree with these previous results. Treating intact nuclei with EGTA resulted in greater than 95% of the NPCs
5. NPCs exposed to transport modifying agents

Figure 5.14: Scanning instabilities were observed on several EGTA treated nuclear envelopes when imaging translocator plugs (red arrows). This effect is attributed to strong attractive forces acting on the tip and may indicate that translocators exhibit a rather hydrophobic surface chemistry. Scale bar in the left image represents 100 nm.

exhibiting a "plug" that frequently protruded over the rim of the NPC (Fig. 5.13). Measurements of the vertical position on the "plug" therefore yielded mean values $\Delta z = 2.9 \pm 2.2$ nm. This effect is accompanied by an increase in pore diameter [14] ($d = 92.9 \pm 1.8$ nm, Fig. 5.16). In contrast, in control samples and in all other conditions where the central pore can be discerned clearly, the percentage of NPCs which have a similarly protruding and plug-like structure is less than 5%. In this respect the interpretation of experiments with the Imp$\beta$ 45-462 construct is more problematic since the central pore region is hidden underneath accumulated material. However, no evidence has been found in these experiments for the presence of the central "plug" structure, in particular for short incubation periods.

The surfaces of the central "translocaters" look rather unnaturally jagged and irregular in the SFM experiments presented here, in contrast to the surface of the surrounding NPC body and the nuclear envelope. (Fig. 5.14). These are characteristics normally ascribed to regions where large adhesion forces are exerted on the tip (the tips material consisted of silicon nitride). Strong attractive forces lead to cantilever instabilities and consequently to irregular height feedback if the force gradient is larger than the force constant of the cantilever (snap-in, 2.3.4). Importantly, in aqueous solutions, large adhesion forces occur mainly because of hydrophobic interactions between tip and sample (see also section 2.1.2).

Our preliminary data thus seem to indicate that the translocator is outfitted with a hydrophobic surface chemistry. It is clear that more involved experiments are needed to unambiguously resolve this issue since it is not $a$ $priori$ obvious how strongly these results are influenced by sample preparation, tip chemistry, and composition of the imaging buffer solutions. Future experiments may involve the mapping of adhesion forces with chemically modified SFM tips.
5.3.4 Discussion: blocked transport

Using SFM different mechanisms of transport block can clearly be distinguished. In Fig. 5.16 central pore characteristics and NPC diameter are plotted for nuclear envelopes incubated in WGA, Impβ 45-462, and EGTA, respectively, in comparison with control conditions. In all three cases, it is the central channel region and the corresponding height measurement which is most clearly affected by the blocking reagent (see profiles taken across typical NPC diameters, Fig. 5.15).

Experimentally, WGA blocks signal-mediated transport, but not passive diffusion of small particles through the NPC. It inhibits nuclear transport presumably by obstructing access to specific binding sites at the nucleoporins. Treatment with WGA on the whole did not seem to induce any gross changes in NPC topology. Only cross-sectional traces through the NPC revealed a significantly reduced pore depression (Fig. 5.12). On average, pore depth decreased by about 50% compared to control values. While some of the pores were possibly obstructed by trapped cargo, most NPCs exhibited a rather inconspicuous pore characteristic with a distinct but shallow pore depression. The central channel surface looked rather smooth, though the detailed structure in the pore depression might not have been accessible to the SFM tip. This finding may indicate that WGA cross-links sugar residues (GlcNAc, section 5.3.2) in the central pore region, characterized here by some kind of "solidified" phase located beneath the NPC rim and barring the pore.

Given WGA's ability to block nucleocytoplasmic transport we sought to determine if WGA could likewise preclude the topological effects imparted by the dominant negative Impβ mutant, 45-462. Nuclear envelopes first exposed to WGA and then to Impβ 45-462 look similar to samples incubated in Impβ 45-462 alone. NPC depth and diameter are also comparable to results from the Impβ fragment (Fig. 5.16). This finding does not necessarily contradict the way WGA is thought to inhibit transport through the NPC. High affinity binding of Impβ 45-462 may simply outcompete WGA binding to the NPC (e.g. the filaments), resulting in an accumulation over the pore in spite of the WGA treatment. The reduction in mean NPC diameter observed with these experiments may partially be attributed to the measurement uncertainty arising from the accumulation of protein material above the pore.

For samples exposed to only Impβ 45-462 translocation is inhibited because

![Figure 5.15: Profiles of NPCs exposed to agents which reduce or block nuclear transport.](image)
Figure 5.16: Block of transport using WGA, Impβ 45-462, and EGTA is mainly characterized by changes in pore depth. While the diameter deviation observed for the WGA+45-462 treatment is attributed to the increased measurement uncertainty associated with the accumulation of protein material above the pore, the increase in NPC diameter for EGTA treated nuclear envelopes confirms previous results [14].

Impβ 45-462 does not bind RanGTP and is thus not released from the nuclear basket. It has been shown that this mechanism of block not only inhibits import of NLS-containing cargo via the Impβ pathway, but also suppresses translocation of receptors and cargo belonging to various other pathways [27]. In our experiments, NPC morphology does not seem to change significantly apart from the accumulation of Impβ fragments. Further, binding and accumulation occurs well after active transport is supposedly blocked by the 45-462 mutant (see Fig. 5.11). This strongly indicates that translocation is inhibited because Impβ 45-462 binds irreversibly to a binding site which is shared among various pathways. Functional sites on other parts of the NPC, as well as initial translocation up to the nuclear basket, are unaffected.

Calcium is a key component of many biological processes, including nuclear transport. Transport for intermediate-size molecules which are normally translocated through the NPC is blocked by depletion of Ca^{2+} from the perinuclear stores.
Whether and how transport of NLS/NES-containing substrates is affected is subject to controversy (cf. [84, 85, 83]). Depletion of Ca\(^{2+}\) induces a drastic change in NPC morphology which is distinctly different from the modifications observed with other experimental conditions. In response to store depletion the pore seems physically plugged by the central “translocator”, an apparently integral component of the NPC, and the NPC diameter is observed to increase by some ten nanometers for plugged pores (Fig. 5.13). Taking into consideration the large errorbars due to the limited sample size, the diameter measurement correlates well with previously reported values (Wang and Clapham, \(d \approx 90\) nm (n=6) [14]).

In conclusion, three different ways of block of nuclear transport have been discerned using SFM. Block of transport is achieved by either 1) restricting access to or cross-linking nucleoporins in the central channel with WGA, 2) permanently occupying a specific binding site common to many transport pathways with Imp\(\beta\) 45-462, or 3) inducing a distinct structural change in NPC morphology by depleting perinuclear Ca\(^{2+}\) stores using EGTA. How and whether these mechanisms are related to each other remains to be answered.

### 5.4 Enhanced transport

#### 5.4.1 1,2-cyclohexanediol, 1,4-cyclohexanediol and 1,6-hexanediol

Amphipathic alcohols have been previously shown to increase nonspecific transport through NPCs [32]. We thus exposed nuclear envelopes to buffer solutions containing 2\% trans-1,2-cyclohexanediol cis/trans-1,4-cyclohexanediol or 1,6-hexanediol for various periods ranging from 5 to 15 minutes. The average NPC depth is similar to values obtained from control experiments (Fig. 5.19). The pore diameter, however, is significantly dilated (\(d = 87.9 \pm 0.6\) nm, 89.2 \(\pm 0.6\) nm, 89.1 \(\pm 0.8\) nm for 1,2-cyclohexanediol, 1,4-cyclohexanediol and 1,6-hexanediol, respectively) as was the case for the Imp\(\beta\) I178D mutant experiments. The increase in diameter compared to control values is statistically significant (see appendix C). Longer incubation times with these alcohols did not dilate the NPC further.

The possibility existed that the amphipathic alcohols damage the nuclear envelope or the NPCs and in this capacity alter nuclear transport. To test this possibility measurements were performed in which the alcohol was removed after 15 minutes and replaced with control buffer for 20 minutes. The average NPC diameter of these samples partially reverted to control values, demonstrating that the effect of the alcohols was largely reversible (Fig. 5.19).

Clearly, at this stage it cannot be excluded that the reversible pore dilation induced by alcohols is connected with modifications in the nuclear envelope rather than specifically with the NPC. Namely, relaxation of the NPC could also be associated with a reversible “dilution” of the lipid membranes or connected cytoskeletal
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Figure 5.17: Nuclear envelopes incubated with 2% 1,6-hexanediol for 5 minutes (upper) and 1,2-cyclohexanediol for 10 minutes (lower images).

networks. On the other hand, strong similarities exist between the alcohol experiments and the Impβ I178D incubations.

Though the alcohol molecules are much smaller than the Impβ I178D protein mutants, their amphiphilic character is used here to mimic the interaction between the I178D mutants and the hydrophobic regions of the nucleoporins. The results presented here are indeed in very good agreement with the Impβ I178D data.

NPCs exposed to 1,2-cyclohexanediol show strongly modified nuclear transport properties [32]. Namely, maltose binding proteins (MBP) lacking any signal sequence (NLS), and larger than the normal exclusion limit (40 kDa) are taken up into the nucleus, where they are normally excluded. The observed effect induced by the alcohols was shown to be reversible, i.e. the NPCs showed the normal transport behavior after the alcohols had been removed from the preparation. The authors attribute these findings to the breakdown of a hydrophobic permeability barrier (originating from FG-containing nucleoporins) induced by the amphipathic alcohols.

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Figure 5.18: Profiles of NPCs with enhanced transport properties. Experiments where alcohols have been removed after 15 min and replaced by control buffer are denoted by "wash". The dashed vertical lines are separated by 80 nm.

Figure 5.19: Impβ I178D mutants and amphipathic molecules increase NPC diameter (circles). These conditions are known to enhance transport through the NPC. Alcohol treatment does not seem to irreversibly affect or damage the NPC structure since the increase in diameter is (partially) reversed after washing out of the alcohols (triangles).
Figure 5.20: Barplots showing the relative occurrences of depth ($\Delta z$) and diameter ($d$) measurements. Solid black line is the corresponding Gaussian. Red dashed lines describe the mean value positions from control experiments to allow comparison with other conditions.

5.4.2 Discussion: enhanced transport

Nuclear transport is enhanced by exposing nuclear envelopes to alcohols (1,2-cyclohexanediol, 1,4-cyclohexanediol and 1,6-hexanediol) and Imp$\beta$ isoleucine mutants (I178D). On the one hand, in the case of the alcohols, this is characterized by the uptake of large macromolecules normally excluded from the nucleus, whereas the translocation rate for transport receptors is not accelerated [32]. On the other hand, the translocation rate of the 1-876 isoleucine mutants is increased [82], presumably resulting from their weak NPC binding affinity.

Remarkably, the mechanism of block usually attributed to N-terminally truncated Imp$\beta$ fragments is completely abolished for the Imp$\beta$ 45-462 I178D mutant. Normally, unmutated 45-462 constructs bind irreversibly to putative terminal sites in the translocation pathway and thus block transport (section 5.3.4). On the other hand, Imp$\beta$ proteins with intact N-termini are released from these sites by interaction with Ran. The Imp$\beta$ 45-462 isoleucine mutant, however, is observed to fully translocate through the NPC despite its inability to bind Ran [82]. The exchange
of the nonpolar isoleucine for an aspartic acid thus apparently severely weakens also the interaction of Imp/3 with the putative terminal sites of translocation, therefore negating the need for Ran-mediated release of the receptor (section 3.5.3).

Experimentally, both the alcohol and the Imp/3 I178D treatments induce a selective and statistically significant dilation in NPC diameter while pore depth is unaffected (Fig. 5.19). It is thus proposed that pore dilation is caused by similar effects in all cases. In fact, the amphipathic character of the alcohols was intentionally used to mimic the effect of introducing an additional charge in the NPC binding region as in the case of the I178D mutant. To further illustrate the dilation of the NPC line traces through individual pores are shown in Fig. 5.18 for treatments using Imp/3 I178D mutants, alcohols, and alcohol wash out experiments. Furthermore, Fig. 5.20 displays the relative frequency (bar plots) for depth and diameter measurements corresponding to the data in Fig. 5.19. The solid black line corresponds to the Gaussian determined by the mean and standard deviation of the measured distribution. Statistical analysis of diameter variations associated with these conditions are presented in Appendix C.

5.5 Molecular structure and function

How intimately structural changes and modifications in transport behavior are correlated cannot yet be fully answered. To this end, a much more detailed insight in the molecular arrangement of the nucleoporins in the central pore is needed. However, transport experiments and crystallographic investigations indicate that hydrophobic interactions within the NPC, and between nucleoporins and transport receptors, strongly influence translocation properties (section 3.6).

Many nucleoporins contain characteristic amino acid sequences which form hydrophobic patches interspersed by hydrophilic linkers (the FG-repeats, see sections 3.2 and 3.6). It is speculated that normally FG-repeats mutually attract each other via hydrophobic interactions. A cargo's ability to interact with these hydrophobic patches is the structural basis for the selective permeability barrier for nuclear transport (section 3.6 and [64, 32]). Here we extend this interpretation by speculating that the mutual attraction between FG-repeats also controls the compactness, i.e. diameter, of the NPC.

WGA and EGTA influence the properties of nucleoporins in the central channel. WGA binds specifically to sugars located between FG-repeats, and thus prevents docking and consequent translocation of import receptors. Expectedly, the NPC diameter is unaffected by this treatment and, in fact, should be locked in a compacted state. Interestingly, when perinuclear calcium stores are depleted using EGTA, the central "plug" structure emerges from the pore channel, and transport is disturbed. According to our data, this structure seems to be fitted with a hydrophobic surface chemistry. It could thus be speculated, that the "plug" actually consists of hydrophobic FG-repeat patches, which are expelled from the depths of the channel.
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Figure 5.21: Speculative molecular model describing the increase in diameter observed with amphipathic alcohols and Impβ I178D mutants, and the appearance of the central "translocator" associated with depletion of perinuclear Ca\(^{2+}\).

upon depletion of Ca\(^{2+}\). It remains unclear what the physiological relevance of such a nucleoporin rearrangement is and how nuclear transport properties are affected in detail.

The nonpolar isoleucine at position 178 of Impβ is crucially involved in the interaction with the NPC. It is at the heart of a site which has a high affinity to likewise nonpolar regions of the FG-repeats (section 3.5.2), [28]). Replacing the isoleucine by an aspartic acid residue, as in 45-462 I178D or 1-876 I178D, is an energetically unfavorable alteration to this hydrophobic interaction. The aspartic acid residue is charged at physiological pH and thus weakens the binding of Impβ to the nucleoporins.

The dilation of the NPC diameter on the order of about 7% (Fig. 5.19) is taken to result from the modified interactions between these mutants and the nucleoporins. Considering the large mass assembled in the body of a NPC, a swelling of this order of magnitude seems to be possible only if the constitution, or the forces holding the
NPC together, are fundamentally altered. Assuming that the molecular arrangement of nucleoporins directly affects NPC diameter, how is it possible that weakly binding transport receptors with a single point mutation are able to cause such a drastic change in the NPC?

First, even though their binding affinity to the NPC is weakened the Imp/3 I178D mutants are still able to penetrate the central pore region. This is because Imp/3 has several sites of interaction with FG-repeats apart from position 178 (section 3.5.2 and [28, 27]). Secondly, the number of Imp/3 I178D mutants, i.e. the number of charged aspartic acid residues, which can be accommodated simultaneously in the central pore should relate to how strongly nucleoporin arrangement is disturbed. For the unmutated 45-462 fragment which accumulates and fills the pore to a large extent, up to a few hundred binding sites are estimated to be available in the pore region. A value of this order of magnitude corresponds well to the estimated local concentration of up to 50 mM of FG-repeats inside the pore (section 3.2, [64]). Therefore the I178D mutants should still be able to interact with the NPC at high densities.

In conclusion, the number of charges introduced by the I178D mutants may be large enough to disrupt hydrophobic interactions between FG-repeats. As to how our mutants interfere in detail with the nucleoporin arrangement will certainly remain speculative. However, the notion that hydrophobic interactions may be important was tested using various amphipathic alcohols. These alcohols are thought to disturb the arrangement of FG-containing nucleoporins [32]. In contrast to the I178D constructs it is the polar -OH groups rather than excess charges which are assumed to interfere with the hydrophobic environment. The results obtained with 1,2-cyclohexanediol, 1,4-cyclohexanediol and 1,6-hexanediol are indeed in close agreement with the Imp/3 I178D data (Fig. 5.19).

To summarize, the point-mutated Imp/3 I178D constructs as well as amphipathic alcohol molecules interfere with the energetically favored arrangement of the FG-repeats in the central pore region. Assuming that interactions between FG-repeats control the radial dimension of the NPC, the dilation of the pore diameter may be understood as a consequence of the weakening of mutual attractions between FG-repeats. This may be understood as follows: under normal conditions, hydrophobic attractions between FG-repeats may exert a net (radially) inwardly directed tension within the lumen of the NPC, compensating the tension that may exist in the plane of the nuclear envelope. Disruption of these interactions may thus induce a relaxation of the NPC body directed radially outwards.

On the other hand, the hydrophobic character of interactions between nucleoporins and wild-type Imp/3 protein is preserved. In other words, normally Imp/3 proteins do not disrupt the hydrophobic environment based on mutual attraction between FG-repeats. In a simple model, even though transport receptors specifically bind to nucleoporins, they may be able to convey mutual attraction by acting as cross-linkers between FG-repeats, thus stabilizing the radial dimension of the
NPCs. Consistent with this interpretation, the pore diameter of NPCs treated with wild-type Impβ constructs is found to be significantly smaller compared to that for I178D mutants, and virtually identical to control values.

Figure 5.21 summarizes experimentally observed changes in NPC topology and how those may be interpreted in terms of a speculative molecular model. Under normal conditions (upper panels) hydrophobic interactions between FG-repeats and transport receptors (Impβ) give rise to an undisturbed NPC structure. Mutual attraction between FG-repeats may be mediated by cross-linking transport receptor molecules (Impβ-FG binding interactions are schematically visualized by blue dots in the right panels). Disruption of FG-repeat interactions may be induced by saturation of hydrophobic interaction sites using amphipathic alcohols, or by Impβ mutants which exhibit partial FG binding but prevent mutual FG-attraction by steric hindrance. These maneuvers are suggested to be responsible for the relaxation of the NPC body due to the disrupted tension normally provided by the mutual attraction between FG-repeats. It may prove worthwhile to investigate how strongly the effect ascribed to the Impβ mutant depends on the introduction of excess charges (or polar groups) rather than sterical inhibition of FG-interactions. In other words, would an Impβ protein with intact NPC binding sites but exhibiting additional charges at other locations be able to induce morphological changes in the NPC similar to the ones observed here?

The lower panel in Fig. 5.21 gives a speculative description as to the origin of the central ”translocator” associated with depletion of perinuclear Ca$^{2+}$. It is assumed that the ”translocator” actually corresponds to hydrophobic FG-containing nucleoporins extruding from the pore. Clearly, a more detailed understanding of the molecular processes in the central pore is needed to be able to unambiguously identify the origin of the experimentally observed changes in NPC topology.

5.6 Conclusions

In summary, SFM investigation of nuclear envelopes provided microscopic information about interactions between NPCs, transport receptors, and other agents known to modify nuclear transport.

Properties of the Impβ import pathway were investigated by analyzing changes in NPC topology induced by known transport receptors. Thereby mutant proteins which block translocation in a dominant-negative fashion (Impβ 45-462) cause an accumulation of material above the NPC, while proteins that fully sustain signal-mediated transport (wild-type Impβ ) only reduce pore depth by 25%. Impβ 1-462 and 1-409 are transport deficient (e.g. deficient in binding of NLS-cargo) and can be distinguished from the above experimental conditions by an increase in pore depth. The observed change in pore depth agrees well with the binding affinity attributed to these mutants; shallower pores correspond to higher affinity interactions between the mutant proteins and the NPC.
With regard to the 45-462 dominant negative construct it is plausible to assume that the accumulated material predominantly consists of bound receptor. This is supported by the observation that by decreasing the concentration of the 45-462 mutant the central bulge disappears and the pore channel once again becomes clearly visible in a concentration dependent manner (Appendix C). It is thus proposed that modulation of pore depth is also related with the binding of Impβ constructs for Impβ 1-876, 1-462 and 1-409. Accordingly, decreasing pore depths should 1) correlate with the number of binding sites available for each of the constructs, 2) reflect the volume of the bound constructs themselves, and 3) correlate with the lifetime of the binding interactions, i.e. with the binding affinity. While our data supports the latter two aspects, we can only assume that the number of binding sites is constant for all constructs. Clearly, at this stage it cannot be excluded that other effects such as pore conformational changes specifically associated with certain constructs may also play a role.

Translocation properties are modified also by exogenous molecules which directly interact with the NPCs (WGA, alcohols) or stimulate physiological processes which regulate nuclear transport (EGTA). Using SFM we can clearly differentiate between these conditions. WGA treated NPCs exhibit pore depths reduced by about 50% compared to controls. This and the associated block of transport is attributed to the binding of WGA to sugar groups located on certain nucleoporin stretches. EGTA induces the extrusion of the central translocator plug by depletion of perinuclear Ca$^{2+}$. The molecular mechanism associated with this behavior is not yet well understood.

Impβ mutants with distorted NPC binding sites (45-462 I178D and 1-876 I178D) dilate the pore diameter by approximately 7%. These mutants have been previously characterized by translocation rates greater than those of wild-type Impβ. Amphipathic alcohols are observed to increase NPC diameter by approximately 4-6%. NPCs exposed to these substances exhibit increased translocation of inert molecules.

It is proposed that the molecular rearrangement of FG-containing nucleoporins imparted by amphipathic alcohols and the Impβ I178D constructs is responsible for the observed increase in NPC diameter. Thereby the introduction of polar groups (alcohols) or excess charges (I178D) is presumably associated with steric inhibition of FG-interactions. In effect, the mutual hydrophobic attraction between FG-repeats is weakened, giving rise to a relaxation of the NPC body directed radially outwards (see also the discussion in section 5.5). Since these same nucleoporins are likely to be involved in nucleo-cytoplasmic transport this effect may illustrate how an increase in transport (which is also associated with a loss in specificity) is manifested in terms of changes in NPC topology.
Chapter 6

Conclusions and outlook

Within the scope of this thesis, the requirements for high resolution SFM investigation of native biological samples have been characterized in detail. Having established optimal conditions, lateral resolution at the scale of nanometers was achieved on nuclear envelope samples. This enabled the study of topological changes in NPCs related to modifications of nucleo-cytoplasmic transport properties induced by various physiological stimulants.

Imaging resolution benefited mostly from dynamic mode operation of the SFM. Shear forces exerted on the samples as are commonly observed with contact mode could be virtually eliminated with this method. On the other hand, it was observed that image quality was strongly dependent on how stably the sample could be adsorbed onto a solid support. In this respect it is important to note that forces similar to those responsible for the stable integration of the sample may also be involved in the interaction between the tip and sample. Consequently, high resolution imaging of biological surfaces in native environments requires sophisticated methods of sample preparation, optimization of substrate surface chemistry, and careful adjustment of imaging conditions such as the sample buffer composition.

As summarized in detail in section 5.6, interactions between the NPC and Impβ transport receptor proteins are associated with characteristic changes in NPC topology. Briefly, an increase in binding affinity is principally responsible for a decrease in NPC depression (1-409, 1-462, 1-876), and in some cases may lead to an accumulation of bound protein over the cytoplasmic surface of the NPC as in the case of the dominant-negative blocking mutant, 45-462. On the other hand Impβ constructs with greatly reduced binding affinity to the NPC (I178D) are characterized by an increase in NPC diameter.

Exogenous molecules can also influence NPC function in a physiologically relevant manner. While WGA treated NPCs exhibit a shallow pore depression, the central translocator plug is observed to protrude from the pore if the nuclear envelope is exposed to EGTA. Amphipathic alcohols are observed to significantly increase NPC diameter in a reversible manner, i.e. pore diameter returned to control values upon the removal of the alcohols.
The increase in NPC diameter associated with amphipathic alcohols and Impβ I178D is proposed to arise from the weakening of the mutual hydrophobic interactions between FG-containing nucleoporins. Disruption of FG interactions may be due to the introduction of excess hydrophilic groups in the hydrophobic environment created by the FG-repeats. Alternatively, FG-attraction may be competed by binding of alcohol molecules or transport receptors. Under normal conditions tension may exist in the plane of the nuclear envelope that is normally offset by the FG interactions within the lumen of the NPC. Interfering with these interactions causes the NPC to give way nuclear envelope tension resulting in dilation.

Further experiments may help to shed light on the issue of changes in NPC topology induced by hydrophilic molecules. In particular, the observation that the increase in transport associated with amphipathic alcohols is prevented by pretreatment with WGA [32] may indicate that the NPC diameter was unchanged. Moreover, WGA alone does not affect NPC diameter. It is not clear a priori how NPC diameter would be affected by Impβ mutants with intact NPC binding affinity but exhibiting additional surface charges. Here the question arises whether additional charges would be sufficient to disturb hydrophobic interactions between FG-repeats and thus lead to dilated NPC diameters, or if Impβ proteins with intact NPC binding sites would offset this effect in their capacity to cross-link FG-domains.

Whether the increase of the NPC diameter is a process relevant in normal, undisturbed nuclear transport might be tested using conjugated gold beads. To this end e.g. nucleoplasmin conjugated beads may be injected into intact oocytes or incubated with Impβ to promote translocation. The optimum experiment would be to check whether pores which exhibit a large gold bead (diameter ~ 25 nm) caught in transit are subject to dilation. Preliminary experiments conducted with gold beads 8 nm in diameter (~ 12 nm including the nucleoplasmin coat) did not show any significant pore dilation.

Clearly, more detailed analyses of molecular interactions in the central pore are needed to explain the structural properties and translocation characteristics of the NPC. SFM may be a valuable tool in this respect because local chemical information could potentially be obtained using appropriately modified probes. This is illustrated e.g. with the observation that the translocater plug protruding from the pore lumen in response to EGTA is apparently outfitted with a surface chemistry different from the rest of the NPC. In light of the previous discussion (section 5.3.3) it is speculated that the increased attraction between translocater and SFM tip is due to hydrophobic interactions. These experiments should be repeated in a more comprehensive study involving 1) careful investigation of the effect of sample preparation and imaging conditions on surface chemistry (i.e. fixed vs. unfixed samples, adjustment of buffer solutions, etc.), 2) examination and reproducible modification of tip surface chemistry, and 3) quantitative measurements of chemical interaction forces on specifically prepared and well characterized surfaces for comparison with experiments on real biological systems (i.e. the NPCs).
Two modes of SFM operation may be used to characterize surface chemistry. On one hand, force measurements could be used to quantify local interaction forces. On the other hand, dynamic mode SFM provides the resolution required to identify variations in surface chemistry within the (lateral) range of single NPCs. Particularly on samples with strongly modulated height characteristics, measurements of cantilever amplitude and deflection are often not sufficient to unambiguously distinguish the influence of interaction forces from variations in sample topography. On the other hand, measurements of the phase lag of the force sensor oscillation may provide valuable quantitative information about characteristic interaction forces (cf. section 2.3.2). Since in liquids the measurement and interpretation of phase signals is impeded by unwanted resonances and viscous interactions or low quality factors, it is suggested to employ Q-control feedback (section 2.2.7) in conjunction with magnetic excitation of the cantilever (section 2.2.6) to maximize contrast arising from variations in surface chemistry.

Carbon nanotube SFM tips may help to increase lateral resolution on nuclear envelopes (which exhibit a rather large height modulation) because of their favorable cylindrical shape, i.e. large aspect ratio. Since the typical lifetime of a SFM tip allowing high resolution imaging is generally limited by tip contamination, it is important that extensive investigations involving several experimental conditions be conducted with a series of tips of reproducible characteristics. Chemical vapor deposition using appropriately optimized catalyst materials allows reproducible growth of nanotubes with a rather narrow diameter distribution, and is thus one of the most promising methods for the production of nanotube SFM tips. Since nanotubes tend to grow along the faces of the supporting tips, their relative orientation is mostly reproducible, but may be improved by lithographic patterning of the catalyst and the application of an electric field during the growth process.
Appendix A

Further experiments

Unfixed nuclear envelope.

Figure A.1: The same region of an unfixed nuclear envelope is shown twice, at which the right scan was taken 15 minutes after the left scan (scale bar 500 nm). The intact sample had been deposited onto a solid substrate. The cytoplasmic membrane (denoted as outer) ruptured during preparation (small arrows), and the nucleoplasmic face of the envelope beneath (denoted as inner) became visible. Contaminating particles (possibly remainders from cellular yolk platelets) are seen to disassemble (upper arrow) and move (middle arrow) with time, presumably under the influence of the scanning tip.
The nuclear lamina.

Figure A.2: The nuclear lamina. This image shows the nucleoplasmic side of an unfixed nuclear envelope where the filamentous lamina is largely left intact (scale bar 500 nm). Only few NPCs are visible.

Dried nuclear envelope.

Figure A.3: Dried nuclear envelope imaged in air (cytoplasmic side). Scale bars represent 200 nm (left) and 100 nm (middle). To the right a profile taken across the diameter of a single NPC is shown. While the NPC diameter $d$ is not affected by drying, the vertical aspect of the pore $\Delta z$ is reduced by a factor of 2 to 3 compared to hydrated samples.
Appendix B

Materials and methods

Buffer solutions.

- Modified Barth’s Solutions (MBS, extracellular buffer, in mM): 1 KCl, 0.82 MgSO₄, 0.41 CaCl₂, 0.33 Ca(NO₃)₂, 2.4 NaHCO₃, 88 NaCl, and 10 HEPES, pH 7.4.

- Mock Intracellular Buffer (MIB, in mM): 90 KCl, 10 NaCl, 2 MgCl₂, 0.75 CaCl₂, 1.1 EGTA, and 15 Tris, pH 7.5.

- Low Salt Buffer (LSB, intracellular buffer, in mM): 1 KCl, 0.5 MgCl₂, and 15 Tris.

Impβ constructs.
Impβ constructs were stored in aliquots of about 3 – 5 µl at a stock concentration of 200 µM with 250 mM sucrose in MIB.

Preparation of solid supports.
Nuclear envelopes were deposited on Falcon (351008) petri-dishes or SuperFrost Plus (Menzel Gläser) glass slides coated with 0.1 % (by weight) poly-L-lysine for 10 min.
Appendix C

Statistical analysis

Sample sizes.
Table C.1 defines the sample sizes (number of radial NPC profiles used for data analysis, second column) and the number of nuclear envelopes from which profiles have been taken (third column) for each of the experimental conditions. The last two columns display mean values and standard deviations for depth and diameter measurements.

Statistical analysis of diameter variations.
The Student’s t-test determines the probability $p$ which describes whether two sets of measurements are likely to have come from statistical populations with the same mean. In other words, $1 - p$ determines whether the difference in mean values between two sets of measurements is statistically significant, i.e. the populations are independent. In table C.2 the probabilities $p$ are given for diameter measurements from the indicated experimental conditions tested against control measurements (upper part), and for the indicated wash experiments tested against the corresponding alcohol conditions (lower part). The data corresponds to the diameter measurements displayed in Fig. 5.19.

Impβ 45-462 concentration dependence
In analogy to experiments where nuclear envelopes were incubated with Impβ 45-462 constructs for various periods of time, Fig. C.1 shows diameter and pore depression characteristics of NPCs exposed to different concentrations of the same construct for 2 minutes. The average pore depth decreases with increasing protein concentration while the NPC diameter is largely unaffected. This is in agreement with the interpretation that Impβ 45-462 accumulates at the central pore channel because of high affinity binding to the nucleoporins.
<table>
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<th>no. of samples</th>
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<th>( \Delta z ) std</th>
<th>( \Delta z ) sem</th>
<th>( d ) mean</th>
<th>( d ) std</th>
<th>( d ) sem</th>
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<td>83.2</td>
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<td>1.5</td>
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Table C.1: Sample sizes and numerical values for pore depth (\( \Delta z \)) and diameter (\( d \)) measurements (std=standard deviation, sem=standard error of mean).
### C. Statistical analysis

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Table C.2: Probabilities $p$ associated with the Student’s t-test for the diameter measurements displayed in Fig. 5.19.

![Figure C.1: Pore depth and diameter for NPCs exposed to Impβ 45-462 at different concentrations for 2 minutes.](image-url)
Appendix D

Viruses

Viruses exploit the cell’s transcriptional and translational mechanism for their own replication. To achieve this the virus’ genetic information must find its way into the host cell’s nucleus [86].

We examined capsids of *Herpes simplex* viruses (HSV1) either deposited on mica or after their docking to the NPCs of *Xenopus* nuclei (Fig. D.1). Capsids are robust protein shells about 120 nm in diameter enclosing the viral DNA. At the cell’s plasma membrane the HSV1 capsid is introduced into the cytoplasm where it moves along cytoskeletal elements until it finally docks at the nuclear envelope of the host cell. The HSV1 viral DNA is threaded through the NPC into the nucleus, while the protein shell remains attached to the cytoplasmic domain of the NPC (Fig. D.1).

The density of capsids docking to the nuclear envelope *in vitro* was found to be rather small. We therefore tried to deposit high concentrations of capsids directly onto freshly cleaved mica substrates.

*Herpes simplex* viral capsids are stored in high salt buffers to prevent aggregation of capsids and to preserve protein-DNA integrity. Unfortunately, this condition was rather incompatible with SFM. High salt buffers screen the highly negative surface charge of the mica, interfering with the attachment of the capsids to the substrate and making the capsids easily displaced by the SFM tip (Fig. D.2). On the other hand, the capsids stuck rather well to mica when deposited in low salt buffers. After approximately a half hour of scanning, however, they were observed to disintegrate due to the unfavorable electrostatic conditions (Fig. D.3).
D. Viruses

Figure D.1: *Herpes simplex* virus capsid docks to a NPC.

Figure D.2: The left image corresponds to the tip moving from left to right (trace), in the right image the tip moves in the opposite direction (retrace). The scan started from the bottom. A viral capsid deposited on mica in high salt buffers was displaced by the SFM tip from right to left approximately half way trough the scan as indicated by the arrow in the right image. Scale bars represent 250 nm.
Figure D.3: Viral capsid adhere well to mica in low salt buffers (left image), but disintegrate after some time (right image). Scale bars represent 200 nm.
Appendix E

Nanotubes

Cantilever tips modified with Carbon Nanotubes (NTs) are desirable for SFM applications because of their large aspect ratio (thin, long geometry) and their resistivity to abrasive wear. Furthermore, NT tips may be well suited for chemical functionalization and thus for investigation of variations in sample surface chemistry.

NT tips may be produced by gluing an individual tube or bundle of tubes to a conventional cantilever tip [87, 88, 60]. After deposition of the NT the tip radius can be estimated by imaging and numerical fitting of radial profiles of single gold beads (diameter \( \sim 5\,\text{nm} \)) deposited on mica (Fig. E.1). The simple geometrical relationship \( r = b^2/16R \) where \( r \) is the NT tip radius, \( R \) the bead radius, and \( b \) the full width of the gold bead as it appears in the SFM image due to tip dilation can be used to quickly estimate the tip radius. Evaluation of profiles from Fig. E.1 gives a NT radius \( r = 16\,\text{nm} \) which is comparable to conventional SFM tips. Note that the foremost tip asperity which might potentially have a smaller radius cannot be evaluated by this method.

NT tips are generally characterized by SFM force measurements, e.g. before and after shortening of the NT via electric breakdown between tip and a conducting surface. By comparing amplitude and deflection data the tube length and the buckling force can be measured (Fig. E.2). Buckling of the NT often seems to occur at very small (Euler) forces \( F_{\text{Euler}} = E\pi^3r^4/4L^2 \) where \( E \) is the NT’s Young’s modulus (\( \sim 1\,\text{TPa} \) for multiwalled NTs), \( r \) the (cylinder) radius and \( L \) the tube length). In the example presented here the buckling force \( F_b = 12\,\text{nN} \) is two orders of magnitude smaller than theoretically expected for a nanotube with radius 16 nm. This may indicate that either a very fine (possibly single-walled) NT protrudes from the larger bundle with an elastic modulus which is much smaller than 1 TPa, or that the NT hits the surface at an inclined angle. Buckling is enhanced for inclined NTs because of shear forces arising from the combined action of repulsive forces at the tip and attractive (e.g. capillary) forces along the shaft of the nanotube. Particularly, when imaging steeply inclined surfaces the shaft of a NT may be more subject to adhesive or attractive forces, resulting in distorted scan images (Fig. E.1, images of the upper side of the gold beads show characteristic distortions). Nonetheless, at typical
Figure E.1: Image of 5 nm gold beads on mica (scale bar 100 nm). From profiles across single beads the NT radius is estimated to 16 nm. Imaging is distorted at the "upper" side of the beads, indicating adhesive interactions between the inclined NT and the gold bead.

Figure E.2: Force measurement for the same NT tip as above. The amplitude decreases abruptly when the nanotube taps on the mica surface. By further approaching the cantilever to the substrate the nanotube buckles (buckling force $F_b = 12$ nN), and thus the oscillation of the cantilever partially recovers. After approaching another 80 nm the nanotube is bent sideways at its full length and the bundle from which it protrudes hits the surface, giving rise to a steep increase of the cantilever deflection. The NT tip fully recovers when the cantilever is retracted above the surface. The deflection signal shows some wavy background with a period of $\lambda/2 = 335$ nm where $\lambda$ is the wavelength of the laser beam. This is due to interference of laser light reflected from the cantilever with light reflected from the mica surface.
amplitude setpoints NT tips are stable for many hours. Finally, for SFM operation in liquids short NTs are preferable since the capillary action of water surfaces is sufficiently strong to rip off or completely bend the NTs.

Better quality tips are in principle obtained by growing NTs directly on SFM tips [89, 90, 91, 92]. With this method NTs with smaller radii are readily produced with the additional advantage that the tube length may be controlled during the growth process. NT growth takes place in a chemical vapor deposition (CVD) process involving a furnace heated to 900 °C, a supply of gaseous carbon feedstock (e.g. methane) and a surface coated by an appropriate catalyst.

Heating is achieved in a large tube furnace or a cold wall reactor equipped with a heating plate or a hot filament. Typical CVD process parameters are given in Table E.1.

<table>
<thead>
<tr>
<th>temperature</th>
<th>gas flow</th>
<th>duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>heat up</td>
<td>Ar (500 ml/min)</td>
<td>15 min</td>
</tr>
<tr>
<td>900 °C</td>
<td>CH4 (11/min) and H2 (300 ml/min)</td>
<td>15 min</td>
</tr>
<tr>
<td>cool down</td>
<td>Ar (&gt; 500 ml/min)</td>
<td>15 min</td>
</tr>
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</table>

Table E.1: CVD process parameters

The quality of the NTs produced depends crucially on the type and purity of the catalyst used. We have investigated NT growth using metallic layers, usually Fe, deposited by electron beam evaporation and liquid catalysts containing ferric nitrate. It has been observed that the NT diameter is closely related to the diameter of the resulting catalyst particles [93]. NTs with smaller radii are obtained by reducing the thickness of the Fe layer, respectively the ferric nitrate concentration, thereby also drastically reducing the density of NTs, which may prove counterproductive. Furthermore, although the growth of contaminating amorphous carbon is generally a large problem it can be minimized by choosing the right catalyst conditions in conjunction with optimized growth temperatures and reactive gas compositions.

NTs are observed to grow in bundles or with rather large diameters (> 50 nm) using metallic catalyst (Fe layer thickness ~ 10 nm deposited on silicon wafers or silicon tips). Moreover, contamination with amorphous carbon is severe. These problems may be related to either the metal coating mechanism (large catalyst grain size), interaction of the catalyst material with the silicon support, or chemical modification of the metallic layer (oxidation).

More convenient production of small diameter NTs with reduced amorphous carbon contamination may be achieved using liquid catalysts such as ferric nitrate dissolved in methanol or ethanol. Surfaces (e.g. SFM tips) may simply be dipped into this solution and are immediately ready for CVD, albeit at small NT yields. Higher yields are obtained by adding to the catalyst solution nanometer sized powder-like materials (aluminium oxide, Aerosil (Degussa)). Typical catalyst compositions used in experiments are given in Table E.2. Using this method the NT tip shown in Fig.
2.13 was produced. The drawback here is that the catalyst material resides on all exposed surfaces, i.e. also on the reflecting side of the cantilever. Thus, in an improved process only the front side (tip) of a cantilever was exposed to the catalyst material by approaching the tip to a small wire loop containing a tiny volume of liquid catalyst. In future this tedious procedure may be avoided by protecting the cantilever surface using a polymer-resist such as PMMA, but leaving the foremost part of the tip free for subsequent coating with liquid catalyst material [90].

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<tr>
<td>Fe(NO₃)₃</td>
<td>301.5 mg</td>
<td>in 15 ml methanol</td>
</tr>
<tr>
<td>MoAcac</td>
<td>70.5 mg</td>
<td>in 15 ml methanol</td>
</tr>
<tr>
<td>aluminium oxide (Aerosil)</td>
<td>78 mg</td>
<td>in 40 ml methanol</td>
</tr>
</tbody>
</table>

Table E.2: Liquid catalyst composition. The three components are dissolved in methanol in an ultrasound bath or magnetic stirrer, and only then mixed to yield a final concentration of 50 mM Fe(NO₃)₃.

In future experiments the NT growth direction will be controlled by applying large electric fields (300 V/cm if discharges can be prevented) which induce electric dipoles in the growing NT and thus may help to control its orientation [94].
Bibliography


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Publications and Presentations

Detailed analysis of forces influencing lateral resolution for Q-control and tapping mode.

Modulation of nuclear pore topology by transport modifiers.
*Biophys. J.*, in press

Investigation of transport-relevant properties of nuclear pore complexes using scanning force microscopy.
*Nanotechnology*, submitted

Metal-insulator transition in a disordered two-dimensional electron gas in GaAs-AlGaAs at zero magnetic field.

Magnetic field dependence of the metal-insulator transition in Ga[Al]As-heterostructures.

Transport signatures for correlated disorder in self-assembled InAs quantum dots on GaAs.

Metal-insulator transition in a two-dimensional electron gas with controlled potential perturbation.
*Proceedings of the Joint Moriond Conference-Nedo Meeting on "Quantum physics at mesoscopic scale", Les Arcs, France,*

**Oral presentations**

**Q-control vs. tapping mode AFM on nuclear envelopes,** 

**The metal-insulator transition in Ga[Al]As-heterostructures,** 

**Poster presentations**

**Transport through nuclear pore complexes: investigation by scanning force microscopy,** 

**Scanning force microscopy on biological membranes,** 
Anglo-Swiss Day of Micro- and Nanotechnology, Zürich (Switzerland), February 7, 2001.

**Scanning force microscopy on nuclear pores in liquids,** 
EMBL Conference on Scanning Probe Microscopy, Sensors and Nanostructures, Heidelberg (Germany), May 29, 2000

**Metallic and insulating behaviour in a disordered two-dimensional electron gas at zero magnetic field,** 
3rd Workshop on Nanoscience, Hasliberg (Switzerland), October 14, 1998.
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