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# Cell Nanobiology

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## 1. Introduction

### 1.1. Cell nanobiology

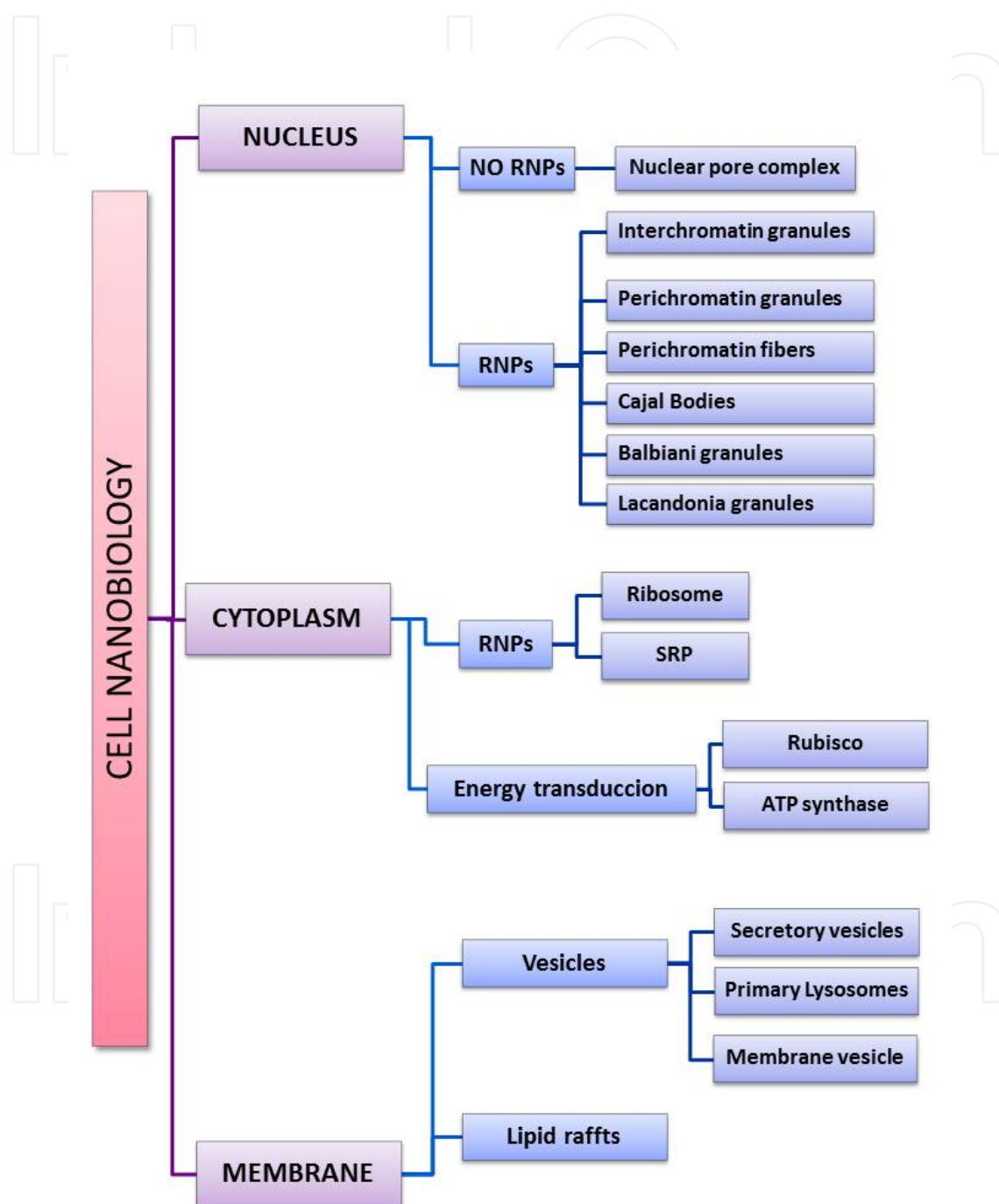
We define cell nanobiology as an emergent scientific area trying to approach the study of the *in situ* cell processes occurring at the nanoscale. Therefore, it is part of cell biology but mainly deals with an interphase between analytical methods such as X-ray crystallography producing models at atomic or molecular resolution, and direct nanoscale imaging with high resolution microscopes such as scanning probe microscopes, electron microscopes and super-resolution microscopes. Several cell structures are involved in nanoscale processes (Figure 1).

#### 1.1.1. An overview of cell structure under a genomic approach of gene expression

The main flow of genetic information represented as the so-called central dogma of molecular biology *in situ*, illustrates the major secretory pathway in the cell (Figure 2).

During this pathway, nanoscale particles represent substrates of different moments. During transcription, nuclear particles are involved in transcription and processing of RNA, both, pre-mRNA and pre-rRNA. pre-mRNA is transcribed and processed in the nucleoplasm while pre-rRNA is transcribed and processed within the nucleolus, the major known ribonucleoproteins structure where ribosome biogenesis and other functions of eukaryotic cell take place. Once in the cytoplasm, translation takes place in the ribosome, also a major ribonucleoprotein

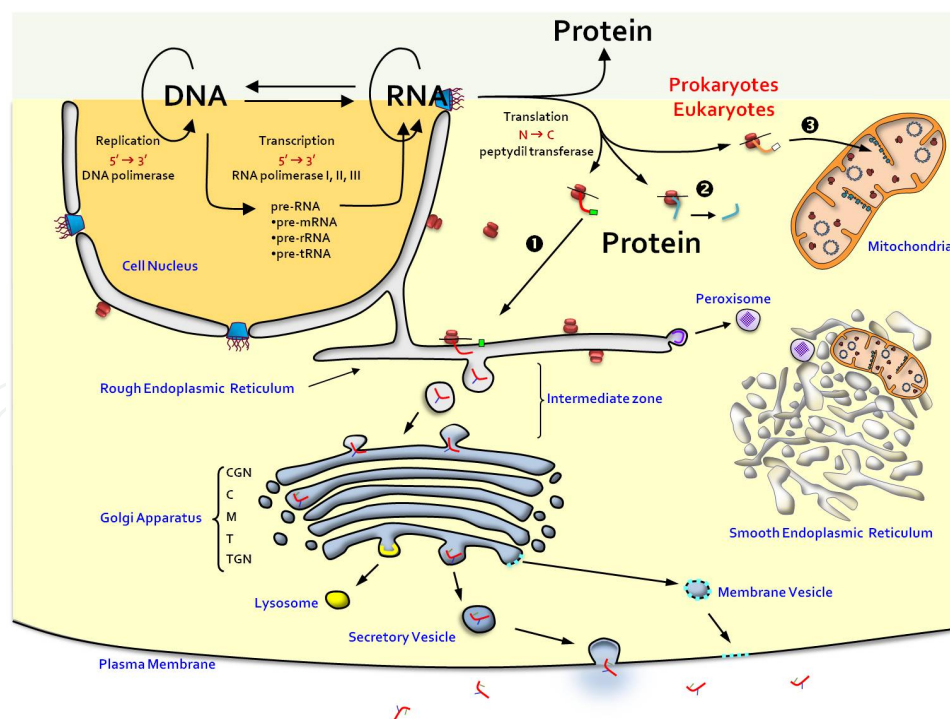
particle of 10-15 nm in diameter. When the synthesized protein contains a signal peptide, it is translocated into the rough endoplasmic reticulum, helped by the signal recognition particle or SRP, another major and conserved ribonucleoprotein. The transport to Golgi apparatus by the intermediated zone and the TGN producing the three derivatives from the Golgi apparatus are mediated by vesicles [see 1].



**Figure 1.** Cell nanobiology proposes to study cell structures using *in situ* high resolution microscopical approaches as electron and atomic force microscopy that could complement molecular and biochemical data to better understand a physiological role at the nanoscale.

## a. Semenogelin

Semenogelin is the most abundant protein in the semen of mammals. It is a glycosylated protein that is responsible for properties such as density. As an example, the semenogelin of the tamarin *Saguinus oedipus* is used to show how the signals in the nucleic acids and proteins determine the intracellular pathways associated to that expression. Its expression includes intranuclear events as transcription by RNA polymerase II from a split gene consisting of 3 exons and 2 intrones, processing of the transcript as 5' end methylation, 3' polyadenylation and splicing. All of them are associated to nuclear particles. Once in the cytoplasm, the mature transcript or mRNA associates to a ribosome that in turns translates the transcript. If the resulting protein contains a signal peptide, the signal recognition particle or SRP -a very well conserved RNA+protein complex- binds to it and associates to the rough endoplasmic reticulum, giving rise to the translocation process that introduces that protein to the lumen. Once there, N-glycosylation takes place at several asparagine residues following the basic rule of adjacent aminoacids showing a basic rule as Asn-X(except proline)-Ser or Asn. In *S. oedipus* semenogelin, there are 14 N-glycosylation sites. The protein then continues flowing through the Golgi apparatus or complex and at the TGN a secretory vesicle forms containing the protein that finally is secreted by the epithelial cell of seminal vesicle. The analysis of the gene sequences, as well as the transcription, processing, translation and post-translational products can predict the cell structures involved in the process [see 1].



**Figure 2.** A general overview of the cell structure and function. The diagram illustrates the *in situ* flow of genetic information of a secretory protein encoded in the genome within the cell nucleus. A gene is copied into a pre-mRNA that is processed to mRNA within the nucleus. mRNA in the cytoplasm may contain a signal sequence that allows entrance to rough endoplasmic reticulum and further to Golgi complex. The protein inside a vesicle is secreted out of the cell.

### 1.1.2. Some nanoscale cell structures

There are many cell structures or products made by cells that could be analyzed under the present approach. Some of them are indicated in Figure 1, but there are others as extracellular matrix components, cytoskeleton elements, etc.; virus are also nanometric structures associated always to cell organelles. Here we will give an overview of some of the cell components, as examples.

#### a. Nuclear particles

In eukaryote cells, transcription and processing mainly takes place within the cell nucleus, associated to nuclear particles that are well known since a method for ribonucleoprotein (RNP) structures was described in 1969 [2]. These particles are few nanometers in diameter or length. To date, several nuclear RNPs have been described including involved in mRNA metabolism: perichromatin fibers, perichromatin granules, interchromatin granules in mammals. In insects, Balbiani ring granules are well known structures [3]. In 1992 Lacandonia granules were described for some plants [4]. In addition, other nuclear bodies around 300-400 nm in diameter have been described involved in gene expression. As for rRNA transcription and processing, the nucleolus is a nuclear organelle containing pre-ribosomes in the granular component that are about 10-20 nm in diameter.

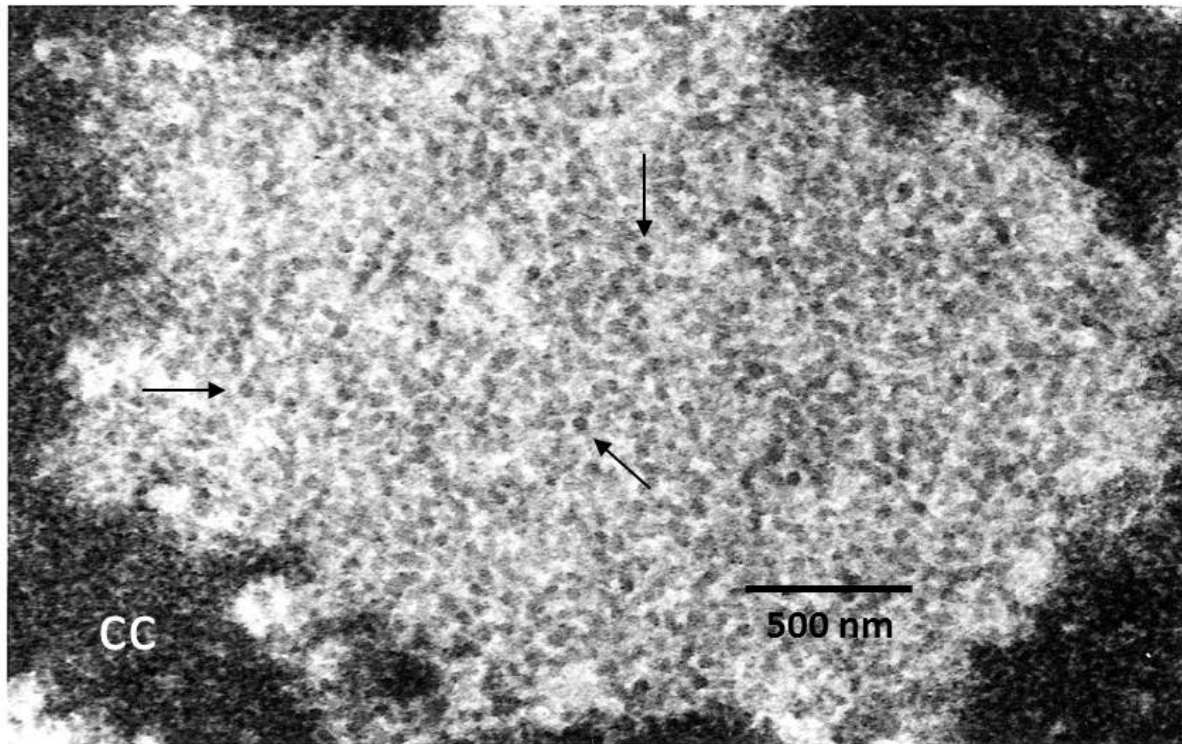
#### b. Rough endoplasmic reticulum particles

##### i. The ribosome

Ribosomes are the universal ribonucleoprotein particles that translate the genetic code into proteins. The shape and dimensions of the ribosome were first visualized by electron microscopy [6-8]. Ribosomes have diameters of about 25 nanometers in size and are roughly two-thirds RNA and one-third protein. All ribosomes have two subunits, one about twice the mass of the other. The ribosome basic structure and functions are well-known. There are 70S ribosomes common to prokaryotes and 80S ribosomes common to eukaryotes. The bacterial ribosome is composed of 3 RNA molecules and more than 50 proteins. In humans, the small ribosome unit has 1 large RNA molecule and about 32 proteins; the large subunit has 3 RNA molecules, and about 46 proteins. Each subunit has thousands of nucleotides and amino acids, with hundreds of thousands of atoms. The small subunit (0.85 MDa) initiates mRNA engagement, decodes the message, governs mRNA and tRNA translocation, and controls fidelity of codon-anticodon interactions and the large subunit catalyzes peptide bond formation.

In 1980, the first three-dimensional crystals of the ribosomal 50S subunit from the thermophile bacterium *Geobacillus stearothermophilus* were reported [9]. Since then, ribosome crystallography advanced rapidly. To date crystal structures have been determined for the large ribosomal subunit from the archaeon *Haloarcula marismortui* at 2.4 Å [10] and the 30S ribosomal subunit from *Thermus thermophilus* [11]. The structure of the entire 70S ribosome in complex with tRNA ligands (at 5.5 Å resolution) emerged shortly after the structures of the initial subunits [12].



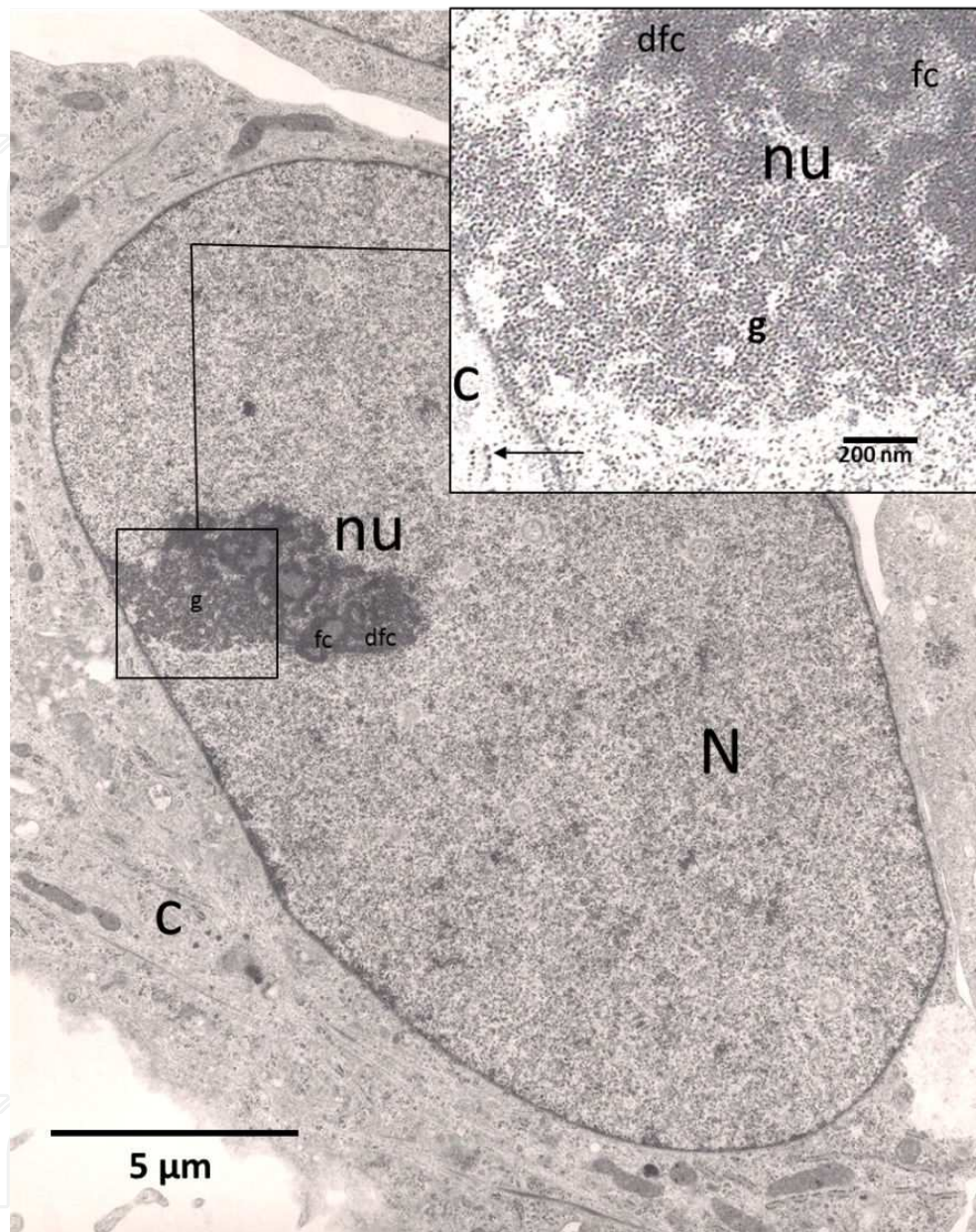


**Figure 3.** Lacandonia granules are nanoscale (32 nm in diameter) and abundant particles (arrows) in the nucleoplasm of the plant *Lacandonia schismatica*. Transmission electron microscopy image of a cell in the teguments of the flower. Cc, compact chromatin.

Snapshots of ribosome intermediates provided by cryo-EM and x-ray crystallography, associated translation factors, and transfer RNA (tRNA) have allowed dynamic aspects of protein translation to be reconstructed. For example, recent cryo-EM reconstructions of translating ribosomes allowed direct visualization of the nascent polypeptide chain inside the ribosomal tunnel at subnanometer resolution [13-15]. The dimension of the ribosomal tunnel in bacterial, archaeal, and eukaryotic cytoplasmic ribosomes is conserved in evolution [16-18]. The ribosomal tunnel in the large ribosomal subunit is ~80 Å long, 10–20 Å wide, and predominantly composed of core rRNA [19]. The tunnel is clearly not just a passive conduit for the nascent chain, but rather a compartment in a dynamic molecular dialogue with the nascent chain. This interplay might not only affect the structure and function of the ribosome and associated factors, but also the conformation and folding of the nascent chain [20]. As the nascent polypeptide chain is being synthesized, it passes through a tunnel within the large subunit and emerges at the solvent side, where protein folding occurs.

Peptide bond formation on the bacterial ribosome and perhaps on the ribosomes from all organisms is catalyzed by ribosomal RNA as well as ribosomal protein and also by the 2'-OH group of the peptidyl-tRNA substrate in the P site. The high resolution crystal structures of two ribosomal complexes from *T. thermophilus* [21] revealed that ribosomal proteins L27 and L16 of the 50S subunit stabilize the CCA-ends of both tRNAs in the peptidyl-transfer reaction,

suggesting that peptide chains from both these proteins take part in the catalytic mechanism of peptide bond formation.

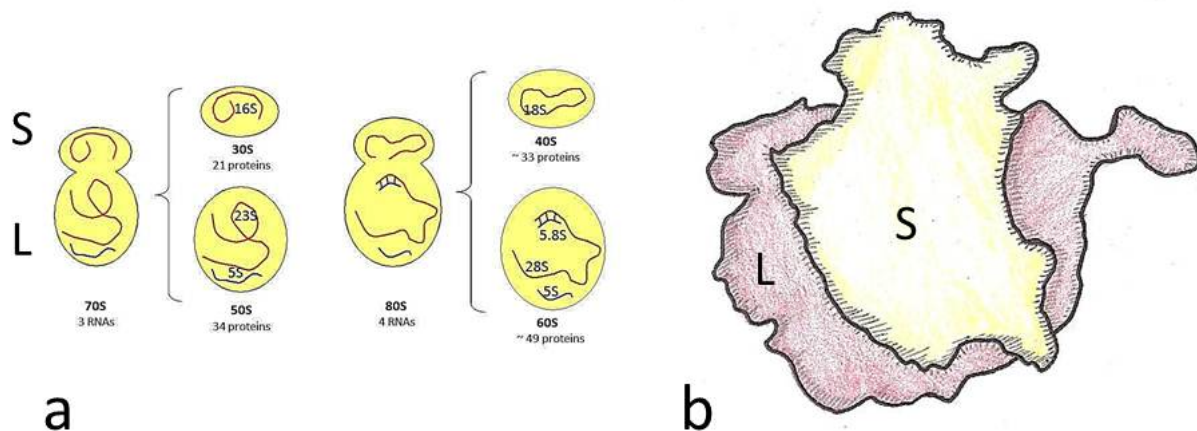


**Figure 4.** Nucleolus of a PtK2 cell. Within the cell nucleus (N), the nucleolus displays three different components named fibrillar center (fc), dense fibrillar component (dfc) and granular components (g). In the inset, a high magnification of the nucleolus shows granular particles or pre-ribosomes in the granular component (g). In the cytoplasm (c), ribosomes are also visible (arrow).

Ribosomes mediate protein synthesis by decoding the information carried by messenger RNAs (mRNAs) and catalyzing peptide bond formation between amino acids. When bacterial ribosomes stall on incomplete messages, the trans-translation quality control mechanism is activated by the transfer-messenger RNA bound to small protein B (tmRNA-SmpB ribo-



nucleoprotein complex). Trans-translation liberates the stalled ribosomes and triggers degradation of the incomplete proteins. The cryo-electron microscopy structures of tmRNA–SmpB accommodated or translocated into stalled ribosomes demonstrate how tmRNA–SmpB crosses the ribosome and how as the problematic mRNA is ejected, the tmRNA resume codon is placed onto the ribosomal decoding site by new contacts between SmpB and the nucleotides upstream of the tag-encoding sequence [22]. Recently, the crystal structure of a tmRNA fragment, SmpB and elongation factor Tu bound to the ribosome shows how SmpB plays the role of both the anticodon loop of tRNA and portions of mRNA to facilitate decoding in the absence of an mRNA codon in the A site of the ribosome [23].



**Figure 5.** Representation of a prokaryotic (a) and a eukaryotic ribosome (a). Each one is an RNP is constituted by two subunits, each containing rRNA and proteins. b) a model to show the nanoscale morphology of a mammalian cytoplasmic ribosome (small [S] and large [L] subunits).

The structure of the ribosome at high resolution reveals the molecular details of the antibiotic-binding sites, explain how drugs exercise their inhibitory effects. Also, the crystal structures help us to speculate about how existing drugs might be improved, or novel drugs created, to circumvent resistance [24]. Recently, ribosome engineering has emerged as a new tool to promote new crystal forms and improve our knowledge of protein synthesis. To explore the crystallization of functional complexes of ribosomes with GTPase, a mutant 70S ribosomes were used to crystallize and solve the structure of the ribosome with EF-G, GDP and fusidic acid in a previously unobserved crystal form [25].

In contrast to their bacterial counterparts, eukaryotic ribosomes are much larger and more complex, containing additional rRNA in the form of so-called expansion segments (ES) as well as many additional r-proteins and r-protein extensions [26]. The first structural models for the eukaryotic (yeast) ribosome were built using 15-Å cryo-electron microscopy (cryo-EM) maps fitted with structures of the bacterial SSU [11] and archaeal LSU [10], thus identifying the location of a total of 46 eukaryotic r-proteins with bacterial and/or archaeal homologs as well as many ES [27].



Ribosome biogenesis is regulated by the conserved protein kinase TOR (target of rapamycin), a member of the ATM-family protein. TOR up-regulates transcription of rRNA and mRNA for ribosomal proteins in both yeast and mammals [28-30]. Recent results indicate that in yeast, conserved kinases of the LAMMER/Cdc-like and GSK-3 families function downstream of TOR complex 1 to repress ribosome and tRNA synthesis in response to nutrient limitation and other types of cellular stress [31].

## ii. The signal recognition particle (SRP)

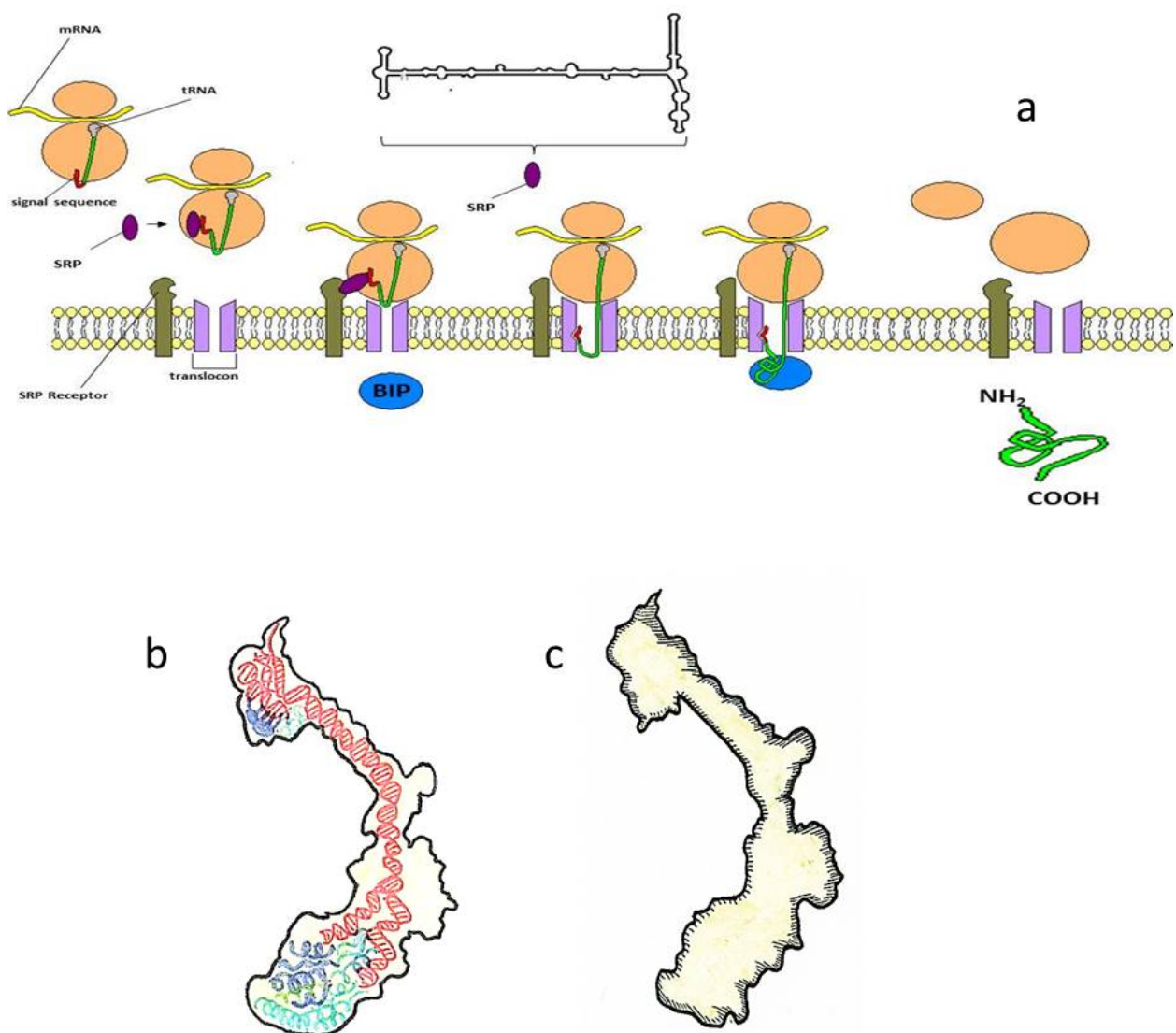
The Signal Recognition Particle (SRP) is an evolutionarily conserved rod-shaped 11S ribonucleoprotein particle, 5–6 nm wide and 23–24 nm long [32]. It comprises an essential component of the cellular machinery responsible for the co-translational targeting of proteins to their proper membrane destinations [33].

Although SRP is essential and present in all kingdoms of life maintaining its general function, structurally it shows high diversity. Vertebrates SRP consists of a single ~ 300-bp RNA (SRP RNA or 7S RNA) and six polypeptides designated SRP9, SRP14, SRP19, SRP54, SRP68 and SRP72. It can be divided into two major functional domains: the Alu domain (comprising the proteins SRP9 and -14) and the S domain (SRP19, -54, -68, and -72). The S domain functions in signal sequence recognition and SR interaction, whereas the Alu domain is required for translational arrest on signal sequence recognition [34]. In Archaea and Eucarya, the conserved ribonucleoproteic core is composed of two proteins, the accessory protein SRP19, the essential GTPase SRP54, and an evolutionarily conserved and essential SRP RNA [35]. SRP54, comprises an N-terminal domain (N, a four-helix bundle), a central GTPase domain [G, a ras-like GTPase fold, with an additional unique  $\alpha$ - $\beta$ - $\alpha$  insertion box domain (IBD)], and a methionine-rich C-terminal domain [36-37]. The N and G domains are structurally and functionally coupled; together, they build the NG domain that is connected to the M domain through a flexible linker [38]. The M domain anchors SRP54 to SRP RNA and carries out the principal function of signal sequence recognition [39-41]. The NG domain interacts with the SR in a GTP-dependent manner [43].

SRP is partially assembled in the nucleus and partially in the nucleolus. In agreement with that, nuclear localization for SRP proteins SRP9/14, SRP68, SRP72 and SRP19 has been determined [44]. After the transport into the nucleus the subunits bind SRP RNA and form a pre-SRP which is exported to the cytoplasm where the final protein, Srp54p, is incorporated [45-47]. Although this outline of the SRP assembly pathway has been determined, factors that facilitate this and/or function in quality control of the RNA are poorly understood [48]. SRP assembly starts during 7S RNA transcription by RNA polymerase III in the nucleolus, by binding of the SRP 9/14 heterodimer and formation of Alu-domain. Prior to transportation to the nucleus SRP9 and SRP14 form the heterodimer in the cytoplasm, a prerequisite for the binding to 7S RNA [49].

The signal recognition particle displays three main activities in the process of cotranslational targeting: (I) binding to signal sequences emerging from the translating ribosome, (II) pausing of peptide elongation, and (III) promotion of protein translocation through docking to the membrane-bound SRP receptor (FtsY in prokaryotes) and transfer of the ribosome nascent

chain complex (RNC) to the protein-conducting channel [50]. Despite the diversity of signal sequences, SRP productively recognizes and selectively binds them, and this binding event serves as the critical sorting step in protein localization within the cell. The structural details that confer on SRP this distinctive ability are poorly understood. SRP signal sequences are characterized by a core of 8–12 hydrophobic amino acids that preferentially form an  $\alpha$ -helix, but are otherwise highly divergent in length, shape, and amino acid composition [51–52]. This and the unusual abundance of methionine in the SRP54 M-domain led to the ‘methionine bristle’ hypothesis, in which the flexible side chains of methionine provide a hydrophobic environment with sufficient plasticity to accommodate diverse signal sequences [53].



**Figure 6.** The signal recognition particle. During protein synthesis of the secretory pathway, the signal peptide binds to SRP, an RNP particle containing a small RNA and 6 different proteins (b, [modified from 60]). A model for SRP at nanoscale is shown in (c).

In the SRP pathway, SRP binds to the ribosome synthesizing the polypeptide, and subsequently also binds an SRP receptor, located next to the machinery that transfers proteins across the membrane and out of the cell. This process begins when a nascent polypeptide carrying a signal sequence emerges from the translating ribosome and is recognized by the SRP. The ribosome-nascent chain complex is delivered to the target membrane via the interaction of SRP with the SRP receptor. There, the cargo is transferred to the Sec61p (or secYEG in archaea and bacteria) translocon, which translocates the growing polypeptide across the membrane or integrates it into the membrane bilayer. SRP and SR then dissociate from one another to enter subsequent rounds of targeting.

During the last years, several structures have been solved by crystallography and cryo-electron microscopy that represent distinct functional states of the SRP cycle. On this basis, the first structure-based models can be suggested that explain important aspects of protein targeting, such as the SRP-ribosome [54], SRP-SRP receptor [55] and SRP-SR interactions. The snapshots obtained by single-particle EM reconstructions enable us to follow the path of a nascent protein from the peptidyl-transferase center, through the ribosomal tunnel, to and across the translocon in the membrane. With new developments in image processing techniques it is possible to sort a biological homogenous sample into different conformational states and to reach subnanometer resolution such that folding of the nascent chain into secondary structure elements can be directly visualized [56].

Molecular biology, biochemistry, and cryo-electron microscopy, have been combined to study the ribosome-protein complexes involved in protein assembly, folding and targeting. These approaches led to obtain structural snapshots of entire pathways by which proteins are synthesized and targeted to their final positions. The link between SRP and its receptor is usually transient and chemically unstable, for this reason, engineered SRP receptor bind more stably to SRP, then introduced to ribosomes and observed the resulting complexes using cryo-electron microscopy (cryo-EM). Cryo-EM can be performed in roughly physiological conditions, providing a picture that closely resembles what happens in living cells. This picture can then be combined with higher-resolution crystallography data and biochemical studies [57-58].

### c. Peroxisomes

The oxidative stress (EO) is a disorder where reactive oxygen species (ROS) are produced. These compounds, that include free radicals and peroxides, play important roles in cell redox signaling. However, disturbances in the balance between the ROS production and the biological system can be particularly destructive. For example, the P450 oxide reductase activity produces  $H_2O_2$  as a metabolite. This enormous family of enzymes is present in the mitochondrial and smooth endoplasmic reticulum (SER) membranes and catalyzes several reactions in the pathway of the biogenesis of steroid hormones [59] and in the detoxification process or in the first stage of drugs or xenobiotics hydrolysis, converting them in the SER, in water-soluble compounds for its excretion in the urine [60-62].

Peroxisomes are single membrane organelles present in practically every eukaryotic cell. Matrix proteins of peroxisomes synthesized in free polyribosomes in the cytoplasm and imported by a specific signal, are encoded in genes present in the cell nucleus genome.

Peroxisomal membrane-bound PEX proteins, also encoded in the nuclear genome, are synthesized by ribosomes associated to rough endoplasmic reticulum since they display signal peptide. Therefore the peroxisome as an organelle derives from the rough endoplasmic reticulum. These organelles participate in ROS generation, as  $H_2O_2$ , but also in cell rescue from oxidative stress by catalase activity. In several biological models for pathological processes involving oxygen metabolites, the role of peroxisomes in prevention of oxidative stress is strongly suggested by de co-localization of catalase and  $H_2O_2$ , and the induction of peroxisomes proliferation [63].

#### d. Mitochondrion and chloroplast particles

##### i. ATP synthase: A rotary molecular motor

To support life, cells must be continuously supplied with external energy in form of light or nutrients and must be equipped with chemical devices to convert these external energy sources into adenosine triphosphate (ATP). ATP is the universal energy currency of living cells and as such is used to drive numerous energy-consuming reactions, e.g., syntheses of biomolecules, muscle contraction, mechanical motility and transport through membranes, regulatory networks, and nerve conduction. When performing work, ATP is usually converted to ADP and phosphate. It must therefore continuously be regenerated from these compounds to continue the cell energy cycle. The importance of this cycle can be best illustrated by the demand of 50 Kg of ATP in a human body on average [64].

Prokaryotes use their plasma membrane to produce ATP. Eukaryotes use instead the specialized membrane inside energy-converting organelles, mitochondria and chloroplasts, to produce most of their ATP. The mitochondria are present in the cells of practically all eukaryotic organisms (including fungi, animals, plants, algae and protozoa), and chloroplasts occur only in plants and algae. The most striking morphological feature of both organelles, revealed by electron microscopy, is the large amount of internal membrane they contain. This internal membrane provides the framework for an elaborate set of electron-transport processes, mediated by the enzymes of the Respiratory Chain that are essential to the process of Oxidative Phosphorylation which generate most of the cell's ATP.

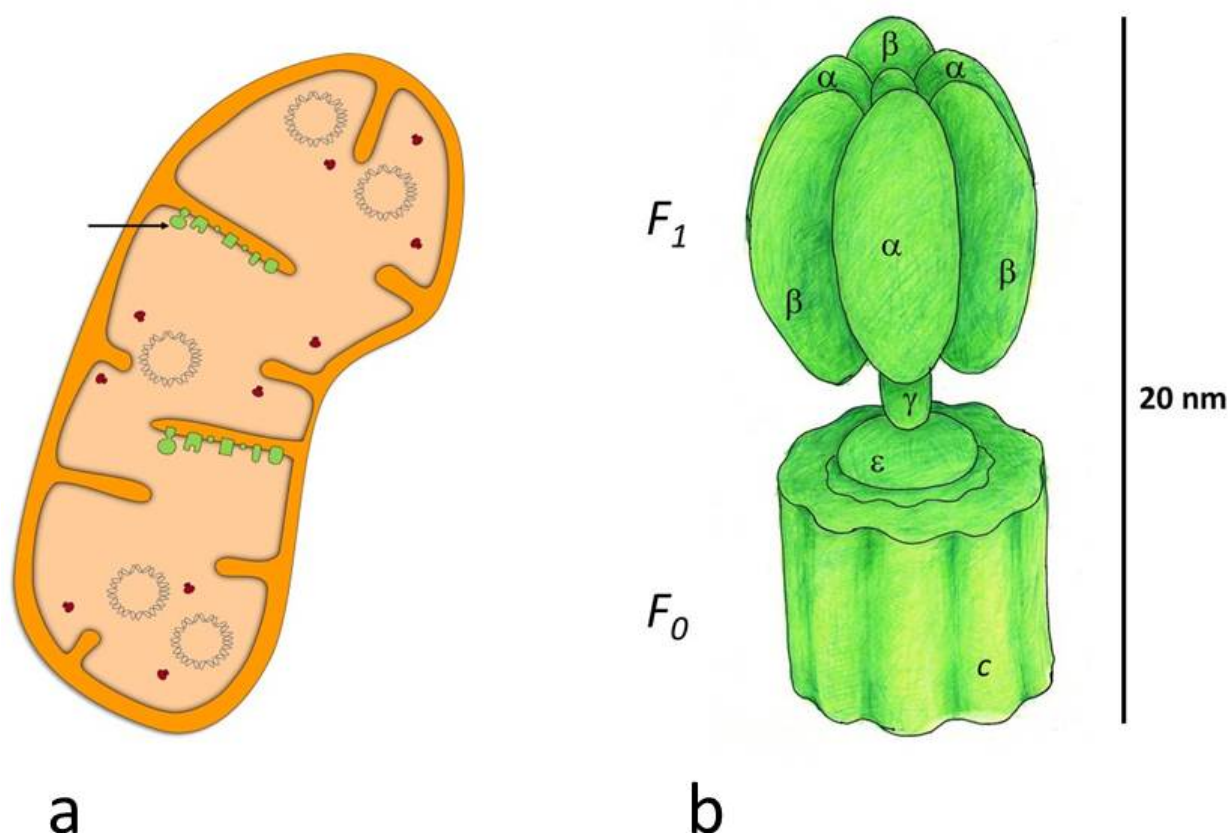
In eukaryotes, oxidative phosphorylation occurs in mitochondria and photophosphorylation in chloroplasts. In the mitochondria, the energy to drive the synthesis of ATP derive from the oxidative steps in the degradation of carbohydrates, fats and amino acids; whereas the chloroplasts capture the energy of sunlight and harness it to make ATP [60].

##### ii. The Chemiosmotic Model of Peter Mitchell

Our current understanding of ATP synthesis in mitochondria and chloroplasts is based on the chemiosmotic model proposed by Peter Mitchell in 1961 [60], which has been accepted as one of the great unifying principles of twentieth century. According with this model, the electrochemical energy inherent in the difference in proton concentration and the separation of charge across the inner mitochondrial membrane (the proton motive force) drives the synthesis of ATP as protons flow passively back into the matrix through a proton pore associated with the ATP synthase (Fig. 7).



Under aerobic conditions, the major ATP synthesis pathway is oxidative phosphorylation of which the terminal reaction is catalyzed by  $F_0F_1$ -ATP synthase. This enzyme is found widely in the biological world, including in thylakoid membranes, the mitochondrial inner membrane and the plasma membrane of bacteria, and is the central enzyme of energy metabolism in most organisms [65].



**Figure 7.** In the mitochondrion (a), ATP synthase (arrow in a; b) is part of the respiratory chain.

Like many transporters the  $F_0F_1$ -ATP synthase (or F-type ATPase) has been a fascinating subject for study of a complex membrane-associated process. This enzyme catalyzes ATP synthesis from adenosine diphosphate (ADP) and inorganic phosphate ( $P_i$ ), by using the electrochemical potential of protons (or sodium ions in some bacteria) across the membrane, i.e. it converts the electrochemical potential into its chemical form. ATP synthase also functions in the reverse direction (ATPase) when the electrochemical potential becomes insufficient: it catalyzes proton pumping to form an electrochemical potential to hydrolyze ATP into ADP and  $P_i$ . Proton translocation and ATP synthesis (or hydrolysis) are coupled by a unique mechanism, subunit rotation. Electrochemically energy contained in the proton gradient is converted into mechanical energy in form of subunit rotation, and back into chemical energy as ATP (Nakamoto RK, et al, 2008).

Mitochondrial ATP synthase is an F-type ATPase similar in structure and mechanism to the ATP synthases of chloroplasts and bacteria. This large complex of the inner mitochondrial membrane, also called Complex V, catalyzes the formation of ATP from ADP and Pi, accompanied by the flow of protons from P (positive) side to N (negative) side of the membrane [66].

### iii. $F_0F_1$ -ATP Synthase Structure and Function

ATP synthase is a supercomplex enzyme with a molecular weight of 500 kDal and consists of two rotary motors. One is  $F_1$  subcomplex (~380 kDal), which is the water-soluble part of ATP synthase.  $F_1$  was identified and purified by Efraim Racker and his colleagues in the early 1960s. When isolated from the membrane portion, it acts as an ATP-driven motor: it rotates its inner subunit to hydrolyze ATP and is therefore term  $F_1$  ATPase. The other rotary motor of ATP synthase is the membrane-embedded  $F_0$  subcomplex (~120 kDal) through which the protons flow.

In the simplest form of the enzyme, in bacteria like *Escherichia coli*,  $F_1$  is composed of five different subunits, in a stoichiometry of  $\alpha_3\beta_3\gamma\delta\epsilon$ , and  $F_0$  consists of three distinct subunits in a stoichiometry of  $ab_2c_{10-15}$ . A newer more mechanically-based division differentiates between the “rotor” (in *E. coli*,  $\gamma\epsilon c_n$ ) and the “stator” ( $\alpha_3\beta_3\delta ab_2$ ). The  $\alpha_3\alpha_3$  ring of the stator contains the three catalytic nucleotide sites, on the  $\beta$  subunits at the interphase to the adjacent  $\alpha$  subunit. The  $a$  subunit contains the static portion of the proton traslocator machinery.  $\alpha_3\beta_3$  and  $a$  are held together by the “stator stalk” (or “peripheral stalk”), consisting of  $b_2\gamma$  [65].

The crystallographic determination of the  $F_1$  structure by John Walker and colleagues [67] revealed structural details very helpful in explaining the catalytic mechanism of the enzyme. The three  $\alpha$ - and  $\beta$ - subunits that constitute the hexameric stator ring are alternately arranged like the sections of an orange. The rotor shaft is the  $\gamma$ -subunit, which is accommodated in the central cavity of the  $\alpha_3\beta_3$ -ring. The  $\epsilon$ -subunit binds onto the protruding part of the  $\gamma$ -subunit and provides a connection between the rotor parts of  $F_1$  and  $F_0$ . The  $\delta$ -subunit acts as a connector between  $F_1$  and  $F_0$  that connects the stator parts.

Catalytic reaction centers for ATP hydrolysis/synthesis reside at the three of the  $\alpha$ - $\beta$  interfaces, whereas the non-catalytic ATP-binding sites reside on the other  $\alpha/\beta$  interfaces. While the catalytic site is formed mainly with amino acid residues from  $\gamma$ -subunit, the non- catalytic sites are primarily within the  $\alpha$ -subunit. Upon ATP hydrolysis on the catalytic sites,  $F_1$  rotates the  $\gamma$ -subunit in the anticlockwise direction viewed from the  $F_0$  side [68].

As mentioned before,  $F_0$  subcomplex ( $o$  denoting oligomycin sensitive) consists of  $ab_2c_{10-15}$  subunits. The number of  $c$  subunits varies among the species and form a ring complex by aligning in a circle. It is widely thought that the  $c$ -ring and the  $a$  subunit form a proton pathway. With the downhill proton flow through the proton channel, the  $c$ -ring rotates against the  $ab_2$  subunits in the opposite direction of the  $\gamma$ -subunit of the  $F_1$  motor [69]. Thus, in the  $F_0F_1$  complex,  $F_0$  and  $F_1$  push each other in the opposite direction. Under physiological condition where the electrochemical potential of the protons is large enough to surpass the free energy of ATP hydrolysis,  $F_0$  forcibly rotates the  $\gamma$ -subunit in the clockwise direction and then  $F_1$  catalyzes the reverse reaction, *i.e.* ATP synthesis which is the principle function of ATP

synthase. In contrast, when the electrochemical potential is small or decreases,  $F_1$  forces  $F_o$  to rotate the  $c$ -ring in the reverse direction to pump protons against the electrochemical potential.

The  $c$  subunit of the  $F_o$  complex is a small (Mr 8,000), very hydrophobic polypeptide, consisting almost entirely of two membrane-spanning  $\alpha$ -helices, that are connected by a small loop extending from the matrix side of the membrane. The crystal structure of the yeast  $F_o F_1$ , solved in 1999, shows the arrangement of the subunits. The yeast complex has 10  $c$  subunits, each with two transmembrane helices roughly perpendicular to the plane of the membrane and arranged in two concentric circles. The inner circle is made up of the amino-terminal helices of each  $c$  subunit; the outer circle, about 55 Å in diameter, is made up of the carboxyl-terminal helices. The  $\epsilon$  and  $\gamma$  subunits of  $F_1$  form a leg-and-foot that projects from the bottom (membrane) side of  $F_1$  and stands firmly on the ring of  $c$  subunits. The  $a$  subunit is a very hydrophobic protein that in most models is composed of five transmembrane helices. Ion translocation takes place through subunit  $a$  and its interface with subunit  $c$ . The  $b$  subunits are anchored within the membrane by an N-terminal  $\alpha$ -helix and extend as a peripheral stalk all the way to the head of the  $F_1$  domain. According to cross-linking studies, the  $b$  subunits contact the C-terminal part of the  $c$  subunit and the loop between helices 4 and 5 of the  $a$  subunit at the periplasmic surface. The  $\delta$ -subunit forms a strong complex with the  $\alpha$ -subunit. In mitochondria, the peripheral stalk consists of more subunits named OSCP (Oligomycin Sensitive Conferring Protein),  $b$ ,  $\delta$  and  $F_6$  [64].

#### iv. Structure of $F_1$ and binding-change mechanism for ATP Synthesis.

The classic working model for  $F_1$  is the “binding-change mechanism” proposed by Paul Boyer [70]. The early stage of this model postulated an alternating transition between two chemical states, assuming two catalytic sites residing on  $F_1$ . It was later revised to propose the cyclic transition of the catalytic sites based on the biochemical and electron microscopic experiments that revealed that  $F_1$  has the three catalytic sites [71-73]. One important feature of this model is that the affinity for nucleotide in each catalytic site is different from each other at any given time, and the status of the three  $\beta$ -subunits cooperatively change in one direction accompanying  $\gamma$  rotation. This hypothesis is strongly supported by X-ray crystallographic studies performed by Walker’s group [67] that first resolved crystal structure of  $F_1$ , which revealed many essential structural features of  $F_1$  at atomic resolution. Importantly, the catalytic  $\beta$ -subunits differ from each other in conformation and catalytic state: one binds to an ATP analogue, adenosine 5’-( $\beta,\gamma$ -imino)-triphosphate (AMP-PNP), the second binds to ADP and the third site is empty. Therefore, these sites are termed  $\beta$ TP,  $\beta$ DP and  $\beta$ Empty, respectively. While  $\beta$ TP and  $\beta$ DP have a close conformation wrapping bound nucleotides on the catalytic sites,  $\beta$ Empty has an open conformation swinging the C-terminal domain away from the binding site to open the cleft of the catalytic site. These features are consistent with the binding-change mechanism. Another important feature found in the crystal is that while the N-terminal domains of the  $\alpha$ - and  $\beta$ -subunits form a symmetrical smooth cavity as the bearing for  $\gamma$  rotation at the bottom of the  $\alpha_3\beta_3$ -ring, the C-terminal domains of the  $\beta$ -subunit show distinct asymmetric interactions with the  $\gamma$ -subunit. Therefore, the most feasible inference is that the open-to-closed transition of the  $\beta$ -subunits upon ATP binding pushes  $\gamma$ , and the sequential conformational change among  $\beta$ -subunits leads the unidirectional  $\gamma$  rotation.

One strong prediction of the binding-change model of Boyer is that the  $\gamma$  subunit should rotate in one direction when  $F_0F_1$  is synthesizing ATP and in the opposite direction when the enzyme is hydrolyzing ATP. This prediction was confirmed in elegant experiments in the laboratories of Masasuke Yoshida and Kazuhiko Kinosita Jr. [74]. The rotation of  $\gamma$  in a single  $F_1$  molecule was observed microscopically by attaching a long, thin, fluorescent actin polymer to  $\gamma$  and watching it move relative to  $\alpha_3\beta_3$  immobilized on a microscope slide, as ATP was hydrolyzed [see 75]. Lately the unidirectional  $\gamma$  rotation was visualized in simultaneous imaging of the conformational change of the  $\beta$ -subunit and the  $\gamma$  rotation.

#### v. New approaches for studying biological macromolecules.

The Atomic Force Microscope (AFM) is a powerful tool for imaging individual biological molecules attached to a substrate and placed in aqueous solution. This technology allows visualization of biomolecules under physiological conditions. However, it is limited by the speed at which it can successively record highly resolved images. Recent advances have improved the time resolution of the technique from minutes to tens of milliseconds, allowing single biomolecules to be watched in action in real time. Toshio Ando and his coworkers at Kanazawa University have been leading innovators in this so-called High-Speed Atomic Force Microscope (HS-AFM) technology [76]. This technology allows direct visualization of dynamic structural changes and dynamic processes of functioning biological molecules in physiological solutions, at high spatial-temporal resolution. Dynamic molecular events appear in detail in an AFM movie, facilitating our understanding of how biological molecules operate to function.

In this regard, the Ando group showed a striking example of molecular motor action in their AFM movies of the isolated subcomplex of the rotary motor protein  $F_1$ -ATPase. Previous single-molecule experiments on parts of this enzyme had measured rotation, but they could only be done if at least one subunit of the rotor was attached. The AFM, however, could visualize the conformational change that the  $\beta$  subunits of the stator undergo when they bind ATP. By imaging at 12.5 frames/s, the authors followed the time dependence of these conformational changes, leading to the surprising conclusion that, contrary to what was widely assumed before, the catalysis on the enzyme maintains its sequential rotary order even in absence of the rotor subunits.

“To directly observe biological molecules at work was a holy grail in biology. Efforts over the last two decades at last materialized this long-quested dream. In high-resolution AFM movies, we can see how molecules are dynamically behaving, changing their structure and interacting with other molecules, and hence we can quickly understand in stunning detail how molecules operate to function. This new approach will spread over the world and widely applied to a vast array of biological issues, leading to a number of new discoveries. The extension of high-speed AFM to a tool for imaging live cells, which allows direct in situ observation of dynamic processes of molecules and organelles, remains an exciting challenge but will be made in the near future because it is a right and fruitful goal” [77].

#### e. Lipid rafts

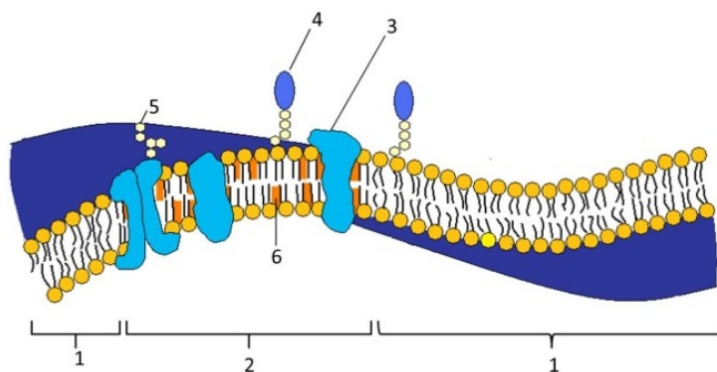
Cell membranes are dynamic assemblies of a variety of lipids and proteins. They form a protective layer around the cell and mediate the communication with the outside world. The



original fluid mosaic model [78] of membranes suggested a homogenous distribution of proteins and lipids across the two-dimensional surface, but more recent evidence suggests that membranes themselves are not uniform and that microdomains of lipids in a more ordered state exist within the generally disorder lipid milieu of the membrane. These clusters of ordered lipids are now referred to as lipid rafts [79] (Pike LJ 2009).

Lipid rafts (LRs), consist of cell membrane domains rich in cholesterol, sphingolipids and lipid-anchored proteins in the exoplasmic leaflet of the lipid bilayer. Because of their ability to sequester specific lipids and proteins and exclude others, rafts have been postulated to perform critical roles in a number of normal cellular processes, such as signal transduction [80], membrane fusion, organization of the cytoskeleton [81-83], lipid sorting, and protein trafficking/recycling, as well as pathological events [84].

LRs are too small to be resolved by standard light microscopy - they range from 10 to about 200 nm - with a variable life span in the order of milliseconds (msec). Detergent resistant membranes, containing clusters of many rafts, can be isolated by extraction with Triton X-100 or other detergents on ice. However, this method involves breaking up the membrane and has limitations in terms of defining the size, properties, and dynamics of intact microdomains [85-88]. Thus, a variety of sophisticated techniques have recently been used to analyze in detail open questions concerning rafts in cell and model membranes including biochemical, biophysical, quantitative fluorescence microscopy, atomic force microscopy and computational methodologies [89-90].



**Figure 8.** Components of a lipid raft. (1) non raft membrane, (2) lipid raft, (3) lipid raft associated transmembrane protein, (4) GPI-anchored protein, (5) glycosylation modifications (glycoproteins and glycolipids).

The raft affinity of a given protein can be modulated by intra- or extracellular stimuli. Saturated fatty acids are preferentially enriched in the side chains of the membrane phospholipids, which allows closer packing and thus increased rigidity, more order and less fluidity of the LR compared to the surrounding membrane [91-92]. Proteins with raft affinity include glycosylphosphatidylinositol (GPI)-anchored proteins [93-94], doubly acylated proteins, such as Src-family kinases or the  $\alpha$ -subunits of heterotrimeric G proteins<sup>8</sup>, cholesterol-linked and palmitoylated proteins such as Hedgehog<sup>9</sup>, and transmembrane proteins, particularly palmitoylated ones [92-95].

Different subtypes of lipid rafts can be distinguished according to their protein and lipid composition. Caveolae are types of rafts that are rich in proteins of the caveolin family (caveolin-1, -2 and -3) which present a distinct signaling platform [96]. The caveolae are enriched in cholesterol, glycosphingolipids, and sphingomyelin. They are the site of several important protein–protein interactions, for example, the neurotrophin receptors, TrkA and p75(NTR), whose respective interactions with caveolin regulates neurotrophin signaling in the brain. Caveolins also regulate G-proteins, MAPK, PI3K, and Src tyrosine kinases.

The most important role of rafts at the cell surface may be their function in signal transduction. Lipid rafts have been implicated as the sites for a great number of signaling pathways. They form concentrating platforms for individual receptors, activated by ligand binding [86]. If receptor activation takes place in a lipid raft, the signaling complex is protected from non-raft enzymes such as membrane phosphatases that otherwise could affect the signaling process. In general, raft binding recruits proteins to a new micro-environment, where the phosphorylation state can be modified by local kinases and phosphatases, resulting in downstream signalling. Individual signaling molecules within the raft are activated only for a short period of time.

Immobilization of signaling molecules by cytoskeletal actin filaments and scaffold proteins may facilitate more efficient signal transmission from rafts [97]. Current evidence supports a role for lipid rafts in the initiation and regulation of The B-cell receptor signaling and antigen trafficking [98-100]. The importance of lipid raft signalling in the pathogenesis of a variety of conditions, such as Alzheimer's, Parkinson's, cardiovascular and prion diseases, systemic lupus erythematosus and HIV, has been elucidated over recent years [101] and makes these specific membrane domains an interesting target for pharmacological approaches in the cure and prevention of these diseases [102]. Rafts serve as a portal of entry for various pathogens and toxins, such as human immunodeficiency virus 1 (HIV-1). In the case of HIV-1, raft microdomains mediate the lateral assemblies and the conformational changes required for fusion of HIV-1 with the host cell [103]. Lipid rafts are also preferential sites of formation for pathological forms of the prion protein (PrPSc) and of the  $\beta$ -amyloid peptide associated with Alzheimer's disease [104].

Plasma membranes typically contain higher concentrations of cholesterol and sphingomyelin than do internal membranous organelles [105-106]. Thus, along the secretion pathway, there are very low concentrations of cholesterol and sphingolipids in the endoplasmic reticulum, but the concentrations of these lipids increase from the cis-Golgi to the trans-Golgi and then to the plasma membrane [107-108]. On the contrary, recent evidence suggests that mitochondria do not contain lipid rafts, and lipid rafts do not contain mitochondrial proteins [109].

Lipid raft domains play a key role in the regulation of exocytosis [110]. The association of SNAREs protein complexes with lipid rafts acts to concentrate these proteins at defined sites of the plasma membrane that are of functional importance for exocytosis [111-114].

#### **f. The nucleolus**

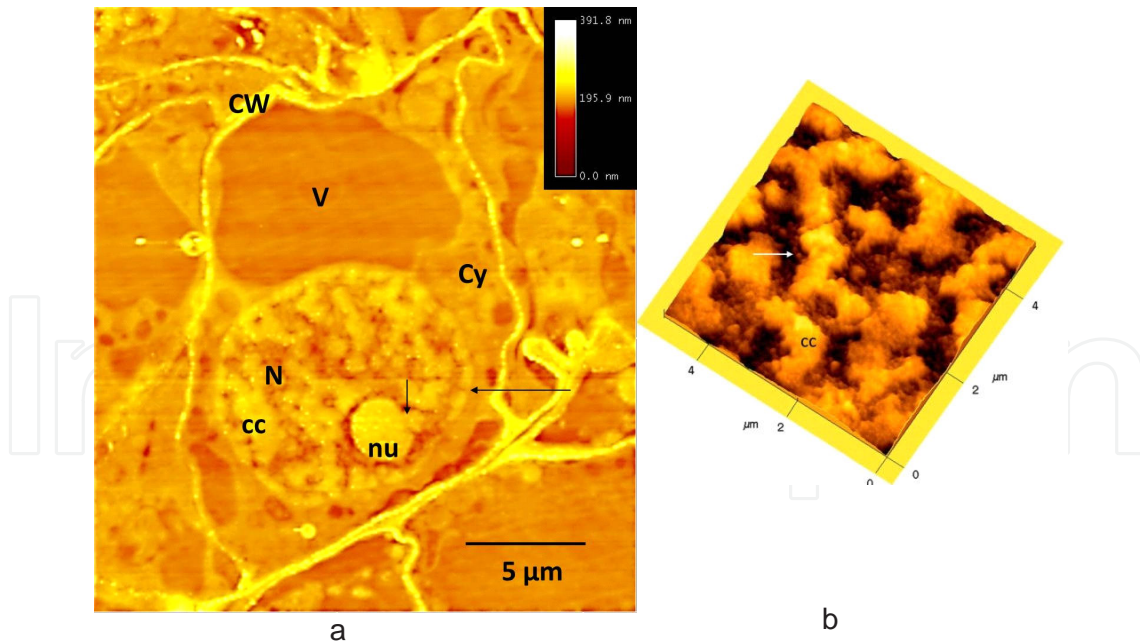
The cell nucleus contains different compartments that are characterized by the absence of delineating membranes that isolate it from the rest of the nucleoplasm [5]. Due to the high

concentration of RNA and proteins that form it, the nucleolus is the most conspicuous nuclear body in cycling cells observed by light and electron microscopy. Nucleoli are formed around nucleolar organizer regions (NORs), which are composed of cluster of ribosomal genes (rDNA) repeat units [115-121]. The number of NOR-bearing chromosomes varies depending on the species, can be found 1 in haploid yeast cells to 10 in human somatic cells (short arms of chromosomes 13, 14, 15, 21 and 22). Nucleolus is the organelle of rDNA transcription by RNA polymerase I, whose activity generates a long ribosomal precursor (pre-rRNA), this molecule is the target of an extensive process that includes removing or cutting the spacers and 2'-O-methylation of riboses and conversions of uridine residues into pseudouridines. The net result of these reactions is the release of mature species of ribosomal RNA (rRNA) 18S, 5.8S and 28S. These particles are assembled with approximately 82 ribosomal proteins and rRNA 5S (synthesized by RNA polymerase III) to form the 40S and 60S subunits; both of these subunits are then exported separately to the cytoplasm and are further modified to form mature ribosomal subunits. Currently, it is widely accepted that nucleolar transcription and early pre-rRNA processing take place in the fibrillar portion of nucleolus while the later steps of processing and ribosome subunits assembly occurs mainly in the granular zone. The architecture of the nucleolus reflects the vectorial maturation of the pre-ribosomes. The nucleolar structure is organized by three canonical subdomains that are morphologically and biochemically different. The fibrillar centers (FC), dense fibrillar component (DFC) and granular component (GC). The FCs are structures with a low electron density, often circular shape of ~0.1 to 1µm in diameter. The FCs are enriched with rDNA, RNA polymerase I, topoisomerase I and upstream binding factor (UBF). DFCs are a compact fibrillar region containing a high concentration of ribonucleoprotein molecules that confer a high electrodensity. This component entirely or partially surrounds the FCs. DFCs contains important proteins such as fibrillarin and nucleolin as well as small nucleolar RNAs, pre-rRNA and some transcription factors. FCs and DFCs are embedded in the GC, composed mainly of granules of 15 to 20nm in diameter with a loosely organized distribution. In the GC are located B23/nucleophosmin, Nop 52, r-proteins, auxiliary assembly factors, and the 40S and 60S subunits that the GC is itself composed of at least two distinct molecular domains. Considering the species, cell type and physiological state of the cell, there is considerable diversity in the prevalence and arrangement of the three nucleolar components.

On the other hand, the current eukaryotic nucleolus is involved in the ribosomal biogenesis but has been described as a multifunctional entity. Extra ribosomal functions include biogenesis and/or maturation of other ribonucleoprotein machines, including the signal recognition particle, the spliceosomal small nuclear RNPs and telomerase, processing or export of some mRNAs and tRNA, cell cycle and cell proliferation control, stress response and apoptosis [116]. The plurifunctional nucleolus hypothesis is reinforced by the description of nucleolar proteome of several eukaryotes. A proteomic analysis has identified more than 200 nucleolar proteins in *Arabidopsis* and almost 700 proteins in the nucleolus of HeLa cells. A comparison of nucleolar proteome from humans and budding yeast showed that ~90% of human nucleolar proteins have yeast homologues. Interestingly, only 30% of the human nucleolar proteome is intended for ribosomal biogenesis [120, 122].

### 1.1.3. Microscopy

Fundamental to approach the cell at the nanoscale in cell nanobiology are the classical and also remarkably new types of microscopy. Three different epochs characterize microscopy: 1) Light microscopy, developed since ca. 1500, where glass lenses and light as source of illumination are used to get resolution of up to 0.2  $\mu\text{m}$ . Different types such as bright field, phase contrast, differential interference contrast (Nomarsky), dark field, polarization, fluorescence, confocal, and super-resolution, are variants of this type of microscopy. 2) Electron microscopy, developed since early 1930s, where electromagnetic lenses and electrons as source of illumination are used to get resolution of up to nm or  $\text{\AA}$ . Transmission and scanning electron microscopy –including the environmental and high resolution modes- are the two forms of this microscopy. 3) Scanning probe microscopy, developed in the early 1980s, where no lenses or illuminations are used, but instead the microscope consists of a fine tip interacting with the samples to potentially obtaining atomic resolution. Scanning tunneling microscopy and atomic force microscopy are the major variants of this type of modern microscopy. Because atomic force microscopy may produce images at high resolution even under liquid, we have been using such microscopy for imaging the cell components. To test this approach, we used several cell types and generated images at low magnification (Figure 9a). Nuclear particles *i.e.* Lacandonia granules were already visualized using this approach (Figure 9b).



**Figure 9.** a) Atomic force microscopy image of a cell from the tegument of the plant *Lacandonia schismatica*. Cell wall (CW), vacuole (V), cytoplasm (Cy), Within the nucleus (N), compact chromatin (cc), nucleolus (nu), nucleolar organizer (small arrow) and nuclear pore (large arrow). b) Atomic force microscopy of Lacandonia granules within the nucleus of a tegument cell of the plant *Lacandonia schismatica*. Three dimensional displaying shows compact chromatin (cc) and associated particles (arrow).



#### 1.1.4. Further research

Further research in our laboratory will focusing in visualizing the nanoscale cell structures involved in fundamental processes as ribosome biogenesis, at a high resolution *in situ* under liquid conditions to perform quantitative analysis.

## 2. Conclusion

A view of the cell emphasizing vertical resolution obtained by atomic force microscopy may represent a way to understand cell structure and function at the nanoscale, an interphase between molecular biology and cell biology.

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