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Visualizing Nuclear Structure *In Situ* by Atomic Force Microscopy

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1. Introduction

The cell nucleus is an organelle where molecules involved in gene expression are highly compartmentalized ***(1)*** in very dynamic ***(2)*** territories. This current notion of functional organization has been possible because of many studies of this organelle that included its cell and molecular organization, and where microscopy played an important role. Light and electron microscopy in conjunction with molecular approaches, such as the construction of both antibodies and nucleic acid probes, as well as molecular construction of genes with green fluorescent protein and fluorescence recovery after photobleaching technology, in addition to high-resolution *in situ* hybridization and immunocytochemistry now offer a more complete knowledge.

Structurally, the mammalian cell nucleus is organized as an organelle surrounded by a double layer of phospholipids called the nuclear envelope, which is interrupted by the nuclear pores. It is covered in the interior by the nuclear lamina. Within the cell nucleus, a nuclear matrix is present as a nuclear skeleton made of several different proteins. DNA is organized as euchromatin and heterochromatin. The ribonucleoproteins are distinguished by their participation in pre-mRNA or pre-rRNA metabolism. In the first case, ribonucleoproteins are visualized as nuclear speckles by light microscopy, which correspond to interchromatin granule clusters and perichromatin fibers observed by electron microscopy. In addition, perichromatin granules are also present. All these structures are related to transcription and splicing of pre-mRNA and transport or storage of intranuclear mRNA. The nucleolus, however, is involved in pre-rRNA transcription and processing as well as ribosome assembly. There are other nuclear structures, such as the Cajal bodies, which contain factors

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involved in both pre-mRNA and pre-rRNA metabolism ***(1–8)***. In plants, there are two major classes of nuclei: (1) the chromocentric and (2) the reticulated types, depending on the arrangement of heterochromatin fibers ***(9–12)***. In the first type, DNA is organized as discrete clumps within the nucleoplasm. In the second one, DNA forms a reticulated strand.

Lacandonia schismatica is a rare plant whose most important feature is the inverted position of the sexual organs, with the androecium in the center, surrounded by the gynoecium ***(13,14)***. In studying the biology of this species, we have analyzed the cell biology, including the interphase cell nucleus, which we have published previously ***(15–20)***. The cell nucleus of *L. schismatica* is reticulated.

To explore the possibility of studying the interphase cell nucleus with high resolution, while at the same time of working in solution, we have been studying the nuclear structure by using an approach whereby a sample is prepared for transmission electron microscopy (TEM) and the surface of the unstained semithin sections is explored with an atomic force microscopy working in contact mode ***(17,19)*** to visualize the interior of the cell. It is a common observation when sectioning with an ultramicrotome that trimming of the plastic blocks to get thin sections many times reveals the profile of the embedded cells over the surface. This observation suggested that even very flat surfaces have a texture corresponding to cell organelles, and indeed this is the case. Therefore, because the microscope is an instrument to analyze surfaces, the surface of each section can be visualized as a representative sample of a portion of the cell interior. Similar approaches also have been used previously ***(21,22)*** to study cell structure *in situ*. As a first step, we have been validating this approach by observing and generating images of already known material. For these purposes we have been visualizing the nuclear structure of *L. schismatica* by using the protocols described in **Subheading 3.** ***(17,19)***.

2. Materials

2.1. Sample Preparation

1. 70% Glutaraldehyde (Polysciences). Toxic. Wear gloves, goggles and work in a chemical fume hood. Store at 4°C.
2. 2% Osmium tetroxide. Toxic. Wear gloves, goggles and work in a chemical fume hood. Store at 4°C. Protect from light.
3. Phosphate-buffered saline (PBS) 0.01 *M*, pH 7.4. Store at 4°C.
4. Deionized water.
5. 100% Ethanol, electron microscopy grade.
6. 100% Propylene oxide, electron microscopy grade.
7. Epoxy resin (glycidether 100, Merck). Plastic resin.
8. DDSA (dodecenylsuccinic anhydride) hardener (Merck).

9. NMA (methylnorbornene-2,3-dicarboxylic anhydride) plasticizer (Merck)..
10. DMP (2, 4, 6-Tris[dimethyl-aminomethyl]phenol) accelerator (Merck)..
11. Toluidine blue.
12. Clean glass slides. Keep within a chamber to avoid dust.
13. Ultramicrotome (Reicher-Jung).
14. Glass knives.
15. Diamond knife.

2.2. Microscope

1. BioScope (Digital Instruments, Santa Barbara, CA).
2. NanoScope IIIa (Digital Instruments, Santa Barbara, CA) control system and the software.
3. Inverted Diaphot 200 microscope (Nikon, NY).
4. Silicone nitride tips, 20–60 nm radius of curvature (model NP).
5. 100-µm Atomic force microscope scanner.
6. Proscan software (version 3.1, Park Scientific, 1997)

3. Methods

3.1. Sample Preparation

Samples are prepared as for standard TEM or histology. For TEM, we have used several protocols, including different types of fixation with aldehydes such as glutaraldehyde or paraformaldehyde at different concentrations, or with embedding media such as glycol methacrylate, London resin white, Lowicryl®, and epoxy resin. Here, we describe the regular protocol for TEM ***(15,23–25)*** because it produced thus far the best results as far as morphology is concerned.

1. Samples are fixed with freshly made 6% glutaraldehyde from 70% vials, buffered with 0.01 *M* PBS at pH 7.4 for 2–6 h at room temperature. PBS is prepared as follows ***(23)***: dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of distilled water. Adjust pH to 7.4 with HCl. Add water to 1 L. Sterilize by autoclaving.
2. After fixation, samples are washed with PBS for three times 10 min each.
3. Postfixation is performed with 1% osmium tetroxide in bidistilled water for 1–2 h. Prepare from a stock solution of 2% made from crystals in bidistilled water.
4. Dehydration is conducted with a series of graded concentration of ethanol, 10 min each of 30, 50, 70, 80, 90, and 96, and three changes of 100% ethanol. Propylene oxide is then used for three times for 10 min each.
5. For preembedding, samples are placed in a mixture of 1:1 of propylene oxide and epoxy resin during 16 h at room temperature. The epoxy resin is made as a stock solution. A recommended proportion of the components for working with plants can be taken from the references ***(23)*** and will contain 9.44 g of epoxy resin, 5 g of DDSA, 10 g of NMA, and 0.2 mL of DMP.

Mix the components thoroughly with a glass rod avoiding making bubbles. Store at 4°C in a small jar avoiding the penetration of air by sealing with parafilm. Prepare in the chemical fume hood. Avoid contact with the skin and to inhale. *See* **Note 1.**

6. Embedding is conducted with epoxy resin for 16 h at 60°C.
7. Semithin sections of about 150–250 nm width are obtained with an ultramicrotome working with glass or diamond knives. *See* **Note 2.**
8. Two sets of sections are placed onto an ethanol-cleaned glass slide. The sections are transferred by placing a platinum loop onto a drop of water. The sections are placed onto a glass slide. Sections are fixed to the glass by gently heating the slide. One set of sections is stained with toluidine blue and rinsed thoroughly with bidistilled water. Toluidine blue stain is prepared as a stock solution by dissolving 1% toluidine blue in 1% sodium borate, to stain the sections but not the plastic. For staining, once the slide reaches room temperature after fixing to the glass, cover the section with one or two drops of the stain and place it on a hot plate until stain starts to dry and obtain a metallic green color at the borders. Rinse the excess stain off the slide with bidistilled water. Sections are stored in a chamber to avoid dust.

3.2. Atomic Force Microscopy

An atomic force microscope model Bioscope (Digital Instruments) is used for observations. The microscope is mounted on an inverted light microscope Diaphot 200 (Nikon). Observations are made in contact mode. The scan size are from 100 to 5 µm at a scan rate varying from 1.969 to 1.285 Hz. Images were generated with the NanoScope IIIa control system. Alternatively, an atomic force microscope from Park Scientific has been used ***(17,19)***. In that case, the microscope is equipped with a scanner of 100 µm and a 100-Å radius silicone nitride tip, mounted on a cantilever of 0.6 µm. A scan rate of 2–3 Hz, a force of 10 nN, and a gain of 0.5 arbitrary units have been used. Images are then generated with Proscan. (*See* **Note 3.**) For the BioScope model, it is suggested to follow the next steps:

1. Fix the slide onto the stage by using the vacuum system provided.
2. Localize the sample with the bright field microscope using a low-magnification objective.
3. Approach the head of the microscope manually.
4. Visualize the cantilever and the tip with the light microscope and align the tip (*see* **Note 4**).
5. Adjust parameters as scan size to 100 µm, scan rate to about 1.5 Hz, and data scale to about 400 nm.
6. Engage the microscope and scan the sample (*see* **Note 5**).
7. Generate the images using the Nanoscope IIIa software provided (**Figs. 1** and **2**; (*see* **Notes 6** and **7**).

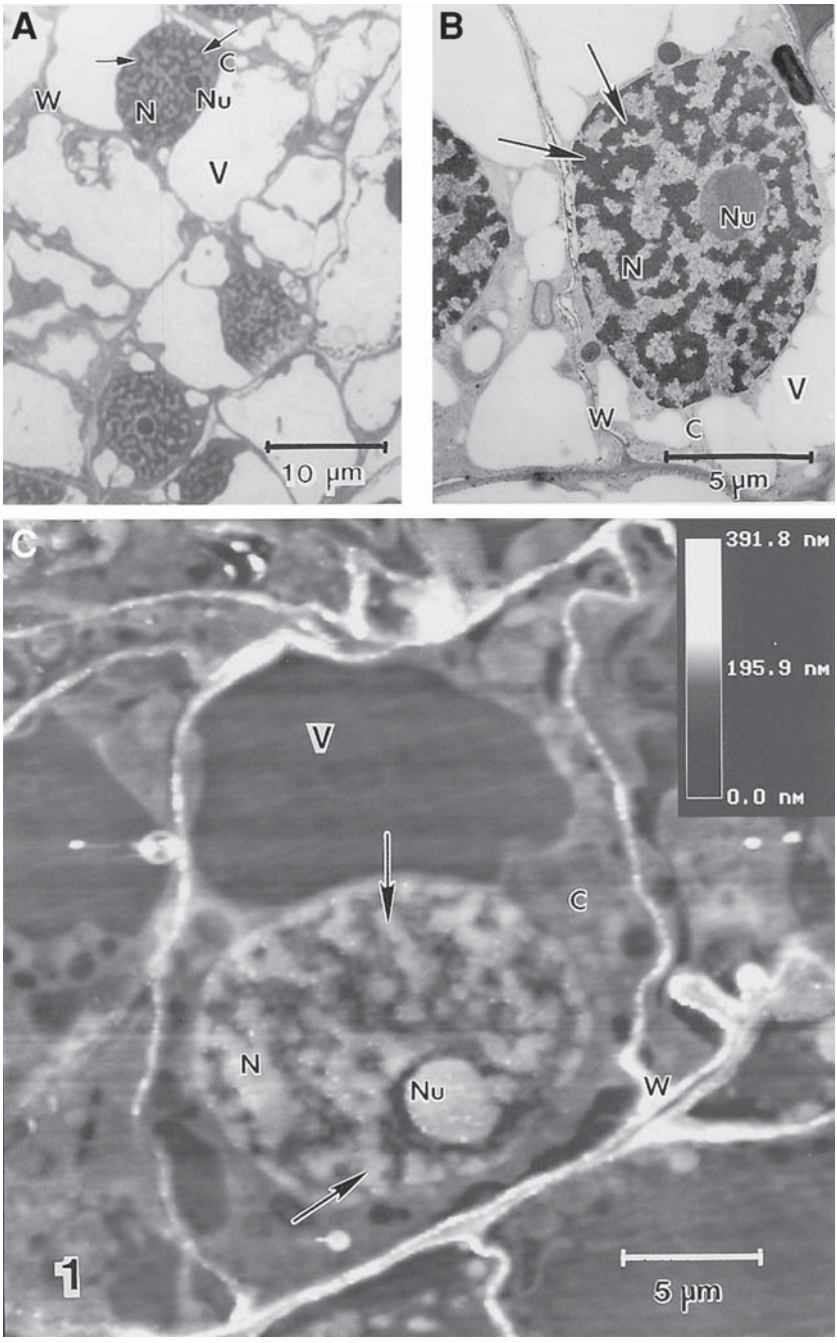

Fig. 1. Light **(A)**, electron **(B)**, and atomic force **(C)** microscopy of cell nuclei from the tegument cells from an ovary of a flower of the plant *Lacandonia schismatica*. The three types of microscopes generate a similar image of the cell nuclei. However, the resolution in every case varies according to the instrument. N, nucleus; Nu, nucleolus; arrows, reticulated compact chromatin; W, cell walls; V, vacuoles; C, cytoplasm.

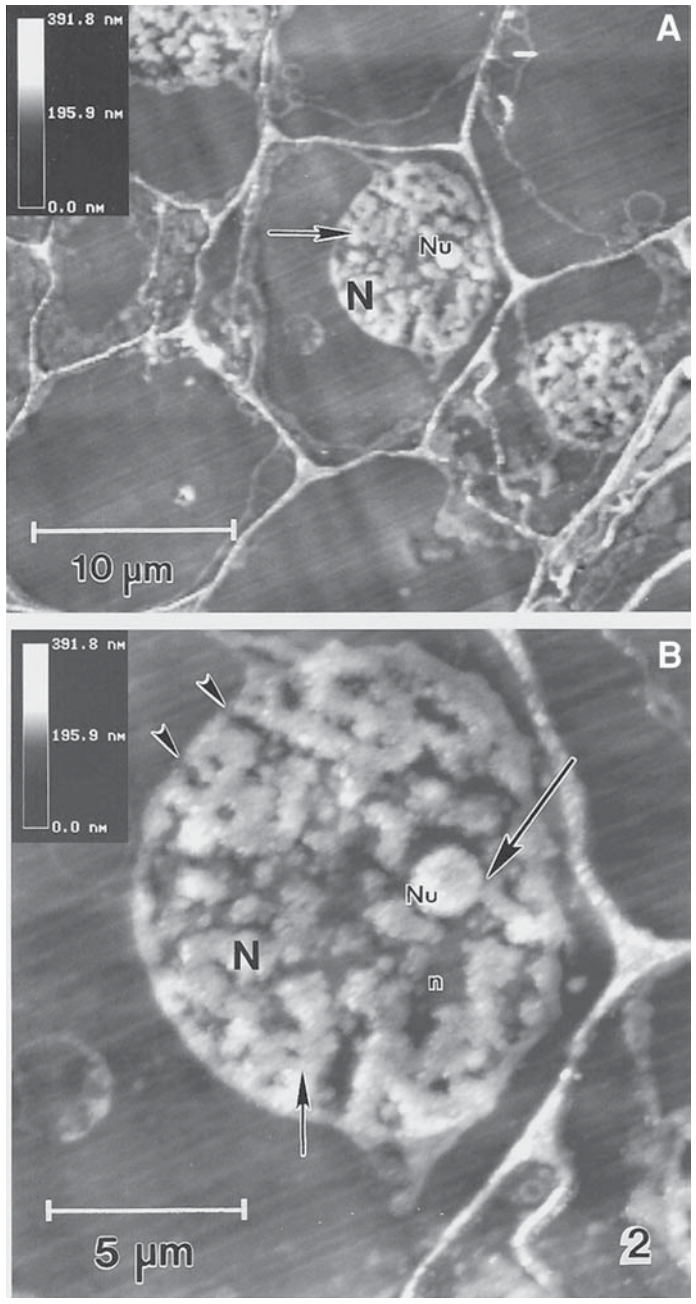

Fig. 2. Atomic force microscopy at low **(A)** and high **(B)** magnification of a *L. schismatica* cell nucleus. N, nucleus; Nu, nucleolus; small arrow, reticulated compact chromatin. In (B), nuclear pores (arrowheads) are observed as discontinuities of the nuclear envelope. The nucleolar organizer region (large arrow) is observed as a portion of compact chromatin strand that makes contact with the nucleolus. The nucleoplasm (n) is seen as texturized material among chromatin strands.

4. Notes

1. Regarding the epoxy resin for embedding, it is better to prepare the hardest mixture, by varying the concentration of the components such as the hardener (DDSA).
2. For obtaining sections it is better to use a diamond knife, although a glass knife also produces good quality material. Use only very clean water during the sectioning.
3. Before observation, it is recommended to place two sets of semithin sections onto the glass slide. One set is intended for staining with toluidine blue and the other will remain unstained. Under a bright-field microscope, the stained sections will be useful in recognizing the area to be scanned with the atomic force microscope on the unstained sections, which will be placed close by the stained sections. After staining, rinse the sections thoroughly with bidistilled water and air dry them.
4. Because the slide is placed sample side up, the sections face the cantilever and the tip of the atomic force microscope. However, the 10, 16, 20, and 40× objectives will image the sample through the width of the slide. Moreover, a fiberglass light will be used to illuminate the sample laterally. In an inverted microscope, it will not be possible to observe with the atomic force microscope and simultaneously perform bright field illumination according to Köhler, because the BioScope microscope is mounted on a special stage and it will interfere with the light path of a halogen lamp. Therefore, although the image visualized under the 10, 16, and 20× objectives will be of relatively good quality, when using the 40× objective, the image will be fuzzy as a result of the width of the glass. In general, however, it will not be necessary to use the 40× objective to localize particular areas of interest.
5. A scanning field of about 100 µm is recommended at the beginning, in order to easily recognize structures at low magnification. Once a good area of interest is obtained, smaller areas can be analyzed. It is better to approach smaller areas of scanning gradually.
6. It is a common practice in our laboratory to generate images of both stained and corresponding unstained samples for comparison.
7. To operate the microscope, it is reccommended to start using the parameters as scan size accordingly (from 100 to 5 mm) The scan rate will be around 1.285, whereas the image data will remain at height and the data scale around 400 nm.

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