Chapter 1
Physical Properties of the Nucleus Studied by Micropipette Aspiration

Amy C. Rowat

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Abstract Understanding the physical properties of the cell nucleus is critical for developing a deeper understanding of nuclear structure and organization as well as how mechanical forces induce changes in gene expression. We use micropipette aspiration to induce large, local deformations in the nucleus, and microscopy to image nuclear shape as well as the response of fluorescently labeled components in the inner nucleus (chromatin and nucleoli) and the nuclear envelope (lamins and membranes). By monitoring the response of nuclear structures to these deformations, we gain insights into the material properties of the nucleus. Here we describe the experimental protocols for micropipette aspiration of nuclei in living cells as well as isolated nuclei. In addition to confocal imaging, deformed nuclei can be imaged by brightfield or epifluorescence microscopy.

1 Introduction

Many fundamental biochemical processes, including DNA replication and gene transcription, are compartmentalized within the cell nucleus. In addition to these biochemical functions, the nucleus is an organelle within the cell that is subject to mechanical forces transmitted by the surrounding cytoskeleton. Despite evidence that physical forces induce changes in gene expression ***(1)***, the mechanical properties of the cell nucleus are not well understood. Understanding the interplay between gene expression and physical environment is critical for a more complete knowledge of phenomena from differentiation to aging. The mechanical properties of the nucleus are also of interest in the context of disease: increasing evidence suggests that nuclear structure and stability are altered in laminopathies, disorders associated with mutations in genes encoding nuclear envelope proteins (reviewed in refs. ***(2–4)***). The mechanism of these tissue-specific disorders is not completely understood, but one hypothesis proposes that nuclei with decreased mechanical stability become damaged in load-bearing tissues, thus giving rise to disease.

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Table 1.1 Components of isolated nuclei and nuclei in living cells are labeled with fluorescent probes and visualized subsequent to micropipette deformation

Structure	Fluorescent probe	Method
Lamins	GFP-Lamin A	Transfection
Membranes	Membrane dye (DiIC18)	Incubation with isolated nuclei
Nuclear pore complexes	Anti-nucleoporin 62 + secondary antibody	Indirect immunofluorescence
DNA	SYTOX orange	Incubation with isolated nuclei
Nucleoli	CFP-NLS-NLS-NLS	Transfection

Transfection of genes encoding fluorophore-tagged proteins is an effective way to stain nuclei in living cells. Isolated nuclei can be additionally stained by indirect immunofluorescence and/or by incubation with fluorescent probes, including DNA dyes and membrane stains (Fig. 1.1)

CFP, cyan fluorescent protein

Mechanical properties of nuclei can be determined experimentally: techniques to investigate how the nucleus responds to mechanical forces involve deforming the cell or nucleus, and monitoring the resultant response ***(4)***. Micropipette aspiration is one such technique that facilitates large, local deformations of cells and nuclei; characterizing the response of nuclei to such external forces yields insights into material properties of nuclei ***(5–8)***.

We couple micropipette aspiration to brightfield as well as confocal microscopy to probe and characterize nuclear physical properties under deformation in three dimensions. To gain detailed information about the response of particular nuclear structures to mechanical perturbation, we label specific nuclear components in isolated nuclei and in living cells, deform nuclei by micropipette aspiration, and image the resulting deformations. These studies reveal that the nuclear envelope behaves as a solid–elastic shell ***(7)***, that nuclear membranes are fluid in nature, and that loss of specific nuclear envelope proteins alters nuclear envelope elastic properties ***(8)*** (*see* Table 1.1).

2 Materials

2.1 Cell Culture and Transfection

1. Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% glutamate, and 1% penicillin/streptomycin (BioWhittaker, Cambrex, East Rutherford, NJ, USA).
2. Carrier DNA: herring testes DNA (Clontech, San Jose, CA, USA).
3. Enhanced GFP conjugated to lamin A construct (eGFP-LamA) (a generous gift from D. Shumaker and R.D. Goldman) ***(9)***.

4. Construct of CFP conjugated to three nuclear localization sequences (CFP-NLS-NLS-NLS) (Clontech).
5. $CaPO_4$ Transfection kit (Invitrogen, Carlsbad, CA, USA).

2.2 *Preparation of Isolated Nuclei*

All salts, creatine phosphokinase, and creatine phosphate are obtained from Sigma (St. Louis, MO, USA). Stocks are stored at 4°C unless otherwise specified. Note that $MgCl_2$ is very hygroscopic, so it is best to buy it in small vials and not to leave open for long periods of time (*see* **Note 1**).

1. Hypotonic buffer: 10 m*M* HEPES-KOH (pH ~7.9), 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5 m*M* DTT (add fresh from a frozen stock solution), protease inhibitor (complete tablets, EDTA-free; Roche, Penzburg, Germany) (1 tablet/50 mL buffer, shake to dissolve). Prepare fresh just prior to isolation.
2. Dounce tissue homogenizer (7 mL) (Wheaton Scientific, Millville, NJ, USA).
3. Refrigerated laboratory centrifuge.
4. Isolated nuclei are resuspended in a physiological buffer whose ionic composition mimics that of the cytoplasm [9] (*see* **Note 2**). Stock solutions are prepared: 1 *M* KCl, 1 *M* Na_2ATP, 1 *M* DTT, and 0.2 *M* Na_2HPO_4 and stored at 4°C (1 *M* KCl and 0.2 *M* Na_2HPO_4) or at –20°C (1 *M* Na_2ATP and 1 *M* DTT). An energy regeneration system is added of creatine phosphokinase (obtain in powder form, store at –20°C, and add just prior to resuspension) and creatine phosphate (1 *M*, store in 1-mL aliquots at –20°C). The final concentrations of components are described in **Section 3.2.4.**
5. SYTOX Orange (Molecular Probes, Eugene, OR, USA).
6. DiIC18 (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (Molecular Probes).
7. Anti-nucleoporin p62 IgG, 250 µg/mL (BD Transduction Laboratories, San Diego, USA).
8. Cy3-conjugated goat anti-mouse IgG, 0.75 mg/mL (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

2.3 *Micropipette Aspiration*

2.3.1 Preparing Pipettes

1. Glass capillaries, 1-mm diameter (World Precision Instruments, Sarasota, FL, USA).
2. Pipette puller (Sutter Instruments Co., Novato, CA, USA).
3. Microforge (MF-900, Narishige, Japan).

4. Silanization solution II (~2% dimethyldichlorosilane in 1,1,1-trichloroethane; Sigma-Aldrich, Denmark).
5. 1-mL plastic syringe fitted with a 97-mm long, 28-gauge backfiller (Microfil, World Precision Instruments, Sarasota, FL, USA).

2.3.2 Micropipette Setup

1. Micropipette setup:
   (a) A home-built micropipette setup consisting of a manometer, a pressure transducer, and micromanipulators or *x*-*y* translation stage ***(11, 12)*** or
   (b) An Eppendorf micropipette system, e.g., Injectman and Cell-Tram Air.
2. Microscope:
   (a) An inverted microscope (Zeiss Axiovert S100, Zeiss LD Achroplan ×40/0.65; Zeiss, Göttingen, Germany) or
   (b) A confocal microscope (Axiovert 200 M laser scanning confocal model 510 equipped with a META polychromatic multichannel detector and Apochromat 403/1.2 W corrected water immersion objective (Zeiss).
3. Charge-coupled device (CCD) camera (XC-85000E Donpisha; Sony, Japan) connected to a computer via a frame grabber (Sigma-SLC; Matrix Vision, Oppenweiler, Germany).

2.3.3 Aspirating Isolated Nuclei

1. Two-well chamber with a borosilicate coverslip bottom (0.17-mm thick) (Nalge Nunc, Slangerup, DK).

2.3.4 Aspirating Nuclei in Intact, Adherent Cells

1. CO_2-independent medium: Minimum Essential Medium without phenol red containing 10% FBS, 1% glutamate, 1% sodium pyruvate, 1% penicillin/streptomycin, and 1% 1 *M* HEPES (Invitrogen).
2. Objective heater (Bioptech, Butler, PA, USA).
3. Coverslips (20 × 0.17 mm) (LaCon, Staig, Germany).

3 Methods

3.1 Cell Culture and Transfection

Cells are grown at 37°C. Transfection is performed with a twofold excess of carrier DNA into HeLa/MEF cells at 70–80% confluency using $CaPO_4$ and cells are then plated on coverslips. This procedure is used to transfect HeLa and MEF cells with

eGFP-Lamin A, as well as HeLa cells with CFP-NLS-NLS-NLS, and mutant lamin proteins (*see* **Note 3**).

3.2 Preparation of Isolated Nuclei

1. Steps 3–6 are carried out on ice, so an ice bucket must be prepared in advance. Both the hypotonic buffer and Dounce homogenizer are cooled on ice before use and the homogenizer is rinsed with hypotonic buffer.
2. Cells are grown to 90% confluency and are harvested by trypsinization, washed three times with PBS, and resuspended in 5 mL of hypotonic buffer. The resuspended cells are incubated on ice for 8–10 min or until the plasma membranes are sufficiently swollen, as observed using phase contrast microscopy. When swollen and looking like they are about to burst, the cells are transferred to the Dounce homogenizer.
3. After ten strokes of the tight pestle in a Dounce homogenizer, 2–3 μL of the nuclear suspension is placed on a microscope slide for inspection: nuclei should appear clean, without too many external membranes. If swollen cells are still visible, a few more strokes of Douncing is required to release the nuclei (*see* **Note 1**).
4. Nuclei are collected by centrifugation (~200×*g*, 4°C) for 5 min, forming a nuclei-enriched pellet. The supernatant is collected and stock solutions (*see* **Section 2.2.4**) are added to give final concentrations of 130 m*M* KCl, 1.5 m*M* $MgCl_2$, 10 m*M* Na_2HPO_4, 1 m*M* Na_2ATP, and 1 m*M* DTT, pH 7.4. Adjusting the concentration of the supernatant rather than using fresh buffer maintains all soluble factors that may be released during nuclear isolation. An energy regeneration system (0.1 mg/mL creatine phosphokinase and 5 m*M* creatine phosphate) is also added to the supernatant and the nuclei-enriched pellet is resuspended in 1 mL of this physiological buffer. A good nuclear isolation for one 10-cm and one 15-cm plate (*see* **Note 1**) typically yields 10^7 nuclei/mL.

3.2.1 Staining of Isolated Nuclei

1. Nuclear pore complexes (indirect immunofluorescence). A volume of 600 μL of isolated nuclei, resuspended in "physiological" buffer, are incubated with 1.5 μg of p62 antibody for 15 min. After centrifugation (100 × *g*), the supernatant is removed and the nuclear pellet is resuspended in physiological buffer and incubated for 15 min with 4.5 μg of secondary antibody (Cy3-conjugated goat anti-mouse IgG). Subsequent centrifugation and resuspension in physiological buffer results in nuclei with labeled nuclear pore complexes and minimal background fluorescence.
2. Membranes. Membranes of isolated nuclei are labeled by incubation with DiIC18 at a final concentration of 0.01 μ*M* in physiological buffer for 15 min. Nuclei that are not cleanly isolated and remain associated with external

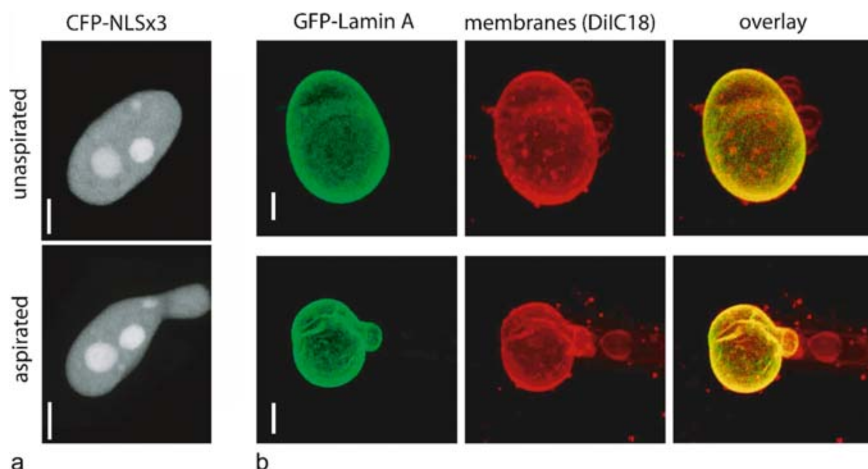

Fig. 1.1 Images of nuclei deformed by micropipette aspiration. Shown here are examples of nuclei both isolated from and in living cells with different stained components. **a** A nucleus in an intact HeLa cell transiently expressing CFP-NLSx3. This fluorophore preferentially localizes to nucleoli. **b** Nucleus isolated from a HeLa cell. The nuclear lamina is visualized in the nuclei of cells transiently expressing GFP-Lamin A. Nuclear membranes of isolated nuclei are stained with the lipophilic probe, DiIC18. Reprinted with permission from [*8*]. To view this figure in color, see COLOR PLATE 1

membranes from, for example, the endoplasmic reticulum, are easily identified because the membrane staining extends beyond the periphery of the nucleus itself; such nuclei should be excluded from analysis. *See* Fig. 1.1b for images of membrane-stained nuclei.

3. Nucleic Acids. SYTOX Orange is used to stain nucleic acids by incubation with isolated nuclei at a final concentration of 0.1 μ*M* for 15 min prior to imaging (*see* ***Note 4***).

3.3 Micropipette Aspiration

3.3.1 Preparing Pipettes

1. Pipettes are pulled from glass capillaries using a pipette puller and are forged to ensure a flat tip with an inner diameter of 1.5–5.0 μm.
2. After pulling and forging the capillaries, the micropipettes are dipped in silanization solution to prevent sticking of biological material to the surface and are then incubated in an oven at 90°C for 120 min. After cooling to room temperature they are inspected on the microforger for clogging that can result from the silanization treatment. The bottom coverslip of the chamber is also silanized.
3. Pipettes are backfilled with physiological buffer using the 1-mL syringe fitted with a backfiller.

3.3.2 Micropipette Setup

The microscope and micropipette setup are mounted on an air-cushioned table that dampens vibrations, ensuring the pipette remains stationary during image acquisition. The pipette is secured in place so that applying an aspiration pressure does not cause it to move. Micromanipulators are used to position the micropipette and pressures are applied to nuclei through the micropipette. Using a custom-built manometer system and a pressure transducer ***(11, 12)***, the aspiration pressure is measured to range from 1 to 7 kPa. Note that pressure transducers are necessary for quantitative studies of deformation as a function of aspiration pressure.

3.3.3 Aspirating Isolated Nuclei

1. Isolated nuclei are visualized at room temperature (25°C) in a chamber that allows for micropipette access: the side of a two-well borosilicate chamber is carefully excised using a razor blade (Fig. 1.2a). A thermostated chamber may also be used to regulate temperature (Fig. 1.2b).
2. A large drop of buffer (~500 µL) is placed in one well of the two-well borosilicate chamber and the micropipette tip is placed inside the drop (Fig. 1.2a).
3. A volume of ~100 µL of resuspended nuclei are pipetted into the drop. Nuclei settle to the bottom of the chamber over several min.
4. The pipette is lowered to the bottom of the chamber into the same focal plane as the nuclei. With the micropipette fixed in position, the chamber is translated until the desired nucleus is located. The micropipette is manipulated so that the edge is as close to the nucleus as possible and an aspiration pressure is applied. Pressures on the order of 1 kPa induce initial deformation of isolated nuclei: the nucleus is drawn toward the mouth of the pipette by the negative pressure and into the pipette, forming a nuclear projection (tongue) inside the pipette. During pipette manipulation, dust and cell debris may block the orifice; if these cannot be removed (see **Note 5**) a fresh pipette must be mounted.
5. To avoid interactions between substrate and nucleus during measurement, the pipette is raised in the vertical direction. Deformed nuclei are imaged either by brightfield, epifluorescence, or confocal microscopy (*see* **Note 5**). Measurements are typically performed either by applying increasing aspiration pressure and analyzing the increase of the tongue length, or by analyzing the tongue length as a function of time at constant pressure.
6. After an aspiration measurement is complete, the nucleus is ejected from the pipette by applying a positive pressure. Gentle tapping of the pipette with a fine object such as a screwdriver or pen can help to dislodge nuclei from the pipette. Larger aggregates of nuclei and/or unwanted particles can be removed by withdrawing the pipette from the drop of buffer and reinserting it through the air-liquid interface. In the case of persistent clogging, it is necessary to load a fresh pipette. Buffer and nuclei should be replaced after no more than 15 min to avoid marked changes in buffer conditions (*see* **Note 6**).

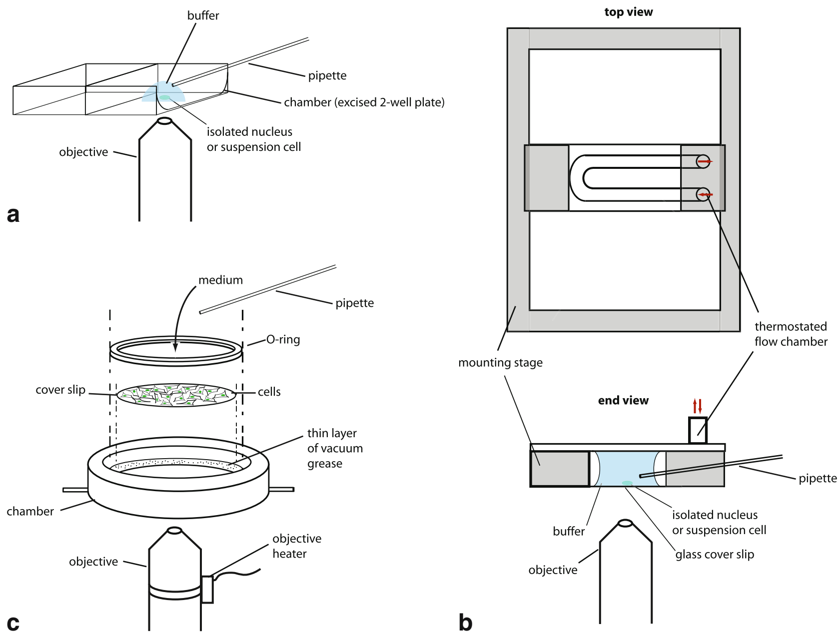

Fig. 1.2 Schematic illustrations of the micropipette aspiration setup. **a** A chamber for isolated nuclei or suspension cells is constructed by excising the side of a two-well chamber with a 0.17-mm-thick coverslip bottom. Nuclei are placed in a drop of buffer, and the pipette enters into the drop. **b** A thermostated chamber constructed of rust-free steel for isolated nuclei or suspended cells is constructed by affixing a glass coverslip to the bottom of the chamber with a small amount of vacuum grease. Buffer is inserted in between the coverslip and top of the chamber. Temperature is controlled by water flowing (*red arrows*) through channels on the upper side of the chamber. **c** Adherent cells plated on a coverslip are immersed in CO_2-independent medium. Nuclei are deformed and imaged at 37°C

3.3.4 Aspirating Nuclei in Intact, Adherent Cells

1. Prior to imaging, the coverslip on which the cells are cultured is mounted in the custom-designed chamber (Fig. 1.2c). A thin layer of vacuum grease is applied to the circumference of the chamber before affixing the coverslip. A rubber O-ring constitutes the third layer and seals the chamber, facilitating the immersion of the cells in CO_2-independent medium. The objective heater is set to 38.5°C, allowing for imaging the cells at 37°C.
2. The pipette is placed in the center of the field of view (aligned with the center of the light beam) and is carefully lowered into the same focal plane as the cells. With the micropipette fixed in position slightly above the cells, the stage is moved in the *x-y* plane until a desirable nucleus is located.
3. The pipette is brought to the cell surface adjacent to the nucleus and aspiration pressure is applied, causing the nucleus to deform into the micropipette. Nuclei are imaged either by brightfield, epifluorescence, or confocal microscopy (*see* **Note 5**).

4. After image acquisition and measurement of a given nucleus, the nucleus can be expelled from the pipette by applying a positive pressure while moving the pipette up and away from the cell. The same pipette can be used to aspirate more than one nucleus; however, the coverslip of cells should be replaced every 15 min or so to avoid excessive buffer evaporation (*see* **Note 6**).

3.4 Image Analysis

To interpret images of deformed nuclei, quantitative image analysis methods must be developed. Such analyses can be performed using image-processing software such as ImageJ (National Institutes of Health, Bethesda, MD, USA) or Zeiss LSM 510 v.3.0 software. For example, to characterize the mechanical properties of the nuclear envelope and inner nucleus, we have analyzed both changes in nuclear shape and subnuclear body position upon deformation, as well as fluorescence intensity distribution of labeled nuclear components (*see* **Note 4**) ***(7, 8)***. Labeling nuclear structures with different fluorophores and imaging the deformations of nuclear components provides a powerful method to probe the physical properties of nuclei both isolated, and in intact, living cells. These methods provide a set of basic tools to further our understanding of the physical nature of the cell nucleus, and how the nucleus responds to its mechanical environment.

4 Notes

Here we add further notes on problems encountered during micropipette aspiration of nuclei.

1. Nuclear isolation. Isolation by Dounce homogenization works best with a large number of nuclei. To isolate a sufficient quantity of nuclei while conserving the amount of DNA used for transfection, only one 10-cm plate is transfected; cells from this small plate harvested together with cells from a 15-cm plate are pooled for nuclear isolation. The success of nuclear isolation and the “cleanliness” of isolated nuclei varies: when not successful, extranuclear membranes remain associated with the nucleus. Unsuccessful nuclear isolation may also result from altered magnesium concentration of the hypotonic buffer.
2. Buffer conditions for isolated nuclei. The physical properties and morphology of isolated nuclei are extremely sensitive to buffer conditions (unpublished observations) ***(5)***, suggesting that salts induce changes in the structure (e.g., chromatin packing) ***(13)*** and mechanical properties of the inner nucleus; buffers should thus be chosen with care.
3. Extension to other systems. This protocol can be adapted for other cell types including mouse embryo fibroblasts as well as isolated plant cell nuclei.

4. Quantitative imaging. SYTOX orange stains nucleic acids very effectively, but is not appropriate for quantitative intensity analysis of DNA concentration. For quantitative analysis of chromatin density, PicoGreen (Molecular Probes) would be a more appropriate choice of dye.
5. Extension to confocal microscopy. Accommodating the confocal microscope setup for micropipette aspiration requires the design of alternative "open" chambers that allow for the entry of the micropipette as nearly horizontal as possible.
6. Timescale. Due to buffer evaporation from the open chamber, it is best to limit the experimental timescale to 10–15 min. Fresh buffer can be added during the experiment, but will alter buffer conditions.

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