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Direct Force Probe for Nuclear Mechanics

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Abstract

We describe a recently reported method for directly applying a known, nanonewton-scale force to the nucleus in a living, intact cell. First, a suction seal is applied on the nuclear surface using a micropipette. Then, the micropipette is translated away from the nucleus. The nucleus deforms and translates with the moving micropipette, then eventually detaches from the micropipette and recovers (roughly) its original shape and position. At the point of detachment, the resisting force (from the deformed nucleus and connected cytoskeleton) balances the suction force. Because the suction force is precisely known and reproducibly applied, this method therefore allows comparisons of nuclear response across disruptions to the cytoskeleton, nucleus or cell. This method is useful for quantifying nuclear elastic properties in its native, integrated environment.

Keywords

nuclear forces; nuclear shaping; nuclear positioning; nuclear mechanics

1 Introduction

A variety of forces act on the nucleus, including mechanical forces generated by molecular motors [1–6], frictional forces in the cytoskeleton [10], and osmotic forces dependent on the concentrations of macromolecules in the nucleus and cytoplasm [11, 12]. These forces, which can be propagated to the nucleus through the linker of nucleus and cytoskeleton (LINC) complex [7–9], act to shape and position the nucleus in the cell. Abnormal nuclear shaping and positioning in the cell characterize multiple human diseases [13–16]. It is therefore important to understand how forces on the nucleus and the mechanical properties of the nucleus become abnormal in human diseases.

Because the nucleus is integrated with the different cytoplasmic structures through the LINC complex, it becomes necessary to probe nuclear properties while it is mechanically integrated with the cell. This is challenging because most methods such as micropipette aspiration [17–20] require either isolated nuclei or nuclei in suspended cells. Under such conditions, the surrounding cytoskeletal network (which exerts stress on the nucleus in a spread, adherent cell) is either removed completely or greatly reorganized relative to its normal state. Atomic force microscopy [20] or applying force to cells adherent to substrates [21] can be used to probe the nucleus. In these methods, force is not applied *directly* to the

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nucleus, but rather is transmitted through the intervening cytoskeletal network. Therefore, the magnitude of the force applied to the nuclear surface is unknown. This makes it difficult to compare nuclear response across different perturbations to the nucleus.

We have recently described a direct force probe, which applies mechanical force directly to the nucleus in an adherent cell[22]. The method is easy to implement with the commonly used Eppendorf microinjection system. A fine micropipette (0.5 μm tip diameter) is suction-sealed against the nuclear surface and moved away from the nucleus (Figure 1A). Motion of the pipette tip deforms and displaces the nucleus. Eventually, the nucleus detaches from the pipette and recovers the original shape and position when the restoring forces exerted by cellular structures and the nucleus itself equal the suction force. We have reported that the extent of nuclear deformation is proportional to the force. One can then perturb the cytoskeleton, chromatin the LINC complex, and determine the contribution of these components to the nuclear deformation response *while it is integrated* with the cell. Here we describe a systematic protocol for using this method to probe the nucleus in adherent cells mechanically.

2 Materials

2.1 Preparation of Cells

1. Adherent cell line, such as NIH3T3, MEF, etc.
2. Cell media (composition dependent on cell line)
3. 35 mm glass-bottomed dishes (World Precision Instruments)
4. Phosphate-buffered saline
5. Fibronectin
6. Trypsin (0.25% w/v)
7. SYTO10/SYTO59 nucleic acid dye (or equivalent; ThermoFisher Scientific)
8. 0.3 mg/ml PLL-g-PEG solution (SuSoS, Dübendorf, Switzerland)
9. Plasmid or siRNA (with appropriate transfection reagents) or small-molecule inhibitor for perturbing components of the LINC complex, nucleus, or cytoskeleton

2.2 Equipment for Probing the Nucleus

1. Femtotips Microinjection capillary tips (0.5 μm -inner diameter; ThermoFisher Scientific)
2. InjectMan® NI2 Micromanipulation system (Eppendorf, Hamburg, Germany; discontinued, current equivalent model is InjectMan® 4)
3. FemtoJet® Microinjection system (Eppendorf)
4. Nikon TE-2000 Eclipse Inverted Microscope (or equivalent inverted epifluorescent or confocal microscope), equipped with a temperature-controlled

environmental chamber (to maintain cells at 37°C and 5% CO₂), and a CCD camera, and connected to a computer with Nikon Elements software

5. Filter cubes corresponding to fluorescent proteins/probes used in the experiment

2.3 Data Analysis

1. Quantitative image-processing software, for example MATLAB (The MathWorks Inc, Natick, MA) or FIJI (open-source, NIH)

3 Methods

3.1 Preparation of Cells and Microscope Set-Up

1. The FemtoJet® Microinjection system installs to the arm of the microscope. Install in accordance with manufacturer's recommendations.
2. Coat sterile 35 mm glass-bottomed dishes with 5 µg/ml fibronectin in PBS for 1 hour at room temperature or overnight at 4° C.
3. Wash the dishes multiple times with PBS.
4. Trypsinize and plate cells on the dishes (roughly 3×10⁵; corresponding to 25% confluency in 35 mm dish). Allow cells to spread overnight.
5. (Optional) Transfect cells with a plasmid or siRNA to perturb the nucleus, cytoskeleton, or LINC complex (for example, overexpression of GFP-KASH4 to disrupt the LINC complex). Allow the appropriate length of time for protein expression, knockdown or drug treatment.
6. (Optional) Treat cells with a small-molecule inhibitor to perturb the nucleus, cytoskeleton, or LINC complex (for example, nocodazole to inhibit microtubule polymerization). Allow the appropriate length of time for inhibition.
7. Wash cells with PBS and change media at least once prior to imaging. Add SYTO dye at manufacturer-recommended concentration (*see Note*¹) 15 minutes prior to the experiment to image the nucleus.
8. Choose the appropriate microscope objective. For most experiments, we used a Plan Fluor oil immersion 40×/1.3 NA objective. Apply a small drop of immersion oil, as needed.
9. Clamp the glass-bottomed dish tightly into the dish holder and load onto the stage. Maintain cells at 37°C and 5% CO₂ at all times while on microscope stage.
10. In order to immerse the micropipette into the culture media (as specified below), we had to modify our closed environmental chamber that supplied the cells with

¹We used the DNA minor groove-binding SYTO dyes because we found that they did not affect nuclear mechanical properties when compared to unlabeled nuclei. Labeling the nucleus with GFP-histone H1 made the nucleus stiffer in fibroblasts [22]. Other small molecule dyes (e.g. Hoechst 33342) may damage DNA or otherwise alter nuclear properties in live cells [23]. Whenever possible, use fluorophores with long wavelengths (i.e. red-shifted) to minimize the deleterious effects of photo-toxicity. Also, minimize exposure time and excitation light power.

humidified CO₂. We did so by removing the hard plastic cover and replacing it with plastic wrap (the transparent wrap maintains the CO₂ concentration inside the chamber).

11. Using DIC microscopy, find the appropriate focal plane to visualize cells. Correct for uneven illumination in the field of view by performing both Kohler illumination (DIC) and shading correction (fluorescence channels).

3.2 Conversion of Micropipette Pressure to Force

A major advantage of this method is that it applies a precisely known suction pressure reproducibly to the nucleus. We begin by initially imposing an external (gauge) pressure P_I using the InjectMan® system to force out liquid from the pipette using a microinjection system. At equilibrium, the pressure P_I plus the fluid head in the micropipette (P_h) balances the capillary pressure (P_c) plus the fluid head in the culture dish (P_H) (Figure 1B). Table 1 shows the magnitude of these pressures; P_I and P_c are approximately equal and opposite for a broad range of values of P_I (Table 1). We then insert the micropipette tip into the cell, touch the micropipette to the nuclear surface and set P_I to zero (gauge pressure) by opening the pipette tubing to atmospheric pressure. A suction pressure ($P_c \approx P_I$) now exists on the nuclear surface. The force on the nuclear surface is calculated by multiplying the pressure by the area of the micropipette tip ($0.20 \mu\text{m}^2$).

In the table, we first chose a value for P_I , which can be set with the Eppendorf system. Next, we calculated the height of the fluid inside the micropipette, h , from the pressure balance $P_I = P_c + P_H - P_h$. $P_h = \rho gh$ is the pressure head in the pipette; ρ is the density of the culture media (approximately 1000 g/L) and g is the gravitational constant (9.8 m/s^2). The pressure head of the fluid in the dish (P_H ; not shown in the table) is constant for the range of P_I , and $\sim 0.2 \text{ hPa}$ in magnitude. $P_c = 4\gamma \cos \theta/d$ is the capillary pressure; γ is the surface tension (72.8 dynes/cm), θ is the wetting angle of water on glass (30°), and d is the diameter of the micropipette, which decreases as the tube tapers towards the tip (accounted for in calculation by approximating the slope of micropipette). Force is calculated by multiplying P_c by the area of the micropipette tip ($0.20 \mu\text{m}^2$).

3.3 Applying Force to the Nucleus

Before the micropipette can be used to probe nuclei, it must be positioned in the field of view (see Note ²).

1. (Optional) To prevent the micropipette from adhering to the cells, immerse the tip in 0.3 mg/ml PLL-g-PEG solution for 1 hour at room temperature (see Note ³).
2. Load the micropipette (0.5 μm diameter tip) into the capillary holder.

²It is very important to avoid lowering the tip of the micropipette too far before the tip is located in the field of view, as the fragile tip can easily break on the surface of the culture dish.

³The blocking step can prevent cell debris and dead cells from adhering to the body of the micropipette. We did not detect nuclear deformation when there is no suction pressure in the capillary (not shown); therefore, we conclude that there is no adhesion between the nucleus and the micropipette tip.

- a. CAUTION: Fine micropipettes are very sharp; use care when handling to avoid personal injury.
3. Peel back the plastic wrap on the environmental chamber (as described above) to allow entry of the micropipette.
4. Lower the micropipette by twisting the joystick that controls the manipulator clockwise until the tip immerses in media. Position the tip as close to the center of the objective as possible (use “coarse” or “fine” mode on the micromanipulator for large or small micropipette movements, respectively).
5. Adjust the focus to a plane above the cells, switch from “DIC” to the “phase” optical configuration on the NIS-Elements software, and remove the polarizer from the light path. This configuration makes the shadow of the micropipette easier to see.
6. Move the micropipette back and forth (along y-axis), watching the screen carefully for a shadow.
7. Locate the tip of the micropipette using fine movements on the micromanipulator.
8. Bring the cells back into focus and lower the micropipette until the shape of the pipette is visible in the focal plane of the cells. Return to the DIC optical configuration and return the polarizer into the optical path.
9. The tip should always be clear of any cell debris and cleaned (*see Note* ⁴) regularly.
10. Set the desired pressure P_I (in hPa) on the microinjector. Allow the height of the fluid in the micropipette to equilibrate (Figure 1B).
11. Insert the tip of the micropipette into the cell, as close to the edge of the nucleus as possible (*see Note* ⁵).
12. Touch the tip of the micropipette to the nuclear surface (*see Note* ⁶) and then seal it to the surface by disconnecting the outlet tube of the microinjector to open the end of the capillary to the atmosphere.

⁴To check whether the pipette is clogged, apply the maximum possible pressure with the microinjector system (ensure the tip is away from live cells). If bubbles emerge from the tip into the cell media, this indicates that the micropipette is clear.

⁵If the micropipette is held in the cell for long times, it may cause cell death. We have confirmed that the cell remains viable within the 30 second timescale of the experiment by adding ethidium homodimer-2, which diffuses into cell and appears red if the membrane is damaged [22].

⁶The relatively small area of the tip (compared to the surface area of the nucleus) prevents substantial flow of water across nuclear pores into the capillary, which would decrease the actual pressure applied. The formula below (derived in [22]) illustrates the higher resistance to flow in the micropipette compared to through nuclear pores:

$$\frac{(P_0 - P_m)}{(P_0 - P_s)} = \frac{1}{1 + \varphi/\varphi_p}$$

In the above equation, P_0 is the hydrostatic pressure inside the nucleus, P_m is the pressure at the outer membrane surface, P_s is the applied suction pressure, φ and φ_p are the filtration coefficients in nuclear pores and in the micropipette, respectively. Using common

13. Set up an .avi acquisition in Nikon Elements software. Set the desired frames per second, then begin recording.
14. Switch to fluorescent microscopy by adding the appropriate excitation and emission filters to the light path.
15. Translate the micropipette away from the nucleus by steadily moving the joystick to the right (laterally) (see Note ⁷).
16. Continue moving the tip and recording until the nucleus eventually detaches from the micropipette. Work quickly to avoid photobleaching.
17. End the .avi acquisition and save the raw data file for later analysis.

3.4 Data Analysis

We use a custom MATLAB script to analyze images, but any other image-processing software (e.g. FIJI) will work as well. The extent of deformation is quantifiable in multiple ways from images of the initial nuclear shape and the deformed shape: length strain (Figure 2A) or maximum nuclear deformation (Figure 2B). The time scale of relaxation is calculated by fitting an exponential decay to a plot of length strain as a function of time.

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literature values, we estimated the ratio in the denominator to be extremely small ($\phi/\phi_p < 10^{-5}$). Therefore, there is negligible pressure drop across the nuclear membrane if a 0.5 μm -diameter micropipette is used, despite the fact that fluid flows through nuclear pores (for the complete derivation, see the supplemental information of [22]).

⁷The pulling rate can be controlled automatically with a computer, but we found it simpler to perform the pulling manually with the micromanipulator joystick. To test whether variations in the manual loading rate could affect the nuclear response, we calculated the loading rate from several experiments by tracking the deformation of the leading nuclear edge over time (loading rate is the maximum slope of the cubic fit; Figure 3B). We found no correlation between nuclear mechanical response and loading rate (Figure 3C).

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Figure 1. Schematic of direct force probe experiments

A) A micropipette is suction-sealed against the nuclear surface of an NIH3T3 fibroblast and moved, deforming the nucleus until it detaches. The region bounded by the white box is shown in a kymograph, displaying the nuclear deformation over time. B) Free-body schematic diagram of the forces in the micropipette.

Figure 2. Useful nuclear properties measurable with the direct force probe
A) Length strain schematic and calculation are shown with average length strain as a function of suction force. Data is mean ± SEM, and all are statistically different at $p < 0.05$. $n = 6, 7, 14$ for 2 nN, 4 nN, and 6 nN respectively (Plot reproduced with permission from PNAS; [22]). B) Schematic explaining how to measure the maximum nuclear deformation and the relaxation of maximum deformation by measuring non-overlapping areas of the nucleus (reproduced with permission from PNAS; [22]).

Figure 3. Deformation is independent of loading rate

A) Front edge nuclear displacements are plotted as functions of time. Data is plotted until detachment of the nucleus from the micropipette in 8 different cells and fit with a cubic polynomial. The slope at maximum displacement is the loading rate of the nucleus. B) Both the length strain of the moved nucleus just before detachment and the dynamics of length strain relaxation were uncorrelated with the loading rate (reproduced with permission from PNAS; [22]).

Table 1

P_l is approximately equal to P_c ; other pressures are negligible, therefore the suction pressure is equal to P_l to a good approximation.

P_l (hPa)	h (mm)	P_h (hPa)	P_c (hPa)	Force (nN)
12.72	3.53	0.347	12.58	0.2516
50	0.88	0.086	50.1	1.002
100	0.44	0.043	100.1	2.002
200	0.21	0.021	200.2	4.004
300	0.14	0.014	300.2	6.004
400	0.11	0.01	400.2	8.004
500	0.08	0.008	500.2	10.004
600	0.06	0.006	600.2	12.004