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New approaches for understanding the nuclear force balance in living, adherent cells

Srujana Neelam [b], Richard B. Dickinson [a], Tanmay P. Lele [a,*]

[a] Department of Chemical Engineering, University of Florida, Gainesville FL 32611, United States
[b] Department of Biomedical Engineering, University of Florida, Gainesville FL 32611, United States

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ABSTRACT

Cytoskeletal forces are transmitted to the nucleus to position and shape it. Linkages mediated by the LINC (linker of nucleoskeleton and cytoskeleton) complex transfer these forces to the nuclear envelope. Nuclear position and shape can be thought to be determined by a balance of cytoskeletal forces generated by microtubule motors that shear the nuclear surface, actomyosin forces that can pull, push and shear the nucleus, and intermediate filaments that may passively resist nuclear decentering and deformation. Parsing contributions of these different forces to nuclear mechanics is a very challenging task. Here we review new approaches that can be used in living cells to probe and understand the nuclear force balance.

1. Introduction

Nuclear shape and structural abnormalities have been associated with a host of pathologies such as cancer, laminopathies and aging [1–4]. Nuclear positioning is also an important cellular function that contributes to cell polarity in critical functions such as wound healing [5]. Therefore, there is much recent interest in understanding how the nucleus is positioned and shaped in the cell. Given the large nuclear size, positioning it and shaping it in the cell requires generation of dynamic mechanical forces on it during cell migration. Cytoskeletal forces can be transferred to the nuclear surface through linkages between the cytoskeleton (and/or cytoskeletal motors) and nuclear envelope proteins [6–8].

Understanding nuclear mechanics is complicated because there are multiple, potentially competing mechanisms for generating nuclear forces. This includes myosin-mediated contractile forces [8–10], microtubule motors like dynein and kinesin [11–13] and passive resistance due to intermediate filaments like vimentin or keratin [14–17]. Parsing contributions of these different forces is a challenging task. Complicating matters further, a given cytoskeletal element may pull [18,19], push or shear [20–22], and the magnitude of these forces may vary depending on the context and cell type.

To enable design and reliable interpretation of experiments to understand nuclear forces, we have taken the view that nuclear position and shape are a result of a balance of competing forces. For example, in a migrating cell, forces generated in between the nucleus and the leading edge will act to generate a net force on the nucleus. This net force must be equal and opposite to a net force generated in the trailing edge. If this view is correct, then it gives rise to interesting questions. Is the net force from one side of the nucleus of a pushing or a pulling type? Of the various types of force generators, is there a dominant source of nuclear force? What is the magnitude of forces that are required to move and shape the nucleus? What are plausible physical explanations for nuclear motions such as nuclear rotations?

Studies in the field of nuclear mechanics have relied on a number of different methods including micropipette aspiration of isolated nuclei [23,24] and of trypsinized, whole cells [25], AFM measurements of nuclei [26], nuclear response to mechanical strain applied to adherent cells [27] and pulling on the cytoplasm [28]. Such approaches have been well-described in recent reviews [29,30]. Here we focus on multiple approaches developed in our laboratories designed to perturb and understand the nuclear force balance in living, adherent cells.

2. Modulating nuclear forces in migrating cells

To test the presence of a 'dominant' force generator and whether the net force acting on one side of the nucleus is tensile

* Corresponding author at: Department of Chemical Engineering, Bldg. 723, University of Florida, Gainesville, FL 32611, United States.
E-mail address: tlele@che.ufl.edu (T.P. Lele).

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or compressive, an approach is required to selectively perturb forces only in the trailing or only in the leading edge of a migrating cell. Selectively inhibiting cytoskeletal forces by administering local doses through (for example) a micropipette to portions of the cell is challenging, considering that cytoskeletal inhibitors can diffuse throughout the small length of the cell much faster than kinetics for drug action. We approached this problem by engineering new lamellipodia in serum-starved non-migrating cells. Originally developed by Klaus Hahn's group [31], this method relies on photoactivation of Rac1 to engineer new lamellipodia [32,33]. The photoactivable Rac1 has a LOV2-Jα sequence fused to the N-terminus of constitutively active Rac1. The LOV2 domain when bound to the Jα helix blocks binding of effectors to Rac1, but when photoactivated, conformation changes cause dissociation of the Jα helix and exposes Rac1 to its effectors. To activate photoactivatable Rac1, an energy pulse from an Argon laser (488 nm) is focused on to a region of interest in cells expressing photoactivable Rac1 at regular intervals (time between intervals can be roughly 10 s). This can be easily accomplished on a conventional laser scanning confocal microscope. Photoactivation causes the formation of lamellipodia in serum-starved cells [10].

Upon engineering a lamellipodium, and then tracking the nucleus, we found that it 'drifts' toward the new lamellipodium (Fig. 1A and B) [10]. Importantly, triggering a new lamellipodium in a cell did not cause significant changes in cell morphology elsewhere over the time-scale of observation. Quantifying nuclear motion revealed that the nucleus undergoes a persistent motion toward the photoactivated spot, with smaller random positional fluctuations (Fig. 1C). This observation suggests that the forces on the nuclear surface may be locally increased due to the formation of a lamellipodium leading to a re-positioning of the nucleus.

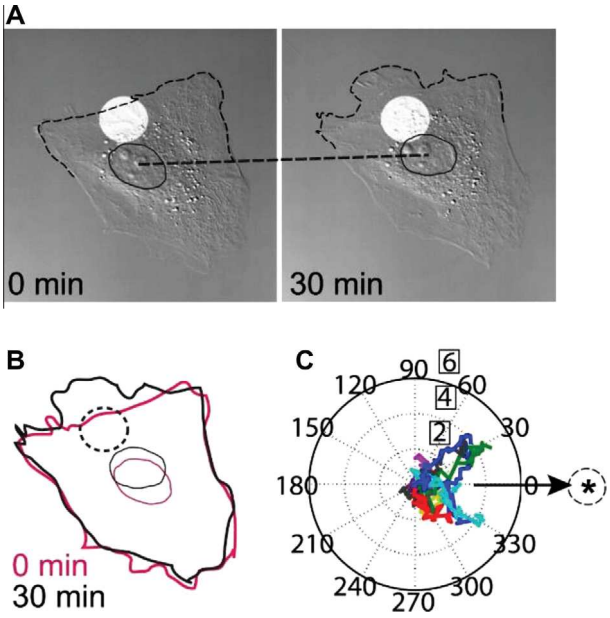

Fig. 1. Photoactivation of Rac1 to induce lamellopodium formation causes directional bias in nuclear translation. (A) DIC images of photoactivation experiment at 0 min and 30 min. Formation of new lamellopodium (black dashed line) at the photoactivation site (bright circular spot). Nucleus (outlined with black line) is observed to move towards the direction of new lamellopodium. (B) The overlap of nuclear and cell outlines at 0 min (red) and 30 min (black) show nuclear displacement. (C) Trajectories of the nucleus upon photoactivation ($n = 11$; angles are in degrees; *represents the photoactivation center, the nucleus-photoactivation center axis is oriented initially along the positive x axis); all trajectories start at the center. Boxed numbers are in microns. (Reprinted from The Biophysical society, volume 106, Actomyosin pulls to advance the nucleus in a migrating tissue cell, 1–9, Copyright (2014), with permission from Elsevier).

We were able to dissect the contributions of different cytoskeletal elements to the nuclear force balance using this approach. Because the assay relies on photoactivating Rac1 to induce lamellipodia, inhibiting myosin activity with blebbistatin, ROCK activity with Y27632, or disrupting microtubules (MT) with nocodazole did not prevent the formation of lamellipodia. This uniquely allowed us to observe nuclear motion in response to creation of a new lamellipodium in the presence and absence of key cytoskeletal force generators. Microtubule disruption did not interfere with nuclear motion toward the new lamellipodium, while myosin activity eliminated nuclear motion. In addition, the nuclear motion required an intact LINC complex as evident from a lack of nuclear motion in KASH4- expressing cells (KASH4 is a domain of nesprin-4 that binds to SUN1/2 proteins in the inner nuclear membrane; by over-expressing GFP-KASH4, endogenous KASH4 linkages with SUN1/2 are competitively inhibited [34]). These observations suggest that at least in the photoactivation assay, actomyosin forces may be increased on the nuclear surface. The net force is likely of a pulling type given that the direction of nuclear motion is toward the newly formed lamellipodium.

To manipulate forces in the trailing edge, we inserted a micropipette with a narrow tip (0.5 μm) under the trailing edge of fibroblasts. The method uses a standard Eppendorf Femtojet microinjection system to lower a micropipette close to the surface of the dish several hundred microns away from the cell. Next, the micropipette is lowered slowly to bend the main shaft as it contacts the surface of the dish, and translated toward the trailing edge. After translation under the trailing edge, the micropipette is raised through a distance of 3–5 μm, which causes detachment of the trailing edge.

Raising the micropipette to detach/rupture the trailing edge caused a rapid retraction of the trailing edge suggesting a dissipation of force from the trailing edge. Detaching the trailing edge caused nuclear motion toward the leading edge. If the net force from the leading edge is tensile as argued above, then the net force from the trailing edge also will be tensile and opposing, and forward nuclear motion in response to the detachment of the trailing edge is then a result of an imbalance of tensile forces between the front and back of the cell. In addition, such a model also predicts that the nucleus transmits tensile forces from the front to the back of the cell.

3. Traction force microscopy as a tool to probe the nuclear force balance

A limitation of the Rac1 photoactivation approach is that triggered lamellipodia may not fully capture the complex signaling events that occur during normal lamellipodial formation. We therefore developed an approach based on traction force microscopy (TFM, [35,36]) to infer the nuclear force balance in a migrating fibroblast [37]. The TFM method originally developed by Dembo and Wang [35] is a well-established technique in which cells are cultured on fibronectin-coated polyacrylamide gels. Fluorescent microspheres of 0.5 μm are suspended in the hydrogel prior to polymerization. Tracking position of the embedded microspheres under and in the vicinity of the cell and before and after complete removal of the cell from the gel allows calculation of traction stresses exerted by the cell. Traction stresses can be calculated from images of fluorescent microspheres using an algorithm developed by Dembo and Wang [35].

If the nucleus transmits internal tension from front to back of the cell, then it should coincide with where the tension is maximum. We therefore measured traction stresses generated by single migrating fibroblasts, and located the point of maximum tension (PMT) along the cell's contractile axis (Fig. 2). After calculating

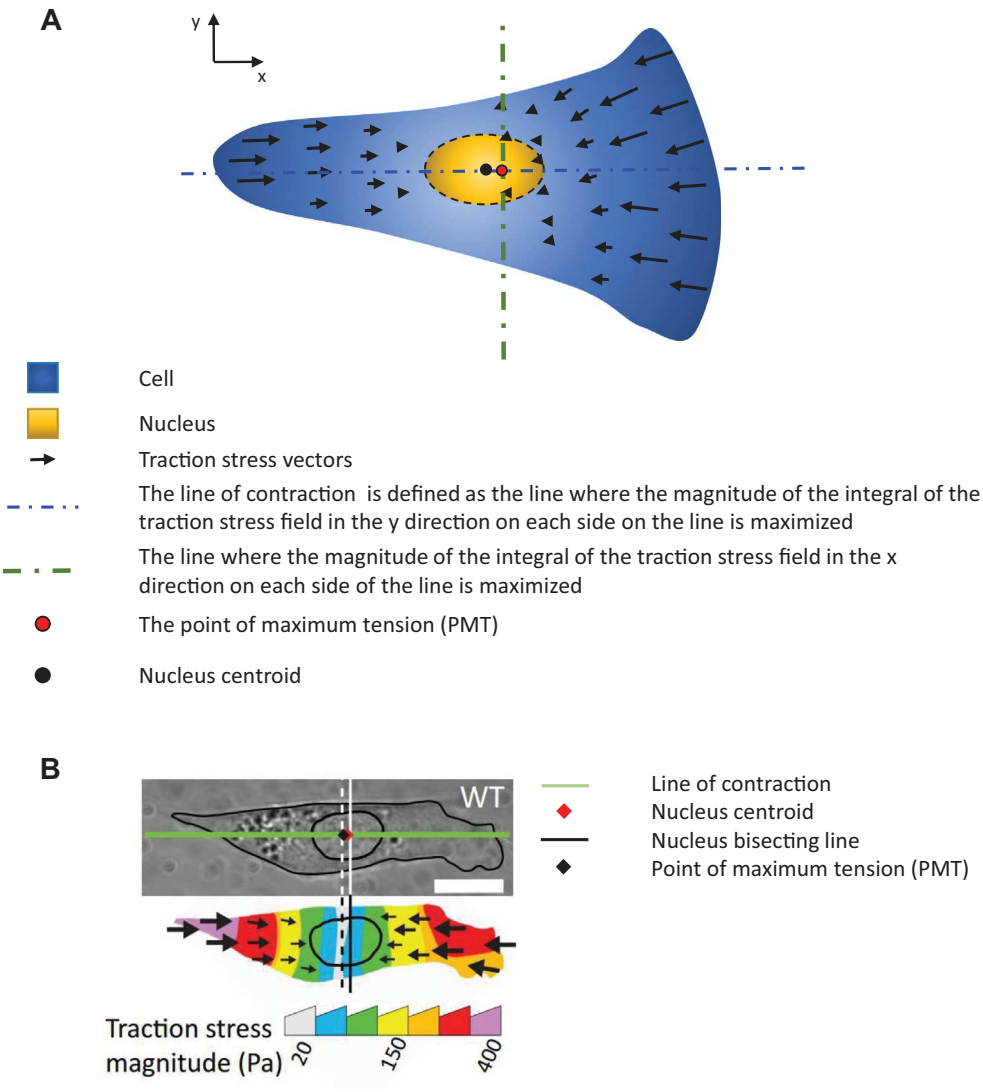

Fig. 2. Nuclear position coincides with the PMT. (A) Pictorial representation of illustrating calculation of the point of maximum tension (PMT). (B) DIC image shows the position of the nucleus in a wild-type fibroblast cell. The cell's corresponding traction stress map is also shown. Vertical solid and dashed lines indicate the positions of the nucleus centroid and the PMT, respectively. (Reprint from the Journal of Cell Science, volume 128 (10), The nucleus is an intracellular propagator of tensile forces in NIH 3T3 firboblasts, 1901–1911, Copyrighted (2015), with permission from Company of Biologists Ltd).

the traction field, the direction of the principal axis of the traction field (PA) is found. The line of contraction is identified as a line parallel to the PA such that the magnitude of integrated traction vectors on each side of the line is maximum. Next, the point on this line where the integrated traction vectors are maximized across another line perpendicular to the PA is located. This unique point is called the PMT. The calculation is illustrated in Fig. 2 [37].

We found that the nuclear centroid does coincide closely with the PMT in control cells with a migratory phenotype. When the trailing edge detaches however, the PMT immediately shifts toward the front of the cell, while the nucleus lags behind. Re-establishment of the migratory shape causes the nucleus to again become repositioned close to the PMT. Importantly, the nuclear centroid lags the PMT in cells with a disrupted LINC complex.

To test the extent to which the nucleus contributes to propagating tensile forces from the front to the back, we devised an approach to measure the decrease in the traction stress at the cell front on detachment of the trailing edge. Using a micropipette to detach the trailing edge while simultaneously measuring cell traction, we found that drop in tension at the cell front was significantly smaller in LINC complex disrupted cells compared to control cells. This provides more direct evidence that nuclear linkages with the cytoskeleton are required for efficient transmission of tension between the front and back of the cell.

Interestingly, naturally occurring protrusions proximal to the nuclear surface in migrating fibroblasts cause nuclear deformation and motion toward the protrusion [37]. This effect is reversible in that the nucleus is restored to its original shape and position on collapse of the protrusion. Traction stress measurements under the newly formed protrusion revealed a transient motion of the PMT and concomitant nuclear motion and deformation, again supporting the concept that protrusions modulate the nuclear force balance.

Collectively, dynamic coincidence between the nuclear position and the PMT during events such as formation of spontaneous protrusions or detachment of the trailing edge, and smaller drop in stress at one end on detachment of the other end in LINC complex disrupted cells strongly suggest that the nucleus is a transmitter of tensile forces from the front to the back of the cell. TFM can thus be very useful in understanding the nuclear force balance.

4. A direct force probe for nuclear mechanical measurements in living, adherent cells

While the above approaches can be used to modulate the nuclear force balance, or to correlate nuclear position with the PMT in migrating cells, methods are needed to directly measure forces on the nuclear surface. We recently developed a new approach [38] in which we first seal the tip of a narrow micropipette (0.5 μm) against the nuclear surface with a specified and known suction pressure (as shown in the schematic, Fig. 3A). If the nucleus were free to move in the cell, moving the micropipette at a known speed away from it would cause motion of the undeformed nucleus. Instead, we found that the nucleus moves only slightly and primarily deforms as the micropipette tip is moved away, finally detaching from the tip (Fig. 3B). At the point of detachment, the resistance to motion and deformation of the nucleus balances force on the outer nuclear surface created by suction. Because suction pressure at the micropipette tip is well-characterized, the actual force on the nuclear surface at the point of release is the suction pressure × the cross-sectional area. This approach allows characterization of deformation and motion of the nucleus in adherent cells under known force, which is a significant benefit in studying the nuclear force balance.

There are some potential caveats that have to be considered. First, the nuclear envelope has pores in it, which will allow flows into the micropipette from inside the nucleus. The existence of such flows means that the actual pressure on the nuclear surface will be smaller than suction pressure in the micropipette. However, a simple calculation shows that resistance across the nuclear envelope to flow is of the order of 10^5 times greater than resistance to the flow in the pipette [38]. Thus, all pressure-drop essentially occurs across the nuclear envelope itself, such that the actual pressure on the nuclear envelope is basically equal to suction pressure in the micropipette tip.

It is also possible that the micropipette tip can become clogged with organelles or cell membrane, partially occluding the pipette tip. Flows from the surrounding cytoplasm into the micropipette tip due to incomplete contact with the nuclear surface can also cause variations in the pressure. These concerns can be addressed by careful inspection of the tip for debris and establishment of good contact between the nuclear surface and the tip. Introducing and moving a micropipette can cause damage to the cell. We confirmed that over short time-scales of our pulling experiments (~30 s), cells remained viable. Damage to membranes was restricted to narrow tethers pulled by the pipette tip itself [38].

We found that the extent of nuclear deformation in our experiments scaled approximately linearly with suction pressure with reasonably small error bars on the measurements (Fig. 3C). In addition, systematic statistically significant differences were observed with perturbations to key molecular players that resist deformation. Forces in the nanoNewton range are required to produce

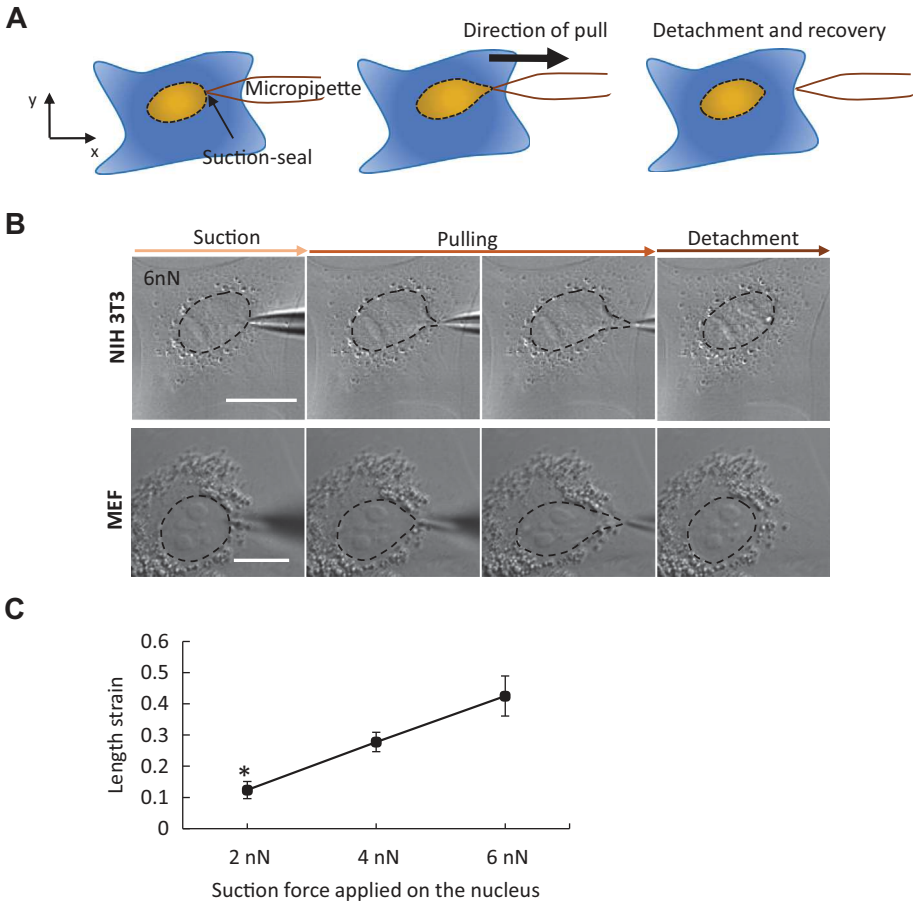

Fig. 3. Nuclear manipulation using the micropipette pulling technique. (A) Schematic shows the micropipette suction-sealed on the nucleus surface in a living spread cell. The micropipette is moved laterally till the nucleus detaches from the pipette tip. (B) DIC images show micropipette manipulation of the nucleus with a 6 nN force in a NIH 3T3 fibroblast cell (panel 1) and in the mouse embryonic fibroblast (MEF) cell (panel 2). The nucleus deforms as the micropipette is moved and recovers completely on detachment. Scale bar is 10 μm. (C) The length strain of the nucleus depends linearly on the suction force (suction force is calculated as the suction pressure × area of cross-section of the micropipette tip) in MEF cells. '*' indicates $p < 0.05$ for the comparison between each force vs the lowest force applied. Error bars indicate SEM. $n = 6$ for 2 nN, $n = 15$ for 4 nN and $n = 7$ for 6 nN suction force.

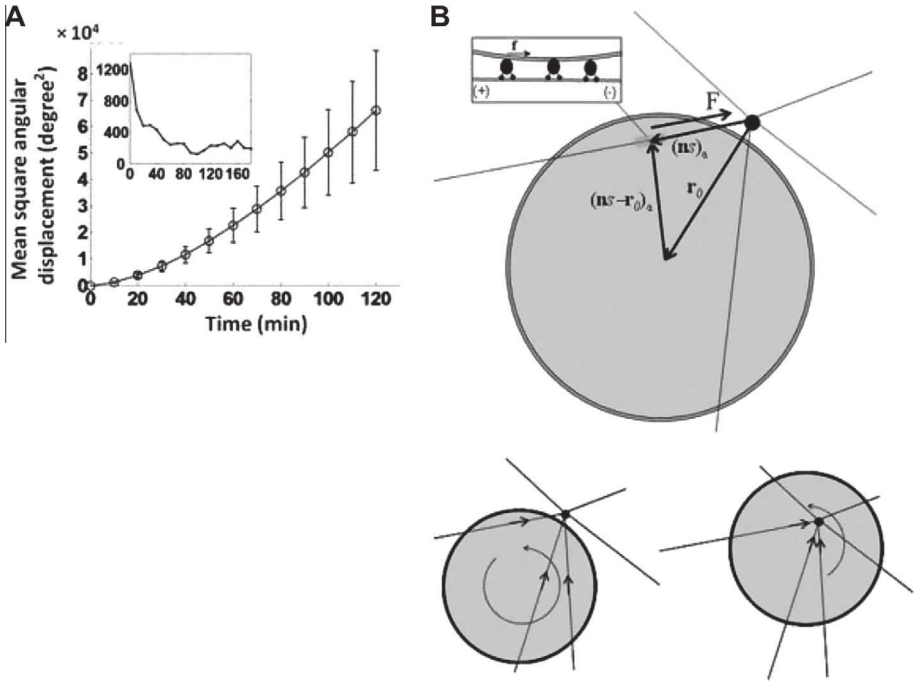

Fig. 4. A physical model for nuclear rotation. (A) Time-dependence of the pooled angular mean-squared displacement calculated from time-lapse imaging of nuclear rotation in NIH 3T3 fibroblasts (n = 25 cells). The MSD shows a parabolic shape at short times followed by a linear dependence at longer times which indicates rotation tis a persistent random walk. Inset shows the averaged autocorrelation of angular displacements over 10-min intervals (degree^2/min^2) indicating a fast decay followed by long time decay, again consistent with the conclusion that the rotation is a persistent random walk. Error bars indicate SEM. (B) Schematic of the nuclear rotation model. Dynein molecules walking on MTs (straight lines) generate forces (f) on the nuclear surface directed toward the centrosome (intersection of straight lines). The resulting mean net force F from the microtubule and the lever arm (vector $ns - r_0$ where s is the position on the contour, n is a unit vector directed toward the MT plus-end, and r_0 is a unit vector directed from the centrosome to the center of the nucleus) create a torque on the nucleus. The magnitude of the torque depends on the centrosome position, because the lever arm length is smaller when the centrosome is closer to the nucleus centroid. (Reprinted with permission from ref 13, copyright 2011 by Wiley-Liss, Inc.).

discernible deformation in the nucleus. These results suggest that for nuclear position and shape to be maintained, magnitude of the local, fluctuating forces acting on it must be lower than the nanoNewton range. Interestingly, upon detachment from the micropipette, the nucleus relaxes toward its original shape very rapidly (less than a second) demonstrating that a large elastic resistance to nuclear deformation is present in the cell.

An important benefit of this approach is that deformation and motion of the nucleus can be quantified and compared under the same force across different perturbations. Through such comparative analysis, we found that intermediate filaments vimentin and lamin A/C, and SUN1-mediated LINC complex linkages but not F-actin and MT are required for the nucleus to resist applied forces. Similarly, an intact nuclear-cytoskeletal linkage was found to be important in aspects of the nuclear mechanical response.

We expect this method to provide important insight into the mechanisms by which nuclear shape and position are maintained in normal cells and become abnormal in a variety of human pathologies.

5. Nuclear dynamical analysis

The force balance on the nucleus is continually changing in time due to continuous assembly and disassembly of the cellular cytoskeleton. As a result of such dynamic forces, the nucleus displays complex motions including rotation [11,13], translation [5,39,40] and deformation. Nuclear rotation in particular is a fascinating type of motion observed in many different cell types. While the physiological relevance of rotation is not clear, it can help understand molecular forces that act on the nuclear surface in a living cell. In particular, we took the approach that analyzing the statistical nature of nuclear motion can help quantitatively test mathematical models for nuclear force generation. We now discuss this combined experimental-mathematical modeling approach to nuclear rotation dynamics.

We started by quantifying the rotational trajectories of the fibroblast nucleus from DIC imaging of living cells [13]. The nucleus rotates like a solid body and quantitative measurements of positions of nucleoli allows calculation of the nuclear rotation angle. Calculating rotational angles from images, and quantifying angular mean squared displacements of nuclei revealed that nuclear rotation is a persistent random walk. That is, the rotation persists in a certain direction over a time, but over long times, the rotation becomes randomized (Fig. 4A). Inhibiting dynein virtually eliminated any nuclear rotation. Together, these two experimental observations were used to develop a molecular model for how nucleus-embedded dynein generates forces on the nucleus.

In the model, individual nucleus-embedded dynein molecules transiently bind, walk on MTs in the vicinity of the nucleus, and pull the nucleus towards MT minus ends. Pulling forces are generated because motion of the head lengthens the dynein 'spring'- one end of which remains attached to the nucleus. Ensemble averaging of the dynein force per MT, and summing up torques over the nucleus accounting for randomly nucleated MTs undergoing dynamic instability allows calculation of a fluctuating rotational angle (Fig. 4B). Importantly, the model accounts for the statistics of dynein binding and unbinding and its force–velocity relation.

The key insight that emerged from the model was that experimentally observed persistence in the nuclear rotation is determined by the time it takes for dynamic instability of MTs to change the microtubule configuration sufficiently enough that the rotational bias changes its direction. Second, the distance between the centrosome and the nucleus determines the level of the torque (as torque depends not only on the force but also on

the lever arm) (Fig. 4B), which was experimentally tested to be true. Thus, a combination of experiments and theory, combined with validation allowed us to explain how nuclear rotation occurs, why it is a persistent random walk, and what determines the extent of torque generation on the nuclear surface.

6. Conclusions

The mechanical properties of the nucleus have been studied with a variety of methods. Isolated nuclei can be mechanically characterized with micropipette aspiration [26,41], AFM [26] and magnetic tweezing of particles adherent to isolated nuclei [42]. Approaches to study nuclear mechanics in living cells include applying strain to flexible cell culture membranes [27], tugging on the cytoplasm [28], micropipette aspiration of whole cells [25], applying forces to magnetic particles inside the nucleus [43,44], particle tracking microrheology [45] of the nucleus and AFM [26]. In this review, we focused on techniques developed in our laboratories that can be used to understand the nuclear force balance in living cells. This is a challenging problem that requires creative combination of engineering methods, mathematical approaches and molecular cell biological tools. Approaches discussed here include mechanically perturbing specific portions of the cell, inferring the nuclear force balance from traction force measurements, applying controlled, known mechanical forces directly to the nuclear surface in the living cell, and using quantitative measurements of nuclear dynamics to test and build mathematical models of nuclear forces.

Such approaches that focus on development of physical principles, physical measurements and theories for nuclear force generation may prove invaluable in explaining how the nucleus becomes dysfunctional in and contributes to disease. For example, nuclear abnormalities are common in cancer and in muscular dystrophies. A molecular understanding of the mechanisms for altered forces on abnormal nuclei can help lead to innovative methods for renormalizing nuclear forces as a means for treating disease. In this context, new techniques that can allow probing nuclear forces in cells in more realistic environments such as in 3D matrices, or *in vivo*, as well as molecular readouts of nuclear forces would be particularly useful for the field.

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