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Beyond lamins: other structural components of the nucleoskeleton

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Abstract

The nucleus is bordered by a double bilayer nuclear envelope, communicates with the cytoplasm via embedded nuclear pore complexes, and is structurally supported by an underlying nucleoskeleton. The nucleoskeleton includes nuclear intermediate filaments formed by lamin proteins, which provide major structural and mechanical support to the nucleus. However other structural proteins also contribute to the function of nucleoskeleton and help connect it to the cytoskeleton. This chapter reviews nucleoskeletal components beyond lamins, and summarizes specific methods and strategies useful for analyzing nuclear structural proteins including actin, spectrin, titin, LINC complex proteins and nuclear spindle matrix proteins. These components can localize to highly specific functional subdomains at the nuclear envelope or nuclear interior, and can interact either stably or dynamically with a variety of partners. These components confer upon the nucleoskeleton a functional diversity and mechanical resilience that appears to rival the cytoskeleton. To facilitate the exploration of this understudied area of biology, we summarize methods useful for localizing, solubilizing and immunoprecipitating nuclear structural proteins, and a state-of-the-art method to measure a newly-recognized mechanical property of nucleus.

I. Introduction

The nucleus houses the genome and is the largest organelle in eukaryotic cells. Its best-known architectural components include the nuclear envelope, nuclear pore complexes (NPCs) and the nucleoskeleton, which is formed primarily by separate networks of nuclear intermediate filaments formed by A- or B-type lamins. The nucleoskeleton is concentrated near the nuclear envelope ('peripheral' nucleoskeleton) but also extends throughout the interior ('internal' nucleoskeleton) with loosely distributed lamins and associated proteins. Chromosomes and chromatin also associate with lamins (Guelen *et al.* 2008; Wen *et al.* 2009), as do most characterized inner nuclear membrane (INM) proteins, suggesting a variety of structures contribute to nuclear architecture (Zastrow *et al.* 2004).

Lamin networks resist deformation and force transmission and are major mechanical elements of the nucleus (Dahl *et al.* 2008). Nuclei reconstituted in lamin-deficient *Xenopus* egg extracts are extremely fragile (Newport *et al.* 1990). Similarly mammalian cells depleted of lamins, particularly A-type lamins, are significantly weaker than their wildtype counterparts (Broers *et al.* 2004; Lammerding *et al.* 2004). Nuclear A- and B-type lamin networks also contribute,

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mechanically or non-mechanically, to many other functions including chromatin organization, transcription, replication, differentiation and signaling (Dechat *et al.* 2008; Gruenbaum *et al.* 2005).

Numerous diseases ('laminopathies') are caused either by perturbed expression of B-type lamins, or by mutations in *LMNA* (encoding A-type lamins) or other genes encoding nuclear envelope membrane proteins (Capell and Collins 2006; Gruenbaum *et al.* 2005). In many cases these mutations alter nuclear mechanics and clinically affect load-bearing tissues (Dahl *et al.* 2008). The spectrum of known laminopathies includes muscular dystrophy, lipodystrophy and diabetes, skeletal dysplasia, skin disorders, neuropathy, leukodystrophy, and progeria (premature aging) (Capell and Collins 2006). It remains unclear how mutations in these proteins, particularly A-type lamins, can produce such widely different diseases. Current evidence points to multiple and varied mechanisms, including perturbed regulation of gene expression and altered nuclear mechanics (Worman and Courvalin 2004). To understand the etiology of laminopathies we must first understand the complexities of nuclear architecture and mechanics, an understudied area of biology.

It is naive to say, since nuclei have many other structural proteins. The cytoskeleton includes multiple "skeletal" elements, each of which contributes uniquely to the structure, dynamics, molecular mechanics and rheological properties of the cytoplasm (Wang *et al.* 1993). For example cytoskeletal actin filaments can be crosslinked either rigidly or flexibly (Gardel *et al.* 2004), and actin filaments can interact with microtubules or cytoplasmic intermediate filament (Stricker *et al.* 2010). This article summarizes evidence that similar interactions are relevant in the nucleoskeleton.

Many proteins with known structural significance in the cytoskeleton are known to either localize specifically in the nucleus, or shuttle in and out of the nucleus. These include β - and γ -(non-muscle) actin (Gieni and Hendzel 2009), and specific isoforms of spectrins, protein 4.1, nesprins (spectrin-repeat proteins) and titin, each of which has one or more demonstrated roles in the nucleus (Table 1). Most of these 'non-lamin' nucleoskeletal proteins interact with lamins and are likely to confer complementary mechanical properties to the nucleoskeleton. Lamins contribute significantly to the viscoelastic stiffness of the nucleus, as shown by several well-characterized methods (Dahl *et al.* 2005; Dahl *et al.* 2004; Lammerding *et al.* 2004; Rowat *et al.* 2004). Here we summarize the 'non-lamin' components of the nucleoskeleton, and describe tools to investigate their contributions to nuclear structure and function. Notably, the nucleoskeleton and cytoskeleton are linked—directly and mechanically—by multi-protein 'LINC' complexes that span the outer nuclear membrane (ONM) and inner nuclear membrane (INM) of the nuclear envelope (Crisp *et al.* 2005; Dahl *et al.* 2008). These connections in living cells, and the existence of both cytoskeletal and nucleoskeletal isoforms of key structural proteins, as well as the structural contributions of chromatin, collectively pose ongoing challenges to studying and understanding the superstructure and function of the nucleoskeleton.

A. Actin in the nucleus

Actin, a major component of the cytoskeleton, actively exchanges between the nucleus and cytoplasm and has numerous functions within the nucleus (Pederson and Aebi 2005). In the nucleus, actin does not polymerize as phalloidin-stainable F-actin, but instead assembles nuclear-specific or other 'unconventional' short polymers (Bettinger *et al.* 2004; Pederson and Aebi 2002). Also, a large fraction of actin in the nucleus is found as G-actin. Fluorescence recovery after photobleaching (FRAP) experiments in cells that transiently express GFP-fused β -actin revealed a dynamic equilibrium between low-mobility versus rapidly-diffusing populations of actin in the nucleoplasm, strongly suggesting the existence of structured/polymeric actin in nucleus (McDonald *et al.* 2006). Under the very specialized conditions of the developing *Xenopus* oocyte nuclei, which are huge and have only NE-associated (not

internal) lamins, nuclear export of actin is blocked to deliberately accumulate actin to enhance structural support for the oocyte nucleus (Clark and Merriam 1977; Parfenov *et al.* 1995).

Actin has many diverse roles in the nucleus including chromatin remodeling and the transcription, processing and export of mRNAs (Bettinger *et al.* 2004). Actin and actin-related proteins (ARPs) are core components of SWI/SNF chromatin remodeling complexes (Olave *et al.* 2002). Actin is also a component of transcription pre-initiation complexes and stimulates transcription by RNA polymerase II (Hofmann *et al.* 2004; Percipalle *et al.* 2001). Actin and nuclear myosin 1c are reported to both associate with and stimulate RNA polymerase I, and actin is required for transcription by RNA polymerase III (Fomproix and Percipalle 2004; Lanerolle *et al.* 2005; Philimonenko *et al.* 2004). However, it remains unclear exactly how actin and myosin function during transcription.

Actin polymers are also required for the integrity of filaments (not lamins) that connect NPC baskets to nucleosomes and Cajal bodies in the *Xenopus* oocyte nucleus interior (Figure 1). These filaments have been visualized by transmission electron microscopy (Arlucea *et al.* 1998) and in three dimensions by field emission scanning electron microscopy (feSEM) (Kiseleva *et al.* 2004; Kiseleva *et al.* 2001; Ris 1997). These 'pore-linked filaments' (PLFs) were destroyed by the actin-depolymerizing drug latrunculin A and altered by treatment with jasplakinolide, which stabilizes actin polymers. With jasplakinolide the filaments became more open and lacy with regularly-spaced short 'struts'. Actin and protein 4.1 each localized on PLFs, as visualized by immunogold labeling and feSEM. The protein 4.1-gold epitopes were spaced at 120-nm intervals and were often located within 60~80 nm of filament 'forks' (Kiseleva *et al.* 2004). The 'backbone' protein of PLFs is thought to be related to Tpr, the NPC basket protein.

Actin interacts functionally with lamin complexes. The inner nuclear membrane protein emerin, which binds lamins directly, is also a pointed-end F-actin capping protein that enhances actin polymerization *in vitro* (Holaska *et al.* 2004). This observation, and associations between emerin, nuclear myosin 1c and spectrin (discussed below), led to a proposal that the nuclear envelope, like the red blood cell membrane, might be supported by a 'cortical' network of membrane (emerin)-anchored spectrin and actin filaments (Gruenbaum *et al.* 2005; Holaska and Wilson 2007). Actin also binds lamin A directly (Simon *et al.* 2010), at two reported sites within the tail domain (Sasseville and Langelier 1998; Zastrow *et al.* 2004). Lamin A is unique in having two actin binding sites, compared to one site in lamin C and one relatively weak site in lamin B, and lamin A can 'bundle' F-actin *in vitro* (Simon *et al.* 2010). Whether this actin-bundling activity is relevant in living cells remains unknown.

The emerging number and variety of structural and non-structural roles for actin in the nucleus complicates the interpretation of studies in which the 'cytoskeleton' is manipulated, e.g., by actin-polymerizing drugs. For example, in many studies measuring nuclear mechanics within a cell, the cytoskeleton is depolymerized so that it does not dissipate applied force. In addition to disrupting cell structure and signaling, perturbing actin may cause unexpected (hence, untested) phenotypes in the nucleus that contribute to the composite phenotype. Better knowledge and new tools are required to understand the structure, function and regulation of actin in the nucleus.

B. Nuclear spectrin

Spectrins, first identified as a major component of the membrane skeleton of red blood cells (erythrocytes), are a well-characterized class of cytoskeletal proteins that line the plasma membrane. Human spectrins are encoded by seven genes, with two encoding α -spectrins (α I and α II) and five encoding β -spectrins (β I through β V) (Bennett and Baines 2001). Spectrins form a structural unit consisting of $\alpha\beta$ tetramers, e.g. (α I β I)₂ in erythrocytes. The α I-spectrin

gene is expressed in erythrocytes whereas α II-spectrin is highly expressed in vertebrate nucleated cells (Cianci *et al.* 1999; Young and Kothary 2005). Importantly α II-spectrin localizes in both the cytoplasm and nucleus; in the nucleus it is best known for its links to Fanconi anemia (FA) since α II-spectrin provides a scaffold that helps recruit DNA repair proteins to sites of DNA damage (McMahon *et al.* 2001; McMahon *et al.* 1999). Cells from FA patients have decreased levels (35%-40% of normal) of nuclear α II spectrin (McMahon *et al.* 2001). α II-spectrin binds directly to FANCG (a component of the FANCA, FANCC, FANCF, FANCG complex) and colocalizes with the cross-link repair protein, XPF, in damage-induced nuclear foci after treatment of cells with DNA interstrand cross-linking agents (Lefferts *et al.* 2009; Sridharana *et al.* 2003).

The phenotypes caused by α II-spectrin depletion support the idea that α II-spectrin functions in both the cytoskeleton and in nuclei. For example siRNA-downregulated lymphoblastoid cells and HeLa cells show chromosomal instability and hypersensitivity to DNA interstrand cross-linking agents (McMahon *et al.* 2009). The α II-spectrin deficiency phenotype in WM-266 human melanoma cells included loss of stress fibers, cell adhesion defects and reduced density of focal adhesions, as well as reduced proliferation with cell cycle arrest at the G1 phase, Rb phosphorylation and elevated expression of the cyclin-dependent kinase inhibitor, p21Cip (Metral *et al.* 2009).

Other nuclear proteins are known to associate with nuclear α II-spectrin, but whether they bind directly or indirectly is unknown. Among the nuclear proteins that co-immunoprecipitated with α II-spectrin are actin, protein 4.1B, β -spectrin (β SpIVS5), lamin A and emerin (Sridharana *et al.* 2006). Independently, chromatographic purification of six proposed emerin-containing complexes from HeLa cell nuclei revealed a proposed 1.5 MDa complex that included emerin, α II-spectrin, actin, nuclear myosin Ic, lamins and SUN2 (Holaska and Wilson 2007). These findings implicate spectrins as significant components of the nucleoskeleton.

In erythrocytes the spectrin-actin-protein 4.1 network is responsible for elasticity and mechanical recovery after deformation (Bennett 1993). Their combined presence in the nucleus suggests similar, possibly essential, contributions to nuclear elasticity that will be important to test. Interestingly protein 4.1 is essential to assemble nuclei *in vitro* in cell-free extracts of *Xenopus* eggs (Krauss *et al.* 2003). In addition to the structural proteins that co-immunoprecipitated with α II-spectrin (Sridharana *et al.* 2006), there were also proteins involved in DNA repair (eg. XPA, XPB, XPF, ERCC1), Fanconi anemia (eg. FANCA, FANCC, FANCD2), chromatin remodeling (eg. actin, BRG1, hBRM and CSB), or transcription and RNA processing (eg. P40, hnRNP A2/B1, PML). These findings suggest α II-spectrin has multiple functions in the nucleus, both at the peripheral and internal nucleoskeleton, most of which await investigation.

C. Nuclear titin

Titins, also known as connectins, are a family of giant elastic proteins first found in vertebrate striated muscle. Titin is the largest known human protein, with a mass of ~3 MDa and contour length greater than 1 μ m (Cola *et al.* 2005). Titins have more than 240 tandem repeats of predominantly immunoglobulin-like (Ig) and fibronectin (FN3)-like domains, each of which has a persistence length of ~10 nm (Lee *et al.* 2007). Skeletal muscle titin localizes in the sarcomere; it has a kinase domain for signaling and also functions mechanically as a molecular spring to provide muscle with elasticity, allow post-contraction recovery, and prevent overextension (Machado and Andrew 2000). Mutations in titin cause dilated cardiomyopathy (Gerull *et al.* 2002); cardiomyopathy is also a clinical phenotype in many lamin-linked syndromes (Capell and Collins 2006). Interestingly, most eukaryotic cells also have a nuclear isoform of titin, which associates with chromosomes and is essential for mitotic chromosome condensation (Machado *et al.* 1998). In *Drosophila*, loss of titin is lethal during mitosis:

chromosomes fail to condense properly during prophase, have defects in sister chromatid cohesion, and missegregate during mitosis (Machado and Andrew 2000).

Titin, which has Ig-folds itself, binds directly to the Ig-fold domain of both A- and B-type lamins *in vitro*, with a slight preference for lamin A (Zastrow *et al.* 2006). Titin binding was mildly but selectively sensitive to specific laminopathy-causing missense mutations in the Ig-fold domain of lamin A, suggesting these mutations might perturb lamin-titin connections. Altering titin-lamin interactions leads to highly dismorphic nuclei (Figure 2). In *C. elegans* embryos, titin co-localized with lamins at the nuclear envelope, and this localization required lamins, suggesting nuclear titin is anchored or organized by lamin filaments (Zastrow *et al.* 2006). Titin is also slightly enriched at the nuclear envelope in human cells (Zastrow *et al.* 2006).

These findings, and the presence of other titin-binding proteins (notably, actin) in the nucleus, implicate titin in the long-range organization, stability, elasticity and mechanics of both the nuclear envelope and chromosomes during interphase, as well as in mitosis. Further studies, sorely needed to test predicted mechanical functions, will be challenging given the enormous size of titin and potential functional overlap with other titin-related genes.

D. LINC complexes at the nuclear envelope

There are direct mechanical connections between the cytoskeleton and nucleoskeleton, mediated by LINC (linker of nucleoskeleton and cytoskeleton) complexes (Dahl *et al.* 2008; Stewart-Hutchinson *et al.* 2008). The basic components of these complexes are KASH-domain proteins (e.g., mammalian nesprins, aka SYNE) and SUN-domain proteins, which are embedded in the outer and inner nuclear membranes and interact (via the SUN and KASH domains) within the luminal space of the nuclear envelope (Razafsky and Hodzic 2009). The discovery of these complexes has ‘coupled’ the disparate research worlds of the cytoskeleton and nucleoskeleton (Hale *et al.* 2008). LINC complexes explain how the nucleus is positioned within cells (Tzur *et al.* 2006), how chromosomes can be moved in the plane of the nuclear envelope (Razafsky and Hodzic 2009), and how mechanical forces can be transduced directly from the cytoskeleton to the nuclear interior (Dahl *et al.* 2008).

The human genome encodes four nesprin genes (SYNE-1, SYNE-2, Nesprin-3 and Nesprin-4). SYNE-1 and -2 are alternatively transcribed and spliced to yield protein isoforms with different sizes, functions and locations, including specific localization to either the ONM, INM, nuclear interior or cytoplasmic organelles including the Golgi complex (Zhang *et al.* 2002; Zhang *et al.* 2005). Nesprin-3 (isoforms α and β) and Nesprin 4 localize at the ONM (Roux *et al.* 2009; Wilhelmsen *et al.* 2005). To localize such proteins specifically requires both epitope-specific tags or antibodies, and methods that can distinguish between the INM and ONM. One classic method, digitonin-permeabilization (described below), is still a powerful approach to localize specific proteins on the nuclear envelope.

The largest (‘giant’) isoforms of nesprin-1, nesprin-2 and nesprin-3 bind either actin (nesprins-1 γ , nesprin-2 γ) or plectin (nesprin-3 α and β) in the cytoplasm and are embedded in the ONM as key components of the LINC complex (Crisp *et al.* 2005; Dahl *et al.* 2008). Other, smaller nesprin isoforms such as nesprin-1 α and nesprin-2 β localize at the INM and directly bind lamins and emerin. The nesprin (nuclear envelope spectrin repeats) polypeptide structure can include an N-terminal actin binding ‘calponin homology’ (CH) domain (present on ‘giant’ isoforms only), variable numbers of tandem spectrin-like repeats (SLRs), and near the C-terminus a unique ‘adaptive’ domain followed by several more SLRs and the C-terminal membrane-anchoring KASH (Klarsicht-Anc1-syne1 homology) domain (Zhang *et al.* 2002). The SLRs in nesprins are similar to typical spectrin repeats, suggesting nesprins (like other spectrin repeat superfamily proteins) have elastic properties, and the ‘adaptive’ domain is

evolutionarily conserved and structurally stabilizes the SLRs in nesprins (Zhong *et al.* 2010). The KASH domain comprises both a transmembrane domain and an evolutionarily-conserved ~30 residue motif that is located in the nuclear envelope lumen and directly binds SUN-proteins (Razafsky and Hodzic 2009).

The ~200 residue SUN-domain (Sad1-UNC-84 homology) is encoded by four mammalian genes (SUN1, 2, 3 and SPAG4). SUN1 and SUN2 are both INM proteins (Tzur *et al.* 2006); their SUN domain is located in the nuclear envelope lumen, and their N-terminal domain is nucleoplasmic and binds lamin filaments. SUN proteins function as dimers. The unique 'adaptive' domain of nesprins may also mediate homodimerization, thereby increasing the mechanical strength and stability of NE-spanning SUN/KASH-mediated LINC complexes (Zhong *et al.* 2010).

E. Roles for nucleoskeletal proteins in mitosis and nuclear assembly

Nearly all mechanical studies of nucleoskeletal proteins have been done in interphase cells, since mitosis involves very rapid, large-scale structural changes that can challenge biophysical measurement. However, many nucleoskeletal proteins appear to have dual roles: they also contribute structurally during mitosis, by organizing the mitotic spindle.

The idea of a "spindle matrix", first proposed in the 1960s (Smetana *et al.* 1963), was that a stiff non-microtubule filamentous structure might help anchor and move chromosomes during mitosis. This idea is supported by recent evidence that lamin B and other nucleoskeletal proteins, including titin, can localize to spindle-like structures during mitosis (Fabian *et al.* 2007; Tsai *et al.* 2006). For example by immunofluorescence, titin co-localizes with microtubules and also with other spindle matrix candidate proteins including (in *Drosophila*) the EAST, skeletor, megator and chromator proteins (Fabian *et al.* 2007). Proposed spindle matrix proteins in vertebrates include NuMA (Nuclear Mitotic Apparatus protein), a conserved 200-240 kDa coiled-coil microtubule-binding protein (Dionne *et al.* 1999; Zeng *et al.* 1994). NuMA was first identified as a mitotic polar component that originated from the nucleus. In *Xenopus*, NuMA associates with dynein-dynactin complexes in centrosomes to anchor spindle microtubules at the poles (Dionne *et al.* 1999). The interphase functions of NuMA are not well understood. However NuMA is known to interact with both structural and regulatory proteins including lamins, protein 4.1, microtubules and the INI1 (chromatin remodeling) complex, suggesting roles in both the nucleoskeleton and chromatin organization (Barboro *et al.* 2001). EAST (Enhanced Adult Sensory Threshold) is a ~250 kDa *Drosophila* protein with seven potential nuclear localization sequences (NLS) and twelve potential PEST proteolytic signals (Wasser and Chia 2000). EAST is an essential, ubiquitous nuclear protein that forms a network throughout the nucleus, and colocalizes with actin. During interphase in *Drosophila*, megator surrounds chromosomes and is enriched at the nuclear envelope, whereas skelator and chromator (which bind each other directly) localize on chromosomes. During mitosis, these three proteins collectively form a "spindle matrix" that underlies the microtubule spindle (Qi *et al.* 2005; Rath *et al.* 2004; Walker *et al.* 2000). Interestingly megator is orthologous to the mammalian NPC-basket protein Tpr; megator interacts with EAST and is proposed to form a nuclear 'endoskeleton' with EAST. We speculate this interior nucleoskeleton might be analogous to the PLFs (pore-linked filaments) visualized in *Xenopus* oocyte nuclei.

II. Methods

A. Localization of endogenous nucleoskeletal proteins by indirect immunofluorescence microscopy

Historically, for proteins now known to function in both the cytoskeleton and nucleoskeleton, nuclear signals were often either disregarded or assumed to be nonspecific. The seemingly

simple task of localizing a proposed endogenous non-lamin nucleoskeletal protein in interphase nuclei, by indirect immunofluorescence, is a litmus test that can pose frustrating challenges. For example staining may be negative because epitopes are inaccessible in assembled structures, accessible only at certain stages of the cell cycle, blocked by association with chromatin or lamin filaments, or masked by posttranslational modifications. Conversely, positive signals provide key information, and can be used to assess the behavior of epitope- (e.g., Green Fluorescence Protein/GFP-) tagged, exogenous versions of the same protein, since tags and overexpression can cause mislocalization or other artifacts. However agreement between these methods supports further experiments using the more-easily-detected tagged protein.

Below we outline a 'typical' indirect immunofluorescence protocol that has been used, with minor modifications, to localize lamins (Dahl *et al.* 2006; Lammerding *et al.* 2006) and several non-lamin nucleoskeletal proteins including LINC proteins, titin and spectrins (Haque *et al.* 2006; Roux *et al.* 2009; Sridharana *et al.* 2003; Zastrow *et al.* 2006) in cultured cells and in some cases also in *C. elegans*, *Drosophila* or tissue samples. We highlight important caveats, and describe alternative approaches that might improve antibody-based detection of endogenous nucleoskeletal proteins. All steps are done at room temperature (22-25°C) unless otherwise noted.

1. Culture cells on coverslips (22×22 mm); wash gently three times with phosphate buffered saline (PBS), then fix 15 min in PBS containing 3.7% formaldehyde (stock solution is 37%, prepare fresh). *Caveat and alternative:* some epitopes are sensitive to fixation. Try other fixation methods, e. g. (a) Paraformaldehyde fixation: fix with 3.5% paraformaldehyde at 4°C for 5 min followed by 10 min at room temperature (DiDonato and Brasaemle 2003). (b) Cold methanol fixation: fix with cold methanol (store at -20°C before use) for 5 min in a pre-chilled glass tray kept on dry ice (Jiang and Serrero 1992). (c) Cold acetone fixation: fix with cold acetone (store at -20°C before use) for 10 min in a pre-chilled glass tray kept on dry ice during fixation (Hammond and Glick 2000). (d) TCA fixation: fix the cells in 10% trichloroacetic acid in water cooled to 4°C for 15 min, then wash with 30 mM glycine in PBS (Haraguchi *et al.* 2004).
2. Wash fixed cells three times with PBS, then permeabilize (all membranes) by incubating 30 min in PBS/0.2% Triton X-100. Permeabilization is not required for samples fixed using organic solvents (e.g. methanol or acetone). Optimal permeabilization is very important for detecting proteins within the nucleus, since intact inner and outer nuclear membranes block antibody access, and potential cytoskeletal signals will overwhelm. Excess or harsh permeabilization can risk solubilizing or destroying endogenous structures.
3. Wash three times with PBS, then block 1 hour in PBS plus a nonspecific blocking protein, such as 2% bovine serum albumin (BSA) or nonspecific serum that does not conflict with your species-specific primary or secondary antibodies.
4. Dilute primary antibodies (dilution is antibody-dependent; test dilution series) in small volume (50-100 µL) of PBS/2% BSA, add to fixed cells and incubate 1 hour (room temperature) or longer at 4°C. Keep coverslips in a moist chamber, or invert onto a 50 µL drop of antibody solution on parafilm. Do not let coverslips dry. *Caveat and alternative:* Epitope(s) can be blocked by posttranslational modifications, bound partners or nearby structures (e.g., lamins, actin, chromatin). Try a different antibody that targets a different region of your protein, or a polyclonal antibody (likely to recognize a variety of epitopes on your protein). Different antibodies can preferentially detect either the nuclear or cytoplasmic isoforms of a given protein (e.g., α II-spectrin; (McMahon *et al.* 1999; Metral *et al.* 2009; Sridharana *et al.* 2003)).

5. Wash three times with PBS, then incubate 1 hour with diluted species-specific conjugated fluorescent secondary antibody.
6. Wash twice with PBS, then once with PBS/DAPI (1:3000 dilution of 1 mg/ml stock).
7. Invert coverslips onto a slide with 4 μ L Vectashield or similar anti-fade reagent. Seal periphery with clear fingernail polish, and visualize by fluorescence microscopy. Confocal microscopy is recommended for high Z-axis resolution and three-dimensional image reconstruction; this is particularly useful to distinguish subnuclear localizations, and visualize nuclear envelope enrichment. Care must be taken to avoid touching or pushing coverslips, which can grossly distort cell and nuclear height. This issue is avoided by immunolabeling cells cultured on glass-bottom dishes (described next).

A-1. Alternative: Immunolabel cells cultured on glass-bottom dishes—To protect cells from mechanical pressure (and avoid air bubbles that can be introduced when mounting coverslips), consider culturing and labeling cells in a glass-bottom dish. Note that these fixed preparations can become contaminated and should be imaged without delay.

1. Culture cells in 35-mm glass-bottom dish (e.g., from Mattek or Warner) and then fix, permeabilize, block and label as described above (A, steps 1-6), taking care to keep cells wet; larger volumes of all reagents, including antibodies, will be required.
2. Fill the dish with 1 ml PBS, and then image fluorescence using an inverted microscope. Dishes wrapped with parafilm can be stored at 4°C for up to one month.

A-2. Alternative: Isolate nuclei prior to immunolabeling—If the cytoskeletal signal overwhelms the nuclear signal, consider labeling isolated nuclei.

Nuclei can be isolated using a kit (several are available from Sigma), or the method described here. Kits are easy but may retain cytoplasmic remnants. The method described here is more labor intensive and gives significantly lower yields than kits, but nuclei are relatively free of cytoskeleton and endoplasmic reticulum. To isolate nuclei (adapted from (Dahl *et al.* 2005; Dean and Kasamatsu 1994):

- (1) Start with $\sim 10^7$ cells; rinse twice with PBS and once with cold (4°C) 10 mM HEPES pH 7.5, 1 mM dithiothreitol (DTT).
- (2) Scrape cells into minimal volume (<1 mL) HEPES/DTT, and incubate 10 min on ice.
- (3) Lyse cells by 10-25 strokes in Dounce homogenizer, or a small bore needle.
- (4) Add lysed cells to one-fifth volume of 5X sucrose-salt solution (1.25 M sucrose, 250 mM Tris pH 7.6, 125 mM KCl, 15 mM MgCl₂, 15 mM CaCl₂, and protease inhibitors) and incubate 10 min on ice. Salt condenses DNA; sucrose will produce a gradient for ultracentrifugation.
- (5) Add 2.3 M sucrose-salt solution (2.3 M sucrose, 50 mM Tris pH 7.6, 25 mM KCl, 3 mM MgCl₂, 3 mM CaCl₂ and protease inhibitor) to achieve a final concentration of 1.6 M sucrose.
- (6) Layer this 1.6 M sucrose-salt-lysate suspension onto a 150 μ L cushion of 2.3 M sucrose-salt and centrifuge at 4°C for 1 hr at 166,000 $\times$ g. Debris will collect at the interface; nuclei are dense and will pellet.
- (7) Resuspend nuclei in PBS. Alternatively, to dilute nuclei, resuspend in 50 mM Tris pH 7.5

Once nuclei are isolated (by either method):

1. Prepare polylysine-coated coverslips by adding a 0.1% (w/v) solution of poly-L-lysine (premade, sterile solutions are available from Sigma) directly to clean, dry coverslips. Allow lysine to adsorb 20 min, then aspirate and dry. (Lysine solution can be reused several times).
2. Dilute isolated nuclei in PBS (or 50 mM Tris pH 7.5) to approximately 10^6 nuclei / mL and place on coverslip.
3. Fix, permeabilize, block and label as described above (A, steps 1-6).

Caveat and alternative: Epitopes can be blocked by chromatin or soluble nuclear components. Before fixing, consider the following strategy to remove most DNA and non-nucleoskeletal components from isolated nuclei (adapted from (Graham and Rickwood 1997)).

- (1) Suspend isolated nuclei in 55 μ L 0.25 M sucrose, 5 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5, then add DNase and RNase to final concentrations of 250 μ g/mL each.
- (2) Incubate 60 min at 4°C, then pellet nuclei (1000xg, 10 min, 4°C).
- (3) Resuspend in 50 μ L 10 mM Tris-HCl pH 7.4, 0.2 mM $MgCl_2$ and then slowly add four volumes of 2 M NaCl, 0.2 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4.
- (4) Add 2-mercaptoethanol to final concentration of 1%, and incubate 15 min at 4°C.
- (5) Pellet nuclei by centrifuging 30 min at 1,600g.
- (6) Repeat step 3 and 5 (skip 4), and resuspend in 50 μ L 10 mM Tris-HCl pH 7.5.

B. Endogenous protein localization at the INM versus ONM

Nuclear envelope membranes and their enclosed luminal space are continuous with the endoplasmic reticulum, but nevertheless represent three highly specialized functional domains: the ONM, the INM and 'pore-membrane' domain around each NPC. Proteins that localize at the pore-membrane domain are readily distinguished by punctate fluorescent staining that overlaps with NPC markers, as recently (and surprisingly) shown for SUN1 in Hela cells (Liu *et al.* 2007). One can also deduce the sub-nuclear envelope localization of candidate proteins by double- or triple-labeling with antibodies against NPC proteins located on either the cytoplasmic (e.g., Nup358 or Nup214) or nucleoplasmic (e.g., Nup153 or Nup98) side of the NPC, or marker proteins specific to the INM (e.g., emerin, MAN1) or nuclear envelope lumen (Schermelleh *et al.* 2008). However, these methods require very high spatial sensitivity in imaging.

Localization can be determined unambiguously by other methods including transmission electron microscopy of immuno-gold-labeled samples (not described here), or the following classic method which distinguishes ONM and INM localization based on indirect immunofluorescence staining of cells made permeable using either Triton-X100 (dissolves all membranes), or Digitonin (affects the plasma membrane but not interior or nuclear membranes).

Digitonin is a steroid glycoside that binds cholesterol and other β -hydroxysterols that are highly enriched in the plasma membrane, relative to intracellular membranes (Fiskum *et al.* 1980). Antibodies incubated with digitonin-permeabilized cells have access only to epitopes that face the cytoplasm, including proteins on the ONM. Proteins located at the INM, nuclear envelope-lumen or nucleoplasm are detected only in Triton-permeabilized cells (See Figure 3).

To apply the digitonin method to fixed cells, incubate a second set of fixed cells 15 min (on ice) in PBS/0.004% digitonin (reported range is 0.002% to 0.005%), rather than PBS/Triton, and then continue as described in section A.

C. Immunoprecipitation of nucleoskeletal proteins

Co-immunoprecipitation (co-IP) is an excellent way to detect protein-protein associations in cells. This procedure can validate biochemical experiments and when coupled to other methods such as mass spectrometry, can be used to identify novel partners or components. The main challenge for lamins, and probably most other nucleoskeletal proteins, is that one must first *dissociate* pre-existing (potentially highly stable) structures, in order to solubilize and study them. Summarized below are examples of methods used to solubilize one or more specific nucleoskeletal proteins. These methods focus specifically on two key steps: cell/nuclear lysis and protein solubilization.

1. Solubilization conditions suitable for recovery of lamins, other nucleoskeletal components and other proteins including nuclear membrane protein emerin, from isolated HeLa cell nuclei—

First, isolate nuclei by hypotonic lysis of cells (Offterdinger *et al.* 2002): Wash cells twice in PBS, harvest by scraping, pellet cells (1,850g, 10 min, 4°C), resuspend in 5 packed-cell-volumes of hypotonic buffer (10 mM Hepes pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, and protease inhibitors). Then recentrifuge, suspend in 3 packed-cell-volumes of hypotonic buffer, incubate on ice 10 min, homogenize (Dounce, type B pestle, 25 strokes) and centrifuge (3,300g, 15 min, 4°C) to pellet nuclei. Wash the nuclear pellet twice with 10 ml hypotonic buffer. Then, to solubilize nucleoskeletal and other components, switch to the following protocol, which allowed subsequent immuno-affinity and chromatographic purification of complexes that included emerin plus specific combinations of other proteins including lamin A, lamin B, α II-spectrin, actin, nuclear myosin 1c, SUN2, histones or transcription regulators (Holaska and Wilson 2007). Resuspend purified nuclei in high-salt/detergent buffer (1 M NaCl, 1% Triton X-100, 20 mM Hepes pH 8.0) to extract lamins and nuclear membrane proteins, and centrifuge (30 min, 40,000g, 4°C) to pellet insoluble material. Dilute the supernatant 10-fold with 20 mM Hepes (pH 8.0), incubate 10 min at 4°C to allow re-formation of complexes that may have dissociated during lysis, and recentrifuge (30 min, 40,000g, 4°C) to yield a clarified supernatant enriched for lamins and other nucleoskeletal proteins, some of which may retain biologically-relevant associations. Protein(s) of interest can be immunoprecipitated or studied by other methods. For quality control, verify and estimate the percent solubilization of nucleoskeletal marker proteins (e.g., lamins, emerin) and protein(s) of interest, by western blot analysis of small aliquots of the starting nuclear pellet, soluble *versus* insoluble nuclear fractions, and each clarified supernatant.

2. Efficient solubilization of lamins—Lamin polymers resist biochemical extraction. This resistance can be exploited by treating cells or isolated nuclei with moderate-salt buffers, to solubilize most nuclear proteins, and then pelleting to obtain a lamin-enriched pellet. Lamins are solubilized relatively efficiently by high salt (e.g., 1 M NaCl) or high pH (pH 8.0 to 9.0) or preferably both as summarized next from (Aebi *et al.* 1986; Herrmann and Aebi 2004). Isolate nuclear envelopes (or nuclei) and wash with 1 M KCl, then incubate 30 min in [300 mM KCl, 2% Triton X-100, 10% sucrose, 20 mM MES-KOH pH 6.0, 2 mM EDTA, 1 mM DTT] and centrifuge (6,000g, 20 min) to obtain a lamin-enriched pellet. Resuspend this pellet in [500 mM KCl, 2% Triton X-100, 20 mM Tris-HCl pH 9.0, 2 mM EDTA, 1 mM DTT] for 30 min on ice, and then centrifuge (200,000g, 40 min, 4°C) to yield a supernatant of solubilized lamins, and an insoluble pellet that may include any contaminating cytoplasmic intermediate filaments (Aebi *et al.* 1986; Herrmann and Aebi 2004). Interestingly the solubility properties of A- and B-type lamins in living cells can be manipulated. E.g., endogenous A- and B-type lamins are more resistant to extraction from cells that overexpress lamin A, whereas endogenous lamins B1 and B2 are more easily solubilized from cells that overexpress lamin B2 (Schirmer and Gerace 2004). The potential effects of these manipulations on other nucleoskeletal components will be interesting to test.

3. Solubilization conditions suitable for nesprins—Several nesprin isoforms, from large to small (e.g., the INM-localized nesprin-1 α) can be immunoprecipitated from mouse C2C12 myoblasts after lysis in [100 mM NaCl, 1% Triton X-100, 20 mM Tris-HCl pH 7.5, 1 mM EDTA-Na₂] (Zhang *et al.* 2001). If cytoskeletal contaminants are a concern, consider first isolating nuclei through a sucrose density gradient (section A-2 part 1).

D. Micropipette aspiration and recoil

Micropipette aspiration (MPA) is a widely used micromanipulation technique for quantifying the viscoelastic properties of cells, isolated nuclei and other biological samples. The mechanical contribution of a target nuclear structural protein can be measured by comparing the micropipette aspiration results of control versus manipulated (overexpressing, or depleted) cell nuclei. Here we introduce a new ‘recoil’ step to the traditional micropipette aspiration assay, which reflects both the energy storage capacity and elasticity of the nucleus. This method enables one to measure both nuclear deformation and recovery.

1. Pull (or purchase) micropipettes of 8- to 10- μ m diameter from 1-mm borosilicate capillaries (WPI, Sarasota, Florida) with commercial pipette pullers (MicroData Instrument Inc or Sutter), also used to make pipettes for microinjection and patch clamping. Recoil assays require larger micropipette diameters (~10 μ m) than those normally used for nuclei MPA (~3-5 μ m). The larger diameter reduces damage to nuclear structure and facilitates recoil by reducing the plasticity of aspiration. Quantitative recoil assays require pipettes with relatively constant tip diameter (~10 μ m) along the entire length contacted by the aspirated nuclei to ensure constant cross section of aspiration.
2. Back-fill the micropipette with 1% BSA in PBS using a syringe with needle to coat the tip and thereby lower static charge and prevent adhesion. New commercial devices are being developed to facilitate micropipette handling and back-filling (e.g., Warner Instruments).
3. Connect the filled micropipette to the pressure system and micromanipulators (e.g., Narishige or Eppendorf) via a waterline to a water-filled reservoir. Aspirate sample buffer into the micropipette to ensure solution compatibility.
4. Cells in suspension can be incubated with both a cell-permeable DNA dye (such as Hoechst 33342) and a cell-impermeable DNA dye (propidium iodide). Thus, nuclear deformation can be specifically tracked in live cells, since ruptured cells become PI-positive. One caveat is that Hoechst 33342 intercalates into DNA and can slightly affect nuclear stiffness. However, it is still useful when comparing samples on this short time scale.
5. Apply and measure aspiration pressures. Since nuclei are typically stiffer than other parts of the cell, negative pressures of 0.1–10 kPa are required for aspiration. Aspiration pressures this high are typically generated by syringe suction. A pressure transducer or manometer in parallel (or within) the apparatus is required to monitor applied pressure.
6. Observe cells and nuclei in brightfield and via the Hoechst 33342 signal, respectively, with a high resolution fluorescent microscope. Check the propidium iodide signal before and after aspiration, to verify cell viability. During aspiration at constant pressure, the nucleus will be gradually sucked into the micropipette forming a ‘projection’. The projection area increases as a function of time.
7. The best strategy is to produce and hold a set aspiration pressure, then watch deformation over time. These “creep” tests reveal viscoelastic deformation. Instantaneous removal of pressure allows nuclei to recoil. To measure recoil

(reversibility of aspiration), release pressure to zero when the projection area of the aspirated nucleus is less than one third of total nuclear area. Aspiration into too-narrow pipettes (<5µm diameter), or release after projections become too large may lead to incomplete recoil.

8. The creep compliance of the micropipette aspiration process can be quantified by the

modified equation: $J(t) = \frac{\pi\Phi\Delta A}{3\Delta P R_p^2}$, where Φ depends on micropipette wall thickness (Theret *et al.* 1988), ΔP is the constant pressure applied, ΔA is aspiration area and R_p is pipette radius (Figure 4). Creep compliance (J) is useful to compare between experimental conditions and to examine the form of the deformation over time (viscoelastic, purely elastic). However, extracting exact measures of viscoelasticity, particularly for large pipette aspiration, is not recommended.

For nuclear aspiration and recoil, time dependant creep compliance matches a power law,

$J(t) = A\left(\frac{t}{t_0}\right)^\alpha$, and the data spots can be linear fit on a log-log scale. We have found that the power law coefficient α is similar for both aspiration and recoil in wildtype HeLa cell nuclei and TC7 cell nuclei (Dahl *et al.* 2005). For cells with nuclear defects or deficiencies (e.g., in lamin A/C or α II-spectrin), the power law coefficient α during aspiration versus recoil can differ dramatically. Thus by measuring recoil explicitly, one can detect new types of mechanical defects, specifically including nuclear elasticity, that were not previously detectable by micropipette aspiration or AFM.

III. Discussion and prospects

Further studies of non-lamin components of the nucleoskeleton are expected to enhance our knowledge of complex nuclear structure, architecture and mechanics. We require this complete insight to better our understanding of nuclear interactions with the cytoskeleton, organization and mechanical access of the human genome, potential disruption in human diseases, as well as the role of nuclear deformation in regulating how cells are able to translocate and negotiate the extracellular matrix.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Actin-based pore linked filaments in the interior of *Xenopus* oocyte nuclei
(A) Nuclear content fixed and visualized by feSEM after “peeling” away the nuclear envelope. Intracellular filaments associated with spherical bodies are seen in the figure. (B) The Immunogold feSEM identify the nuclear actin in filamentous network (pseudo-colored yellow). Adapted from (Kiseleva et al. 2004)

Figure 2.

Indirect immunofluorescence microscopy indicate that cells expressing GFP-NLS (nuclear localization signal) fused titin fragment M-is7 had a high frequency of aberrantly-shaped nuclei and nuclear envelope herniations (arrowheads) that contained GFP-is7, but not chromatin or lamin B. Negative control use cells transfected with GFP-NLS fused to pyruvate kinase. Bar: 2 μ m. Adapted from (Zastrow *et al.* 2006)

Figure 3. Altered membrane permeabilization allows visualization of INM versus ONM proteins
 Digitonin permeabilizes the plasma membrane but not the nuclear membrane whereas Triton X-100 permeabilizes all membranes. During immunocytochemistry this allows for altered antibody accessibility to different regions of the nucleus. INM proteins cannot be detected with digitonin permeabilization (A and A'), but can be detected with Triton X-100 treatment (B and B'). ONM protein can be detected both with digitonin (C and C') and Triton X-100 (D and D') treatment. This has been shown functionally with the nucleoskeleton protein lamin A/C which is inside the nucleus, and the ONM protein Nesprin 4 (A' through D' adapted from (Roux *et al.* 2009)).

Figure 4. Micropipette aspiration (MPA) of nuclei and cells to show subtle nuclear structural and mechanical changes

(A) Schematic of aspirated cell showing nuclear deformation into the pipette of ΔA (crosshatched) under applied pressure ΔP . (B) Aspiration of cells into the micropipette under constant pressure shows nuclear deformation into the pipette. (C) When pressure released to zero, the wide type (WT) cell recoiled back gradually, while cell with loss of α II spectrin (KD α II-sp) failed to recoil.

Table 1

Non-lamin structural proteins in nucleus and their partners and functions.

Structural Protein	Associated Protein	Associated Function
actin	<i>CH doamin proteins:</i> α II-spectrin, α actinin-4, β II-spectrin, filamin A, Dystrophin, vav, nesprin-2, L-plastin; <i>Chromatin remodeling complexes:</i> BAF, SWI-SNF, BAP, NuA4, TIP60, PBAF, p400, SWR1, INO80 complexes; <i>Ribonucleoprotein complexes proteins:</i> hrp36, hrp65, DBP40; <i>Other proteins:</i> emerin, titin, protein 4.1, lamin A, MAL, profilin, capG, exportin-6, Zyxin, Myopodin, Nrf2, NDHII, DNase I.	Nucleoskeleton, Chromatin remodeling, RNA transcription and processing, Nuclear export
α II-spectrin	<i>Structural proteins:</i> actin, protein 4.1, β SpIVS5, lamin A, emerin; <i>DNA repair proteins:</i> hHR23B, XPA, RPA32, RPA70, XPB, XPD, XPG, XPF, ERCC1, MRE11, RAD50, RAD51, XRCC2, Ku70, Ku80; <i>Chromatin remodeling proteins:</i> actin, FANCA, BRG1, hBRM, CSB; <i>Fanconi anemia proteins:</i> FANCA, FANCC FANCD2, FANCF, FANCG, FANCI; <i>Transcription and RNA processing proteins:</i> p40, PML (hnRNP) A2/B1.	DNA repair, Fanconi Anemia, Nucleoskeleton
titin	lamin A, lamin B, actin, nuclear myosin I	Spindle matrix
nesprin	lamin A, lamin B, actin, emerin, SUN proteins	LINC complex
protein 4.1	actin, α II-spectrin, NuMA, U2AF, SC35	Nucleoskeleton, pre-mRNA processing
NuMA	Dynein, dynactin, protein 4.1, lamins, Arp1, GAS41, INI1, LGN, tubulin	Nucleoskeleton, Spindle matrix
EAST	actin, CP60, megator,	Endonucleoskeleton
megator	EAST	Spindle matrix
skeletor	chromator	Spindle matrix
chromator	skeletor	Spindle matrix