

# Chapter 17

## Wound-Healing Assays to Study Mechanisms of Nuclear Movement in Fibroblasts and Myoblasts

Wakam Chang, Susumu Antoku, and Gregg G. Gundersen

### Abstract

The rearward positioning of the nucleus is a characteristic feature of most migrating cells. Studies using wounded monolayers of fibroblasts and myoblasts have shown that this positioning is actively established before migration by the coupling of dorsal actin cables to the nuclear envelope through nesprin-2G and SUN2 linker of nucleoskeleton and cytoskeleton (LINC) complexes. During nuclear movement, these LINC complexes cluster along the actin cables to form adhesive structures known as transmembrane actin-associated nuclear (TAN) lines. Here we described experimental procedures for studying nuclear movement and TAN lines using wounded monolayers of fibroblasts and myoblasts, the acquisition of data using immunofluorescence microscopy and live-cell imaging, and methods for data analysis and quantification.

**Key words** LINC complex, SUN protein, Nesprin, TAN line, Retrograde actin flow, Nuclear lamina, Nuclear positioning, Centrosome orientation, Cell migration

### Abbreviations

CH	Calponin homology
DAPI	4',6-Diamidino-2-phenylindole
GFP	Green fluorescent protein
KASH	Klarsicht, Anc1 and Syne homology
LINC	Linker of nucleoskeleton and cytoskeleton
LPA	Lysophosphatidic acid
MEFs	Mouse embryo fibroblasts
miniN2G	Mini-nesprin-2G
MT	Microtubule
MRCK	Myotonic dystrophy-related, Cdc42-binding kinase
SUN	Sad1 and Unc83
TAN lines	Transmembrane actin-associated nuclear lines

## 1 Introduction

The nucleus maintains its proper position in most cells and undergoes active movement during various cellular and developmental processes, including migration, fertilization, cell division, and differentiation in animal cells [1]. Abnormal nuclear positioning is associated with diseases such as muscular dystrophy, cardiomyopathy [2], and lissencephaly [3]. The nucleus is moved and positioned by forces generated by the actin and microtubules networks [1]. In most cases this movement is mediated by the linker of nucleoskeleton and cytoskeleton (LINC) complex, which is composed of inner nuclear membrane SUN (Sad1 and *Unc83* homology) domain proteins and outer nuclear membrane KASH (*Klarsicht*, *Anc1* and *Syne* homology) domain proteins [4]. The SUN domain projects into the perinuclear space where it interacts with the KASH domain, establishing a protein complex that spans the two nuclear membranes. KASH proteins (termed nesprins in mammals) interact with cytoskeletal elements in the cytoplasm through direct and indirect means whereas SUN proteins bind to the nuclear lamina and other inner nuclear membrane proteins [5].

Various systems have been developed to study nuclear movement and positioning, including fertilized eggs [6], embryonic *C. elegans* hypodermal cells [7], *Drosophila* ommatidia [8, 9], neuronal progenitors and migrating neurons [10], developing myotubes [11, 12], junction formation in epithelial clusters [13–15], and wounded monolayers of fibroblasts and myoblasts polarizing for migration [16–22]. The wounded monolayer systems have been particularly useful for understanding the molecular mechanisms of nuclear movement and offer the ease of manipulating tissue culture cells. Additionally, with serum-starved wounds, nuclear movement can be synchronously triggered by adding serum or the serum factor lysophosphatidic acid (LPA). The latter induces movement of the nucleus toward the cell rear without inducing membrane protrusion or migration, thus allowing nuclear movement to be studied separately from cell migration [5, 18]. LPA-stimulated rearward nuclear movement generates cell polarity by orienting the centrosome, and this occurs through two Cdc42 pathways: an actin/myosin pathway that moves the nucleus and a MT/dynein pathway that maintains the centrosome in the cell center [18, 23]. Inhibition of the actin/myosin pathway impairs nuclear movement so both the nucleus and centrosome stay near the centroid of the cell. On the other hand, inhibition of the MT/dynein pathway disrupts centration of the centrosome, resulting in its rearward movement with the nucleus [18]. The mechanism of actin-dependent nuclear movement has been extensively studied [17–22]. Stimulated by Cdc42, myotonic dystrophy-related, Cdc42-binding kinase (MRCK) activates myosin IIA and IIB by

phosphorylating their regulatory light chains [18, 20]. Active myosin IIA is responsible for the formation of dorsal actin cables and their movement, whereas myosin IIB interacts with emerin, a nuclear envelope protein, to regulate the directionality of actin movement [20]. Moving dorsal actin cables are captured by the LINC complex proteins nesprin-2G (the giant isoform of nesprin-2) via its N-terminal CH domains and by interacting with the formin FHOD1, while SUN2 mediates the anchorage of nesprin-2G via its C-terminal KASH domain [19, 22]. Nesprin-2G, FHOD1, and SUN2 accumulate along dorsal actin cables to form transmembrane actin-associated nuclear (TAN) lines [19]. TAN lines are anchored to the nuclear lamina through SUN2 interaction with A-type lamins [21]. The inner nuclear membrane proteins, Samp1 and emerin, are also found in TAN lines and contribute to their function [17, 20].

Nuclear movement in fibroblasts was first studied using NIH3T3 fibroblasts [18], mouse embryo fibroblasts (MEFs), and human fibroblasts [21]. More recently it was found that undifferentiated C2C12 myoblasts utilize a similar mechanism for nuclear movement [5]. In all of these studies, modified wound-healing assays were used. In brief, a confluent monolayer of cells is serum starved to deprive the cell of polarization stimuli. The monolayer is then wounded, and LPA is added to stimulate nuclear movement. Cells are then fixed, permeabilized, and stained with a combination of DAPI, phalloidin, and anti-centrosome/tubulin antibodies to visualize the nucleus, dorsal actin cables, and the centrosome. Fluorescence images of cells at the wound edge are acquired and subjected to analysis of centrosome orientation and nuclear and centrosomal positions. Data analysis and quantification can be done manually using *Metamorph* (Molecular Devices, Sunnyvale, CA) or *ImageJ* (NIH, Bethesda, MD) but are quite time-consuming, so we developed custom software, *Cell Plot*, to quantify nuclear and centrosomal positions in fixed cell images, which automates the analysis [20]. Live-cell imaging using phase contrast or DIC microscopy is useful to study movement of the nucleus, because it minimizes photodamage. However, to study the dynamics of actin flow and the movement of TAN lines, fluorescent live-cell microscopy is necessary. This is usually accomplished by co-expressing a fluorescent protein-tagged actin probe (e.g., LifeAct-mCherry) and GFP-mini-nesprin-2G (GFP-miniN2G), an engineered form of nesprin-2G which lacks the spectrin repeats 3–54 but contains the actin-binding CH domains and the KASH domain and rescues nuclear movement in cells depleted of nesprin-2G [19]. The velocity and directionality of TAN lines and nuclear movement are then measured from kymographs prepared from the movies. Here, we describe these methods in detail.

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## 2 Materials

NIH3T3 fibroblasts are cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10 % calf serum. Other cell lines including mouse C2C12 myoblasts, MEFs, and primary human fibroblasts are cultured in DMEM containing 10 % fetal bovine serum [5].

### 2.1 General

1. Serum-free medium: DMEM with 10 mM HEPES (pH 7.4) (*see Note 1*).
2. Growth medium: Serum-free medium with 10 % calf serum.
3. NIH3T3 fibroblasts, MEFs, human primary fibroblasts, or C2C12 mouse myoblasts: cultured in growth medium.
4. 0.25 % Trypsin-0.9 mM EDTA in Hank's Balanced Salt Solution (HBSS).
5. LPA (Avanti Polar Lipids, Alabaster, AL): 1 mM solution in 100 mM NaCl, 1 % fatty acid-free Bovine Serum Albumin, 10 mM HEPES, pH 7.4 (*see Note 2*).
6. Microscope slides and acid-washed coverslips (22 mm×22 mm) (*see Note 3*).
7. Glass-bottom imaging dishes (*see Note 3*).
8. siRNA transfection reagent, e.g., Lipofectamine RNAiMax (Life Technologies).
9. siRNA: 20 μM stock solution in RNase-free water.
10. Micromanipulator for microinjection (*see Note 4*).
11. Recording medium: HBSS supplemented with essential and non-essential MEM amino acids, 2.5 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, and 20 mM HEPES (pH 7.4).

### 2.2 Nuclear Movement and TAN Line Detection in Fixed Cells

1. Fixation buffer: 4 % paraformaldehyde in phosphate buffered saline (PBS).
2. Blocking/permeabilization buffer: 0.3 % Triton X-100, 5 % normal goat serum or 5 % BSA in PBS.
3. Staining reagents: Alexa-488 phalloidin, DAPI, anti-tubulin, and/or anti-pericentrin antibodies. We use rat-monoclonal anti-tyrosinated-tubulin (YL1/2, the European Collection of Animal Cell Cultures, Salisbury, UK) and/or mouse monoclonal anti-pericentrin antibodies (BD Biosciences, San Jose, CA) (*see Note 5*).
4. Anti-fade mounting and sealing medium.
5. GFP-miniN2G plasmid [19]: 5 mg/mL in HKCl buffer (140 mM KCl, 10 mM HEPES, pH.7.4) (*see Note 6*).
6. Epifluorescence microscope equipped with high NA oil immersion 60× objective and fluorescence filter cubes for three or four color imaging.

### **2.3 Nuclear Movement (Live-Cell Phase Contrast Microscopy)**

1. Phase contrast microscope with temperature control and motorized stage (*see* **Notes 7 and 8**).

### **2.4 Actin Flow and TAN Line Movement (Live-Cell Fluorescent Microscopy)**

1. GFP-miniN2G and LifeAct-mCherry plasmids [19]: 5 mg/mL GFP-miniN2G plasmids and 25 mg/mL LifeAct-mCherry plasmids in HKCl buffer (*see* **Note 6**).
2. Epifluorescence microscope with temperature control and motorized stage (*see* **Note 9**).

### **2.5 Data Analysis**

1. *ImageJ* software.
2. *Cell Plot* (WC, Gundersen Laboratory: <http://www.columbia.edu/~wc2383/software.html>).

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## **3 Methods**

The standard protocol that has been developed to study nuclear movement in NIH3T3 fibroblasts [24] can also be applied to human fibroblasts, MEFs, and myoblasts. With each of these systems, proper optimization of the starting cell density before serum starvation and the length of serum starvation is required to obtain a confluent, but not overcrowded, monolayer of cells. For instance, for NIH3T3 fibroblasts and C2C12 myoblasts, the optimal starting cell density for serum starvation is ~80 and ~40 %, respectively. Length of starvation time is also critical. Without sufficient starvation, cells at the wounded monolayer edge can move their nuclei or even migrate in the absence of LPA or serum. A useful criterion to confirm proper serum starvation is to stain cells for filamentous actin. In well-starved cells, there should almost be no visible actin cables. Starvation times vary from 2 days for NIH3T3 fibroblasts (*see* **Note 10**) to 4 days in C2C12 cells. Microinjection of purified proteins and plasmids is an efficient way to introduce proteins into cells at the wound edges. This technique and RNAi-mediated depletion of proteins are invaluable in deciphering the molecular pathway of nuclear movement.

### **3.1 Preparation of Cells with siRNA Knockdown**

1. Transfect NIH3T3 fibroblasts with siRNAs of interest (40 nM) using siRNA transfection reagent according to the manufacturer's instruction (*see* **Note 11**). Transfected cells are plated onto a 22-mm square acid-washed coverslip in a 35-mm dish (for immunostaining) or on a glass-bottom coverslip dish (for live-cell imaging). Initial cell density is ~20 % so that it reaches ~80 % in 2 days. For assaying nuclear position in fixed cells, prepare two coverslips for each siRNA: one for LPA stimulation and the other without LPA stimulation (this also serves as

a check on the serum starvation, since incompletely starved cells will move their nuclei without added LPA).

2. Incubate cells for 2 days.
3. Remove medium from the dish and briefly wash three times with serum-free medium.
4. Add 2 mL of serum-free medium to each dish and incubate cells for 2 days.
5. Wound the monolayers with a small pipette tip or a jeweler's screwdriver and incubate cells for at least 30 min to allow them to recover (*see* **Note 12**).

### **3.2 Expression of Proteins in Wound Edge Cells by Microinjection**

1. Prepare the plasmid expressing the protein of interest in HKCl buffer, back-load this into a glass micropipette, and inject into the nuclei of serum-starved cells at the wound edge using a micromanipulator (*see* **Notes 4, 6, 13, 14**).
2. Incubate the cells for 1–4 h, depending on the construct and the desired level of expression, to allow the cells to express the protein of interest.

### **3.3 Assay of Nuclear Position in Fixed Cells**

1. Add 10  $\mu\text{M}$  LPA to the serum-starved wounded monolayer of cells. Reserve one dish without LPA stimulation to serve as a negative control.
2. Incubate cells for 2 h.
3. Briefly wash the coverslips with PBS and fix the cells with 4 % PFA at room temperature for 10–20 min.
4. Wash the coverslips three times with PBS, each for 5 min.
5. Permeabilize with 0.5 % Triton X-100 in PBS for 5 min and block with blocking buffer at room temperature for 30 min.
6. Follow standard immunostaining procedure and mount the coverslips on pre-cleaned glass slides. DAPI and anti-tubulin and/or anti-pericentrin antibodies should be used to visualize the nucleus and the centrosome (*see* **Note 5**).
7. Mount the slides onto a microscope and acquire images of cells at the wound edges. Avoid imaging isolated cells and only image cells within a continuous wound edge.

### **3.4 TAN Line Detection (Fixed Cells)**

1. Prepare serum-starved cells expressing GFP-miniN2G or use untransfected cells if endogenous TAN lines are to be detected (*see* **Note 15**).
2. Add 10  $\mu\text{M}$  LPA to the dishes and incubate cells for 1 h (*see* **Note 16**).
3. Fix, permeabilize, and block the coverslips as described in Subheading **3.3, steps 3–5**.

4. Follow standard immunostaining procedure and mount the coverslips to slides. Use DAPI, phalloidin, and anti-GFP (or anti-nesprin-2G) antibody staining to visualize the nucleus and TAN lines.
5. Mount the slides onto a microscope and acquire images of injected cells. TAN lines will be found on the dorsal surface of the cells (*see Note 17*).

### **3.5 Nuclear Movement (Live-Cell Phase Contrast Microscopy)**

1. Add conditioned recording medium to a wounded monolayer of serum-starved cells on glass-bottom imaging dishes (*see Note 18*).
2. Mount dishes on a phase contrast microscope with temperature control (37 °C) (*see Notes 19 and 20*).
3. Locate cells at the wound edge and record their positions for multi-position acquisition using microscope control software.
4. Add 20  $\mu$ M LPA to induce nuclear movement.
5. Acquire time-lapse images for each position for at least 2 h at an acquisition rate of 5 min/frame.

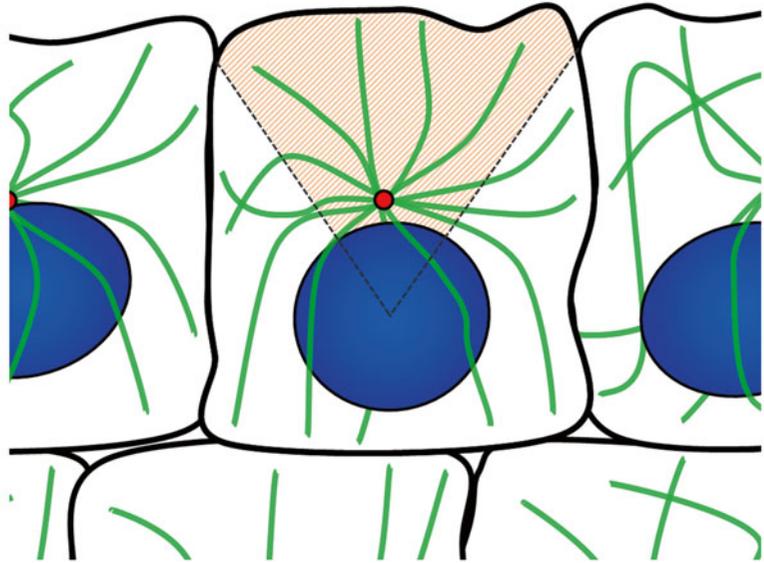
### **3.6 Actin Flow and TAN Line Movement (Live-Cell Fluorescent Microscopy)**

1. Add conditioned recording medium to a wounded monolayer of serum-starved cells in a coverslip dish that were previously microinjected with GFP-miniN2G and LifeAct-mCherry (*see Note 18*).
2. Mount the dish onto an epifluorescence microscope with temperature control (37 °C) (*see Notes 19 and 20*).
3. Locate expressing cells at the wound edge and record their positions for multi-position acquisition using microscope control software.
4. Add 20  $\mu$ M LPA to induce nuclear movement.
5. Acquire two-channel image stacks for each position for at least 2 h. An acquisition rate of 3–5 min/frame is sufficient to capture the movement of TAN lines. TAN lines are on the dorsal side of the nuclei so include the top of nuclei in the range of the Z stacks. Retrograde actin cables originate from the leading edge, so the bottom of the cells should also be included if retrograde actin movement is of interest (*see Note 16*).

### **3.7 Data Analysis: Centrosome Orientation**

Analysis of centrosome orientation has been previously described in detail [24, 25] and is summarized here. This measurement can be done with an image program or judged by eye.

1. Using the drawing tool in the imaging program, draw lines from the center of the nucleus to the two sides of the cell's leading edge where it contacts neighboring cells (Fig. 1).



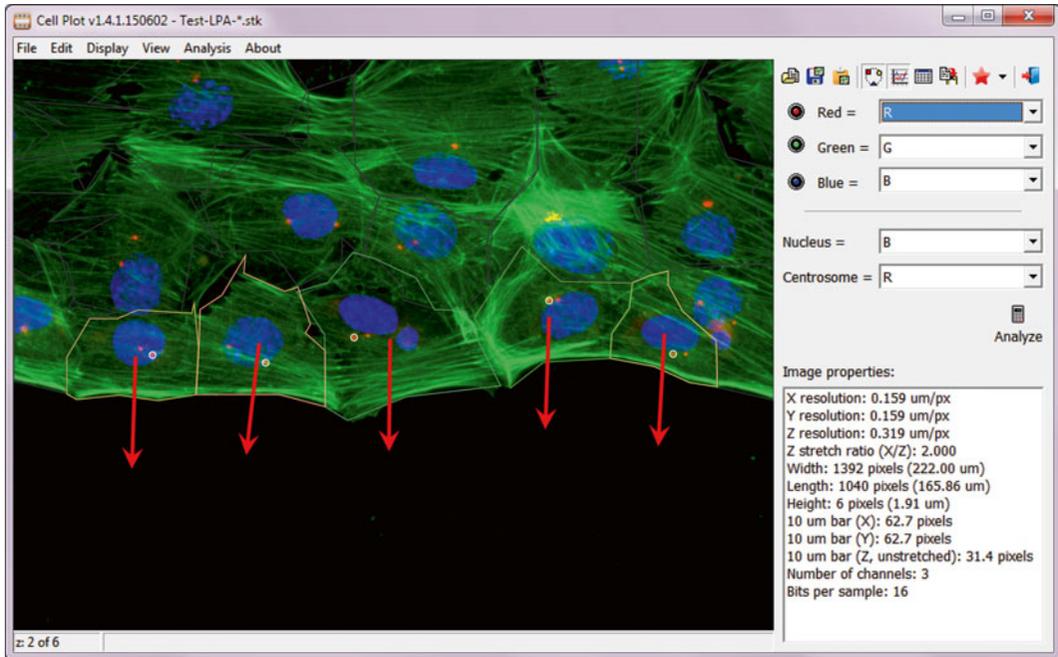
**Fig. 1** The centrosome is considered “oriented” if and only if it is located within the shaded area (excluding the nucleus) and is not on the nucleus

2. The centrosome is considered “oriented” if: (1) it lies between these two lines and the leading edge and (2) it is not located on top of the nucleus (*see* **Notes 21** and **22**).
3. For each experimental condition at least 30 cells should be counted. In the absence of LPA and serum ~33 % of cells have oriented centrosome because of random position of the centrosome. The number increases to 60–70 % of the cells after induction with serum or LPA.

### **3.8 Data Analysis: Analyzing Nuclear and Centrosome Positions with ImageJ**

Nuclear and centrosome positions can be manually measured using *ImageJ*, *Metamorph*, or similar software. We have developed *Cell Plot* freeware (Fig. 2) to semi-automate these measurements. Here we include a brief description of how this is done manually with *ImageJ*, because it is helpful to know the nature of these measurements to understand results from *Cell Plot*.

1. Open *ImageJ* and make sure that both “Area” and “Centroid” are selected in the “Set Measurements” dialog.
2. Open the images of interest in *ImageJ*. Merge the channels if necessary.
3. Select a cell at the wound edge and rotate the image so that the leading edge of the cell is parallel to bottom of the image window and the wound is at the bottom.
4. Use the freehand selection tool to draw the boundary of the cell creating a region of interest (ROI) and click menu item



**Fig. 2** The user interface of *Cell Plot*. After analysis is finished, identified cells are marked by *gray* boundaries. Double-clicking on a cell highlights it and includes it in the output results. When a highlighted cell is selected, its boundary can be modified by moving the points that define the boundary. The direction of wound edge (*red arrows*) and centrosome position (*white circles*) can also be adjusted with a mouse

“Measure (Ctrl+M).” The area of the ROI and the (*x, y*) position of the centroid ROI should be logged.

5. Use the oval selection to draw a circle to represent the nucleus and click “Measure (Ctrl+M).”
6. Use the point selection to mark the centrosome and “Measure (Ctrl+M).”
7. Repeat **steps 2–6** for every cell to be analyzed. We typically analyze 30–40 cells for each condition.
8. Export the data to a spreadsheet program such as *Microsoft Excel*.
9. Calculate the relative position of the nucleus and centrosome by subtracting the Y position of the cell centroid from their Y positions (in pixels).
10. Calculate the average radius of the cell from its area:  $r = \sqrt{\frac{\text{Area}}{\pi}}$  (in pixels).
11. Normalize the relative position of the nucleus and centrosome to the radius (no unit, expressed as %). By this measurement, positive values represent positioning toward the leading edge, and negative values represent rearward location.

### 3.9 Data Analysis: Analyzing Nuclear and Centrosome Positions with Cell Plot

1. Open *Cell Plot* (Fig. 2). A window with an empty image should appear.
2. Drag and drop image files into *Cell Plot* or use the “Load images...” function to browse for the images. *Cell Plot* currently accepts TIFF stack files (\*.tif files from *ImageJ* or \*.stk files from *MetaMorph*). All frames in the files are analyzed together. See **Note 23** for file name requirements.
3. Select the “nucleus” and “centrosome” channels using the drop-down lists on the right panel.
4. Click “Analyze.”
5. Double click on a cell to include/exclude it from analysis.
6. Use the mouse to correct the cell boundary (polygons), the direction or wound edge (red arrows), and the position of centrosomes (white circles) (see **Note 24**).
7. Use PageUp and PageDown to move between frames.
8. Click “Export to Excel...” to open *Microsoft Excel* with all results entered.

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## 4 Notes

1. 1× Penicillin and streptomycin can be added to growth medium and serum-free medium.
2. It is important to use fatty acid-free BSA (for example, Sigma-Aldrich #A6006), as regular BSA contains LPA.
3. Coverslips are treated with 2 M HCl for 5 min and washed with running tap water for 15 min, ddH<sub>2</sub>O for 5 min (three times), and 95 % ethanol for 5 min before drying. Glass-bottom imaging dishes should also be acid washed.
4. Microinjection is preferred for introducing plasmid DNA into cells over liposome transfection because the former gives immediate expression, better control of the protein expression level, and less trauma to cells.
5. Antibody to a junctional/plasma membrane protein (e.g.,  $\beta$ -catenin) or phalloidin staining of actin may be used to stain the cell boundary. Efficient depletion of the protein of interest should be confirmed by immunofluorescence staining and western blotting with an appropriate antibody.
6. DNA concentration needs to be optimized for each plasmid. Plasmid DNA is diluted in HKCl buffer, and aggregates in the solution are removed by centrifugation ( $\geq 15,000 \times g$ , 30 min).
7. A microscope with motorized  $x$ - $y$  stage is preferred because it allows recording several samples/positions sequentially. With our Proscan II  $x$ - $y$  stage (Prior), we are able to record more

than 50 positions at a rate of 5 min per frame with autofocus on. Autofocus or a focus-maintaining system is necessary for live-cell phase contrast microscopy.

8. A dry objective is preferable for phase contrast microscopy. We use a Nikon 40× ELWD Plan objective (NA 0.6) and a CoolSNAP HQ CCD camera on a Nikon TE300 microscope. In some circumstance a 20× objective gives acceptable resolution.
9. For live-cell fluorescence microscopy, a 60× or 100× Plan Apo oil objective should be used to provide optimal resolution. Photodamage from the fluorescence illumination can lead to imaging artifacts and cell toxicity; to limit photodamage, use neutral density filters, limit exposure times, and/or reduce the number of focal planes and positions.
10. Starvation time may vary for NIH3T3 fibroblasts from different sources.
11. When using liposome transfection, we use a “reverse transfection” protocol. In this protocol, trypsinized cells and medium are added to pre-plated transfection complexes.
12. The monolayer can be wounded multiple times to increase the number of the cells at the wound edges.
13. Only inject cells that are part of a continuous wound edge. Microinjection should take less than 15 min to reduce cell damage due to pH changes in low CO<sub>2</sub> environment outside of the incubator.
14. When injecting multiple coverslips, it does not affect the results if the monolayers are wounded all at once since without serum or LPA addition, there will be no response from the cells if they have been properly serum starved.
15. The expression of GFP-miniN2G enhances detection of TAN lines compared to immunofluorescence staining of endogenous N2G. TAN lines can be detected in 30–40 % of GFP-miniN2G expressing cells, while endogenous TAN lines are detectable in ~15 % cells at the wound edge. Note that the number of cells exhibiting TAN lines (30–40 % of wound edge cells) is similar to the percentage of cells that actively move their nucleus [18, 19].
16. It is important to note that TAN lines are transient structures and are optimally detected 30–60 min after LPA stimulation of NIH3T3 fibroblasts and C2C12 myoblasts.
17. Nuclear membrane folding may also cause linear nesprin-2 signals so we only consider linear nesprin-2G colocalizing with actin cables as TAN lines. An additional criterion is the colocalization of SUN2 with the nesprin-2G and actin filaments.

18. Using recording medium reduces background fluorescence relative to DMEM. Care is needed when changing the serum-free medium of serum-starved cells as application of fresh medium can lead to detachment of the cells. If medium must be replaced, use conditioned serum-free medium prepared by harvesting medium from separate batches of serum-starved cells.
19. Pour enough mineral oil on top of the medium to reduce evaporation of water if a humidity chamber is not available.
20. Live-cell imaging may also be done at 34–35 °C. Cells are in general healthier, and it also reduces focal drift on microscopes without a focus controlling system.
21. For centrosomes located on the boundary of the shaded area (*see* Fig. 1), consider half of them as oriented.
22. In some cells, the two centrosomes are separated and can be distinguished. In this case, use the MT channel to determine which one is the dominant MT organization center.
23. Multichannel images should be saved as multiple single-channel TIFF files, and they should be named as “filename-G.tif”, “filename-R.tif”, “filename-B.tif”, and “filename-I.tif”. For example, when a file named “3T3-LPA-B.tif” is opened, *Cell Plot* looks for “3T3-LPA-G.tif”, “3T3-LPA-R.tif”, and “3T3-LPA-I.tif” and opens them all at once.
24. The cell boundary can be modified by moving the points with a mouse. Alternatively, a boundary can be directly drawn by holding the “Alt” key and moving the mouse.

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