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# Examining the Dynamics of Cellular Adhesion and Spreading of Epithelial Cells on Fibronectin During Oxidative Stress

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**TITLE:**

# Examining the Dynamics of Cellular Adhesion and Spreading of Epithelial Cells on Fibronectin During Oxidative Stress

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**KEYWORDS:**

cell adhesion, cellular spreading, oxidative stress, fibronectin, epithelial cells, ATM inhibition, ruffles, stress fibers, cell circularity

**SUMMARY:**

This method is useful for quantifying the early dynamics of cellular adhesion and spreading of anchorage-dependent cells onto the fibronectin. Furthermore, this assay can be used to investigate the effects of altered redox homeostasis on cell spreading and/or cell adhesion-related intracellular signaling pathways.

**ABSTRACT:**

The adhesion and spreading of cells onto the extracellular matrix (ECM) are essential cellular processes during organismal development and for the homeostasis of adult tissues. Interestingly, oxidative stress can alter these processes, thus contributing to the pathophysiology of diseases such as metastatic cancer. Therefore, understanding the mechanism(s) of how cells attach and spread on the ECM during perturbations in redox status can provide insight into normal and disease states. Described below is a step-wise protocol that utilizes an immunofluorescence-based assay to specifically quantify cell adhesion and spreading of immortalized epithelial cells on fibronectin (FN) in vitro. Briefly, anchorage-dependent cells are held in suspension and exposed to the ATM kinase inhibitor Ku55933 to induce oxidative stress. Cells are then plated on FN-coated surface and allowed to attach for predetermined periods of time. Cells that remain attached are fixed and labeled with fluorescence-based antibody markers of adhesion (e.g., paxillin) and spreading (e.g., F-actin). Data acquisition and analysis are performed using commonly available laboratory equipment, including an epifluorescence microscope and freely available Fiji software. This procedure is highly versatile and can be modified for a variety of cell

lines, ECM proteins, or inhibitors in order to examine a broad range of biological questions.

**INTRODUCTION:**

Cell-matrix adhesions (i.e., focal adhesions) are large and dynamic multimolecular protein complexes which mediate cell adhesion and spreading. These processes are critical for tissue development, maintenance, and physiological function. Focal adhesions are composed of membrane-bound receptors, such as integrins, as well as scaffolding proteins that link cytoskeletal actin to the extracellular matrix (ECM)[1]. These complexes are capable of responding to physiochemical cues present in the extracellular environment through the activation of various signaling transduction pathways. As such, focal adhesions serve as signaling centers to propagate extracellular mechanical cues into a number of cellular processes including directed migration, cell cycle regulation, differentiation, and survival[1,2]. One group of signaling molecules that regulate and interact with focal adhesions includes members of the Rho family of small GTPases. Rho GTPases are key proteins that regulate cell migration and adhesion dynamics through their specific spatiotemporal activation[3]. Not surprisingly, dysregulation of Rho protein function has been implicated in a number of human pathologies such as metastasis, angiogenesis, and others. Of particular interest, cellular redox status plays a predominant role in the modulation of cell migration and adhesion. Alterations in redox homeostasis, such as increases in reactive oxygen species (ROS), have been demonstrated to regulate Rho protein activity, as well as adhesion, in a number of cell types and human diseases[4-8]. For example, individuals suffering from the neurological disorder ataxia-telangiectasia (A-T), which is caused by a mutation in the DNA damage repair serine/threonine kinase A-T-mutated (ATM), have an increased risk of metastatic cancer[9,10]. Loss of ATM kinase activity in these patients and cell lines, either through genetic mutation or chemical inhibition, results in high levels of oxidative stress due to dysfunction of the pentose phosphate pathway[7,11,12]. Moreover, recent studies from the laboratory have highlighted a pathophysiological role for ROS in A-T by altering cytoskeletal dynamics (i.e. adhesion and spreading) as a direct result of activating Rho family GTPases in vitro[5]. Ultimately, these alterations in cytoskeletal dynamics caused by Rho family activation may lead to the increased risk of metastatic cancer noted in A-T patients[5,13]. Therefore, understanding the interplay between cell-matrix interactions during oxidative stress can provide insights into the regulation of adhesion and spreading. These studies can also set the stage for further investigations into a possible role for Rho family GTPases in these signaling processes.

Described herein is a protocol to study the early cellular dynamics of adhesion assembly and spreading during oxidative stress caused by inhibition of ATM kinase activity. This assay is based on the well-characterized mechanism of anchorage-dependent cells adhesion to the ECM protein fibronectin (FN). When cells maintained in suspension are plated onto FN, several Rho GTPases coordinate the control of the actin cytoskeletal remodeling[3,14]. Morphological changes are observed as cells shift from round and circular in appearance to flattened and expanded. Concomitant with these observations is the development of numerous matrix adhesions with the ECM. These changes are attributed to the biphasic activation of RhoA with Rac1 during the first hour as cells adhere and spread [15,16].

A variety of methods have been utilized to examine adhesion morphology and dynamics as well

as cell spreading. However, these methods rely on sophisticated long-term, live-imaging total internal reflection fluorescence (TIRF) or confocal microscopy systems. Thus, users must have access to specialized equipment and software. Furthermore, the set-up time required by these bio-imaging systems makes capturing early adhesion events challenging, especially when testing multiple inhibitors or treatment conditions concurrently.

The methods detailed, herein, provide a straightforward, economical, yet quantitative way to assess parameters that govern the adhesion assembly and spreading in vitro. The protocol is performed using commonly available laboratory equipment, such as an epifluorescence microscope and CCD camera. This assay involves applying anchorage-dependent cells to an FN-coated surface after a period of oxidative stress caused by chemical inhibition of ATM kinase activity, which has been demonstrated previously[5]. Following plating, cells are allowed to attach and adhere for specified lengths of time. Unattached cells are washed away, while attached cells are fixed and labeled with fluorescence-based antibodies to markers of adhesion (e.g., paxillin) and spreading (e.g., F-actin)[2,5]. These proteins are then visualized and recorded using an epifluorescence microscope. Subsequent data analysis is performed using freely available Fiji software. Moreover, this method can be adapted to examine adhesion dynamics under a wide range of conditions including different ECM proteins, treatment with various oxidants/cell culture conditions or a variety of anchorage-dependent cell lines to address a broad range of biological questions.

**PROTOCOL:**

**1. Preparations**

NOTE: The protocol described below has been optimized for the use with REF52 cells and $ATM^{+/+}$ or $ATM^{-/-}$ human fibroblasts. Other cell types may require further optimization as described in the notes and troubleshooting sections below.

1.1. Make 500 mL of complete cell culture medium for REF52 cells. To 500 mL of high-glucose containing Dulbecco's modified Eagle's medium (DMEM) add 10% FBS, 2 mM L-glutamine, and 100 units/mL penicillin-streptomycin.

1.2. Prepare a 25 μg/mL solution of fibronectin (FN) by adding 300 μL of 1 mg/mL FN solution to 12 mL of sterile 1x phosphate buffered saline (PBS), pH 7.4. Mix well.

1.3. Prepare a 0.5% (w/v) delipidated (i.e., fatty acid free) bovine serum albumin (dlBSA) solution in serum free DMEM cell culture medium. Add 0.5 g of dlBSA to 100 mL of serum free DMEM medium. Mix the solution well, but do not vortex. Sterile filter the solution into a new sterile container, using a 0.22 μM syringe filter before use. Store at 4 °C.

1.4. Make 3.7% paraformaldehyde solution by dissolving 3.7 g of paraformaldehyde in 100 mL of 1x PBS. Use gentle heat and stirring to get the paraformaldehyde into solution.

NOTE: The paraformaldehyde solution is light sensitive and should be protected from light. It is good for up to one week when stored at 4 °C.

CAUTION: Paraformaldehyde is toxic, flammable, corrosive and a health hazard. Review the material safety data sheet for paraformaldehyde prior to use. Use the appropriate personal protective equipment when handling including eye shield, face shield, full-face particle respirator, gloves, and lab coat.

1.5. Prepare the permeabilization solution containing 0.2% non-ionic surfactant in 1x PBS (v/v)**.** For 100 mL, slowly add 0.2 mL of Triton X-100 to 100 mL of 1x PBS, while stirring.

1.6. Make the immunofluorescence blocking buffer containing 2.5% BSA, 5% goat serum, and 0.05% non-ionic surfactant (w/v/v) dissolved in 1x PBS solution. For 100 mL, add 5 mL of goat serum, 2.5 g of BSA and 0.05 mL of Triton X-100 in ~ 95 mL 1x PBS, while stirring.

1.7. Grow REF52 cells in DMEM complete culture medium in a 10 $cm^2$ (or any other vessel size) cell culture-treated plate in a cell culture incubator at 37 °C and 5% $CO_2$.

**2. Coating cell culture plates with the extracellular matrix protein fibronectin**

NOTE: Perform this section using aseptic technique and sterile reagents in a BSL-2 certified laminar flow hood. Refer to **Figure 1A** for an overview of key steps prior to beginning.

2.1. Using a tissue culture certified 24-well plate, place one glass coverslip (12-Cir-1) in each well. Label the plate according to **Figure 1B**.

2.2. Pipette 500 μL of the 25 μg/mL FN solution to each well of a 24-well plate.

2.3. Pipette the solution over each coverslip a few times to ensure even coating and complete submersion. Place the lid back on the plate.

2.4. Incubate the plate in a cell culture incubator at 37 °C and 5% $CO_2$ for 1 h.

NOTE: Alternatively, incubate overnight at 4 °C.

2.5. After 1 h, remove the plate from the incubator and aspirate the FN solution from the wells.

2.6. Wash wells three times with 500 μL of 1x PBS. Aspirate the final wash of 1x PBS.

2.7. Block wells with 500 μL of dIBSA solution for a minimum of 15 min at 37 °C and 5% $CO_2$.

2.8. Aspirate the dIBSA solution prior to plating cells in step 3 below.

NOTE: If storing plates, add 500 μL of 1x PBS to each coverslip after aspiration of the dIBSA

solution. Plates can then be kept at 4 °C for up to one week.

**3. Preparing anchorage-dependent cells for the adhesion assay**

NOTE: Perform this section using aseptic technique and sterile reagents in a BSL-2 certified laminar flow hood.

3.1. At least 30 min prior to the cell plating, pre-warm the following solutions: DMEM complete medium, dIBSA solution, 1x PBS, 0.5% trypsin-EDTA solution, and trypsin neutralizing serum (TNS) in a 37°C water bath.

3.2. Starting with a confluent monolayer of REF52 cells in a 10 $cm^2$ dish, wash cells twice with 6 mL of warm 1x PBS. Serum starve the cells for at least 1 h (depending on cell type) in 6 mL of warm dIBSA solution.

3.3. Wash cells with 6 mL of warmed 1x PBS, aspirate PBS and add 1.5 mL of warm 0.5% Trypsin-EDTA solution.

3.4. Place cells in a cell culture incubator at 37 °C and 5% $CO_2$ for ~2 min.

3.5. Observe cells under a light microscope to ensure the detachment is complete. If cells are still adherent after tapping the plate on the bench top, return to the 37 °C incubator for an additional 2 min. Trypsinize the cells for as little time as is necessary.

3.6. Gently detach cells from the dish using 1.5 mL of warm trypsin neutralizing serum (TNS). Pipette the solution up and down over the bottom of the plate numerous times to remove all remaining adherent cells. If cells appear clumpy, further triturate the cell suspension by gently pipetting up and down over the back of the dish.

3.7. Count cells using trypan blue exclusion and a hemocytometer under a light microscope. Alternatively, use an automated cell counter.

3.8. Remove an appropriate amount of cells to create a 1.0 - 3.0 x $10^4$ cells/mL cell suspension in 5 mL of dIBSA in a 15 mL conical tube.

3.9. Centrifuge cells at ~ 300 *x g* for 5 min using a fixed angle rotor in a table-top centrifuge.

3.10. Aspirate the supernatant from the cell pellet, and resuspend cells in a total of 7 mL of warm dIBSA solution. Do not allow the cells to be overly confluent upon coverslip plating, but evenly distributed with few cells touching one another.

3.11. Evenly divide the cell suspension into two 15 mL conical tubes, one for the vehicle alone control (DMSO) and one for Ku55933 (ATM kinase inhibitor, oxidant)[5]. Ensure each tube contains 3.5 mL of the cell suspension.

3.12. Using a tube rotator, revolve the tubes at 37 °C for 90-120 min in a cell culture incubator.

3.13. 30 min before plating, add a final concentration of 10 μM Ku55933 and DMSO (1:1,000) to each respective tube. Place the cell suspension back on the rotator for the remaining time.

3.14. After revolving the cell suspension for 90-120 min, remove 500 μL of cell suspension from each treatment group and add to one FN coated coverslip in the 24-well plate from step 2 as illustrated (**Figure 1B**). Return the plate to the 37 °C and 5% $CO_2$ cell culture incubator and the cell suspension back to the rotation.

3.15. After plating the cell suspension on the FN covered-coverslips, allow cells to adhere for the desired length of time (e.g., 10 min, 15 min, 20 min, 30 min) and then immediately proceed to step 4.

**4. Cell fixation and antibody staining for immunofluorescence**

NOTE: The following steps are performed under non-sterile conditions and at room temperature unless otherwise stated.

4.1. After the desired time for adhesion has passed, aspirate the cell solution from each coverslip in the plate.

4.2. Using the sides of the well, gently dispense 500 μL of 3.7% paraformaldehyde solution onto each coverslip and wait 10-15 min.

4.3. Remove the paraformaldehyde solution and wash each coverslip with 500 μL of 1x PBS for a total of two times.

NOTE: Dispose of paraformaldehyde waste responsibly, according to the institution's environmental health and safety plan.

4.4. Aspirate the PBS, and permeabilize cells on each coverslip with 500 μL of 0.2% Triton X-100 in 1x PBS (v/v) for 10-15 min at room temperature.

4.5. Wash each coverslip with 500 μL of 1x PBS three times.

4.6. Block cells on each coverslip with 500 μL of immunofluorescence blocking buffer containing 5% goat serum, 2.5% BSA and 0.05% Triton X-100 dissolved in a 1 x PBS solution for 30-60 minutes.

4.7. Dilute the primary anti-paxillin antibody (1:250) in the blocking buffer. Mix well and add 200 μL of the antibody solution to each coverslip. Incubate at room temperature for at least 1 h.

NOTE: Alternatively, the primary antibody solution can be incubated overnight at 4 °C. There are many common focal adhesion markers that could be used for staining adhesion complexes and subsequent FA analysis. These include antibodies against the following proteins: integrin subunits ($\beta$1, $\alpha$5, or $\alpha$V), talin, or vinculin[2].

4.8. Aspirate the antibody solution, and wash each coverslip with 500 µL of 1x PBS three times for 10 min each. Protect the samples from light from this point forward.

4.9. Dilute the phalloidin F-actin probe conjugated to the red fluorescent Alexa 594 dye (1:1000) and goat-anti mouse 488 fluorescent secondary antibody (1:400) in the same blocking buffer solution. Mix well and add 200 µL of the antibody solution to each coverslip for 30 min.

NOTE: Fluorescently conjugated secondary antibodies from other species may be used as well. However, the use of antibodies from other species will require modification of the blocking buffer serum.

4.10. Aspirate the antibody solution, and wash each coverslip with 500 µL of 1x PBS three times for 10 min each.

4.11. Aspirate the 1x PBS and rinse one time with 500 µL of $dIH_2O$.

4.12. Fix coverslips onto microscope slides using anti-fade mounting medium containing DAPI.

4.13. Leave microscope slides to set overnight in the dark at room temperature.

4.14. Store microscope slides in the dark at 4 ˚C for the long-term storage and until imaging.

NOTE: Image using standard immunofluorescence techniques. It is recommended to use a high-powered oil immersion 60x objective lens to ensure enough resolution to note the focal adhesions and peripheral ruffles at cell edges. Acquire images of 20-30 cells in multiple fields of view for each coverslip under each treatment condition and time. From combined replicates, this should yield at least 60 cells in order to perform statistical analysis. Save and export fluorescence images as a .TIFF file with a minimum of 300 dpi resolution.

**5. Quantifying stress fibers, cell circularity, and focal adhesion formation**

NOTE: The following image analyses are performed using the latest version of the open source imaging processing package Fiji Is Just Image J (Fiji), which can be downloaded free of charge at (http://fiji.sc/).

**5.1. General image processing**

NOTE: All images will need to be prepared for computational analyses by performing steps 5.1.1-

5.1.5 below (**Figure 2**). Afterward, any or all subsequent quantification procedures may be selected.

5.1.1. Open the .TIFF fluorescence image using Fiji. Ensure the images are 8-bit and grayscale.

5.1.2. Select **Image-Adjust-Window/level** and select **Auto** (**Figure 2A**).

5.1.3. Select **Process-Subtract Background** to subtract the background fluorescence. Check **Sliding Paraboloid** and select the option of a **Rolling Ball Radius** of 50 pixels (**Figure 2B**).

NOTE: To verify the proper size for the rolling ball radius, select the **Line Tool** and draw a radius on the largest adhesion in the image. Select **Measure** to verify the length of the line drawn. If the value of the radius is too large, features including adhesions will be lost in the image. If the radius is too small, it will give rise to artifacts in the processed image due to background noise.

5.1.4. Select **Image-Adjust- Brightness/Contrast** to check the intensity of the adhesion over the background. Adjust if necessary.

NOTE*:* To optimize the brightness/contrast and avoid saturating the signal, use the lookup tool of the image to examine its histogram to adjust the brightness/contrast.

5.1.5. Select the following parameters under **Analyze-Set Measurements**: Area, Mean Gray Value, Shape Descriptors, and Integrated Density.

**5.2 Stress fiber formation analysis**

NOTE: Stress fibers can be quantified multiple ways depending on the phenotype.

5.2.1. Count the number of cells with stress fibers as a percentage over the total number of cells. This analysis is best if there are visual differences in the number of stress fibers formed under different experimental conditions.

5.2.2. Count the number of stress fibers that transverse the cell. This analysis allows for the comparison of the number of stress fibers formed per cell.

5.2.3. Measure the total fluorescence intensity given by the phalloidin (e.g., F-actin) staining per cell[17,18]. This method will highlight drastic increases/decreases in fluorescence intensity due to F-actin staining.

5.2.3.1. Set the measurement parameters in step 5.1.5 above.

5.2.3.2. Select the **Freehand Tool** in the Fiji toolbar and manually trace the cell(s) of interest. Select **Analyze-Measure**. A new window will appear showing the selected measurement parameters.

5.2.3.3. Select the **Freehand Tool** in the Fiji toolbar and manually trace an empty space with no cells present. Select **Analyze-Measure**. This measurement will serve as the background fluorescence.

5.2.3.4. Use the equation below to determine the total F-actin fluorescence per cell:

$$F - actin\ Fluorescence\ Per\ Cell = (Integrated\ Density\ of\ Measured\ Cell) - (Area\ of\ Measured\ Cell\ x\ Mean\ Fluorescence\ of\ the\ Background)$$

NOTE: The resulting measurement can be normalized and compared to other cells to give F-actin fluorescence per cell.

**5.3. Cell circularity analysis**

NOTE: Information on cell area (an indicator of cell spreading over time), as well as, the circularity can also be recorded. This measurement is given as a ratio between 0 to 1 as a way to quantify cells that are elongated to round, respectively.

5.3.1. Select the **Freehand Tool** in the Fiji toolbar, and trace an individual cell. Select **Image-Measure** and record the cell area and perimeter measurements for each cell. Repeat this procedure for each cell.

NOTE: Under the **Set Measurements** function, circularity is provided as the **Shape Descriptors** measurement (step 5.1.5).

5.3.2. Manually count actin-enriched ruffling or protrusions per cell as depicted in **Figures 3 and 4.**

**5.4. Focal adhesion analysis**

NOTE: Before performing focal adhesion analysis, install the Mexican Hat Filter plugin on the latest version of Fiji. The following protocol has been modified from previous studies[19-21].

5.4.1. Select **Process-Enhance Local Contrast (Clahe)** using a block size of 19, histogram bins 256, and a maximum slope of 6, with no mask and not fast. (**Figure 2C**)

5.4.2. Select **Process-Filters- Gaussian Blur** with a Sigma (Radius) of 2.0 to filter the image (**Figure 2D**).

5.4.3. Select **Plugins-Mexican Hat Filter (Mhf)** with a Radius of 2.0 (**Figure 2E**).

5.4.4. Run **Threshold** and select **Dark Background** and **Over/Under** using either **Huang** or **Isodata** as the thresholding method. Select **Auto-Threshold**.

NOTE: This step ensures that adhesions are highlighted, but also distinct from one another.

5.4.5. Select **Analyze-Analyze Particles** with the following parameters selected: size=20, pixels-infinity and circularity=0.00-0.99. Check the outlines to ensure the proper detection and separation of focal adhesions.

NOTE: These results yield the number, area, and shape description of individual focal adhesions.

**REPRESENTATIVE RESULTS:**

**A general schema of the experimental set-up**

**Figure 1** represents the general schema for the cell adhesion and spreading protocol beginning with serum starvation of REF52 cells and ending with computational analysis of acquired fluorescence images. Key steps in the protocol are illustrated in the timeline. Of note, step 2 of the protocol describes the preparation of the FN-coated coverslips, which should be performed concurrently with step 3: serum starving REF52 cells prior to placing them in suspension (**Figure 1A**). An example of a mock-labeled 24-well plate indicating treatment groups and duration of cell adhesion prior to fixation of samples for fluorescence microscopy (**Figure 1B**).

**Immunofluorescence image processing for focal adhesion quantification**

REF52 cells were held in suspension for 90 minutes, plated on FN, and allowed to adhere for an additional 15 minutes. After fixation and staining with an anti-paxillin antibody, fluorescent 8-bit grayscale images of the cells were acquired. Image processing analysis was performed according to the protocol delineated in Step 5. Shown are representative images of each distinct processing step including the original image (**Figure 2A**), and images following background subtraction (post-Rolling Ball) (**Figure 2B**), CLAHE (**Figure 2C**), Gaussian Blur (**Figure 2D**) and Mexican Hat Filter (Post-MHF) (**Figure 2E**) filtering steps. After completing all image processing steps, individual focal adhesions should be prominent, in focus, and readily distinguishable from one another (**Figure 2E**). After the images are filtered, the focal adhesions can be quantified and their area measured (steps 5.1 and 5.4).

**Visualization of cell adhesion and spreading on FN after oxidative stress**

A representative grayscale fluorescence image of anti-paxillin (focal adhesion marker) (**Figure 3**, top panel) and phalloidin F-actin probe staining (**Figure 3**, bottom panel) of REF52 cells after plating on FN with or without Ku55933 (ROS-inducing agent) treatment. Prior to the assay, REF52 cells were serum starved for 1 h. Following serum starvation, cells were held in suspension while being treated with either vehicle alone or 10 μM Ku55933 to induce oxidative stress. Cells were plated on FN-coated coverslips for the indicated times, fixed, and then stained with an antibody to focal adhesions and phalloidin to detect F-actin proteins. Prominent focal adhesions and stress fibers should be readily visible in REF52 cells after being allowed to adhere for 20-30 min on FN. Plated cells should not overlap with one another to permit full cellular spreading after adhesion. Notice the clear, distinct cell edges as well as space for individual cells to spread (**Figure 3**). F-actin enriched ruffles at the leading edge of cell membranes are visible and indicated with an

arrow (**Figure 3**, bottom panel).

**Graphical representation of quantified fluorescence images of stress fibers and the degree of cell spreading**

Examples of quantified images displayed in bar graph form representing the percentage of cells with stress fibers and the degree of cell spreading with and without Ku55933 treatment at various times after adhesion. Fluorescent images of phalloidin F-actin probe and anti-paxillin staining, similar to images shown in **Figure 3**, were analyzed for the percentage of stress fibers and cell spreading (i.e., circularity index) using the image analysis procedures described in step 5 of the protocol. Notably, oxidant treatment caused a significant increase in stress fiber formation at all adhesion time points examined (**Figure 4A**) and a decrease in cell spreading following 15 minutes of cell adhesion to FN (**Figure 4B**).

**Non-quantifiable immunofluorescence images due to cellular over confluency**

Serum-starved REF52 cells were held in suspension for 90 minutes, during which time they were treated with 10 μM Ku55933 to induce ROS formation. Cells were then plated on FN and allowed to adhere for 20 minutes, after which they were fixed and stained with anti-paxillin or phalloidin-Alexa 594 F-actin probe. Plating at higher cell densities leads to cellular crowding, which prohibits cells from fully spreading due to over confluency. Notice cell edges are indistinguishable from adjacent cells (yellow arrows) (**Figure 5A**). As a result, quantification of individual cells is precluded, and spreading circumference cannot be accurately determined. In Figure 5B, a separate cell line, mouse embryonic fibroblasts (MEF), were held in suspension and then plated on FN for 30 minutes. Cells were then fixed and stained with an anti-paxillin antibody. Out of focus cells are denoted by red arrows (**Figure 5B**). Furthermore, the cross-reactivity of the anti-paxillin antibody with cellular debris (blue arrow) will alter thresholding during quantitative image analysis (Part 5) and should not be included in the analysis (**Figure 5B**).

**FIGURE LEGENDS:**

**Figure 1**: **Time-line of protocol and example 24-well plate set-up.** (**A**) The time-line highlights key steps in the cell adhesion and spreading procedure. (**B**) Representative labeled 24-plate, illustrating treatment groups and times for cell adhesion.

**Figure 2: Examples of representative immunofluorescence images following image-processing.** REF52 cells were held in suspension for 90 min, plated on FN, and allowed to adhere for 15 min. Cells were fixed and stained with an anti-paxillin antibody. (**A**) Original image and images following (**B**) Background subtraction (post-Rolling Ball), (**C**) CLAHE, (**D**) Gaussian Blur and (**E**) Mexican Hat Filter (Post-MHF) filtering steps. Bar, 20 μm.

**Figure 3: Representative immunofluorescence images of anti-paxillin and phalloidin F-actin probe stained REF52 cells plated on FN.** Prior to the assay, REF52 cells were serum starved for 1 h. Following serum starvation, cells were held in suspension while treated with either vehicle alone or 10 μM Ku55933 to cause oxidative stress. Cells were plated on FN-coated coverslips for the indicated times, fixed and stained with an antibody to focal adhesions and phalloidin to detect F-actin proteins. F-actin enriched ruffles at the leading edge of cell membranes are

indicated with an arrow. Bar, 40 μm. This figure has been modified from Tolbert et al.[5]

**Figure 4**: **Quantification of immunofluorescence images.** Graphs illustrating (**A**) the percentage of cells exhibiting stress fibers and (**B**) cell circularity measurements. Cell circularity was defined as the cell area divided by the cell perimeter. Values ranged from 0-1.0 indicating an elongated or rounded morphology, respectively. Error bars indicate S.E.M. Student's *t*-test for paired samples *$p \leq 0.01$ from experiments performed in triplicate. This figure has been modified from Tolbert et al.[5]

**Figure 5: Non-quantifiable immunofluorescence images.** (**A**) Serum-starved REF52 cells were held in suspension for 90 min, while treated with 10 μM Ku55933. Cells were plated on FN and allowed to adhere for 20 min. Cells were fixed and stained with anti-paxillin or phalloidin-Alexa 594 F-actin probe. Cell edges are shown by yellow arrows. (**B**) MEF cells were held in suspension and then plated on FN for 30 min. Cells were fixed and stained with an anti-paxillin antibody. Out of focus cells are denoted by red arrows and cross-reactivity of anti-paxillin antibody with cellular debris are denoted with blue arrows. Bar, 30 μm.

**DISCUSSION:**

The protocol described here is a versatile and economical way to rapidly screen a number of anchorage-dependent cell types for dynamic cytoskeleton remodeling during cell spreading. In particular, this method quantitatively examines stress fiber and focal adhesion formation during oxidative stress when cells adhere to FN (**Figure 1A**). Moreover, these cellular phenotypes may suggest a regulatory role for members of the Rho family of small GTPases since they have documented roles during cell attachment and spreading[15,16,22]. However, additional biochemical techniques would be required to identify the possible involvement of GTP-bound, active Rho family proteins.

The presented protocol utilizes immunofluorescence detection of F-actin and paxillin to specifically examine cell adhesion and spreading of immortalized epithelial cells on FN after oxidative stress induced by ATM kinase inhibition (**Figures 2-4**). However, this procedure can also be adapted for use on other ECM proteins and/or for other adherent cell types. When adapting to other cell lines, it is important to optimize the experimental conditions, particularly: cell number/density, time of serum starvation, ECM protein concentration, and oxidative stress treatment conditions. When testing the effects of an unknown stimulus on adhesion and spreading dynamics, it is necessary to include both negative and positive control samples to verify that the assay is functioning correctly. Negative control samples can include an untreated or vehicle-only sample, while a positive control should induce oxidative stress (e.g., $H_2O_2$). Furthermore, although not discussed here, it is also essential to utilize the proper antibody controls. It is recommended that three separate controls be used for each antibody to verify its specificity and to identify any potential fluorescence bleed-through[23,24]. These include: 1) a primary antibody control to ensure specific binding of the primary antibody to the antigen and to confirm that antigen binding occurs under the fixation conditions used, 2) a secondary antibody control (for non-secondary conjugated-antibodies) that shows specificity to the primary antibody, and 3) fluorophore controls that ensure the fluorophore added is not the result of

endogenous fluorescence or bleed-through from another antibody.

While this assay is useful for analyzing the early kinetic events of adhesion assembly and spreading, it is not suitable for the detailed examination of adhesion disassembly or adhesion strength and cellular reinforcement. The latter requires the use of long-term imaging bio-stations or single-cell force spectroscopy techniques. The latter techniques include atomic-force microscopy, optical tweezers, tension sensors of adhesion proteins such as vinculin[25] or talin[26,27], and 3D-force microscopy[28].

Critical steps in the protocol include thoroughly coating coverslips with FN. This is necessary for the uniform spreading and adhesion of cells. It is therefore important to pipette the FN solution up and down over the coverslips multiple times prior to incubation. Coverslips must remain fully submerged in the FN solution during the incubation time. FN coated coverslips can be stored for up to 2 weeks at 4°C.

Cell density is also important, as cells that are plated too densely will not achieve maximum spreading circumference. Furthermore, it would not be possible to distinguish individual focal adhesions or cellular ruffles for each individual cell. It is, therefore, necessary to count the cells using a hemocytometer or automated cell counter prior to placing the cells in suspension. While an estimate of cell density is provided for REF52 cells, this will need to be empirically determined for other cells under study. Cells should be plated sparsely enough that few cells overlap, allowing them to spread fully (**Figure 5**).

Other critical steps in the protocol to consider are fluorescently conjugated phalloidin-Alexa 594 F-actin probe and secondary antibodies are light sensitive. Therefore, samples should be minimally exposed to light after the application of these reagents. Furthermore, a number of agents that induce oxidative stress have short half-lives. It is, therefore, necessary to test the chosen oxidant for optimal dose and exposure time to achieve peak activity.

The following sections contain trouble-shooting tips with regards to FN concentration, serum starvation conditions, and cell detachment methodologies. These tips are useful when adapting the protocol for other cells lines and/or treatment conditions.

For consistent FA analysis, optimal attachment and spreading conditions are necessary, which will vary depending upon the ECM ligand. For FN, begin using a dynamic range of 10-30 μg/mL. At higher concentrations of FN, there is little difference in cell attachment. However, some cell types do not efficiently spread at higher concentrations of ECM.

Each cell type responds differently to conditions of serum starvation. REF52 cells can easily be starved overnight without any loss of viability, however, this is not true for all cell lines. Therefore, it is necessary to determine the degree to which the cell line under study can withstand serum starvation.

For spreading assays, cells need to be trypsinized for as little time as possible for reproducible

results[29]. Following cell detachment by trypsinization, cell surface receptors, their cognate ligands, and Rho GTPase activity require a recovery period to return to steady-state levels[14,15]. The trypsinization procedure outlined in this protocol should enable cells to retain most of their cell surface FN-binding receptors[29]. However, certain cell types may require alternative methods of cell detachment to completely prevent digestion of cell surface receptors. These methods include chelating cells using EDTA-based solutions (e.g., Versene) or milder enzymatic dissociation solutions (e.g., Accutase). Use of these dissociation solutions may leave cell-cell adhesions intact resulting in cell clumps. It is therefore important to completely resuspend individual cells to ensure experimental reproducibility.

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**DISCLOSURES:**

The authors declare that they have no competing financial interests.

**REFERENCES:**

1 Geiger, B., Bershadsky, A., Pankov, R., Yamada, K. M. Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. *Nature Reviews: Molecular Cell Biology.* **2** (11), 793-805, (2001).

2 Geiger, B., Yamada, K. M. Molecular architecture and function of matrix adhesions. *Cold Spring Harbor Perspectives in Biology.* **3** (5), pii: a005033 (2011).

3 Lawson, C. D., Burridge, K. The on-off relationship of Rho and Rac during integrin-mediated adhesion and cell migration. *Small GTPases.* **5** e27958 (2014).

4 Heo, J., Campbell, S. L. Mechanism of redox-mediated guanine nucleotide exchange on redox-active Rho GTPases. *Journal of Biological Chemistry.* **280** (35), 31003-31010 (2005).

5 Tolbert, C. E., Beck, M. V., Kilmer, C. E., Srougi, M. C. Loss of ATM positively regulates Rac1 activity and cellular migration through oxidative stress. *Biochemical and Biophysical Research Communications.* **508** (4), 1155-1161 (2019).

6 Hobbs, G. A. et al. Redox regulation of Rac1 by thiol oxidation. *Free Radical Biology and Medicine.* **79** 237-250 (2015).

7 Zhang, Y. et al. Mitochondrial redox sensing by the kinase ATM maintains cellular antioxidant capacity. *Science Signaling.* **11** (538), (2018).

8 Hobbs, G. A., Zhou, B., Cox, A. D., Campbell, S. L. Rho GTPases, oxidation, and cell redox control. *Small GTPases.* **5** e28579 (2014).

9 Shiloh, Y., Ziv, Y. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nature Reviews: Molecular Cell Biology.* **14** (4), 197-210 (2013).

10 Lang, L. et al. ATM-Mediated Phosphorylation of Cortactin Involved in Actin Polymerization Promotes Breast Cancer Cells Migration and Invasion. *Cellular Physiology and Biochemistry.* **51** (6), 2972-2988 (2018).

11 Peter, Y. et al. Elevated Cu/Zn-SOD exacerbates radiation sensitivity and hematopoietic abnormalities of Atm-deficient mice. *European Molecular Biology Organization Journal.* **20** (7), 1538-1546 (2001).

12 Takao, N., Li, Y., Yamamoto, K. Protective roles for ATM in cellular response to oxidative stress. *Federation of European Biochemical Societies Letters.* **472** (1), 133-136 (2000).
13 Jansen, S., Gosens, R., Wieland, T., Schmidt, M. Paving the Rho in cancer metastasis: Rho GTPases and beyond. *Pharmacology & Therapeutics.* **183** 1-21 (2018).
14 Berrier, A. L., Martinez, R., Bokoch, G. M., LaFlamme, S. E. The integrin beta tail is required and sufficient to regulate adhesion signaling to Rac1. *Journal of Cell Science.* **115** (Pt 22), 4285-4291 (2002).
15 Arthur, W. T., Petch, L. A., Burridge, K. Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism. *Current Biology.* **10** (12), 719-722 (2000).
16 Arthur, W. T., Burridge, K. RhoA inactivation by p190RhoGAP regulates cell spreading and migration by promoting membrane protrusion and polarity. *Molecular Biology of the Cell.* **12** (9), 2711-2720 (2001).
17 Chandra, S., Kalaivani, R., Kumar, M., Srinivasan, N., Sarkar, D. P. Sendai virus recruits cellular villin to remodel actin cytoskeleton during fusion with hepatocytes. *Molecular Biology of the Cell.* **28** (26), 3801-3814 (2017).
18 Fitzpatrick, M. *Measuring Cell Fluorescence Using ImageJ*, <https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html> (2014).
19 Berginski, M. E., Vitriol, E. A., Hahn, K. M., Gomez, S. M. High-resolution quantification of focal adhesion spatiotemporal dynamics in living cells. *PLoS One.* **6** (7), e22025 (2011).
20 Horzum, U., Ozdil, B., Pesen-Okvur, D. Step-by-step quantitative analysis of focal adhesions. *MethodsX.* **1,** 56-59 (2014).
21 Elosegui-Artola, A. et al. Image analysis for the quantitative comparison of stress fibers and focal adhesions. *PLoS One.* **9** (9), e107393 (2014).
22 Meller, J., Vidali, L., Schwartz, M. A. Endogenous RhoG is dispensable for integrin-mediated cell spreading but contributes to Rac-independent migration. *Journal of Cell Science.* **121** (Pt 12), 1981-1989 (2008).
23 Donaldson, J. G. Immunofluorescence Staining. *Current Protocols in Cell Biology.* **69** (4 3), 1-7 (2015).
24 Burry, R. W. Controls for immunocytochemistry: an update. *Journal of Histochemistry and Cytochemistry.* **59** (1), 6-12 (2011).
25 Grashoff, C. et al. Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature.* **466** (7303), 263-266 (2010).
26 Kumar, A. et al. Correction: Talin tension sensor reveals novel features of focal adhesion force transmission and mechanosensitivity. *Journal of Cell Biology.* **214** (2), 231 (2016).
27 Kumar, A. et al. Talin tension sensor reveals novel features of focal adhesion force transmission and mechanosensitivity. *Journal of Cell Biology.* **213** (3), 371-383 (2016).
28 Friedrichs, J., Helenius, J., Muller, D. J. Quantifying cellular adhesion to extracellular matrix components by single-cell force spectroscopy. *Nature Protocols.* **5** (7), 1353-1361 (2010).
29 Brown, M. A. et al. The use of mild trypsinization conditions in the detachment of endothelial cells to promote subsequent endothelialization on synthetic surfaces. *Biomaterials.* **28** (27), 3928-3935 (2007).

**Figure 1**

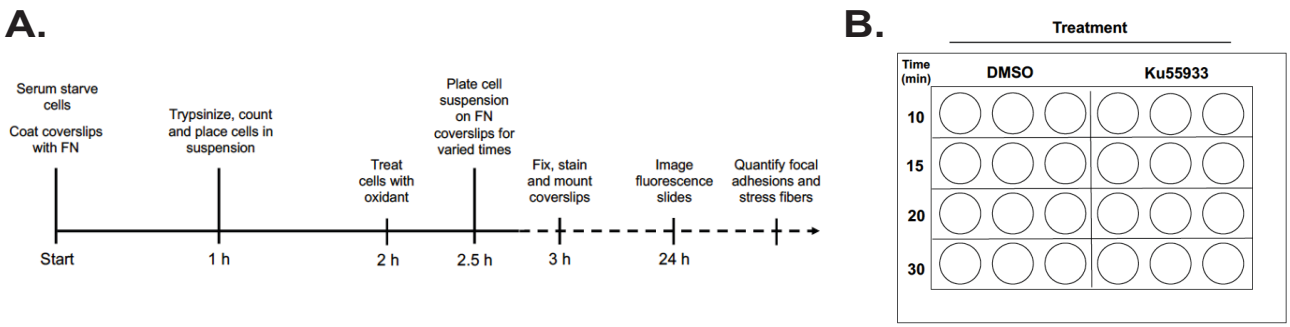

Figure 2

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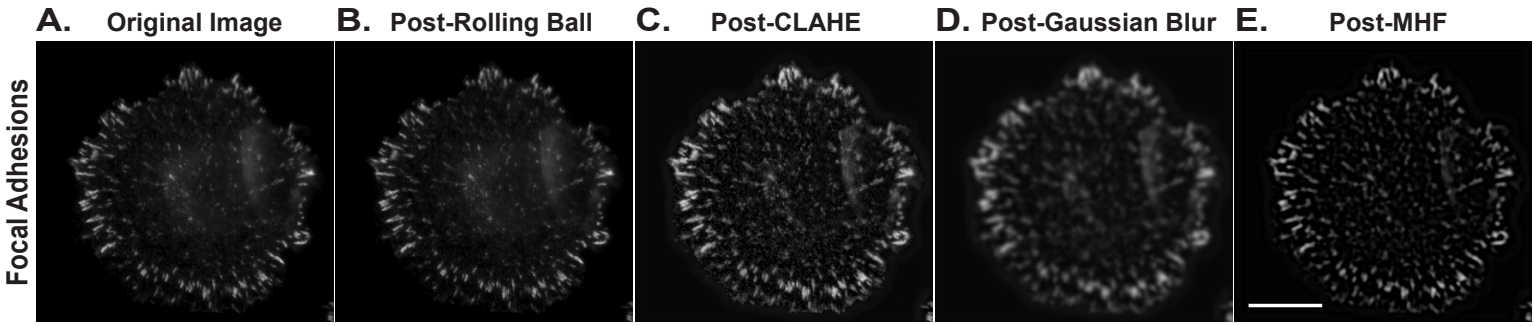


Figure 3



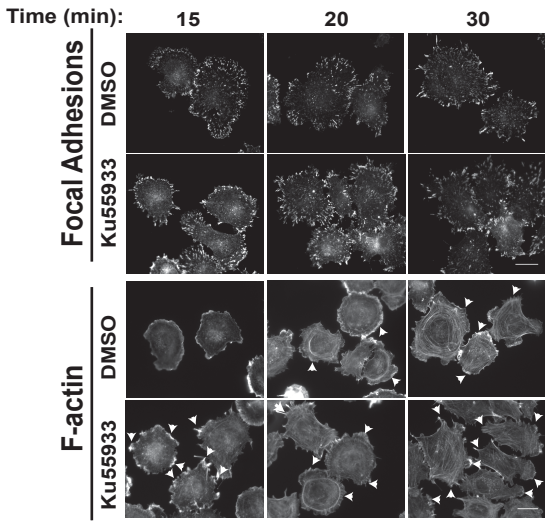
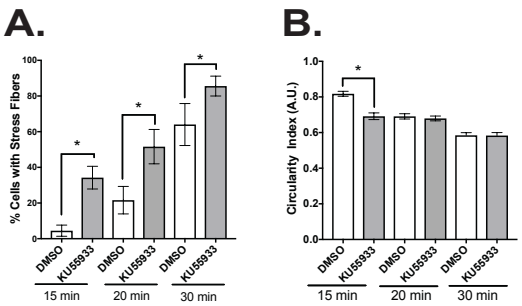


**Figure 4**



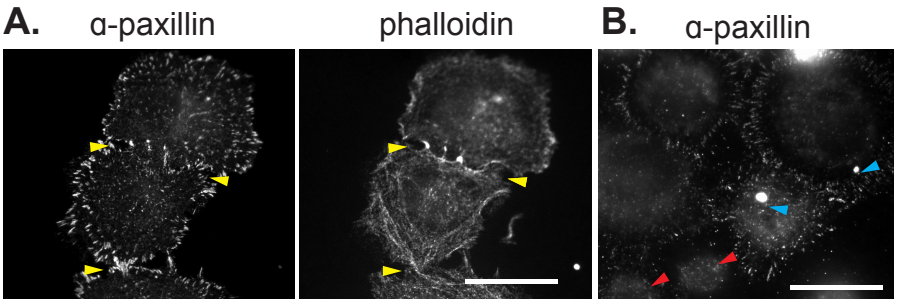


Table of Materials

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| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| 0.05% Trypsin-EDTA (1x) | Gibco by Life Technologies | 25300-054 |
| 10 $cm^2$ dishes | Cell Treat | 229620 |
| 15 mL conical tubes | Fisher Scientific | 05-539-5 |
| 1X Phosphate Buffered Saline | Corning Cellgro | 21-031-CV |
| 24-well cell culture treated plates | Fisher Scientific | 07-200-740 |
| 4°C refrigerator | Fisher Scientific | |
| Mouse IgG anti-paxillin primary antibody (clone 165) | BD Transduction Laboratories | 610620 |
| Aspirator | Argos | EV310 |
| Biosafety cabinet | Nuair | NU-477-400 |
| Delipidated Bovine Serum Albumin (Fatty Acid Free) Powder | Fisher Scientific | BP9704-100 |
| Dimethyl Sulfoxide | Fisher Scientific | BP231-100 |
| Dulbecco's Modified Eagle Media, High Glucose | Fisher Scientific | 11965092 |
| Fetal bovine serum | Fisher Scientific | 16000044 |
| Fiji | National Institutes of Health | http://fiji.sc/ |
| Filter syringe | Fisher Scientific | 6900-2502 |
| Glass coverslips (12-Cir-1.5) | Fisher Scientific | 12-545-81 |
| Goat anti-mouse IgG secondary antibody Alexa Fluor 488 | Invitrogen | A11001 |
| Goat Serum | Gibco by Life Technologies | 16210-064 |
| Hemocytometer | Fisher Scientific | 22-600-107 |
| Human Plasma Fibronectin | Gibco by Life Technologies | 33016-015 |
| IX73 Fluorescence Inverted Microscope | Olympus | |
| Ku55933 | Sigma-Aldrich | SML1109-25MG |
| L-glutamine | Fisher Scientific | 25-030-081 |
| Monochrome CMOS 16 bit camera | Optimos | |
| Paraformaldehyde | Sigma-Aldrich | P6148-500G |
| Penicillin-streptomycin | Fisher Scientific | 15-140-122 |
| Alexa Fluor 594 phalloidin (F-actin probe) | Invitrogen | A12381 |
| ProLong Gold Anti-fade reagent with DAPI | Invitrogen | P36941 |
| REF52 cells | | |
| Stir plate with heat control | Corning Incorporated | PC-420D |
| Syringe | BD Biosciences | 309653 |
| Tissue culture incubator | Nuair | |
| Triton X-100 | Fisher Scientific | BP151-500 |

| | | |
|---|---|---|
| Trypan Blue Solution | Fisher Scientific | 15-250-061 |
| Trypsin Neutralizing Solution (1x) | Gibco by Life Technologies | R-002-100 |
| tube rotator | Fisher Scientific | 11-676-341 |
| water bath | Fisher Scientific | FSGPD02 |

| Comments/Description |
| --- |
| cell dissociation |
| sterile, tissue culture treated |
| sterile |
| PBS, sterile, free of Mg2+ and Ca2+ |
| sterile, tissue culture treated |
| |
| marker of focal adhesions |
| |
| Class II, Type A, series 5 |
| dlBSA |
| organic solvent to dissolve Ku55933 |
| REF52 base cell culture medium |
| certified, cell culture medium supplement |
| image analysis program |
| 0.2 µM, sterile |
| autoclave in foil to sterilize |
| fluorescent secondary antibody, light sensitive |
| component of blocking solution for immunofluorescence |
| for cell counting |
| FN |
| microscope to visualize fluorescence, cell morphology, counting and dissociation |
| ATM kinase inhibitor, inducer of reactive oxygen species |
| cell culture medium supplement |
| |
| PFA, fixative for immunofluorescence |
| P/S, antibiotic solution for culture medium |
| marker of F-actin, light sensitive |
| cover slip mounting media including nuclear dye DAPI, light sensitive |
| Graham, D.M. et. al. Journal of Cell Biology 2018 |
| |
| 60 mL syringe |
| |
| detergent used to permeabilize cell membranes |

for cell counting
TNS, neutralizes trypsin instead of fetal bovine serum

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