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Corresponding Author:	Hava Gil-Henn, PhD Bar-Ilan University Safed, Safed ISRAEL
Corresponding Author's Institution:	Bar-Ilan University
Corresponding Author E-Mail:	Hava.Henn@biu.ac.il
Order of Authors:	Hava Gil-Henn, PhD
	Nikola Lukic
	Trishna Saha
	Stefanie Lapetina
	Gilad Lehmann
	Anthony Koleske
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TITLE:

Measuring Cell-Edge Protrusion Dynamics during Spreading using Live-Cell Microscopy

AUTHORS AND AFFILIATIONS:

Nikola Lukic[1,#], Trishna Saha[1,#], Stefanie Lapetina[1], Gilad Lehmann[1], Anthony J. Koleske[2,3], Hava Gil-Henn[1,*]

[1]The Azrieli Faculty of Medicine, Bar-Ilan University, Safed 1311502, Israel.
[2]Department of Molecular Biophysics and Biochemistry, Yale University, New-Haven, CT 06520, USA.
[3]Department of Neuroscience, Yale University, New-Haven, CT 06520, USA.

[#]These authors contributed equally to this work.

Email addresses of co-authors:
Nikola Lukic (lukicznikola@gmail.com)
Trishna Saha (trishna.saha27@gmail.com)
Stefanie Lapetina (lapetinastefanie@gmail.com)
Gilad Lehmann (giladleh@gmail.com)
Anthony Kolaske (anthony.koleske@yale.edu)
Hava Gil-Henn (Hava.Henn@biu.ac.il)

*Corresponding author:
Hava Gil-Henn (Hava.Henn@biu.ac.il)

KEYWORDS:

Cell migration, lamellipodia, protrusions, retractions, ruffles, live imaging, kymography.

SUMMARY:

This protocol aims to measure the dynamic parameters (protrusions, retractions, ruffles) of protrusions at the edge of spreading cells.

ABSTRACT:

The development and homeostasis of multicellular organisms rely on coordinated regulation of cell migration. Cell migration is an essential event in the construction and regeneration of tissues, and is critical in embryonic development, immunological responses, and wound healing. Dysregulation of cell motility contributes to pathological disorders, such as chronic inflammation and cancer metastasis. Cell migration, tissue invasion, axon, and dendrite outgrowth all initiate with actin polymerization-mediated cell-edge protrusions. Here, we describe a simple, efficient, time-saving method for the imaging and quantitative analysis of cell-edge protrusion dynamics during spreading. This method measures discrete features of cell-edge membrane dynamics, such as protrusions, retractions, and ruffles, and can be used to assess how manipulations of key actin regulators impact cell-edge protrusions in diverse contexts.

INTRODUCTION:

Cell migration is a critical process that controls the development and function of all living organisms. Cell migration occurs in both physiological conditions, such as embryogenesis, wound healing, and immune response, and in pathological conditions, such as cancer metastasis and autoimmune disease. Despite differences in cell types that take part in different migratory events, all cell motility

events share similar molecular mechanisms, which have been conserved in evolution from protozoa to mammals, and involve common cytoskeletal control mechanisms that can sense the environment, respond to signals, and modulate cell behavior in response[1].

An initial stage in cell migration can be the formation of highly dynamic protrusions at the leading edge of the cell. Behind the lamellipodium one can find the lamella, which couples actin to myosin II-mediated contractility and mediates adhesion to the underlying substrate. Lamellipodia are induced by extracellular stimuli such as growth factors, cytokines, and cell adhesion receptors and are driven by actin polymerization, which provides the physical force that pushes the plasma membrane forward[2,3]. Many signaling and structural proteins have been implicated in this; among them are Rho GTPases, which act coordinately with other signals to activate actin-regulating proteins such as the Arp 2/3 complex, WASP family proteins, and members of the Formin and Spire families in lamellipodia[2,4,5].

In addition to actin polymerization, myosin II activity is required for generating contractile forces at the lamellipodium and the anterior lamella. These contractions, also defined as cell-edge retractions, can also result from depolymerization of dendritic actin at the cell periphery and are critical for developing the lamellipodial leading edge and allowing the protrusion to sense the flexibility of the extracellular matrix and other cells and determine the direction of migration[6–8]. Cell edge protrusions that cannot attach to the substrate will form peripheral membrane ruffles, sheet-like structures that appear on the ventral surface of lamellipodia and lamella and move backward relative to the direction of migration. As the lamellipodium fails to attach to the substrate, a posterior lamellipodium forms underneath it and mechanically pushes the first lamellipodium toward the upper ventral surface. The actin filaments in the ruffle that were formerly parallel to the substrate now become perpendicular to it, and the ruffle is now positioned above the advancing lamellipodium. The ruffle that moves backward falls back into the cytosol and represents a cellular mechanism for recycling lamellipodial actin[9,10].

Here, we describe an assay for the measurement of cell-edge protrusion dynamics. The protrusion assay uses time-lapse video microscopy to measure single cell-edge protrusion dynamics for 10 min during the spreading phase of the cell. Protrusion dynamics are analyzed by generating kymographs from these movies. In principle, a kymograph imparts detailed quantitative data of moving particles in a spatiotemporal plot to yield a qualitative understanding of cell edge dynamics. The intensity of the moving particle is plotted for all image stacks in a time versus space plot, where the X-axis and the Y-axis represent time and distance, respectively[11]. This method uses a manual kymograph analysis with ImageJ to get detailed quantitative data, enabling retrieving information from movies and images in case of low signal-to-noise ratio and/or high feature density, and the analysis of images acquired in phase-contrast light microscopy or poor image quality.

The cell-edge protrusion dynamics assay described herein is a fast, simple, and cost-effective method. As such, and because it has been shown to directly correlate with cell migration[11,12], it can be used as a preliminary method for testing cytoskeletal dynamics involved in cell motility before deciding to perform more resource-demanding methods. Moreover, it also enables quantitative measurement of how genetic manipulations (knockout, knockdown, or rescue constructs) of cytoskeletal proteins impact cytoskeletal dynamics using a straightforward platform. The assay is an instructive model for exploring cytoskeletal dynamics in the context of cell migration and could be used for elucidation of the mechanisms and molecules underlying cell motility.

PROTOCOL:

All methods described in this protocol have been approved by the institutional Animal Care and Use Committee (IACUC) of Bar-Ilan University.

NOTE: A step-by-step graphical depiction of the procedure described in this section appears in **Figure 1**.

1. Cell culture

NOTE: The cells used in the protocol are mouse embryonic fibroblasts (MEFs) that were generated from E11.5–13.5 embryos of wild-type C57BL/6 mice. Primary MEFs were generated according to the Jacks laboratory protocol[13]. Cells from five different embryos were pooled together and immortalized by infection with a retroviral vector expressing SV40 large T antigen followed by selection with 4 mM Histidinol for 3 weeks.

1.1. Culture cells in tissue culture plates containing Dulbecco's Modified Eagle Medium (DMEM) containing 1 g/L glucose, 1% glutamine, 1% penicillin-streptomycin, and 10% fetal bovine serum (FBS) (see **Table of Materials**), in a humidified incubator with 5% CO_2 at 37 °C.

1.2. Culture the cells until 90%–95% confluency and split at a ratio of 1:5 every 2–3 days.

2. Glass-bottom dish coating

NOTE: Glass-bottom dish coating should be performed in the tissue culture hood in sterile conditions.

2.1. Add 2 mL of 1N HCl solution at the center of a glass-bottom dish (**Table of Materials**) and incubate for 20 min at room temperature (RT).

NOTE: This stage is meant to remove residue from the glass that may interrupt imaging later.

2.2. Wash the glass-bottom dish three times with 2 mL of 1x PBS (**Table of Materials**) each.

2.3. Dilute fibronectin (see **Table of Materials**) to 10 µg/mL in 1x PBS. Add 200 µL of the diluted fibronectin solution to the glass center of the dish. Incubate at 37 °C (tissue culture incubator) for 1 h.

NOTE: Alternatively, the glass-bottom dish can be incubated overnight at 4 °C on a flat surface.

2.4. During fibronectin coating incubation time, prepare 1% BSA (**Table of Materials**) solution in 1x PBS, filter through 0.2 µm, and denature by incubating at 70 °C for 30 min in a pre-warmed water-bath.

2.5. Wash the coated glass-bottom dish three times with 2 mL of 1x PBS each.

2.6. Add 2 mL of denatured BSA solution to the glass center and incubate for 1 h at 37 °C (tissue culture incubator).

NOTE: Alternatively, the glass-bottom dish can be incubated overnight at 4 °C on a flat surface.

2.7. Wash the glass-bottom dish 3 times with 2 mL of 1x PBS each.

NOTE: Incubation of glass-bottom dishes with fibronectin should be performed for at least 1 h at 37 °C to coat the surface properly. Shorter incubation time may not produce proper coating, and as a result, cell phenotype may not be related to integrin activation. The BSA layer is inert and will not affect protrusion dynamics and is meant to block free potential sites for integrin-independent cell adhesion. The BSA must be denatured before coating to prevent it from inducing cell apoptosis, which can influence cell-edge dynamics.

3. Preparation of cells for imaging

3.1. Before 16–18 h of the experiment, plate the cells to reach 70%–80% confluence on the following day. For MEFs, plate 0.7 x 10^6 cells per 10 cm diameter tissue culture plate a day before performing the experiment.

NOTE: For a successful protrusion experiment, it is important that the cells will be used during their logarithmic growth phase. To achieve this, cells should reach 70%–80% confluence on the day of the experiment. A higher density of cells will result in a longer spreading time and/or impediment in attachment during the experiment.

3.2. On the experiment day, add 2 mL of trypsin solution (**Table of Materials**) per 10 cm diameter tissue culture plate and incubate for 2–3 min in a tissue culture incubator until cells detach. Inactivate trypsin by adding 5 mL of the complete medium.

3.3. Count the cells using a hemocytometer and plate 20,000 cells in 2 mL of the complete medium on a glass-bottom dish coated as above (section 2).

NOTE: The number of cells for plating depends on the size and type of cells. For larger or more spread cells, plate only 10,000 cells to have enough cells for imaging. It is important to pick single cells that do not touch as cell-to-cell contact can alter cell-intrinsic spreading behaviors.

3.4. Incubate the glass-bottom dishes with plated cells in the tissue culture incubator for 15 min.

NOTE: During this stage, cells adhere to the fibronectin substrate and spread over it. When measuring protrusions during cell spreading, cells should be allowed to spread for 15 min after plating and before imaging. Imaging can be performed within a time window between 15 min to ~1 h following plating, and in any case, before the cells start migrating.

4. Microscope setup and imaging

NOTE: Various live-cell microscopy systems are available. The system used here is a Leica AF6000 inverted microscope equipped with CO_2 and heating units and is attached to an ORCA-Flash 4.0 V2 digital CMOS camera.

4.1. Turn on the heating unit at least 1 h before imaging and set it at 37 °C.

4.2. Turn on the CO_2 unit at least 10 min before imaging and set it at 5% CO_2.

4.3. Switch on the microscope, camera, and computer.

4.4. Open the microscope acquisition software (see **Table of Materials**). Choose the folder to save captured images and type a file name. Save every movie as a new file.

4.5. Set the magnification on a 40x dry lens, phase-contrast. Set the time interval on 5 s, total movie duration 10 min.

4.6. After 15 min incubation of the plated cells, place the glass-bottom dish with adhered cells into the adapter and fix it. Insert the adapter with the dish into its slot in the microscope stage.

4.7. Take off the dish cover and place the CO_2 lid instead. Open the CO_2 valve.

NOTE: Make sure the CO_2 cover is clean before placing it on top of the glass-bottom dish. A dirty cover will reduce the quality of movies. Wipe the underside of the CO_2 lid with a lint-free wipe soaked with 70% ethanol to remove dust and dirt. Wipe a second time with a dry lint-free wipe.

4.8. View the cells and find an appropriate cell for imaging. Make sure the cell is in focus and start movie acquisition.

5. Image analysis

NOTE: Image analysis is performed using ImageJ (**Table of Materials**) as following:

5.1. Open the acquired movie in ImageJ.

5.2. Using the **Straight** tool in the main toolbar, make eight lines of 20 arbitrary units each perpendicular to the protrusions, including the lamella and cell edge, in a radial arrangement every 45°, as shown in **Figure 2A**.

5.3. In the main toolbar, go to **Image** > **Stacks** > **Reslice**. This will yield a kymograph picture, which describes the movement of single points within the cell membrane (**Figure 2B**). This action should be performed for every line out of the eight lines separately.

5.4. Extract and manually count from the respective kymograph images the number of protrusions, retractions, and ruffles in each of the eight regions in the cell, marked by the grid lines. These numbers represent the frequency of protrusions, retractions, and ruffles per 10 min (**Figure 2C, D**).

NOTE: Ruffles can be distinguished from other structures based on their dark appearance in phase-contrast microscopy and their centripetal movement, which starts at the cell edge and ends at the border of the cell body, which can be observed in the acquired movies. Of note, when quantifying protrusion, retraction, and ruffle frequency, movies should be observed as a control for quantification and especially for defining ruffles.

5.5. Determine the protrusion persistence, distance, and velocity by kymography analysis. For each of the generated kymographs, the X-axis represents distance, and the Y-axis represents time.

5.6. To measure protrusion distance, follow steps 5.6.1–5.6.2.

5.6.1. Draw a perpendicular line from the base of the protrusion to the highest peak of the protrusion. Press M in ImageJ to measure the length of the line in pixels.

5.6.2. To convert the length from pixels to μm, ensure that the pixel to μm ratio is known.

NOTE: The μm to pixel ratio is the physical length of a pixel on the CCD camera / total magnification. The pixel size is characteristic of each type of camera. For example, for the camera used in this study, the pixel size is 6.5 μm x 6.5 μm, the physical length is 6.5 μm and the magnification we used is 40x. Therefore, the μm to pixel ratio of our camera is 0.1625 μm/pixel. In the analysis, for a line in the length of 30 pixels, the protrusion distance would be 30 pixels x 0.1625 μm/pixel = 4.875 μm (**Figure 3**).

5.7. To measure protrusion time (persistence; the amount of time a protrusion spends protruding before retracting), follow steps 5.7.1–5.7.2.

5.7.1. Draw a horizontal line from the beginning of the protrusion (left to right) to the region of the highest peak. Press **M** in ImageJ to measure the length of the line in pixels.

5.7.2. To convert the length from pixels to minutes, calculate the pixel to min ratio. This value depends on the interval between images.

NOTE: In this example, the interval between images is 5 s, the min/pixel ratio is 0.0833, and the horizontal line length is 8 pixels. Therefore, the protrusion time is 8 pixels x 0.0833 min/pixel = 0.6664 min.

5.8. Measure and calculate the retraction time similarly to protrusion time for a line drawn horizontally at the base of the protrusion from where the peak of the protrusion is to its base on the right (line X2 in **Figure 3**).

5.9. Calculate the protrusion velocity by dividing protrusion distance by protrusion time. Calculate the retraction velocity by dividing protrusion distance by retraction time. In this example, protrusion velocity is calculated as 4.875 μm/0.6664 min = 7.315 μm/min., and the retraction velocity is identical because the line representing time is at the same length.

NOTE: In comparing different cell types, i.e., cells expressing wild type and mutant constructs of the same protein, it is imperative to perform a blinded analysis, so no bias is introduced.

REPRESENTATIVE RESULTS:

In the experiment described in **Figure 2**, immortalized MEFs were plated on glass-bottom dishes pre-coated with fibronectin to activate integrin-mediated signaling, blocked by denatured BSA, to block free potential sites for cell adhesion which is not dependent on integrin activation. To reach the logarithmic growth phase at 70%–80% confluence of cells on the day of the experiment, 0.7 x 10^6 MEFs were plated in a 10 cm diameter tissue culture plate 16 h before the experiment. On the experiment day, cells were trypsinized and counted, and 20,000 cells were plated on a fibronectin/BSA-coated glass-bottom dish. The dish was incubated for 15 min at 37 °C to allow attachment and spreading of the cells before imaging. Following incubation, the plate was placed in a microscope incubator chamber (37 °C, 5% CO_2), and single cells were imaged using a 40x dry lens in phase light. Imaging was performed between 15 min to 1 h following plating before the cells

started migrating. Images were acquired every 5 s for 10 min, which yielded 121 images per cell (**Supplementary Movie 1**).

Image analysis was performed using ImageJ. Using the **Straight** tool in the main toolbar, 20 arbitrary unit-long straight lines were made on a radial grid in the same places every 45 degrees in all cells (**Figure 2A**). To generate a kymograph, we used the **Image** > **Stack** > **Reslice** commands, which yielded a kymograph picture describing the movement of single points within the cell membrane (**Figure 2B–D**). The number of protrusions, retractions, and ruffles formed during 10 min of the movie in each of the eight regions in the cells, which are marked by the grid lines, was extracted, manually counted from the respective kymograph images, and plotted in a graph as protrusions/retractions/ruffles frequencies per 10 min. The average frequencies obtained were 5.1/10 min for protrusions and retractions and 2.1/10 min for ruffles (**Figure 2E**).

The protrusion distance, protrusion time, retraction time, protrusion and retraction velocities were calculated from the generated kymographs. In the representative kymograph in **Figure 3**, protrusion distance was 30 pixels x 0.1625 μm/pixel = 4.875 μm, protrusion time was 8 pixels x 0.0833 min/pixel = 0.6664 min, retraction time was 8 pixels x 0.0833 min/pixel = 0.6664 min. Protrusion and retraction velocities were calculated as 4.875 μm/0.6664 min = 7.315 μm/min.

When measuring cell-edge protrusion, it is important to choose cells that are in their spreading phase. An example of a proper cell for analysis appears in **Figure 2A** and **Supplementary Movie 1**. Following kymography analysis, the protrusions, retractions, and ruffles can be easily distinguished in this experiment. An example of a wild-type fibroblast that is not appropriate for analysis is shown in **Figure 4**. Following kymography analysis, in lines (slices) 1, 3, 5, 7, for instance, clear protrusions cannot be distinguished. In this case, the cell finished spreading but did not start moving yet, and therefore not many membrane movements can be observed.

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental stages of the protrusion assay. (A) A 1N HCl solution is added to the glass-bottom dish for 20 min. **(B)** Following washes in PBS, 10 μg/mL fibronectin solution is added to the glass part of the dish and incubated for 1 h at 37 °C. **(C)** The dish glass bottom is blocked by incubation in 1% denatured BSA for 1 h at 37 °C. **(D)** A tissue culture plate of 70%–80% confluent fibroblasts is trypsinized and counted **(E)** 20,000 cells are plated in a glass-bottom dish and **(F)** incubated for 15 min at 37 °C to allow cells to spread. **(G)** The plate is placed in a microscope humid chamber with 37 °C in 5% CO_2, and live imaging is performed by phase-contrast light microscopy. **(H)** Cell movies and images are subjected to kymography analysis by Image J.

Figure 2: Protrusion assay image analysis. (A) Representative image of MEF in ImageJ with indicated eight membrane cross-sections for quantification during 10 min. Scale bar, 20 μm. **(B)** Generation of a kymograph in Image J. **(C)** Representative kymograph from cross-section on which protrusions, retractions, and ruffles can be distinguished. **(D)** Resulting kymographs after image analysis during 10 min in Image J using the **Reslice** command. **(E)** Quantification of protrusions, retractions, and ruffles frequencies per 10 min from the analyzed movie and kymograph. Average protrusion frequency per 10 min = 5.1, average retraction frequency per 10 min = 5.125, average ruffles frequency per 10 min = 2.1. Eight kymographs were analyzed from one movie.

Figure 3: Analysis and quantification of protrusion persistence, distance, and velocity. In the representative kymograph, the X-axis represents time in min (left to right), and the Y-axis shows the distance in μm. X1 represents protrusion time (persistence), X2 represents retraction time, and Y

represents the protrusion distance. Protrusion velocity is calculated by dividing protrusion distance (Y) by protrusion time (X1). Retraction velocity is calculated by dividing protrusion distance (Y) by retraction time (X2). In this example, protrusion distance was 30 pixels x 0.1625 μm/pixel = 4.875 μm, protrusion time was 8 pixels x 0.0833 min/pixel = 0.6664 min., retraction time was 8 pixels x 0.0833 min/pixel = 0.6664 min. Protrusion/retraction velocity was calculated as 4.875 μm/0.6664 min. = 7.315 μm/min.

Figure 4: Example of a cell that should be excluded from the analysis. (A) An example of a cell that is not appropriate for analysis. Scale bar, 20 μm. **(B)** The kymography analysis of this cell shows, especially in re-slice 1,3,5,7, no clear protrusions. In this case, the cell finished spreading but did not start moving yet, and therefore not many membrane protrusions can be observed.

Supplementary Movie 1. MEF was plated on a fibronectin-coated glass-bottom dish and imaged using time-lapse phase-contrast video microscopy for 10 min using 40x/1.4 NA dry objective. Time is indicated in seconds. Scale bar, 10 μm.

DISCUSSION:

Cell-edge protrusion dynamics, comprised of protrusions, retractions, and ruffles, is both a prerequisite and a potential rate-limiting event in cell motility. Here we describe a fast and simple method for measuring the dynamics of cell-edge protrusions during spreading. This method enables short-time imaging, generates a significant amount of data, does not require fluorescent labeling of cells or expensive fluorescent microscopy equipment, and could be used as a preliminary method for testing cytoskeletal dynamics involved in cell motility before deciding to perform more resource-demanding methods. Moreover, one can use knockout or knockdown cells and/or protein mutants in this assay as a fast and simple tool to identify critical proteins and potential signaling mechanisms involved in cytoskeletal dynamics.

Of note, it is important to choose the correct cells for protrusion analysis. Cells are incubated for 15 min before movies are acquired to allow spreading. If one decides to measure protrusion during spreading (as opposed to measuring protrusions during migration), then only cells that are in their spreading phase during movie acquisition should be imaged. Cells that did not start spreading not be appropriate for kymography analysis. Cells that completed their spreading phase but did not start moving (**Figure 4**) will not be appropriate for analysis as well. The movement of their nucleus can distinguish these cells: during spreading, the nucleus is stationary, while during cell migration, the nucleus is dynamic and localizes at the rear side of the cell to construct a leading edge-centrosome-nucleus axis towards the direction of migration. Another common issue in the later stages of imaging is a situation in which cells touch each other. Such movies should be excluded from analysis, as interactions and signals from neighboring cells can interfere with cell-edge protrusion dynamics.

In this manuscript, we describe the analysis of cell-edge protrusion dynamics using phase-contrast light microscopy. This method can be expanded to measure the dynamics of intracellular components with fluorescence microscopy as well. Such common usage of fluorescent kymography is often described for measuring the dynamics of cytoskeletal structures within cells. For example, Dogget and Breslin have used kymography of GFP-actin transfected HUVEC cells to analyze actin stress fiber dynamics and turnover[14].

This protocol and several other previous papers used fibroblasts plated on fibronectin for the cell-edge protrusion assay as well as for two-dimensional cell motility assays. Fibroblasts are commonly used for motility assays and other related assays such as the cell-edge protrusion dynamics assay

because they are mesenchymal and motile and have clear cytoskeletal structures such as lamellipodia, filopodia, and focal adhesions. Although we do not describe the assay for other cell types and substrates, this method could easily be modified. For example, in the first documentation of the lamella dynamics assay, which we and others modified to become the cell-edge protrusion dynamics assay[11], the authors used keratinocytes stimulated to migrate by EGF in a scratch assay, demonstrating that other cell types and other stimulations could be applied to this assay. Moreover, although we describe the measurement of cell-edge protrusion dynamics during cell spreading, the same method could be used by measuring the dynamics of protrusions during migration of cells, as demonstrated, for example, in Bear et al.[12] and Hinz et al.[11].

Indeed, several labs have used this assay on MEFs to elucidate cytoskeletal dynamics and signaling mechanisms in the past. For example, Miller et al. had previously used the protrusion assay to demonstrate that Abl2/Arg mediates the contact between actin and microtubules at the cell-edge[15]. Bryce et al. demonstrated using the assay that cortactin knockdown cells have impaired cell motility which co-insides with impairment in the persistence of lamellipodial protrusions. This defect results from impairment in the assembly of new adhesions in protrusions[16]. Lapetina et al. used the cell-edge protrusion assay in Abl2/Arg knockout and cortactin knockdown cells rescued with mutants of the two proteins to elucidate an Abl2/Arg-mediated regulation mechanism cell-edge protrusions[17]. Using the same assay, Miller et al. have also demonstrated that Arg regulates N-WASP-mediated actin polymerization and consequent cell-edge protrusion dynamics[18]. We have recently used the cell edge protrusion assay to demonstrate that the non-receptor tyrosine kinase Pyk2 regulates the dynamics of protrusions and subsequent cell migration via direct and indirect interactions with the adaptor protein CrkII. In this paper, we have used Pyk2-WT and Pyk2$^{-/-}$ and Crk-WT and knockdown MEF, rescue mutants, and epistasis experiments to elucidate the molecular interactions and signaling hierarchy between the two proteins during cell-edge protrusion. This novel complex regulation mechanism enables fine-tuning of cell-edge protrusion dynamics and consequent cell migration on the one hand together with tight regulation on cell motility on the other hand[19]. Using the cell-edge protrusion assay, the above papers and others that followed have significantly increased our knowledge of the regulation mechanisms of cell-edge protrusions in particular and cell migration in general.

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DISCLOSURES:

The authors have no conflict of interests to disclose.

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Figure 1

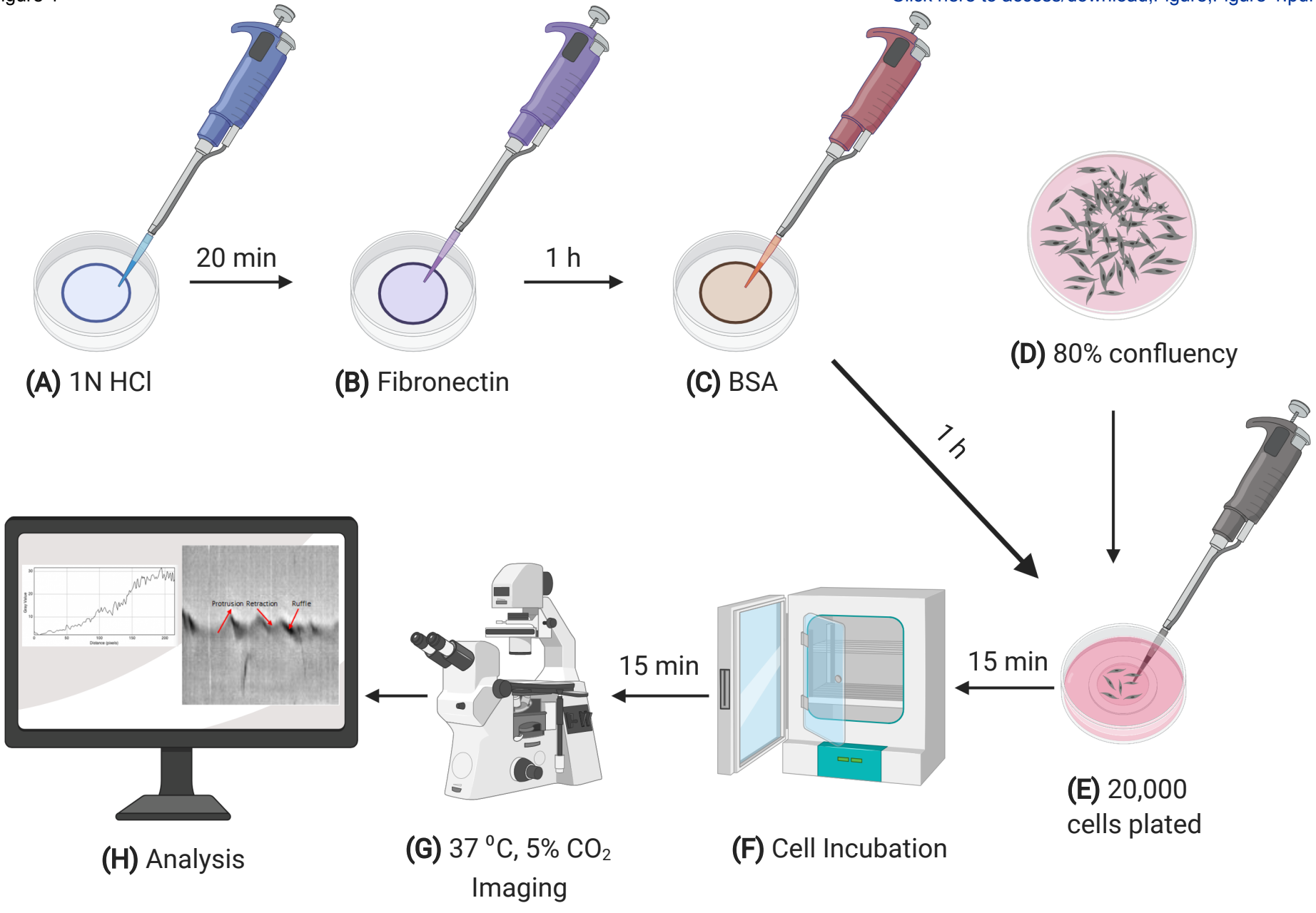


Figure 2

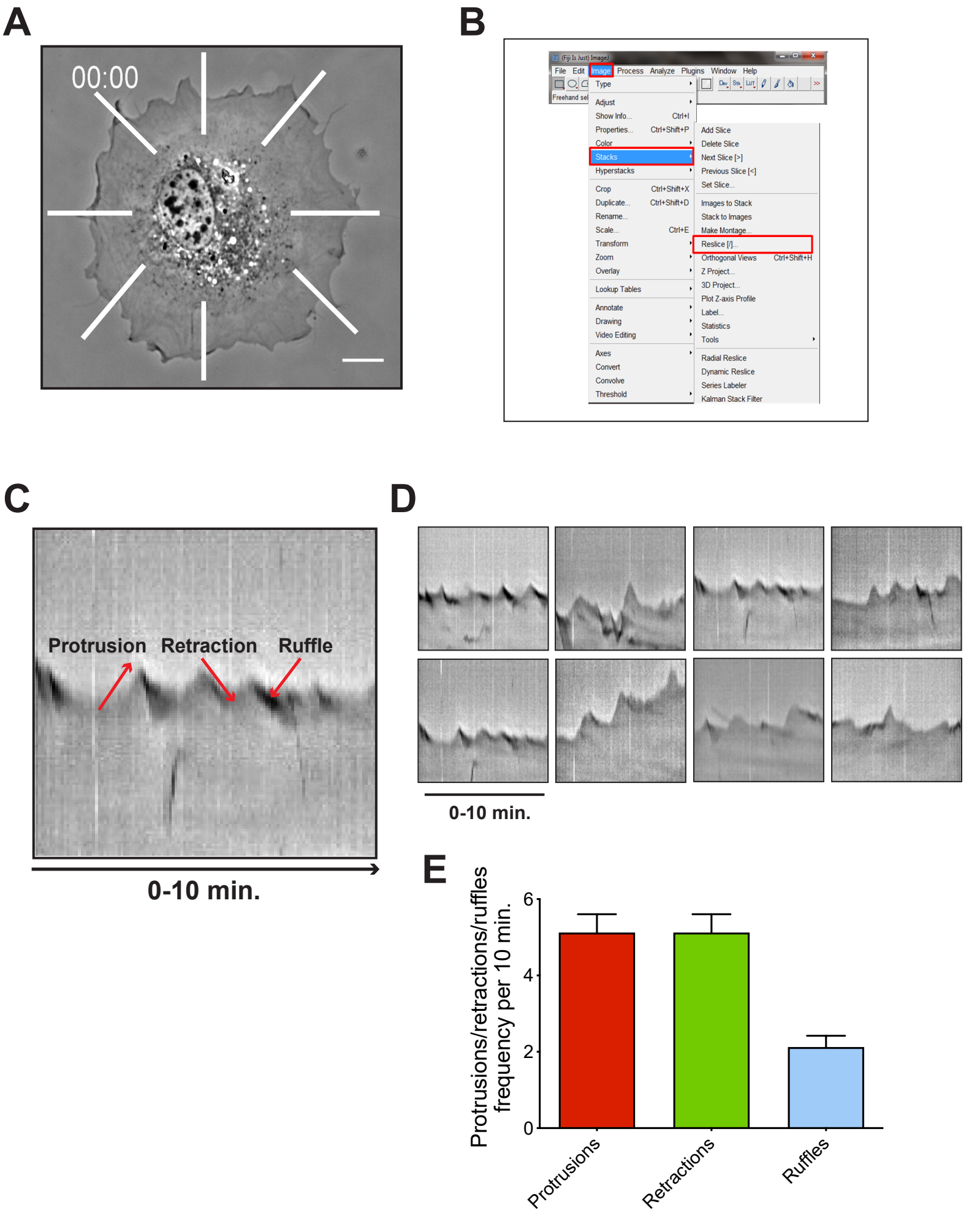


Figure 3

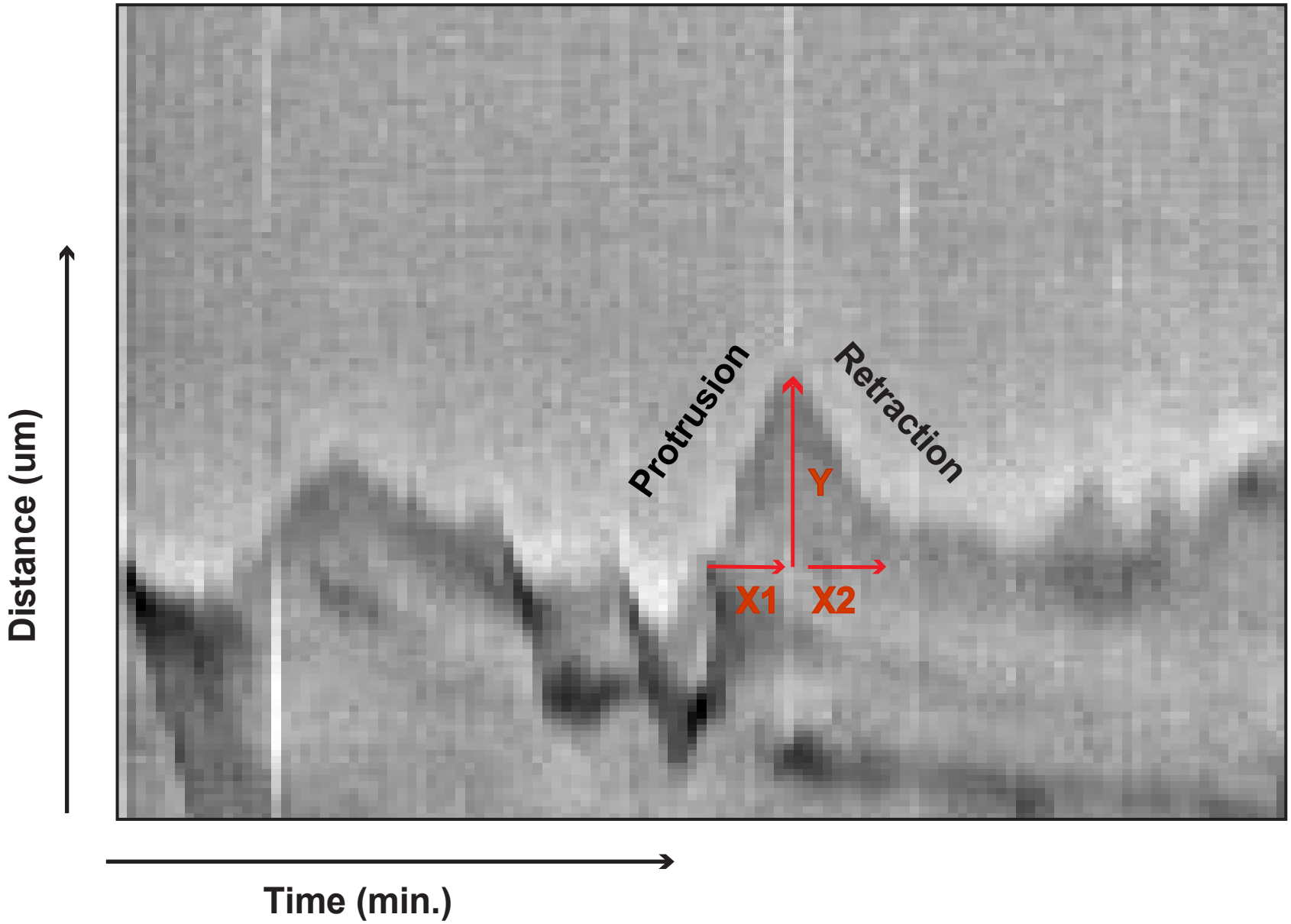
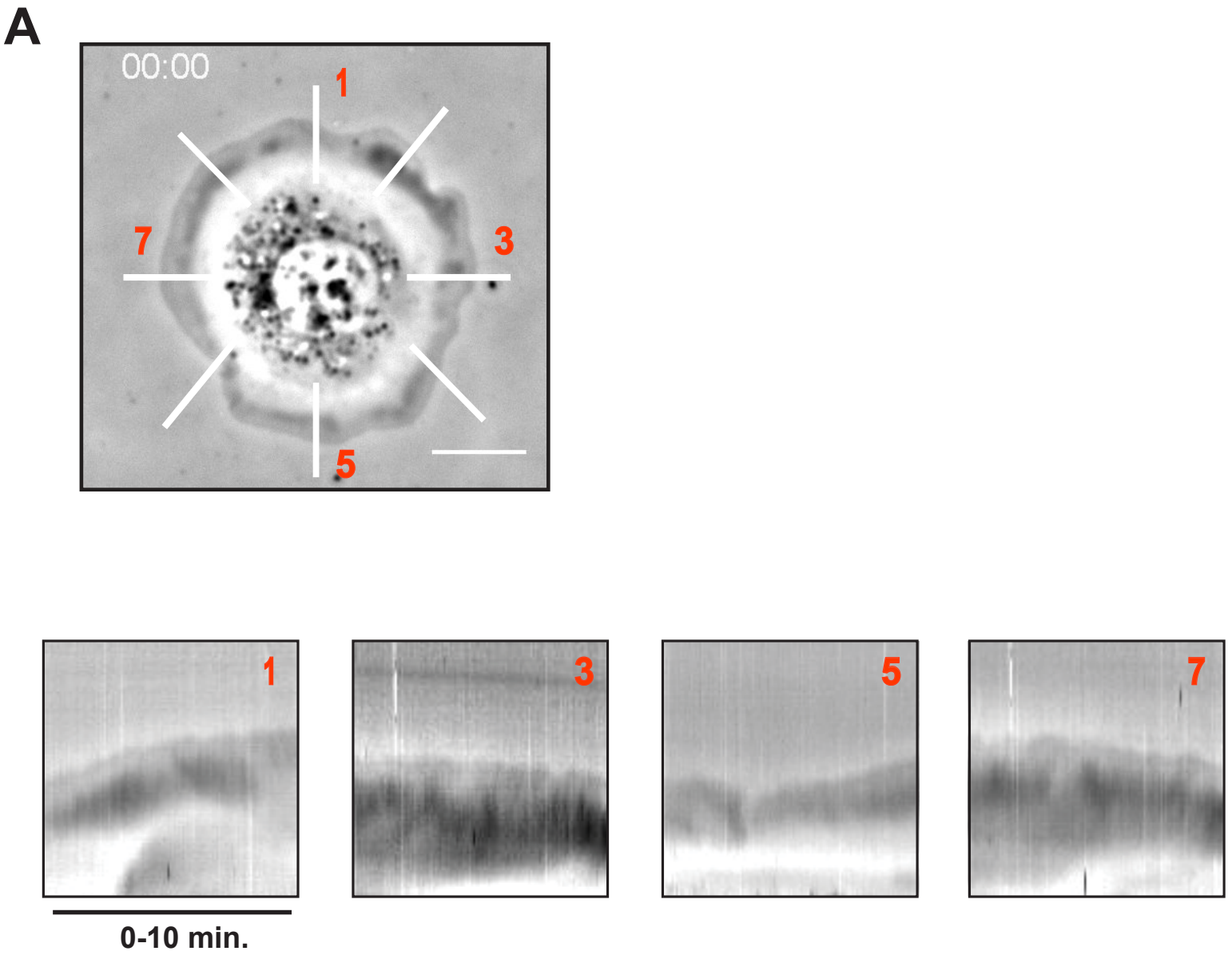


Figure 4



Video

Click here to access/download

Video or Animated Figure

WT protrusion.mp4

Editorial comments:

We thank the editorial staff and editor for their constructive comments and suggestions. We have addressed each of these comments, which has significantly improved the quality of our manuscript. We provide a point-by-point response of these changes here:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The revised manuscript has been extensively edited both manually and using an editing software following this suggestion.

2. Please note that there are two manuscript files. Please remove one during resubmission. However, please feel free to upload a manuscript file with track changes and a clean manuscript file during resubmission.

We have now uploaded two manuscript files, a revised manuscript in which the changes are tracked and a second clean manuscript file, for reviewers' convenience.

3. Please provide an institutional email address for each author.

The email addresses of the corresponding author and all co-authors have now been included in the title page.

4. Please revise the following lines to avoid previously published work: 40-43, 48-50, 52-54.

The lines have been revised as suggested, to avoid similarity to previously published work (lines 47-50, 54-60 in the revised manuscript).

5. Please provide suitable references, wherever applicable (e.g., for the following lines: 67-71, 233-239).

Reference for lines (revised numbers) 69-76: Lee JM et al. Morgan and Claypool, Life Sciences, 2013; Chhabra ES et al., Nature Cell Biology 2007. These references are mentioned at the end of the paragraph.

Reference for lines 233-239: This part has been removed from the revised version, because it repeats an explanatio of kymography that has been included in other parts of the manuscript.

6. Please revise the introduction to include all the following:
a) The rationale behind the development and/or use of this technique
b) The advantages over alternative techniques with applicable references to previous studies
c) A description of the context of the technique in the wider body of literature
d) Information to help readers to determine whether the method is appropriate for their application

The introduction of our manuscript has been revised accordingly. Specifically, the biggest advantage of the method described in our manuscript is that it is fast, simple, cost-effective, and does not require expensive reagents or equipment. As such, and because it has been shown to directly correlate with cell migration, it can be used as a preliminary method for testing cytoskeletal dynamics involved in cell motility before deciding to perform

more resource-demanding methods. It also enables testing of the effect of genetic manipulations (knockout, knockdown, or rescue constructs) of cytoskeletal proteins on cytoskeletal dynamics in a relatively simple and short experimental time (lines 88-96 in the revised manuscript).

A description of the context of the technique in the literature is included in the discussion section of our manuscript (lines 382-401 in the revised manuscript).

7. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

An ethics statement has been included at the beginning of "PROTOCOL" section as follows: "All methods described in this protocol have been approved by the institutional Animal Care and Use Committee (IACUC) of Bar-Ilan University" (lines 101-102 in the revised manuscript).

8. Use SI units as much as possible and abbreviate all units: L, mL, µL, cm, kg, etc. Use h, min, s, for hour, minute, second.

All units throughout the manuscript have been abbreviated as suggested above.

9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We have now added more details to existent parts of the protocol, and additional steps in the protocol describing in detail the microscope setup and imaging stages (lines 182-207 in the revised manuscript).

10. Lines 82-85: Please provide more details on generation of MEFs. In case it is a well-established protocol, a citation would suffice.

For the generation of MEFs, we used a protocol which is described in the following link: http://jacks-lab.mit.edu/protocols/making_mefs. A reference to this link has now been included in the text (line 107-108 in the revised manuscript).

11. Step 1.1: How were the cells cultured? Was a tissue culture flask or well plate used?
Cells were cultured in 10 cm diameter tissue culture plates. These details were added to section 1.1 (line 112 in the revised manuscript).

12. Line 107-113: Please merge the notes in to one "NOTE".
Lines 107-113 were merged into one "NOTE" accordingly (lines 146-151 in the revised manuscript).

13. Step 3.2: How was trypsinization and cell inactivation done? How much trypsin was added? How much medium was added to neutralize trypsin?
Cells were trypsinized in 1 ml/10 cm tissue culture plate and were inactivated by adding 5 ml complete medium. These details were added to section 1.1 (lines 164-166 in the revised manuscript).

Commented [KA1]:

Commented [KA2]: I would emphasize this. It is a facile platform with which to investigate how manipulations of cytoskeletal regulators control cell edge dynamics.

Commented [KA3]: You do use min. Think you must want to use min.

14. Step 3.6: How was the plate set up for imaging? How were the images acquired? Please elaborate the steps for acquiring images. If this step needs to be filmed, please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screen shots, etc. Please keep in mind that software steps without a graphical user interface (GUI) cannot be filmed.

We have now added a separate "microscope setup and imaging" section (new section 4) to the protocol, detailing all necessary steps for these stages.

15. 4.3: Please format the commands/clicks to Images > Stacks > Reslice to emphasize the commands/options (bold letters with the initial letter capitalized).

The commands were formatted as required (lines 219 and 280 in the revised manuscript).

16. Step 4.4 NOTE: How is the spreading phase of the cell determined? How is their nucleus different from cells which just started moving and cells which completed movement?

The spreading phase in which imaging is performed is defined as 15 min following plating (when cells adhere to the substrate) and until 1 hr following plating, when cells start migrating. The migration of cells can be observed by movement of their nucleus: during spreading the nucleus is stationary, which during cell migration the nucleus is dynamic and localizes at the rear side of the cell to construct leading edge-centrosome-nucleus axis towards the direction of migration. This explanation has now been added to the discussion lines 352-363 in the revised manuscript.

17. Please remember that our scripts are directly derived from the protocol text. Please include all actions associated with each step.

The protocol section has now been revised and all actions associated with each step have been included.

18. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

As required, we have now moved several notes about the protocol into the discussion section.

19. Please include a single line space between all the steps and sub-steps.

A single line space was included between all the steps and sub-steps in the manuscript.

Please highlight up to 3 pages of the protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted protocol steps will remain in the manuscript, and therefore will still be available to the reader.

As suggested, we have now highlighted essential steps of the protocol for the video.

20. Please ensure that the Results section focuses on the effectiveness of your technique backed up with data.

As suggested, we have now revised the results section to focus on the effectiveness of the technique. The results section is backed up with example data (Figure 2).

21. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The discussion section has been revised to include all points above.

22. Please do not use the &-sign or the word "and" when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

Although we used the JoVE format of references in Endnote in the submitted version, we now manually corrected the references as suggested above.

23. Please ensure that the table of materials contains all the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

The table of materials has been revised accordingly, changes are marked in red.

24. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.).

Each figure has been submitted separately as an Illustrator file (.ai) via the submission website as requested.

25. Please ensure to include scale bar in all the images of the panel, wherever necessary.

A scale bar has been included in all images and movie as necessary.

26. Figure 1: Maintain a single space between the numeral and (abbreviated) unit, except in cases of %, x, and ° (i.e., the degree sign; excluding temperature). (e.g., 5 mL, 10%, 3°, 100 °C, 3x SSC). Revise "1 hour" to "1 h", "37°C" to "37 °C", "5 %" to "5%".

The above changes have now been included in Figure 1.

Reviewers' comments:

We thank the reviewers for their instructive and constructive comments and suggestions, which have significantly improved the quality of our manuscript. Each comment was addressed by us and appears in the revised manuscript as follows:

Reviewer #1:

Issue 1: More than half of the protocol is how to culture and plate cells to perform microscopy. This protocol is standard in most labs.

We appreciate the reviewer's note on this issue. In their instructions for authors, JoVE specifically requires "a detailed description to enable the accurate replication of the presented method by both experts and researchers new to the field." Moreover, some of the editorial comments for this manuscript specifically required more details about cell plating and culturing that we provided accordingly.

Issue 2: The most interesting part might be the analysis. However, also this part provides no novelty at all. The method has been well documented (there are plenty of YOUTUBE movies on the internet with stepwise instructions) and publishing such a method via JoVE seems excessive (also the plugin for ImageJ exist already for a long time).

We understand the reviewer's concern about this issue. Indeed, the method of cell-edge protrusion dynamics measurement was developed some time ago and was used by our groups and others in several past publications (for example, Bear *et al.*, Cell 2002, PMID: 12086607; Miller *et al.*, JCB 2004, PMID: 15138293; Bryce *et al.*, Current Biology 2005, PMID: 16051170; Lapetina *et al.*, JCB 2009, PMID: 1941460; Miller *et al.*, Biochemistry 2010, PMID: 20146487; Lukic *et al.*, MBOC 2021, PMID: 34432482). However, a single well-resourced detailed, and updated description of the method has not been published within the past 22 years, neither in the materials and methods sections of these papers nor as a methods paper. Therefore, scientists that wish to learn this approach had to visit labs that use this method. The primary aim of our detailed step-by-step methods manuscript is to enable these scientists to use the technique in their laboratories. Moreover, as this is also the primary goal of JoVE, we were specifically noted by the editor that novelty is not a requirement for publication in this journal.

Issue 3: points 4.1 - 4.3 are unclear despite the images provided. It is not clear how one places the lines correctly (length, angle) and whether one needs to press reslice for each and every line or whether there is a bulk reslice for all of the lines that can automatically generate the images in a certain sequence.

We thank the reviewer for this constructive comment and apologize for the unclarity. Eight lines of 20 arbitrary units each, including the lamella and cell edge, are placed in a radial arrangement every 45 degrees. Each line is analyzed separately, so the "Reslice" command should be pressed for every line. These details have been added to the "Image analysis" section of the protocol (section 5, points 5.2 and 5.3; lines 215-221 in the revised manuscript).

Issue 4: The discussion is not inspiring. The kymograph analysis can be used in so much more inspiring ways (e.g., fluorescence profiles over time etc.). Instead, authors just describe the use of it for the simplest phase-contrast analysis for cell edge ruffling during cell spreading.

We thank the reviewer for this insightful comment. Indeed, kymography analysis could be used for many more applications than mentioned in our manuscript and with fluorescence microscopy as well. Such applications are described in detail elsewhere, such as Doggett and Breslin, JoVE 2011 (PMID: 22126853). The authors describe kymography analysis of actin stress fiber dynamics and turnover in GFP-actin transfected HUVEC cells. Our manuscript describes a fast and cost-effective method that can be used as a primary method for testing cytoskeletal dynamics related to cell motility, which can assist scientists in deciding whether to perform more resource-demanding techniques and to test the effect of genetic manipulations of cytoskeletal proteins quickly. Following this reviewer's comment, we have now added a note on the different applications of the kymography assay and the above example in the discussion (lines 364-369 in the revised manuscript).

Issue 5: The provided movie does not open in Quick Time Player.

We apologize for the inconvenience. In the instructions for authors, JoVE specifically requires movies to be uploaded in .mov, .mp4, or .m4v formats. We have provided the movie in .mov format, which should open in QuickTime player. In our revised version, we have added a .mp4 version of the movie to overcome such difficulties that may occur to the readers.

Reviewer #2:

Issue 1: In the title and the abstract, it should be specified that the measurements are performed in the spreading phase: I suggest extending the phrase "protrusion dynamics" into "protrusion dynamics during spreading" or something similar (line 33). This has been adequately stated, e.g., in the one-sentence summary.

We are thankful to the reviewer for this thoughtful comment. Indeed, the measurements described in our methods manuscript are performed in the spreading phase. However, a similar analysis could be performed at the edge of migrating cells (which is less common).

Based on this comment, we have now changed the title of the manuscript to read: "Measuring cell-edge protrusion dynamics during spreading using live-cell microscopy." We have also modified the sentence describing the method in the abstract into the following: "...imaging and quantitative analysis of cell-edge protrusion dynamics during cell spreading". We have also mentioned, in the discussion section, that although our manuscript describes measurements during cell spreading, this method could be applied to migrating cells as well, as was performed, for example, in the original method that was modified by us (Hinz *et al*., Experimental Cell Research 1999, PMID: 10438589) and in Bear *et al.* (Cell 2002, PMID: 12086607) (lines 379-381 in the revised manuscript).

Issue 2: The sentence starting with "The assay is an instructive model for exploring actin dynamics..." is not clear; please reformulate (lines 75-76).

We thank the reviewer for this thoughtful note. Indeed, the cell edge protrusion assay is not limited to measuring actin dynamics, and more cytoskeleton components and processes, in addition to actin, are necessary for regulating protrusions. As requested by the reviewer, the sentence has been changed to: "The assay is an instructive model for exploring cytoskeletal dynamics in the context of cell migration and could be used for elucidation of the mechanisms and molecules underlying cell motility" (lines 94-96 in the revised manuscript).

Issue 3: The noun "plate" is used ambiguously to designate the glass-bottom dishes used for microscopy, and the plastic cell-culture dishes (or plates) used to cultivate the cells. Please review the text and introduce a unified designation.

We are grateful to the reviewer for drawing our attention to this issue. As requested by the reviewer, we have revised the protocol section to distinguish between cell culture plates and glass-bottom dishes.

Issue 4: Instead "Image cells using 40X dry lens in phase light" write "Image cells by phase-contrast light microscopy using a 40x dry objective" (line 135).

We are thankful to the reviewer for this comment. This part has now been revised into new section 4 (microscope setup and imaging).

Issue 5: Line 169: does 10 cm refer to the diameter of the dish?

We thank the reviewer for drawing our attention to this point. Indeed, 10 cm refers to the diameter of the cell culture plate. Following the reviewer's comment, we have now changed the text to read: ".....$0.7x10^6$ MEFs were plated in a 10 cm diameter cell culture plate...." (line 270 in the revised manuscript).

Issue 6: Line 204: ...live imaging is performed by phase-contrast light microscopy.

We thank the reviewer for this comment. The text was corrected as suggested by the reviewer (line 309 in the revised manuscript).

Issue 7: Line 220: Time-lapse phase-contrast video microscopy...

We thank the reviewer for this comment. The text was corrected as suggested by the reviewer; additionally, the figure legend was revised to become more detailed (lines 338-340 in the revised manuscript).

Issue 8: Table of materials: Is 664160 a company? What is Cellstar?
Do the glass-bottom dishes have a 1.5 mm thick glass bottom (hint: they don't)?
For performing experiments described in this study, an inverted bright field microscope equipped with phase-contrast optics is needed!

We appreciate the reviewer's notes on these points and sincerely apologize for these errors in the materials table. All errors have been corrected in the table as suggested by the reviewer; all changes appear in red within the revised table.

Issue 9: A description of Figure 2E is lacking.

We are grateful to the reviewer for drawing our attention and apologize for this error. Following the reviewer's note, we have now added a legend to Figure 2E (lines 316-320 in the revised manuscript).

Issue 10: Finally, whereas the author used their measurements to quantify the frequency of the occurrence of protrusions and retractions during cell spreading, it should be mentioned that the speed of protrusions and retractions can also be easily calculated from the slopes of the corresponding features in kymographs.

We are grateful to the reviewer for this critical comment, which has significantly improved the utility and comprehensiveness of the protocol described in our manuscript. Indeed, more than just the frequency of protrusion/retractions/ruffles could be measured by kymography of spreading or migrating cells. We have now added a section in the protocol (lines 241-267 in the revised manuscript) and a new figure (now new Figure 3) describing how to calculate additional parameters of cell-edge protrusion dynamics, namely persistence (protrusion time; the amount of time that a protrusion spends protruding before retraction), protrusion distance (distance from protrusion base to the highest peak), and protrusion/retraction velocity (protrusion distance divided by protrusion/retraction time).

Reviewer #3:

Issue 1: The technique is a very standard and straightforward one, so it is not clear how useful this visualized protocol would be compared to, for example, simply reading a text-based method description.

We understand the reviewer's concern about this issue. Indeed, the method of cell-edge protrusion dynamics measurement was developed some time ago and was used by our groups and others in several past publications (for example, Bear *et al.*, Cell 2002, PMID: 12086607; Miller *et al.*, JCB 2004, PMID: 15138293; Bryce *et al.*, Current Biology 2005, PMID: 16051170; Lapetina *et al.*, JCB 2009, PMID: 1941460; Miller *et al.*, Biochemistry 2010, PMID: 20146487; Lukic *et al.*, MBOC 2021, PMID: 34432482). However, a single well-resourced detailed, and updated description of the method has not been published within the past 22 years, neither in the materials and methods sections of these papers nor as a methods paper. Therefore, scientists that wish to learn this approach had to visit labs that use this method. The primary aim of our detailed step-by-step methods manuscript is to enable these scientists to use the technique in their laboratories. Moreover, as this is also the primary goal of JoVE, we were specifically noted by the editor that novelty is not a requirement for publication in this journal.

Issue 2: The potential utility, as well as the demonstrability of the protocol's efficacy (which is a requirement for publication), could be significantly enhanced if the authors were to show the ability of this technique to discern different cellular phenotypes (e.g., a wild-type cell versus a cell in which a particular adhesion, cytoskeletal, or regulatory component has been genetically lost or pharmacologically inhibited). The latter option might be of particular strength, as one could depict the 'before' and 'after' kymographs and statistics for an individual cell.

We appreciate the thoughtful comment of the reviewer on this topic. The main goal of our methods manuscript is to provide a step-by-step protocol for applying the cell-edge protrusion assay. Indeed, several examples of previous papers which use the method in wild type *vs.* knockout or knockdown fibroblasts are described in the discussion section of the manuscript. In addition, we have recently used the technique to elucidate the role of the non-receptor tyrosine kinase Pyk2 in protrusion dynamics and consequent cell migration (Lukic *et al.*, MBOC 2021, PMID: 34432482). A detailed paragraph describing the ability of the technique to discern different cellular phenotypes using Pyk2-WT and Pyk2-knockout cells is included in the last paragraph of the discussion section as an example of the power of the cell-edge protrusion technique to discern different cellular phenotypes (lines 392-401 in the revised manuscript).

Issue 3: The authors should explain in more detail how "the number of protrusions, retractions, and ruffles formed during 10 minutes of the movie in each of the eight regions in the cell, marked by the grid lines, is extracted and manually counted from the respective kymograph". Specifically, guidance should be supplied as to whether it is important to discern between protrusions or retractions that are short (in duration and/or distance) versus those that are more protracted. Also, it would be helpful and important to include guidance regarding how to 'threshold' (either manually/subjectively or using ImageJ) phase-dark kymograph signals that are interpreted (or rejected) as ruffles (i.e. is every dark spot along the edge considered a ruffle?).

We thank the reviewer for this constructive comment. In the protocol, we demonstrate and explain the frequency of protrusions/retractions/ruffles. For this measurement, we focus on protrusion peaks at the cell edge; namely, if one observes such a peak, it would be counted as a protrusion followed by a retraction. In this aspect, because the number is determined, it is not necessary to distinguish between short vs. long protrusions/retractions. We also explain how to determine the time, distance, and velocity of protrusions and retractions, where it could be relevant to distinguish between short and long, however in our opinion, it is not necessary to distinguish between values in this case. Such differences (i.e., two or more groups of values) do not usually occur when using knockout cells. Still, they may arise when using knockdown or transfection of cells, when not all cells express the desired construct, or differences in expression levels may occur. In these cases, the existence of a more heterogeneous population will be expressed by a more significant error bar. We usually overcome this obstacle, which occurs in many different systems when using knockdown or overexpression by transfection, to quantify only the cells that are transfected and expressing. This could be performed by adding a fluorescent tag or using a dual expressing vector that expresses fluorescence when using knockdown or overexpression and quantifying only the cells that express fluorescence.

Additionally, in our analysis, we distinguish ruffles from other structures within the cell based on their dark appearance in phase-contrast microscopy and by their centripetal movement, which starts at the cell edge and ends at the border of the cell body, which can be observed in the movies. Of note, when quantifying protrusions, retractions, and ruffles, movies should be observed as a control for quantification and to define ruffles. Based on the reviewer's comment, we have now added a more detailed explanation in the protocol section on how to recognize and define ruffles (lines 228-232 in the revised manuscript).

Issue 4: Addition of text and/or demonstration of how edge velocity (protrusion/retraction speed) can be calculated would also increase the utility and comprehensiveness of the protocol.

We are grateful to the reviewer for this critical comment, which has significantly improved the utility and comprehensiveness of the protocol described in our manuscript. Indeed, more than just the frequency of protrusion/retractions/ruffles could be measured by kymography of spreading or migrating cells. We have now added a section in the protocol (lines 234-263) and a new figure (now new Figure 3) describing how to calculate additional parameters of cell-edge protrusion dynamics, namely persistence (protrusion time; the amount of time that a protrusion spends protruding before retraction), protrusion distance (distance from protrusion base to the highest peak), and protrusion/retraction velocity (protrusion distance divided by protrusion/retraction time).

Issue 5: The authors state that "Cells that completed their spreading phase and started moving are also not appropriate for kymography analysis." This is, at best, quite misleading or, at worst, wrong. The same general approach (save for the eight symmetrical radial lines) can easily be (& has been) used to analyze leading-edge dynamics in polarized and actively migrating cells, not just cells that have been adhered and spreading for 15 minutes. Indeed, the authors begin their Introduction with the words "Cell migration..." and end it by suggesting that their "assay is an instructive model for exploring actin dynamics in the context of cell migration", so the text should be re-thought and re-written to (1) clarify the similarities and distinctions between edge dynamics during cell spreading versus those during migration and (2) broader utility of kymographic edge analysis for actual cell migration.

We appreciate the thoughtful comment of the reviewer on this topic. Although our manuscript describes measurements during cell spreading only, this method could be applied to migrating cells as well, as was performed, for example, in the original method that was modified by us (Hinz *et al*., Experimental Cell Research 1999, PMID: 10438589), or in Bear *et al.*, Cell 2002 (PMID: 12086607). These publications also demonstrated a direct correlation between cell translocation and the frequency and persistence of protrusions, but not with their velocity. Following this constructive comment of the reviewer, we have now added a note in the discussion explaining that, although we measure protrusions during spreading only in our protocol, this method could also be used for measuring protrusion dynamics in migrating cells, including the references above as examples (lines 379-381 in the revised manuscript).

Issue 6: While MEFs on fibronectin are used here, the authors should make clear that different cells and different ECMs can be used to similar effect.

We appreciate the reviewer's thoughtful note on this specific issue. Indeed, this methods manuscript and most of the previously published papers mentioned in the discussion section of our manuscript have used fibroblasts for cell-edge protrusion assay as well as for two-dimensional (2D) cell motility assay. Fibroblasts are commonly used for 2D motility assays and other related assays such as the cell-edge protrusion dynamics, as they are mesenchymal, they move fast and have clear cytoskeletal structures such as lamellipodia, filopodia, and focal adhesions. Although we have not tried to use the cell edge protrusion assay with other cell types and other ECM types in our laboratories, this could be applied. Moreover, in the first documentation of the lamella dynamics assay, which was modified by us to become the cell edge protrusion dynamics assay (Hinz *et al.*, Experimental Cell Research 1999, PMID: 10438589), the authors used keratinocytes stimulated to migrate by EGF, demonstrating that other cell types and other stimulations could be used. Following the reviewer's note on this issue, we have added this information in the discussion section (lines 374-379 in the revised manuscript).

Issue 7: The authors suggest that edge retraction is a completely contractile/myosin-driven process and do not mention the considerable importance of * depolymerization* of dendritic actin at the periphery.

We thank the reviewer for this constructive comment. Indeed, as mentioned at the end of our introduction section, edge retraction could result from a contractile myosin-driven process. Additionally, as noted by the reviewer, retraction at the cell edge could also result from depolymerization of dendritic actin. As suggested by the reviewer, we have added this note to our introduction in the paragraph that discusses retraction (line 66 in the revised manuscript).

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Title of Article: Measuring cell-edge protrusion dynamics by using live-cell microscopy.

Author(s): Nikola Lukic, Trishna Saha, Stefanie Lapetina, Gilad Lehmann, Anthony J. Koleske, Hava Gil-Henn

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CORRESPONDING AUTHOR

Name:	Hava Gil-Henn
Department:	Faculty of Medicine
Institution:	Bar-Ilan University
Title:	PhD (Principal investigator)
Signature:	Hawaffenn
Date:	July 28th, 2021

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