Cell & Systems Biology
UNIVERSITY OF TORONTO

March 26th, 2021

Dear Dr Myers,

We are hereby re-submitting our manuscript entitled "Quantitative analysis of cell edge dynamics during cell spreading" (manuscript number: JoVE62369) to JoVE. We appreciate the consideration of the paper and the reviewers' helpful suggestions and insightful criticisms. We are encouraged by the positive comments and have worked hard to address the major concerns in the revised manuscript.

One major issue raised by Reviewer #2 is regarding the quantification of protrusion speed in the kymograph analysis section. In the previous manuscript, we quantified protrusion speeds of the spreading cells by taking the slope between the first local minimum and the last data point, which means that the length of the image acquisition would affect the measurement of the protrusion speed. To overcome this problem, we incorporated a curve-fitting function in our analysis such that we can now derive the plateau phase of the protrusion, which is used to compute the average protrusion speed. With this new implementation, we found a statistical difference in the protrusion speeds of control and Arp2/3-inhibited cells.

Another major issue raised by all 3 reviewers concerns the recovery step after trypsinization. Reviewers questioned the necessity of the recovery step and the method by which we identified the duration of the recovery. We regret having not addressed these questions in the previous manuscript. In the revised manuscript, we added a figure (Figure 2) that demonstrates how the length of the recovery step changes the rate and variability in cell spreading speed. We found that cells recovering for 45 mins exhibited faster and more synchronized cell spreading than cells which were allowed to recover for shorter periods of time.

To address reviewers' suggestions, we have refined the manuscript so that it now reflects live cell spreading assays present in the literature, contains fewer over-generalized statements, and presents a more fluid, less cluttered protocol.

We are immensely grateful for the reviewers and their comments, and we hope that our responses summarized in the following pages alleviate the concerns and make the manuscript acceptable for publication in JoVE.

Sincerely,

Sergey V Plotnikov, PhD
Assistant Professor
Department of Cell & Systems Biology
University of Toronto

25 Harbord St., Toronto, ON M5S 3G5 Canada
Fax: +1 416 978-8532 • www.csb.utoronto.ca

Reviewer comments:

Reviewer #1:

1. ***"Others have used live cell spreading assays for years (MP Sheetz). It would be good to mention these and not just those endpoint assays that examine fixed cells in the introduction."***

   We are aware of a large body of papers on cell spreading and lamellipodia dynamics published by the Sheetz lab and we apologize for not citing a few of these papers in the manuscript. As the reviewer suggested, we expanded and revised the introduction to acknowledge the contribution of the Sheetz group. We also briefly described the advantages and limitations of the protocols described in those papers. The following text was added (page 2):

   "To determine the molecular mechanisms that control lamellipodia dynamics, the Sheetz group pioneered the use of quantitative analysis of live spreading cells and uncovered many fundamental properties of cell edge protrusions[11, 12, 22]. These studies have demonstrated that the live-cell spreading assay is a robust and powerful technique in the toolbox of a cell biology laboratory. Despite that, a detailed protocol and open-source computational tool for a live-cell spreading assay are currently unavailable for the cell biology community."

2. ***"What are the advantages and disadvantages of this approach vs. manual or in house analysis approaches used in the past."***

   We apologize for the vague description of the advantages and disadvantages of automated segmentation. We added the following text to the discussion section (page 15):

   "Our image processing and analysis software performs a streamlined analysis of the spreading cells, from cell segmentation to data quantification. Manual image analyses of cell spreading usually involve biased selection of a threshold value or applying an automated segmentation algorithm, which is not suited for high-throughput experiments where many images need to be analyzed. Our software is, therefore, designed to detect and segment spreading cells in an automatic fashion, in addition to quantifying protrusion dynamics and morphological descriptors. Together, these features make the described protocol amenable for large-throughput screenings of signaling pathways and molecular players that regulate lamellipodia."

3. ***"The introduction reads like the innovation is live cell protrusion assays, but isn't the innovation the specific analysis approach?"***

   We agree with the reviewer that the novelty of spreading assay was overstated in the initial submission. We modified the introduction as follows (page 2):

   "These studies have demonstrated that the live-cell spreading assay is a robust and powerful technique in the toolbox of a cell biology laboratory. Despite that, a detailed protocol and open-source computational tool for a live-cell spreading assay are currently unavailable for the cell biology community. To this end, our protocol outlines the procedures of imaging live spreading cells and provides an automated image analysis tool."

4. ***"Abstract: …the cell spreading assay or … cell spreading assays."*** *And* ***"There was an instance of using trypsin-EGTA instead of trypsin-EDTA. Was that intentional?"***

   We apologize for the typos in the manuscript. All typos were corrected in the revised manuscript.

5. ***"Perhaps both trypsin and not proteolytic cell adhesion blockers could be mentioned as there is less chance for integrin cleavage. Is there much difference between allowing cells to "rest" before spreading and using non-proteolytic approaches for detaching cells?"***

   We are very grateful to the reviewer for this suggestion. We did not mention proteolytic cell adhesion blockers in the original submission because this cell detachment technique is rarely used for strongly adherent fibroblasts. However, we agree that non-proteolytic cell detachment might be a good alternative to trypsin for the readers working with weakly-adherent cells. Thus, the revised protocol contains a note suggesting non-proteolytic cell detachment for weakly adherent cell. The following note was added to page 5 under Step 2.6:

   "**NOTE:** If applicable, trypsin can be replaced by a non-proteolytic cell adhesion blocker."

   We included an additional figure to demonstrate the importance of the recovery period for the analysis of cell spreading ***(Figure 2)****. We plated cells recovered for 0 to 45 minutes on fibronectin-coated coverslips and let them to spread for 15 minutes. Then we fixed the cells and analyze the distribution of the cell area. We found that cells recovered for longer time (up to 45 mins) exhibited dramatically lower cell-to-cell variability in cell area compared to their counterparts that did not recover after trypsinization or recovered for a short period of time. The following text was added to the Results section to describe these data (pages 11):*

   "During the recovery step, cells replenished their integrin receptors on the plasma membrane as indicated by the fast and synchronous attachment of the recovered cells to the fibronectin coated coverslips **(Figure 2)**. Without the recovery, cells spread for 15 minutes exhibited a broad distribution of cell size indicating a high variability in the onset of cell spreading **(Figure 2A and B)**."

Reviewer #2:

1. ***"However, the major scientific issue that I have is in the "protrusion speed" measurement. The authors are taking the lowest point in the kymograph in the first half of the movie and going to the terminal time point. Therefore, the length of the imaging is critical in determining their "protrusion speed", and their protrusion speed includes retractions. This is very difficult to avoid. But as you can see in Figure 4, some cells have a steady spreading rate that plateaus, and some cells edges appear to still be spreading at the end of the imaging, especially the CK666 cell. I wonder if some tweaking or addition here would more accurately measure aspects of protrusion?"***

   We thank the reviewer for pointing out this fundamental flaw in our analysis. To address this concern, we implemented a curve fitting module that detects the rapid expansion and the plateau phases of the protrusion and excludes the latter phase when computing the average

*protrusion speed **(Supplemental Figure 1)**. Using this modified analysis, we revealed a statistically significant difference in the cell protrusion speed between control and CK-666 treated cells. Therefore, we modified the Results section as follows (page 13):*

"The analysis of cell edge speed revealed a moderate but significant decrease in the average protrusion speed of Arp2/3-inhibited cells compared to control (Control: 37.1 ± 12.87 nm/s vs. CK-666: 28.7 ± 13.4 nm/s, p = 0. 9 x 10^{-3}) **(Figure 5C)**."

2. ***"Line 89: The authors are using PH-Akt-GFP. They might inform the reader why they are using this probe, and what other reporters would also be suitable. Would a diffuse fluorophore work or do we need cortical or membrane fluorophore? Perhaps a number of Addgene #s to guide the user to some appropriate examples would also be useful."***

We agree with the reviewer that membrane targeted fluorophore is not required for the described assay. Soluble GFP or any other fluorescent marker evenly distributed in the cytoplasm will work as well as PH-Akt-GFP. Our rationale to perform these experiments on MEFs with genetically integrated PH-Akt-GFP was (i) homogeneous cell population with similar expression of the fluorescent marker in every cell, and (ii) even distribution of the fluorophore in the cytoplasm with barely noticeable enrichment at the protruding cell edge. In preliminary experiments, we also tested fluorescent markers localized on the plasma membrane. We found the fluorescent proteins (eGFP and mCherry) tagged with CAAX domain tend to aggregate in the cytoplasm or retain in the membrane compartments, creating extremely bright spots in the perinuclear area of cells. Since our segmentation algorithm is optimized for images with bimodal distribution of pixel intensities (i.e. background and foreground pixels), such bright spots creating another mode on the intensity histogram decrease the accuracy of cell segmentation. Thus, we do not recommend using CAAX-eGFP and CAAX-mCherry in the cell spreading assay. The following note was included in the Protocol section to address this reviewer's concern (page 3):

"**NOTE:** The described cell spreading protocol was performed using mouse embryonic fibroblasts (MEFs) expressing PH-Akt-GFP (a fluorescent marker for PIP_3/$PI(3,4)P_2$). This cell line was generated by genomically integrating an expression construct for PH-Akt-GFP (Addgene #21218) by CRISPR-mediated gene editing. However, other fluorescent markers that are expressed transiently or integrated in the genome can also be used in this assay. For optimal image segmentation, we recommend using fluorescent markers that are evenly distributed in the cytoplasm, *e.g.,* cytosolic GFP."

3. ***"Can the authors provide a sample data set? I think many users would find this useful to get started. I would also like to test out the code on my system."***

We greatly appreciate reviewer's interest in our software and the suggestion to provide a sample dataset. It is a great idea that we did not think about! We added two sample datasets to a publicly accessible cloud storage at: https://www.dropbox.com/sh/dik21h1fnbaqhlg/AAB314RZT7AF6o6mLadLimYaa?dl=0. We provide link to the sample dataset in the README file in the Github repository.

4. ***"Line 113 section 1.9: When we want individual cells plated without clumping we filter them through a 100 um filter, commonly used in FACS facilities. This might be helpful for some readers."***

   As the Reviewer suggested, we added the following to the Protocol section (page 3):

   Step 1.8: "Pipette 1 mL of the trypsinized cells into the 15 mL centrifuge tube in order to dilute the cells. Pipette the contents of the tube up and down to ensure an even distribution of cells within the media. For cell types with high aggregation propensity, filtering cells through a cell strainer (100 µm mesh size) is recommended to minimize the occurrence of cell clumping."

5. ***"Line 219 section 4.2: Is it possible to state what resolution might be the minimum to enable the kymographs? This might help orient some readers that want to use lower NA air objectives for high throughput."***

   We tested movies with different pixel size generated by binning our existing images and resolved protrusions and quantified their dynamics even when the pixel size was as large as 0.5mm. So, we believe that 20x objective would be sufficient for the cell spreading assay and should definitely be considered for high-throughput experiments. However, since most low magnification objectives have relatively low numerical aperture, such objective might be unable to provide desired image quality (signal-to-noise ratio) and/or result in excessive photobleaching of the cells. In our experiments, 60X/1.4NA objective combined with the large field of view spinning disk scanner (Crest V2) and 25mm sCMOS camera (Prime 95b) was an optimal choice allowing us to image 2-4 spreading cells per field of view with high frame rate and moderate photobleaching. We modified the protocol to reflect these considerations (page 6):

   "**NOTE**: We use a 60X, 1.4 N.A. oil immersion objective in this protocol because of its reasonably large field of view and outstanding light collection efficiency. If a larger field of view is required, a lower magnification objective can be used as long as the signal-to-noise ratio of the images is greater than 2.5.

6. ***"Line 242 section 4.8: How did the authors come to the 6 second sampling frequency? In my experience, most cells are going to have a different spreading rate. I think instead of specifying here, the authors might guide the readers what they might do to determine the optimal sampling frequency, and advise them that the authors use 6 seconds as the starting point. For example, if someone wanted to use this protocol for immune cells spreading on activated coverslips, they'll need to image much faster I think. Those cells are crazy fast spreaders. Stupid fast. Crazy."***

   We thank the reviewer for this suggestion. We agree that different cell types exhibit very different spreading speed, and this point should be addressed in the protocol. Therefore, we added the following note to section 4.7 of the Protocol (page 6 - 7):

   "**NOTE:** Due to the high variability of lamellipodia protrusion velocity among different cell types, the optimal frame rate should be determined empirically. The imaging interval of 6 seconds used in our experiments is a good starting point for the analysis of many

mesenchymal and epithelial cells. However, cells that spread very quickly (*e.g.*, immune cells) may require a much higher frame rate (shorter imaging interval). The optimal frame rate for cell spreading movies ensures a 2-5 pixels displacement of the protruding cell edge between subsequent frames. Considering the accuracy of curve fitting used to identify the plateau of cell spreading, the optimal frame rate should also ensure 50-100 measurements of cell edge displacement during the rapid expansion phase of cell spreading. The number of fields of view should be adjusted depending on the exposure time, the distance between acquisition points, and the stage movement speed. Users are advised to determine the maximum number of fields of view that can acquired with the desired frame rate."

7. ***"Line 263 section 5.1: I think stating what the goal here is would help users expedite the process. "Images need to be in tiff format and the pixel size in microns is needed. You can export your images from your acquisition software as tiff or use the following steps in FIJI. If your files are already in tiff format then skip to step XX.***

   As the reviewer suggested, we added the following text to the protocol to help users expedite the process (page 7):

   "**NOTE:** The software requires an image in .tiff format and a pixel size as the input parameters. Both requirements can be fulfilled using the acquisition software or Fiji (in this protocol). If these requirements are fulfilled, proceed to step 5.2."

8. ***"Is there a reason the functions can't be in a single python file? The GUI build is great. However, there seems to be some points where the authors might want to change components in the code but not built into the GUI. Perhaps the authors could look through and make sure these points are carefully annotated for what they might change and why?"***
   The python script we developed is modular, composed of the GUI, the segmentation algorithm, the data analysis module etc. In our opinion, such structure of the script makes any further edits and modifications easier, so we would like to keep it. We apologize for not including annotations (or comments) in the initial submission. We provide the revised scripts with docstrings and comments as supplemental files for this manuscript. We also uploaded these files to the Github repository.

Reviewer #3:

1. ***"In the intro and throughout the manuscript, the authors seem to make the point that lamellipodia are essential for cell spreading or constitute the structural organelle to mediate specific spreading. This is actually a misleading statement and thus cannot stand as the authors put it at present! Indeed, the authors themselves show that inhibition by CK666 of Arp2/3 complex (which is considered to be essential for the protrusion of lamellipodia) has only very modest effects on spreading efficiency, at least if considering these very short time points (up to 15 minutes), which the authors claim are the only relevant ones for spreading. The authors also fail to refer in this context to a study they are even citing (ref 10), in which it is shown very clearly that cells lacking lamellipodia (due to genetic removal of Rac1 GTPase) can still spread efficiently. I see the authors' point that the latter study looked at spreading with much longer time points (and thus may have missed***

subtle differences up to the 15 minute-time point, for instance). However, spreading was by no means completed in these conditions and cell type after 15 minutes in the experiments described in ref 10, and thus the data used in the present and aforementioned study cannot be directly compared! Indeed, most studies published on spreading efficiency so far look at much longer time periods than the authors here, so to claim that everything is finished (plateauing by 15 minutes), is certainly misleading and too generalized."

We apologize for the confusion about the role of lamellipodia in cell spreading. We certainly do not think lamellipodia are required for cell spreading, nor cell migration and we agree with the reviewer that the correct interpretation of cell spreading assay results might (and often does) require additional experiments, such as immunostaining for known lamellipodia and filopodia markers. However, description of these techniques and experimental design for studying lamellipodia dynamics is certainly outside the scope of this protocol.

We are aware that many published protocols of cell spreading assay were performed on a much longer timescale. The timescale of cell spreading assay described here was determined empirically but is also supported by a large body of published studies. Cai et al., 2006 have showed previously that the average time for MEF cells to reach to their fully spread stage on fibronectin is ~16 min. Similarly, in our hands, MEFs reached a plateau phase within first 15 mins of cell spreading. Several other studies published by the Sheetz group (Dubin-Thaler.Sheetz.2008., Wolfenson.Sheetz.2014) have also reported that isotropic expansion of spreading cells detected by a rapid (≈5mm/min) protrusion of the cell edge occurs within the first 15 minutes after plating. Following this rapid expansion, cells polarize and downregulate their protrusive activity. The decrease in protrusion speed during the late stages of cell spreading was previously attributed to an increase in membrane tension (Raucher.Sheetz.2000, Gauthier.Sheetz.2011, Wolfenson.Sheetz.2014). Since membrane tension is an important, but indirect, regulator of actin dynamic at the cell leading edge and lamellipodia protrusions, we restricted our analysis by the first 15 min of cell spreading when the endogenous activity of actin polymerization machinery is mostly unaffected by external factors.

2. ***"In the intro (lines 59/60), the authors word that "Compared to the highly variable lamellipodia of migrating cells, the lamellipodia of spreading cells are much more uniform and stereotypical..." This will definitely depend on cell type and conditions, so overgeneralized statements of this kind should be omitted (think of the efficiently migrating keratocytes using highly uniform lamellipodia or hemocytes in Drosohila perhaps, so oversimplified statements of this kind are certainly not helpful)."***

   We agree with the reviewer that the morphology and dynamics of protruding cell edge vary greatly among different cell types. But we would argue that fish keratocytes and Drosophila hemocytes are rather rare exceptions in the multitude of cell types with highly variable lamellipodia. We are certain that for a generic mammalian cell variability of lamellipodia protrusions is much higher for migrating rather than for spreading cells.

3. ***"The authors also make a huge point about trypsin sort of digesting off extracellular portions of transmembrane proteins, such as integrins, for instance, and thus recommend to force cells to stay in suspension for a quite long period of time (at least 45 minutes as far***

as I understand) before seeding. The authors should be informed that such a treatment is not useful for various cell types, and might lead to exaggerated cell-to-cell sticking etc, so cannot be recommended as general rule for spreading assays in all cell types and conditions! I do admit, though, that it is possible that the rapid plateau in cell spreading (after roughly 15 minutes) observed by the authors here might derive from the fact that keeping the cells in suspension before seeding allows them to recover perhaps from potential adverse effects of trypsin. However, this is sole speculation at present, and if this is a realistic possibility, it should be discussed and experimentally tested in fact! In other words, it would be much more convincing if the authors actually tested whether the plateau is reached later if this forced time in suspension is omitted."

We appreciate the reviewer's concern for the necessity of the recovery step after trypsinization. Also, we are aware that a prolong maintenance of cells in suspension is deadly for non-transformed adherent cells. To address this concern and inform users on the biological effects of cell of this consideration, we included the following (page 4):

"**NOTE:** The duration of recovery time may vary for different cell types. Although in our experiments 45-minute-long recovery had a negligible effect on cell viability, some cell types may undergo anoikis when maintained in suspension for too long. Therefore, we recommend determining the optimal recovery time empirically. The optimal recovery time enables fast and synchronous cell spreading with no dead or apoptotic cells in the sample."

*Furthermore, we included an additional figure to demonstrate the importance of the recovery period for the analysis of cell spreading **(Figure 2)**. We plated cells recovered for 0 to 45 minutes on fibronectin-coated coverslips and let them to spread for 15 minutes. Then we fixed the cells and analyze the distribution of the cell area. We found that cells recovered for longer time (up to 45 mins) exhibited dramatically lower cell-to-cell variability in cell area compared to their counterparts that did not recover after trypsinization or recovered for a short period of time. The following text was added to the Results section to describe these data (pages 11-12):*

"During the recovery step, cells replenished their integrin receptors on the plasma membrane as indicated by the fast and synchronous attachment of the recovered cells to the fibronectin coated coverslips **(Figure 2)**. Without the recovery, cells spread for 15 minutes exhibited a broad distribution of cell size indicating a high variability in the onset of cell spreading **(Figure 2A and B)**."

Finally, we added a note suggesting non-proteolytic cell detachment for the readers working with weakly-adherent cells. Thus, the revised protocol contains a note suggesting non-proteolytic cell detachment for weakly adherent cell. The following note was added to page 5 under Step 2.6:

"**NOTE:** If applicable, trypsin can be replaced by a non-proteolytic cell adhesion blocker."

4. ***"The authors employ a 60x oil immersion objective, and propose to select 4 independent cells (fields of viee, meaning very low throughput) for filming cells during the spreading process. Indeed, using an oil immersion objective is limiting the field of view to a very small***

area on the coverslip, as the oil will not easily spread over long distances on the bottom coverslip surface. So I wonder why the authors don't use high NA air objectives, which may lead to much better results, although the reason perhaps includes the fact that air objectives are usually much less light-sensitive, and may thus be of limited use for fluorescence as used here. This then actually brings me back to the point that the necessity of the method to require fluorescence is actually a real limitation here - aside from the points already mentioned in this context above."

We tested movies with different pixel size generated by binning our existing images and resolved protrusions and quantified their dynamics even when the pixel size was as large as 0.5mm. So, we believe that 20x objective would be sufficient for the cell spreading assay and should definitely be considered for high-throughput experiments. However, since most low magnification objectives have relatively low numerical aperture, such objective might be unable to provide desired image quality (signal-to-noise ratio) and/or result in excessive photobleaching of the cells. In our experiments, 60X/1.4NA objective combined with the large field of view spinning disk scanner (Crest V2) and 25mm sCMOS camera (Prime 95b) was an optimal choice allowing us to image 2-4 spreading cells per field of view with high frame rate and moderate photobleaching. We modified the protocol to reflect these considerations (page 6):

"**NOTE**: We use a 60X, 1.4 N.A. oil immersion objective in this protocol because of its reasonably large field of view and outstanding light collection efficiency. If a larger field of view is required, a lower magnification objective (*e.g.*, 20x) can be used as long as the signal-to-noise ratio of the images is greater than 2.5."

5. ***"In the discussion (line 563), the authors state that the employed "…image processing routine reduces bias in data analysis", which is certainly not justified, because I wonder why and in which way would there be less bias using this method as compared to simply fixing cells at given time points and staining them to assess spreading efficiency...? Such misleading statements should definitely be avoided, as they will confuse the reader!"***

We apologize for such an ambiguous statement. We clearly have no data to prove such statement. Therefore, we modified the discussion in page 15 as follows:

*"*Our image processing and analysis software allows for a streamlined analysis of the spreading cells, from cell segmentation to data quantification. Manual image analyses of spreading cells often involve manually determining a global threshold value or applying an automated segmentation algorithm in an image analysis software, such as ImageJ, and then measure different aspects of cell spreading. While such manual workflow is applicable to low throughput experiments, it is not suited to higher throughput experiments where a large number of images need to be analyzed. Our software is, therefore, designed to detect and segment spreading cells automatedly and to quantify the morphological descriptors and protrusion dynamics. Together, these features make the described protocol amenable for large-throughput screenings of signaling pathways and molecular players that regulate lamellipodia.*"*

6. ***"In lines 610/611 of the discussion, the authors state that "…it is also important to highlight that this live cell spreading protocol is a robust and powerful tool to study the dynamics of lamellipodia". Again, this statement is entirely misleading if assuming that spreading can also occur efficiently in the absence of lamellipodia. In other words, the authors must consider that lamellipodium protrusion and cell spreading can definitely be functionally separated, as shown by others in the field previously. So in conclusion, spreading certainly constitutes an interesting parameter to look at, but if readers would like to study lamellipodia dynamics, they should be advised to study cells and conditions forming lamellipodia in a robust and reproducible fashion, and not just spreading, which may or may not involve lamellipodium protrusion!"***

   We agree with the reviewer that lamellipodial protrusion and cell spreading can be functionally separated as seen in our experimental data. However, we don't think that these data prevent us from using cell spreading as a simple model to study lamellipodia protrusion. as long as we understand the limitations of this assay. In fact, a substantial body of published work have shown that lamellipodia formed during migration and cell spreading are structurally identical (Gauthier.Sheetz.2011, Wolfenson.Sheetz.2014, Cai.Sheetz.2006, Giannone.Sheetz.2004, Raucher.Sheetz.2000). These lamellipodia also consist of the same molecular species (e.g., actin, focal adhesion components, myosin, etc.) and exhibit very similar pattern of protrusive activity.