STAR Protocols
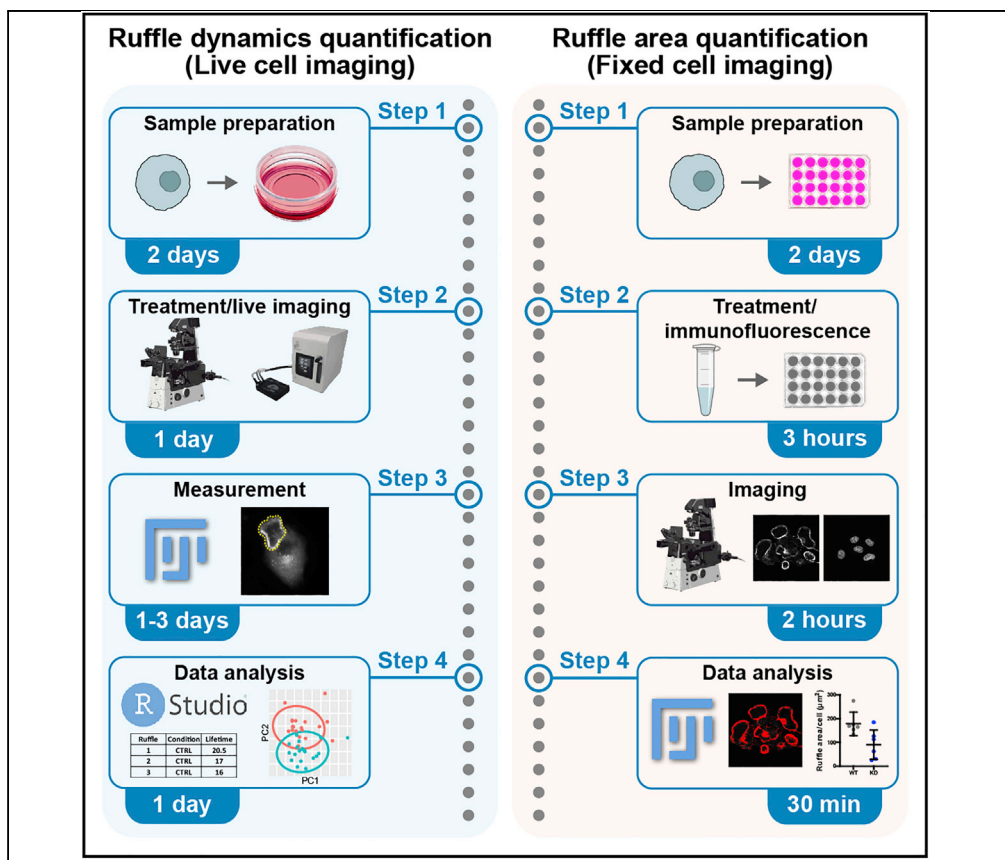
Protocol

Quantification of ruffle area and dynamics in live or fixed lung adenocarcinoma cells

Ruffle dynamics quantification (Live cell imaging)	Ruffle area quantification (Fixed cell imaging)
Step 1 — Sample preparation — 2 days	Step 1 — Sample preparation — 2 days
Step 2 — Treatment/live imaging — 1 day	Step 2 — Treatment/immunofluorescence — 3 hours
Step 3 — Measurement — 1-3 days	Step 3 — Imaging — 2 hours
Step 4 — Data analysis — 1 day	Step 4 — Data analysis — 30 min

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Highlights

Induction of actin-rich ruffles in lung adenocarcinoma cells by growth factors

Analysis of ruffle dynamics in live cells

Quantification of ruffle area in fixed samples

Ruffles are actin-rich membrane protrusions implicated in actin reorganization and initiation of cell motility. Here, we describe methods for measuring and analyzing ruffle dynamics in live cells and average ruffle area per cell in fixed samples. The specific steps described are for the analysis of A549 lung adenocarcinoma cells, but the protocol can be applied to other cell types. The protocol has applications for dissecting the signaling events linked to ruffling.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Quantification of ruffle area and dynamics in live or fixed lung adenocarcinoma cells

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SUMMARY

Ruffles are actin-rich membrane protrusions implicated in actin reorganization and initiation of cell motility. Here, we describe methods for measuring and analyzing ruffle dynamics in live cells and average ruffle area per cell in fixed samples. The specific steps described are for the analysis of A549 lung adenocarcinoma cells, but the protocol can be applied to other cell types. The protocol has applications for dissecting the signaling events linked to ruffling.
For complete details on the use and execution of this protocol, please refer to Cooke et al. (2021).

BEFORE YOU BEGIN

In before you begin steps 1–18, and step-by-step method details 1–15, we describe the protocol for analyzing ruffles in live lung adenocarcinoma cells treated with growth factors, such as EGF (Cooke et al., 2021). However, the basic principles can be adapted to other cell types or even other structures, as well as to different treatments. For example, this protocol was inspired by similar methods used to analyze focal adhesion dynamics (Berginski et al., 2011). Since this protocol utilizes quantitative microscopy, best practices for reproducibility in imaging must be considered (Lee and Kitaoka, 2018; Montero Llopis et al., 2021; Sticker et al., 2020).

We also describe in before you begin steps 19–42, and step-by-step method details 16–22, a method for analyzing average ruffle area per cell in fixed samples using rhodamine-phalloidin as a marker for F-actin. The rearrangement of the actin cytoskeleton during ruffle formation in lung adenocarcinoma cells can be measured by thresholding for only the signal in the ruffles. Thresholding is an easy to implement method to quantify large rearrangements of the actin cytoskeleton. While this protocol focuses on circular ruffles, it can also be used to quantify other types of ruffles, such as edge ruffles and membrane ruffles, that can be segmented based on intensity (Figures 1A–1C). The method described in this section could be used to analyze changes in the F-actin staining intensity distribution within any cell type and in response to various treatments.

Live cell imaging: Initial preparation

⏱ Timing: 9 h
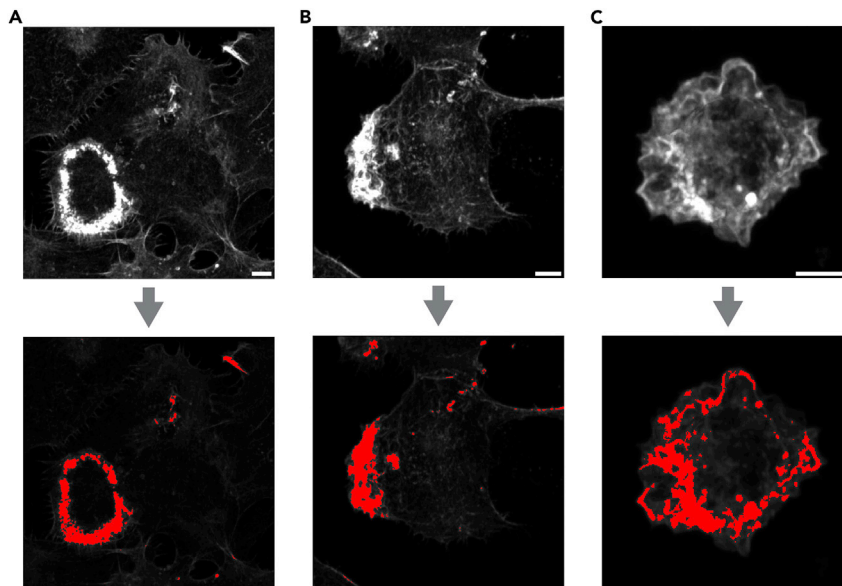
Figure 1. Types of ruffles that can be quantified using protocol 2

Representative micrographs of rhodamine-phalloidin stained cells forming different types of ruffles. Increased actin density within ruffles can be segmented by thresholding as represented by the red pixels.

(A) H1299 cells treated with 500 ng/mL Gas6, a strong inducer of ruffle formation, forming a circular ruffle. Scale bar: 5 μm.

(B) H1299 cells treated with 500 ng/mL Gas6 forming an edge ruffle. Scale bar: 5 μm.

(C) U937 macrophage treated with 200 nM PMA for 24 h forming membrane ruffles. Scale bar: 5 μm.

1. Culture A549 cells in RPMI media containing 10% fetal bovine serum (FBS) and 1× Penicillin-Streptomycin.

2. Split A549 cells into glass-bottom 35 mm dish at the desired concentration and let cells attach for 8 h.

   Note: We have found that A549 lung adenocarcinoma cells require 8 h to fully attach. This should be tested for every cell line used as some cell lines attach much faster and others slower. Alternatively, the cells can be allowed to attach overnight for 16–24 h, however, this will add an additional day to the protocol.

   Note: We do not use any extra-cellular matrix coating for this protocol. However, it should be noted that the substrate stiffness on which the cells are grown will likely impact the dynamics of circular ruffles (Zeng et al., 2011).

3. Wash cells twice with 25°C 1× DPBS and replace media with 37°C serum-free media (same as growth media but with the 10% FBS omitted).

4. Infect cells with mCherry-Cortactin adenovirus as a marker for ruffles (Goicoechea et al., 2017).

   Note: This protocol is dedicated to the analysis of single cells. However, it would also be possible to adapt this protocol to the analysis of ruffles in polarized epithelial cells (Abella et al., 2010).

   Note: We use mCherry-Cortactin adenovirus as our marker of ruffles in live cells. However, any marker for ruffles could be used such as Lifeact for actin or CAAX for the cell membrane. Variations of these ruffle markers can all be purchased from Addgene (Cortactin-pmCherryC1, #27676; pLifeAct_mScarlet_N1, #85054; mCherry-CaaX(Hras), #108886). The

ruffle marker can be expressed using any standard transfection method if adenovirus is not an option.

Note: Care should be taken to use the minimum amount of adenovirus (or transfected DNA) that still provides enough signal for imaging. This is to minimize the effects of over-expression. This can be determined by infecting cells with serial dilutions of the virus to determine the minimum amount of virus that can still achieve a signal-to-noise ratio of 1:10 using the imaging parameters as described in before you begin step 14. Additionally, the impact of the ruffle marker on ruffle formation can be determined by using the assay in the fixed cell imaging section to compare the ruffle formation of infected cells to uninfected cells. This method can also be followed for transfection to determine the optimal amount of DNA to transfect.

5. Incubate the cells in the 37°C serum-free media for 16–24 h.

Live cell imaging: Preparation on the day of imaging

⏱ Timing: 1–2 h

6. Startup the microscope and stage-top incubator so that the temperature of the equipment can stabilize.

Note: Usually, at least 1 h is required for proper warming with longer times being better if possible (we use a Tokai Hit STX stage top incubator: top heater 42°C; stage heater 38°C; bath heater 37°C; lens heater 37°C; sample temp 37°C; feedback ON).

7. Wash cells twice with 25°C 1× DPBS and replace media with 900 μL of 37°C serum-free media without phenol-red.
8. Add 1:100 Oxyfluor reagent and 10 mM dl-lactate to reduce oxygen free radicals and minimize photodamage.

Note: Oxygen scavenging is optional but recommended when imaging for under 1–2 h with high acquisition frequency. For longer experiments with less frequent acquisition it is best to omit this step. The effectiveness of the oxygen scavenging can be improved by covering the dish. However, we skip this step to allow for the drug-delivery tubing (before you begin step 12).

9. Prepare 150–200 μL of 1 mg/mL EGF diluted in the imaging media in a 1.5 mL Eppendorf tube.

Live cell imaging: Image acquisition

⏱ Timing: 2–8 h

10. Use a high numerical aperture 60× oil objective.
11. Place cells in the stage-top incubator.
12. Feed drug delivery tubing into the chamber making sure that the tubing is aligned at the side of the well. This minimizes the risk of disturbing the cells during injection.
13. Allow the temperature to stabilize for 30 min.

Note: We use a temperature feedback thermocouple included with the Tokai Hit STX stage top incubators which decreases the time required for sample temperature stabilization to 5–10 min.

14. During temperature stabilization, the acquisition parameters can be set up.

    a. 15–30 s intervals for 30–60 min will generally be a good starting point.

Note: The intervals and length of acquisition required will vary based on cell type and experimental setup. Generally, the imaging should be frequent enough to have at least several frames for the shorter-lived ruffles and the imaging should be long enough to not stop short before the longer-lived ruffles disassemble.

    b. Since ruffles are comparatively large structures, 2 × 2 binning is recommended as it will allow for faster imaging and less light intensity.
    c. Minimize light intensity to prevent damage to the cells and/or photobleaching while maintaining an exposure time of 800 msec or less and a signal to noise ratio greater than 10:1.
    d. The number of fields of view should be maximized within the constraints of the acquisition interval and exposure time to get the most out of each plate of cells.

Note: Shorter exposures times will increase the number of fields of view that can be acquired. A good starting value is 500 msec. Exposure time can be decreased to allow for more data collection if the light intensity required to achieve a good signal to noise ratio is not damaging the cells.

15. Start the acquisition and confirm that the system is maintaining focus and not drifting.
16. Acquire images for 5–10 frames then, using a syringe, inject 100 μL of 1 mg/mL EGF slowly through the drug delivery tubing so as not to disturb the cells or the system. Record the frame that the EGF was injected.
17. After imaging, discard the cells and save the time-series in the microscopes systems proprietary file format or as TIFFs with metadata.
18. Repeats steps 11–17 with additional samples as required, maintaining the same acquisition settings within and between conditions.

Fixed cell imaging: Cell preparation

    ⏲ Timing: 2 days

19. Gently insert a 22 mm diameter round coverslip into each well of a 12-well plate.
20. Add 10,000 A549 cells per well in 1 mL of culture 37°C media with 10% serum and 1× antibiotics.

Note: For optimal post-image processing, cells should not be in contact with each other. The number of cells may need to be adjusted for different cell lines.

21. Make sure cells are homogenously distributed and avoid air bubble formation.
22. Incubate plate at 37°C for 24 h.
23. Wash cells twice with 25°C 1× DPBS and replace media with 37°C serum-free media.
24. Incubate the cells in serum-free media for 24 h.

Fixed cell imaging: Growth factor treatment and immunofluorescence

    ⏲ Timing: 3 h

25. Aspirate media and replace with 37°C serum-free media containing 100 ng/mL EGF for 5 min.
26. For fixation, aspirate the media and add 100 μL of 4% formaldehyde solution to each well.
27. Incubate the plate for 30 min at 25°C.
28. Wash three times with 1 mL of 1× DPBS and leave on the final wash.

⫿⫿ **Pause point:** At this stage the coverslips can be stored at 4°C for a maximum of 72 h.

29. For permeabilization, remove the 1× DPBS and add 1 mL of 0.1% Triton 100-X in PBS to each well. Place the plate in a benchtop shaker for 15 min at 25°C.
30. Wash three times with 1× DPBS.

   Note: Prepare blocking solution (3% BSA in 1× DPBS) during permeabilization and store at 4°C.

31. For blocking, add 1 mL of pre-cooled blocking solution to each well and place the plate in a benchtop shaker for 30 min at 25°C.
32. Wash three times with 1× DPBS.

   Note: Prepare the fluorescence phalloidin staining solution by diluting the stock rhodamine-phalloidin solution 1/10,000 in blocking solution. Protect from light.

33. For actin staining, add 250 μL of fluorescence phalloidin staining solution to each well. Protect from light.
34. Cover the plate with aluminum foil and place in a benchtop shaker for 60 min at 25°C.
35. Wash three times with 1× DPBS.

   Note: Prepare the DNA staining solution by diluting the stock DAPI solution 1/10,000 in blocking solution. Protect from light.

36. For DNA staining, add 250 μL of DAPI solution to each well.
37. Cover plate with aluminum foil and place in a benchtop shaker 10 min at 25°C.
38. Wash three times with 1× DPBS.
39. For mounting the slides:
   a. Add 1 drop of mounting solution per coverslip to glass slides.
   b. Gently remove each coverslip from the 12-well plate and invert on the drop of mounting solution.

   Note: Be aware that the cell surface must face toward the drop of mounting solution.

   c. Store the slides in dry conditions for 16 h. Protect from light.

Fixed cell imaging: Image acquisition

⏱ **Timing: 2 h**

40. Visualize the DAPI and phalloidin staining by fluorescence microscopy making sure to use appropriate filters (DAPI and TRITC have excitation/emission peaks of 360/460 nm and 540/565 nm respectively).
41. Image the nuclei and actin channels in at least 5 random fields for each condition while avoiding cells overlapping with the edge of the field of view to simplify analysis.

   ⚠ CRITICAL: For this analysis to work, it is essential to maintain the same acquisition settings within and between conditions so that that pixel intensity values are comparable.

42. Save the images in the microscopes systems proprietary file format or as TIFFs with metadata.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Sodium DL-lactate	Sigma-Aldrich	L1375-100ML
EGF	R&D	P01133
Fluoromount-G™ Mounting Medium	Invitrogen	50-187-88
Formaldehyde solution 4%, buffered, pH 6.9	Sigma-Aldrich	1004960700
Gas6	R&D	NP_00081
Oxyfluor	Oxyrase Inc.	OF-0005
phorbol 12-myristate 13-acetate (PMA)	LC Laboratories	P-1680
Rhodamine Phalloidin	Invitrogen	R415
4′,6-Diamidino-2-phenylindole (DAPI)	Thermo Scientific	62248
Experimental models: Cell lines		
A549	ATCC	CCL-185
H1299	ATCC	CRL-5803
U-937	ATCC	CRL-1593.2
Recombinant DNA		
mCherry-Cortactin adenovirus	Garcia-Mata's lab	Goicoechea et al. (2017)
Software and algorithms		
FIJI/ImageJ	Available from NIH	https://imagej.net/software/fiji/
RStudio version 1.4.1106	RStudio	https://www.rstudio.com
Prism 9	GraphPad Software	https://www.graphpad.com
Other		
Drug delivery tubing	Tokai Hit Co Ltd	KTSF
STX stage top incubator	Tokai Hit Co Ltd	tokai-hit-stxg
Tissue culture 12-well plate	TPP	92012
Single frosted slides, clipped corners 75×25 mm	VVR	10118-596
Cover glasses, round 22 mm	VVR	48382-063
DPBS 10×	Sigma-Aldrich	11666789001
Triton™ X-100	Sigma-Aldrich	X100
Bovine Serum Albumin	Sigma-Aldrich	A9418
Nikon Ti2 Microscope	Nikon	ECLIPSE Ti2-E
Stage top incubator	Tokai Hit	TOKAI-HIT-STXF
ORCA-Flash4.0 V3 Digital CMOS camera	Hamamatsu	C13440-20CU
SPECTRA X Light Engine	Lumencor	SPECTRA X
NIS-Elements Advanced Research	Nikon	Version 5.11.03

MATERIALS AND EQUIPMENT

The system used for the is protocol is as follows: a Nikon Eclipse Ti2 microscope equipped with a Tokai Hit STX stage top incubator, 60× NA1.49 oil immersion objective, Hamamatsu ORCA-Flash 4.0 camera, Lumencor SPECTRA X solid-state light source, and NIS-Elements software. The system requirements to follow this protocol are highly flexible. Any high-resolution fluorescent microscope system capable of live-cell imaging will likely be appropriate. For live imaging, a widefield system is optimal to image ruffles quickly with low light intensity, although a confocal system can also be used.

STEP-BY-STEP METHOD DETAILS

For quantification of live cell ruffles, follow steps 1–15; for quantification of fixed cell ruffles, follow steps 16–22.

Live cell quantification: Initial image processing (optional)

🕐 Timing: 5–15 min

Here we outline the steps for initial processing of the data collected from before you begin steps 1–18. Initial image processing is performed in FIJI/ImageJ (see key resources table for download link). While image processing is not always necessary for analysis, it can make analysis easier and is highly recommended for display purposes. The following steps can be adapted as necessary as some may be unnecessary based on the quality of the image and/or the system used.

1. Drift/jitter correction: Often during live imaging there can be problems with drift if the system is not very stable (temperature or stage/dish stability).
   a. To correct drift, run the FIJI/ImageJ plugin: Plugins ⟩ Registration ⟩ Correct 3D drift.
   b. After drift/jitter correction, crop out the black bars around the stack by drawing a rectangular ROI and running Image ⟩ Crop.

   Note: Jittering in the movies can occur during multi-point image acquisition, especially if the imaging regions are far apart. Both problems can be corrected using the Correct 3D drift plugin. The default settings work well in most scenarios, however, if processing speed is slow and/or the results are unsatisfactory, draw a rectangular region of interest (ROI) around a relatively unmoving part of the time series (e.g., a non-migratory cell) to calculate the stabilization on the contents of the ROI.

2. Background subtraction: The following background subtraction script can be run using the FIJI/ImageJ macro tool: Plugins ⟩ New ⟩ Macro.

```
waitForUser("Draw an ROI to indicate the background, then press OK");

for (i=1; i<=nSlices; i++) {

    setSlice(i);

    getStatistics(area, mean);

    run("Select None");

    run("Subtract...", "value="+mean);

    run("Restore Selection");

    }
run("Select None");
```

   Note: Subtracting the background of a time-series is often critical for distinguishing the signal from the background noise and is necessary before proper bleach correction.

3. Bleach correction: After background subtraction, bleach correction can then be used as necessary: Image ⟩ Adjust ⟩ Bleach Correction.

   Note: The Bleach Correction plugin provides three methods: Simple Ratio, Exponential Fit, and Histogram Matching. Exponential Fit is the typically preferred method; however, results can vary depending on the experimental setup.

   ⚠ CRITICAL: Because bleach correction is changing the values of your data, care must be taken to preserve its meaning. It is usually necessary to crop the time series that is to be analyzed before bleach correction to eliminate any debris or cells moving in and/or out of the field of view. If drift correction is required, it is almost always necessary to use at least some cropping before bleach correction (see step 1).
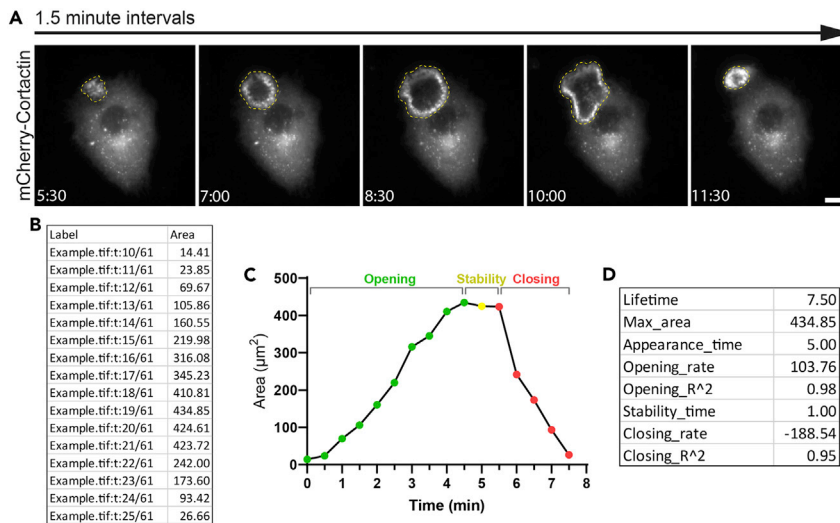
Figure 2. Example of ruffle and quantification
(A) Ruffle area can be measured manually in FIJI/ImageJ using the freehand selection tool. Scale bar: 10 μm.
(B) Example area measurements of a single ruffle.
(C) Plot showing area measurements over time with the opening, stability, and closing phases colored green, yellow, and red respectively.
(D) Example calculations for the dynamics of a single ruffle.

4. Save the processed images as a separate file from the original data using the TIFF file format to preserve the original data and the quality of the processed data.

Live cell quantification: Measurement of ruffles

⏱ Timing: 1+ h

Here we outline the method for measuring the data collected from before you begin steps 1–18. While the actual measurement of ruffles is very simple, due to the difficulty in automating the process it can be time consuming. A large amount of information about ruffles, or many biological structures for that matter, can be derived by measuring the size of the structures over time. In the instance of ruffles, the simplest method is measuring the area. Here we outline a workflow for analysis using FIJI/ ImageJ and a spreadsheet software (only basic functionality is required so any spreadsheet software will be appropriate, including free and open-source options).

5. Open FIJI/ImageJ.
6. Drag an image set into FIJI such as the preprocessed data from the previous section and identify a cell forming or disassembling a ruffle within the duration of the time-series.
7. Set-up the measurements in FIJI: Analyze ⟩ Set Measurements...
   a. Select "Area" and "Display label".
   b. Use an appropriate number of decimal places.
8. Go to Analyze ⟩ Set Scale... to check if the pixel to distance conversion scale was imported. If not, the conversion can be entered.
9. Select the Freehand selections tool in FIJI.
10. For each frame of the ruffle, draw an ROI around the ruffle (Figure 2A) and then press Ctrl + M or M to run the Measure plugin (also Analyze ⟩ Measure).
11. Copy the data from the Results table into a spreadsheet (Figure 2B).

Table 1. Example of raw data from ruffle dynamics experiment

Ruffle	Condition	Lifetime (min)	Max area (μm^2)	Time of appearance (min)	Opening rate (μm^2/min)	Closing rate (μm^2/min)	Stability (min)
1	CTRL	20.5	129.6606	6.5	20.948	-17.9616	6.5
2	CTRL	17	735.7603	4.5	180.32	-143.108	1
3	CTRL	16	165.8078	9.5	41.284	-40.464	0.5
4	CTRL	14.5	135.8572	8	70.852	-14.9148	0
5	CTRL	14	179.4686	6.5	27.4556	-76.252	1
6	CTRL	13.5	210.3111	2	42.632	-56.496	2
7	CTRL	13	139.8944	5	74.804	-35.8556	4
8	CTRL	12	244.1581	2.5	59.432	-65.996	2.5
9	CTRL	12	142.5233	2.5	35.5412	-23.5316	0
10	CTRL	11.5	381.4236	3	93.664	-93.78	0
11	CTRL	11.5	209.56	9	44.56	-61.108	1.5
12	CTRL	10.5	284.5772	13.5	173.32	-349.08	6.5
13	CTRL	10.5	153.3206	18	49.584	-49.492	0
14	CTRL	9.5	327.1558	12	203.308	-153.508	3
15	CTRL	9.5	219.9817	1.5	101.828	-110.896	1.5
16	CTRL	9.5	144.0725	4	55.624	-68.22	4.5
17	CTRL	8.5	122.525	16	77.628	-171.44	5
18	CTRL	8.5	255.3778	1	146.844	-40.504	0
19	CTRL	8	187.8717	3.5	106.524	-64.544	0
20	CTRL	7.5	387.01	16.5	183.5	-315.864	0
21	KD	8.5	166.2772	10.5	58.688	-90.864	1
22	KD	8	363.8664	12	121.98	-250.564	0
23	KD	8	117.455	8	31.0208	-139.424	4.5
24	KD	7.5	159.5642	16.5	45.192	-160.476	0
25	KD	6.5	114.4036	20	60.596	-141.208	3
26	KD	6	478.3169	18.5	351.988	-277.212	0
27	KD	6	223.6903	20.5	144.644	-191.648	0
28	KD	5.5	83.51417	14	92.82	-128.16	2.5
29	KD	5.5	103.3247	0	35.0956	-46.548	0
30	KD	5	174.8211	21	123.144	-205.956	1
31	KD	5	90.32111	11	40.108	-66.844	0
32	KD	4.5	111.9156	5.5	52.916	-198.2	1
33	KD	3.5	172.38	18	144.308	-136.816	0
34	KD	3.5	153.4614	1.5	50.964	-115.144	0
35	KD	3.5	46.14639	14	28.2604	-24.3924	0.5
36	KD	3	123.2761	5	71.544	-117.36	0.5
37	KD	3	70.69833	25	83.092	-97.456	0.5
38	KD	3	47.32	3	23.0968	-63.376	0.5
39	KD	3	47.22611	20	52.392	-44.408	0
40	KD	2.5	69.10222	12.5	72.952	-111.26	0

Live cell quantification: Processing data

⏱ Timing: 1+ h

Once the ruffle area data has been collected as described in the previous section, it can then be processed to gain information on the dynamics of the ruffle formation. Data management is key to successful analysis, and Table 1 can be referred to for an example of formatting. For processing the data, we use a combination of basic spreadsheet functionality and the R programing language through the RStudio Integrated Development Environment (IDE). Here, we will describe the data processing steps using spreadsheet software; however, experienced users of RStudio can accelerate this process by performing the calculations in RStudio.

12. The lifetime, maximum area, and time of appearance variables are relatively self-explanatory and are easily acquired from the measurements made in the previous section ("Measurement of ruffles").

    a. Calculate lifetime by taking the total number of frames the ruffle present and converting the value to time (e.g., when imaging every 30 s, a ruffle present for 15 frames will have a lifetime of 7.5 min).

    b. Maximum area is simply the largest area value for each ruffle, regardless of time point.

    c. Calculate time of appearance from the first row of the "Label" column of the ruffle area data (for example, t:10/61 in Figure 2B indicates that the ruffle appeared in frame 10).

    Note: Make sure to convert the value from frames to time and account for any frames acquired before growth factor treatment. Time 0 is the frame at which the growth factor was added.

13. The opening and closing rates are calculated by linear regression analysis of the linear portions of the ruffle opening and closing.

    a. Opening and closing rate are both calculated using the same principles. The opening and closing phases are defined as the linear portions of values starting from the first and last time-points respectively (Figure 2C).

    b. To find the opening phase, determine the approximate region by eye and then calculate the R^2 value for points before and after the approximate point. The end of the opening phase will be represented by a drop-off in the R^2 value as the ruffle reaches maturity and the area stops increasing.

    c. Once the opening phase is determined using R^2 values, the slope of the resulting trend line is the rate of opening (Figure 2D).

    d. Calculate closing rate in the same way but instead start from the final value and work backward.

    Note: It may be necessary to define a minimum number of frames acceptable for an opening or closing phase as well as a minimum R^2 value. While this will exclude some data, the resulting measurements will likely be more accurate as it will exclude ruffles that do not have sufficient temporal resolution or have too much variability to calculate an accurate rate. For example, a starting criterion could be a minimum of 4 frames for the opening and closing phases with an R^2 value of 0.7 or greater.

14. The stability phase is defined as the time range between the final frame of the opening phase and the first frame of the closing phase (see Figure 2C).

15. For each measurement, enter the value in a spreadsheet using clean data principles (see Table 1 for example) and saved as a file in CSV format.

    Note: For experienced users of R, or other programing languages, these data processing steps can be automated.

Fixed cell quantification: Analysis of ruffle area

⏱ Timing: 15–30 min

After collecting the images of actin and nuclei staining as outlined in before you begin steps 19–42, the cells can be analyzed to quantify the average ruffle area/cell. Ruffles are characterized by a prominent increase in actin density. This allows for unbiased quantification of ruffles by means of a basic threshold which is set to include ruffles but exclude other actin structures such as stress fibers. While thresholding may occasionally separate other structures besides ruffles, the effect is generally insignificant.
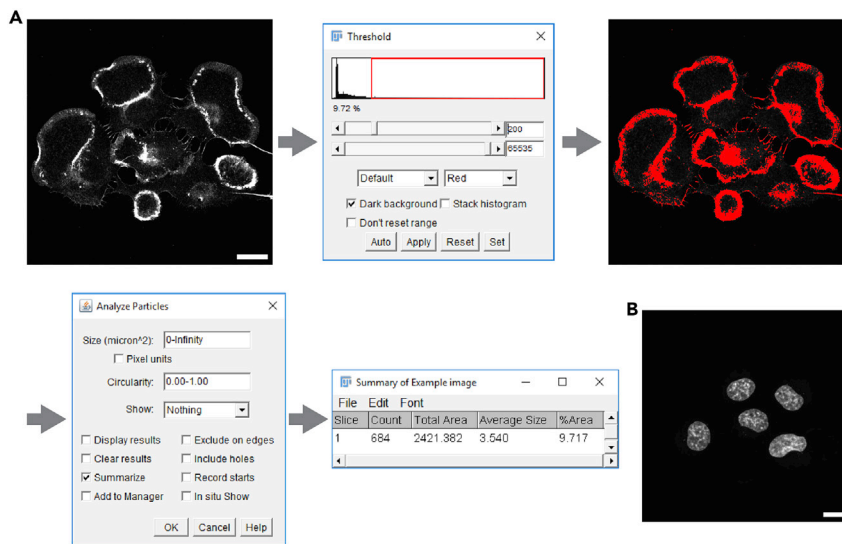
Figure 3. Example analysis of ruffle area
(A) Workflow of image analysis used to calculate area covered by ruffles. Scale bar: 20 μm.
(B) Image of nuclei used to count the number of cells. Scale bar: 20 μm.

16. Open each image individually by dragging it into FIJI.
17. Set-up the measurements in FIJI: Analyze ⟩ Set Measurements...
    a. Select "Area" and "Display label".
    b. Use an appropriate number of decimal places.
18. Go to Analyze ⟩ Set Scale... to check if the pixel to distance conversion scale was imported. If not, the conversion can be entered.
19. Run Image ⟩ Adjust ⟩ Threshold... (or Ctrl + Shift + T).
    a. Make sure "Dark background" is selected.
    b. For the first image analyzed, adjust the top slider to set the threshold for only the ruffles and make note of the threshold values (Figure 3A).
    c. For subsequent images, click "Set" and manually enter the threshold values used in the previous step.

Optional: If images are noisy, use of a median filter (Process ⟩ Filters ⟩ Median...) before thresholding can increase the accuracy of the analysis by reducing the noise while preserving edges.

20. Run Analyze ⟩ Analyze Particles...
    a. Set the size range of particles. Usually the default setting of 0-Infinity is appropriate unless there is noise that needs to be excluded based on size. Keep this value consistent across conditions.
    b. Select "Summarize" and leave the other options unselected.
    c. Click "OK".
    d. If analyzing a multichannel image, select "No" when prompted to process all images.
21. From the resulting Summary window, copy the "Total Area" value into a spreadsheet along with the number of cells (Figure 3B) for the image as determined by the number of nuclei.

Note: The units of the total area value will be in microns^2 if the scale conversion was set in step 18 or imported with the image metadata. Otherwise, the area will be in pixels.

22. In the spreadsheet software:
    a. Sum all total area values and cell number values for each condition.
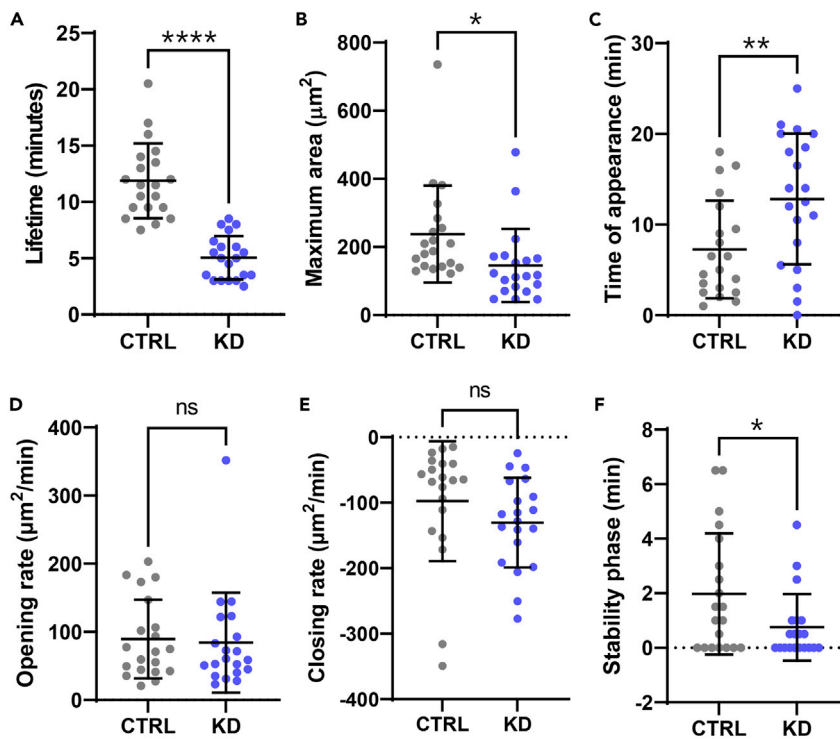
Figure 4. Individual plots of ruffle dynamics data
(A–F) Plots of the ruffle data from Table 1 comparing CTRL and KD conditions. Data are represented as mean ± SD. ns = not significant; *p < 0.05; **p < 0.01; ****p < 0.0001.

    b. Divide the summed ruffle area by the summed number of cells to calculate the average ruffle area/cell for each condition.

EXPECTED OUTCOMES

After processing the ruffle data from the live imaging analysis, you should have a data set like in the example Table 1. The results can be displayed in a simple bar-plot style graph like in Figures 4A–4F. Here, we can see that the conditions have differences in lifetime, maximum area, time of appearance, and the stability phase but not the opening or closing rates. From this we can conclude that the KD cells have smaller, shorter-lived ruffles that are slower to appear after EGF treatment. These differences can be summarized with a principal component analysis (PCA). Figure 5C shows that the CTRL and KD conditions form two distinct populations of ruffles.

After following the method and analysis described for the fixed ruffle samples, the results can be displayed as shown in Figure 6. In this example, H1299 cells were treated with Gas6 in CTRL and KD conditions. These results show that there is a reduction in average ruffle area per cell in the KD cells as compared to the CTRL cells.

QUANTIFICATION AND STATISTICAL ANALYSIS

We have included a sample dataset in Table 1 that can be used to reproduce the analysis described for live cell imaging. The table can be copied into a spreadsheet and saved as a CSV file to reproduce the results shown in Figures 4 and 5.

Since the data acquired from the ruffle analysis has many dimensions, it can be difficult to visualize the differences between conditions using only a series of bar plots. PCA is a straightforward and
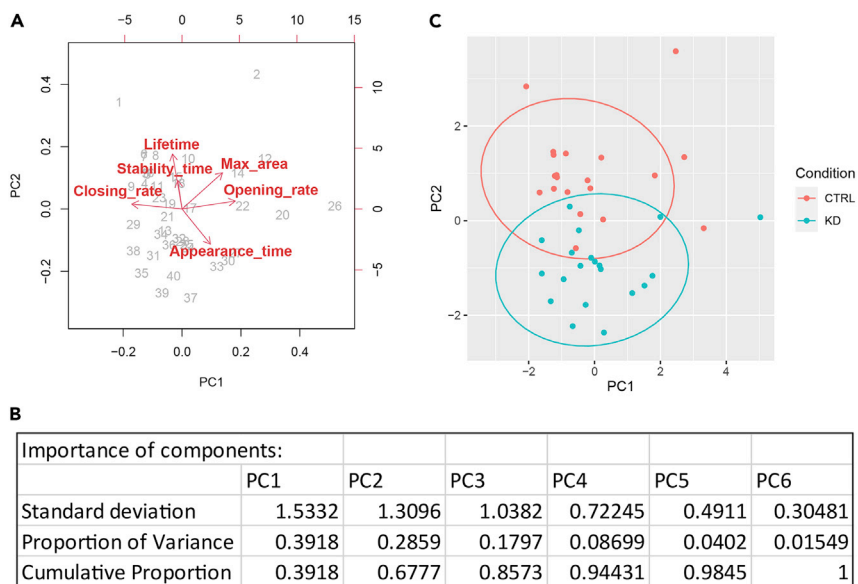
Figure 5. Principal component analysis of ruffle dynamics data

(A) PCA biplot showing the individual ruffles as numbers and the variables as vectors indicating the direction of positive correlation.

(B) Summary of the importance of each of the principal components.

(C) The proportion shows how much of the variance in the data is accounted for by each principal component with PC1 and PC2 accounting for 68% of the variance. Plot of the first two principal components with CTRL ruffles in red and KD ruffles in blue. Ellipses represent 95% confidence.

relatively easy method by which one can visualize many variables of a data set in fewer dimensions (Jolliffe, 2002; Jolliffe and Cadima, 2016). The following code can be used to perform a principal component analysis in RStudio on the results from the data analysis. The results shown in Figures 5A–5C were acquired using the example data from Table 1 which the readers can use to test the analysis for themselves.
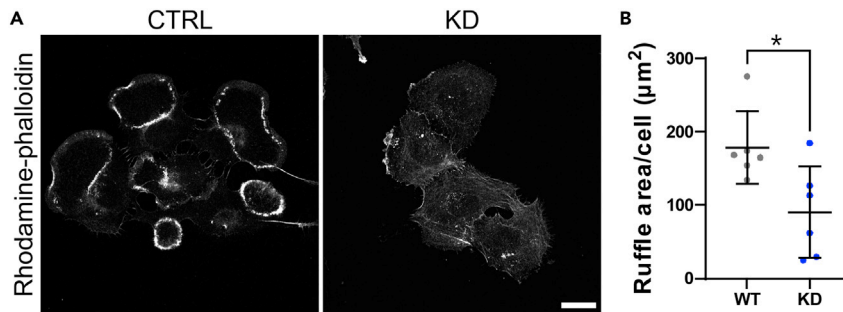
```
# Load libraries

library(ggplot2)

# Load data

df <- read.csv(file.choose())

# Principal component analysis, summary, and biplot

pca <- prcomp(df[, 3:8], scale = TRUE)

summary(pca)

biplot(pca)

# Add PCA scores to the data frame

df_pca <- cbind(df, pca$x[, 1:2])

# Plot data with ggplot2

ggplot(df_pca, aes(PC1, PC2, col = Condition)) +

  stat_ellipse() +

  geom_point()
```

Figure 6. Images and plot of ruffle area analysis

(A) Representative micrographs of rhodamine-phalloidin stained H1299 cells treated with 500 ng/mL Gas6, a strong inducer of ruffle formation. Scale bar: 20 μm.

(B) Example plot of ruffle area analysis with each point representing an analyzed image. Data are represented as mean ± SD. *p < 0.05.

The plots in Figures 4A–4F and 6B were generated with Prism 9. The statistical analysis was performed in Prism 9 with crossbars and error bars representing mean and standard deviation respectively. The Student's t-test was used to determine significance.

LIMITATIONS

The major limitation of for the live cell ruffle analysis is the requirement for manual measurement of ruffle area. This prevents the adaption of this protocol to high throughput conditions. Once the measurements are made, the rest of the analysis can be automated (Berginski et al., 2011). Future development of an automated methodology for measuring ruffle size will allow this protocol to be adapted to high-throughput scenarios. An automated method would facilitate comparison of receptor ligands (e.g., different growth factors) or test drugs that block ruffle formation, which could be conceptually useful for diseases involving deregulated cell migration.

The fixed sample analysis is limited in its ability to distinguish ruffles from other signals such as prominent stress fibers, cell debris, and dividing cells. It may be possible to reduce these effects by using a more sophisticated segmentation approached. However, unless the cells being analyzed have a very modest ruffle phenotype, this limitation will likely not be a problem.

TROUBLESHOOTING

Problem 1

No or little ruffle formation (before you begin, steps 16–18).

Potential solution

This problem can arise from several issues. One is that the cells were insufficiently washed before starvation and that the time of starvation was not long enough. This can be resolved by increasing washes or time of starvation. This problem can also arise from mishandling of the cells, i.e., from overgrowing/high confluency and/or excessive trypsinization.

Problem 2

Cells dying during imaging (before you begin, steps 15–18).

Potential solution

Solutions include decreasing laser/light power and compensating with exposure time as necessary, increasing intervals between imaging, using 2 × 2 binning, and/or using Oxyfluor or similar product to scavenge oxygen free radicals. Also, make sure that the fluorophore used is matched appropriately with the light source and/or filter sets used.

Problem 3

Difficulty achieving a good threshold value between images and conditions (step-by-step method details, step 19).

Potential solution

Make sure that the images acquisition settings are consistent between images. Expose cells to the minimum possible amount of light before imaging to prevent photobleaching which will cause differences in signal strength between images. If the problem is the result of uneven sample illumination, it may be necessary to correct the signal via flat-field correction by dividing the images by an averaged background image (no cells).

Problem 4

Difficulty segmenting the ruffles from other cell structures such as actin stress fibers (step-by-step method details, step 19).

Potential solution

If the ruffles being segmented are not very prominent, segmentation can become more difficult. It may be possible to solve this problem by using another marker for ruffles, such as cortactin, that does not stain stress fibers. If that still is not sufficient, multiple ruffle markers can be used for the same sample and the thresholded images can be masked (in ImageJ: click apply on the Threshold window after step 19) and multiplied together (in ImageJ: Process ⟩ Image Calculator…) to create a binary mask that only includes overlapping signal between the different ruffle markers. Apply a new threshold to the masked image and continue with step 20.

Problem 5

Low transfection efficiency (before you begin, step 4).

Potential solution

If using transfection for the ruffle marker, make sure that the transfection reagent used is suitable for the cell type. Also, use highly purified and contaminant free DNA, check the ratio between the DNA and transfection reagent, and use actively dividing cells ≥80% confluent.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Rafael Garcia-Mata (rafael.garciamata@utoledo.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The published article includes all data and code generated or analyzed during this study.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

AUTHOR CONTRIBUTIONS

Conceptualization, G.K.-L., M.C., S.M.G., R.G.M., and M.G.K. Investigation, G.K.-L., M.C., and S.M.G. Methodology, G.K.-L., M.C., and S.M.G. Formal analysis, G.K.-L., M.C., R.G.-M., and

M.G.K. Writing, G.K.-L., M.C., S.M.G., R.G.-M., and M.G.K. Visualization, G.K.-L. and M.C. Resources, R.G.-M. and M.G.K. Funding acquisition, R.G.-M. and M.G.K. Supervision, R.G.-M. and M.G.K. Project administration, G.K.-L., M.C., R.G.-M., and M.G.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Abella, J.V., Parachoniak, C.A., Sangwan, V., and Park, M. (2010). Dorsal ruffle microdomains potentiate Met receptor tyrosine kinase signaling and down-regulation. J. Biol. Chem. *285*, 24956–24967. https://doi.org/10.1074/jbc.M110.127985.

Berginski, M.E., Vitriol, E.A., Hahn, K.M., and Gomez, S.M. (2011). High-resolution quantification of focal adhesion spatiotemporal dynamics in living cells. PLoS One 6, e22025. https://doi.org/10.1371/journal.pone.0022025.

Cooke, M., Kreider-Letterman, G., Baker, M.J., Zhang, S., Sullivan, N.T., Eruslanov, E., Abba, M.C., Goicoechea, S.M., García-Mata, R., and Kazanietz, M.G. (2021). FARP1, ARHGEF39, and TIAM2 are essential receptor tyrosine kinase effectors for Rac1-dependent cell motility in human lung adenocarcinoma. Cell Rep. *37*, 109905. https://doi.org/10.1016/j.celrep.2021.109905.

Goicoechea, S.M., Zinn, A., Awadia, S.S., Snyder, K., and Garcia-Mata, R. (2017). A RhoG-mediated signaling pathway that modulates invadopodia dynamics in breast cancer cells. J. Cell Sci. *130*, 1064–1077. https://doi.org/10.1242/jcs.195552.

Jolliffe, I.T. (2002). Principal Component Analysis, 2nd Edition (Springer).

Jolliffe, I.T., and Cadima, J. (2016). Principal component analysis: a review and recent developments. Philos. Trans. R. Soc. A Math. Phys. Eng. Sci. *374*, 20150202. https://doi.org/10.1098/rsta.2015.0202.

Lee, J.Y., and Kitaoka, M. (2018). A beginner's guide to rigor and reproducibility in fluorescence imaging experiments. Mol. Biol. Cell *29*, 1519–1525. https://doi.org/10.1091/mbc.E17-05-0276.

Montero Llopis, P., Senft, R.A., Ross-Elliott, T.J., Stephansky, R., Keeley, D.P., Koshar, P., Marqués, G., Gao, Y.-S., Carlson, B.R., Pengo, T., et al. (2021). Best practices and tools for reporting reproducible fluorescence microscopy methods. Nat. Methods *18*, 1463–1476. https://doi.org/10.1038/s41592-021-01156-w.

Sticker, M., Elsässer, R., Neumann, M., and Wolff, H. (2020). How to get better fluorescence images with your widefield microscope: a methodology review. Microsc. Today *28*, 36–43. https://doi.org/10.1017/s155192952000156x.

Zeng, Y., Lai, T., Koh, C.G., LeDuc, P.R., and Chiam, K.H. (2011). Investigating circular dorsal ruffles through varying substrate stiffness and mathematical modeling. Biophys. J. *101*, 2122–2130. https://doi.org/10.1016/j.bpj.2011.09.047.