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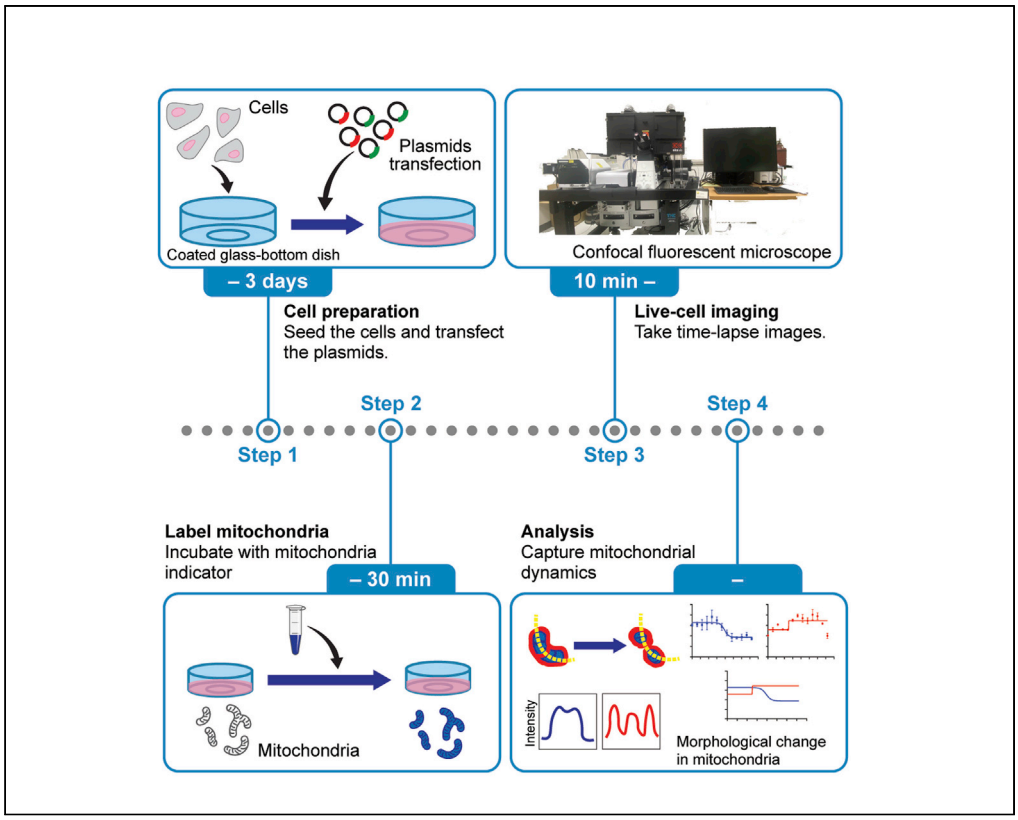
Live-cell imaging and analysis of actin-mediated mitochondrial fission



Current approaches, such as fixed-cell imaging or single-snapshot imaging, are insufficient to capture cytoskeleton-mediated mitochondrial fission. Here, we present a protocol to capture actin-mediated mitochondrial fission using high-resolution time-lapse imaging. We describe steps starting from cell preparation and mitochondria labeling through to live-cell imaging and final analysis. This approach is also applicable for analysis of multiple cytoskeleton-mediated organelle events such as vesicle trafficking, membrane fusion, and endocytic events in live cells.

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

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Highlights

Real-time imaging of actin organization around mitochondria causing fission

Localize actin attractant GJA1-20k which identifies the specific point of fission

High-resolution time-lapse imaging and processing of organelle dynamics

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Live-cell imaging and analysis of actin-mediated mitochondrial fission

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SUMMARY

Current approaches, such as fixed-cell imaging or single-snapshot imaging, are insufficient to capture cytoskeleton-mediated mitochondrial fission. Here, we present a protocol to capture actin-mediated mitochondrial fission using high-resolution time-lapse imaging. We describe steps starting from cell preparation and mitochondria labeling through to live-cell imaging and final analysis. This approach is also applicable for analysis of multiple cytoskeleton-mediated organelle events such as vesicle trafficking, membrane fusion, and endocytic events in live cells.

For complete details on the use and execution of this protocol, please refer to Shimura et al. (2021).[1]

BEFORE YOU BEGIN

Generally, dynamin-related protein 1 (DRP1) is known as a major mitochondrial fission mediator.[2] In addition to DRP1, the actin cytoskeleton has been shown to play an important role in mitochondrial fission events.[3,4] Actin can assemble around mitochondria[5,6] and finalize fission by recruiting and interacting with DRP1.[7–9] Inhibiting actin organization results in disruption of mitochondrial fission.[10,11] We recently identified that the small peptide named GJA1-20k, introduced below, is also a mitochondrial fission mediator that organizes the actin cytoskeleton around mitochondria and at scission points to achieve this function.[1] GJA1-20k activity for mitochondrial fission is either downstream of, or independent of, DRP1.

The protocol below describes the specific steps and workflow for analysis of actin and mitochondrial dynamics using cell lines. We focus on HEK293 cells and have also used this protocol in HeLa cells with the same procedures as HEK293 cells. The protocol focuses on mitochondrial fission induced by GJA1-20k, which is a N-terminus truncated isoform of Connexin 43 formed by internal translation.[12,13] We have recently identified that GJA1-20k localizes around mitochondria inducing protective mitochondrial fission.[1,14] GJA1-20k recruits actin to mitochondrial membrane, to effect the fission event. To observe how GJA1-20k and actin cooperate and induce mitochondrial fission, time-lapse live-cell imaging is performed with high frequency acquisition of the dynamic events which capture in real time the interaction of fluorescently labeled mitochondria, actin, and GJA1-20k. GFP-tagged GJA1-20k and LifeAct-mCherry (labeling actin) are transfected into HEK293 cells. Mitochondria are labeled with Mitotracker dye. Although actin-associated mitochondria can be seen even under basal conditions,[6] the phenomenon of actin surrounding mitochondria is greatly enhanced, as is the occurrence of fission, in the presence of GJA1-20k.[1] Moreover, to prevent the involvement of other mitochondrial regulators such as DRP1, we have knocked-down DRP1 or introduced its dominant negative form (K38A) and then analyzed mitochondrial dynamics. Of note, DRP1-mediated mitochondrial fission can also be observed using a GFP-tagged DRP1 plasmid as described previously.[15]

Prepare the culture and imaging medium

Timing: 30 min (prior to initiating experiments)

1. Remove and store for other uses 61 mL of DMEM from a 500 mL DMEM container.
2. Add 50 mL of FBS, 5 mL of amino acids, 5 mL of sodium pyruvate, and 1 mL of MycoZap to the 500 mL DMEM container.
3. Mix well and store at 4°C (Pre-warm in 37°C water bath before use in culture below).

Note: Please refer to materials and equipment for the recipe.

Coat the glass-bottom dish

Timing: 2 h at 37°C or 16 h at 4°C (prior to cell seeding)

4. Make gelatin/fibronectin solution by following recipe in the materials and equipment section.
5. Add 2 mL of the gelatin/fibronectin solution to cover the glass section of glass-bottom dishes.
6. Incubate for 2 h at 37°C or 16 h at 4°C.

Prepare the plasmid transfection solution

Timing: 30 min (immediately before transfection)

7. Warm up OptiMEM at 37°C in water bath.
8. Make the plasmid solution for transfection by following the recipe in materials and equipment section.
9. Incubate the plasmid solution for 10 min at 20°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific	Cat# 11965-092 Cat# 21063-029
Fetal bovine serum (FBS)	Thermo Fisher Scientific	Cat# 26140-079
MEM non-essential amino acids	Thermo Fisher Scientific	Cat# 11140-050
Sodium pyruvate	Thermo Fisher Scientific	Cat# 11360-070
MycoZap™ Plus-CL	Lonza	Cat# VZA-2012
Dulbecco's phosphate buffered saline (PBS)	Thermo Fisher Scientific	Cat# 14190-144
Opti-MEM™ I Reduced Serum Medium	Thermo Fisher Scientific	Cat# 31985-062
FuGene HD	Promega	Cat# E2312
Lipofectamine RNAiMAX	Thermo Fisher Scientific	Cat# 13778150
Mitotracker™ Deep Red FM	Thermo Fisher Scientific	Cat# m22426
Gelatin solution	Sigma-Aldrich	Cat# G1393
Human fibronectin	Corning	Cat# 356008
Experimental models: Cell lines		
HEK293FT cell line	Thermo Fisher Scientific	Cat# R70007
Recombinant DNA		
Plasmid: GFP-tagged GJA1-20k	N/A	Fu et al.[16]
Plasmid: LifeAct-mCherry	Addgene	Cat# 40908
siRNA for Drp1 knock-down	Thermo Fisher Scientific	ID: 19561
siRNA control (stealth RNA)	Thermo Fisher Scientific	Cat# 12935300

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
NIS-Elements	Nikon Instruments Inc.	https://www.microscope.healthcare.nikon.com/products/software/nis-elements
ImageJ Fiji	Johannes Schindelin, Ignacio Arganda-Carreras, Albert Cardona, Mark Longair, Benjamin Schmid, and others	https://imagej.net/software/fiji/downloads
GraphPad Prism 9	GraphPad	https://www.graphpad.com
Other		
35 mm glass-bottom culture dish	MatTek Corporation	Cat# P35G-1.0-14-c
Water bath	Precision	Model# 2864
Microscope	Nikon	Eclipse Ti2 (Inverted)
Microscope incubator for gas and temperature control	Okolab	https://www.oko-lab.com/
Objective	Nikon	100×/1.49 Apo TIRF
Spinning disk confocal unit with 486, 561, and 647 nm diode-pumped solid-state lasers (DPSSL)	Yokogawa	CSU-W1 SoRa
CMOS Disk camera	Photometrics	Prime BSI

MATERIALS AND EQUIPMENT

Culture and Imaging Medium		
Reagent	Final concentration	Amount
Dulbecco's Modified Eagle Medium (without phenol red for imaging) (1×)	N/A	439 mL
MEM Non-Essential Amino Acids (100×)	1×	5 mL
Sodium Pyruvate (100 mM)	1 mM	5 mL
FBS	10%	50 mL
MycoZap™ (500×)	1×	1 mL
Total	**N/A**	**500 mL**

Note: Use phenol red free medium during imaging. Keep refrigerated at 4°C for up to 3 months. Pre-warm in water bath at 37°C before use.

Gelatin/Fibronectin solution (For 5 × 35 mm glass-bottom dish)		
Reagent	Final concentration	Amount
2% Gelatin solution	0.1%	0.5 mL
Human Fibronectin (1 mg/mL)	20 μg/mL	0.2 mL
MycoZap™ (500×)	1×	0.02 mL
PBS	N/A	9.28 mL
Total	**N/A**	**10 mL**

Note: Make it at the day of use. Use 2 mL per dish. Adjust the volume as following dish types and the number of samples.

Plasmid solution for transfection per 35 mm glass-bottom dish		
Reagent	Final concentration	Amount
Plasmid (GFP-tagged GJA1-20k)	0.5 μg/dish	N/A
Plasmid (LifeAct-mCherry)	1.0 μg/dish	N/A
FuGene HD	1:3 ratio (total plasmids μg : FuGene μL)	4.5 μL (for 1.5 μL of total plasmids)
OptiMEM	N/A	Bring up volume to 125 μL
Total	**N/A**	**125 μL per dish**

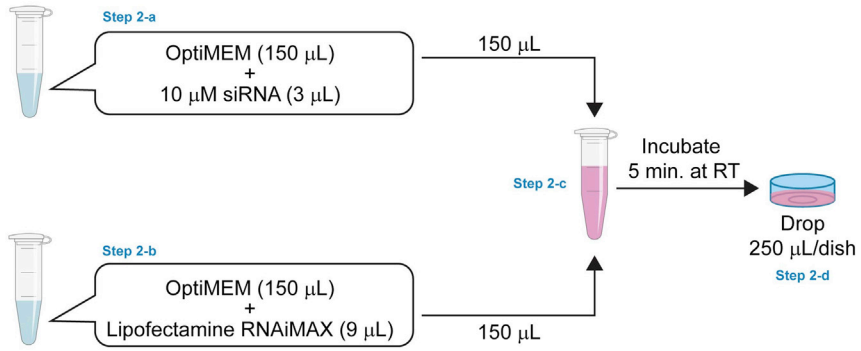


Figure 1. Schematic steps for siRNA preparation for one 35 mm glass-bottom dish

Note: Make Plasmid Solution on the day of use. Adjust the volume as following dish types and the number of samples.

Alternatives:

- FuGene HD (Promega, Cat# E2312) can be replaced by an equivalent product for plasmid transfection such as Lipofectamine™ 2000 (Thermo Fisher Scientific, Cat# 11668019).[16]
- Mitotracker™ Deep Red FM (Thermo Fisher Scientific, Cat# m22426) can be replaced by any other fluorescent types or other indicators of mitochondria that can be visualized by live microscopy.
- MycoZap™ (Lonza, Cat# VZA-2012) can be replaced by an equivalent product for antibiotics.
- Imaging medium can be replaced by PBS containing 10% FBS and 1× MycoZap™, with the same conditions as regular cell culture recommended during the imaging procedure.
- LjfeAct-mCherry can be replaced by any other indicator as long as applicable for live cell such as CellLight actin or CellMask Actin (Thermo Fisher Scientific).
- Similar to Mitotracker and LifeAct, any types of tag for GJA1-20k will be applicable. Based on multiple published studies, the tag will not affect the function of GJA1-20k and its cellular distribution.[1,12,17–19]

STEP-BY-STEP METHOD DETAILS

Prepare the cells for imaging

Timing: 3 days

This step describes the cell preparation for imaging. Different from regular cell culture, glass-bottom dishes are required to allow subsequent high-resolution imaging on an inverted microscope. In addition, a gelatin/fibronectin coating on the glass supports cell spreading allowing cells to be flatter, which improves the imaging of individual organelles and associated cytoskeleton. The transfection protocol follows that of our previous study.[1]

1. Cell seeding onto 35 mm glass-bottom dish with gelatin/fibronectin coat:
   a. Remove gelatin/fibronectin solution (see before you begin) and rinse the dish with 1.5 mL of PBS 3 times.
   b. Seed the cells with 2.0 × 10^5 cells/dish in 2 mL of total medium volume (approximately 20%–30% confluency).
   c. Incubate/Allow growth of the cells at 37°C, 5% CO_2 for 16 h.
2. DRP1 knock-down by siRNA transfection following manufacturer instructions (Figure 1):
   a. Dilute siRNA by mixing 3 μL of siRNA (10 μM in stock) and 150 μL of OptiMEM for each 35 mm glass-bottom dish.

b. Dilute Lipofectamine RNAiMAX by mixing 9 μL of Lipofectamine RNAiMAX and 150 μL of OptiMEM for each 35 mm glass-bottom dish.
c. Mix 150 μL of each diluted solution prepared by steps 2-a and 2-b and incubate 5 min at 20°C.
d. Add 250 μL of the mixture prepared in step 2-c above to the cells incubated on 35 mm glass-bottom dish, and gently swirl for 2 s to mix solutions.
e. Incubate at 37°C, 5% CO_2 for 16 h.

Note: It is required to scale the total amount of solution to number of samples (dishes). Final amount of siRNA will be 25 pmol per 35 mm dish. Knock-down efficiency should be confirmed by Western blot as described in our previous study.[1]

3. Transfection of plasmids (GFP-GJA1-20K and LifeAct-mCherry):
   a. Add 125 μL of the plasmid solution (see before you begin) per dish with incubated cells.
   b. Gently swirl for 2 s.
   c. Incubate at 37°C, 5% CO_2 for 16 h.

Preparation for live-cell imaging (mitochondria labeling)

Timing: 30 min

This step describes mitochondrial labeling prior to imaging fission events. This step follows that of our previous study.[1]

4. Warm up the culture media and imaging media (see before you begin) at 37°C in water bath.
5. Dilute Mitotracker (1 mM of stock) to final concentration of 200 nM with warmed culture media (required 2 mL final volume per dish).
6. Remove culture media from 35 mm glass-bottom dish and add the new media with Mitotracker.
7. Incubate at 37°C and 5% CO_2 for 20 min. Troubleshooting 1.
8. Gently, wash out Mitotracker by rinsing twice with imaging media (2 mL per rinse). Final volume of imaging media should be 2 mL per dish.
9. Subject to live-cell imaging.

Note: To avoid color contamination, Phenol Red Free culture medium is recommended as imaging medium. Aggressive wash cycles can affect cell adhesion.

Time-lapse live-cell imaging

Timing: 10 min or more (depending on sample size or experimental preference)

This step provides details on visualizing and acquisition of mitochondrial dynamics regulated by GJA1-20k and actin.

10. Set up the imaging incubator connected to the stage of the confocal microscope (Microscope Incubator for gas and temperature control from Okolab; see key resources table).
    a. Set incubator at 37°C and 5% CO_2.
    b. Set the excitation laser power at 50% of 488 nm for GFP (GJA1-20k), 80% of 561 nm for LifeAct-mCherry (actin), and 40% of 647 nm for Mitotracker Deep Red (Mitochondria). Troubleshooting 2.
    c. Set the exposure time for 100 ms for all channels.
11. Focus 100× oil immersion objective on a cell with well distributed mitochondria. Image stacks will be over time at the same z-depth (i.e., once a mitochondria is in focus, maintain the same focal plane).

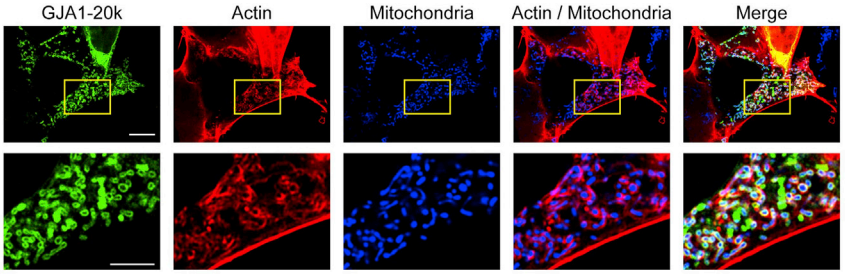


Figure 2. GJA1-20k and actin localize around mitochondria

Representative single plane images of GJA1-20k (GFP; green), actin (LifeAct-mCherry; red), and mitochondria (Mitotracker; blue). Bottom panels are enlarged images. Scale bar, 10 microns (above) and 5 microns (bottom). These images are HEK293 cell with siDRP1 and GJA1-20k overexpression. Confocal fluorescent microscope described in key resources table was used for the imaging.

12. Obtain time-lapse images every 3 s (each color is taken in series and without delay, so there are three 100 ms images per 3 s imaging acquisition) for a total duration of 3–5 min. Approximately 10–20 areas containing one to three cells each are recommended to be captured per dish to observe distinct dynamics.

⚠ CRITICAL: Channel settings (laser power and exposure time) must be maintained between different samples to make sure the experiment is performed under the same conditions. Also, confirm the focal plane is at the fission point to capture the full fission event in a 2D image and without the need for time consuming z-stack imaging.

Note: The imaging parameters (laser power, exposure time, and imaging duration) may need to be optimized depending on the experimental design and laser strength. High power, long exposure duration, or rapidly acquiring imaging without an interval of more than a second, will improve imaging resolution yet also induce photobleaching and cellular toxicity.

EXPECTED OUTCOMES

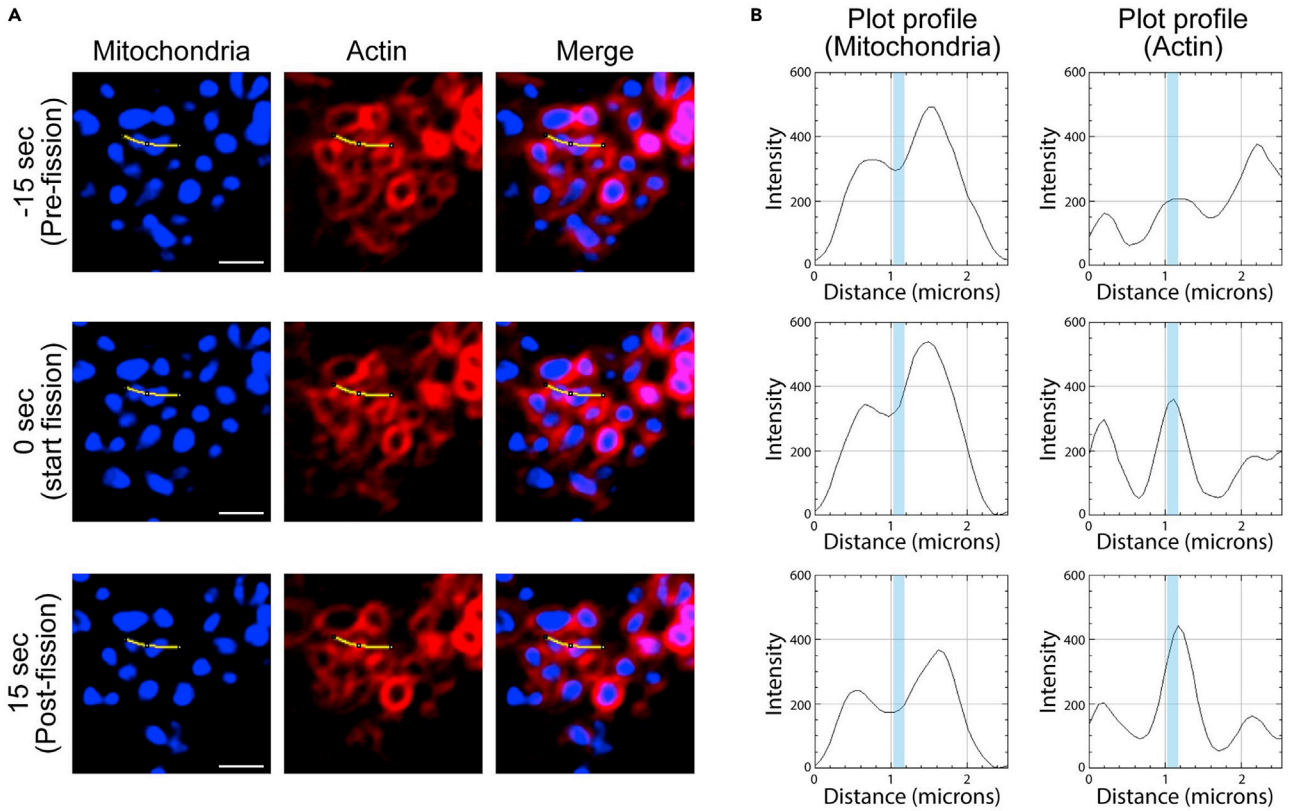
We expect to obtain time-lapse images demonstrating clear colocalization of GJA1-20k around mitochondria, accumulation of actin, and instances of actin localizing across mitochondria and initiating fission (Figure 2). From our experience, we usually observe one or two mitochondria fission events in a single three-minute clip (when cells express exogenous GJA1-20k and DRP1 is knocked-down).

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of mitochondrial fission process

This step describes the technical in silico (computer based) analysis of mitochondrial morphological change (fission) subsequent to GJA1-20k mediated actin assembly. For simplicity, we focus on actin and mitochondrial signals only. For GJA1-20k signals, the actin steps can be repeated but with the GJA1-20k images.

1. Open the image array in ImageJ Fiji software as an image stack, and use the time bar to identify mitochondria that undergo fission during the three-minute imaging period.
2. Define a spatial region of interest (ROI) in which mitochondrial fission is occurring.
3. Extract the time period covering the full mitochondrial fission process, beginning just before actin starts assembling at the fission point (e.g., from 15 s (5 frames) before the actin assembles).
4. Draw a line crossing the mitochondrial fission point across which the mitochondria divides into two separate mitochondria (Figure 3A; yellow line).
5. Save the line as a ROI (Edit/Selection/Add to Manager) for analysis of different time points yet at same position in each frame.



C

Plot Profile at 15 second (bottom panel of Fig. 3A and B)

Distance	Mitochondria Intensity	Actin Intensity
⋮	⋮	⋮
0.975	155.084	310.724
1.04	160.015	353.27
1.105	185.698	392.363
1.17	219.264	416.113
1.235	251.019	406.436
1.3	275.912	358.997
⋮	⋮	⋮

Extract each intensity from all frames to Table1.
at fission point (correspondig to blue square in Fig. 3B)
and calculate average for Fig. 3D

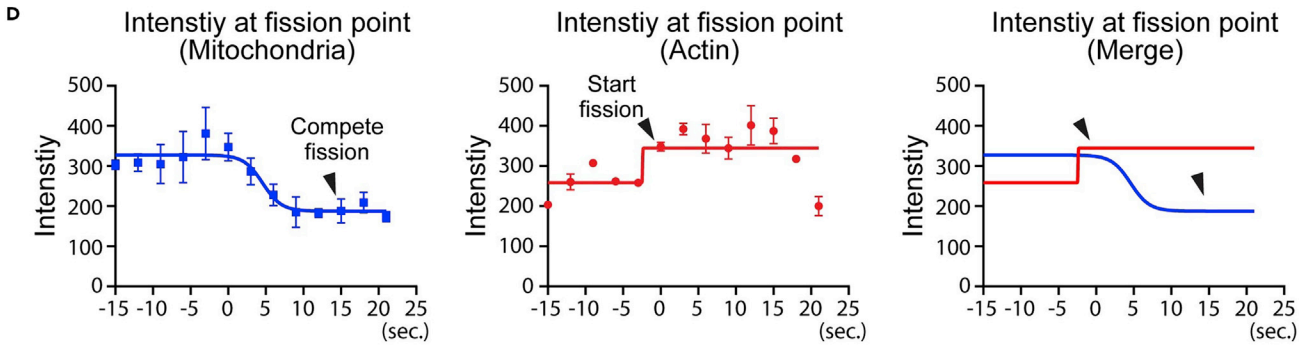


Figure 3. Data analysis of actin and mitochondrial fission by real-time imaging

(A) Find and select the mitochondria which is moving forward to fission. Draw a linear ROI for the plot to reveal intensity between mitochondria indicated by yellow line. Save the linear ROI and run plot profile in each continued image at same position. Set as 0 s just before the actin intensity starts increasing (middle panel) and analyze before and after 15 s (top and bottom panel, respectively). Scale bar, 2 microns.

(B) Profile plot of the ROI. Light blue square indicates the presumed fission site.

Figure 3. *Continued*

(C) The representative data table of mitochondria and actin intensity at fission point. At 1.04–1.17 data point on linear ROI, selected in (A), is defined as fission point. The fission point corresponds to the blue square described in (B). Extract these data sets to Table 1 to generate graph shown (D).
(D) The intensity of mitochondria and actin at each time point, with curve fitting proved by regression analysis. Black arrows indicate fission completion (mitochondria) and fission initiation (actin). Actin (LifeAct-mCherry) is in red, and Mitochondria (Mitotracker) is in blue.

6. Run "Plot profile" to obtain the profile of intensity in ROI for each channel (both actin and mitochondria signals) (Figure 3B).
7. Repeat "Plot profile" for each frame / time point of the mitochondrial fission event (Figure 3B).
8. Identify the exact spatial occurrence of mitochondrial fission event by noting the simultaneous occurrence of the highest intensity of actin together with a decrease in the intensity of mitochondria (Figure 3B, blue square). Mitochondrial signal should remain low at the fission point in subsequent frames to confirm that fission has occurred (Figure 3D).
9. Extract the intensity of actin and mitochondria at the fission point from the results of "Plot Profile" (Figure 3C).
10. Calculate the average intensity of actin and mitochondria at the fission pixel and two spatially surrounding pixel to limit sampling error). Graph the results (Figure 3D; Table 1).
11. Fit the curve with a nonlinear regression (four parameter logistic).
12. Define the time point of "fission initiation" at the time point when actin intensity increased.
13. Define "fission completed" at the time point where the mitochondria intensity visually drops and plateaus to background in the graph. Verify physical separation of mitochondria in the corresponding movie at the designated time point.

Note: The background of the image should be subtracted prior to image analysis. To subtract background, select a non-mitochondrial and non-actin area, measure the average gray value of each color channel, and subtract that pixel value from each pixel in the image (Figure 4). We normally use 16-bit image format for analysis.

Distance (in microns) shown Figure 3 and Table 1 are from the length of the line (ROI) for plot profile analysis. In this protocol, the length of the ROI is 2.535 micron and the fission points are between 1.04 and 1.17.

LIMITATIONS

This protocol is optimized for HEK293 cell line with GJA1-20k transfection. However, the technique should be universal for different proteins of interest and cell types. The major limitation would be to confirm that the time windows of image acquisition are appropriate for mitochondrial fission events

Table 1. Example of raw data of plot profile at fission point

	Mitochondria intensity			Actin intensity		
	Distance (micron)			Distance (micron)		
Time (sec)	1.04	1.105	1.17	1.04	1.05	1.17
-15	295.36	297.85	316.48	198.12	206.49	206.08
-12	291.84	301.01	333.40	239.24	263.38	278.45
-9	263.51	292.13	358.60	302.77	313.51	305.60
-6	268.37	305.84	393.25	259.70	268.21	257.39
-3	321.95	370.79	450.40	250.47	268.65	255.61
0	318.63	337.47	385.37	345.79	360.05	338.11
3	257.95	279.38	322.59	392.93	406.22	377.18
6	206.34	219.72	257.86	328.39	377.59	398.59
9	150.17	178.55	225.26	313.60	354.09	365.13
12	173.18	180.35	194.26	347.71	413.23	443.77
15	160.02	185.70	219.26	353.27	392.36	416.11
18	186.09	204.06	236.59	314.16	323.16	315.77
12	163.78	169.07	187.57	176.51	200.47	224.01

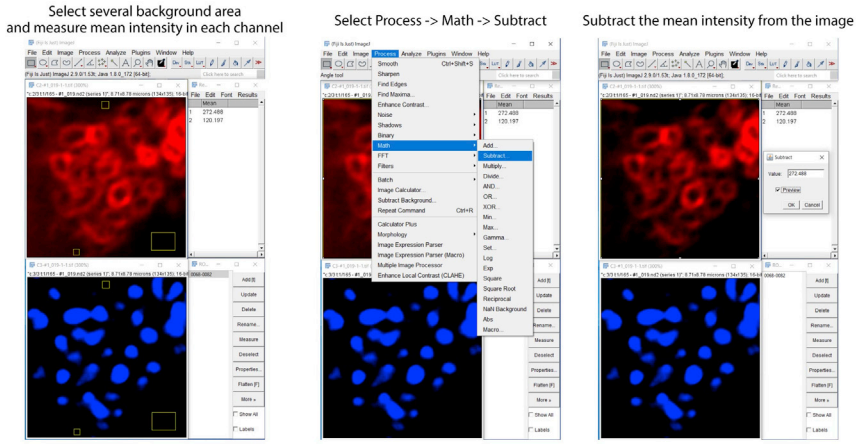


Figure 4. Representative screenshots for subtracting background intensity

Open images and follow the procedure described in the main text. If image has multiple channels, then split channels and perform background subtraction for each channel.

in different cells. Additionally, imaging parameters will need to be re-optimized for different proteins that may be more or less intense than the proteins studied above. If mitochondria are unusually thick, a z-stack before and after the fission event may be helpful to confirm that the imaging captured a complete fission and did not exclude out-of-plane signal.

TROUBLESHOOTING

Problem 1

Mitochondria signal is weak (section "preparation for live-cell imaging (mitochondria labeling)", step 6).

Potential solution

Increase the concentration of Mitotracker (remaining within a 25–500 nM range) or incubation with Mitotracker for a longer period (remaining within a 15–45 min range). Mitotracker is taken up into mitochondria based on mitochondrial membrane potential. So other dyes such as CellLight (Thermo Fisher Scientific; see alternatives) which targets mitochondrial pyruvate dehydrogenase could be useful as alternates. Note, however, that higher concentrations of Mitotracker could stain other organelles. Additionally, increased laser power could increase captured signal; or increased gain and/or frame acquisition rate can help reducing potential of photobleaching.

Problem 2

Actin around mitochondria cannot be visualized (section "time-lapse live-cell imaging", step 9).

Potential solution

The actin intensity around mitochondria tends to be less intense relative to polymerized actin elsewhere in cells such as in stress fibers. If actin signal around mitochondria is too low, consider increasing laser power (laser wavelength correlating to actin emission fluorescence) or exposure time (i.e., from 100 ms to 150 or 200 ms). In general, overexpression of LifeAct may also result in high levels of cytosolic actin (not polymerized) which may make it hard to visualize polymerized actin around mitochondria.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Robin M. Shaw (Robin.Shaw@hsc.utah.edu).

Materials availability

Plasmids are available at the non-profit plasmid repository Addgene, under Robin Shaw Lab. The Shaw lab can provide additional published plasmids or other reagents not yet listed in Addgene. All commercially available products are listed in the key resources table.

Data and code availability

This protocol does not include datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

D.S. performed the experiments and analyzed the data. D.S. and R.M.S. designed the research, interpreted the results, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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