Chapter 1

Discovery and Quantitative Dissection of Cytokinesis Mechanisms Using *Dictyostelium discoideum*

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Abstract

The social amoeba *Dictyostelium discoideum* is a versatile model for understanding many different cellular processes involving cell motility including chemotaxis, phagocytosis, and cytokinesis. Cytokinesis, in particular, is a model cell-shaped change process in which a cell separates into two daughter cells. *D. discoideum* has been used extensively to identify players in cytokinesis and understand how they comprise the mechanosensory and biochemical pathways of cytokinesis. In this chapter, we describe how we use cDNA library complementation with *D. discoideum* to discover potential regulators of cytokinesis. Once identified, these regulators are further analyzed through live cell imaging, immunofluorescence imaging, fluorescence correlation and cross-correlation spectroscopy, micropipette aspiration, and fluorescence recovery after photobleaching. Collectively, these methods aid in detailing the mechanisms and signaling pathways that comprise cell division.

Key words *Dictyostelium discoideum*, cDNA library complementation, Cytoskeletal network, Mechanoresponsive, Protein dynamics

1 Introduction

Cytokinesis is the last step during cell division in which a cell dramatically changes shape to separate into two daughter cells. For this process to occur, the cell utilizes a complex signaling pathway system with biochemical and mechanical signals and regulatory feedback loops. Thus, cytokinesis is fundamentally a mechanical process and a robust one, making it ample for many essential cellular functions such as cell proliferation, cell survival, and tissue formation. Understanding the mechanisms of cytokinesis allows for insight into these fundamental processes and into diseases where these mechanisms are dysregulated, such as cancer.

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Studies on cytokinesis have occurred in various mammalian cell types and other model systems including yeast and *Drosophila* [1–2]. We focus on using the social amoeba, *Dictyostelium discoideum*, as a model for cytokinesis. With its genetic tractability, rapid growth rate, ease of handling, and genetic homology to mammalian cells, *D. discoideum* is a suitable model for studying cytokinesis at a fundamental level. Through molecular genetic techniques, individual proteins responsible for cytokinesis have been identified through *D. discoideum* such as myosin II, cortexillin I, racE, and coronin [3–8]. Nonetheless, mapping out the complex biochemical network and pathways behind cytokinesis requires methods that integrate interaction genetics. One such approach is cDNA library complementation and high copy suppression, in which genetic suppressors of a *D. discoideum* cytokinesis-deficient mutant are recovered and identified. Through this method, we uncovered various genetic suppressors of genes like cortexillin I and myosin II, including dynacortin and enlazin [9–10].

As regulators of cytokinesis are identified via genetic selection (or other methods such as mass spectrometry [11]), we follow a workflow to examine if and how these proteins affect cytokinesis. Genetic mutant cell lines (cells expressing fluorescently tagged proteins or knockdown/knockout cell lines) are generated. They are then imaged for cytokinetic phenotypes, including multinucleation and furrow ingression defects. If there are noticeable cytokinetic phenotypes, the behaviors of these regulators during cytokinesis are expanded upon by methods like fluorescence correlation spectroscopy (FCS), fluorescence recovery after photobleaching (FRAP), and micropipette aspiration (MPA). These methods offer insight into protein movement, localization, and cell mechanics. Protein-protein interactions can also be studied if multiple cytokinetic regulators are observed in mutant cell lines. The previously mentioned methods can be used with multiple regulators along with another assay, fluorescence cross-correlation spectroscopy (FCCS), which specifically involves examining the interactions between proteins tagged with two differently colored fluorophores.

In this chapter, we detail the workflow of identifying potential regulators of cytokinesis and examining how they contribute to the cell division process. We first describe *D. discoideum* cell culture and the different approaches in creating mutant cell lines. From there, we expand on imaging these cells and using them in these quantitative assays: FRAP, FCS, FCCS, and MPA. The materials needed for each assay are also described.

2 Materials

2.1 D. discoideum *Cell Culture and Mutant Cell Line Generation*

2.1.1 General Cell Handling

1. 1.5× HL-5 medium: For 2 L of medium, mix 30.0 g proteose peptone, 0.255 g $Na_2HPO_4{\cdot}7H_2O$, 7.8 g Bacto™-yeast extract, 0.255 g KH_2PO_4, and 6.0 g glucose in deionized and double distilled water (*see* **Note 1**). Adjust pH to 6.5 and aliquot into 500 mL bottles. Autoclave and store at room temperature.
2. Hans' Enriched HL-5 medium:
   (a) ForMedium (FM): For 2 liters, mix 38 g FM with ~1500 mL of water. Dissolve by stirring rapidly. Raise volume to 2 L. Aliquot into 500 mL bottles and cover bottles with foil because FM is sensitive to light. Autoclave and store at room temperature. Add 43 mL of FM per 500 mL of 1.5× HL-5, which yields Hans' Enriched HL-5 medium (1.4× HL-5, 8% FM).
   (b) 500× Penicillin/Streptomycin (P/S): Dissolve 1.00 g Penicillin G and 1.56 g Streptomycin sulfate in 156 mL water. Filter sterilize and aliquot into 15 mL tubes. Store at −20 °C.
3. 10 cm Petri dishes.

2.1.2 Identification of Cytokinetic Mutants via cDNA Library Screening

2.1.3 Screening of Cytokinetic Mutants

1. Hemocytometer.
2. Electroporation (E-pore) buffer: 17.115 g/L sucrose and 10 mL/L 1 M potassium phosphate buffer, pH 6.5. Filter sterilize and store at room temperature.
3. Gene Pulser®/MicroPulser™ Electroporation Cuvettes, 0.4 cm gap (Bio-Rad, #1652088).
4. Gene Pulser Electroporator (Bio-Rad, model #1652076).
5. 100 mg/mL G418: Add 81.6 μL into 500 mL of Hans' Enriched HL-5 for 15 μg/mL G418. Store G418 stock solution at −20 °C.
6. 150 mL Erlenmeyer flasks.

Monitoring Growth Rate of D. discoideum

1. Hans' Enriched HL-5 medium (*see* Subheading 2.1.1, **items 1** and **2**)
2. 150 mL Erlenmeyer flasks
3. Hemocytometer

Multinucleation

1. Hans' Enriched HL-5 medium (*see* Subheading 2.1.1, **items 1** and **2**).
2. Coverslips (Fisher Scientific).
3. 10% paraformaldehyde: Dissolve 1 g PFA powder in 65 °C water. Add 1 M NaOH dropwise and vortex to dissolve PFA.
4. 100 μg/μL Hoechst dye.
5. 1× PBS: 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4-$7H_2O$, and 0.24 g/L KH_2PO_4 in ~800 mL of water. Adjust pH to 7.4 with HCl or NaOH and bring volume up to 1 L.
6. 1× PBST: 0.1% (v/v) Tween 20 in 1× PBS.
7. Triton X-100.
8. Fixation solution: 8 mL Hans' Enriched HL-5 medium, 2 μL Triton X-100, 2 mL 10% PFA, and 2 mL water.
9. Anti-fade mounting media.

2.1.4 Development of Cell Lines Expressing Fluorescently-Labelled Proteins

1. PCR reagents:
   (a) Primers are obtained from Integrated DNA Technologies (IDT).
   (b) Q5® High-Fidelity 2× Master Mix (New England Biolabs).
2. Transformation materials (*see* Subheading 2.1.3, **items 2**–**5**).

2.1.5 Generation of D. discoideum Cytokinetic Knockdown/Knockout Cell Lines

Homologous Recombination

1. Transformation materials (*see* Subheading 2.1.3, **items 2**–**5**).

RNA Hairpin

1. PCR reagents (*see* Subheading 2.1.4, **item 1**).
2. Expression vector for cloning.
3. Transformation materials (*see* Subheading 2.1.3, **items 2**–**5**).
4. RNA extraction reagent such as TRIzol® (Thermo Fisher Scientific).
5. RT-qPCR reagents such as the Verso 1-step RT-qPCR Kit with SYBR Green (Thermo Fisher Scientific).

CRISPR

1. *D. discoideum* strains growing at exponential growth phase in 10 mL of Hans' Enriched HL-5 media on 10 cm Petri dish.
2. SM-5 plates: 1.0 g/L Bacto™-tryptone, 1.0 g/L Bacto™--yeast extract, 1.0 g/L glucose, 0.95 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.1 g $MgSO_4$-$7H_2O$, and 20 g/L Bacto™-agar. Adjust pH to 6.4 with 2.5 M NaOH to be more basic or 1 M KH_2PO_4 to be more acidic. Autoclave the mixture, allow it to

cool to approximately 50 °C, and pour 30 mL of the mixture in each 10 cm Petri dish. Store the plates at 4 °C.

3. *Klebsiella aerogenes* culture: Add approximately 0.5 mL of *K. aerogenes* in 50 mL of LB medium and grow in suspension at 37 °C overnight to be used the next day.
3. LB medium: 5 g/L Bacto™-tryptone, 2.5 g/L Bacto™-yeast extract, 2.5 g/L sodium chloride, and 100 µL/L 5 M NaOH in water. Autoclave the medium and store at room temperature.
4. Transformation materials (*see* Subheading 2.1.3, **items 2**–**5**).
5. All-in-one CRISPR-Cas9 pTM1285 vector was obtained from NBRP Nenkin.
6. We selected gRNA guide sequences targeting the gene of interest using Cas-Designer [12]. Customized RNA oligos were obtained from Integrated DNA Technologies (IDT).
7. Forward and reverse primers complementary to gene regions within 50 nucleotides away from *Bbs* I-cut sites/gRNA insertion sites on pTM1285 vector were customized and obtained from IDT.
8. Fermentas FastDigest *Bbs* 1 restriction enzyme, FastDigest Buffer (10×), and FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific).
9. QIAquick gel extraction kit (Qiagen).
10. Quick ligation kit (NEB).
11. T4 PNK (NEB).
12. T4 ligation buffer (NEB).

2.2 Assessing Cytokinesis via Cell Imaging

1. Glass coverslips (Fisher Scientific).
2. Glass bottom imaging chamber.

2.2.1 Live Cell Imaging/ Furrow Measurements

2.2.2 Fixed Cell Imaging

1. Glass coverslips and glass slides (Fisher Scientific).
2. 4% paraformaldehyde.
3. Acetone.
4. 10× PBS: For 1 L, dissolve 80 g NaCl, 14.4 g $Na_2HPO_4{\cdot}7H_2O$, 2.0 g KCl, and 2.4 g KH_2PO_4 in ~800 mL water. Adjust pH to 7.4 with HCl or NaOH. Raise volume to 1 L. Autoclave and store at room temperature.
5. Blocking buffer: 1× PBS with 0.05% Triton X-100 and 0.5% BSA.

6. 1× PBST: Dilute 10× PBS 10-fold in water and then add Triton X-100 for final concentration of 0.05%.
7. Primary and secondary antibodies compatible with immunofluorescence.
8. Anti-fade mounting media.

2.3 Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Cross-Correlation Spectroscopy (FCCS)

1. Confocal microscope with a fluorescence correlation and dual color cross-correlation capabilities.
2. A high numerical aperture, water-immersion objective.
3. Analysis software such as Zen Blue or Zen Black software for acquisition and data analysis.
4. Glass bottom imaging chambers (Thermo Fisher Scientific).
5. Coverslips (Fisher Scientific).
6. Low fluorescence media: For 1 L, pour ~600 mL water into a glass container. Dissolve 3.85 g glucose, 1.20 g Na_2HPO_4-$12H_2O$, 1.78 g Bacto™ Proteose-Peptone, 0.485 g KH2PO4, and 0.450 g Bacto™-yeast extract. Adjust pH to 6.5 using 1 M KH_2PO_4 or 1 M K_2HPO_4. Bring volume up to 1 L. Autoclave.
7. Rhodamine 6G.

2.4 Fluorescence Recovery After Photobleaching (FRAP)

1. Zeiss Axiovert 200 inverted microscope with LSM510-Meta confocal module and a 63× (NA 1.4) objective.
2. Glass bottom imaging chambers (Thermo Fisher Scientific).
3. MES starvation buffer: 50 mM MES, pH 6.8, 2 mM $MgCl_2$, and 0.2 mM $CaCl_2$ in deionized water.

2.5 Micropipette Aspiration (MPA)

This list briefly details our materials used for MPA, but an MPA system can be designed in various ways with different motorization settings.

1. Microscope: Olympus IX81 or IX71 capable of DIC and fluorescence imaging, with 10×, 40× (NA1.3), and 60× (NA1.45) objectives and a 1.6× Optovar.
2. Micromanipulator: Sutter Instrument MP225.
3. Micropipette puller: MicroData Instrument PMP-102.
4. Microforge: MicroData Instrument MFG-5 Microforge-Grinding Center.
5. Custom-built rectangular imaging chambers made of anodized aluminum with a thickness of about 1.5–2 mm.
6. Vacuum grease (Dow Corning high-vacuum grease) for attaching cover glass to imaging chamber.
7. Cover glass (No. 1.5 thickness, 22 × 60 mm, Fisher Scientific).
8. MES starvation buffer: *see* **item 3** in Subheading 2.4.

9. Micropipette needles: Micropipettes needles are made with a micropipette puller and microforge. Thin-wall borosilicate glass tubing (1 mm outer diameter, 0.75 mm inner diameter, 10 cm length, Sutter Instrument) can be used with the micropipette puller. When pulling pipettes, the inner diameter should ideally be in the 4–6 μm range. The micropipette tip should be straight and polished with the microforge. Pre-Pulled Glass Pipettes are also commercially available through World Precision Instrument.
10. 1 μm blue polystyrene microbeads (Polysciences, #15712) for calibrating the MPA system.

3 Methods

3.1 D. discoideum Cell Culture and Mutant Cell Line Generation

3.1.1 General Cell Handling

Vegetative *D. discoideum* cells are routinely grown in Hans' Enriched HL-5 (1.5× HL-5 medium supplemented with ForMedium (FM, 8% final concentration), penicillin (60 U/mL), and streptomycin sulfate (60 mg/mL)). Cells are grown at 22 °C on polystyrene Petri dishes and passaged by simple pipetting before cells reach the stationary phase (between 2×10^6 and 6×10^6 cells/mL). Cells can also be grown in suspension in Erlenmeyer flasks at 180 rpm at 22 °C.

3.1.2 Identification of Cytokinetic Mutants via cDNA Library Screening

While proteins involved or required in cytokinesis in *D. discoideum* have been identified with common molecular genetic methods, uncovering complex genetic interactions with these methods are more difficult to do because *Dictyostelium* are primarily haploid organisms that are not easily genetically crossed. To bypass this issue, we focus on using cDNA library complementation and high-copy suppression methods to probe for more complex genetic interactions during cytokinesis. Numerous genes have been identified to play a role in cytokinesis using this method [9–10, 13] by transformation of the cDNA library into cell lines with identified cytokinetic aberrations, including high nuclei/cell ratio and a lower saturation density. The generation of the cDNA library in the pLD1A15SN expression vector is detailed in reference 9. Below, we describe the transformation of the cDNA library and identification of genetic suppressors in a cell line with a cytokinesis defect resulting in multinucleation and decreased growth rate.

3.1.3 Screening of Cytokinetic Mutants

1. Grow mutant cell line on 20 10-cm Petri dish plates until they reach late log phase growth, generally between 2×10^6 and 6×10^6 cells/mL.
2. Pool cell lines and determine cell density with a hemocytometer.

3. Spin down cells at 2000 rpm for 5 min and wash with 10 mL ice-cold electroporation buffer.
4. Decant supernatant and resuspend pellet in 10 mL ice-cold electroporation buffer. Keep cells on ice.
5. Spin down cells at 2000 rpm for 5 min and resuspend cells at a density of 10^7 cells/350 μL of electroporation buffer.
6. Transform 1 μg of plasmid cDNA library per 350 μL of cells using a Gene Pulser. Add DNA into cuvette with cells. Keep cuvettes on ice.
7. Pulse cells one time in 0.4-cm cuvettes at 3 μF and 1.3 kV for ORF 7–3 cells (HS1000 cells: (Ax3: Replicase orf+)) (*see* **Note 2**). Pour cells from the cuvette into a petri dish.
8. Allow cells to grow in enriched Hans' HL-5 media for 24 h at 22 °C. Replace media with Hans' enriched HL-5 with G418 (typically 10–15 μg/mL but determined using kill curves and/or optimization of transformation efficiency with the empty plasmid). Change media every 2–3 days until clones are harvested.
9. When clones reach near confluency on 10 cm plates, harvest cells. About 1000–1500 clones/μg of DNA can be recovered from ORF+-derived strains. These cells can be combined into pools as large as 30,000–35,000 clones per pool as was originally done, but we find that smaller pool sizes (&3000 clones/pool) allow for much more rapid identification of genetic interactors (*see* **Note 3**).
10. To begin genetic selection, grow cells in suspension in at 1×10^5 cells/mL in 10 mL cultures in 125 mL Erlenmeyer flasks at 180 rpm at 22 °C. Allow cells to grow to $5–6 \times 10^6$ cells/mL before splitting cells into new flasks at 1×10^5 cells/mL. Culture cells over 3–4 growth cycles for smaller pool sizes.
11. Monitor cell growth rates (as detailed in the next section). During log phase growth, aliquot cells from suspension cultures for nuclei/cell ratio determination (as described in the Multinucleation section below).
12. Identify cultures with wild-type cell morphology (deemed as winners) and harvest DNA.
13. Transform harvested DNA into STBL2 *E. coli* cells (*see* **Note 4**). Perform DNA clean-up and appropriate restriction endonuclease digestion on isolated bacterial clones for DNA sequencing. Identify genes from DNA sequencing via sequence database (i.e., Dictybase BLAST).
14. To confirm that the suppression phenotype can be recapitulated, transform recovered cDNA into original mutant cell line and monitor cell growth rates and nuclei/cell ratio.

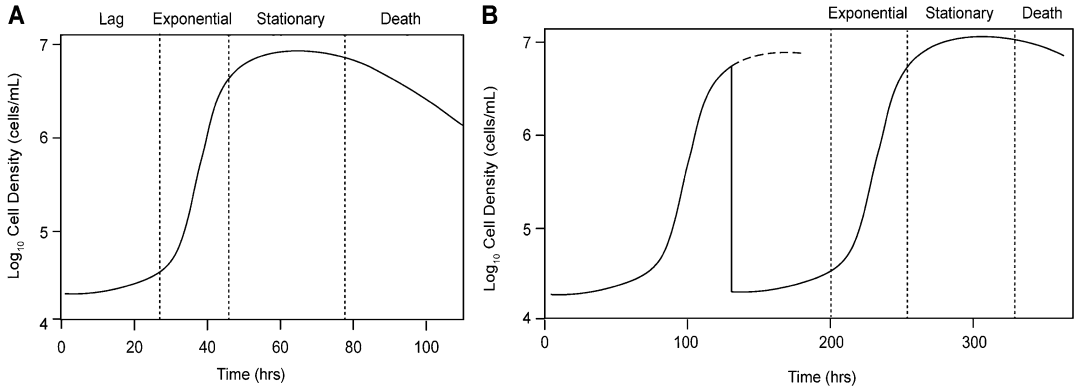

Fig. 1 Relative *D. discoideum* growth rates can be determined by plotting cell densities versus time. (**a**) Sample *D. discoideum* growth curve. (**b**) Example growth curves over multiple cycles of growth. Cells are split within the exponential phase of growth before being subjected to a subsequent cycle of growth

Monitoring Growth Rate of D. discoideum

1. Seed cells in 10 mL of Hans' Enriched HL-5 medium in 150 mL Erlenmeyer flasks at a starting concentration of 1×10^5 cells/mL (*see* **Note 5**).
2. Shake cells in 22 °C incubator at 180 rpm. Cell concentration is measured and recorded roughly every 24 h using a hemocytometer. It is important to note that because of the time lag required for the winner cells to populate the cultures, we normally acquire and analyze data from second and third cycles of suspension culture.
3. When cells reach the later point of exponential growth phase (about 5–6 × 10^6 cells/mL), split down cultures to a concentration of 0.5–1 × 10^5 cells/mL (*see* **Note 6**). Continue to measure roughly every 24 h, recording the exact times cell density is measured.
4. Relative growth rates are determined by plotting cell densities versus time (Fig. 1a). The resulting exponential phase curve is fitted to a single exponential equation using a data analysis software such as KaleidaGraph (Synergy Software). If cell concentrations are measured throughout multiple cycles of suspension culture, growth rates can be obtained from each cycle (Fig. 1b). Growth rate (k) can be determined for each growth curve through the exponential growth equation:

$$n(t) = n_0 e^{kt}$$

where $n(t)$ = population at time t, n_0 = initial population concentration, and k = relative growth rate.

5. Relative growth rates between cell lines can be compared to their respective control lines to determine if there are any growth defects.

Multinucleation

1. Seed cells at approximately 70% confluency on coverslips in Hans' Enriched HL-5 media and allowed to adhere for 15–30 min.
2. Remove media and fix cells with ice-cold fixation solution for 10 min.
3. Wash cells quickly with 1× PBS and stain nuclei with 10 μg/uL Hoechst for 10 min. Afterward, wash cells with 1×PBST three times, 10 min each.
4. Before visualization, mount cells on coverslips using an anti-fade of choice (*see* **Note 7**).
5. Image nuclei using a fluorescence microscope and count nuclei through an imaging software such as ImageJ.

3.1.4 Development of Cell Lines Expressing Fluorescently Labeled Proteins

Generating *D. discoideum* cell lines expressing fluorescently labeled cytokinetic proteins allows for further examination into their potential role in cytokinesis. With fluorescent tags, protein localization throughout cytokinesis can be assessed as shown in Fig. 2 [14]. Protein-protein interactions of cytokinetic players can also be examined by expressing fluorescent proteins in different background strains (e.g., GFP-cortexillin I in a *myosin II*-null strain). In addition, behavioral changes based on protein expression levels can also be assessed (e.g., overexpression, rescue cell lines).

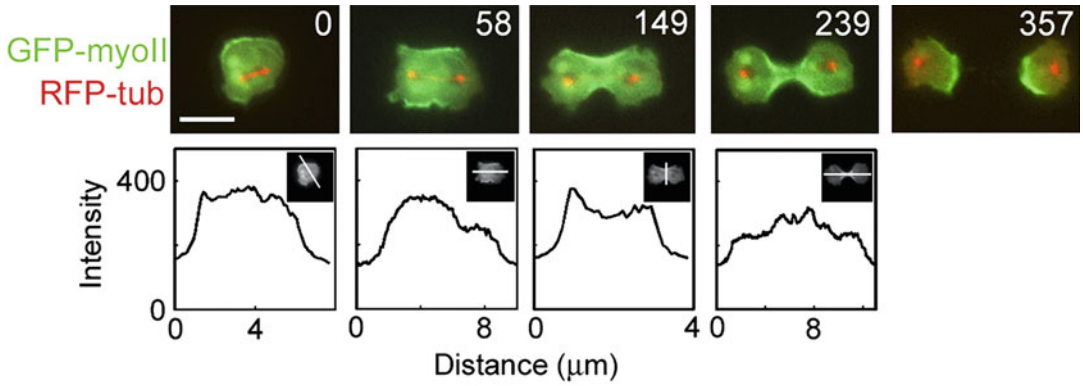

Fig. 2 Fluorescently labelled proteins can be used to assess protein localization during cytokinesis. Proteins with fluorescent tags such as GFP-myosin II and RFP-tubulin (a mitotic spindle marker) can be visualized during cell division over time. GFP-myosin II concentrates in the cleavage furrow and bridge as the cell divides as indicated by the time lapse images and corresponding line scans. (Reproduced from ref. 14 with permission from Elsevier)

D. discoideum readily take up plasmid DNA [15]. Transformants are isolated via drug selection and expression of recombinant protein is confirmed via fluorescence microscopy or Western analysis. The generation of these cell lines is detailed below.

3.1.5 Cloning and Transformation of Recombinant Protein

1. Amplify gene of interest via PCR. The amplified gene of interest should contain restriction enzyme sites for insertion into expression vectors.
2. Insert gene of interest into expression vectors containing sequences for fluorescent proteins (e.g., GFP, mCherry, etc.). We use a variety of expression vectors including pLD1A15SN, pDM181, pDRH, and pBIG. A list of expression vectors can be found on the *D. discoideum* database, Dictybase.
3. Transform cells by electroporation (*see* Subheading 3.1.3, **steps 1–8**, for more details on transformation), and select for transformants with supplemented Hans' Enriched HL-5 medium containing G418 (10–15 μg/mL) or hygromycin B (35–40 μg/mL) (*see* **Note 8**).
4. Change out drug media every 2–3 days. Transformants will appear as colonies on the petri dish. The amount of time needed for colonies to appear can vary depending on which genes are being expressed (*see* **Note 9**).

3.1.6 Generation of D. discoideum Cytokinetic Knockdown/Knockout Cell Lines

In addition to generating cell lines with fluorescently labeled proteins, knocking down or knocking out potential cytokinetic proteins creates another avenue for examining their role in cytokinesis. Our studies and others [4–11] detail gene knockout and knockdown in *D. discoideum* with various methods, including homologous recombination and RNAi. As regards to RNAi technology, both short hairpin (25 nt) and long RNA hairpin (300–500 nt) have been used in *D. discoideum* previously. More recent findings utilize CRISPR technology in *D. discoideum* to knockout genes using a pTM1285 vector, mapped in Fig. 3 [16–17]. We detail various methods of gene knockdown/knockout utilized in our studies below.

3.1.7 Homologous Recombination

In *D. discoideum*, gene knockout can occur through homologous recombination by simply transforming a plasmid containing a drug selection marker with flanking regions from the gene of interest, ideally from the 5′ and 3′ UTRs [3]. *See* Subheading 3.1.3 for general transformation and isolation of mutant cells.

RNA Long Hairpin

1. Determine a target region for knockdown. Both coding sequence DNA and 3′ UTR can be targeted. While it is tempting to target the untranslated regions (UTRs) to allow for rescue experiments, *Dictyostelium* UTRs have very high AT

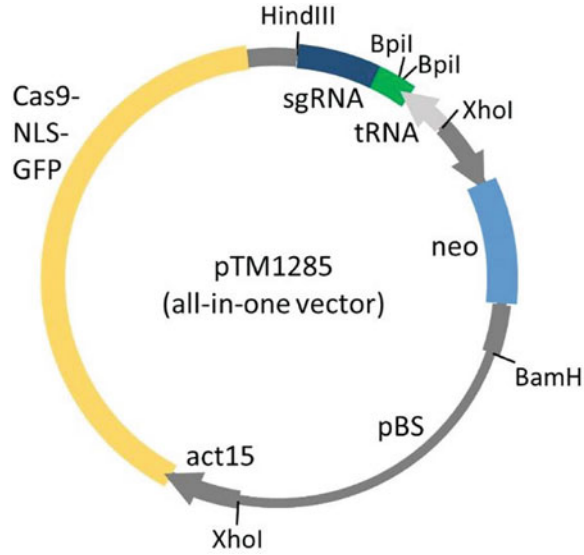

Fig. 3 Map of the pTM1285 plasmid. (Reproduced from ref. 17)

content, which leads to a very low complexity. To increase specificity, we have had to focus on coding sequences to identify regions where extensive DNA sequence searching reveals no, or very little, homology across >15 bp to any other region in the genome.

2. Once the target region is determined, use PCR to amplify both the anti-sense and sense strand DNA templates for cloning into an expression vector. We routinely use pLD1A15SN, a G418-resistant expression vector that is readily titratable, to achieve different levels of knockdown.
3. Determine the spacer sequence. For long hairpin structure, the spacer sequence is generally between 100 and 300 nucleotides. We routinely use the sequence immediately upstream of targeting coding sequence as the spacer to simplify cloning procedure. However, because this stretch is included in the antisense portion, we also apply the same standard that it should not include any stretch >15 bp with homology to any other region of the genome. Only two PCR runs are needed to amplify both antisense and sense strands (Fig. 4).
4. Introduce the hairpin RNA plasmid into cells via transformation. For transformation by electroporation, *see* Subheading 3.1.3, **steps 1–8**, for more details. For pLD1A15SN vectors expressing hairpin RNA, drug select for transformed cells with G418 (*see* **Note 10**). Empty vector-containing cells also need to be generated to serve as the control comparator.
5. To determine the level of knockdown, extract RNA using commercial extraction reagents (such as TRIzol®) from cells

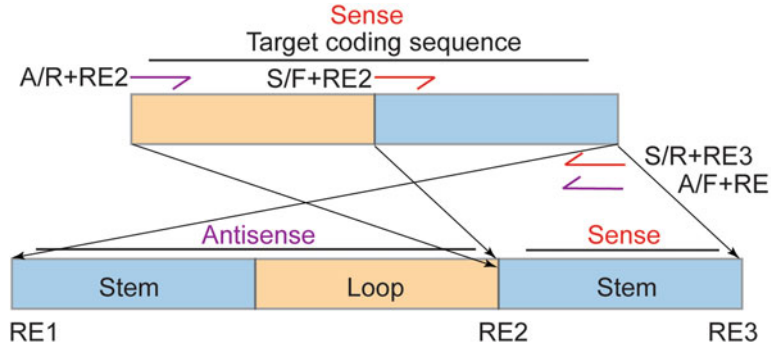

Fig. 4 Hairpin RNA can be generated to knock down genes in *D. discoideum*. Hairpin RNA for *D. discoideum* can be created by amplifying the target coding sequence containing the gene of interest (blue regions) and an upstream spacer region (beige regions). Restriction enzyme recognition sites (RE2, RE3) are added to sense strand primers (sense forward (S/F) and sense reverse (S/R)) to amplify the sequence encoding the sense fraction of the RNA hairpin. Likewise, restriction enzyme recognition sites (RE1, RE2) are added to the antisense strand primers (antisense forward (A/F) and antisense reverse (A/R)) to amplify the antisense fraction of the RNA hairpin plus spacer. The stem is generated by combining the antisense and sense strands with the antisense spacer loop separating the regions containing the gene of interest

expressing the RNA hairpin vector and cells expressing the empty vector. Perform qRT-PCR with the extracted RNA to quantify the relative amount of RNA for the gene of interest in each cell line. Primers for qRT-PCR should be targeting a sequence of 100–200 base pairs outside the sequence included in the RNA hairpin. A housekeeping gene such as GAPDH could be used as a normalization factor during knockdown level quantification.

CRISPR

1. To begin the process of inserting the gRNA into the pTM1285 vector (Fig. 3), digest 1 μg of pTM1285 plasmid with 1 μL FastDigest *Bbs* 1, 1 μL FastAP, 2 μL 1× FastDigest Buffer, and water to a final reaction volume to 20 μL total. Incubate the reaction for 30 min at 37 °C. After the digestion is completed, load the entire reaction volume on a 1% DNA gel. Gel-purify the plasmid using a QIAquick Gel Extraction Kit and elute it in EB buffer.
2. Phosphorylate 1 μL of complementary gRNA oligos by 0.5 μL T4 PNK in a 10 μL reaction volume containing 1 μL 10× T4 ligation buffer and water. Anneal the pair of oligos in a thermocycler for 30 min at 37 °C, followed by 5 min at 95 °C, and then ramped down to 25 °C at 5 °C/min.

3. Ligate 50 ng of the vector and 150 ng of the annealed gRNA oligos with 1 μL Quick Ligase in an 11 μL reaction volume containing 2× Quick Ligation Buffer and water. Incubate the ligation reaction for 30 min at room temperature.
4. Transform 2 μL of the ligation reaction from **step 3** into 50 μL of STBL2 competent cells. Heat shock the competent cells for 28 sec at 42 °C and plate on ampicillin-containing LB plates (*see* **Note 11**). Incubate plates at 30 °C for 16 h and isolate individual clones for mini-prep. Mini-prepped recombinant pTM1285 plasmids should be confirmed using pTM1285 forward and reverse primers. The correct pTM1285 with the corresponding gRNA can be maxi-prepped for future use.
5. With the correct pTM1285 plasmid, transform 1–10 μg of plasmid into *Dictyostelium* cells by electroporation and drug select with G418. *See* Subheading 3.1.3, **steps 1–8**, for more details on transformation.
6. After drug selection, plate any remaining live cells on SM-5 plates with cultured *K. aerogenes*, and incubate at room temperature for 3–4 days until visible, isolated plaques form (*see* **Notes 12–13).** Transfer individual plaques separately into 48-well plates containing Hans' Enriched HL-5 media without G418. Check for colony growth after approximately 2–3 days and transfer them to 10 cm Petri dishes. Since the cells should be expressing only Cas9 and gRNA transiently, it is important to test a subpopulation of these cells for G418 sensitivity to ensure the absence of pTM1285 plasmid. Retention of the plasmid can potentially induce off-target gene modifications. The tentative KO cell lines can be identified using gene sequencing and Western analysis.

3.2 Assessing Cytokinesis by Imaging Cells

Time-resolved microscopic imaging of live *D. discoideum* is a powerful way to study cytokinesis and identify if and how it is affected in mutant cell lines. The morphological changes and dynamics during cytokinesis reflect multiple aspects of the cell, including mechanics, actomyosin contraction, adhesion, polar protrusive forces, and abscission machineries. Cells can simply be imaged during cell division, allowing for the observation of furrow ingression and bridge formation (Fig. 5). Cytokinesis dynamics and alterations to furrow ingression can be observed, quantified, and even modeled [18–19]. With fluorescently tagged proteins, fluorescence microscopy can be useful for assessing protein localization during cell division. If proteins are difficult to visualize live and antibodies for a protein of interest are available, *D. discoideum* can be fixed for immunofluorescence (IF) imaging. Fluorescence imaging is also particularly helpful for identifying multinucleated mutants, a sign of a cytokinetic defect, using nuclear stains such as DAPI and

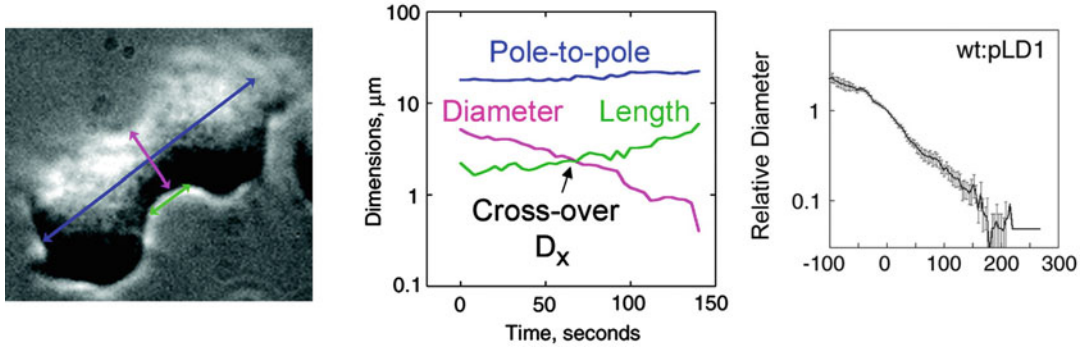

Fig. 5 *D. discoideum* cytokinesis furrow measurements can be observed and quantified. During *D. discoideum* cell division, the diameter of the division furrow, which later becomes the intercellular bridge, is measured (indicated by purple line with arrows over cell). The furrow length is also measured (indicated by green line with arrows over cell). Both values are plotted over time to find the point where diameter equals length (crossover). In the end, at each time point, relative diameter is calculated by dividing measured furrow diameter by crossover value and plotted over time. Different strains and different genetic makeup could result in apparent variations in relative diameter dynamics over time. (Reproduced from reference 18 with permission from the National Academy of Sciences. Copyright (2005) National Academy of Sciences, USA)

Hoechst (detailed in the Multinucleation section in Subheading 3.1.3). Below we detail how we use imaging to assess cytokinesis in *D. discoideum*.

3.2.1 Imaging Cell Division and Furrow Measurements

1. Seed cells on a glass bottom imaging chamber in Hans' Enriched HL-5 media and allowed to adhere for 15–30 min.
2. Put the imaging chamber on the microscope stage. Begin imaging cells using the DIC channel with the 40× objective. Scan the field of view for dividing cells. There is not an efficient cell synchronization method for imaging many dividing cells at once, but it is not necessarily required to synchronize cells beforehand. The doubling time of *D. discoideum* is 8–12 h when grown in culture media under drug selection, whereas mitosis through cytokines usually takes around 15 min. That means 2–3% of the cells are dividing at a given time during exponential growth phase. During exponential growth phase when observed under 40× objective, usually 80–120 cells can be seen within the same field of view. Thus, it is not difficult to spot one dividing cell after scanning two different fields of view, and when starting with 15 min time lapse video acquisition, it is very common to find a second and third divider within a single movie acquisition.
3. After spotting a cell undergoing mitosis, capture time lapse videos until the cell finishes dividing. A time lapse video of 15 min with 2 s intervals is recommended when imaging cell division using differential interference contrast (DIC)

microscopy. Mitosis usually starts with rounding up of the cell, followed by elongation and furrow ingression, and ends with intercellular bridge thinning and abscission. It is easier to spot cells during furrow ingression stage since there is a significant morphological difference, although rounded-up cells are also relatively easy to find.

4. Analyze the time lapse videos with imaging software such as ImageJ. Cell lines exhibiting cytokinesis failure may display furrow regression and failure in abscission (*see* **Note 14**). A variety of parameters can be considered to assess cytokinesis with these videos. Some to consider include success/failure of division, symmetry of daughter cells, total time required to divide, distance between poles, and furrow ingression and intercellular bridge-thinning dynamics. The ratio of success/failure of division can be used as a phenotype for a mutant cell line and may be related to the function of the modified gene. The symmetry of the daughter cells reflects the cell's ability to correctly position contractile machinery, which is especially challenging in multicellular cells. Total time required to finish cell division and the distance between two poles are additional parameters to consider in order to spot differences in elasticity and polar protrusive forces.
5. Finally, the kinetics of furrow ingression and intercellular bridge thinning dynamics reflect a cell's cortical viscoelasticity, active contractile forces, adhesion, protrusive forces, passive fluid mechanics, strain stiffening of the network, and the resistive stresses generated through the cortex and cytoplasm from the emerging daughter cells. To assess the furrow ingression dynamics and intercellular bridge thinning, quantify the diameter and length of furrow and intercellular bridge over time [18]. Find the crossover distance when the diameter equals length, and then normalize the bridge diameter to cross over distance to get relative furrow diameter.

3.2.2 Fixed Cell Imaging

1. Grow cells on coverslips in Hans' Enriched HL-5 media and allow to adhere for at least 1 h.
2. Gently aspirate off media and fix cells in ice-cold 4% paraformaldehyde for 10 min, and then permeabilize with −10 °C acetone for 3 min.
3. Wash cells with 1× PBS three times.
4. Block cells in 1× PBS + 0.05% Triton X-100 + 0.5% BSA for 45 min at room temperature. Follow with three washes in 1× PBST (1× PBS + 0.05% Triton X-100).
5. Incubate with primary antibody in PBST overnight at 4 °C. Wash cells with PBST five times.

6. Incubate with secondary antibody in PBST for 60 min at room temperature, away from light. Wash cells with PBST five times.
7. Mount coverslips onto glass slides. Allow sections to cure for at least 24 h at room temperature in the dark.
8. Proceed to image slides on a fluorescence microscope. We routinely image on confocal microscopes on 40× or 63× oil-immersion objectives with excitation lasers at 488 for GFP, 561 for mCherry, and 633 for far-red fluorescent proteins.

3.3 Fluorescence Correlation Spectroscopy (FCS) and Fluorescence Cross-Correlation Spectroscopy (FCCS)

Fluorescence correlation spectroscopy (FCS) and fluorescence cross-correlation spectroscopy (FCCS) are common methods used to measure the concentration and diffusion coefficient of one molecular species or the interaction between two molecular species [20, 21]. Both methods involve capturing sensitive fluorescence fluctuations within a small confocal volume, which can be used to extract diffusion and mobility parameters (Fig. 6). Whereas FCS utilizes autocorrelation analysis, FCCS utilizes cross correlation between two fluorescently tagged species. In *D. discoideum*, these methods have been used to quantify the mobility and concentration of proteins and to reveal protein-protein interactions within the cytoskeleton and the mechanosensitive network [11]. Below are detailed steps for FCS and FCCS experiments geared toward live *D. discoideum* cells, but the same methods can also be applied to molecules in vitro. More information on FCS/FCCS are also detailed in 20 and 21.

3.3.1 Cell Line Preparation

1. For in vivo FCS and FCCS, introduce fluorescently tagged molecules of interests into cells. One of the major concerns about FCS and FCCS is the brightness of the fluorescent

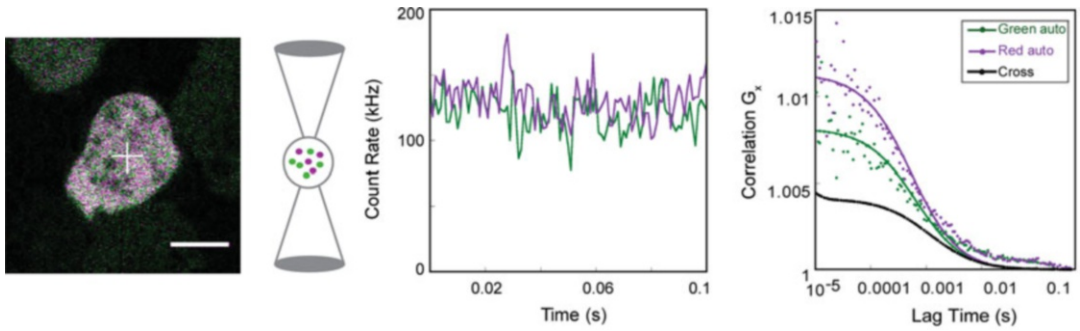

Fig. 6 Fluorescence cross-correlation spectroscopy (FCCS) can be used to probe the interaction between two molecular species. Two proteins are labeled with two different colored fluorophores and exogenously expressed in *D. discoideum* cells. Within a small confocal volume, fluctuations of both fluorophores are recorded and reported as count rates in kHz. Using auto-correlation and cross-correlation calculations, diffusion coefficients of both proteins and in vivo K_D between the two proteins can be calculated. (Reproduced from reference 11 with permission from the Company of Biologists)

probes since the effective confocal volume is relatively small. Probes should be bright enough to indicate fluctuations, but the concentration cannot be too high as it will saturate the signal, making it difficult to assess the fluctuations in signal intensity. Another factor to consider when choosing fluorescent probes is the triplet state since it directly affects the correlation calculation. For FCCS, having spectral separation between the two probes is also an important factor to be considered. Overlapping excitation and emission spectra should be minimized to avoid noise in detecting signals.

3.3.2 Microscope Setup and Calibration of System

1. Prepare a solution of rhodamine 6G with a concentration between 50 nM and 200 nM. Load into a glass imaging chamber.
2. In imaging software such as Zen, select appropriate dichroic and emission filters for the dye pair to be used. A laser power that is too low may result in count rates that are undetectable, whereas a very high laser power may cause photo damage to the cells and oversaturate the signals. Ideally, laser powers for both channels should be kept consistent.
3. Find the image plane by using line scan. Move the objective up from the load position to identify both edges of the coverslip. Stop when two spikes of signals appear. These correspond to the two edges of the coverslip. Move the focus up 200 μm into the solution.
4. To compensate for suboptimal refractive indexes of the specimen, adjust the correction collar of the objective until the maximal count rate is achieved.
5. Adjust the pinhole. We usually set the pinhole at 1 Airy unit, although a smaller size can be considered. Adjust the coarse lateral pinhole position first and then the axial position. Repeat the sequence when adjusting the fine pinhole position.
6. Acquire measurements for rhodamine 6G every 5 s for ten repetitions.
7. Select the correct model to fit the data. The equation we use to fit fluorescence fluctuations over time to obtain an autocorrelation value (G_x) for FCS is given below:

$$G_x = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$

where t is time and $F(t)$ is the fluorescence fluctuation detected.

Fluorescence fluctuations relay information on the mobility of a species, but they may also occur to a change in intrinsic fluorescence. To correct for the change in intrinsic fluorescence, a triplet-state component must be added to the model. Set the model by putting 1 for the triplet state, the triplet

relaxation time as 8 μs, and the structural parameter as 6 (a well-aligned system will have a structural parameter between 5 and 7). The diffusion time of rhodamine 6G should be between 20 and 30 μs in water. If the diffusion time largely deviates from the expected value, readjust the focal plane, the correction collar, and pinhole position to achieve fine measurements. Note that the number of components and triplet-state relaxation time might vary between different molecules of interest and different fluorescent probes used in the experiment.

The setups for FCS and FCCS are similar, but an additional laser will be used for FCCS. The second laser will excite the second fluorophore and create a second confocal volume at the same time as the first laser. Fluorescence fluctuations from both channels are recorded. Thus, three plots will be generated. Each channel will have its own autocorrelation plot and there will also be a cross-correlation plot. The model for FCCS is as follows and relays information on the cross-correlation function, G_x, a measure of how well the fluorescence fluctuations of two channels correlate over time:

$$G_x = \frac{\langle \delta F_A(t) \cdot \delta F_B(t+\tau) \rangle}{\langle F_A(t) \rangle \langle F_B(t) \rangle}$$

where t is time and $F(t)$ is the fluorescence fluctuation in either the green or red channel.

3.3.3 Obtaining Traces for Individual Cells

1. Seed cells into an imaging chamber on a coverslip at least 30 min before imaging. The temperature should be at 22–25 °C to maintain cell viability.
2. Right before imaging, change the media to water or low fluorescence media. *D. discoideum* wild-type cells are relatively motile and photosensitive; therefore acquisition should be made within a short time frame. Select the desired light path and set the acquisitions to take images every 2 s with ten repetitions.
3. Make acquisitions from the background. Select cells and regions of cells with expression levels that are significantly stronger than background but are not oversaturated. Count rates below 50 or over 1000 should be avoided.
4. Acquire images of cells before and after taking FCS/FCCS measurements. The images are taken afterward to ensure cells do not move out of the imaging volume during acquisition. For each experiment, we recommend collecting at least 20 measurements to be able to draw conclusions from the acquired data.

3.3.4 Data Analysis

1. Open measure files in analysis software such as Zen Black [22]. Manually inspect each trace. Avoid traces with noticeable downward trends, which indicate photobleaching, and large broad peaks, which indicate aggregates. Significant deviation in count rate indicating active movements should also be avoided.
2. Take the average of the remaining trace measurements, and in the analysis tab, fit the averages to the model established from the system calibration.
3. Evaluate the fitted data and compare to acquired traces. If there is a large deviation between the correlation curves and fits in general, the fitting parameters might be inappropriate. Discard fits with significant deviations.
4. From the fitted data, parameters such as the diffusion times can be derived. The diffusion time is extracted by the negative slope of the fitted data.

3.4 Fluorescence Recovery After Photobleaching (FRAP)

With cell lines expressing fluorescently tagged proteins, fluorescence recovery after photobleaching (FRAP) is a quantitative method that can be used to uncover protein turnover dynamics. As diffusion is generally much faster, these dynamics are frequently dominated by the off-rates of a protein from the structures with which it is associated. The method entails a fluorescent region of interest (ROI) in a cell being photobleached with a high intensity laser. The fluorescence in the ROI recovers over time due to the movement of fluorescent molecules into the photobleached area. The time it takes for the fluorescence to recover is measured against the fluorescence intensity to generate a curve (Fig. 7). From the data, the mobile and immobile fractions of the protein can be identified, and kinetic parameters can be calculated such as diffusion times and time to dissociate from binding interactions [23].

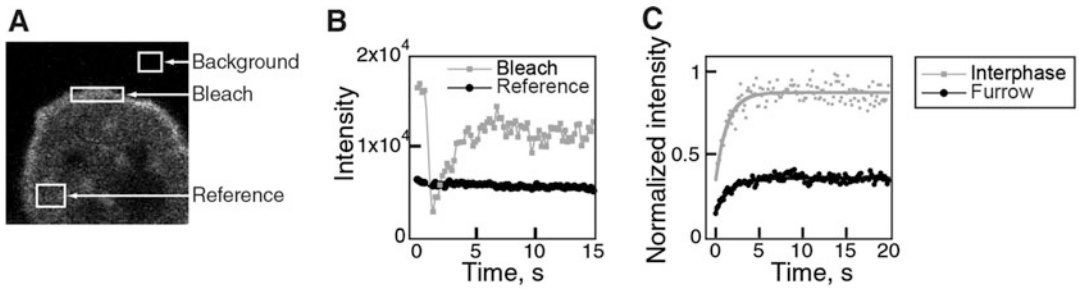

Fig. 7 Fluorescence recovery after photobleaching (FRAP) measures protein turnover dynamics. (**a**) Example image of a *D. discoideum* cell-expressing GFP-cortexillin I. The average intensities for the labeled regions are collected for FRAP calculations. (**b**) An example graph of fluorescence intensity over time for the bleached and reference regions. (**c**) After background subtraction, the intensity is fit to an exponential curve. The reference region stays consistent while the bleached region regains fluorescence intensity over time. (Reproduced from reference 25 with permission from Elsevier)

Applying this method to cytokinesis, FRAP can be used to characterize and quantify protein mobility during cell division. For example, a study utilizing FRAP in *D. discoideum* demonstrated that myosin II, a protein enriched at the cleavage furrow, is largely recruited from a cytoplasmic pool while some myosin II also flows along the cortex [24]. Interactions between different cytokinesis proteins can also be analyzed using FRAP. We measured the recovery times for various cytoskeletal proteins through FRAP including actin and proteins at the cleavage furrow such as myosin II, cortexillin I, and IQGAPs 1 and 2 [25]. These cleavage furrow proteins have slower recovery times in the cortex than in the cytoplasm, indicating that their recovery in the cortex is highly dependent on unbinding events rather than diffusion. We continued to build upon the idea of the cleavage furrow proteins participating in a meshwork by expressing these proteins in and using FRAP on mutant cell lines lacking myosin II, cortexillin I, and the IQGAPs.

Below, we detail the experimental setup for FRAP in *D. discoideum* and how to analyze the data output.

3.4.1 Experimental Setup

1. Grow cells expressing a fluorescently tagged protein in a glass bottom imaging chamber for 1 h.
2. Replace culture medium with MES starvation media before imaging.
3. Conduct experiment using a confocal microscope with a 63× objective. Take images of cells before photobleaching for later calculations.
4. Pick a small region of the cell and photobleach with a laser of the excitation wavelength of the fluorescent protein.
5. Measure time of recovery until the region is saturated (150 frames, 45–150 ms/frame depending on protein).

3.4.2 Data Analysis

1. For each frame, measure the average intensity of the bleached region, a reference (unbleached) region, and the background with analysis software such as ImageJ. Subtract the average background intensity from the average intensities of the bleached region and the reference region.
2. To correct for photobleaching, calculate the reference theoretical intensity (RTI) by fitting the background subtracted reference intensity with the following equation:

$$\mathrm{RTI}(t) = A - B \cdot \mathrm{e}^{-C \cdot t}$$

   where A, B, and C are fitting parameters.
3. Normalize the background subtracted bleached region intensity to RTI.

4. Calculate the normalized intensity (NI) by normalizing values in **step 2** to the pre-bleach intensity (the average of 4 pre-bleach images) to the following equation:

$$\mathrm{NI}(t) = m_1\left(1 - m_2 \cdot \mathrm{e}^{-kt}\right)$$

where m_1, m_2 are fitting parameters and k is the recovery rate.

5. The recovery time (τ) and the immobile fraction, F_i, can be measured with the following equations:

$$\tau = \frac{1}{k}$$

$$F_i = \frac{1 - m_1}{1 - m_1 + m_2}$$

3.5 Micropipette Aspiration (MPA)

While cytokinesis is a process that involves the biochemical interactions between various molecules, it also depends on the generation of force and the reaction of mechanosensitive components in the cell to this force [26–27]. Methods such as atomic force microscopy (AFM) [28], optical traps [29], and cell compression [30] can be used to study how cells respond to force, but they come with several caveats. AFM requires the cells to be attached to a surface, which can be problematic as *Dictyostelium* cells tend to be fairly weakly adhered to substrates. Optical traps lead to localized increases in heat from the laser light. Generating sufficient forces with an optical trap to deform a *Dictyostelium* cell can lead to lethality due to the heat. Cell compression is a fast, high-throughput method for applying mechanical stresses to cells, which can be very useful for many types of studies [31]. Micropipette aspiration (MPA) remains one of our preferred techniques as it allows for a well-controlled, specific amount of force in the nanoNewton (nN) range to be applied to a well-defined surface area (up to tens of μm^2) of the cell. The forces applied through MPA mimics the forces generated by a cell during cytokinesis, making it useful for observing how cytokinetic proteins sense and respond to mechanical perturbations. Proteins that do respond, which are mechanoresponsive, can change protein localization when a force is applied to the cell. In a previous study, we identified such mechanoresponsive cytokinetic proteins including myosin II and cortexillin I [32]. In addition, we used MPA as a tool for uncovering and expanding on the pathways leading to mechanosensory responses. Mitotic regulator proteins such as kif12 (*Dictyostelium* kinesin-6) and INCENP are also mechanoresponsive and are dependent on myosin II because they are non-mechanoresponsive in knockout cell lines lacking myosin II [33].

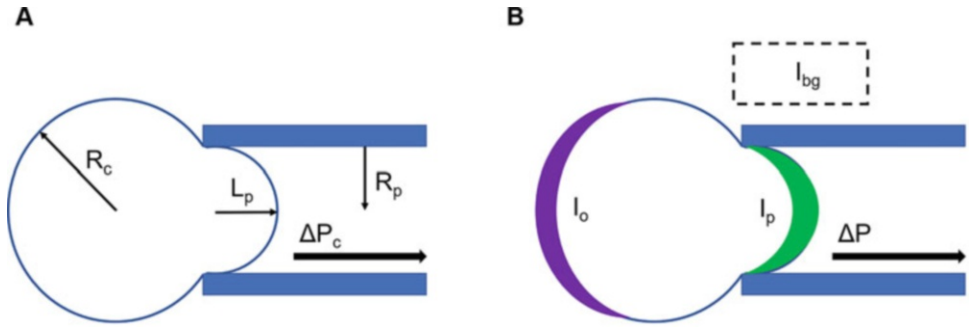

Fig. 8 Micropipette aspiration (MPA) is a tool for measuring cortical tension and mechanosensitivity. (**a**) A pipette aspirates on a cell with radius, R_c, until the critical pressure (ΔP_c) is reached, which is the point at which the length of the cell deformation into the pipette, L_p, equals R_p, the radius of the pipette. From this pressure, the cortical tension may be calculated. (**b**) To measure the mechanoresponse of a fluorescent protein, the mean intensities of the region inside the pipette (I_p), the cortex from the opposite side of the cell (I_o), and a background region outside of the cell (I_{bg}) are collected. The cell is aspirated on by a pipette with a fixed pressure (ΔP) set prior to contacting the cell

MPA can also be used to measure mechanical parameters in the cell, such as cortical tension. As shown in Fig. 8a, as the cell, with radius R_c, is aspirated upon by a pipette with radius, R_p, it will form a hemispherical deformation inside the pipette at the equilibrium pressure (ΔP_c). At the equilibrium pressure, the radius of the hemisphere, L_p, equals to R_p. With these parameters, the cortical tension can be measured with the following equation:

$$\Delta P_c = 2T_c\left(\frac{1}{R_p} - \frac{1}{R_c}\right)$$

3.5.1 Preparing the MPA System

The setup of an MPA system and data analysis have been previously detailed in refs. 34 and 35, but briefly, we proceed with the following steps for MPA.

3.5.2 Loading and Locating the Micropipette

1. Before loading micropipette tip, loosen micropipette holder and push out drops of water, leaving a drop hanging out of the opening of the micropipette tip. This will ensure that air bubbles will not be introduced into the micropipette holder when loading the tip.
2. Fill the micropipette with MES starvation buffer or growth medium. Leave an excess droplet at the opening of the micropipette.
3. Load the micropipette midway into the micropipette holder. Insert the micropipette into the holder droplet to avoid introducing an air bubble. Tighten the micropipette holder.
4. Place imaging chamber with cells onto the microscope stage. Select the 40× magnification and set up the DIC channel.

Move the micropipette into the light path and lower the tip until it is immersed. Adjust valve so that water manometer is connected to the micropipette.

5. Using the micromanipulator, move the micropipette close to the surface where cells are and position the tip into the center of the imaging field.

Calibrating the MPA System

1. To calibrate the system, locate the micropipette and connect it to the manometer.
2. Position the two water tanks so that they are the same level (home position).
3. Add ~20–50 μL of diluted bead solution (1 μm polystyrene beads diluted in 1:500 filtered-sterilized water) to the imaging chamber.
4. Move micropipette next to a free-floating microbead. The system is balanced when the bead does not move toward or away from the micropipette opening.
5. If the bead does move, adjust the manometer by raising it if the bead is going toward the pipette opening. Lower the manometer if the bead moves away from the pipette.

3.5.3 Cortical Tension Measurements

1. After loading the micropipette and calibrating the system, set up time acquisition using the DIC channel with 5 s intervals.
2. On a healthy suspended cell, apply pressure to the cell, starting with low pressures (around 10–15 mm of water pressure).
3. Increase the pressure by 5 mm every five frames until cell is deformed into pipette until $L_p = R_p$. Finer step sizes are required to home in on the final pressure at which point $L_p = R_p$.
4. Collect at least three measurements per cell. Wait for 5 min between each measurement for the cell to regain stability or aspirate on a different region of the cell.

3.5.4 Protein Accumulation Quantification

1. Put cells in MES starvation buffer on imaging slide and place sample on microscope imaging stage.
2. Calibrate system.
3. Set up image acquisition with DIC channel and channels for fluorescent proteins. Set up time intervals for 15–30 s.
4. Increase pressure in micropipette prior to contacting the cell. Keep pressure fixed and aspirate the cell.
5. Using an image analysis software such as ImageJ (https://imagej.nih.gov/ij), collect the mean gray values from the cell region inside the pipette (I_p), the cortex from the opposite side

of the cell (I_o), and a background region outside of the cell (I_{bg}) (Fig. 8b). Calculate the mechanosensitive protein response with the following equation:

$$\text{Normalized } I = (I_p - I_{bg})/(I_o - I_{bg})$$

4 Notes

1. The water used in the materials and methods mentioned is always deionized and double distilled.
2. The voltage settings can vary based on strain of *D. discoideum*. The electroporation settings (3.0 μF and 1.3 kV) are optimized for our lab's ORF+7–3 (Ax3:Rep orf+ (HS1000)) cells (from reference 9). We use 1.1 kV for 11–5.1 cells [9].
3. For a larger-scale screen, more pools of 30,000–35,000 clones can be analyzed. We have analyzed up to 240,000–280,000 independent clones [9].
4. STBL2 *Escherichia coli* are generally used for cloning because *D. discoideum* DNA sequences are AT-rich, making them unstable in many other *E. coli* strains.
5. For cells in drug media or mutants that may have growth defects, it is important to start suspension culture at a higher initial cell density (at least ~1×10^5 cells/mL).
6. Initial cell densities from the second or third cycle can be lower than those of the first cycle, roughly $0.5–1 \times 10^5$ cells/mL, as cell growth becomes more consistent.
7. After being mounted, coverslips can be sealed with nail polish to avoid dehydration and stored at −20 °C for later visualization.
8. Transforming with multiple plasmids is possible if each individual expression vector confers resistance to different selection markers.
9. The amount of time for colonies to appear can vary based on which genes are being transformed. Colonies usually form after 5–7 days but some can appear after 4 weeks.
10. G418 concentrations for drug selecting RNAi plasmids typically fall between 7.5 μg/mL and 15 μg/mL. For genes that are critical to cell survival and growth, a high concentration of G418 selection for 1 or 2 days, followed by a sudden drop and a slow ramp-up of drug concentration, is sometimes helpful for transformed cells to adjust and grow into colonies. The time frame and drug concentration of selection regime have to be empirically determined.

11. Ampicillin-containing LB plates should not be in 30 °C for more than 20 h to avoid the breakdown of ampicillin and growth of non-transformants.
12. SM-5 plates should be stored at 4 °C and used within 30 days to avoid dehydration.
13. During drug selection, dead floating cells can be gently washed with Hans' Enriched HL-5 media containing 15 μg of G418 once every 2 days. It is important to eliminate as many floating cells as possible before plating transformants on SM-5 plates to avoid growth of non-transformants.
14. Additional markers including tubulins or nuclear proteins should be considered in order to differentiate from pseudopod protrusion and retraction.

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