Chapter 4

Imaging of the Cytoskeleton Using Live and Fixed *Drosophila* Tissue Culture Cells

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Abstract

In recent years, the convergence of multiple technologies and experimental approaches has led to the expanded use of cultured *Drosophila* cells as a model system. Their ease of culture and maintenance, susceptibility to RNA interference, and imaging characteristics have led to extensive use in both traditional experimental approaches as well as high-throughput RNAi screens. Here we describe *Drosophila* S2 cell culture and preparation for live-cell and fixed-cell fluorescence microscopy and scanning electron microscopy.

Key words Fluorescence microscopy, S2 cells, Transfection, Microtubule, Actin, Electron microscopy

1 Introduction

The marriage of Drosophila tissue culture and cytoskeletal research has been a fruitful one. Numerous cytoskeletal proteins have been discovered using S2 cells [1], which is a testament to their practicality. Major advances in our understanding of cytoskeletal dynamics and function have been achieved using S2 cells, an indication of their versatility and strength as a model system [2–10]. Drosophila cells are cultured at room temperature in a medium that does not require buffering with CO_2. RNAi is both effective and easily administered [11]. Drosophila cells do not produce an interferon response to dsRNAs. Therefore, dsRNAs can be produced in vitro, eliminating the need to buy siRNAs. S2 cells readily incorporate dsRNAs when added to tissue culture medium. dsRNAs can also be generated against the 5′ and 3′ untranslated regions (UTRs) allowing for the depletion of endogenous target genes, and testing of exogenous genes for their ability to rescue RNAi phenotypes. RNAi protocols can involve as few as 3 days, depending upon the turnover of a given protein and frequently lead to substantial protein knockdown [12]. The Drosophila genome represents a pared

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down form of the mammalian genome and while approximately 75 % of human disease causing genes are retained in the Drosophila genome, there is far less functional redundancy [13–15]. Whereas the human or mouse genome may have three genes that are functionally interchangeable, the Drosophila genome will often have one. Collectively, these qualities have led to hundreds of RNAi screens of the full Drosophila genome at a fraction of the cost of screens preformed in mammalian tissue culture systems.

The usefulness of Drosophila S2 cells as tools for cytoskeletal research goes beyond their use in RNAi screens. S2 cells in particular adopt a flat, fried-egg-like morphology when plated on a concanavalin A (Con A)-coated surface [16]. This conformation makes them highly amenable to high-resolution microscopy [17], and their sessile nature eliminates the problems produced by motile cells. S2 cells form a circumferential actin-rich lamellipodium with characteristic fast actin dynamics where proteins such as the Arp2/3 complex, Capping Protein, and Ena/VASP proteins localize [5]. This lamellipodium is followed by a contractile network highly reminiscent of a lamella with its own characteristic slower actin dynamics and signature proteins such as tropomyosin, non-muscle myosin II, and alpha-actinin (Fig. 1) [18, 19]. This is even more evident as these actin-rich zones of S2 cells display similar phenotypes to those of migrating cells following RNAi depletion of these key actin-binding proteins [5, 20–22]. Thus, despite being non-motile, the behavior and dynamics of the actin-rich cell periphery of S2 cells mimics that of migrating cells. S2 cells have also been quite useful in studying microtubule dynamics. Our current

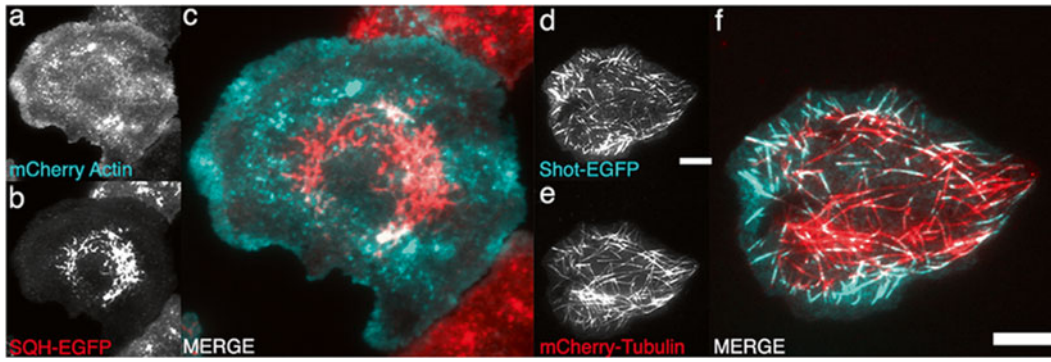

Fig. 1 Live imaging of cytoskeletal probes in *Drosophila* S2 cells. (**a**–**f**) Shown is a single time point from a live-cell image sequence of S2 cells imaged by TIRF-M. (**a**) An S2 cell co-expressing mCherry-Actin and (**b**) the regulatory chain of non-muscle myosin II (Spaghetti-Squash, Sqh), tagged with EGFP. (**c**) Shown at higher magnification, the merged image of (**a**) and (**b**) where mCherry actin pictured in (**a**) is shown in *cyan* and EGFP-Sqh pictured in (**b**) is shown in *red*. (**d**) An S2 cell co-expressing EGFP-Short stop (Shot) and (**e**) mCherry-α-Tubulin. (**f**) The merged image of (**d**) and (**e**) where Shot-EGFP pictured in (**d**) is shown in *cyan* and mCherry-α-Tubulin pictured in (**e**) is shown in *red*. Scale bars in both low and high magnification images are 10 μm

understanding of spindle formation, in particular poleward flux, kinetochore attachments, and the role of molecular motors during mitosis owe much to experiments performed in S2 cells [16, 23–30]. In addition, the acentriolar interphase microtubule array offers a unique system to study with great detail the dynamics of the microtubule plus-end and minus-end as well as interactions between the actin and microtubule cytoskeletons [16, 31–36].

S2 cells are easily transfected with tagged protein (fluorescent or otherwise), but are naturally non-adherent and must be induced to attach to a substrate. If the cells have not been attached to a substrate, they will be washed away during fixation, permeabilization, and staining. There are two main coatings that will induce cell attachment, concanavalin A (Con A) and polylysine. These two coatings differ in how the cell responds to them. Con A is a lectin (sugar binding protein) that is commonly purified from the jackbean (*Canavalia ensiformis*), and it presumably induces cell spreading in a Rac and Arp2/3-dependent manner as cells try to engulf the Con A coated substrate [5]. Due to the strong inducement of spreading as well as the engagement of the phagocytic machinery to the basal cortex of the cell, many membrane trafficking events (including cytokinesis) are disrupted in Con A plated cells, and these side effects should be considered when deciding whether to use Con A to induce adherence. Polylysine is a positively charged polymer of lysine that electrostatically interacts with negatively charged cell membranes and induces cell attachment and mild amounts of cell spreading. Polylysine is available as both L and D forms and available in a range of molecular weights. We have found that Drosophila cells need higher concentrations of polylysine than mammalian cells to attach to the substrate, and they tend to be highly sensitive to the oxidizing effects of thimerosal. Therefore, it is not recommended to purchase premade polylysine that uses this chemical as a preservative.

2 Materials

2.1 Culturing and Transient Transfection of Drosophila S2 Cells

1. Schneider's Drosophila Medium (Gibco/Life Technologies).
2. Fetal bovine serum, heat inactivated.
3. 100× antibiotic/antimycotic (Gibco/Life Technologies).
4. pMT/V5 His, pIZ/V5, and/or pAc5.1 vectors (Life Technologies).
5. $CuSO_4$: 100 mM aqueous solution.
6. FugeneHD (Promega).
7. Amaxa Kit V (Lonza) (optional).
8. Nucleofector Machine (Lonza) (optional).

2.2 Fixation and Staining of Drosophila S2 Cells for Immunofluorescence

1. 1 mg/mL Polylysine: Dissolve Poly-D-Lysine or Poly-L-lysine (70,000–150,000 average molecular weight) in 50 mM Tris–HCl, pH 8.0. Aliquot and freeze at −20 °C.
2. 0.5 mg/mL concanavalin A(Con A): Dissolve Con A (Type IV-S, tissue culture grade) at 0.5 mg/mL in distilled water. Filter the Con A solution through a 0.22 μm filter into a fresh tube or centrifuge at 18,000 × *g* (14,000 RPM) for 5 min to pellet aggregates. Retain the supernatant, and store it in 0.5 mL aliquots in 1.5 mL tubes at −20 °C.
3. 1 M Tris–HCl, pH 8.0.
4. #1.5 glass coverslips, glass-bottom or optical plastic multi-well plates.
5. Humidified chambers.
6. Paraformaldehyde: EM Grade packed in ampoules or made fresh.
7. Glutaraldehyde: EM Grade.
8. PHEM Buffer pH 6.9: 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$. This buffer can be made as a 2× stock solution, autoclaved, and stored until use.
9. Sodium borohydride: aqueous solution 1 mg/mL.
10. Saponin: 10 mg/mL aqueous stock solution.
11. Donkey or goat serum.
12. Bovine serum albumin.
13. Triton X-100.
14. Mounting medium: (Dako, ProLong (Life Technologies), Fluoromount (Sigma), VECTASHIELD (Vector Laboratories), or a solution of 90 % glycerol, 20 mM sodium bicarbonate (from 0.5 M aqueous solution, pH 9), 3 % *n*-propyl gallate.
15. CoverGrip (Biotium) or nail polish.

2.3 Live Cell Imaging

1. #1.5 glass-bottom dishes.
2. Titanium Step Drill Bit—1/4″ to 1-3/8″ Increments, ten steps (Neiko) (optional).
3. 120 V 10-in. Drill Press (optional).
4. Norland Optical Adhesive 81 (Norland) (optional).
5. UV transilluminator (optional).
6. Cell-Tak (Corning) (optional).
7. Poly-lysine (optional).

2.4 Fixation and Preparation of Drosophila S2 Cells for Scanning Electron Microscopy

1. Silver paint.
2. Double-sided conductive tape.
3. Critical point dryer.
4. Coverslip holder for critical point dryer.
5. 200 proof ethanol.
6. Sputter coater.

3 Methods

3.1 Culture of Drosophila S2 Cell (See* Note 1*)

1. Maintain *Drosophila* S2 cells at room temperature (preferably between 19 and 27 °C) and in semi-suspension in 25-cm or 75-cm flasks with plug-end caps.
2. Passage cells by pipetting them from the culture medium, making sure that the cells are adequately resuspended and that any cells lightly adhered to the tissue culture plastic are removed. Generally, a 1:4 split of the cells into fresh cell culture medium will yield a cell density that will require passage every 3–4 days. It is important to note that S2 cell viability decreases when the cell density falls below 5×10^5 cells/mL.

3.2 Transient Transfection of Drosophila S2 Cells (See* Notes 2 *and* 3*)

1. Plate S2 cells at 40–80 % confluency in a 6 or 12-well tissue culture plate 15–30 min prior to transfection. Alternatively, plate cells a day in advance of transfection. This step is needed as the transfection protocol calls for the exchange of medium, and the cells will need this time to loosely adhere to the tissue culture plastic.
2. If using the Fugene HD transfection reagent (Promega) (*see* **Note 3**), prepare the DNA–Fugene HD complex by diluting the desired amount of DNA in water or serum free Schneider's medium to a final volume of 100 μL. Next, add 6–8 μL of Fugene HD reagent directly to the diluted DNA with special care taken to avoid pipetting the reagent down sides of the microfuge tube. Incubate the DNA–Fugene HD mixture for 15–20 min at room temperature.
3. While the incubation is in progress, gently remove the cell culture medium from the tissue culture plate, taking precaution not to disturb the weakly adherent cells. Replace the cell culture medium with 900 μL of fresh cell culture medium. Add the 100 μL DNA–Fugene HD complex dropwise to the 900 μL of medium containing the cells. The transfection is carried out overnight, and the medium is replaced with fresh medium the following day.
4. Alternatively, transiently transfect S2 cells by using electroporation systems such as Amaxa nucleofector from Lonza. The Amaxa nucleofector kits are tailored to specific cell lines. The manufacturer suggests Kit V for Drosophila S2.

5. The Amaxa kit provides a nucleofection solution and a supplement solution. Mix these two solutions at a ratio of 4.5:1 prior to use. The mixed nucleofector reagent can be stored at 4 °C for up to 3 months.
6. Prepare DNA for electroporation by diluting the desired amount of DNA, typically between 0.1 and 2 μg of DNA, in the nucleofector reagent to a final volume of 100 μL in a microfuge tube. This can be prepared and set aside until the cells are ready.
7. Determine the appropriate volume of cells and pellet them by gentle centrifugation at 300 × *g* at room temperature for 5–10 min.
8. Next, remove the supernatant (tissue culture medium) (*see* **Note 4**).
9. Resuspend the pellet of cells in the diluted DNA–nucleofector mixture, and transfer the mixture to the electroporation cuvette (*see* **Note 5**).
10. After electroporation, transfer cells to fresh medium, and allow them to recover prior to $CuSO_4$ induction if using pMT/His-5 vectors (*see* **Note 6**).

3.3 Fixation and Staining of Drosophila S2 Cells for Immunofluorescence (See **Note 7***)*

3.3.1 Preparing the Surface of Coverslips (See **Notes 8** *and* **9***)*

1. For flame cleaning, use fine tipped forceps to grab an individual coverslip, and dip it into absolute ethanol. Allow most of the ethanol to run off the edge of the coverslip, and then wave the coverslip through an open flame. As the ethanol burns, continue to tilt and move the coverslip to prevent the ethanol from pooling in a single corner and superheating the glass.
2. Place flamed coverslips in a rack and rinse thoroughly in distilled water. Then place the coverslip rack over a heat block to allow them to dry thoroughly. Cover and store to block dust accumulation.

3.3.2 Coating of Substrates

Con A Coating of Coverslips

1. Cover the bottom of a broad shallow chamber with Parafilm, and then place the cleaned coverslips onto the Parafilm.
2. For 22 × 22 mm coverslips, put a 50 μL drop of Con A on the center of the coverslip and then evenly smear the drop over the surface of the glass.
3. For 18 × 18 mm coverslips use a 35 μL drop.
4. Place the coverslips in an out-of-the-way location where they will not accumulate dust, and allow to dry overnight.

Con A Coating of 96-Well Plates

1. Dilute Con A to 0.05 mg/mL and then:
2. Add 80 μL of Con A to each well and allow to dry over at least 2 days.

Polylysine Coating of Coverslips

1. Place coverslips into the Parafilm lined chamber.
2. Spread 50 μL of polylysine onto coverslips and let sit at room temperature for 3 h.
3. Rinse coverslips three times with distilled deionized water, and then aspirate excess water.
4. Allow the coverslips to dry, and then store at 4 °C until needed.

Coating of Coverslip-Bottom Imaging Chambers

1. Add a 100 μL drop of Con A or polylysine to the center of the coverslip, and spread it evenly over the surface.
2. Use the tip of a p100 pipette to remove any excessive fluid accumulation that might pool in the interface between the glass bottom and plastic side.
3. Allow Con A coated chambers to dry overnight. Allow lysine-coated chambers to sit for 3 h before rinsing them 3× with distilled deionized water. Dry them by aspirating any leftover fluid and then store them at 4 °C.

3.3.3 Plating of Cells (See **Note 10***)*

1. Resuspend cells that have been growing in a 96-well plate or 6-well dish.
2. Place prepared coverslips into a 6-well plate or 35 mm dish.
3. Place a drop of 100–200 μL of fresh medium onto the center of the coverslip.
4. Place a drop of the cell suspension into the fresh medium and pipette to mix. This step is very empirical. Allow the cells to settle to the surface and observe their density. Initially, add fewer cells to the coverslip, plate, or chamber than you think you will need. After 20 min the cells can be checked for density, and more cells can be added to achieve the desired density.
5. Add additional medium to the wells after the cells have attained the desired density and have begun to attach to the coverslip.
6. Initiate fixation, observation, or further manipulation after 1 h.

3.3.4 Fixation and Staining (See **Note 11***)*

1. Remove the culture medium from each well of the six-well plate or 35 mm dish, and fix cells for 10 min by adding in a solution of 0.3 % glutaraldehyde, 4 % formaldehyde in 1× PHEM supplemented with saponin (1 mg/mL).
2. Remove fixative and incubate cells with PHEM-T (1× PHEM + 0.5 % Triton X-100) for 5 min.
3. Rinse cells in PHEM.
4. Reduce unreacted aldehydes in the sample by three treatments for 10 min with a solution of 1 mg/mL sodium borohydride dissolved in PHEM (Make immediately before use).

5. Wash the coverslips three times in PHEM-Wash (1× PHEM + 0.1 % Triton X-100)—5 min per wash.
6. Block nonspecific antibody binding sites and reduce background by incubating cells in a blocking solution (PHEM-Wash + 5 % Boiled donkey serum) for 30 min to 1 h at room temperature (*see* **Note 12**).
7. Incubate cells in primary antibody for 1 h at room temperature or overnight at 4 °C if antibody staining is desired. Dilute antibodies to the appropriate concentration with blocking solution; determine concentrations empirically for each antibody. Dot 50 μL drops of antibody solution onto a Parafilm sheet in a humidified chamber, and place a coverslip on top of each drop. Ensure that the cell-adhering side of the coverslip makes contact with the drop of antibody solution.
8. Wash cells 4 × 5 min in PHEM-Wash.
9. Perform secondary antibody incubation as described for primary antibody using fluorescent secondary antibodies and additions such as DAPI or fluorescent-labeled phalloidin diluted in blocking buffer.
10. Wash coverslips 4 × 5 min in PHEM-Wash.
11. Rinse cells 1× in PHEM and 1× in distilled water to remove salts.
12. Wick away excess fluid from coverslips and then mount them (cell side down) on slides using one drop of a mounting media.
13. Use vacuum aspiration to remove any excess mounting media from around the edges of the coverslips and then seal them with a hardening material such as nail polish, VALAP, or CoverGrip (Biotium).

3.4 Live Cell Imaging (See Note 13*)*

1. Plate S2 cells on glass-bottom dishes (*see* **Note 14**) that have been coated with a lectin solution, concanavalin A (con A). Con A should be applied to the glass surface and allowed to incubate for 1–2 min.
2. Remove con A, carefully ensuring that the reagent is completely removed, and allow the dishes to dry before the addition of cell culture medium containing cells. Allow 30–60 min for S2 cells to fully attach and spread on the con A coated surface.
3. Alternatively, plate S2 plate on Polylysine or Cell-tak (Corning Cat. No. 354240) coated glass. See manufacturer's instructions for coating glass-bottom dishes with Cell-tak.
4. Perform imaging in Schneider's Insect Medium supplemented with 10 % heat-inactivated FBS and antibiotic. Imaging is conveniently carried out at room temperature without CO_2.

3.5 Fixation and Preparation of Drosophila S2 Cells for Scanning Electron Microscopy

3.5.1 Fixation (See **Note 15)**

1. Equilibrate fixative to the same temperature as the cell culture. Remove the cell medium from the coverslips, and replace it with a solution of 4 % glutaraldehyde with 4 % paraformaldehyde in 0.1 M Phosphate Buffered Saline.
2. Incubate the sample in the fixative for at least 30 min at room temperature.
3. Store samples for a maximum of 2 weeks at 4 °C if desired.

3.5.2 Dehydration and Critical Point Drying (See **Note 16)**

1. Wash the sample three times for 5 min in distilled water to remove fixative.
2. Exchange distilled water for 30 % ethanol for 5 min, and continue through dehydration with 5 min steps in 50, 70, 90, 100 % ethanol, using three washes at each ethanol concentration.
3. Under 100 % ethanol, stack coverslips in a CPD holder with a washer between each.
4. Fill the CPD chamber with enough dehydrated ethanol to cover the holder. Place the holder in the chamber. Operate the CPD as directed by the manufacturer's instructions.

3.5.3 Mounting and Sputter Coating (See **Note 17)**

1. Remove dry coverslips from the CPD chamber and adhere onto SEM stubs with double-sided conductive tape.
2. To further facilitate conductivity, apply silver paint to create a bridge from the surface of the coverslip to the conductive tape or SEM stub.
3. After the silver paint has dried, place the samples in a sputter coater chamber.
4. Operate the sputter coater according to manufacturer's instructions.
5. Image the coverslips in a SEM (Fig. 2).

4 Notes

1. S2 cells can be maintained in either Schneider's Drosophila medium (available from many suppliers) supplemented with 10 % FBS and antibiotics, or serum free media that are designed for SF9 cell culture. However, live cell imaging should be performed in Schneider's medium as serum free media both produces background fluorescence and contains trace amounts of divalent ions, which tend to induce expression from pMT/V5-His vectors. S2 do not require CO_2, and they are tolerant of and perform well at high cell densities. Cells should be passaged when they become confluent. S2 cells are loosely adherent on tissue culture plastic that has been treated for cell culture and thus do not require trypsin/EDTA treatments.

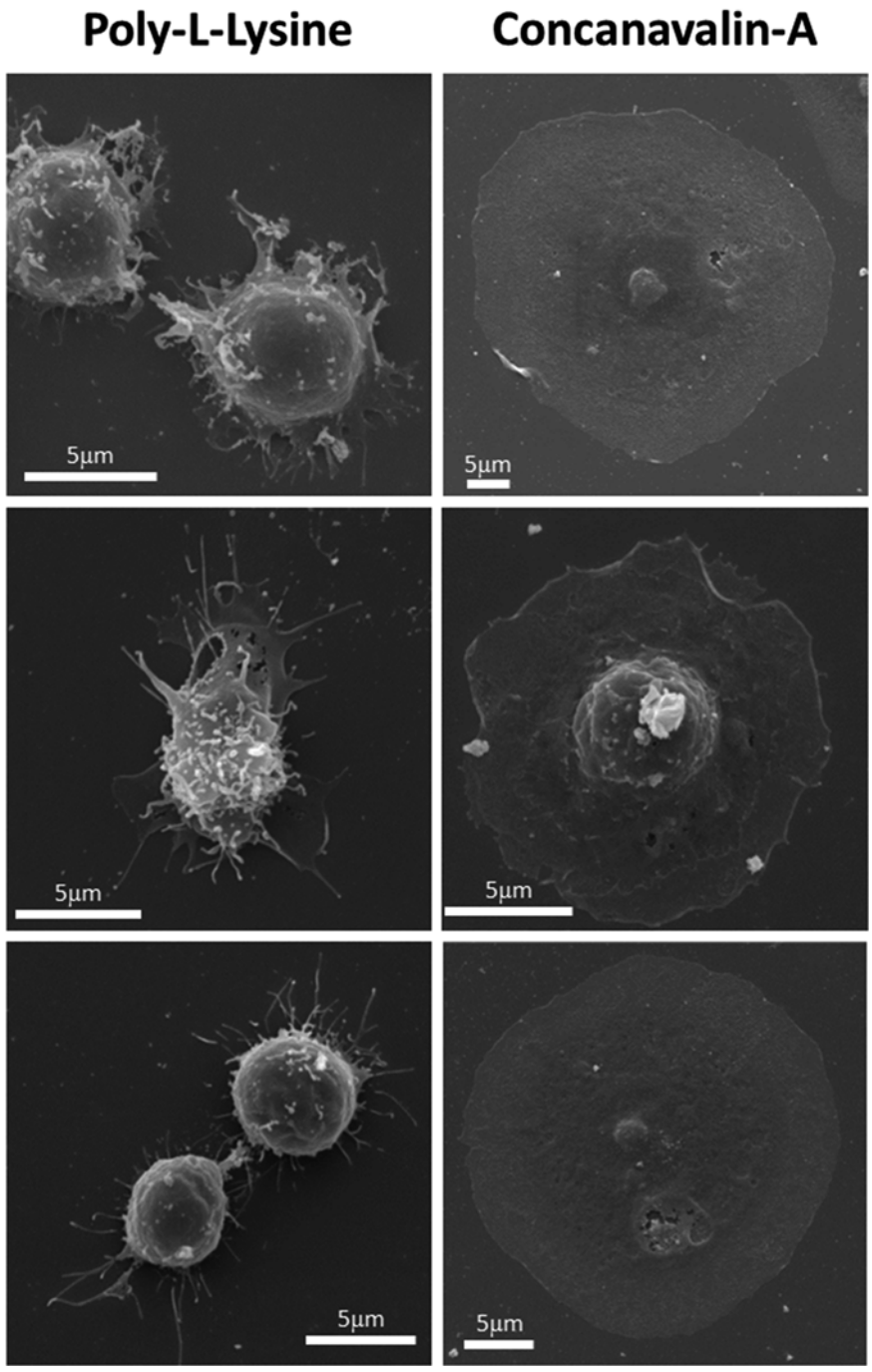

Fig. 2 Scanning electron micrography of *Drosophila* S2 cells. Shown are representative images of S2 cells allowed to adhere to glass coverslips coated with either poly-L-lysine or concanavalin-A. Cells were allowed to adhere for 1 h prior to fixation and processing for SEM. While the size and surface morphology for cells was somewhat variable, nearly all cells grown on concanavalin-A coated coverslips displayed a smooth dorsal surface morphology with few projections, a smooth-edge morphology and were well-spread. Scale bars are 5 µm

2. The amount of plasmid DNA transfected depends on the cytoskeletal protein being imaged and the *Drosophila* expression vector used. pMT/V5 His vectors that are copper inducible are typically transfected at a range of 0.1–2 μg DNA. An advantage of using pMT/V5-His vectors is that the amount of protein expressed can be titrated with the amount of $CuSO_4$ supplemented in the tissue culture medium prior to imaging. Successful imaging experiments have been carried out using vectors that have constitutively active promoters such as the pIZ/V5 vector, which uses the OpIE2 promoter and the pAc5.1/V5 which uses the *Drosophila* actin 5C promoter. However, it is often best to titrate the amount of DNA transfected in order to achieve optimal protein expression levels with these vectors. It is also possible to transfect in vectors containing the UAS promoter, which are usually generated for producing transgenic flies. To work with UAS vectors, one can co-transfect a pMT-Gal4 to allow for inducible expression of the UAS promoter. For live cell imaging of fluorescently tagged cytoskeletal proteins good results have been achieved by an overnight induction of 25–600 μM $CuSO_4$. It is particularly important to use the low end of this range of $CuSO_4$ when imaging microtubule plus-end tracking proteins (+TIPS) that are sensitive to mis-localization as a consequence of overexpression. Expression of other cytoskeletal proteins such as tubulin, actin, or actin-binding proteins that are less sensitive to the artifacts generated by overexpression, can be achieved by overnight induction at the higher end of this range.

   Transient transfection of cytoskeletal proteins is generally carried out 1–2 days prior to live-cell imaging. The exact timing is highly dependent on the vector and the expression of the protein of interest and an effort should be made to optimize this timing. For visualizing mitosis, it is usually best to wait 3 or more days before imaging. Mitosis is a rare event in a cells lifetime, and only 5 % of cells will be in mitosis at any point in time. If the transfection efficiency is 50 %, a culture will only have 2.5 % of cells transfected and in mitosis at any point during imaging. For this reason, stably transfected cells are usually preferred for studies of mitosis.

3. We have achieved good results using Fugene HD transfection reagent (Promega). It is always best to follow the manufacturer's instructions while preparing both the plasmid DNA and cells for electroporation. Lonza recommends 2×10^6 cells per transfection and from 0.1 to 2 μg of DNA per transfection.

4. It is important to remove as much tissue culture medium as possible without disturbing the pellet as residual media can interfere with the electroporation.

5. The electroporation machine provided by the Lonza system contains a preset S2 cell protocol, G-030 (or G-30 for Nucleofector I device).
6. Electroporation does lead to cell death but in general if the protocol is adhered to, the amount of cell death does not interfere with the progress of the experiment. Very high transfection efficiencies can be achieved with electroporation, however, transfection efficiency is highly dependent on plasmid DNA quality and the health of the cells prior to electroporation.
7. Drosophila S2 cells cannot be fixed and stained like mammalian cells. They are non-adherent and must be induced to adhere to coverslips or multiwell plates; they do not tolerate low-density cultures and therefore must be seeded carefully in order to function optimally. And they do not tolerate hypertonic buffers and therefore require specific buffers for washing and fixation.
8. In order to plate cells on glass, the hydrophobic coating found on most coverslips must be removed. There are several ways to do this: plasma cleaning, hydrogen peroxide + sulfuric acid soaking, and flame cleaning. Flame cleaning does not produce any chemical waste that requires storage and disposal or require a special piece of equipment.
9. S2 cells are only weakly adherent to glass surfaces, which must be functionalized in order to keep cells immobilized for fixation and staining.
10. S2 cells are extremely sensitive to plating density. If plated at too low of a concentration, they will not form extensive microtubule networks. Additionally, if one wishes to visualize mitotic cells, a too low density will make it very difficult to observe enough cells. However, plating at a too high density will inhibit their spreading and lead to cells stacking upon each other, resulting in out of focus noise in images. It is advisable to empirically determine how many cells to plate for any given application.
11. Many existing protocols for cell fixation work well for S2 cells. Several methods may need to be tested to find one that is compatible with both fixing the structure you wish to observe and that preserves the antigenicity of the molecules you wish to localize. Fixatives that work for S2 cells include –80 °C methanol, a mixture of methanol and formaldehyde (90 % methanol, 3.2 % formaldehyde, 5 mM sodium bicarbonate, pH 9 (from a 0.5 M stock) chilled to –80 °C prior to fixation), 4–10 % formaldehyde, and ice-cold acetone. The protocol in Subheading 3.3.5 uses a common fixation method that contains a mild detergent to give simultaneous fixation and permeabilization. Simultaneous fixation/permeabilization works well for many

antigens and structures as the detergent speeds up the access of the fixative while allowing some loosely bound protein to leave the cells, thereby reducing background. Alternatively, fixation followed by permeabilization or rapid fixation (10–60 s) followed by permeabilization and then 10 min of a second fixation can be utilized as well.

12. We use donkey serum and the highly cross-subtracted donkey secondary antibodies from Jackson ImmunoResearch diluted in 1× TBS + 0.1 % Triton X-100 + 2 % BSA.
13. Imaging is performed on an inverted microscope. Both the actin and microtubule cytoskeletons are sensitive to phototoxicity. Exposure to excess fluorescent light can quickly lead to a slowing or altering of the natural filament dynamics. Imaging should be performed to minimize the fluorescent light exposure. Both spinning-disk confocal and total internal reflection (TIRF) microscopy lend themselves to limiting the amount of light exposure while providing a good signal-to-noise ratio ideal for imaging proteins that are expressed at low levels. The speed of image acquisition by both these types of microscopy is also ideal for capturing the dynamics of the actin and microtubule cytoskeletons of live cells.
14. Glass bottom dishes can be made using a step drill bit (Neiko Titanium Step Drill Bit—1/4″ to 1-3/8″ Increments, 10 steps) installed in an upright drill press (SKIL 3320-01 120 V 10-in. Drill Press); however, commercial dishes with their superior flatness are highly recommended for TIRF. The bit is used to bore a circular opening in the bottom of a 35 mm plastic dish 1.5–2 cm in diameter. Cover-slips can then be glued to the dish, using any number of quick drying plastic compatible adhesives. We prefer an ultraviolet curing adhesive available from Norland (Norland Optical Adhesive 81) which quickly cures following a brief exposure (5–10 min) to ultraviolet light from a transilluminator.
15. Chemical fixation halts deterioration of the sample by crosslinking proteins. This crosslinking also provides some support for dehydration and critical point drying. Chemical fixatives are hazardous. Gloves and a fume hood should be used during fixation.
16. Samples must be dry to withstand the high vacuum of a scanning electron microscope. Air-drying creates enormous surface tension that causes samples to crumple losing their original surface shapes. To preserve a sample's structure, dehydration in ethanol and critical point drying (CPD) in carbon dioxide are used to dry the sample. During CDP 100 % ethanol, which is miscible with water and carbon dioxide, is exchanged for liquid carbon dioxide. Heat and pressure are applied to the

sample to reach the point where liquid carbon dioxide changes into gaseous carbon dioxide (the critical point). The sample is now dry. Having never been exposed to surface tension the original structures are preserved.

17. Scanning Electron Microscopy (SEM) requires conductivity throughout the system. Biological samples lose their conductivity once water is removed. Mounting samples on aluminum stubs facilitates conductivity from the SEM stage to the sample and provides a stable surface. Conductivity must continue across the surface of the sample. This requires the deposition of a thin layer of metal. Sputter coating uses a heavy inert gas (typically Argon) to create a plasma field around a metal target. The plasma field erodes the target so that metal atoms are ejected onto the surface of the sample.

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