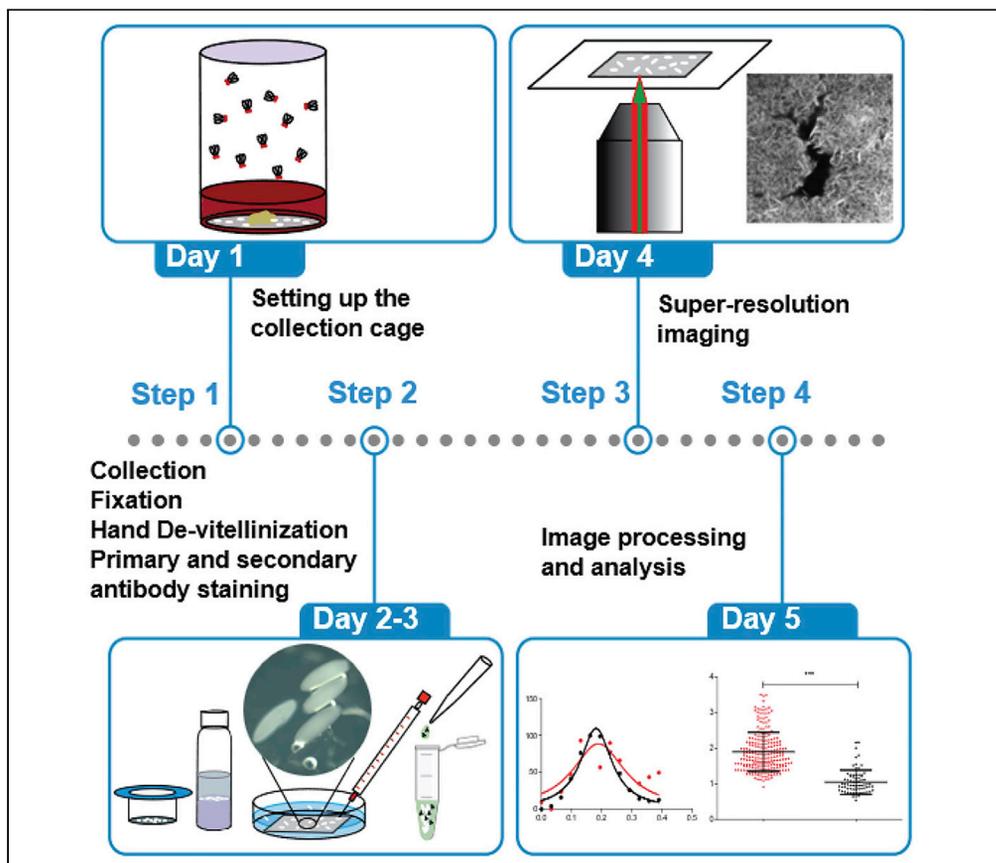


Protocol

Imaging and quantification of apical microvilli in the syncytial blastoderm of *Drosophila* embryos



The syncytial *Drosophila* blastoderm embryo contains apical microvilli with filamentous actin that are remodeled during nuclear division cycles 10–13. Here, we describe a protocol for preparing whole embryo samples and capturing images of microvilli using confocal and super-resolution STED microscopy. This protocol enables visualization and quantification of lengths and numbers of microvilli oriented along the imaging plane. We provide information on identifying different nuclear division cycles and examples of quantification from the interphase and metaphase of cycle 12.

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

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Highlights

Embryo sample preparation for imaging microvilli using confocal and STED microscopy

Identification of interphase and metaphase stages for nuclear division cycles

Visualization of apical microvilli using confocal and STED microscopy

Quantification of microvilli lengths and numbers from confocal and STED images

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Protocol

Imaging and quantification of apical microvilli in the syncytial blastoderm of *Drosophila* embryosDebasmita Mitra,^{1,4,*} Amruta Swaminathan,^{1,2} Gayatri Mundhe,^{1,3} and Richa Rikhy^{1,5,*}¹Biology, Indian Institute of Science, Education and Research, Pune 411008, India²Present address: Stowers Institute for Medical Research, Kansas City, MO 64110, USA³Present address: IBDM, Aix-Marseille Université, CNRS UMR 7288, Campus de Luminy, Marseille, France⁴Technical contact⁵Lead contact*Correspondence: mitra.debasmita@students.iiserpune.ac.in (D.M.), richa@iiserpune.ac.in (R.R.)
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SUMMARY

The syncytial *Drosophila* blastoderm embryo contains apical microvilli with filamentous actin that are remodeled during nuclear division cycles 10–13. Here, we describe a protocol for preparing whole embryo samples and capturing images of microvilli using confocal and super-resolution STED microscopy. This protocol enables visualization and quantification of lengths and numbers of microvilli oriented along the imaging plane. We provide information on identifying different nuclear division cycles and examples of quantification from the interphase and metaphase of cycle 12.

For complete details on the use and execution of this protocol, please refer to Sherlekar et al. (2020).

BEFORE YOU BEGIN

Drosophila embryo development begins as a syncytium wherein nuclei divide deep in the interior of the embryo until nuclear cycle 9. Nuclei then migrate to the periphery and nuclear cycle 10–13 occur at the cortex (Foe and Alberts, 1983). Each cortical nucleus is encompassed partially by the plasma membrane and an actin-rich cap is present between each nucleus and the plasma membrane during interphase of the syncytial division cycle. This actin cap bears filamentous actin protrusions termed apical microvilli (Turner and Mahowald, 1976; Warn et al., 1980; Foe and Alberts, 1983; Warn et al., 1984; Karr and Alberts, 1986; Mavrikis et al., 2009). The caps expand in area from interphase to metaphase of each nuclear division cycle (NC) and their microvilli are remodeled simultaneously, undergoing a concomitant reduction in lengths and numbers (Sherlekar et al., 2020). The apical cap remodeling is accompanied by extension of the plasma membrane in the form of furrows in between the nuclei and spindles. In this protocol, we use confocal and super-resolution STED microscopy to visualize apical microvilli present on the surface of embryos derived from the Canton-S wild-type strain of *Drosophila melanogaster*. We further show downstream image processing methods to resolve these structures using deconvolution. Finally, we demonstrate image analyses to elucidate the changes in microvilli architecture from interphase to metaphase in NC12.

Institutional permissions (if applicable)

Obtain appropriate institute biosafety permissions for import, maintenance and disposal of *Drosophila melanogaster* stocks and crosses.

Preparation of fly media and embryo collection cages

⌚ Timing: 45 min



1. Maintain the desired *Drosophila* stocks at 25°C in bottles containing fly media (see [materials and equipment](#) for the standard cornmeal agar recipe for fly media).
2. Prepare sucrose-agar plates for the embryo collection cages (see [materials and equipment](#) for the recipe).
3. Set up the embryo collection cages with at least 100 Canton-S flies per cage, as in shown in [Figure 1A](#).
 - a. Smear a dollop of yeast paste on the surface of a sucrose-agar plate.
4. Fix the plate on the mouth of the cage and invert the cage with the plate at the bottom.
5. Leave the cage undisturbed at 25°C for at least 24 h to allow the flies to acclimatize to the cage.

Preparation of fixative, buffers, blocking agent and mounting media

⌚ Timing: 3–4 h

Prepare the solutions listed below. See [materials and equipment](#) for recipes.

6. 10× PBS (Phosphate-buffered saline), pH 7.4.
 - a. Dilute the 10× stock with deionized water to make a 1× working solution.
7. 0.3% PBST (Phosphate-buffered saline containing 0.3% [v/v] Triton X-100).
8. 2% PBTA (PBS containing 0.3% [v/v] Triton X-100, 2% [w/v] bovine serum albumin (BSA), and 0.02% [w/v] sodium azide), 4°C.
9. 4% [w/v] Paraformaldehyde (PFA) solution for fixation, 4°C.
10. Mowiol-DABCO solution for mounting embryo samples, –20°C.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--|------------------------------|--------------------------------|
| Antibodies | | |
| Phospho-histone H3 (pH3) | Invitrogen, Molecular Probes | RRID: AB_2533589 |
| Alexa Fluor Anti-Rabbit 488 nm, 568 nm | Invitrogen, Molecular Probes | RRID: AB_2534114, AB_143157 |
| Alexa Fluor Phalloidin 488 nm, 568 nm | Invitrogen, Molecular Probes | catalog number: A12379, A12380 |
| Hoechst 33258 405 nm | Invitrogen, Molecular Probes | RRID: AB_2651133 |
| Chemicals, peptides, and recombinant proteins | | |
| Agar | local purchase | non-molecular biology grade |
| Yeast | local purchase | non-molecular biology grade |
| Malt | local purchase | non-molecular biology grade |
| Cornflour | local purchase | non-molecular biology grade |
| Sucrose | local purchase | non-molecular biology grade |
| Propionic acid | local purchase | non-molecular biology grade |
| Orthophosphoric acid | local purchase | non-molecular biology grade |
| 5% methyl-para-hydroxybenzoate | Loba Chemie | catalog number: 04660 |
| Ethanol | Jiangsu | catalog number: 1170 |
| NaCl | Qualigens | catalog number: Q15918 |
| KCl | Qualigens | catalog number: Q13305 |
| Na ₂ HPO ₄ | HiMedia | catalog number: MB024 |
| K ₂ HPO ₄ | HiMedia | catalog number: MB044 |
| Triton X-100 | Sigma | catalog number: T8787 |
| Sodium Azide | Sigma | catalog number: S2002-25G |
| Paraformaldehyde | HiMedia | catalog number: GRM3660 |
| BSA | HiMedia | catalog number: MB083 |
| Mowiol | Sigma-Aldrich | Product number: 81381 |
| DABCO | Sigma-Aldrich | catalog number: 290734 |
| HCl | Fisher Scientific | catalog number: 29505 |

(Continued on next page)

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--|---|---|
| NaOH pellets | Qualigens | catalog number: Q15895 |
| 4% Sodium Hypochlorite (bleach) | Sigma | catalog number: 1056142500 |
| Glycerol | HiMedia, India | catalog number: MB060 |
| n-Heptane | Merck | catalog number: 1.04365.2521 |
| Experimental models: Organisms/strains | | |
| Canton-S strain of <i>Drosophila melanogaster</i> | L. S. Shashidhara, IISER Pune, India | RRID: DGGR_105666 |
| Software and algorithms | | |
| ImageJ/Fiji - Bio-Formats plugin | (Schindelin et al., 2012); (Rueden et al., 2017) | RRID: SCR_002285 |
| Microsoft Excel | https://www.microsoft.com/en-in/microsoft-365/excel | RRID: SCR_016137 |
| GraphPad Prism | https://www.graphpad.com/scientific-software/prism/ | RRID: SCR_002798 |
| Leica LAS X software (version 3.5.3) | Leica Microsystems | RRID: SCR_013673 |
| Huygens Professional (version 17.04) | https://svi.nl/Huygens-Microscopy-Analysis-Software | RRID: SCR_014237 |
| MATLAB R2021b | https://www.mathworks.com | RRID: SCR_001622 |
| MATLAB code for calculating the Full-Width at Half-Maxima | Patrick Egan (2006). MATLAB Central File Exchange. Retrieved April 17, 2022 | https://www.mathworks.com/matlabcentral/fileexchange/10590-fwhm |
| Other | | |
| Incubator at 25°C for <i>Drosophila</i> stocks and crosses | Panasonic | MIR-554-PE |
| Stereomicroscope | Olympus | SZX10 |
| STED microscope | Leica Microsystems, Mannheim, Germany | Inverted TCS SP8 3x |
| Embryo collection cages | Flystuff.com | Small (Cat # 59-100): 5.6 cm (D) × 7.6 cm (H) |
| Cell strainer | Corning | catalog number: CLS431751 |
| Scintillation vials 6 mL or 20 mL | Sigma | catalog number: M1152 and V7130 |
| <i>Drosophila</i> culture vials and bottles | Laxbro, India | catalog number: BT-30PSBK and FLBT-20 |
| BD Ultra fine 40 IU/31G Syringe | Becton Dickinson (I) Pvt. Ltd. | Ref: 324902 |
| Paintbrush | Camlin, India | 00 number |
| Petri plates | Tarsons, India | 60 mm |
| Soft tissue | Local purchase | non-molecular biology grade |
| Coverslips | VWR | 22 mm × 22 mm, 22 mm × 40 mm #1.5 |
| Glass slides | VWR | Slide Vista 25 × 75 mm |
| Micropipettes and tips | Tarsons | P1000, P200, P2 |
| Conical tubes | Falcon | 50 mL, 15 mL |
| Microcentrifuge tubes | Eppendorf | 1.5 mL, 0.6 mL |

MATERIALS AND EQUIPMENT

Fly Media (1 L)

| Reagent | Amount for 3% / 1 L | Amount for 6% / 1 L |
|--------------------------------|---------------------|---------------------|
| Agar | 10 g | 10 g |
| Yeast | 15 g | 24 g |
| Malt | 30 g | 60 g |
| Cornflour | 75 g | 75 g |
| Sugar | 80 g | 80 g |
| Deionized water | 900 mL | 900 mL |
| Propionic acid | 5 mL | 5 mL |
| Orthophosphoric acid | 1 mL | 1 mL |
| Ethanol | 5 mL | 5 mL |
| 5% Methyl-para-hydroxybenzoate | 0.7 g in ethanol | 1.7 g in ethanol |

Weigh the required amounts of agar, yeast, malt, cornflour and sugar. Add them to deionized water and mix well by stirring. Autoclave the mixture of at 121°C and 15 psi for 15–20 min. Mix propionic

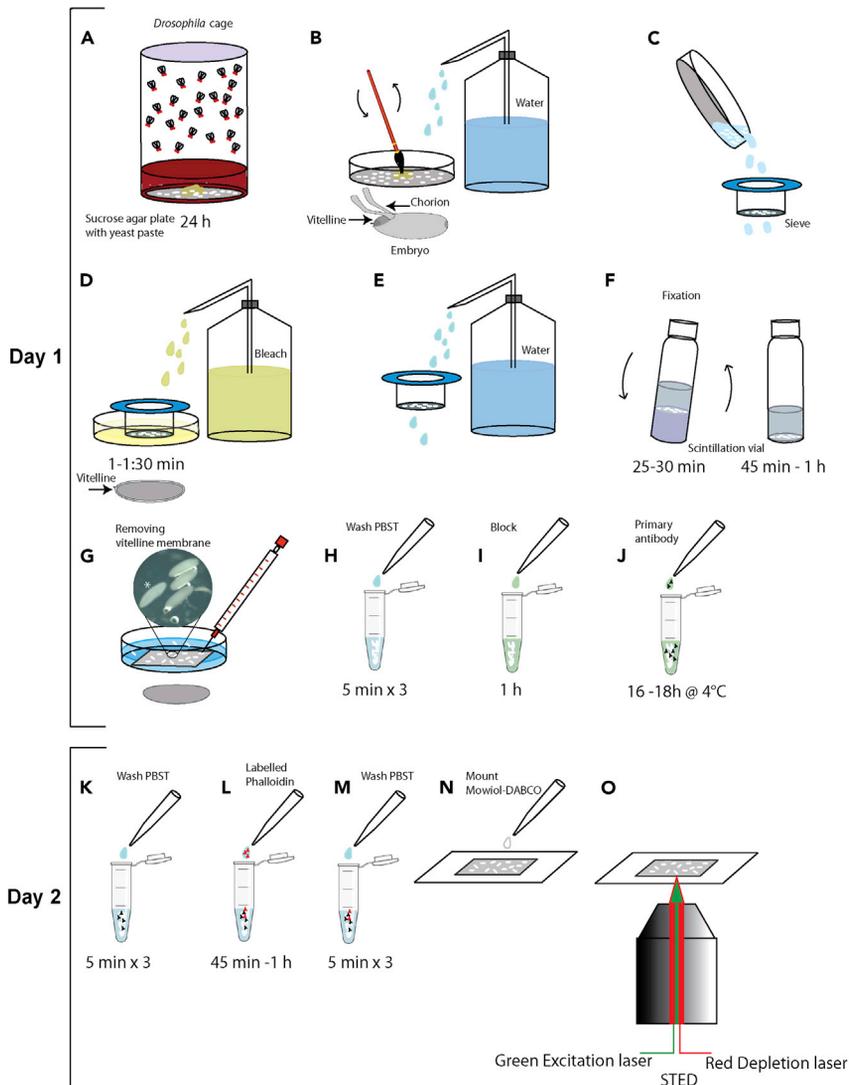


Figure 1. Schematics depicting the protocols for setting up cages, collecting embryos, hand-devitellinization, immunostaining and super-resolution imaging

(A) Carry out embryo collection in a sucrose agar plate containing yeast paste attached to a fly cage.
 (B and C) Wash the embryos with water to remove residual yeast (B) and transfer the embryos into a sieve (C).
 (D and E) Add bleach to dechorionate the embryos (D) and wash them in water (E).
 (F) Transfer the embryos to a scintillation vial containing n-heptane. Add a 1:1 mixture of 4% PFA in PBS and n-heptane, cap the scintillation vial and shake it. The embryos settle at the interface of the two layers in the middle of the scintillation vial.
 (G) Remove the PFA solution at the bottom. Transfer the embryos to a Whatman filter paper and overturn the filter paper on a double-sided sticky tape in a Petri plate and add PBS.
 (H and I) Devitellinize the embryos (Methods video S1) using an insulin needle. Add the embryos in PBST in an Eppendorf tube, wash them 3 times (H) and add the blocking solution for 1 h (I).
 (J) Add the anti-phospho histone antibody for 16–18 h at 4°C.
 (K and L) Wash the embryos with PBST 3 times (K) and add fluorescently coupled phalloidin for 45 min to 1 h at room temperature (L).
 (M and N) Finally wash with PBST 3 times (M) and mount in Mowiol-DABCO (N).
 (O) Mount the slide on the 100× objective of the STED microscope. The time required for steps is mentioned below each schematic.

acid and orthophosphoric acid in a 15 mL conical tube and keep aside. Mix ethanol and methyl p-hydroxybenzoate in a 15 mL conical tube (to make a 5% solution) and keep aside. Mix the autoclaved media continuously for a few minutes, preferably with a motor-driven stirrer. Add the propionic acid and orthophosphoric acid mixture to the autoclaved media. Add the ethanol and methyl p-hydroxybenzoate mixture. Mix well and start pouring in vials/bottles. Cover the mouths of the vials/bottles with a thin clean cotton cloth and allow them to dry overnight.

Plug the mouths of the vials/bottles with tight cotton plugs and store at 4°C until further use.

Sucrose-Agar Plates (450 mL)

| Reagent | Final concentration | Amount |
|-----------------|---------------------|-------------------|
| Sucrose | 3% [w/v] | 13.50 g |
| Agar | 2.5% [w/v] | 11.25 g |
| Deionized water | N/A | Make up to 450 mL |

Weigh the required amounts of sucrose and agar and dissolve them in deionized water. Make the volume up to 300 mL. Heat the solution in a microwave oven for 5–6 min. Add deionized water to the solution to make the volume up to 450 mL. Pour approximately 5 mL of the solution per 60 mm Petri plate. This will make approximately 90 plates. Let the sucrose-agar solution set in the plates at room temperature until it solidifies. Store the plates at 4°C for up to 3–4 days.

10× PBS, pH 7.4 (1 L)

| Reagent | Final concentration | Amount |
|----------------------------------|---------------------|------------------------------------|
| NaCl | 1.37 M | 80 g |
| KCl | 27 mM | 2 g |
| Na ₂ HPO ₄ | 100 mM | 14.4 g |
| KH ₂ PO ₄ | 18 mM | 2.4 g |
| Deionized water | N/A | Make up to 1 L |
| NaOH | N/A | As needed for pH adjustment to 7.4 |
| HCl | N/A | As needed for pH adjustment to 7.4 |

Autoclave the solution and store it at room temperature for up to 3–4 months.

0.3% PBST (Phosphate-buffered saline containing 0.3% [v/v] Triton X-100) (200 mL)

| Reagent | Final concentration | Amount |
|-----------------|---------------------|-------------------|
| 10× PBS | 1× | 20 mL |
| Triton X-100 | 0.3% [v/v] | 600 μL |
| Deionized water | N/A | Make up to 200 mL |

Add a magnetic bead to the solution and place it on a magnetic stirrer to ensure that the Triton X-100 dissolves completely. Store the solution at room temperature for up to 3–4 weeks.

2% PBTA (PBS containing 0.3% [v/v] Triton X-100, 2% [w/v] bovine serum albumin (BSA), and 0.02% sodium azide) (10 mL)

| Reagent | Final concentration | Amount |
|-----------------------------------|---------------------|--------------------|
| 0.3% PBST | 0.3% | 10 mL |
| Bovine serum albumin (BSA) powder | 2% [w/v] | 0.2 g |
| 10 mg/mL Sodium Azide solution | 1.2 [w/v] | #202122; 200 μL |

Weigh the required amount of BSA powder and dissolve it in 0.3% PBST with sodium azide in a 15 mL conical tube. Place the tube on a rocker at 4°C to ensure that the BSA dissolves completely. Store the solution at 4°C for up to 1 week.

4% PFA (50 mL)

| Reagent | Final concentration | Amount |
|------------------|---------------------|----------|
| Paraformaldehyde | 4% [w/v] | 2 g |
| 1× PBS | 1× | to 50 mL |

Weigh the required amount of PFA powder and dissolve it in 1× PBS in a glass beaker. Make the volume up to 50 mL. Cover sides and top of the beaker with aluminum foil. Add a magnetic bead to the solution and place the beaker on a hot plate magnetic stirrer set to 60°C and 100 rpm till the PFA powder dissolves completely. Allow the solution to cool to room temperature. Store the solution at –20°C in 1 mL aliquots.

△ CRITICAL: Paraformaldehyde is a potential carcinogen and a strong respiratory and skin irritant. Ensure you wear a lab coat, safety goggles, mask and gloves while handling PFA.

PFA is light-sensitive. Ensure that the PFA powder and the solution formed after dissolving it in PBS is protected from light exposure. Cover the glass beaker with aluminum foil from all sides.

Mowiol-DABCO (20 mL)

| Reagent | Final concentration | Amount |
|-------------------|---------------------|--------|
| Mowiol | 2.5% [w/v] | 2.4 g |
| Glycerol | 30% [w/v] | 6 g |
| Tris-HCl (pH 8.5) | N/A | 12 mL |
| DABCO | 2.5% [w/v] | 0.5 g |
| Deionized water | N/A | 6 mL |

Measure the required amounts of Mowiol powder and glycerol. Dissolve them in 6 mL of deionized water mixed with 12 mL of 0.2 M Tris-Cl (pH 8.5) in a 50 mL conical tube. Place the tube in a water bath set to 50°C for several hours. Centrifuge the solution at 5,000 g for 15 min and transfer the supernatant to a fresh 50 mL conical tube. Add DABCO to the supernatant to achieve a final concentration of 2.5% [w/v] of DABCO. Store the resultant solution at –30°C in 1 mL aliquots for up to 5–6 months.

STEP-BY-STEP METHOD DETAILS

Collection, fixation, and de-vitellinization of syncytial *Drosophila* embryos

⌚ Timing: 3–4 h

1. Place a fresh sucrose-agar plate smeared with fresh yeast paste on the mouth of a cage containing Canton-S flies.
2. Replace the cage at 25°C and collect the sucrose-agar plate after 2.5 h.

Note: The plate now contains embryos, the majority of which are in the syncytial blastoderm stages.

3. Pour deionized water into the plate and carefully with the help of a paint brush, dissolve the yeast, taking care not to break the sucrose-agar layer (as shown in [Figure 1B](#)).

Note: This dislodges embryos and suspends them in the water-yeast solution.

- Carefully pour the contents of the plate into a cell strainer (as shown in [Figure 1C](#)).

Note: The water-yeast mixture passes through the strainer and the embryos remain in the strainer.

- Wash the embryos multiple times with deionized water to get rid of any debris.
- Pour 5–6 mL of 4% bleach into an empty 60 mm Petri plate. Immerse the cell strainer in bleach for 1–1.5 min such that the embryos are submerged, to remove the chorion, as shown in [Figure 1D](#).

Note: Fly embryos are encased in two semi-transparent membranes - an outer chorion and an inner vitelline envelope, as shown in [Figure 1B](#). These membranes need to be removed for this protocol.

△ CRITICAL: Avoid increased exposure to bleach as this may damage the embryos. Ensure that the bleach is fresh and unexposed to air and sunlight. Bleach loses its efficiency with time and exposure to air.

To ensure the chorion has been removed, observe the embryos under a stereomicroscope, once before adding bleach and once after the embryos have been soaked in bleach for 1 min. Each embryo possesses a pair of protrusions on the anterior end called the dorsal appendages which are part of the chorion. Before bleaching, the dorsal appendages are intact, whereas after bleaching, they are shed off along with the chorion.

- Wash the embryos thrice with deionized water ([Figure 1E](#)).
- Dry them by placing the cell strainer on a piece of soft tissue.
- Add 1 mL of n-heptane to a scintillation vial.
- Carefully, using a fine paint brush, swipe the embryos up from the walls of the strainer.
- Transfer them into the scintillation vial containing n-heptane.

Note: The embryos sink to the bottom of the scintillation vial.

- Add a 1: 1 mixture of 4% PFA and n-heptane (1 mL each) to the scintillation vial. 4% PFA and n-heptane are immiscible and separate into two phases - 4% PFA below and n-heptane above.

△ CRITICAL: The 4% PFA solution should be brought to room temperature before it is added to the scintillation vial to ensure efficient fixation.

- The embryos come to rest at the interface of the two liquids, as shown in [Figure 1F](#).
- Place the scintillation vial on a rocker at room temperature for 25 min.
- Carefully remove and discard the 4% PFA (bottom) layer of the solution in the scintillation vial.
- Remove the n-heptane.
- Add fresh n-heptane and incubate the embryos at 4°C for a minimum of 40 min.
- Cut rectangular pieces of double-sided tape (~ 6 cm long) and Whatman filter paper (~ 6 cm long, and as wide as the double-sided tape).
- Remove the plastic covering from one side of the tape and stick the tape to a 60 mm Petri plate along its diameter, as shown in [Figure 1G](#).
- Transfer the embryos and the n-heptane using a 200 µL pipette onto the filter paper rectangle.
- Allow the n-heptane to evaporate, leaving the embryos on the filter paper.
- Remove the plastic covering on the exposed surface of the tape.
- Invert the filter paper rectangle such that the embryos face downwards, and touch it lightly on the sticky surface of the tape. The embryos will get immobilized on the tape.

24. Pour enough 1 × PBS into the Petri plate containing the immobilized embryos so as to submerge the embryos.
25. Place the Petri plate under a stereomicroscope.
26. Using a 1 mL insulin syringe needle, poke each embryo at one end of its long axis to rupture its vitelline envelope (Figure 1G).
27. Give a slight push to each embryo with the needle to release it from its vitelline envelope as shown in the [Methods video S1](#).

Note: The vitelline envelope remains stuck to the tape and the embryo floats in the 1 × PBS. Embryos still encased in the vitelline envelope glisten under the light of the stereomicroscope, and are easily distinguished from de-vitellinized embryos. In Figure 1G, in the inset image, an asterisk marks the embryo that has been de-vitellinized whereas the others are not. If the embryos are soft and disintegrate when touched with the needle, please see [troubleshooting step 2](#).

28. Transfer the de-vitellinized embryos in 1 × PBS solution into a 0.6 mL microcentrifuge tube. Let the embryos settle at the bottom of the tube.
29. Without disturbing the embryos, remove the 1 × PBS and add 300 μL 0.3% PBST to the tube.
30. The de-vitellinized embryos can be stored in 0.3% PBST solution for upto 24 h at 4°C.

Staining of microvilli and nuclei in syncytial *Drosophila* embryos

⌚ Timing: 18–20 h

31. Remove the 0.3% PBST from the microcentrifuge tube containing de-vitellinized embryos.
32. Add 300 μL of fresh 0.3% PBST.
33. Wash the embryos in 0.3% PBST for 5 min by placing the tube on a rocker at room temperature.
34. Allow the tube to stand so that the embryos settle at the bottom. Using a 200 μL pipette, remove the 0.3% PBST carefully without disturbing the embryos.
35. Add 300 μL of fresh 0.3% PBST and repeat the wash step twice more for a total of 3 washes.
36. Remove the 0.3% PBST after the last wash.
37. Add 300 μL of 2% PBTA to the tube.
38. Incubate the tube on a rocker at room temperature for 1 h for the blocking process.
39. Allow the tube to stand so that the embryos settle at the bottom. Using a 200 μL pipette, remove the 2% PBTA carefully without disturbing the embryos.
40. For the primary antibody staining, dilute the primary antibody in fresh 2% PBTA (1:200 for Phospho-Histone H3 in rabbit).
41. Remove the 2% PBTA used for the blocking step and add the diluted primary antibody solution to the tube containing the embryos.
42. Incubate on a rocker at 4°C for a minimum of 16 h.
43. Allow the tube to stand so that the embryos settle at the bottom. Using a 200 μL pipette, remove the primary antibody solution carefully without disturbing the embryos.
44. Add 300 μL of fresh 0.3% PBST to the tube.
45. Place the tube on a rocker at room temperature for 5 min. Repeat the wash step twice more for a total of 3 washes.
46. Dilute Alexa 488 phalloidin (1:100) and Alexa 568-coupled anti-rabbit secondary antibody (1:1,000) in 300 μL of 0.3% PBST.
47. Allow the tube to stand so that the embryos settle at the bottom. Using a 200 μL pipette, remove the 0.3% PBST carefully without disturbing the embryos.
48. Add the secondary antibody/dye solution to the tube.
49. Wrap the microcentrifuge tube in aluminum foil to protect the fluorophores (conjugated to the antibody/dye) from photobleaching.
50. Incubate the tube on a rocker at room temperature for 1 h.

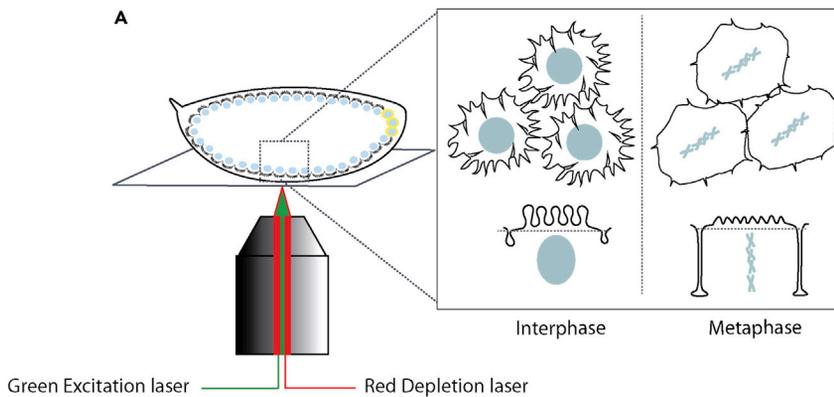
51. During the incubation step, thaw an aliquot of Mowiol-DABCO on ice.
52. Allow the tube containing the embryos to stand so that the embryos settle at the bottom. Using a 200 μ L pipette, remove the secondary antibody/dye solution carefully without disturbing the embryos.
53. Add 300 μ L of fresh 0.3% PBST to the tube and place it on a rocker at room temperature for 5 min.
54. Allow the tube to stand so that the embryos settle at the bottom. Using a 200 μ L pipette, remove the 0.3% PBST carefully without disturbing the embryos.
55. Repeat this wash step twice more for a total of 3 washes.
56. After removing the 0.3% PBST from the final wash, add 100 μ L of fresh 0.3% PBST to the tube.
57. Transfer the solution with embryos onto a glass slide.
58. Drain the excess 0.3% PBST by standing the slide on its side.
59. Space the embryos on the slide by gently pushing them apart from each other using a fine brush.
60. Excess liquid can be drained out using a thin piece of Whatman filter paper by dabbing the paper lightly at the edges of each of the embryo(s).
61. Using a cut 1 mL pipette tip, add 2 drops of Mowiol-DABCO solution to the slide.
62. Carefully place a 22 \times 40 mm, #1.5 coverslip on the slide, while avoiding formation of air bubbles and ensuring that the Mowiol-DABCO solution covers the embryos.
63. Seal the edges of the coverslip with transparent nail polish.
64. Protect the slide from light by covering it with aluminum foil and allow the nail polish to set for 15 min at room temperature.
65. Store the slide in an opaque box at 4°C and image them within 1–2 days of preparation to minimize the degradation of fluorophore signal.

Confocal and super-resolution STED imaging to obtain high-resolution images of microvilli

⌚ Timing: 3–4 h

66. Mount the slide on the stage of an inverted TCS SP8 3 \times microscope containing the super-resolution STED module (Leica Microsystems, Mannheim, Germany) operated by the Leica LAS X software (version 3.5.3).
67. Load the 100 \times /1.4 NA oil immersion (refractive index 1.518) objective (Leica HC PL APO CS2-STED WHITE).
68. Switch to fluorescence mode and toggle to the rhodamine/RHOD (red) filter while looking through the eyepiece.
69. Move the stage until an embryo becomes visible as an orange glow.
70. Use the coarse and fine focus knobs to bring the surface of the embryo in focus.
71. Identify the mitotic stage of the syncytial division cycle based on the phospho-histone H3 label as shown in [Figure 2B](#).

△ CRITICAL: Hoechst 33258 cannot be used for labeling samples for super-resolution STED imaging as it might have a negative influence on image quality (background), especially with the 592 nm STED laser. Thus, for super-resolution STED imaging we have used phospho-histone H3 (green) only. We have shown nuclei co-labeled with phospho-histone H3 (green) and Hoechst 33258 (blue) using confocal microscopy in [Figure 2B](#), for each of the mitotic stages (interphase, prophase and metaphase). Interphase nuclei appear as rounded and smooth with non-punctate chromatin in Hoechst, however phospho-histone H3 does not label interphase nuclei at all. Prophase nuclei in both appear as round structures with partially condensed chromatin resembling puncta. Metaphase nuclei in both cease to be rounded and have fully condensed chromatin that appears as thick coiled strands.



STED

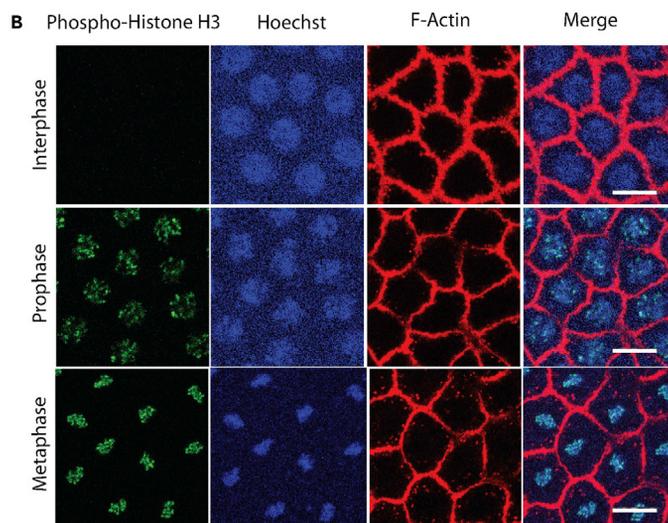


Figure 2. Setup for imaging and identification of the cell cycle stage in the syncytial blastoderm embryo

(A) Syncytial *Drosophila* embryos imaged using an inverted confocal and STED microscope (left). The schematic of the syncytial nuclear cycle (right, magnified inset), showing an apical view and a sagittal view (rotated by 180° to show the apical side on top) at interphase and metaphase. Interphase embryos possess short furrows between cortical nuclei (blue) and long apical microvilli (black) whereas metaphase embryos have long furrows and fewer and shorter microvilli. The dashed line indicates the plane of the membrane shown in the top panel.

(B) Grazing sections of confocal images showing the nuclei, labeled using both phospho-histone H3 (green) and Hoechst 33258 (blue), for each of the mitotic stages (interphase, prophase and metaphase). Cortical F-actin (red) is labeled using Alexa 568 phalloidin at different mitotic stages of a single nuclear division cycle 12. Scale bar: 5 μm .

Use the laser settings shown in [Table 1](#) for exciting fluorophores and detecting emitted light for confocal imaging to identify the nuclear staining at different cell cycle stages.

72. Identify the nuclear division cycle number (10–13) based on the area of the apical caps as shown in [Figure 3](#).
73. Apical caps are larger than 240 μm^2 during syncytial cycle 10, 120–240 μm^2 during syncytial cycle 11, 80–120 μm^2 during syncytial cycle 12 and 40–80 μm^2 during syncytial cycle 13. Cycle 14 is identified by small caps with area less than 40 μm^2 .

Table 1. Laser settings for confocal microscopy

| Fluorophore | Labels | Excitation wavelength | Excitation laser intensity | Detector | Spectral window for detection | Gain | Line averaging |
|----------------------|----------------------|-----------------------|---------------------------------------|----------------------------|-------------------------------|-----------|----------------|
| Hoechst 33258 | Dividing nuclei | 405 nm | 70% (90 MHz pulsed white light laser) | Photomultiplier tube (PMT) | 443–466 nm | 800–1,000 | 2 |
| Phospho-Histone H3 | Dividing nuclei | 488 nm | 70% (90 MHz pulsed white light laser) | Hybrid detector (HyD) | 506–539 nm | 100–200 | 2 |
| Alexa 568 Phalloidin | Microvilli (F-actin) | 561 nm | 70% (90 MHz pulsed white light laser) | Hybrid detector (HyD) | 585–623 nm | 100–150 | 2 |

74. Move through a few z-planes near the surface of the embryo and find an optical plane which has the greatest number of microvilli in focus.
75. Set the pinhole size to 1.00 AU and capture a standard confocal image at the optical plane determined above. Use the standard mode with a zoom of 3.5× and scan speed 200 Hz.
76. To obtain STED images of the microvilli (labeled with Alexa 488 phalloidin), use the 592 nm pulsed depletion laser (Leica Microsystems) at 90% laser power in 2D STED mode.
77. Keep the gain at 100 and line averaging at 4.
78. Use the laser settings in [Table 2](#) for exciting fluorophores and detecting emitted light for super-resolution STED imaging.
79. Export the images as 8 bit, 1024 × 1024 TIFF format images with a pixel size of about 30–40 nm.

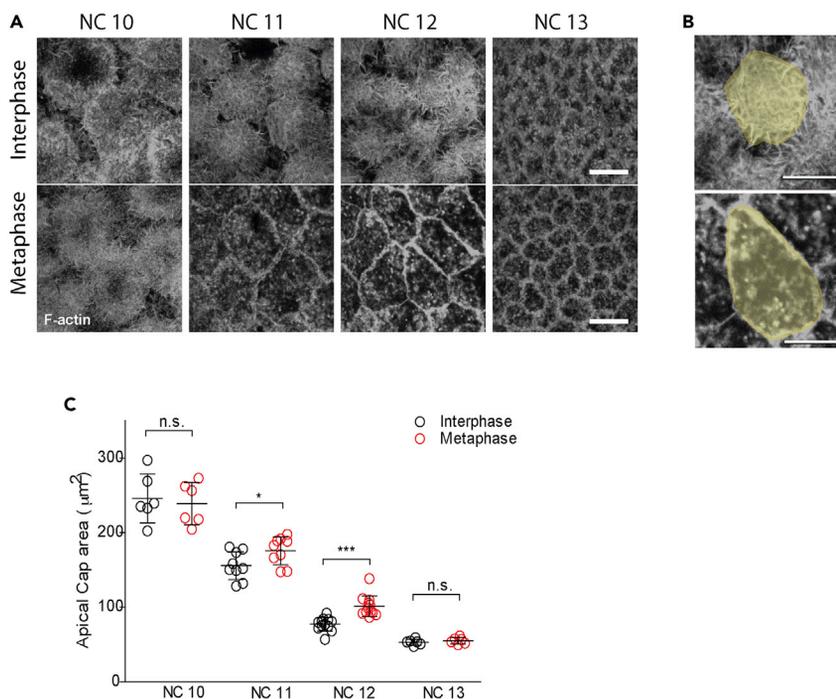


Figure 3. Apical villi images in the syncytial *Drosophila* blastoderm in interphase and metaphase of nuclear cycles 10–13

(A) Super-resolution STED images of apical actin caps at interphase and metaphase of nuclear cycles 10–13 in the syncytial *Drosophila* embryo. Scale bar: 5 µm.

(B) Image showing an ROI drawn (yellow) along the boundaries of the apical cap to measure its area using ImageJ. Scale bar: 5 µm.

(C) Quantification of apical cap areas at interphase and metaphase in nuclear division cycles 10–13 in the syncytial *Drosophila* embryo. Each circle represents the area obtained from individual caps from at least 3 embryos. NC 10, n = 6, 3 caps from 2 embryos each; NC 11, n = 9, 3 caps from 3 embryos each; NC 12, n = 12, 3 caps from 4 embryos each; NC 13, n = 6, 3 caps from 2 embryos each. Data are represented as mean ± SD, *p < 0.05, **p < 0.01, and ***p < 0.001, two-tailed, unpaired Student's t test.

Table 2. Laser settings for STED super resolution microscopy

| Fluorophore | Labels | Excitation wavelength | Excitation laser intensity | Detector | Time gate | Spectral window for detection | Gain | Line averaging |
|----------------------|----------------------|-----------------------|---------------------------------------|-----------------------|-----------|-------------------------------|---------|----------------|
| Alexa 488 Phalloidin | Microvilli (F-actin) | 488 nm | 70% (90 MHz pulsed white light laser) | Hybrid detector (HyD) | 0.3–6 ns | 507–542 nm | 100–120 | 2 |
| Phospho-Histone H3 | Dividing nuclei | 561 nm | 70% (90 MHz pulsed white light laser) | Hybrid detector (HyD) | 0.3–6 ns | 584–633 nm | 50–60 | 2 |

Deconvolution of images obtained from STED microscopy

⌚ Timing: 1–2 h

- Deconvolve the images using the STED module on Huygens Professional version 17.04 (Scientific Volume Imaging, Netherlands).
- Estimate the average background using the In/near object module by controlling the radius parameter.
- Use the Classical Maximum Likelihood Estimation (CMLE) algorithm and set the maximum iterations at 40. Set the Signal to Noise Ratio at 7 and Quality Threshold at 0.1 in the ‘fast’ iteration mode with possible bleaching correction.
- Save the deconvolved images as high-quality TIFF images.

EXPECTED OUTCOMES

This protocol describes the steps for staining and imaging microvilli composed of F-actin present on the apical caps of the syncytial *Drosophila* blastoderm embryo. A greater density of microvilli are visible at the periphery of each apical cap as compared to the center (Sherlekar et al., 2020). Microvilli are visible with both confocal and super-resolution STED microscopy. This protocol enables the documentation of density and length of microvilli in each actin cap, and recommends an analysis of microvilli remodeling in syncytial cycles 11 and 12. Since the remodeling process takes place from interphase to metaphase of each syncytial cycle, a comparison of density and length of microvilli in control and mutant animals at interphase and metaphase of syncytial cycles 11 and 12 provides an estimate of the extent of microvilli remodeling.

QUANTIFICATION AND STATISTICAL ANALYSIS

Identification of the nuclear division cycle number in fixed and stained embryos.

- Draw a region of interest (ROI) as shown in Figure 3B, along the boundaries of the apical caps to form a closed area shown in yellow using the “Polygon Selection” tool in ImageJ.
- Save the ROI and measure the area covered by the ROI using ROI Manager.

Measure at least 3–5 caps for each embryo and a minimum of 3 embryos at each stage.

- Use the cap area information in Table 3 for estimating the apical cap areas in each nuclear cycle to determine the nuclear division cycle number for each embryo.

Note: Since the actin cap is a dome-shaped structure, the cap area will vary based on the optical plane of imaging.

Table 3. Apical cap area range in syncytial nuclear cycles 10–13

| Nuclear cycle | 10 | 11 | 12 | 13 |
|-----------------------------|----------------------|-------------------------|------------------------|-----------------------|
| Cap area range ^a | >240 μm^2 | 120–240 μm^2 | 80–120 μm^2 | 40–80 μm^2 |

^aArea ranges identified are approximate.

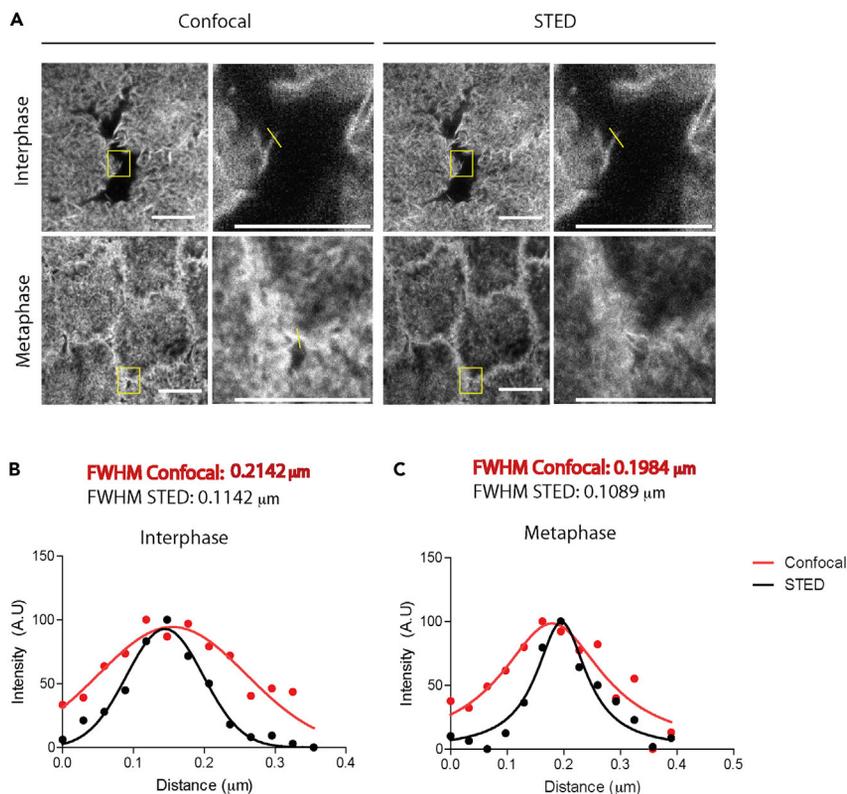


Figure 4. Comparison of confocal and STED super resolution images of apical caps

(A) Comparison of confocal and non-deconvolved super-resolution images of apical actin caps in nuclear cycle 12 of the syncytial *Drosophila* embryo, Scale bar: 5 μm . A section is zoomed in to show the apical microvilli. Scale bar: 5 μm . An ROI is drawn across a single microvillus and used to plot its intensity profile.

(B) Intensity profile of one microvillus at interphase obtained from confocal (red) and STED (black) imaging. Full-width at half-maximum (FWHM) values are calculated for both.

(C) Intensity profile of a microvillus at metaphase obtained from confocal (red) and STED (black) imaging. Full-width at half-maximum (FWHM) values are calculated for both.

Quantification of Full-Width at half maximum (FWHM)

4. Draw an ROI across a single microvillus (yellow line in Figures 4A and 5A) using the “Segmented Line” tool in ImageJ.
5. Select the “Plot Profile” option under the “Analyze” tab which plots the intensity of the pixels along the length of the ROI. Save the data of coordinates (x) and intensities (y).
6. Plot the data in GraphPad Prism as a scatter and fit a Gaussian distribution to the scatterplot (Figures 4B and 5B).
7. Input the data into MATLAB R2021b and run the code available publicly at <https://www.mathworks.com/matlabcentral/fileexchange/10590-fwhm>.

The output is the Full-Width at Half-Maximum (FWHM) of the Gaussian distribution waveform $y(x)$ and its polarity.

Quantification of microvilli length and number

8. Draw an ROI along the length of the microvilli (yellow lines in Figure 6A) using the “Segmented Line” tool in ImageJ.
9. Count as many microvilli as can be observed in sharp focus emanating from one apical cap.

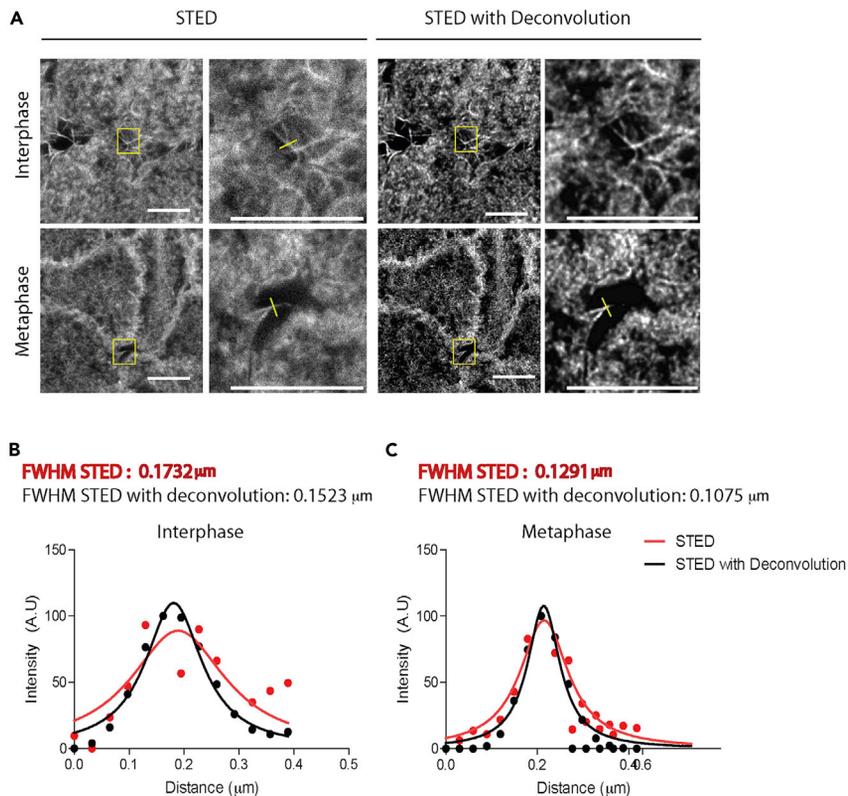


Figure 5. Analysis of STED super resolution images with deconvolution

(A) Comparison of acquired and deconvolved super-resolution images of apical actin caps in nuclear cycle 12 of the syncytial *Drosophila* embryo, Scale bar: 5 μm . A section is zoomed in to show the apical microvilli. Scale bar: 5 μm . An ROI is drawn across a single microvillus and is used to plot its intensity profile.

(B) Intensity profile of a microvillus at interphase obtained from STED imaging (red) and the STED image with deconvolution (black). Full-width at half-maximum (FWHM) values are calculated for both.

(C) Intensity profile of a microvillus at metaphase obtained from STED imaging (red) and the STED image with deconvolution (black). Full-width at half-maximum (FWHM) values are calculated for both.

Note: This quantification is possible from the deconvolved STED images and confocal images.

- Repeat this for each apical cap that is fully visible in the image from the deconvolved super-resolution images and confocal images (Figure 6).
- Save the ROIs and measure the length of each ROI. These are the length measurements for each microvillus.
- Note also the number of ROIs per cap. These are the number of microvilli for each cap.
- Save the measurements and plot the microvilli lengths and microvilli numbers per cap at interphase and metaphase in GraphPad Prism (Figures 6B–6E).

LIMITATIONS

The limitation of this method is the ability to only capture apical microvilli in a single focal plane of the apical cap which is a three-dimensional dome-shaped structure. The dimensions of the microvilli are dependent upon the orientation of the microvilli and the optical plane of imaging.

TROUBLESHOOTING

Problem 1

Very few embryos are obtained from embryo collections (step 1 of [step-by-step method details: collection, fixation, and de-vitellinization of syncytial *Drosophila* embryos](#)).

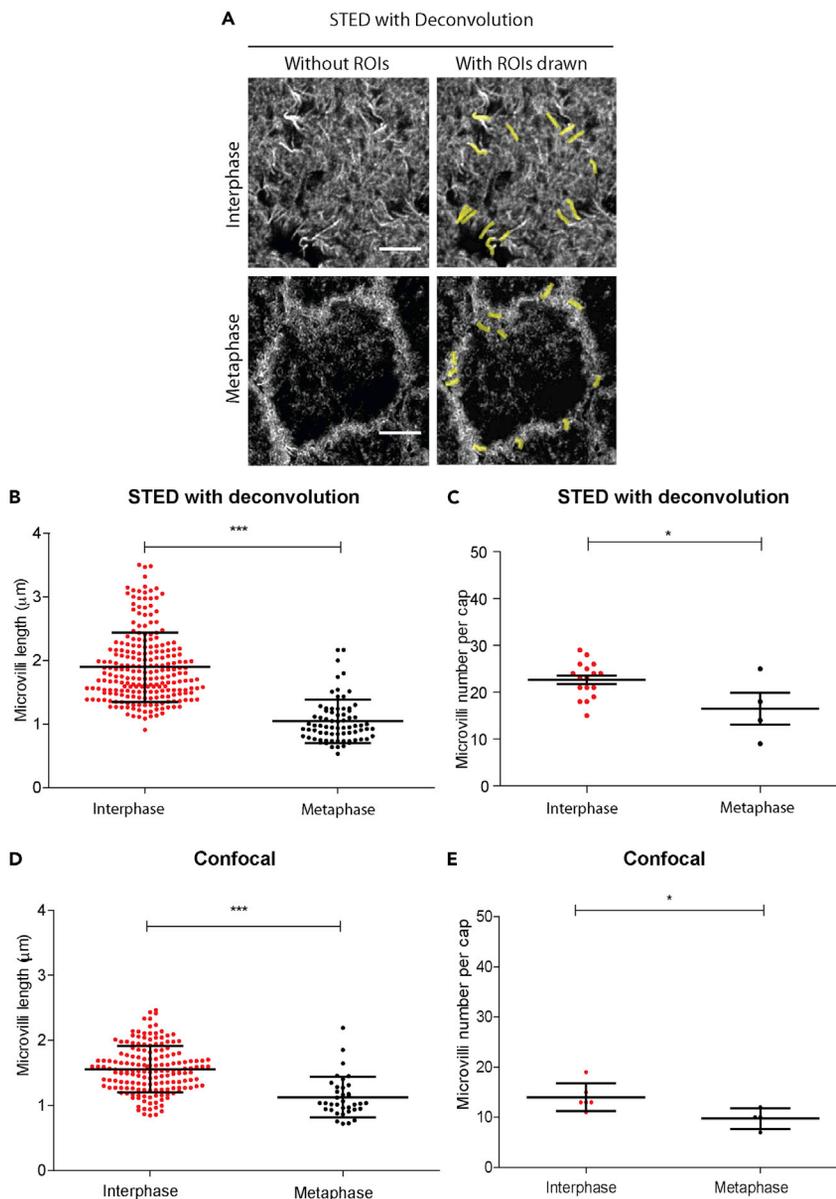


Figure 6. Quantification of microvilli lengths and number from deconvolved STED and confocal images

(A) Quantification of microvilli lengths and numbers using de-convolved super-resolution images of apical actin caps in nuclear cycle 12 of the syncytial *Drosophila* embryo. ROIs are drawn as shown in yellow along the length of each microvillus. (B and C) The lengths of microvilli (B) and numbers of microvilli per cap (C) are measured and plotted for interphase and metaphase in the deconvolved STED images. $n = 2\text{--}4$ cells each from 3 embryos. The range of microvilli length at interphase and metaphase as quantified from the de-convolved super-resolution images are $1.69 \pm 0.342 \mu\text{m}$ and $1.04 \pm 0.341 \mu\text{m}$, respectively (B). The range of microvilli numbers at interphase and metaphase as quantified from the de-convolved super-resolution STED images are 21.17 ± 3.710 and 16.50 ± 6.758 respectively (C). Data are represented as mean \pm SD, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, two-tailed, unpaired Student's *t* test. Scale bar: $5 \mu\text{m}$. (D and E) Quantification of microvilli lengths and numbers using confocal images of apical actin caps in nuclear cycle 12 of the syncytial *Drosophila* embryo. The lengths of microvilli (D) and numbers of microvilli per cap (E) are measured and plotted for interphase and metaphase. $n = 2\text{--}3$ cells each from 3 embryos. The range of microvilli length at interphase and metaphase as quantified from the confocal images are $1.45 \pm 0.331 \mu\text{m}$ and $1.12 \pm 0.312 \mu\text{m}$ (D). The range of microvilli numbers at interphase and metaphase as quantified from the confocal images are 14.00 ± 2.757 and 9.75 ± 2.062 respectively (E). Data are represented as mean \pm SD, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, two-tailed, unpaired Student's *t* test. Note that the numbers of villi and their lengths are reduced in the confocal images in interphase as compared to deconvolved STED images with a significance of $p < 0.001$ possibly due to the reduced resolution.

Potential solution

Add more flies to the cage. The numbers of female flies may be increased to twice as many as the males in the cage for increasing the numbers of eggs in the collection. Also, transfer the flies into a fresh cage 6–7 days after the cage is set up. Post-transfer, allow the flies to get acclimatized to the cage for about 2–3 h before resuming egg collections.

Problem 2

Fixed embryos are too soft and disintegrate when touched by the syringe needle during de-vitellinization (step 18 of [step-by-step method details: collection, fixation, and de-vitellinization of syncytial *Drosophila* embryos](#)).

Potential solution

Allow the PFA solution to reach room temperature before using it to fix the embryos. Warmer temperatures are more likely to speed up the fixation process as the formaldehyde-methylene glycol equilibrium shifts towards formaldehyde. Formaldehyde is the active molecule that effectively facilitates tissue fixation ([Winkelman et al., 2002](#)). Fixing the embryos using a concentration of PFA that is greater than 4%, such as 8% PFA, can also be attempted. If the problem still persists, then prepare and use a fresh solution of 4% or 8% PFA.

Problem 3

The STED image has too much background noise (step 13 of [step-by-step method details: confocal and super-resolution STED imaging to obtain high-resolution images of microvilli](#)).

Potential solution

Increase the line averaging.

Problem 4

The STED image has been photobleached (step 13 of [step-by-step method details: confocal and super-resolution STED imaging to obtain high-resolution images of microvilli](#)).

Potential solution

Lower the power of the STED depletion laser by reducing its percentage.

Problem 5

The lateral resolution of microvilli in the STED images is not sufficient (step 13 of [step-by-step method details: confocal and super-resolution STED imaging to obtain high-resolution images of microvilli](#)).

Potential solution

This can be done by either increasing the line averaging up to 16 and power of STED depletion laser. Tweaking the STED beam like generating an azimuthally polarized light could also help achieve higher lateral resolution ([Yang et al., 2016](#)).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Richa Rikhy, richa@iiserpune.ac.in.

Materials availability

The materials used in the protocol are listed along with their source of procurement.

Data and code availability

The protocol includes all datasets generated or analyzed during this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101736>.

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AUTHOR CONTRIBUTIONS

D.M., A.S., G.M., and R.R. designed the experiments, performed experiments, and carried out the data analysis for the project. D.M., A.S., and R.R. wrote the manuscript. R.R. obtained funding for the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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