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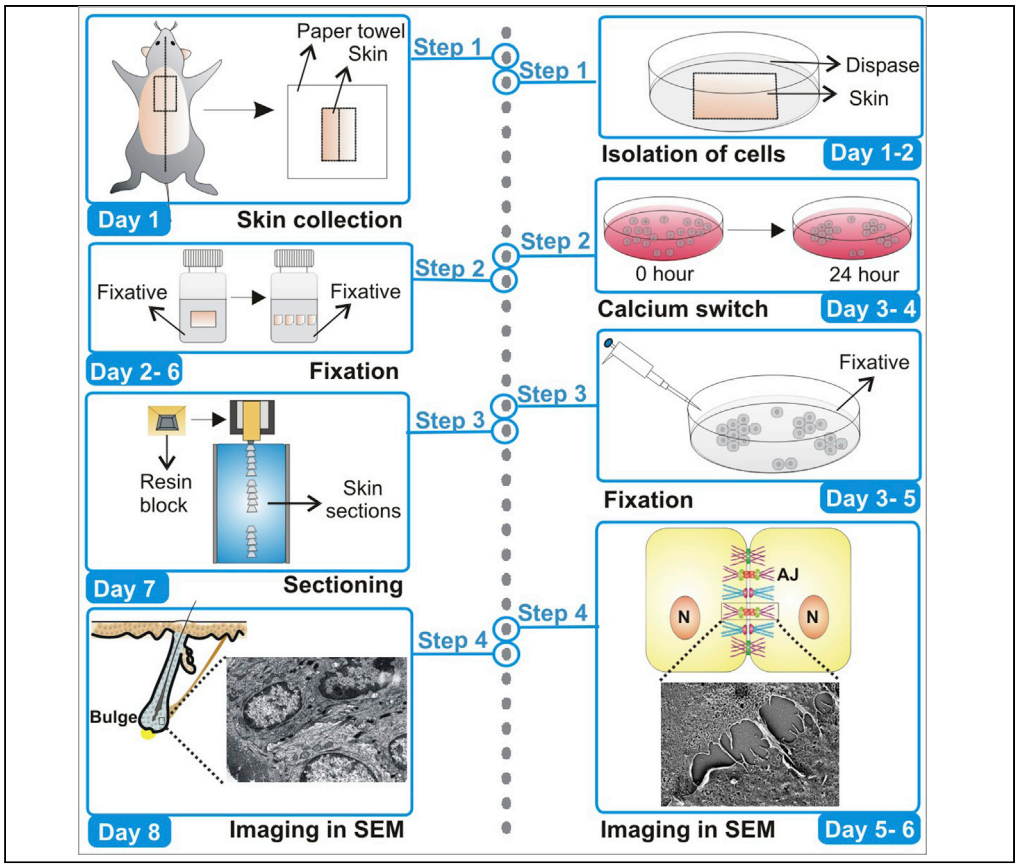
# Scanning electron microscopy of murine skin ultrathin sections and cultured keratinocytes




Generating high-quality EM images of the skin and keratinocytes can be challenging. Here, we describe a simple protocol for scanning electron microscopy (SEM) of murine skin. The protocol enables characterization of the ultrastructure of the epidermis, dermis, hair follicles, basement membrane, and cell-cell junctions. We detail the specific steps for sample preparation and highlight the critical need for proper orientation of the sample for ultrathin sectioning. We also describe the isolation and preparation of primary keratinocyte monolayers for SEM.

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## Highlights

Visualizing adherens junctions in ultrathin sections of murine skin using SEM

Generating nanometer scale sections of murine skin using an ultramicrotome

Protocol adaptable for cultured keratinocytes

Banerjee et al., STAR Protocols 2, 100729
September 17, 2021 
https://doi.org/10.1016/j.xpro.2021.100729

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# Scanning electron microscopy of murine skin ultrathin sections and cultured keratinocytes

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https://doi.org/10.1016/j.xpro.2021.100729

## SUMMARY

**Generating high-quality electron microscopy images of the skin and keratinocytes can be challenging. Here we describe a simple protocol for scanning electron microscopy (SEM) of murine skin. The protocol enables characterization of the ultrastructure of the epidermis, dermis, hair follicles, basement membrane, and cell-cell junctions. We detail the specific steps for sample preparation and highlight the critical need for proper orientation of the sample for ultrathin sectioning. We also describe the isolation and preparation of primary keratinocyte monolayers for SEM.**

**For complete details on the use and execution of this protocol, please refer to Biswas et al. (2021).**

## BEFORE YOU BEGIN

Obtaining electron microscopy (EM) data involves the use of specialized equipment such as electron microscopes and ultramicrotomes. The use of electron microscopes and ultramicrotomes requires training and practice to obtain routinely good quality sections that will produce good quality images. If the person using this protocol has never done EM before, we recommend that they contact an experienced EM specialist who can guide them and provide the necessary training.

The protocol describes the collection and fixation of skin samples in steps 1–13 in the before you begin section and the post fixation, dehydration, embedding, sectioning and imaging in steps 1–44 in the step by step section.

The protocol also describes the procedure for culturing of primary keratinocyte monolayers and fixation for SEM in steps 14–35 in the before you begin section and the post fixation, dehydration and imaging in steps 45–59 in the step by step section.

### Fixative preparation

Timing: 1–2 h

The purpose of this step is to stabilize the biological sample in a way such that the ultrastructure of tissues or cells are preserved. This section describes the fixative preparation for skin samples.

1. Prepare 0.2M Na-Cacodylate buffer and adjust the pH to 7.2.
2. Prepare 0.2M $CaCl_2$ solution and autoclave the solution.
3. Prepare the fixative to a final concentration of 2% Glutaraldehyde, 4% Formaldehyde, and 2 mM $CaCl_2$ in 0.05M Na-Cacodylate buffer (pH 7.2).

*Note:* Follow the Recipes for common reagents and Recipes for skin sample preparation sections under materials and equipment

### Collection of back skin from mice

In this protocol, both males and females were used for experiments as no obvious differences were observed between genders. Newborn pups were used for cell monolayer protocol and 3–8 week old animals were used for skin sample protocol. Mice were housed in NCBS/inStem Animal Care Resource Centre. Animals were handled, bred and euthanized in compliance with the guidelines and procedures approved by the inStem IACUC (Institutional Animal Care and Use Committee) (Biswas et al., 2021). Animals were regularly monitored for any health concerns. All animals for experiments were housed in a specific pathogen free (SPF2) facility in ventilated cages kept under 12-h light and dark cycle and were given unlimited food and water. The temperature in the facility was maintained at 21°C.

*Day 1*

**Timing: Step 4–7: 30 min**

**Timing: Step 8–11: 10–15 min**

**Timing: Step 12: 1–2 h**

**Timing: Step 13: 10 min**

4. Before beginning the collection procedure, keep the following materials ready: Fixative, glass scintillation vials, sharp scissors, forceps, paper towel, fresh razor blades, 70% ethanol, Veet® hair removal cream, hair trimmer. After euthanizing the mouse using $CO_2$, carefully shave the back skin of the mouse using a hair trimmer (Figures 1A and 1B).
5. Apply hair removal cream on the shaved region and leave it for 2 min.
6. Wipe off the hair removal cream and thoroughly wash the region with water. This will remove any remaining hair found on the shaved region of the skin.
7. Pat the skin dry.
8. Draw a line with a thin sharpie marking the midline on the dorsal anterior region of the mouse (Figure 1C).
9. Using forceps, loosen the skin by pinching the desired region of excision.
10. Cut a rectangular piece of skin alongside the midline of the back using a dissecting scissors and place it dermis side down on a paper towel. (In this study, the anterior region of the mouse back skin was used) (Figures 1B and 1C).

*Note:* Keeping the shape rectangular will aid in the orientation of the back skin later.

*Note:* Work gently and fast. Avoid stretching the back skin too much as this might lead to ruptures within the tissue. The dissected skin should be kept dermis side down on the paper towel for less than a minute.

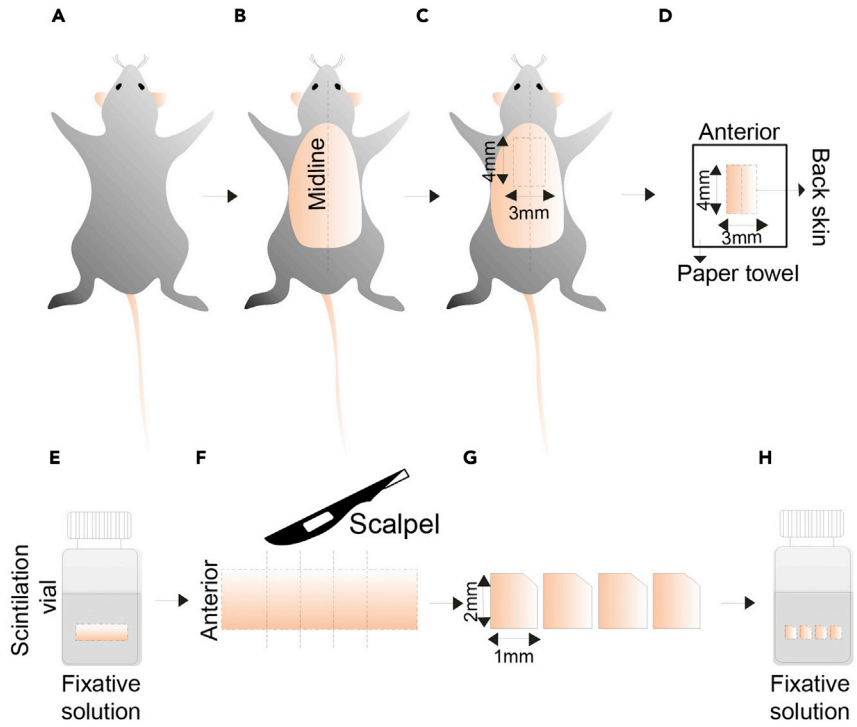


**Figure 1. Illustration of collecting mouse back skin and initial fixation**

(A–D) Stepwise illustration of collection of mouse back skin.

(E) Initial fixation of the back skin in fixative solution.

(F–G) Stepwise illustration of trimming of back skin into pieces.

(H) Fixation of samples overnight at 4°C.

11. Cut a region of skin from the dissected rectangular piece (3×4 mm) and insert this specimen (together with the paper towel underneath it) into a vial containing the freshly prepared fixative solution for skin samples (Figure 1D).

⚠ CRITICAL: Ensure that the specimen is completely submerged in the fixative solution (Figure 1E).

*Note:* Marginal fixation will occur if the specimen is not completely submerged or if the basal side is placed upwards in the fixative solution. This happens due to the barrier function of the epidermis; aqueous solutions can only penetrate from the basal side of the skin samples.

12. Fix the sample for 1–2 h at room temperature (RT) (Figure 1E).

⚠ CRITICAL: Perform the fixation as soon as the mouse is euthanized. If you have several mice to process, work with one mouse at a time. Ultrastructural changes can occur rapidly (within minutes).

13. Trimming:
    a. Place a piece of parafilm on the dissection scope stage.
    b. Put the specimen in a drop of fixative on the parafilm.
    c. Separate the skin from the paper towel and orientate the skin with the basal side facing up.
    d. Using a new razor blade, cut smaller rectangle pieces (1×2 mm) while holding the specimen with blunt forceps (Figures 1F and 1G).

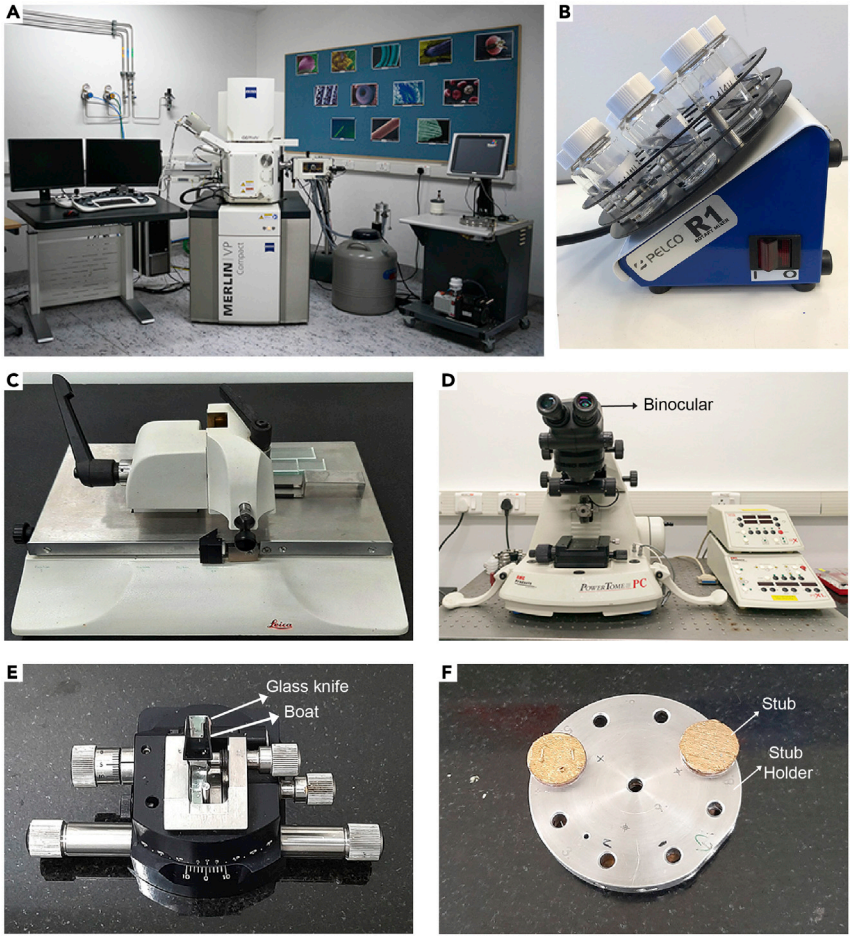


**Figure 2. Electron Microscopy facility, equipment and parts**
(A) Merlin VP Compact, Scanning Electron Microscope.
(B) Photograph of Pelco Rotator.
(C) Leica EM KMR3 glass knife maker.
(D) RMC Ultramicrotome Powertome PTPC.
(E) Photograph of glass knife holder with a plastic boat attached to the glass knife.
(F) Photograph of stub holder.

*Note:* Cut the skin with smooth movements of the blade without applying pressure.

e. Place the specimen back in a new vial containing fresh fixative solution (Figure 1H).
f. Fix the samples overnight at 4°C on a rotator (6–8 h) (Figure 2B).

*Note:* Continue on to steps 1–44 in step-by-step section.

## Isolation of primary keratinocytes

Keratinocytes were isolated from mouse skin as described in (Raghavan et al., 2003). The following steps were performed to isolate the keratinocytes.

## Media preparation

Timing: Variable

### E media

Follow the Recipes for cell monolayer section under materials and equipment, for preparing E media for culturing primary keratinocytes. To prepare supplemented serum (FBS), add the following to 500 mL serum: 37.5 mL of 100× Cocktail + 375µL cholera toxin + 375µL hydrocortisone

The supplements for the E media are as listed:

14. 100× HYDROCORTISONE

Prepare stock solution 4 mg/mL by adding 6.25 mL of 95% ETOH to 25 mg.

Filter-sterilize and aliquot into a sterile centrifuge tube and store at −20°C.

15. 100× CHOLERA TOXIN

Prepare $10^{-6}$ M stock solution by dissolving 1 mg vial of cholera toxin in 11.9 mL of distilled water. Filter-sterilize and aliquot into a sterile centrifuge tube and store at 4°C.

16. 5 mg/mL Insulin (minimum M.W. = 6000 g, approx. 8.3 × 10−4 M)

Prepare a stock solution by adding 250 mg of Insulin to 50 mL of 0.1N HCl. Dissolve completely

Store in 10 mL aliquots in 15 mL Falcon tubes at 4°C.

17. 5 mg/mL Transferrin

Prepare the stock solution by adding 250 mg of Transferrin to 50 mL sterile Phosphate Buffer Saline (PBS). Aliquot into 10 mL portions in 15 mL falcon tubes and store at −20°C.

18. $2 \times 10^{-8}$ M T3 (3,3′,5-Triiodo-L-Thyronine)

Weigh 13.6 mg of T3 and dissolve into 100 mL of 0.02N NaOH to prepare $2 \times 10^{-4}$ M T3, store at −20°C. Serially dilute the solution to obtain $2 \times 10^{-8}$ M T3: Take 0.1 mL of $2 \times 10^{-4}$ M T3 + 9 mL Sterile PBS to prepare $2 \times 10^{-6}$ M T3, store at −20°C. Take 1.0 mL of $2 \times 10^{-6}$ M T3 + 99 mL Sterile PBS=$2 \times 10^{-8}$MT3. Store at −20°C.

*Note:* All of these compounds stick to glass. It is necessary to use plastic. Add all the ingredients together in a large plastic flask, mix well, filter sterilize and store in 50ml falcon tubes.

*Note:* After preparing the individual components, make the 100× cocktail following the Recipes for cell monolayer section under materials and equipment.

### F media

19. Follow the Recipes for cell monolayer section under materials and equipment, for preparing F media for culturing J23T3 feeder cell line.

### Monolayer preparation

Timing: Variable

20. To isolate primary mouse keratinocytes from P-0 pups, euthanize the mice, remove the back, and belly skin. Place the skin dermis side down in dispase (1 mg/mL in 1×PBS) overnight at 4°C which will aid in the separation of the epidermis from the dermis (Raghavan et al., 2003).
21. On the same day treat a confluent J23T3 feeder plate (10 cm) with mitomycin C (0.4 mg/mL, using 80μL in 4 mL F media), for 1.5–2 h at 37°C. After treatment, wash the plate with 1×PBS, 4 times and add E media to the plate. Keep in a 32°C incubator overnight.

⚠ CRITICAL: Mitomycin C is carcinogenic. Please handle it with care.

22. Following day, using fine forceps, separate the epidermis from the dermis and place it in a 3.5 cm dish containing 2 mL of 0.05× trypsin for 5 min at RT. After incubation, detach the cells from the epidermis by pipetting up and down with a 2 mL pipette. This will help to form a cell suspension.
23. To stop the enzymatic reaction of trypsin, add 1 mL of E media. Collect the cells and filter through a cell strainer (70 μm) into a 50 mL falcon tube. Spin the cells at 400×g, for 5 min at RT. Resuspend the cell pellet in 5 mL of fresh E media.
24. Plate the cells on the mitomycin C treated fibroblast plate (10 mL E media in a 10 cm plate).

*Note:* Grow the keratinocytes on mitomycin C treated J23T3 fibroblast feeder layer. Keratinocytes can be passaged 8 times on feeders and subsequently can be passaged in a feeder independent manner.

25. Use the high calcium E-media (1.2 mM $Ca^{2+}$, in this protocol we refer to it as E media), (as described in Recipes for cell monolayer section under materials and equipment) for culturing the keratinocytes at 32°C, 5% $CO_2$.
26. In this protocol, feeder independent keratinocyte culture was used.

### Calcium switch

In this study, we performed calcium switch assays to induce junction formation. E cadherin-based AJs form in the presence of extracellular calcium, hence we grow the cell in low calcium media and replenish the media with high calcium E media.

*Day 1*

Timing: 2 h + 5 min

27. The following protocol is used for preparing the cell monolayer. Before you begin, prepare the following:
    a. Clean the glass coverslips by soaking it in 70% Ethanol, for an hour followed by rinsing it with distilled water, 4 times and finally autoclave.
    b. Coat the coverslips with 10 μg/mL of fibronectin, overnight at 4°C. Before plating the cells, air-dry the coated coverslips for 5 min to aid in adsorption of the fibronectin.

*Note:* The coated coverslips can be stored at 4°C for up to a month. OtherECM components like collagen, laminin or poly-L-lysine can also be used.

*Day 2*

Timing: 30 min

28. Seed enough primary keratinocytes in low calcium E-media to obtain a cell monolayer with 50% confluence on the day of fixation.

*Note:* Low calcium E media contains 0.05mM $Ca^{2+}$. This is prepared like the high calcium E media except using calcium-free DMEM/F12 (3:1) powder and chelating the calcium from the FBS using chelex. Once the media is prepared, $CaCl_2$ is added back to a final concentration of 0.05mM (Nowak and Fuchs, 2009). Follow Recipes for cell monolayer section under materials and equipment

*Day 3*

Timing: Step 29–31 (Day of fixation for 0 hours post calcium switch): 30 min

*Day 4*

Timing: Step 29–31 (Day of fixation for 24 hours post calcium switch): 30 min

29. Switch the culture media to high calcium once the cells have adhered and well spread on the coverslips (approximately after 24 h).
30. Aspirate the media gently (do not use vacuum pump suction) and wash twice with PBS containing $Ca^{2+}$ and $Mg^{2+}$ by pipetting slowly along the wall of the culture dish.
31. Add the fixative solution immediately, as detailed in the next section.

⚠ CRITICAL: Do not wash the cells harshly with PBS, since this will lead to disruption and loss of AJs.

*Note:* In this study, we fixed cells at 0-hour and 24-hour post calcium switch to study the ultrastructure of AJs.

### Fixative preparation

Timing: 1–2 h

32. Prepare 0.2M Na-Cacodylate buffer and adjust the pH to 7.2 with 0.2N HCL.
33. Prepare 0.2M $CaCl_2$ solution.
34. Prepare the fixative to a final concentration of 2% Glutaraldehyde, 2 mM $CaCl_2$ in 0.08M Na-Cacodylate buffer (pH 7.2).

*Note:* Follow the Recipes for cell monolayer section under materials and equipment.

35. Add 3 mL of fixative to the 3.5 cm culture dish and fix the cells for at least 20 min at RT.

*Note:* Continue on to steps 45–59 in step-by-step section.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| Sodium cacodylate buffer 0.2M | Fisher Scientific | 11652 |
| Glutaraldehyde 25% EM grade | VWR | 16220 |
| $CaCl_2$ | Fisher Scientific | C79-500 |
| 16% Formaldehyde solution | Pierce | 28908 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Potassium ferricyanide ($K_3Fe(CN)_6$ | Rasayan Laboratory | 401501 |
| Osmium Tetroxide ($OsO_4$) | Sigma-Aldrich | 19150 |
| Uranyl Acetate | Electron Microscopy Sciences | 22400 |
| Fibronectin | Sigma | F1141-5MG |
| Chelex | Biorad | 1422842 |
| Dulbecco's Modified Eagle's Medium (DMEM) | Gibco | 12100046 |
| Ham's F12 | Gibco | 21700075 |
| Sodium bicarbonate (NaHCO3) | Sigma | S3817 |
| Penicillin-Streptomycin Solution | Gibco | 15140122 |
| TC grade water | Sigma | W3500 |
| Fetal Bovine Serum | Gibco | 16000044 |
| Bovine Calf Serum | Thermo Fisher Scientific | 10371029 |
| Trypsin-EDTA | Sigma | T4049-500mL |
| Mitomycin C | Sigma | M4287-2MG |
| Dispase | Thermo Fisher Scientific | 17105-041 |
| Hydrocortisone | Sigma | H0888-1g |
| Cholera enterotoxin | Sigma | c8052-2mg |
| apo-Transferrin | Sigma | T2252-500mg |
| Insulin | Sigma | I1882-600mg |
| T3 (Triiodothyronine) | Sigma | T2752-100mg |
| Malic acid | Sigma | M0375-500G |
| Ethanol molecular grade | Merck | 100983 |
| Molecular Sieves, 4A, beads, 8–12 mesh | Sigma-Aldrich | 208604 |
| Propylene oxide | Sigma-Aldrich | 110205 |
| Toluidine Blue | Sigma-Aldrich | 89640 |
| TC grade Water | Sigma | W3500-6X1L |
| DMEM/F12 (3:1) without calcium | Life Technologies | 90–5010 |
| Critical commercial assays/kits | | |
| Araldite/Embed Embedding kit (Epon 812, Araldite 502, DDSA, DMP-30) | Electron Microscopy Sciences | 13940 |
| Experimental models: cell lines | | |
| VCL Ctrl | (Biswas et al., 2021) | N/A |
| Experimental models: organisms/strains | | |
| VCL Ctrl mice (VCL Flx/Flx; Cre-) | (Zemljic-Harpf et al., 2007) | N/A |
| Software and algorithms | | |
| Image J | NIH | ImageJ.net |
| BioRender | N/A | https://biorender.com |
| Adobe Photoshop | Adobe | https://www.adobe.com/ |
| Others | | |
| 35 mm Culture Dishes | Corning | CLS430165-500EA |
| Resin mold | Electron Microscopy Sciences | 70901 |
| Single Crystal Silicon wafer | Macwin India | 07500306232 |
| MERLIN Compact VP | Carl Zeiss | N/A |
| Ultramicrotome Powertome PTPC | RMC | N/A |
| Glass Knife maker EM KMR3 | Leica | N/A |
| Ultra-Diamond knife 45° (wet) | DiATOME Ltd. | N/A |
| EM CPD300 | Leica | N/A |
| Sputter Coater K550X | EMITECH | N/A |

## MATERIALS AND EQUIPMENT

### Recipes for common reagents

| 0.2M Na-Cacodylate solution | |
|---|---|
| Na-Cacodylate | 4.2806g |
| Distilled $H_2O$ | Adjust volume to 100mL |

*Note:* Before making up the volume with distilled $H_2O$, adjust pH to 7.2 with 0.2N HCL.

| 0.1M Na-Cacodylate solution | |
|---|---|
| 0.2M Na-Cacodylate buffer | 50mL |
| Distilled $H_2O$ | 50mL |

| 0.2M $CaCl_2$ solution | |
|---|---|
| Calcium Chloride | 0.221g |
| Distilled $H_2O$ | Adjust volume to 10mL |

| 0.2M Maleate buffer stock | |
|---|---|
| Maleic Acid | 2.32g |
| 1N NaOH | 20mL |
| Distilled $H_2O$ | To 100mL |

| 0.05M Maleate buffer (pH5.2) | |
|---|---|
| 0.2M Maleate buffer | 25mL |
| 0.2N NaOH | ~4.2mL |
| Distilled $H_20$ | To 100mL |

*Note:* Adjust the pH to 5.2 with 0.2N NaOH.

| UA (1% UA in 0.05M Maleate buffer pH5.2) | |
|---|---|
| Uranyl Acetate | 0.1g |
| 0.05M Maleate buffer | 10mL |

*Note:* After making UA (1% UA in 0.05M Maleate buffer pH5.2), spin it at 21000 ×g for 10 minutes at RT. Perform the preparation inside a chemical fume hood wearing appropriate PPE. UA is a slightly radioactive material and all necessary precautions should be taken when disposing it. To avoid precipitates during staining, filter the solution through a 0.22μm filter.

## Recipes for skin sample preparation

| Fixative | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Glutaraldehyde 25% | 2% | 1.6mL |
| Formaldehyde 16% | 4% | 3.2mL |
| Na-Cacodylate buffer 0.2M | 0.05M | 5mL |
| $CaCl_2$ 0.2M | 2mM | 0.2mL |
| $H_20$ add to complete | N/A | 11mL |
| Total | N/A | 20mL |

⚠ CRITICAL: Perform the preparation inside a chemical fume hood wearing appropriate Personal Protective Equipment (PPE). Glutaraldehyde is available in ampoules or vials. For ampoules, we prefer to open one ampoule for the experiment and discard the unused aldehyde after use. Do not open the glutaraldehyde vials. Instead, use a syringe and needle to take the desired volume. In this way, the vials remain sealed. Preferably, do not reuse formaldehyde ampoules. If ampoules are not available, formaldehyde can be prepared from paraformaldehyde powder. Do not use formalin. The fixatives should always be freshly prepared, close to the time of sample collection.

| **EPON Resin** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| EPON 812 | 25% (v/v) | 25 mL/27.50g |
| Araldite | 15% (v/v) | 15 mL/17.00g |
| DDSA | 55% (v/v) | 55 mL/55.00g |
| DMP-30 | 1.45% (v/v) | 1.45 mL/1.80g |
| Total | N/A | 100mL |

*Note:* The materials listed above are viscous in nature. Mix these components by measuring their mass according to the desired total volume of EPON resin needed. Mix these components thoroughly (at least 15 min) to get a homogeneous mixture. The resin components can be placed in an oven at 60°C for 15–30 min. This will make the resin less viscous and easier to mix, without affecting its properties. The catalyst DMP-30 must be added and mixed just before use.

## Recipes for cell monolayer preparation

| **E media** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| DMEM | 63.1%(v/v) | 300ML |
| Ham's F12 | 21.05%(v/v) | 100 mL |
| Supplemented FBS | 15%(v/v) | 70 mL |
| Pen-strep solution | 1% (v/v) | 5 mL |
| Total | N/A | 475mL |

| **100× COCKTAIL for E- media** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Insulin | 0.5 mg/mL | 20mL |
| Transferrin | 0.5 mg/mL | 20mL |
| $T_3$ | $2 \times 10^{-9}$ M | 20mL |
| PBS (sterile) | 1× | 140mL |
| Total | N/A | 200mL |

| **F media** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| DMEM | 65.9%(v/v) | 300mL |
| Ham's F12 | 21.9%(v/v) | 100 mL |
| Bovine Calf Serum | 10%(v/v) | 50 mL |
| Pen-strep solution | 1% (v/v) | 5 mL |
| Total | N/A | 455mL |

| Fixative | | |
|---|---|---|
| Reagent | Final concentration | Amount (ml) |
| Glutaraldehyde 25% | 2% | 1.6 |
| Na-Cacodylate buffer 0.2M | 0.08M | 8 |
| $CaCl_2$ 0.2M | 2mM | 0.2 |
| $H_20$ (mL): add to complete | N/A | 10.2 |
| Total | | 20 |

## STEP-BY-STEP METHOD DETAILS

### Skin sample: post fixation

This section describes the secondary fixation steps following the formaldehyde/glutaraldehyde fixation. The main purpose of this process is to increase the contrast in the sample.

*Day 2*

Timing: Step 1–4: 4–5 h

Timing: Step 5: 1.5 h

1. Wash the fixed samples 3 times for 5 min each with 0.1M Na-Cacodylate buffer (pH 7.2) at RT on a rotor (Figure 2B).
2. Post-fix with 1% Osmium Tetroxide in 0.1M Na-Cacodylate buffer (pH7.2) for 3–4 h on ice. Agitate gently from time to time or use a rotor.

   *Note:* Perform the preparation inside a chemical fume hood wearing appropriate PPE.

3. Wash 3 times in succession with 0.1M Na-Cacodylate buffer (pH7.2).
4. Wash 4 times for 5 min each with 0.05M Maleate buffer (pH5.2).

   *Note:* The use of Maleate buffer in this step is critical to avoid precipitation of UA in the presence of trace amounts of Na-Cacodylate.

5. En bloc staining/fixation with Uranyl Acetate (UA). Stain the samples with UA (1% UA in 0.05M Maleate buffer pH5.2) for 1.5 h at RT in the dark.

   *Note:* Follow the Recipes for common reagents and Recipes for skin sample preparation sections under materials and equipment.

### Skin sample: dehydration

The following dehydration steps helps to remove water from the sample and preserve the morphology of the specimen.

*Day 2*

Timing: 45 min

During dehydration, it is recommended to fill at least half the scintillation vial volume with dehydration solvent. Make sure to gently invert the vial a couple of times to remove any sample and water that could be stuck to the lid.

6. The following will describe the dehydration of the samples in a scintillation vial in a stepwise manner.

a. 30% Ethanol, 15 min with rotation at 4°C.
b. 50% Ethanol, 15 min with rotation at 4°C.
c. 70% Ethanol, 15 min with rotation at 4°C.

**Pause point:** If necessary, the samples can be kept overnight at Step 6c at 4°C.

*Day 3*

**Timing: 1.5 h**

d. 95% Ethanol, 15 min with rotation at RT.
e. 100% Ethanol, 3 times for 10 min each with rotation at RT

*Note:* Store the ethanol with molecular sieves to prevent moisture buildup.
f. Propylene oxide, 3 times for 15 min each with rotation at RT.

*Note:* Propylene Oxide is very toxic and volatile. Always work under the chemical hood with proper PPE.

**Skin sample: embedding**

The following section describes the steps for the embedding of skin sections using Epon-Araldite mixture.

*Day 3*

**Timing: 12 h**

7. After dehydration, infiltrate the samples with EPON resin embedding solution. Prepare the EPON resin as described in Recipes for skin sample preparation section under materials and equipment. Incubate the sample with 100% Propylene oxide: EPON resin (2:1) for 2 h at RT with gentle agitation in a scintillation vial.

*Note:* For agitation, a rotor can be used.

8. Next, remove the sample and incubate it in a vial containing 100% Propylene oxide: EPON resin (1:1) for 2 h at RT with gentle agitation.
9. Remove the sample from the 1:1 mixture and incubate it in a new vial containing 100% Propylene oxide: EPON resin (1:2) for 2 h at RT with gentle agitation.
10. Remove the sample from the 1:2 mixture and incubate it in a new vial containing 100% EPON resin for 6 h at RT with gentle agitation.

*Note:* At step 12, the samples can also be incubated overnight at RT

*Day 4*

**Timing: 6 h**

11. After 6 h of incubation (Step 10), continue the incubation with fresh 100% EPON resin for at least another 6 h with agitation.

*Day 3*

**Timing: 1 h**

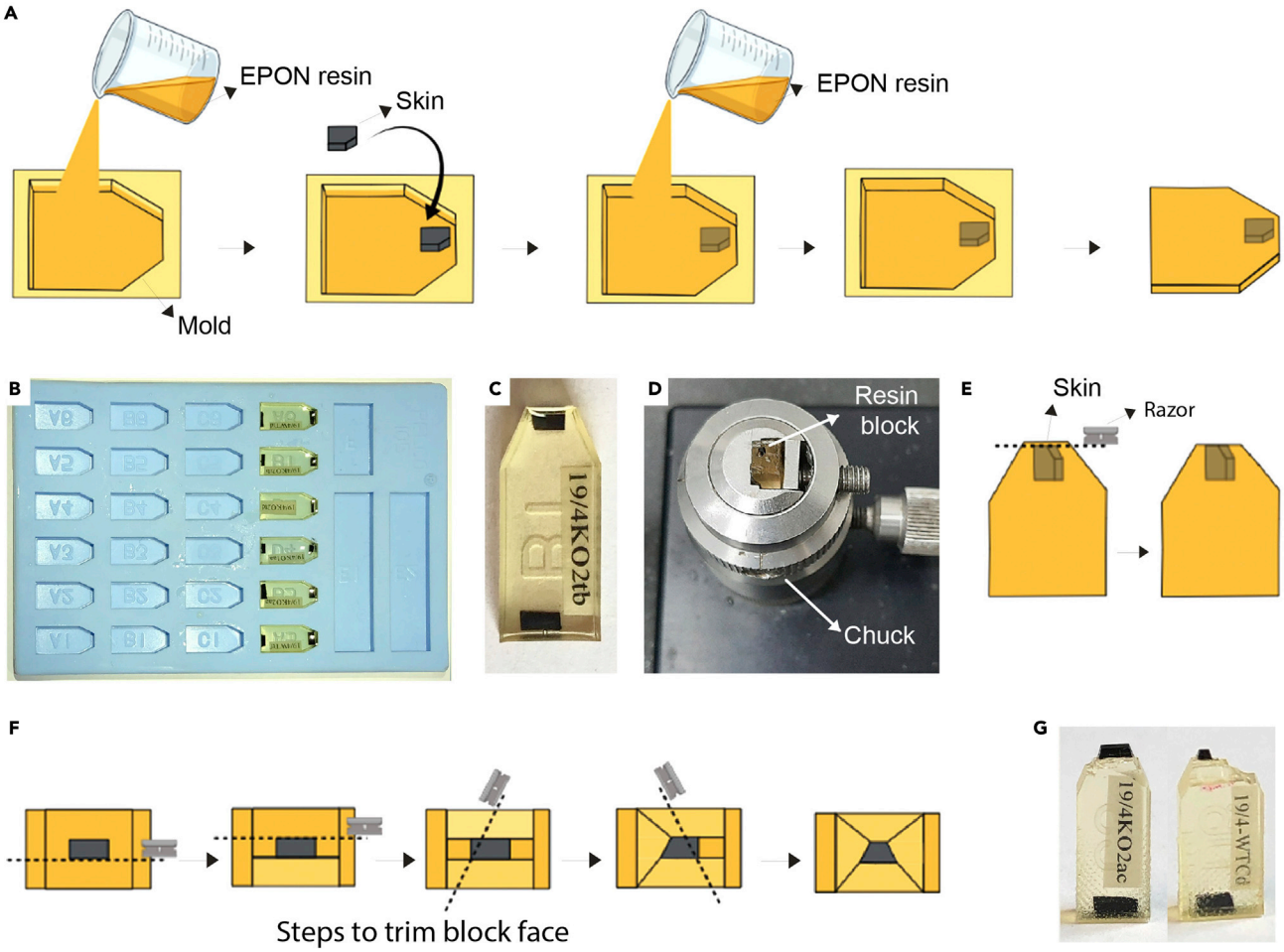


**Figure 3. Procedure for embedding sample in resin and trimming of resin block embedded with the sample**

(A) Stepwise illustration of embedding of the sample.

(B) Photograph of the resin mold containing polymerized EPON resin with the sample.

(C) Photograph of the side view of the EPON resin block with the sample.

(D) Photograph of EPON resin block inserted onto the chuck before trimming.

(E) Illustration of trimming of excess resin from the top of the EPON resin block using a single-edge razor blade.

(F) Illustration of the top view of the EPON resin block trimmed to a trapezoid shape using a single-edge razor blade.

(G) Photograph of EPON resin block at different stages of trimming; initial trimming (left) and final trimming (right).

12. Next embed the sample in silicone molds. For proper orientation and to prevent the sample from sinking to the bottom, fill half the mold with resin and cure it at 60°C in the oven overnight (Figure 3A).

*Note:* For this protocol, flat embedding molds were used (Figure 3B).

⚠ CRITICAL: This step must be performed the day before the final embedding of the samples due to the long curing time.

*Day 4*

Timing: 1 h

13. Place the sample in an orientation in the previously prepared EPON resin bed inside the mold (Figure 3A).

*Note:* It is important to embed the sample near to the edge of the mold.

*Day 5–6*

Timing: 48 h

14. Fill the rest of the mold with fresh EPON resin. Avoid bubbles and be careful not to disturb the position of the sample. Leave the EPON resin to polymerize for 48 h at 60°C (Figures 3A–3C).

**Skin sample: sectioning of EPON resin block**

The following section describes the steps to obtain ultrathin sections using the RMC Ultramicrotome Powertome PTPC (Figure 2D) with a glass/diamond knife from EPON resin molds containing the skin section.

*Day 7*

Timing: Step 15–20: 1 h

Timing: Step 21–24: 45 min

Timing: Step 25–35: 3–4 h per sample

Timing: Step 36–39: 30 min

15. Place the flat resin molds containing the back skin sample onto the chuck (Figures 3C and 3D).
16. If the skin sample is not embedded at the edge of the mold, first trim away the excess surface resin using a single-edge razor from the top until the sample can be seen (Figure 3E).

*Note:* Maintain a flat surface while trimming the excess resin away.

17. Use a razor blade to trim the top of the mold into a trapezium block shape with sloping sides. From top view: Make sure the top and bottom of the trapezium shape are parallel to each other with the bottom being longer than the top (Figure 3F).
18. Make final adjustments to the trapezoid by trimming away as much excess resin as possible. The final trapezoid should be as small as possible and mostly contain only the Region of Interest (ROI) of the skin sample (Figure 3G).

*Note:* The trapezoid shape is advantageous as it makes the orienting of the block to the knife easier, minimizing the compressive forces on the samples while sectioning.

19. Place and secure the chuck containing the trimmed resin onto the cantilever arm of the ultramicrotome (Figures 3D and 5D).
20. Place a freshly prepared glass knife onto the knife holder (Figure 2E).

*Note:* 2 knives are prepared from one glass square using a Leica EM KMR3 glass knife maker (Figure 2C). One knife will be of higher quality than the other. The knives can be stored in a knife holder box to prevent dust from collecting on its edge.

21. Orient the EPON resin block/glass knife such that the longer side of the trapezoid is parallel to the knife-edge (Figure 4A).

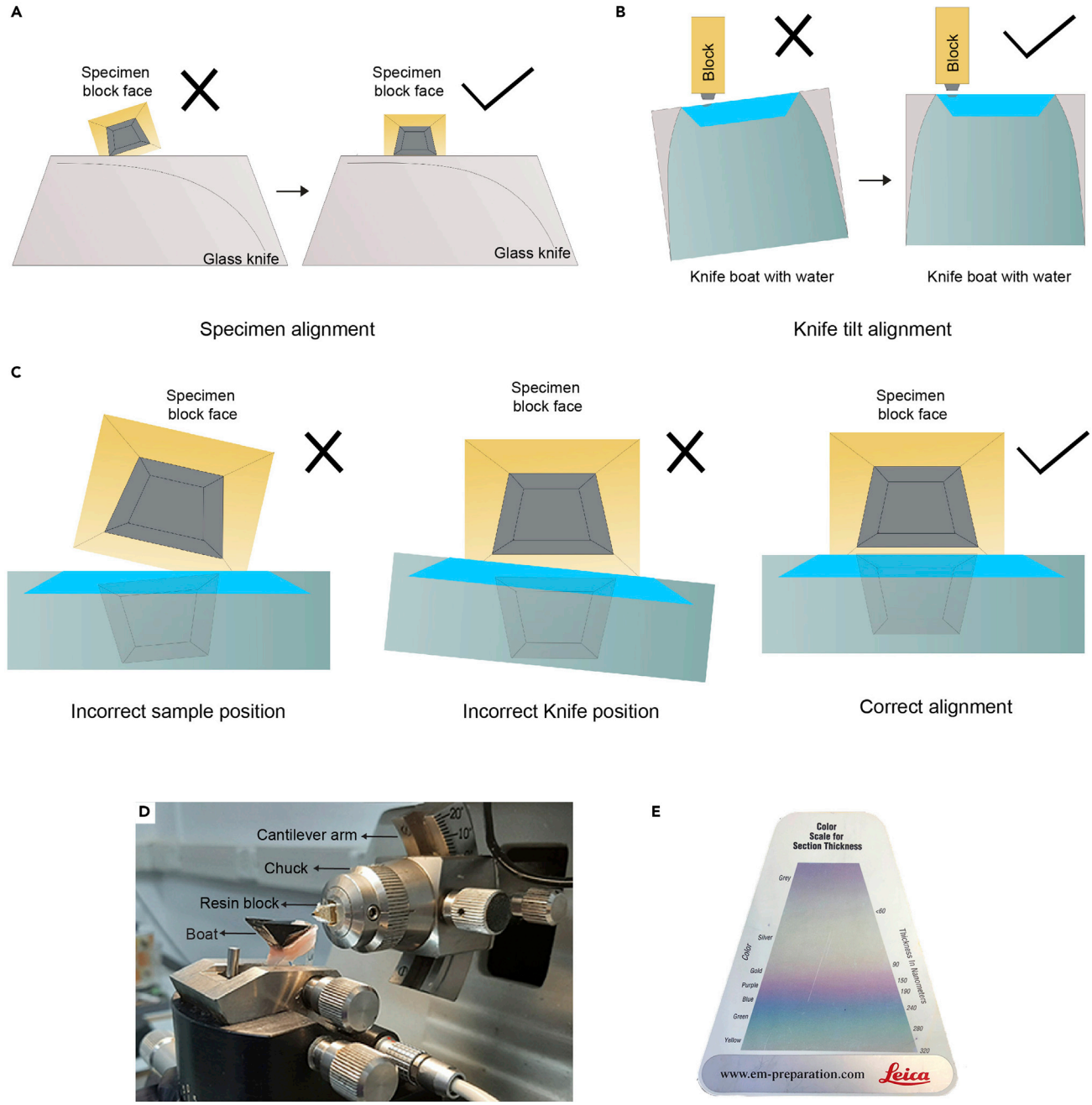


**Figure 4. Illustration for alignment of block face with the knife**

(A) Illustration of the front view of the alignment of the block face with respect to the knife-edge.
(B) Illustration of the alignment of the knife tilt with respect to the block face.
(C) Illustration of the orientation of the block face and knife.
(D) Photograph of ultramicrotome set up with the knife and the EPON resin block on the chuck.
(E) Photograph of interference card from Leica

*Note:* When aligning, move the block away from the knife-edge to protect the block surface and the knife-edge from any form of damage.

22. Trim the surface of the trapezoid block until the block face is completely flat with a mirror-like quality.

23. Prepare a fresh glass knife and attach a plastic boat to it using wax. Test to ensure that the seal does not leak using water (Figure 2E and Figure 4D).

*Note:* After testing it for leakage, dry the set up for at least 30 minutes before usage.

24. Replace the existing glass knife with the freshly prepared glass knife/plastic boat or a diamond knife suitable for semi thin sections.

*Note:* While changing the knife, move the block away from the knife-edge to protect the block surface and the knife-edge from any form of damage.

25. Set the clearance angle for the knife on the ultramicrotome. This is usually at 6°.
26. Using a 1 mL syringe, fill the boat with deionized water and adjust the water level to the knife-edge such that the knife-edge is also wet.

*Note:* Achieving a correct water level will create a uniform silvery surface near the knife-edge

⚠ CRITICAL: Continuously check the water level to ensure that it does not evaporate to below its original level. It is important to maintain the correct water level as it will be crucial for determining the thickness of the sections.

27. Orient the EPON resin block/glass knife again such that the longer side of the trapezoid is parallel to the knife-edge (Figure 4A and Figure 4B).

*Note:* These alignments can be achieved by aligning the shadow cast by the knife-edge to the longer side of the trapezoid using the binoculars of the ultramicrotome (Figure 2D and Figure 4A–Figure 4C).

28. Carefully bring the block to the knife-edge, leaving a gap of 3–4 mm between the block and the knife-edge.
29. Make finer adjustments to the alignment between the longer side of the trapezoid and the knife-edge by a) Specimen rotation alignment b) Knife lateral alignment c) Knife tilt alignment d) Specimen tilt alignment (Figure 4A–Figure 4C).

*Note:* These adjustments rely on observing the shadow cast by the knife-edge on the block face using the binoculars.

30. From the block face, section 500 nm–1 μm sections, stain with toluidine blue and examine under a light microscope.

*Note:* This step is crucial to determine the quality of sample preparation and to select the area of interest.

31. For ultrathin sections, trim the block face even smaller which contains only the ROI and switch to a diamond knife or a freshly prepared glass knife for further sectioning after performing all the necessary adjustments (Step 23–31).
32. On an automated ultramicrotome, set the cutting window by defining the top position (specimen block 1 cm above the knife-edge) and the bottom position (specimen block 1 cm below the knife-edge) (Methods video S1).

33. Set the sectioning speed from 0.5 mm/s to 1.0 mm/s.

*Note:* When using a diamond knife, use the speed as recommended by the manufacturer.

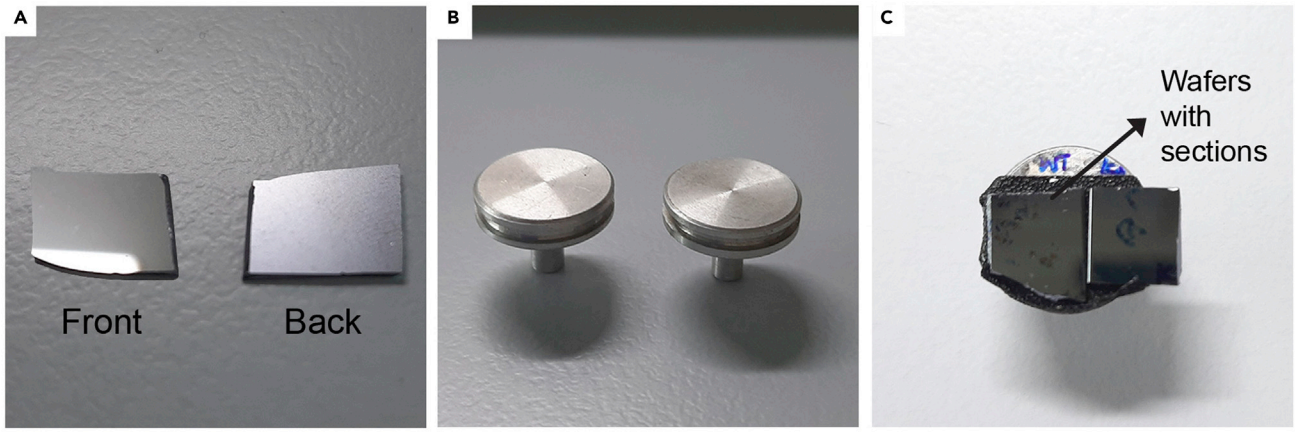


**Figure 5. Photographs of specimen mounts**

(A) Photograph of a silicon wafer with front (left) and back (right) view.

(B) Photograph of stubs.

(C) Photograph of silicon wafers with ultra-thin sections mounted on the stub.

34. Set the section thickness from semi-thin (100nm–120 nm) to ultra-thin (60 nm) depending on the desired imaging technique (SEM vs TEM).

*Note:* Stain with toluidine blue to focus on the correct ROI and sample orientation.

35. Section thickness can be determined with the help of an interference card that provides a color spectrum that reflects its respective section thickness. For example, for semi-thin to ultra-thin sections, the color of the sections will vary from a gold to silver color (Figure 4E).
36. With the aid of an eyelash brush, the sections can be detached from the knife-edge. To ease the collection of the sections, the brush can also be used to guide the sections towards the end of the boat.
37. Before collection, flatten the section with the isopropanol vapors.

*Note:* One can use a cotton wick soaked in isopropanol to flatten the section.

38. Scoop the sections from water with the help of a loop and place them on the front side of a silicon wafer. Representation of the front side of silicon wafer and sections attached on the wafer is shown in (Figure 5A and Figure 5C).
39. Dry the sections overnight under a light bulb.

### Skin sample: imaging

The following section describes the different SEM image acquisition parameters applicable for the ultrathin skin sections using the MERLIN Compact VP scanning electron microscope (Figure 2A).

*Day 8*

**Timing: 3–4 h (variable depending on requirements of the experiment)**

Imaging with an electron microscope will require assistance from someone who is well trained on the SEM or TEM. Electron microscope imaging particulars vary from sample to sample. The steps that are described here are focused only on imaging of skin samples.

40. On the day of imaging, place the dried silicon wafer on the SEM pin stub (12.56 mm diameter) using the carbon adhesive tape (Figure 5B and Figure 5C). Place this onto the stub holder (46 mm diameter) that is suitable for the specific microscope (Figure 2F).
41. Place the sample into the microscope and wait until a vacuum has been attained before adjusting the parameters. Under a lower magnification mode, try to locate the ROI.
42. After finding a suitable ROI, increase the magnification and take individual images.
43. Imaging is done in InlensDUO mode maintaining EHT at 2 kV, probe current 5–10pA and aperture size between 30–60 microns. Images are captured while maintaining a scan speed of 6–9 with a resolution of 3072×2304 pixel. Line integration mode is used for noise reduction.
44. Image processing is done using ImageJ or Adobe photoshop.

### Cell monolayer: post fixation

This section describes the secondary fixation steps following the formaldehyde/glutaraldehyde fixation. The main purpose of this process is to increase the contrast in the sample.

*Day 3- 0-h coverslip*
*Day 4- 24-h coverslip*

**Timing: Step 45–48: 1.5 h**

**Timing: Step 49: 1.5 h**

45. Pipette out the fixative solution and wash the fixed cells with 0.1M Na-Cacodylate buffer (pH7.2) 3 times for 5 min each at RT.
46. Fix the cells with 0.8% Potassium Ferricyanide ($K_3Fe(CN)_6$), 1% Osmium Tetroxide ($OsO_4$) in 0.1M Na-Cacodylate buffer (pH7.2) for 30 min on ice.
47. Wash the fixed cells with 0.1M Na-Cacodylate buffer (pH7.2) 3 times for 5 min each at RT.
48. Wash the fixed cells with dd $H_2O$, 5 times for 5 min each at RT.
49. Stain the cells with UA (1% UA in 0.05M Maleate buffer pH5.2) for 1.5 h at RT in the dark.

*Note:* Follow the Recipes for common reagents and Recipes for cell monolayer preparation sections under Materials and Equipment.

### Cell monolayer: dehydration

The following dehydration steps helps to remove water from the sample and preserve the morphology of the specimen.

*Day 3- 0-h coverslip*
*Day 4– 24-h coverslip*

**Timing: Step 50–51c: 25 min**

**Timing: 51d–51e 1 h**

50. After staining, directly transfer the coverslips onto a glass petri dish.

51. The following will describe the dehydration of the cells in a stepwise manner.

*Note:* All these steps should be done on ice

a. 30% Ethanol, 7 min.
b. 50% Ethanol, 7 min.
c. 70% Ethanol, 7 min.

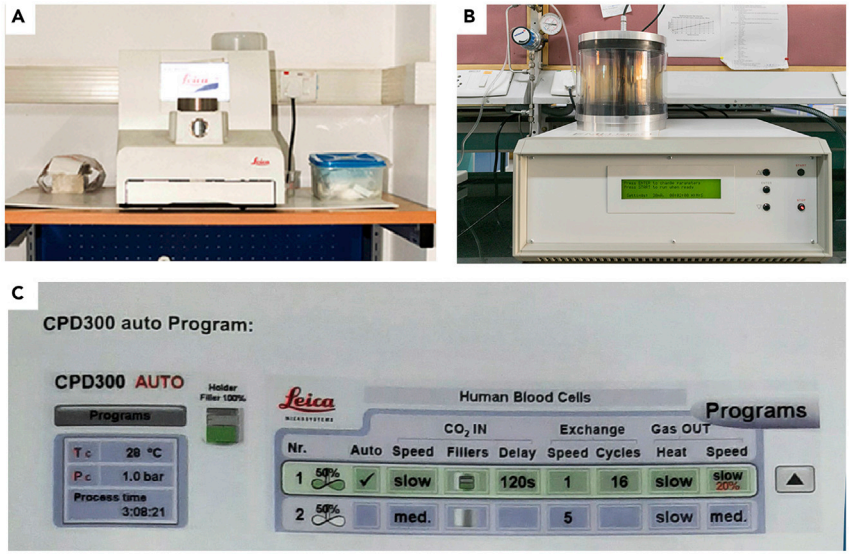


**Figure 6. Equipment for cell monolayer protocol**
(A) Leica EM CPD300 Critical Point Dryer.
(B) EMITECH K550× sputter coater.
(C) Parameters used for Critical Point Drying.

**Pause point:** If necessary, the samples can be kept overnight at Step 51c at 4°C.

d. 95% Ethanol, 7 min at RT.
e. 100% Ethanol, 3 times for 7 min each at RT.

*Note:* Store the ethanol with molecular sieves.

### Cell monolayer: critical point drying (CPD)

The following steps describes the procedure of critical point drying for the cell monolayer. These steps help to preserve the surface structure of the cell monolayer by removing the liquid from the sample while avoiding any damage caused by surface tension that occurs due to the transition from liquid $CO_2$ to gaseous $CO_2$

*Day 4- 0-h coverslip*
*Day 5- 24-h coverslip*

**Timing: 3.1 h**

52. For CPD, use the CPD300 auto program for human blood cells, which is a preset program in the instrument (Figure 6A and Figure 6C). Place the dehydrated coverslips into the pressure chamber of the instrument and fill it with the exchange fluid such as ethanol to prevent air-drying.
53. The program performs the subsequent steps automatically, which include filling of the liquid into the pre-cooled pressure chamber, purging the $CO_2$ for 16 cycles at a slow speed, with a delay of 120 s. After the purging cycles, the exchange fluids are replaced with liquid $CO_2$ and a heating process is initiated at 28°C to generate supercritical $CO_2$.
54. Subsequently the supercritical $CO_2$ is converted to a gaseous state by maintaining a constant temperature of 31°C. Finally, the pressure in the chamber is reduced by slowly purging the gas in the chamber at a speed of 20% (Gas-Out phase). This entire program runs for 3 h, 8 min and 21 s. After the Gas-out phase, take out the coverslips for sputter coating and imaging.

### Cell monolayer: imaging

The following section describes the different SEM image acquisition parameters applicable for the critical point dried cell monolayer using the MERLIN Compact VP scanning electron microscope (Figure 2A).

*Day 5- 0-h coverslip*
*Day 6- 24-h coverslip*

**Timing:** 3–4 h (variable depending on requirements of the experiment)

The steps described here are focused only on imaging of keratinocyte monolayer.

55. On the day of imaging, place the dried coverslips onto the SEM pin stub (12.56 mm diameter) using carbon adhesive tape. Place this onto a sputter coater (Figure 6B) and coat it with gold/palladium at 20–30 mA for 30 s using argon at $1\times10^{-1}$ mbar with the stage rotation enabled to obtain a 5–10 nm thick coating. Once coated, place the SEM pin stub onto the stub holder (46 mm diameter) that is suitable for the specific microscope (Figure 2F).
56. Place the sample into the microscope and wait until a vacuum has been attained before adjusting the parameters. Under a lower magnification mode, try to locate the ROI.
57. After finding a suitable ROI, increase the magnification and take individual images.
58. Imaging is done in SE mode maintaining EHT at 2 kV, probe current 5–10pA and aperture size between 30 microns. Images are captured while maintaining a scan speed of 6–9 with a resolution of 2048×1536 pixel. Line integration mode is used for noise reduction.
59. Image processing is done using ImageJ or Adobe photoshop.

## EXPECTED OUTCOMES

Following this protocol will help to successfully visualize the ultrastructure of the mammalian skin including the epidermis, dermis and appendages such as the hair follicle (Figure 7A) and sebaceous glands. Subcellular structures can also be studied according to the researcher's interests such as cell junctions, including adherens junctions (AJs), desmosomes (Figure 7A′, Figure 7C, Figure 7D, and Figure 7D′), hemidesmosomes and the basement membrane (Figure 7B), mitochondria, cell nuclei, lysosomes, etc. under normal and pathological conditions. Following this protocol will also help to successfully visualize the filopodia extensions at the cell-cell junctions in the primary keratinocyte cell monolayer (Figure 8A and Figure 8A′).

## LIMITATIONS

One of the major limitations of SEM is the resolution. The optimal spatial resolution that can be achieved from SEM is around 10 nm, compared to that of TEM, which is around 0.2 nm. For example, finer structures like lamellar granules in the upper layers of the epidermis can be better visualized using TEM (Wertz, 2018). In addition, visualizing finer structures like actin and actin-binding proteins also requires advanced EM techniques like the cryo techniques, light microscopy with super resolution as well as Correlative Light and Electron Microscopy (CLEM) (Jung et al., 2020). Fine structures like individual actin filaments are difficult to visualize from SEM. SEM only provides imaging of the cell surface, unless permeabilization methods are applied. Furthermore, coating with heavy metals may obscure some fine surface details in some specimens. However, SEMs equipped with field emission guns can resolve this issue (Muscariello et al., 2005; Van Meerbeek et al., 2000).

## TROUBLESHOOTING

### Problem 1

Issue with tissue handling. Excision of tissue can result in mechanical damage to the superficial skin layers, causing the cells to look empty or having ruptured membranes (associated with: "Before you begin" section Steps 10–13).

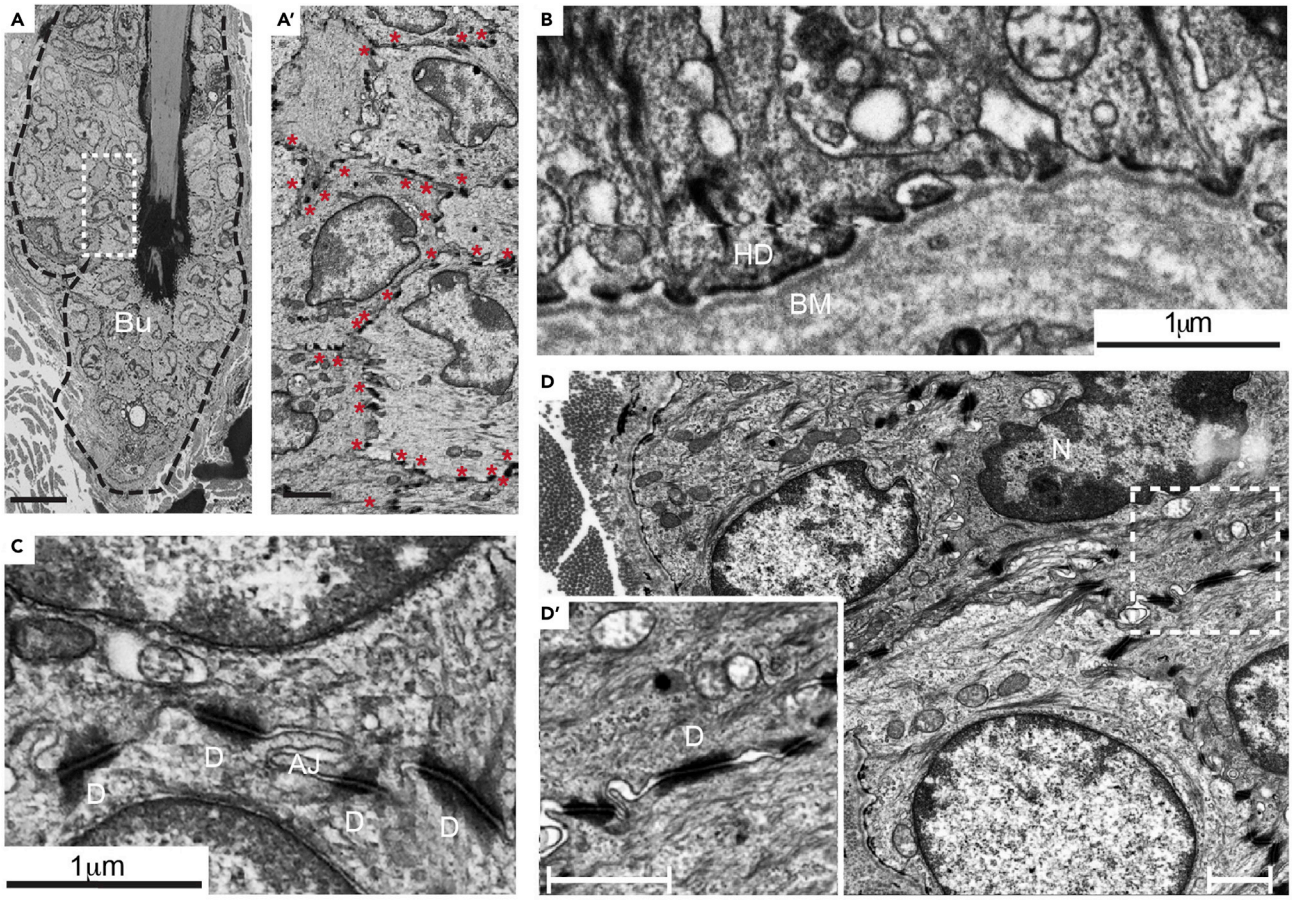


**Figure 7. SEM images of ultrathin skin sections**

(A) Electron micrograph of Hair Follicle and Bulge region (Bu).

(A′) Magnified image highlighting desmosomes from (A). Red asterisks mark the desmosomes.

(B) Electron micrograph of Inter Follicular Epidermis (IFE), with basement membrane (BM) and hemidesmosome (HD) clearly visible.

(C) Electron micrograph of adherens junction (AJ) between two Desmosomes (D) on the plasma membrane of two adjacent cells.

(D and D′) (D) Electron micrograph of cell-cell junctions in the bulge region of HF (D′) Inset high magnification image of the region demarcated in the white dashed box.

Scale bar: (A′): 10 μm, (A): 2 μm, (B, C, D, and D′): 1 μm

Figure reprinted with permission from (Biswas et al., 2021).

### Potential solution 1

This damage can be caused by delays in placing the sample in fixative due to early postmortem changes, therefore, work quickly. Avoid stretching the back skin too much as this might lead to ruptures within the tissue. Cut the skin with smooth movements of the blade without applying pressure.

### Problem 2

Issue with fixative. Problems may arise due to gradient fixation during the fixation procedure, which can later lead to issues while imaging. Similarly, during osmium fixation, issues such as gradient fixation may arise in the adipose tissues, which may lead to the loss of lipid droplets from the sample (associated with: "step-by-step methods" section step 2) (Figure 9A, compared to Figure 9B).

### Potential solution 2

Keep the sample size small, optimally 1 mm × 2 mm, to avoid problems from gradient fixation.

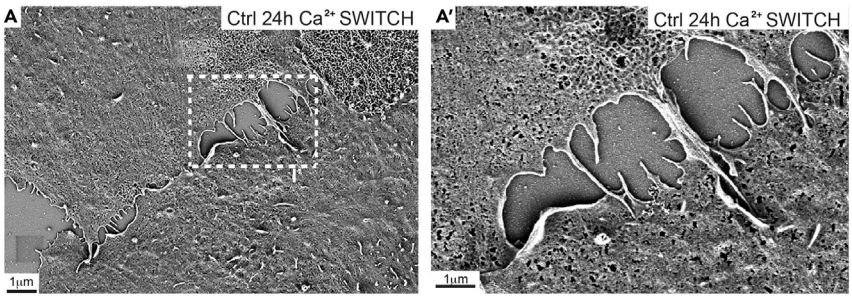


**Figure 8. SEM image for cell monolayer protocol**

(A) Electron micrograph of junction formation after 24 h of calcium switches in keratinocytes.

(A′) Higher magnification of dashed box in (A).

Scale bar: (A, A′): 1 μm

Figure reprinted with permission from (Biswas et al., 2021)

### Problem 3

Issue with dehydration. Prolonged dehydration and deviation from the recommended temperature (associated with: "step-by-step methods" section step 6) may destroy the ultrastructure of the sample whereas shortened dehydration times may cause holes in the section.

### Potential solution 3

Perform the dehydration step at 4°C until the 70% ethanol and do not extend or reduce the dehydration time from the recommended duration.

### Problem 4

Issues while sectioning. Insufficient mixing of resin components (associated with: "step-by-step methods" section step 7). Insufficient infiltration time of the resin into the sections (associated with: "step-by-step methods" section steps 7–11). Difficulty sectioning due to large sample size after trimming (more than 1 mm in all dimensions) (associated with: "step-by-step methods" section step 18). Dull knife, scratches on the knife (associated with: "step-by-step methods" section steps 23 and 24) (Figure 9C). Sectioning above the recommended thickness can result in holes in the sample and issues with contrast during imaging (associated with: "step-by-step methods" section steps 32–35).

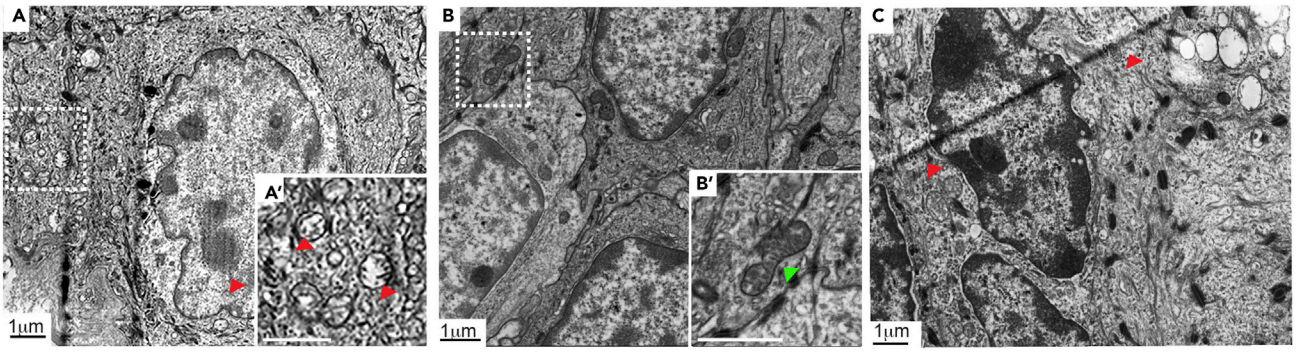


**Figure 9. SEM images for troubleshooting for mouse skin ultrathin sections**

(A and A′) (A) Electron micrograph showing abnormal mitochondria without cristae, suggesting an issue with fixation, inset (A′) high magnification image of the region demarcated by white dashed box showing improper fixation of mitochondria (red arrowhead). Knife marks are also visible in the nucleus as indicated by the red arrowhead.

(B and B′) (B) Electron micrograph showing properly fixed mitochondria with cristae, inset (B′) high magnification image of the region demarcated by white dashed box indicating proper fixation of mitochondria (green arrowhead).

(C) Electron micrograph showing knife marks as indicated by the red arrowheads.

Scale bar: (A-C): 1 μm, Inset (A′, B′): 1 μm.

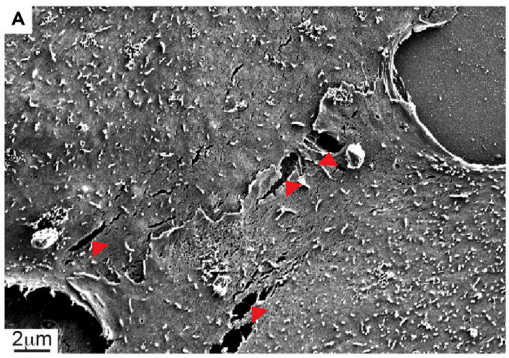


**Figure 10. SEM images for troubleshooting for cell monolayer**

(A) Electron micrograph showing issues with CPD. Red arrowheads indicating cracks on the cell surface.

### Potential solution 4

Mix for at least 15 min to prepare a homogeneous resin mixture. Do not reduce the resin infiltration time from the recommended duration. Trim away as much excess sample such that the final dimension is no more than 1 mm. Always prepare and use a fresh knife. Always refer to the interference card to monitor the thickness of the sections.

### Problem 5

Over drying of samples, insufficient dehydration or $CO_2$ exchange during CPD could lead to cracks and holes in the sample (associated with: "step-by-step methods" section steps: 52–54) (Figure 10).

### Potential solution 5

Processing time should not exceed 3 h for the cell monolayer. If cracking still occurs, reduce the drying duration. Alternatively, other drying methods such as hexamethyldisilazane (HMDS) can be used (Braet et al., 1997). After coating, samples should be kept in a dry environment to prevent changes as a result of water absorption or possible cracking of the coated layer.

### Problem 6

Masking of details in the sample due to overcoating of the sample using the sputter coater and the charging in SEM due to undercoating of the sample using the sputter coater (associated with: "Step-by-Step methods" section Step 55).

### Potential solution 6

A 5–10 nm layer of coating is typically applied for conventional gold/palladium sputtering. If final details are obscured, apply a thinner layer of gold/palladium, or change to a finer grain of element with a lower atomic number. If there is a charging issue while imaging, apply a thicker coat.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by lead contact, Srikala Raghavan (srikala@instem.res.in and srikala_raghavan@asrl.a-star.edu.sg)

### Materials availability

Unique materials generated in this study are available from the lead contact upon request.

### Data and code availability

This study did not generate any data sets and code.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100729.

## ACKNOWLEDGMENTS

We would like to thank members of the Raghavan Lab and Ramanuj DasGupta for providing critical feedback on the work and manuscript. We thank the EM facility and the animal facility at NCBS/inStem. This work is supported by grants from the Science & Engineering Research Board (SERB) EMR/2016/003199, Department of Science and Technology (DST), India, and Institute for Stem Cell Science and Regenerative Medicine (InStem), India core funding to S.R The work at Singapore was supported by the AMBM grant (A18A8b0059) to S.R. R.B. is supported by a DST-SERB WOS-A predoctoral fellowship (SR/WOS-A/LS-255/2017). A.B. was funded through a NPDF postdoctoral funding from DST SERB (PDF/2017/000860). A.B. and R.L. are funded through the AMBM grant (A18A8b0059). Animal work was partially supported by the National Mouse Research Resource (NaMoR) grant (BT/PR5981/MED/31/181/2012; 2013–2016) from the DBT.

## AUTHOR CONTRIBUTIONS

H.A.P. and S.R. conceived the manuscript. S.R. arranged for the funds. A.B., R.B., R.L., H.A.P., and S.R. wrote/edited the manuscript.

## DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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