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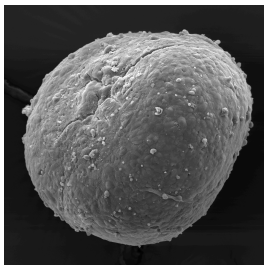
Preparation and gold sputter coating of whole-mount spheroids for scanning electron microscopy analysis



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We use this protocol and it's working

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Abstract

Three-dimensional spheroid models provide a more physiologically relevant platform for cancer research compared to conventional two-dimensional cell cultures, as they closely mimic features of the tumour microenvironment, including hypoxia and acidic pH. These models are essential for studying tumour heterogeneity, cancer stem cell dynamics, and therapy resistance. However, the small size and fragility of spheroids make sample handling and preparation for ultrastructural analysis challenging.

This protocol provides a detailed, step-by-step workflow for the preparation and gold sputter coating of whole-mount spheroids, optimized for scanning electron microscopy (SEM) surface analysis. The method is demonstrated using spheroids derived from normal (SV-HUC-1) and cancerous (T24) human urothelial cell lines but can be readily adapted to spheroids generated from other cell types and tissue origins. Following this protocol enables reproducible, high-resolution imaging of spheroid surface ultrastructure.

Materials

Materials

- **1 mL and 200 µL micropipette tips** (Brand, Germany, Cat. No.: 732012 and 732028)
- **10 mL glass vials** (SGD Pharma, Germany)
- **Bovine Serum Albumin (BSA)** (Sigma-Aldrich, USA, Cat. No.: A2153)
- **Cacodylic acid sodium salt trihydrate** (Serva, Germany, Cat. No.:1554002)
- **Microcentrifuge tubes 0.5 mL and 1.5 mL** (LLG Labware, United Kingdom, Cat. No.: 9409026 and 6.490 852)
- **Distilled water** (Institute of cell biology, ULMF, Slovenia)
- **Ethanol** (100 %) (Carlo Erba Reagents, France, Cat. No.: 4146072)
- **Glutaraldehyde** (Serva, Germany, Cat. No.: 23114.01)
- **Hexamethyldisilazane (HMDS)** (Sigma-Aldrich, USA, Cat. No.: 440191)
- **Metal stubs** (12.7 diameter) (Mikrolux, Croatia)
- **Osmium tetraoxide** (Roth, Germany, Cat. No.:8371.3)
- **Paraformaldehyde** (Sigma-Aldrich, Germany, Cat. No.: 158127)
- **Pasteur pipette** (3 mL) (Brand, Germany, Cat. No.:747765)
- **Phosphate buffer saline** (PBS):
  - Potassium chloride (Merck (Sigma-Aldrich); Germany, Cat. No.:104936)
  - Disodium hydrogen phosphate (Merck (Sigma-Aldrich); Germany, Cat. No.:106580)
  - Potassium dihydrogen phosphate (Merck (Sigma-Aldrich); Germany, Cat. No.:104873)
  - Sodium chloride (Chem-Lab, Belgium, Cat. No.:CL00.1429)
- **Sterile 96-well ultra-low attachment U-shaped bottom microplates** (Corning, USA, Cat. No.: 7007)
- **Ultrasmooth carbon discs** (SPI supplies, USA, Cat. No.:04967-B1)

Equipment

- **Bench-mounted fume cupboard System Delta 30** (Wesemann, Germany)
- **Centrifuge MyFuge Mini** (Benchmark Scientific, USA)
- **Laminar air-flow cabinet M182** (II) (Iskra Pio, Slovenia)
- **Scanning electron microscope Vega 3** (Tescan, Brno, Czech Republic)
- **Sputtering coating machine SCD 040** (Balzers Union, Liechtenstein)

Protocol for the formation of normal and cancer urothelial spheroids

1w

1 SV-HUC-1 and T24 spheroids were grown in 96-well ultra-low attachment U-shaped bottom microplates (Corning, New York, NY, USA) and incubated in a humidified incubator at 5% CO2 and 37°C. Seeding densities of 100,000 cells per well (in 200 µL of culture medium) were used to generate spheroids. Spheroids were grown for 7 days prior to their preparation for scanning electron microscopy analysis.

1w

Note

During seeding, ensure that pipette tips do not touch the bottom or sides of the wells to avoid damaging the surface coating of the ultra-low attachment U-shaped bottom microplates.

Note

Spheroid loss during washing, solution exchanges, and procedures such as dehydration is expected and correlates with operator experience level and handling technique precision. Always process 20–30% excess spheroids beyond experimental requirements to compensate for potential losses.

Spheroid transfer and collection

30m

2 Room temperature

30m

2.1 Use a 1 mL micropipette fitted with a tip or a 3 mL plastic Pasteur pipette to transfer the spheroids from the 96-well ultra-low attachment U-bottom cell culture microplate to a 1.5 mL microcentrifuge tube filled with cold 2% formaldehyde (w/v) and 2% glutaraldehyde (v/v) in 0.2 M cacodylate buffer (pH 7.4).

Note

To prevent spheroids from adhering to the pipette wall and to ensure maximal transfer, pre-coat the micropipette tips or Pasteur pipettes with 1% BSA in PBS by dipping their full length of the tip in 1% BSA in PBS.

2.2 Quickly aspirate the culture medium and spheroid with the pipette. You should be able to see the spheroid inside the tip. If it is not there, return the medium to the well and

aspirate again until you can visually confirm the presence of the spheroid inside the tip of the pipette.

2.3 Allow the spheroid to settle at the bottom of the tip, then pipette it into the microcentrifuge tube containing fixative.

Note

You can transfer several spheroids (3-5) into a single microcentrifuge tube or work with individual spheroids.

Fixation

1h 30m

3

1h 30m

3.1 Fix the spheroids with cold 2% formaldehyde (w/v) and 2% glutaraldehyde (v/v) in 0.2 M cacodylate buffer (pH 7.4) for 1 hour at 4 °C ensuring that the spheroids are completely submerged in the fixative.

3.2 Rinse the samples with 0.2 M cacodylate buffer three times for 10 minutes each at Room temperature.

Note

To change the solution in which the spheroid is submerged in a microcentrifuge tube, gently aspirate as much of the solution as possible with a 200 µL/1 mL micropipette fitted with a tip without disturbing the spheroid and add the appropriate volume of the new solution. However, to prevent accidental spheroid loss, maintain 10-20µL of residual liquid at the container bottom during solution exchanges.

Note

When performing solution exchanges, first allow spheroids to settle at the container bottom. For accelerated settling, tubes may be gently spun at 2000×g (6000 rpm) for 5 seconds to ensure complete spheroid sedimentation. Visually confirm the presence of spheroids after each step.

3.3 Transfer the samples to glass vials and post-fix the samples in 1% (w/v) osmium tetroxide in 0.2 M cacodylate buffer for 1 hour at Room temperature.

3.4 Rinse samples in 0.2 M cacodylate buffer for three times for 10 minutes each at Room temperature .

Dehydration

25m

4 Room temperature 25m

4.1 Submerge the samples in 30% ethanol for 5 minutes.

4.2 Submerge the samples in 50% ethanol for 5 minutes.

4.3 Submerge the samples in 70% ethanol for 5 minutes.

4.4 Submerge the samples in 90% ethanol for 5 minutes.

4.5 Submerge the samples in 100% ethanol twice for 5 minutes each.

Drying

1d

5 Room temperature 1d

5.1 Submerge the samples in acetone twice for 10 minutes each.

5.2 Submerge the samples in hexamethyldisilazane (HMDS) with a 1:1 ratio of acetone:HMDS for 10 minutes.

5.3 Submerge the samples in HMDS twice for 10 minutes each.

5.4 Aspirate the HMDS and air-dry the samples overnight at room temperature (open the vial/microcentrifuge tube with the spheroids immersed into remaining HMDS and leave it in the fume hood overnight).

Mounting

1h

6 Room temperature 1h

Mount spheroids on metal stubs (Fig 1) using a sticky carbon disc (apply double-sided conductive tape to the stub and remove the protective layer) with tweezers (do not squeeze the spheroids to avoid damaging them), or simply by dropping spheroids from glass vials onto the sticky carbon disc (by turning the vial upside down so that the spheroids fall onto the conductive tape).

Note

Several spheroids can be mounted on individual metal stub (Fig 1A).

Sputter Coating

1h

7 Sputter-coat the samples with gold according to the manufacturer's instructions (Fig 1B). 1h

Note

The SCD 040 sputter coating machine (Balzers Union) with Baltec gold sputter target (50 mm) was used in our experiments to apply a gold layer approximately 16 nm thick, operating at 30 mA for 1 minute.

Note

Samples should be stored in an air-tight container at Room temperature .

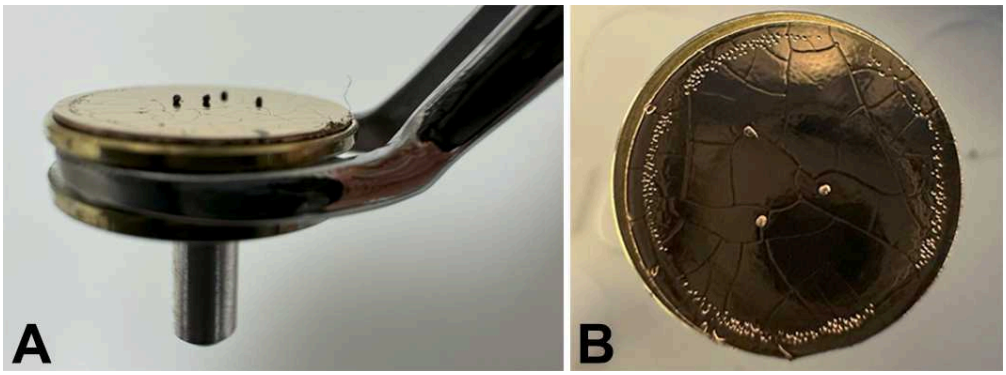

Fig 1. SV-HUC-1 spheroids mounted on metal stubs and sputter-coated with gold. A: side view. **B**: top view.

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