

LAB PROTOCOL

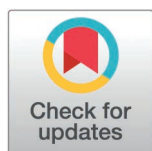
Integrated light and electron microscopy workflow for morphological, molecular and ultrastructural analysis of spheroids

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Data availability statement: Documentation describing the data, including a detailed README file, is publicly available in the Repository of the University of Ljubljana (RUL): <https://repozitorij.uni-lj.si/lzpisGradiva.php?id=178001&lang=eng> All datasets required

Abstract

Traditional two-dimensional cell cultures are limited in their ability to accurately model cancer progression and treatment response, which has prompted researchers to develop three-dimensional *in vitro* models, such as spheroids. These models better mimic the cellular interactions and microenvironment found in solid tumors, including features like hypoxic cores and acidic pH, which activate survival pathways and closely mirror *in vivo* tumor cell behavior. To fully harness the potential of spheroids in cancer research, comprehensive characterization at the morphological, molecular, and ultrastructural levels using various microscopy workflows is essential. Given the challenges associated with traditional handling and processing methods due to the small size of spheroids, we have developed and present here detailed protocols for light and electron microscopy analysis of spheroids formed from the human noncancerous urothelial cell line SV-HUC-1 and the malignant urothelial cancer cell line T24. Spheroids were analyzed by light microscopy using histological staining and immunofluorescence labelling, following either cryosectioning or paraffin embedding. Whole-mount immunofluorescence combined with optical clearing and confocal microscopy enabled visualization of protein expression and localization throughout the entire spheroid. Scanning and transmission electron microscopy provided high-resolution insights into surface morphology and internal ultrastructure, respectively. Each imaging modality, paired with optimized sample preparation, contributed to a comprehensive workflow offering distinct and complementary views of spheroid morphology, protein distribution, and cellular organization. This integrated approach, combining various light and electron microscopy workflows, enables accurate and thorough characterization of spheroids and establishes a comprehensive baseline for downstream functional investigations. The described protocols are adaptable to spheroids derived from

to reproduce the analyses and support the conclusions of the study, including processed datasets as well as full raw image data exported as unmodified TIFF files, are openly available at this link without access restrictions. Data processing and analysis pipelines are described in detail in the Methods section, and all results can be reproduced using the deposited data and accompanying documentation.

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Abbreviations: 2D/3D, two-/three-dimensional; HE, haematoxylin–eosin; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

various cell types and tissue origins, making them a versatile tool for a broad range of applications.

Introduction

In recent years, the use of spheroids, three-dimensional (3D) *in vitro* models, has emerged as a promising tool for studying the intricacies of cancer. Unlike traditional two-dimensional (2D) cultures, spheroids provide a more realistic representation of the tumor microenvironment, cellular behavior, and drug penetration. These models facilitate the investigation of factors necessary for the development of more effective cancer treatments, such as tumor heterogeneity, cancer stem cell behavior, and mechanisms of therapy resistance [1,2]. Additionally, 3D cancer models offer ethical and economic advantages by enabling prediction of tumor responses to chemotherapy or radiation, thus minimizing the reliance on animal testing in preclinical studies [3].

Spheroids possess a layered structure, with proliferative cells located in the outer zone and a quiescent region in the interior. This organization results from limited oxygen and nutrient supply, which creates an acidic pH environment within the spheroid's core, mimicking the hypoxic core of solid tumors, thereby replicating the physiological gradients observed in real tumors [4]. This hypoxic microenvironment activates genes involved in cell survival, providing a more accurate reflection of tumor cell behavior [4].

To date, several bladder cancer spheroids have been successfully generated from cell lines of various carcinoma grades (such as RT4, 5637, T24, J82, etc.) and patient-derived tumor tissues [5–7]. Spheroids from normal urothelial cell lines such as SV-HUC-1 [5,7] and primary human urothelial cells isolated from surgical ureter specimens [5,8,9] have also been successfully established. The transition from 2D to 3D culture significantly impacts the molecular phenotype of bladder cancer cells: cell lines cultured as spheroids exhibit altered expression of proteins associated with epithelial-mesenchymal transition, including changes in E-cadherin and N-cadherin levels [10], compared to 2D conditions. Additionally, cell lines cultured as spheroids exhibit reduced cell growth and metabolism, while their resistance to chemotherapeutic agents increases compared to traditional 2D cultures [4], highlighting the importance of using physiologically more relevant 3D models such as spheroids in bladder cancer research.

However, the use of spheroids requires advanced microscopy visualization and analysis techniques, regardless of the spheroid generation method: scaffold free (forced floating, agitation, hanging drop, liquid overlay, magnetic levitation) or scaffold-based, using natural polymers (e.g., collagen, alginate) or synthetic ones (e.g., polyethylene glycol) [2]. Light and electron microscopy are essential tools in this context, enabling detailed characterization of spheroids at the morphological, molecular, and ultrastructural levels.

Spheroid imaging presents several challenges. Firstly, the limited penetration depth of light in microscopy can reduce the image quality due to a reduced signal-to-noise ratio or even result in a complete loss of signal in deeper regions of the

spheroid. This is exacerbated in larger spheroids due to the increased number of cellular layers and the presence of a dense extracellular matrix, which cause light scattering and absorption, thereby obscuring internal structures, especially those in the spheroid core. Secondly, due to their small diameter, spheroids are difficult to manipulate, particularly during sample preparation steps such as cryosectioning and paraffin embedding. Moreover, some preparation techniques can be destructive, potentially compromising the 3D integrity of the spheroid.

To address these challenges, we have developed a set of accessible, user-friendly, and detailed protocols for spheroid analysis. Their applicability was demonstrated on spheroids generated from normal, ureter-derived, human urothelial cells and human cancer urothelial cells derived from the urinary bladder. The workflows integrate multiple sample preparation techniques including chemical fixation, followed by paraffin or cryo-embedding to preserve morphology and stabilise the integrity of spheroids for downstream sectioning, histological staining, and immunofluorescence labelling. These steps are optimized for both light and fluorescence microscopy, enabling detailed analysis of protein localization and tissue structure (Fig 1). Furthermore, we present a workflow for optical clearing combined

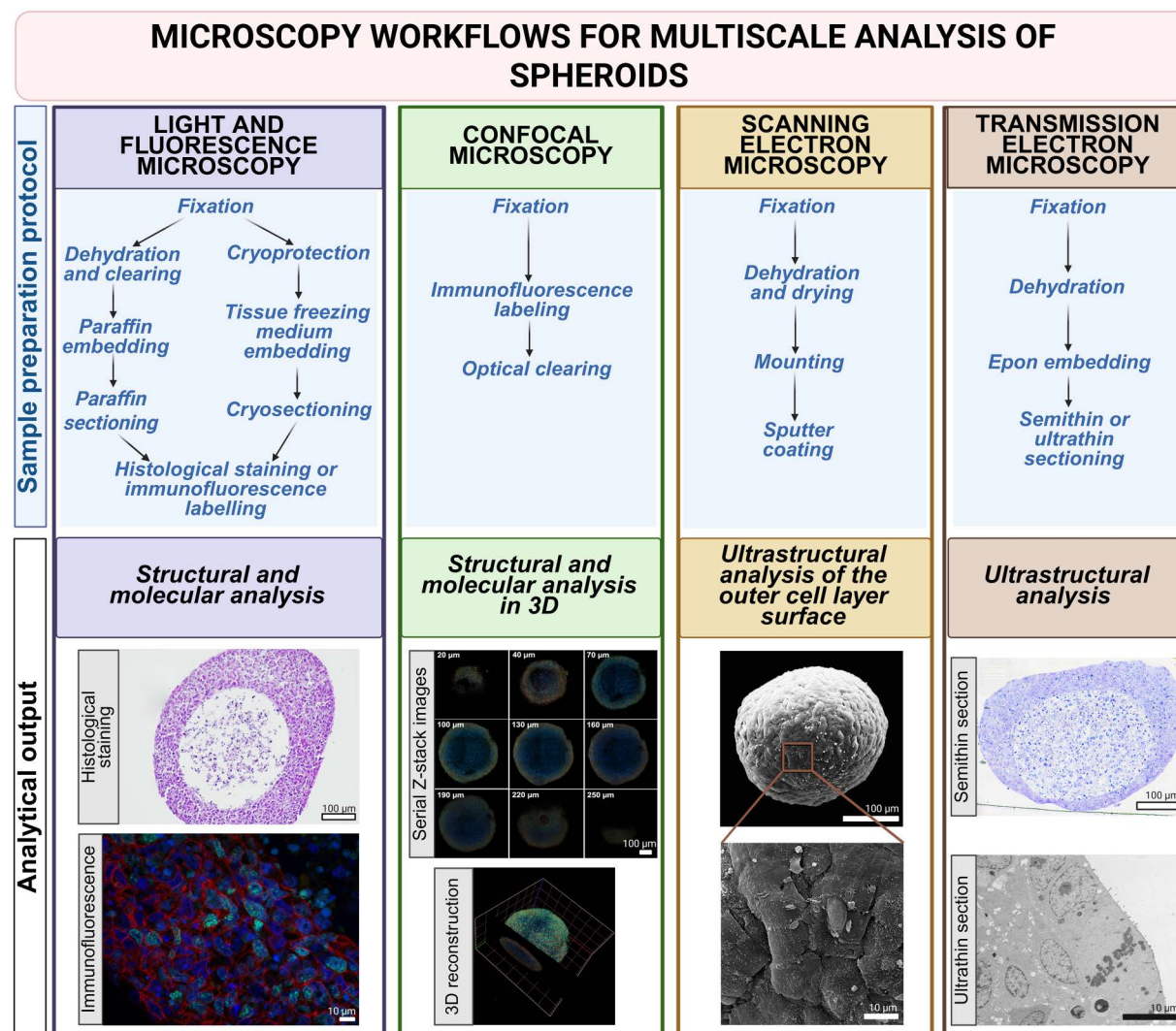


Fig 1. Schematic overview of the microscopy workflows presented in this study, integrating multiple imaging modalities.

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with immunofluorescence labelling of whole-mount spheroids for confocal microscopy, allowing volumetric analysis of molecular markers without the need for sectioning (Fig 1). Additionally, dedicated workflows for transmission (TEM) and scanning electron microscopy (SEM) have also been developed. The TEM protocol includes fixation, resin embedding and ultramicrotomy, while the SEM protocol involves whole-mount spheroid sputter-coating and surface imaging (Fig 1). Representative results are provided to illustrate the strengths and limitations of each technique, highlighting the importance of combining light and electron microscopy for comprehensive morphological, molecular, and ultrastructural characterization of spheroids.

Materials and methods

Protocols detailing the generation of spheroids, together with general instructions for their handling, preparation, and fixation for downstream applications, are provided in this section. The following protocols described in this peer-reviewed article are published on protocols.io: (i) the protocol entitled “*Preparation of cryo- and paraffin sections of spheroids for histological and immunofluorescence analysis*” is published on protocols.io (dx.doi.org/10.17504/protocols.io.ewov11oyovr2/v1) and is included for printing purposes as [S1 File](#); (ii) the protocol entitled “*Whole-mount immunofluorescence labelling and optical clearing of spheroids*” is published on protocols.io (dx.doi.org/10.17504/protocols.io.8epv5kj95v1b/v1) and is included for printing purposes as [S2 File](#); (iii) the protocol entitled “*Preparation and gold sputter coating of whole-mount spheroids for scanning electron microscopy*” is published on protocols.io (dx.doi.org/10.17504/protocols.io.x54v95yoql3e/v1) and is included for printing purposes as [S3 File](#); (iv) the protocol entitled “*Epon embedding and ultrathin sectioning of spheroids for transmission electron microscopy analysis*” is published on protocols.io (dx.doi.org/10.17504/protocols.io.4r3l21opjg1y/v1) and is included for printing purposes as [S4 File](#).

[Fig 1](#) provides an overview of these microscopy protocols for multiscale spheroid analysis described in this article. The detailed lists of materials and equipment used in these workflows are provided in [Tables 1](#) and [2](#).

Cell lines and general culture conditions

Normal human urothelial SV-HUC-1 cells (CRL-9520) and muscle-invasive human bladder cancer urothelial T24 cells (HTB-4) were purchased from ATCC (Manassas, VA, United States) and cultured in a 1:1 mixture of A-DMEM medium (Gibco, Thermo Fisher Scientific, Waltham, MA, United States) and F12 (Sigma-Aldrich, St. Louis, MO, United States), supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA, United States) and 4 mM GlutaMAX (Gibco, Thermo Fisher Scientific, Waltham, MA, United States). Cell cultures were maintained according to ATCC recommendations and harvested using standard cell culture methods. Both cell types were repeatedly tested negative for mycoplasma infection using the MycoAlert mycoplasma detection kit (Lonza, Basel, Switzerland), ensuring their suitability for experimentation.

Protocol for the formation of normal and cancer urothelial spheroids

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.

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.

All protocols for microscopy analysis described below used a seeding density of 100,000 T24 or SV-HUC-1 cells per well. Spheroids were grown for 7 days prior to analysis.

NOTE: For cryosectioning and paraffin embedding and cutting, a higher seeding density (50,000–100,000 cells per well) is recommended to obtain larger spheroids that are easier to handle.

Table 1. Materials used in the experiments.

Material	Manufacturer
1 mL and 200 µL micropipette tips	Brand, Germany, Cat. No.: 732012 and 732028
10 mL glass vials	SGD Pharma, Germany
35 mm glass-bottom dish	MatTek Life Sciences, Cat. No.: •P35G-1.5-14-C
Anti-E-cadherin antibody	BD Biosciences, USA, Cat. No.: 610182
Anti-Ki-67 antibody	Abcam, United Kingdom, Cat. No.: 16667
Anti-N-cadherin antibody	Cell Signaling Technology, USA, Cat. No.: 13116
Benzyl-alcohol	Sigma-Aldrich, USA, Cat. No.: 305197
Benzyl-benzoate	Sigma-Aldrich, USA, Cat. No.: B6630
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
Cover glasses	Brand, Germany, Cat. No.: 470060
Distilled water	Institute of cell biology, ULMF, Slovenia
Entellan new mounting medium	Merck (Sigma-Aldrich); Germany, Cat. No.: 107961
Eosin	Merck (Sigma Aldrich), Germany, Cat. No.: 1.09844
Epon;	
Glycid ether 100	Serva, Germany, Cat. No.: 21045
Methylnadic anhydride	Serva, Germany, Cat. No.: 29452
2-Dodecenylsuccine acid anhydride	Serva, Germany, Cat. No.: 20755
2,4,6-Tris(dimethylaminomethyl)phenol	Serva, Germany, Cat. No.: 36975
Ethanol (100%)	Carlo Erba Reagents, France, Cat. No.: 4146072
Glutaraldehyde	Serva, Germany, Cat. No.: 23114.01
Goat anti-mouse Alexa Fluor 488 and 555	Molecular Probes, Thermo Fisher Scientific, USA, Cat. No.: A11001 and A21422
Goat anti-rabbit Alexa Fluor 488 and 555	Molecular Probes, Thermo Fisher Scientific, USA, Cat. No.: A11008 and A21428
Goat Serum (Normal)	Agilent Technologies, Singapore, Cat. No.: X0907
Hexamethyldisilane (HMDS)	Sigma-Aldrich, USA, Cat. No.: 440191
Hoechst 33342	Thermo Fisher Scientific, Cat. No.: 62249
Hydrophobic barrier pen	Invitrogen, USA, Cat. No.: R3777
Lead citrate	
Lead (III) nitrate	Merck, Germany, Cat. No.: 1.07398
Tri-Sodium citrate dihydrate	Merck, Germany, Cat. No.: 1.06447
Mayer's Haematoxylin solution	Merck, Sigma-Aldrich, Germany, Cat. No.: 54275
Metal stubs (12.7 diameter)	Mikrolux, Croatia
Microscope slides	Epredia, Menzel-Thermo Scientific, Germany, Cat. No.: AA00000112E01 MNZ10
Microscope slides racks and jars	Merck Brand, Sigma Aldrich, USA
Micro-slide 8 well glass bottom slides	Ibidi, Germany, Cat.No.:80826
Mounting medium with DAPI – Aqueous Fluoroshield	Abcam, United Kingdom, Cat. No.: AB104139
Osmium tetroxide	Roth, Germany, Cat. No.:8371.3
Paraffin (Histosec pastilles)	Merk, Germany, Cat. No.: 115161
Paraformaldehyde	Sigma-Aldrich, Germany, Cat. No.: 158127
Pasteur pipette (3 mL)	Brand, Germany, Cat. No.:747765

(Continued)

Table 1. (Continued)

Material	Manufacturer
Phosphate buffer saline (PBS); Potassium chloride Disodium hydrogen phosphate Potassium dihydrogen phosphate Sodium chloride	Merck (Sigma-Aldrich); Germany, Cat. No.:104936 Merck (Sigma-Aldrich); Germany, Cat. No.:106580 Merck (Sigma-Aldrich); Germany, Cat. No.:104873 Chem-Lab, Belgium, Cat. No.:CL00.1429
Potassium hexacyanoferrate(II) trihydrate	Merck (Sigma-Aldrich); Germany, Cat. No.:14459-95-1
Silicon moulds 11–039 and 11–035	Balzers Union, Liechtenstein
Sodium citrate dihydrate	Merck, Germany, Cat. No.:106447
Spatulas	Sigma-Aldrich, USA
Sterile 96-well ultra-low attachment U-shaped bottom microplates	Corning, USA, Cat. No.: 7007
Sterile surgical blades BB510	B Braun, Cat. No.: 4512201760
Sucrose	Sigma-Aldrich, Germany, Cat. No.: S9378
TEM grids	Micro to Nano BV, Netherlands
Tissue freezing medium	Leica, Germany, Cat. No.: 14020108926
Triton X-100	Sigma-Aldrich, USA, Cat. No.: T8787
Tween-20	Sigma-Aldrich, USA, Cat. No.: P7949
Ultrasmooth carbon discs	SPI supplies, USA, Cat. No.:04967-B1
Uranyl acetate	Merck, Germany, Cat. No.:8473
Vial stopper	Neolab, Germany
Watch glasses	Sigma-Aldrich, USA
Xylene	Carlo Erba Reagents, France, cat. no.: 492301

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Spheroid formation and growth were assessed using an inverted Leica DM-IL microscope (with objective lenses C Plan 10×/0.22 Ph1 and L40×/0.50 Ph2) equipped with a Basler acA1300-200 µm camera (Basler, Germany) (Fig 2).

General instructions for handling, preparation and fixation of spheroids for downstream applications

Spheroid transfer and container selection. All protocols for microscopy analysis described below include chemical fixation of spheroids as the first step, followed by changing the solutions in which the spheroids are submerged. Spheroids can be transferred from the 96-well low-attachment U-shaped-bottom microplates to appropriate processing containers for fixation and subsequent handling or can be fixed in microplates and then transferred.

Option 1: Transfer before fixation

1. Transfer spheroids from 96-well low-attachment U-shaped-bottom microplates to processing containers filled with cold fixative (4°C), ensuring that the spheroids are completely submerged in the fixative.
2. Recommended containers
 - a) 10 mL glass vials, and
 - b) Microcentrifuge tubes (500 µL or 1.5 mL).

NOTE: We recommend using microcentrifuge tubes, as they enable better visualization of spheroids compared to larger 10 mL glass vials. However, researchers should note that certain steps for TEM and SEM sample preparation require glass vials exclusively. If using microcentrifuge tubes initially, transfer spheroids to glass vials before specific TEM/SEM procedures.

Table 2. Equipment used in the experiments.

Equipment	Manufacturer
acA1300-200 um camera	Basler, Germany
AMT camera	Advanced Microscopy Techniques Corp., Woburn, MA, USA
Bench-mounted fume cupboard System Delta 30	Wesemann, Germany
Camera DS-Fi3	Nikon Instruments Inc, Japan
Camera GXCAM-U3-18	Gt vision, Great Britain
Centrifuge MyFuge Mini	Benchmark Scientific, USA
Confocal microscope LSM900	Carl Zeiss, Germany
Cryostat CM1950	Leica, Germany
Axiomager. Z1 to Axio Imager Z1 Fluorescence microscope Axiomager. Z1 equipped with ApoTome	Carl Zeiss, Germany
Inverted microscope DM-IL	Leica, Germany
Laminar air-flow cabinet M182 (II)	Iskra Pio, Slovenia
Light microscope Eclipse Ci	Nikon Instruments Inc, Japan
Microtome RM 2135	Leica, Germany
Microwave oven	GoldStar, LG Electronics, South Korea
Optimal Cutting Temperature (OCT) compound, also known as tissue freezing medium	Tissue-Tek® O.C.T. Compound
Orbital Shaker KS 260	IKA, Germany
Scale AE 163	Mettler Toledo, Swiss
Scanning electron microscope Vega 3	Tescan, Brno, Czech Republic
Sputtering coating machine SCD 040	Balzers Union, Liechtenstein
Stereomicroscope SMZ800	Nikon, Japan
Thermoblock	Marijan Krokter s.p., Slovenia
Thermostat	Kambič, Slovenia
Thermostat (oven)	Instrumentaria, Croatia
Transmission electron microscope CM100, equipped with AMT camera	Philips, The Netherlands
Ultramicrotome EM UC6	Leica, Germany
VorTemp 56 Microplate Shaking Incubator	Labnet International, Inc., USA

<https://doi.org/10.1371/journal.pone.0342659.t002>

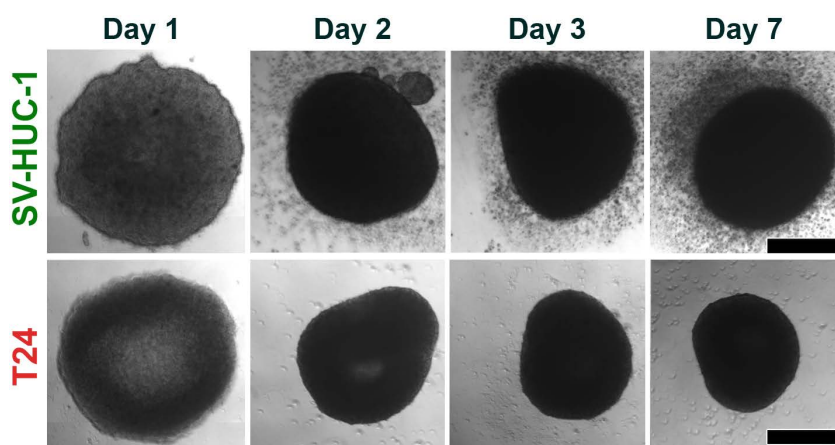


Fig 2. Growth of SV-HUC-1 and T24 spheroids over 7 days. Phase-contrast images were taken on days 1, 2, 3, and 7 to monitor spheroid development before further processing. Scale bars, 200 μ m.

<https://doi.org/10.1371/journal.pone.0342659.g002>

Option 2: Fixation of spheroids in 96-well low-attachment U-bottom microplates

1. Without removing culture medium, add 50 μ L cold fixative (4°C) to each well.
2. Incubate for 1 minute at room temperature.
3. Remove medium/fixative mixture using a multichannel pipette.
4. Add 200 μ L fresh cold fixative per well.
5. After fixation completion, transfer spheroids to microcentrifuge tubes via pipetting.

All described protocols specify transfer of spheroids to microcentrifuge tubes for fixation.

Critical transfer procedures.

1. Spheroid transfer from 96-well microplates to processing containers should be performed with either:
 - a) a standard 1 mL micropipette fitted with a tip, or
 - b) a 3 mL plastic Pasteur pipette.
2. To prevent spheroids from adhering to the pipette wall and to ensure efficient transfer, it is advisable to pre-coat pipette tips or Pasteur pipettes by immersing their full length in 1% BSA in PBS.
3. Fixative temperature note

In the experiments we used a cold fixative (4°C), because it improves preservation of phosphorylated proteins and other sensitive antigens in tissue for immunolabelling, compared to warm fixation (protocol adapted from Lerch *et al.* [11]).

Solution exchange protocols.

1. Prefer solution exchange over transfer: For the steps following fixation, which include changing the solutions in which spheroids are immersed, we advise changing liquids instead of transferring spheroids from one solution to another to minimize handling damage, unless specified otherwise (see »Whole-mount immunofluorescence labelling and optical clearing of spheroids« section).
2. When performing solution exchanges, first allow spheroids to settle at the container bottom.
3. To accelerate settling, tubes may be gently spun at 2000 $\times$ g (6000 rpm) for 5 seconds to ensure complete spheroid sedimentation.
4. Use a 200 μ L or 1 mL pipette to carefully aspirate the maximum volume of the existing solution without disturbing the settled spheroids.
5. Visually confirm spheroid position *before*, *during*, and *after* aspiration.
6. Maintain 10–20 μ L residual liquid at the container bottom during solution exchanges to prevent accidental aspiration of spheroids.
7. Add fresh solution to achieve target volume.

Loss mitigation strategies.

1. Spheroid loss during washing, solution exchanges, and procedures such as paraffin embedding is expected and correlates with:
 - a) Operator experience level, and
 - b) Handling technique precision

2. Always process 20–30% excess spheroids beyond experimental requirements to compensate for potential losses.
3. Visual verification of spheroids is required at every process milestone:
 - a) Before/after solution exchanges,
 - b) Following washing steps, and
 - c) Post-handling procedures
4. Check for:
 - a) Presence confirmation, and
 - b) Structural integrity

Preparation of cryo- and paraffin sections of spheroids for histological and immunofluorescence analysis

[Protocols.io DOI: dx.doi.org/10.17504/protocols.io.ewov11oyovr2/v1](https://doi.org/10.17504/protocols.io.ewov11oyovr2/v1)

Cryosections. The preparation of cryosections involved fixation in cold 4% formaldehyde (w/v) in PBS (pH 7.2–7.4) for 15 minutes at room temperature, ensuring spheroids were fully submerged. Washing was done three times in PBS for 5 minutes each. Cryoprotection was achieved by submerging spheroids in 30% sucrose in PBS for at least 2 hours at room temperature or overnight at 4°C. For embedding, spheroids were sequentially immersed twice in tissue freezing medium (Optimal Cutting Temperature (OCT) compound) on a watch glass for 5 minutes each at room temperature. A small amount of freezing medium was applied to the cryostat specimen disk and partially hardened before transferring 1–2 spheroids on top (not submerged) of the medium using a scalpel or spatula. The embedded spheroids were left in the cryostat for 15 minutes to complete hardening. Sectioning was performed at –20°C ambient cryostat temperature to cut 5–10 µm sections that were mounted onto microscope slides and dried at room temperature for 1 hour.

Paraffin sections. For paraffin sections, spheroids were fixed in cold 4% formaldehyde (w/v) in PBS (pH 7.2–7.4) for 30 minutes at room temperature, followed by four washes with PBS. Dehydration and clearing were performed at room temperature on an orbital shaker at 100 rpm (0.056 × g, orbital diameter 10 mm), by sequential incubation of the samples in increasing concentrations of ethanol: 50% for 5 minutes, 70% for 5 minutes, 90% for 5 minutes, and finally in 100% ethanol for 5 minutes. Spheroids were then transferred to glass vials and cleared by incubation in ethanol–xylene mixtures: first in a 2:1 (v/v) mixture of 100% ethanol and xylene for 5 minutes, then in a 1:1 (v/v) mixture for 5 minutes, and finally in 100% xylene for 5 minutes. Spheroids were then embedded in paraffin by transferring them into rubber vial stoppers, removing excess xylene on a warm plate, and adding liquid paraffin pre-warmed to 60°C. Embedding was completed by incubating at 58°C for 2 hours; then paraffin was solidified at room temperature or on a cold plate. Paraffin blocks were sectioned at 6–7 µm thickness using a microtome RM 2135 (Leica, Germany). Sections were collected on slides after flattening in a water-heated thermoblock at 60°C and adhered by incubation at 65°C for 10–20 minutes.

Histological staining. The cryosections were warmed to room temperature and rinsed with PBS for 5 minutes. Paraffin sections underwent deparaffinization at room temperature by immersion in xylene for 5 minutes, followed by sequential immersion in 100% and 70% ethanol for 5 minutes each. Sections were then rinsed twice in distilled water for 5 minutes at room temperature.

The histological staining procedure applied to both cryosections and paraffin sections was performed entirely at room temperature. Sections were first incubated for 15 seconds in Mayer's hematoxylin solution, followed by a submersion wash in distilled water. The sections were then rinsed under running water for 1 minute to remove excess stain, after which they were incubated in eosin for 2 minutes.

Dehydration, clearing, and mounting steps were also conducted at room temperature. The sections were submerged twice in 90% ethanol for 1 minute each, followed by two 1-minute immersions in 100% ethanol. Subsequently, the sections were cleared by submersion in xylene for 5 minutes. Finally, a drop of Entellan new inclusion mounting medium was applied to the samples, which were then covered with coverslips. Samples were examined using a light microscope Eclipse Ci (Nikon Instruments Inc., Japan).

Immunofluorescence labelling. Cryosections were equilibrated to room temperature and rinsed with PBS for 5 minutes. Paraffin sections were deparaffinized as described in the “Histological staining” protocol, followed by antigen retrieval via microwave heating in 10 mM sodium citrate buffer (pH 6.0). Slides were heated until the buffer reached boiling point, then maintained at a sub-boiling temperature for 10 minutes. This was achieved by heating at full power (600 W) for 2.5–3 minutes, then reducing to 20–30% power. Slides were cooled at room temperature for 30 minutes and rinsed with PBS for 5 minutes.

Both cryo- and paraffin-processed sections were blocked with 1% (w/v) BSA in PBS for 1 hour at room temperature. Primary antibody incubation was performed overnight at 4°C using mouse anti-E-cadherin, rabbit anti-N-cadherin, or rabbit anti-Ki-67 (all diluted 1:100 in 1% BSA/PBS). A hydrophobic barrier pen was used to confine the antibody solution (100–200 μ L per slide). Negative control sections were processed identically, omitting the primary antibody.

Following three PBS washes (5 minutes each), sections were incubated for 90 minutes at room temperature with goat anti-rabbit or goat anti-mouse secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 555 (both diluted 1:400 in 1% BSA/PBS). After final washes in PBS (3–5 times, 5 minutes each), sections were mounted with DAPI-containing mounting medium for nuclear counterstaining. Samples were examined using a fluorescence microscope Axio Imager Z1 equipped with ApoTome (Carl Zeiss, Germany).

Whole-mount immunofluorescence labelling and optical clearing of spheroids. [Protocols.io DOI: dx.doi.org/10.17504/protocols.io.8epv5kj95v1b/v1](https://doi.org/10.17504/protocols.io.8epv5kj95v1b/v1)

The following protocol is adapted from [12], originally developed for light sheet microscopy, and optimized to preserve fluorescence for confocal imaging using a Zeiss LSM900 microscope.

Fixation was conducted by fully immersing spheroids in cold 4% (w/v) formaldehyde in PBS (pH 7.2–7.4) for 30 minutes at room temperature. After fixation, spheroids were washed five times with sterile PBS for 5 minutes each, on a shaker set to 800 rpm (1.07 $\times$ g, orbital diameter 3 mm) at room temperature. For immunofluorescence labelling, spheroids were blocked by incubating in blocking buffer containing 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20, and 10% normal goat serum in PBS for 90 minutes at room temperature on the shaker. After removing the blocking buffer, primary antibodies (mouse anti-E-cadherin, rabbit anti-N-cadherin, or rabbit anti-Ki-67) diluted 1:100 in 1% BSA in PBS were applied for 20 hours at 37°C with shaking at 800 rpm. Spheroids were then washed three times with sterile PBS for 10 minutes each at room temperature in darkness on the shaker. Next, samples were incubated with secondary antibodies goat anti-rabbit or goat anti-mouse conjugated to Alexa Fluor 488 or Alexa Fluor 555, both diluted 1:400 in 1% BSA in PBS for 6 hours at 37°C in darkness on the shaker. After this, spheroids were washed three times with sterile PBS. For nuclear staining, spheroids were incubated in 100 μ g/mL Hoechst 33342 diluted in 1% BSA in PBS for 16 hours at 37°C in darkness on the shaker. Following nuclear staining, spheroids were washed three times in PBS. For clearing, increasing ethanol concentrations (30%, 50%, 70%, 90%, 96%, and 100%) in deionized water were prepared in separate wells of an 8-well chamber slide (Ibidi μ -slide 8 well). Spheroids were serially transferred between these wells. Dehydration-induced shrinkage necessitates confirming spheroid presence visually, preferably with a stereomicroscope, at each step. Subsequently, spheroids were carefully moved from 100% ethanol into a 1:2 (v/v) solution of benzyl alcohol and benzyl benzoate (BABB) in a 35 mm glass-bottom dish and incubated until transparent, typically within 20 minutes. Transparency was monitored under a stereomicroscope SMZ800 (Nikon, Japan).

Imaging was performed using the Zeiss LSM900 confocal microscope system, ensuring all instruments and components were initialized. A 20 $\times$ Plan-Apochromat objective with numerical aperture 0.8 was used to optimize resolution and

field of view. The sample was positioned on the stage and centered in the live view mode. Depending on the spheroid size, a tiling layout was configured: a 3 × 3 tile grid for spheroids up to 300 µm in diameter, or larger grids such as 4 × 3 for bigger spheroids. The Z-stack acquisition was adjusted to cover the entire spheroid volume, with slice intervals of approximately 2–3 µm to capture structural detail. Laser power was modulated at different depths (lower at shallow regions, medium in the middle, and higher at deeper layers) to compensate for signal attenuation. Gain was adjusted to preserve the signal-to-noise ratio, the pinhole size was set around 1 Airy unit for optimal resolution and penetration, and gamma or histogram settings were tuned to avoid pixel saturation. Z-stack acquisition commenced, producing a 3D image stack; this process can take hours and requires stable sample positioning to prevent movement. Following acquisition, image processing, including stitching of tiles was performed as needed.

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

Protocols.io DOI: [dx.doi.org/10.17504/protocols.io.x54v95yoql3e/v1](https://doi.org/10.17504/protocols.io.x54v95yoql3e/v1)

Spheroids were fixed by complete submersion in cold 2% (w/v) formaldehyde and 2% (v/v) glutaraldehyde prepared in 0.2 M cacodylate buffer (pH 7.4) for 1 hour at 4°C. Following fixation, samples were rinsed three times with 0.2 M cacodylate buffer for 10 minutes each at room temperature. Samples were then transferred to glass vials and post-fixed in 1% (w/v) osmium tetroxide in 0.2 M cacodylate buffer for 1 hour at room temperature, followed by three rinses in the same buffer for 10 minutes each. For dehydration, all steps were performed at room temperature. Spheroids were sequentially immersed in 30% ethanol for 5 minutes, 50% ethanol for 5 minutes, 70% ethanol for 5 minutes, 90% ethanol for 5 minutes, and 100% ethanol twice for 5 minutes each. For drying, samples were immersed twice in acetone for 10 minutes each, then placed in a 1:1 (v/v) mixture of acetone and hexamethyldisilazane (HMDS) for 10 minutes, followed by two immersions in 100% HMDS for 10 minutes each. The HMDS was then aspirated, and the samples were left to air-dry overnight at room temperature inside a fume hood, with the vial or microcentrifuge tube left open. Dried spheroids were mounted on metal stubs prepared with double-sided conductive carbon tape by either gently placing spheroids onto the adhesive surface with tweezers, avoiding mechanical pressure, or by inverting the glass vial so that spheroids fell directly onto the tape. Finally, sputter coating was performed to deposit a gold layer. A Balzers Union SCD 040 sputter coater equipped with a 50 mm Baltec gold target was used to apply an approximately 16 nm-thick gold coating at 30 mA for 1 minute. Samples were stored in airtight containers at room temperature until imaging with a scanning electron microscope Vega 3 (Tescan, Brno, Czech Republic) at 25–30 kV.

Epon embedding and ultrathin sectioning of spheroids for transmission electron microscopy analysis

Protocols.io DOI: [dx.doi.org/10.17504/protocols.io.4r3l21opjg1y/v1](https://doi.org/10.17504/protocols.io.4r3l21opjg1y/v1)

The fixation of spheroids was carried out by immersing them in a cold solution containing 4% formaldehyde (w/v) and 2% glutaraldehyde (v/v) in 0.1 M cacodylate buffer (pH 7.2–7.4) for 30 minutes at room temperature. Following fixation, samples were rinsed with 0.33 M sucrose prepared in 0.1 M cacodylate buffer through three consecutive 10-minute washes at room temperature. After rinsing, the samples were transferred to glass vials and subjected to post-fixation in a mixture of 1% (w/v) osmium tetroxide and 0.8% (w/v) potassium ferrocyanide in 0.2 M cacodylate buffer for 30 minutes at room temperature in the dark. Subsequently, the samples were rinsed with 0.1 M cacodylate buffer for 5 minutes, followed by rinsing in distilled water for 2 minutes at room temperature. Dehydration was performed entirely at room temperature by sequential incubation of the samples in increasing concentrations of ethanol: 50% for 5 minutes, 70% for 5 minutes, 90% for 5 minutes, and finally twice in 100% ethanol for 5 minutes each. For Epon resin embedding, spheroids were first transferred into silicon resin embedding molds with larger cavity sizes containing a 1:1 mixture of Epon resin and ethanol and incubated for 1 hour at room temperature. Subsequently, the samples were moved to silicon molds with smaller cavity sizes optimized for sectioning. Polymerization of the Epon was carried out over a period of 5 days with a gradual temperature increase every 24 hours through the following stages: 35°C, 45°C, 60°C, 70°C, and 80°C. For section preparation,

1 μm semithin sections were initially prepared for localizations of spheroids and cells using a light microscope. Ultrathin sections of 60 nm thickness were then cut and mounted onto grids for TEM. These ultrathin sections were contrast-enhanced by staining with a saturated solution of uranyl acetate for 20 minutes, followed by a 10% solution of lead citrate for 3 minutes. The samples were examined using a transmission electron microscope CM100 (Philips, The Netherlands) at 80 kV.

Results and discussion

Representative outcomes of the protocols described in the Methods section combined with appropriate imaging techniques, namely light microscopy, confocal microscopy, SEM, and TEM, are presented. Each technique has unique advantages and limitations, which are summarized in Table 3. Combined, these protocols enable a comprehensive characterization of normal and cancer spheroids in terms of their morphology, structure, composition, and cellular ultrastructure.

Histological characterization of spheroids by light microscopy

Both cryosections and paraffin sections enabled histological evaluation of spheroid morphology (Fig 3). Both methods produce sections of similar thickness (5–7 μm), although paraffin sections can be made thinner if necessary (2–3 μm) and provide information on the structure, organization, and morphology of the spheroids. Necrotic cores were evident in spheroids of both cell types, with SV-HUC-1 cells displaying tighter intercellular connections than T24 cells (Fig 3).

Comparing cryo- and paraffin sections reveals several advantages of paraffin embedding over freezing the samples. Namely, better preserved spheroid morphology (Fig 3), more reproducible sections, and lower variability within the results. Furthermore, long-term storage of such paraffin sections is easily done at room temperature. Cryosections, on the other hand, show aberrant spheroid morphology, which is likely damage due to ice crystals formed during freezing (Fig 3), and are more difficult to store (at -80°C , can last up to a year). Nevertheless, such sections can be more suitable for immunolabelling (see next section) and can be obtained much faster and in fewer steps compared to embedding in paraffin.

Immunofluorescence characterization of spheroid cryosections and paraffin sections by fluorescence microscopy

In our experiments, both paraffin and cryosections have been utilized successfully for immunofluorescence labelling and analysis of SV-HUC-1 and T24 spheroids. Here, we demonstrate the immunolabelling of spheroids for E- and N-cadherin, accompanied by the counterstaining of cell nuclei with DAPI in paraffin spheroid sections (Fig 4), which provides valuable insight into the localizations of these proteins. Specifically, E-cadherin positive signals were observed on the plasma

Table 3. Summary of microscopy techniques combined with spheroid preparation for analysing and characterizing spheroids.

Sample preparation	Microscopy	Key Strengths	Limitations
Histological staining and immunolabelling of cryosections or paraffin sections	Conventional light and fluorescence microscopy	Enables examining both the structural and molecular characteristics of spheroids and localizations of specific proteins or other molecules within the spheroids.	Provides only 2D information. Technical challenges and artefacts (antigen retrieval for paraffin sections, morphology limitations in cryosections).
Immunofluorescence labelling and optical clearing of whole-mount spheroids	Confocal microscopy	Visualization of spatial cell distribution and cell-to-cell interactions within the whole spheroids.	Antibody penetration can be difficult. Limited resolution due to whole-mount imaging.
Epon embedding and ultrathin sectioning	Transmission electron microscopy	High-resolution imaging of cells in spheroids.	Requires ultrathin sections; limited to small sample areas.
Sputter coating of whole-mount spheroids	Scanning electron microscopy	High-resolution surface imaging.	Limited internal structure analysis.

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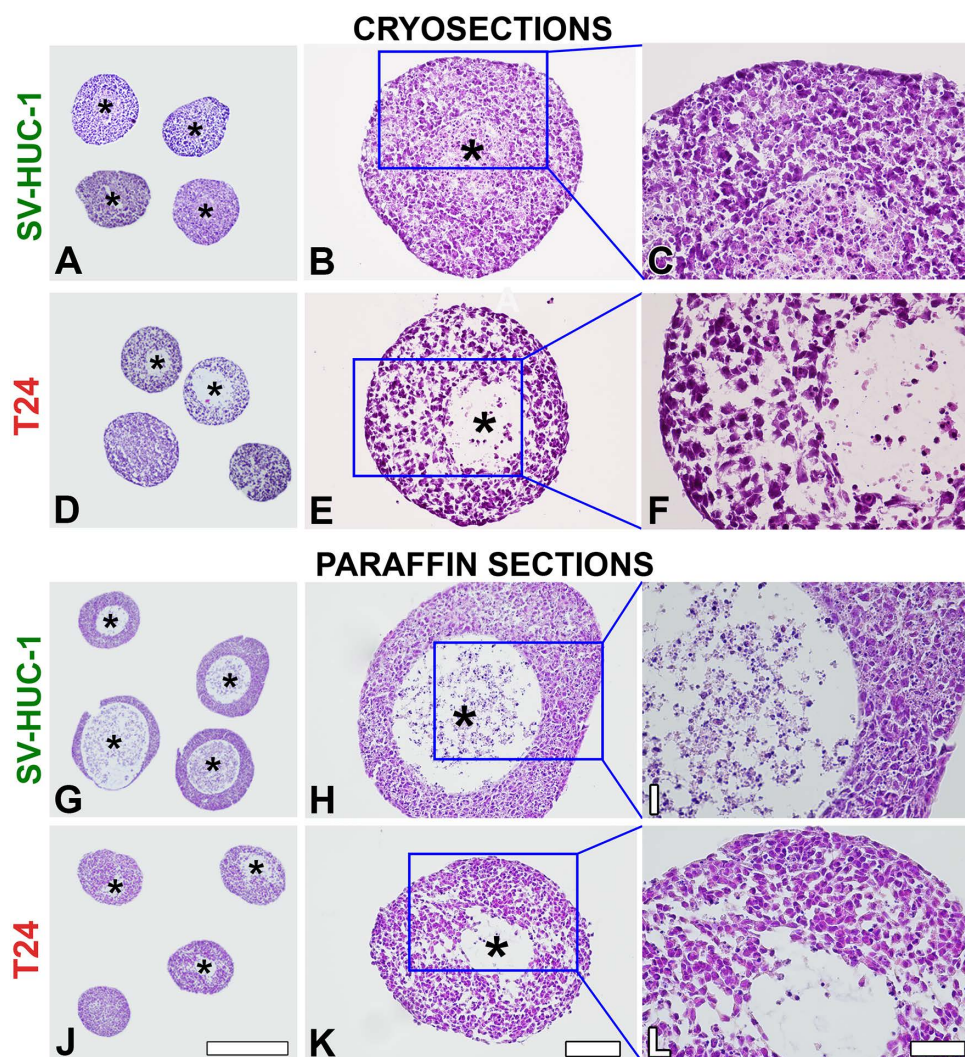


Fig 3. Superior morphological preservation in paraffin sections compared to cryosections. Representative haematoxylin–eosin (HE)-stained sections of SV-HUC-1 and T24 spheroids prepared by cryosectioning (A–F) and paraffin embedding (G–L). A necrotic core (indicated by asterisks, A–B, D–E, G–H, J–K) is visible in most cross sections, except those obtained from peripheral regions. Scale bars: 500 μ m (A, D, G, J), 100 μ m (B, E, H, K), 50 μ m (C, F, I, L).

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membrane of cells in SV-HUC-1 spheroids. Similarly, the plasma membrane of T24 cells was labelled with N-cadherin (Fig 4). A few E-cadherin-positive SV-HUC-1 cells were also N-cadherin positive (Fig 4). On the other hand, T24 cells did not express E-cadherin (Fig 4). The paraffin sections were the preferred choice for immunolabelling of selected adherent junction proteins, as cryosections tend to show reduced morphological integrity. Many antibodies require antigen retrieval for paraffin sections, complicating the protocol. In contrast, cryosections generally preserve antigenicity, eliminating the need for such retrieval steps. Overall, both approaches offer specific advantages that should be considered during the design of experiments.

Immunofluorescence labelling on both cryosections and paraffin sections offers good antibody penetration and information of protein distribution in 2D planes. To obtain information on protein expression within the entire spheroid, the preferred method is whole-mount (*in toto*) immunofluorescence labelling (Fig 5). To better illustrate the strengths and

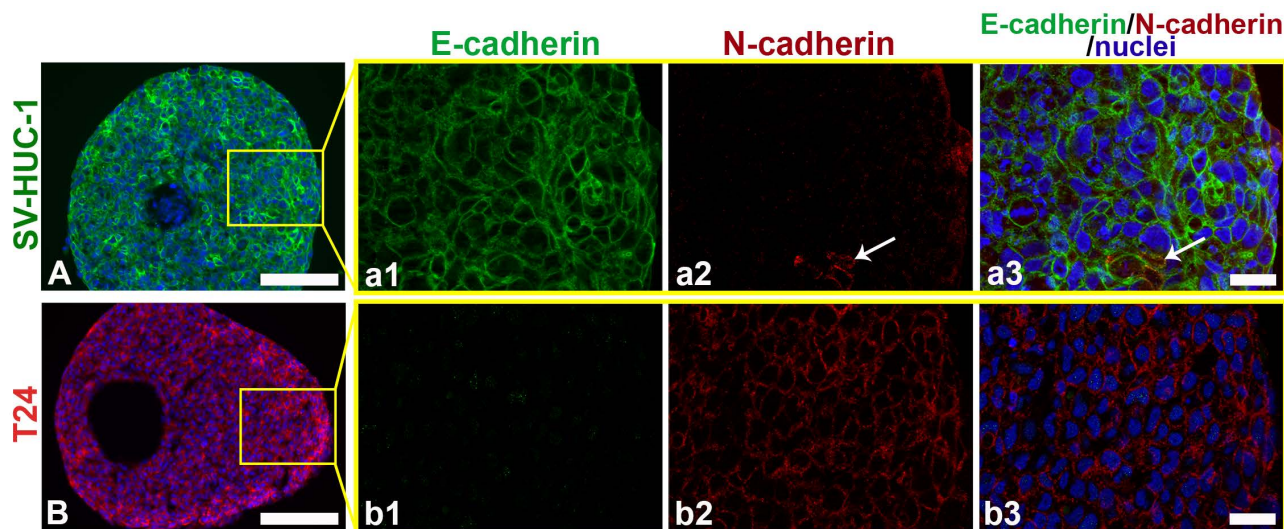


Fig 4. Double immunofluorescence labelling in paraffin sections of SV-HUC-1 and T24 spheroids reveals distinct adhesion molecule expression patterns between normal and cancerous urothelial cells in 2D. Representative images of paraffin sections show E-cadherin (green) in the plasma membrane in cells of SV-HUC-1 spheroids (**A-a3**) and N-cadherin (red) in the plasma membrane of T24 spheroids (**B-b3**). Some SV-HUC-1 cells are also positive for N-cadherin (arrows, **a2** and **a3**). Yellow insets (in **A** and **B**) are magnified (**a1-a3** and **b1-b3**) and display individual and merged channels of E-cadherin, N-cadherin, and DAPI-stained nuclei. Scale bars: 100 μm (**A**, **B**), 20 μm (**a1-a3**, **b1-b3**).

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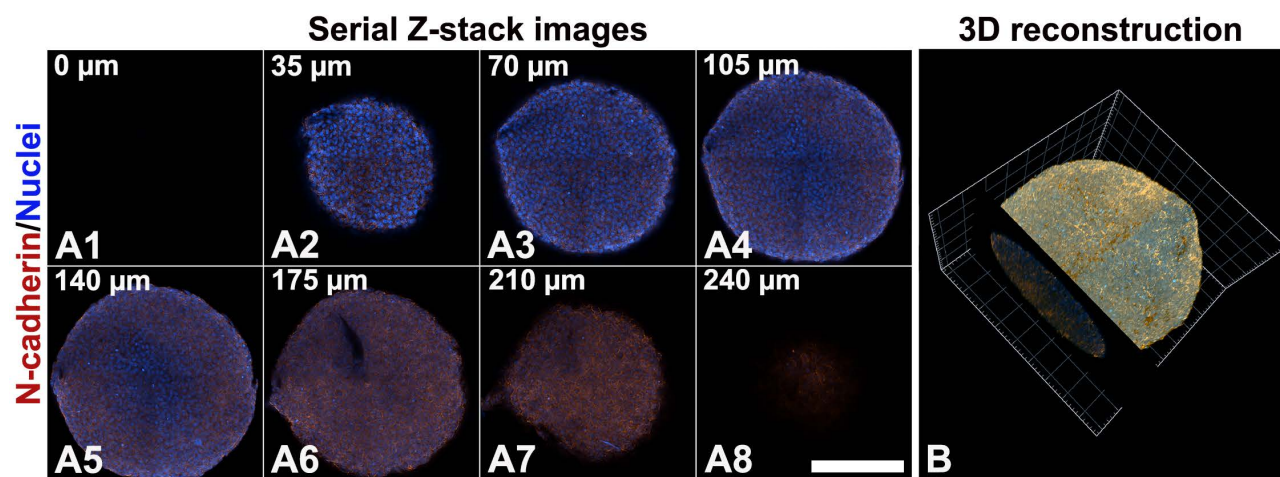


Fig 5. Whole-mount immunofluorescence reveals protein expression throughout entire spheroids, preserving their 3D structure. (**A1-A8**) Shown are single confocal images at different z-depths showing N-cadherin immunolabelling (red) and nuclei labelling with Hoechst stain (blue). Numbers in the upper left corner indicate the depth (in μm) of the optical sections within the Z-stack. (**B**) 3D reconstruction of a whole spheroid made from serial Z-stack images. The Z-stack comprised 274 optical sections, each taken with a 0.896 μm step. Scale bar, 200 μm .

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limitations of each technique, we compared all three methods of sample preparation for double immunofluorescence analysis of spheroids: cryosectioning, paraffin embedding, and whole-mount immunofluorescence (Fig 6, see next section).

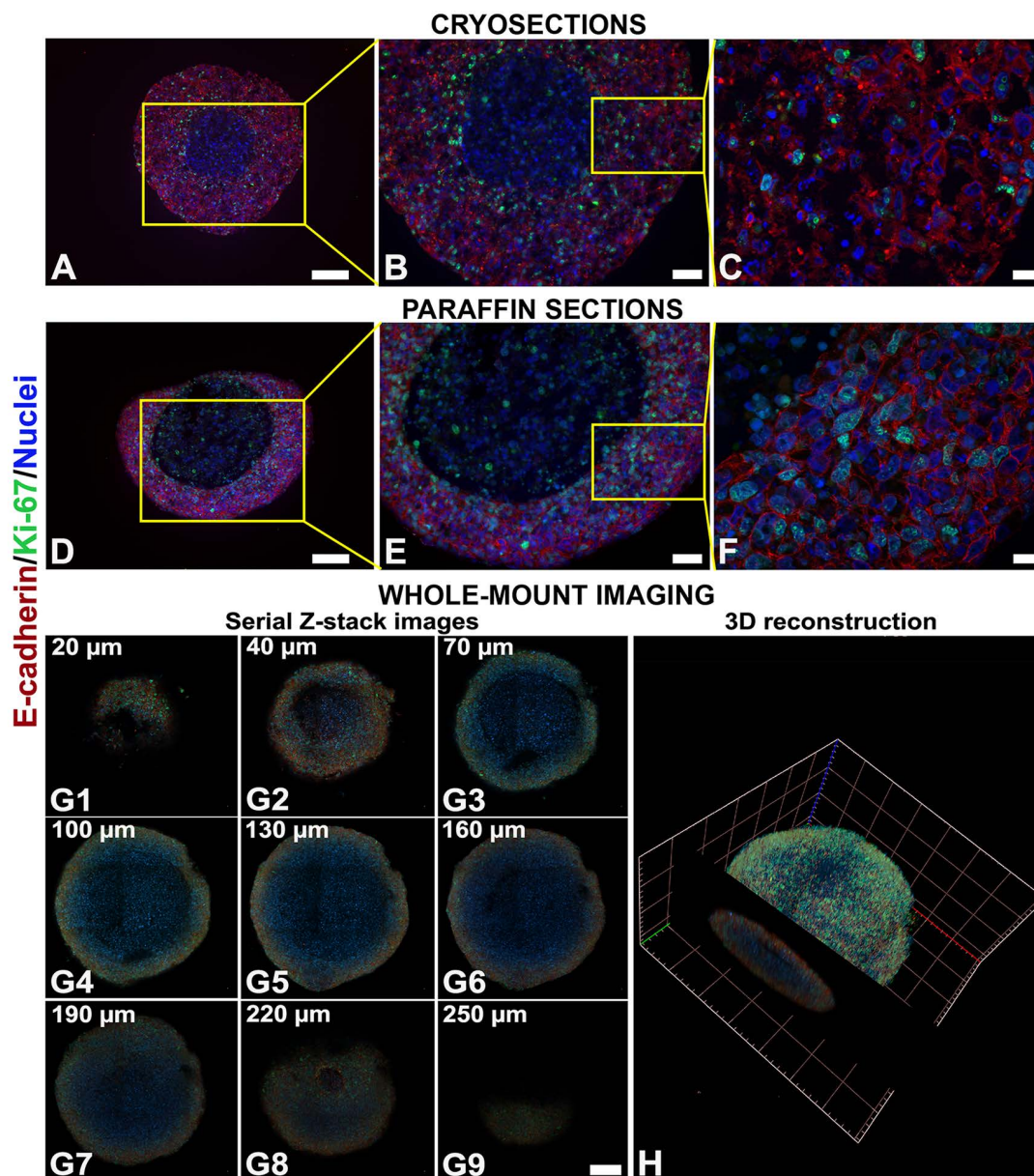


Fig 6. Comparison of double immunofluorescence labelling on cryosections, paraffin sections, and whole-mount SV-HUC-1 spheroids, highlighting 3D protein distribution and spatial context revealed by whole-mount imaging beyond traditional sectioning methods. Cryosections (A-C) and paraffin sections (D-F) demonstrated E-cadherin-positive cell membranes and Ki-67-positive cell nuclei in individual cryosections (A-C) and paraffin sections (D-F), while whole-mount immunofluorescence (G1-G9) revealed the spatial distribution of E-cadherin and Ki-67 within the complete spheroid volume. (G1-G9) Numbers in the upper left corner represent the focus position of the optical section. (H) 3D reconstruction of the whole spheroid made from serial Z-stack images. The Z-stack comprised 168 optical sections, each with a 2 µm thickness. Scale bars, 100 µm (A, D), 40 µm (B, E), 10 µm (C, F), 100 µm (G1-G9).

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Immunofluorescence characterization of whole-mount spheroids by confocal microscopy and comparative analysis with cryosection and paraffin section methods

While immunofluorescence on cryosections and paraffin-embedded sections is considered the gold standard for many histological analyses, whole-mount immunofluorescence provides a more comprehensive approach for studying spatial distribution and protein interactions within the intact 3D structure. This is particularly important for studying processes such as cell-cell interactions, tissue organization, and morphological changes. In comparison to section-based approaches (Fig 4), which may miss specific regions of interest during sectioning, whole-mount imaging reveals the expression of N-cadherin throughout the entire structure while preserving its structural integrity (Fig 5).

Despite its advantages, whole-mount imaging faces challenges in antibody penetration through millimeter-sized spheroids, which necessitates longer permeabilization and incubation times. While whole-mount imaging preserves 3D structure and morphology, dehydration and BABB clearing steps can induce shrinkage and tissue distortions. These effects result from the extraction of water and lipids, leading to compaction of cellular and extracellular components and, consequently, a loss of fine structural details that are better resolved in thin tissue sections [13,14]. Moreover, the extent of shrinkage is size-dependent, with smaller samples exhibiting proportionally greater changes. These effects may therefore compromise fine morphological details in spheroids relative to thin tissue sections [15]. Additionally, whole-mount 3D imaging may require specialized protocols and equipment, making it less accessible than traditional sectioning-based approaches. The large volume of generated data also requires additional computational tools for analysis and visualization.

To compare double immunofluorescence labelling on cryosections, paraffin sections, and whole-mount spheroids, we performed E-cadherin and Ki-67 immunofluorescence on samples of SV-HUC-1 spheroids. On cryosections and paraffin sections, E-cadherin positive signals were clearly observed on the cell membranes and Ki-67-positive cell nuclei in SV-HUC-1 spheroids. Morphological integrity was best preserved in paraffin sections (Fig 6). On the other hand, whole-mount immunofluorescence provided additional information about the spatial distribution of E-cadherin and Ki-67 in the entire spheroid (Fig 6, S5 File). Moreover, the morphology of the spheroid was also well preserved in whole-mount labelled spheroids.

Ultrastructural characterization of spheroids with scanning electron microscopy

SEM was utilized to analyse morphology, surface topography, and cellular interactions on the surface of SV-HUC-1 and T24 spheroids (Fig 7). SEM revealed that SV-HUC-1 and T24 spheroids have round morphology (Fig 7). Cells at the surface of SV-HUC-1 spheroids display tight junctions and microvilli, while wide intercellular spaces are present between T24 cells and less microvilli are observed on the surface of T24 cells (Fig 7). Although preparation time for SEM analysis is shorter in comparison to TEM and the integrity of entire spheroids can be examined, it only enables the analysis of their surface. TEM is necessary for characterizing the internal structures of the spheroid, although it is limited to a small sample area spanning across a few cells (see next section “Ultrastructural characterization of spheroids with transmission electron microscopy”).

Ultrastructural characterization of spheroids with transmission electron microscopy

TEM revealed that cells in the outermost layer of the SV-HUC-1 spheroids are more cuboidal while cells in T24 spheroids have an elongated shape. Both spheroids exhibit cell junctions in the outermost cell layer, creating a tight network at the periphery (Fig 8). Underneath the outermost cell layer, significantly smaller intercellular spaces are observed in SV-HUC-1 spheroids compared to T24 spheroids (Fig 8). Consistent with observations in histological sections (Fig 3), TEM revealed necrotic zones in both types of spheroids, filled with necrotic cells (Fig 8). Both the necrotic zone and the spheroid diameter are larger in SV-HUC-1 compared to T24 as revealed by semithin section analysis (Fig 8). TEM enables the analysis of spheroid ultrastructure in different layers, providing deeper understanding of the cellular network within spheroids.

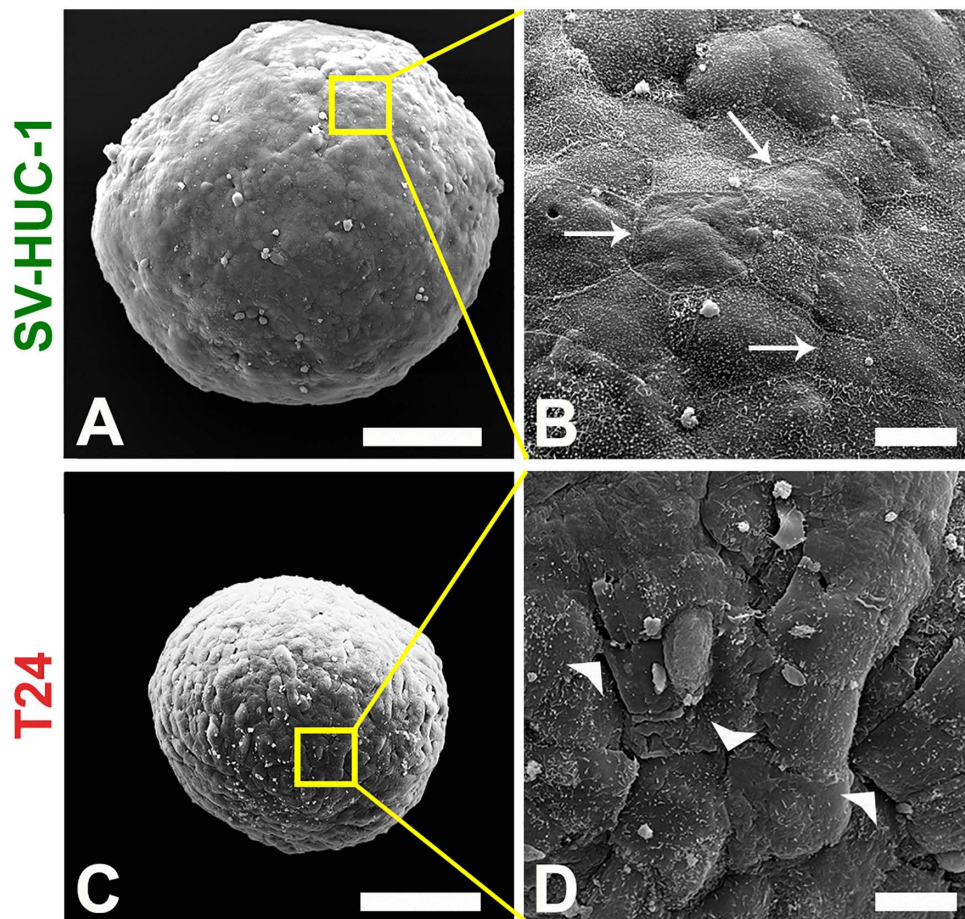


Fig 7. SEM reveals distinct surface ultrastructure of SV-HUC-1 and T24 spheroids. SV-HUC-1 (A) and T24 cells (C) form spheroids with a spherical morphology. (B) SV-HUC-1 cells are tightly attached to each other (arrows), and microvilli are seen on their surface. (D) T24 cells are loosely attached, displaying wider intercellular spaces (arrowheads) and have fewer microvilli. Scale bars: 100 μ m (A, C), 10 μ m (B, D).

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Additional considerations for spheroid characterization

While the microscopy workflows presented here enable comprehensive morphological, molecular, and ultrastructural study of spheroids, researchers should be aware that complete spheroid characterization also requires quantitative assessment of spheroid size and viability. Spheroid diameter and volume directly influence the cellular organization patterns observed with microscopy, including the extent of hypoxic cores visible in TEM analysis (Fig 8) and the proliferation zones visualized by Ki-67 immunofluorescence (Figs 4–6).

Spheroid size can be readily assessed using phase-contrast microscopy during the culture monitoring steps described in our protocols (Fig 2) or by confocal microscopy following nuclear or cytoplasmic labeling [16,17], which improves spheroid boundary detection and enables semi-automated size analysis. Alternatively, spheroid size and volume can be calculated from 3D confocal Z-stacks obtained through whole-mount imaging (Figs 5,6). Additionally, light-sheet fluorescence microscopy (LSFM) provides an advanced imaging approach, allowing for rapid, low-phototoxicity imaging of intact spheroids [18] and accurate volumetric reconstruction after optical clearing [12].

Beyond optical microscopy, high-resolution X-ray micro-computed tomography (μ CT) coupled with a suitable contrasting agent such as osmium tetroxide, iodine potassium iodide, or phosphotungstic acid, represents a

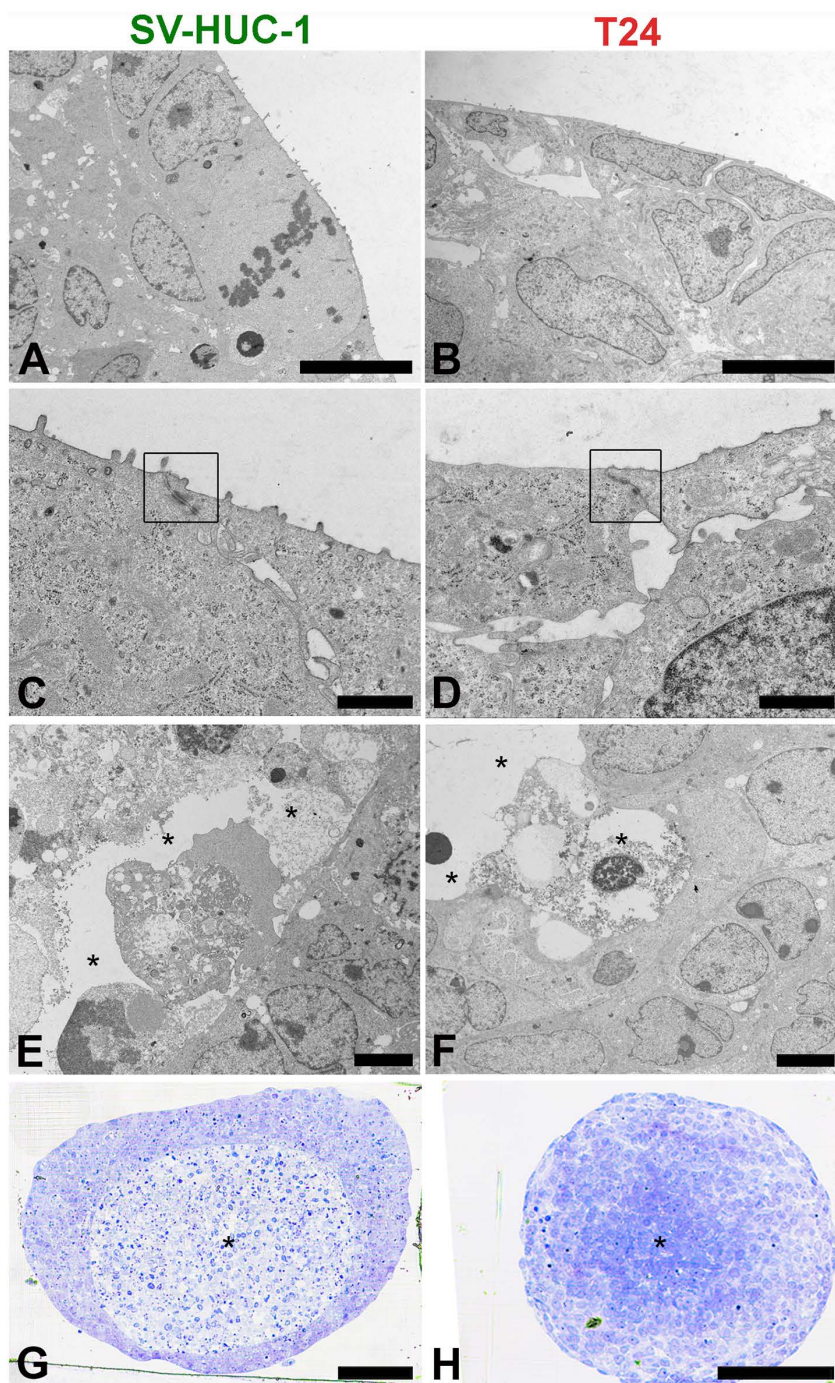


Fig 8. TEM reveals distinct ultrastructure of SV-HUC-1 and T24 spheroids. Outermost cells in SV-HUC-1 spheroids display cuboidal morphology (A), whereas T24 cells are more elongated (B). The presence of cell junctions in the outermost cell layer of SV-HUC-1 (C) and T24 spheroid (D) (boxed regions) indicates a tight cellular network of the outermost cell layer. The central necrotic zone (asterisks) is filled with necrotic cells in SV-HUC-1 and T24 spheroids (E, F), as clearly demonstrated on semithin sections (G, H), prepared before ultrathin sectioning. Scale bars: 100 μ m (G-H), 10 μ m (A-B), 5 μ m (E-F), 1 μ m (C-D).

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complementary volumetric micro-imaging approach that enables non-destructive 3D assessment of spheroid size at the micrometer scale [19]. Collectively, these size-assessment approaches provide essential reference data for interpreting microscopy findings, enable standardization, and ensure comparability across studies employing different imaging modalities.

Similarly, viability assessment complements the morphological and molecular characterization achieved through the presented microscopy workflows. While necrotic cores are evident in histological sections (Fig 3), direct viability mapping can be achieved through live/dead staining, such as Calcein-AM [20] or propidium iodide [21], applied to whole-mount spheroids. Furthermore, analysis of Ki-67 proliferation patterns (Figs 4–6) or EdU incorporation assays [22] enables the discrimination between viable, proliferating regions and quiescent zones.

These complementary viability assessments provide quantitative data that support the morphological observations and clarify the microenvironmental organization observed with the microscope. For conducting downstream functional studies, establishing baseline size and viability parameters supports reproducibility and meaningful comparison of treatment effects in 3D spheroid models.

Conclusions

A comprehensive analysis of structural and molecular features of (bladder) cancer spheroids requires integration of multiple complementary approaches that extend beyond microscopy analysis alone. The quantitative characterization of spheroid size and viability distribution, whether through live/dead staining, functional assays, morphometric analysis from sectioned material, or 3D volumetry, provides essential information that substantially enhances the interpretation of the morphological, molecular, and ultrastructural data obtained through the microscopy workflows presented here. Together with these quantitative parameters, integration of various multi-modal microscopy approaches facilitates comprehensive study of tumors at different organizational levels and protein expression. This integrated, multi-parameter characterization approach is necessary to obtain novel insights and make informed decisions when advancing translational bladder cancer research, particularly when investigating cell-to-cell interactions, tissue organization, disease progression, and treatment response in 3D culture systems.

Supporting information

S1 File. Step-by-step protocol for preparation of cryo- and paraffin sections of spheroids for histological and immunofluorescence analysis, also available on protocols.io.

(PDF)

S2 File. Step-by-step protocol for whole-mount immunofluorescence labelling and optical clearing of spheroids, also available on protocols.io.

(PDF)

S3 File. Step-by-step protocol for preparation and gold sputter coating of whole-mount spheroids for scanning electron microscopy, also available on protocols.io.

(PDF)

S4 File. Step-by-step protocol for epon embedding and ultrathin sectioning of spheroids for transmission electron microscopy analysis, also available on protocols.io.

(PDF)

S5 File. Whole-mount immunofluorescence of E-cadherin and Ki-67 in an SV-HUC-1 spheroid, visualized as a three-dimensional optical reconstruction.

(WMV)

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Writing – review & editing: Larisa Tratnjek, Aleksandar Janev, Nataša Resnik, Uroš Cerkenik, Mateja Erdani Kreft.

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