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Culture of Bladder Cancer Organoids as Precision Medicine Tools

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Corresponding Author:	Elizabeth Williams Queensland University of Technology IHBI: Queensland University of Technology Institute of Health and Biomedical Innovation Brisbane, Queensland AUSTRALIA
Corresponding Author's Institution:	Queensland University of Technology IHBI: Queensland University of Technology Institute of Health and Biomedical Innovation
Corresponding Author E-Mail:	ed.williams@qut.edu.au
Order of Authors:	Patrick Thomas
	Mahasha Perera
	Saeid Alinezhad
	Andre Joshi
	Paria Saadat
	Clarissa Nicholls
	Caitlin Devonport
	Alivia Calabrese
	Abby Templeton
	Jack Wood
	Nathan Mackenzie
	Penny Jeffery
	Ian Vela
	Elizabeth Williams
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TITLE:

Culture of Bladder Cancer Organoids as Precision Medicine Tools

AUTHORS AND AFFILIATIONS:

Patrick B. Thomas[1,2,3], Mahasha P.J. Perera[1,2,3,4,5], Saeid Alinezhad[1,4], Andre Joshi[1,4,5], Paria Saadat[1,2], Clarissa Nicholls[1,2], Caitlin P. Devonport[1,2,4], Alivia R. Calabrese[1,2,3,4], Abby R. Templeton[1,2,3], Jack R. Wood[1,2], Nathan J. Mackenzie[1,2], Penny L. Jeffery[1,2,3,4], Ian Vela[1,2,3,4,5*], Elizabeth D. Williams[1,2,3,4*]

[1]School of Biomedical Sciences, Faculty of Health, Queensland University of Technology (QUT) at Translational Research Institute, Woolloongabba, Queensland 4102, Australia
[2]Queensland Bladder Cancer Initiative (QBCI), Brisbane, Queensland 4102, Australia
[3]Centre for Personalised Analysis of Cancers (CPAC), Brisbane, Queensland 4102, Australia
[4]Australian Prostate Cancer Research Centre - Queensland, Brisbane, Queensland 4102, Australia
[5]Department of Urology, Princess Alexandra Hospital, Brisbane, Queensland 4102, Australia

*These authors contributed equally.

Corresponding Author:

Elizabeth D. Williams (ed.williams@qut.edu.au)

Email addresses of co-authors:

Patrick B. Thomas (pb.thomas@qut.edu.au)
Mahasha P.J. Perera (mahasha.perera@gmail.com)
Saeid Alinezhad (saeedalinezhad@gmail.com)
Andre Joshi (andre.joshi@health.qld.gov.au)
Paria Saadat (paria.saadat@hdr.qut.edu.au)
Clarissa Nicholls (clarissa.nicholls@connect.qut.edu.au)
Caitlin P. Devonport (caitlin.devonport@connect.qut.edu.au)
Alivia R. Calabrese (alivia.calabrese@hdr.qut.edu.au)
Abby R. Templeton (abby.templeton@hdr.qut.edu.au)
Jack R. Wood (j11.wood@qut.edu.au)
Nathan J. Mackenzie (nathan.mackenzie@hdr.qut.edu.au)
Penny L. Jeffery (p.jeffery@qut.edu.au)
Ian Vela (ianvela@me.com)
Elizabeth D. Williams (ed.williams@qut.edu.au)

SUMMARY:

Patient-derived organoids (PDOs) are a powerful tool in translational cancer research, reflecting both the genetic and phenotypic heterogeneity of the disease and response to personalized anti-cancer therapies. Here, a consolidated protocol to generate human primary bladder cancer PDOs in preparation for the evaluation of phenotypic analyses and drug responses is detailed.

ABSTRACT:

Current *in vitro* therapeutic testing platforms lack relevance to tumor pathophysiology, typically employing cancer cell lines established as two-dimensional (2D) cultures on tissue culture plastic. There is a critical need for more representative models of tumor complexity that can accurately predict therapeutic response and sensitivity. The development of three-dimensional (3D) *ex vivo* culture of patient-derived organoids (PDOs), derived from fresh tumor tissues, aims to address these shortcomings. Organoid cultures can be used as tumor surrogates in parallel to routine clinical management to inform therapeutic decisions by identifying potential effective interventions and indicating therapies that may be futile. Here, this procedure aims to describe strategies and a detailed step-by-step protocol to establish bladder cancer PDOs from fresh, viable clinical tissue. Our well-established, optimized protocols are practical to set up 3D cultures for experiments using limited and diverse starting material directly from patients or patient-derived xenograft (PDX) tumor material. This procedure can also be employed by most laboratories equipped with standard tissue culture equipment. The organoids generated using this protocol can be used as *ex vivo* surrogates to understand both the molecular mechanisms underpinning urological cancer pathology and to evaluate treatments to inform clinical management.

INTRODUCTION:

Bladder cancer is the most prevalent urinary tract cancer and the tenth most common human malignancy worldwide[1]. It encompasses a genetically diverse and phenotypically complex spectrum of disease[2]. Urothelial non-muscle-invasive forms of bladder cancer (NMIBC) are the most common bladder cancer diagnoses (70%-80%), and these cancers display considerable biological heterogeneity and variable clinical outcomes[2-4]. Patients with NMIBC typically experience a high risk of disease recurrence (50-70%) and one-third of cancers will progress and develop into significantly more aggressive muscle-invasive bladder cancer (MIBC)[2]. Although 5-year survival rates for NMIBC are high (>90%), these patients must undergo long-term clinical management[5]. On the other hand, locally advanced (unresectable) or metastatic MIBC is generally considered incurable[6]. Consequently, bladder cancer has one of the highest lifetime treatment costs within cancer care and is a significant burden for both the individual and the healthcare system[3,7]. The underlying genetic aberrations in advanced disease renders therapeutic management of bladder cancer a clinical challenge, and therapeutic options for invasive urothelial tumors have only recently improved since the approval of immunotherapies for both advanced and high-risk NMIBC[8,9]. Currently, clinical decision-making has been guided by conventional clinical and histopathological features, despite individual bladder cancer tumors showing large differences in disease aggressiveness and response to therapy[10]. There is an urgent need to accelerate research into clinically useful models to improve the prediction of individual patient prognosis and identification of effective treatments.

Three-dimensional (3D) organoids show great potential as tumor models due to their ability to self-organize and recapitulate the original tumor's intrinsic *in vivo* architecture and pharmacogenomic profile, and their capability to mirror the native cellular functionality of the original tissue from which they were derived[11-13]. Although established bladder cancer cell lines are readily available, relatively cost-effective, scalable, and simple to manipulate, the *in vitro* cell lines largely fail to mimic the spectrum of diverse genetic and epigenetic alterations observed in

clinical bladder cancers[12,14] and were all established and maintained under 2D, adherent culture conditions. Additionally, cell lines derived from primary and metastatic bladder tumors harbor significant genetic divergence from the original tumor material. [8,15].

An alternative approach is to use genetically engineered and carcinogen-induced mouse models. However, while these models recapitulate some of the natural oncogenic cascades involved in human neoplasia (reviewed in refs[16-18]), they lack tumor heterogeneity, are expensive, poorly represent invasive and metastatic bladder cancer, and are not viable for rapid term drug testing as tumors can take many months to develop[14,19]. Patient-derived models of cancer (including organoids, conditionally reprogrammed primary cell culture, and xenografts) provide invaluable opportunities to understand the effects of drug treatment before clinical treatment[20]. Despite this, few groups routinely use these patient-proximal models due to limited access to fresh primary patient tissue and the extensive optimization required to reproducibly generate patient-derived organoid (PDO) culture conditions. In an *in vivo* setting, oncogenic cells can interact and communicate with various compositions of the surrounding constituents, including stromal cells, tissue infiltrating immune cells, and matrix[12]. Similarly, for PDOs grown in a 3D format, cellular/matrix complexity can be customized to include other relevant components. PDOs can be rapidly generated and are often able to be passaged extensively or cryopreserved for later use, despite having a finite lifespan[21-23]. Pharmacodynamics (i.e., response to a drug) can be evaluated using multiple read-outs, including organoid viability and morphology, and characterization of immunohistochemistry targets or transcriptional changes.

Here, the procedures for the establishment of bladder cancer organoids from patient material collected from transurethral resection of bladder tumor (TURBT) or surgical removal of the bladder (radical cystectomy) are described. The method to generate PDOs is illustrated, using readily available wet laboratory materials and tools. Endpoints include changes in cell morphological characteristics and viability. These were measured using fluorescent microscopy, *in vitro* viability (metabolic and cell membrane integrity) assays, and histopathological analysis. **Figure 1** shows the workflow for establishing human bladder cancer PDOs from clinical material obtained during elective surgery.

PROTOCOL:

Patients have consented following their admission under the Urology team at the Princess Alexandra Hospital, Brisbane, Australia. This study was performed in accordance with the principles of the Declaration of Helsinki and within ethical and institutional guidelines (ethics number HREC/05/QPAH/95, QUT 1000001165).

NOTE: As eligibility criteria, patients must be aged ≥ 18 with cancer, and able to understand and provide consent. Those who are not able to give informed consent were excluded. Those having a primary language other than English were excluded as the provision of interpreters is not possible due to logistical and budgetary considerations. Also excluded were patients whose tumors are not accessible to biopsy or unlikely to be available in adequate amount after routine pathology.

1. Organoid Medium Preparation

NOTE: Human bladder cancer organoid medium requires growth factors that aid in the survival, growth, and continuous expansion of organoids derived from dissociated clinical material (**Table 1**). For complete details of each supplement used in this procedure, please refer to the **Table of Materials**.

1.1. Thaw frozen ingredients on ice or in a refrigerator at 2–8 °C. Avoid freeze/thaw and work from frozen aliquots (stored at -20 °C).

1.2. Basal medium: Prepare basal medium by supplementing advanced Dulbecco's Modified Eagle Medium/Ham's F-12 (adDMEM/F12) with HEPES (10 mM) and glutamine (2 mM). This is also used as a transport medium, and during organoid washing steps.

NOTE: Advanced DMEM/F-12 is used as the basal medium to aid in the expansion of organoids in the presence of limited serum; however, it requires supplementation with HEPES and L-glutamine.

1.3. Briefly centrifuge FGF-10, FGF-2, EGF, SB202190, PGE2, and A 83-01 before opening to ensure components are at the bottom of the vial.

1.4. Complete organoid medium: Supplement basal medium with human Noggin- and R-spondin 1-conditioned media at a final concentration of 5% v/v, human EGF (50 ng/mL), human FGF-2 (5 ng/mL), human FGF-10 (20 ng/mL), A 83-01 (500 nM), SB202190 (10 µM), B27 (1x), Nicotinamide (10 mM), N-acetylcysteine (1.25 mM), Y-27632 (10 µM), Prostaglandin E2 (PGE2) (1 µM) and a broad-spectrum antibiotic formation at the concentration indicated by the manufacturer.

1.5. Store organoid media at 4 °C in the dark and use it within 2 weeks (1 month at most). Do not freeze. Avoid extended exposure to light sources.

NOTE: The organoid medium is prepared serum-free; however, serum and penicillin/streptomycin could be supplemented on a user-to-user basis as required.

2. A day before procedure outlined in 3

2.1. Thaw growth factor reduced basement membrane (BME; see **Table of Materials**) overnight for at least 12 h before use in a 4 °C fridge or cold room. If required, dispense BME into 1 mL aliquots in a 1.5 mL polypropylene single-use tube to avoid freeze-thaw cycles.

2.2. Place filtered pipette tips into a 4 °C fridge or cold room.

NOTE: This section refers to pipette tips that will be used when handling BME to prevent premature polymerization and reduce the coating of BME on the surface of the tips.

2.3. Sterilize all surgical equipment required for the procedure.

3. Generation of bladder tumor organoids

NOTE: This is an initial step for PDO establishment from primary patient tumors. This procedure is adapted for bladder cancer tissues from methods established by Gao et al.[24].

3.1. Use a class II biohazard hood to prepare the specimen. Retrieve dry and wet ice and print *Patient Specimen Processing Sheet* (**Supplemental File**).

3.2. Call research personnel to the operating room when surgical resection is near complete.

NOTE: Confirm that the patient meets the eligibility criteria and has signed the participant consent form. Tissue is provided for research only if surplus to requirements for clinical histopathologic assessment.

3.3. Collect a fresh macroscopically viable tumor specimen from surgery. Ensure that the sample is submerged in transport medium (either 1x adDMEM/F12 or 1x Dulbecco's phosphate-buffered saline (DPBS)) in a sterile 50 mL conical tube or urine specimen jar during transit.

NOTE: At some clinical centers, tissue may need to be transported to a pathology laboratory for allocated research. Under these circumstances, it is recommended that antibiotics and antimycotics be added to the transport medium. Tissue can be stored at 4 °C in basal medium for up to 24 h post-surgery and still generate viable organoid cultures.

3.4. Record specimen details, including tissue weight (g or mg), sample description, and details regarding any blood and urine samples on the *Patient Specimen Processing Sheet* (**Supplemental File**).

3.5. Carefully remove the transport medium and replace it with 10 mL of basal media. Allow the tumor tissue to settle by gravity.

NOTE: Transport medium is considered as clinical waste and must be collected in an appropriately labeled waste container in an appropriate amount of decontamination solution. Once the liquid has been chemically decontaminated, it can be disposed of according to institutional guidelines for hazardous waste.

3.6. Remove the tumor tissue with forceps and place it in a sterile 90 mm Petri dish (**Figure 2A**). Record the weight of the tissue in mg or g on the clinical specimen processing sheet and dissection grid (**Figure 2B**).

3.7. Remove non-cancerous tissue (including adipose tissue) and macroscopically visible necrotic regions using sterile forceps and disposable scalpel blade mounted to scalpel handle (**Figure 2C**). Wash tumor pieces 1–2 times with cold 1x DPBS. Collect the tumor pieces and transfer them to

a new sterile 90 mm Petri Dish.

NOTE: As scalpel blades are sharp, take caution when manually slicing. Tumor-adjacent adipose tissue can be identified by distinctly soft, gelatinous, pale areas immediately adjacent to the visual tumor perimeter. Macroscopic adipose tissue, and focally dark regions representing areas of necrosis will require personal judgment when dissecting from an excisional bladder tumor biopsy.

3.8. Take a photograph, draw a diagram of tissue, and plan tissue dissection on a clinical processing grid (**Figure 2B** and **Figure 2C**).

NOTE: It is important to keep a visual record and rough sketch of the individual macroscopic tumor characteristics and dissection for each specific case.

3.9. Dissect tumor tissue pieces and allocate for histopathological **(Figure 2D)** and molecular analyses (**Figure 2E**).

3.9.1. For histological analyses: Place approximately 50 mg of tumor tissue into a labeled disposable plastic histology cassette (**Figure 2D**). Submerge histology cassette into a container with 5x to 10x volume of 10% neutral buffered formalin (NBF). Incubate overnight.

3.9.2. Remove 10% NBF and replace with 70% (w/w) ethanol the next day for storage at 4 °C until tissue can be processed using routine tissue processing protocol

3.9.3. For molecular analyses: Snap freeze at least one 1–3 mm^3 tumor piece in an RNase/DNase-free 1.5 mL cryovial using liquid nitrogen and store at -80 °C (**Figure 2E**).

3.10. Dispense 5 mL of organoid medium (**Table 1**) in the 90 mm Petri dish containing the remaining tumor piece(s).

3.11. Mechanically mince tissue as finely as possible (0.5–1 mm^3 pieces or smaller) with a sterile #10 scalpel blade.

NOTE: Larger fragments or whole chunks of tissue (>3 mm^3) will take considerably longer to digest and will decrease the viability of the specimen. Omit the above step if the tissue is disaggregated and fragments are small enough to pipette with a 5 mL serological pipette tip.

3.12. Transfer finely minced tissue to a 50 mL conical tube and add 4 mL of organoid medium, 1 mL of 10x collagenase/hyaluronidase, and 0.1 mg/mL deoxyribonuclease 1 (DNase 1) to avoid cell clumping.

NOTE: Enzyme solution should be freshly prepared each time. Increase the volume of the medium to be approximately 10x the visible amount of the tumor fragments for large samples.

3.13. Incubate the minced tumor tissue and enzyme solution for 1–2 h on an orbital shaker or rotator (150 rpm) in an incubator (37 °C, 5% CO_2) to dissociate fragments into a cell suspension and break down collagens. This can be checked with histological analysis (**Figure 3A**).

NOTE: If the amount of tissue is large or no obvious dissociation is observed after 1–1.5 h, increase the incubation time checking the level of dissociation every 30 min. Timing for this step depends on the sample and must be determined empirically each time. A noticeably clearer solution with tissue fragments indiscernible to the eye (or very few fragments) indicates successful digestion.

3.14. Terminate the digestion with the addition of 2x volume (20 mL) of basal medium to the sample.

3.15. Centrifuge the sample at 261 x *g* for 5 min at room temperature (RT), aspirate, and discard the supernatant.

3.16. To lyse contaminating red blood cells (RBCs), resuspend the pellet obtained from the above step in 5 mL of ammonium-chloride-potassium (ACK) buffer. Incubate the tube at RT for 3 min or until the complete lysis of the RBCs is seen (suspension becomes clear).

NOTE: If RBCs are not observed as a small red clump in the pellet, this step may be omitted.

3.17. Add 20 mL of the basal medium into the tube. Centrifuge the tube at 261 x *g* for 5 min at RT and aspirate the supernatant.

3.18. At this step, place a 10 mL aliquot of 2x and 1x organoid medium in a 37 °C water bath to warm.

3.19. Filter the sample through a pre-wet reversible 100 µm strainer into a new 50 mL tube to remove large insoluble material.

NOTE: Large undigested material (>100 µm) collected by the filter may contain cells of interest and can be cultured in 90 mm Petri dish or 6-well cell culture plate in organoid medium to derive two-dimensional (2D) cultures (**Figure 1 (step 5)** and **Figure 3B**).

3.20. Filter the eluate through a pre-wet reversible 37 µm strainer to collect single cells and small clusters for single-cell and immune cell isolation (Tube **37-1; Figure 1 (step 6)** and **Figure 3B**).

NOTE: The yield of tumor cells during this step may be increased by passing the filtered cell suspension through the strainer again.

3.21. Reverse the 37 µm strainer and use 10 mL of basal media to collect small and moderate-sized clusters (37-100 µm) (tube **70-1**; **Figure 3B**).

3.22. Top up each of the new 50 mL tubes (tubes **70-1** and **37-1**) with DPBS to 40 mL. Centrifuge the suspension at 261 x *g* for 5 min at RT. Aspirate and discard the supernatant.

3.23. Add 10 mL of basal media to tube **37-1** and count the cells using trypan blue exclusion dye and an automated cell counter (as per the manufacturer's specifications). Determine the cell number and viability of cells.

3.24. Centrifuge the remainder of the sample at 261 x *g* for 5 min at RT. Aspirate the medium and replace it with cell freezing solution or basal media containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 10% dimethyl sulfoxide (DMSO).

3.25. Place samples in 1.5 mL cryovials and store them in a cell-freezing container. Immediately transfer the container to a -80 °C freezer for optimal rate of cooling. Following overnight freezer storage at -80 °C, transfer the cryovials into cryogenic liquid or air phase storage (-196 °C) for long-term storage.

3.26. Resuspend cells from tube **70-1** with 500 µL of pre-warmed 2x organoid medium.

NOTE: Seeding density must be high for successful organoid propagation. The volume of the organoid medium and, subsequently, BME should be altered empirically on the number of cells isolated from the filtration step. This step provides a relevant starting point based on studies in our laboratory.

3.27. Add BME to cells (at a 1:1 ratio with 2x organoid medium) with ice-cold P1000 sterile filter pipette tips and mix gently to suspend cells. Quickly and carefully pipette 100 µL of reconstituted cells/ BME mixture to wells of an ultra-low attachment flat-bottom 96-well plate. Place the 96-well plate in an incubator (37 °C, 5% CO_2) for 20–30 min to solidify.

3.28. Add 1:2 ratio of 1x organoid medium on top of BME cell suspension depending on the empirically assessed volume. In the relevant starting point, add 50 µL of 1x organoid medium media on top of 100 µL of reconstituted cells/ BME mixture.

3.29. Place the 96-well plate in an incubator (37 °C, 5% CO_2) for 20 min to equilibrate.

3.30. Take the cell culture plate out of the incubator and assemble it on a specimen holder on the stage of a microscope. Assess organoids visually under phase contrast or brightfield settings.

NOTE: Images are best acquired by an inverted phase-contrast microscope equipped with differential interference (DIC) optics, digital camera, and associated software to observe the formation of spherical PDOs in preparation for endpoint analysis.

3.30.1. If distinct clusters are visible, place the cell culture plate into an onstage electronically heated microscope chamber (37 °C, 5% CO_2) for live-cell imaging over the first 24–72 h.

3.30.2. Ensure the flexible heated collar is attached to the lens to reduce thermal drift and the humidifier is filled with dH_20.

3.30.3. Perform the initial imaging using a 4x or 10x objective lens (N.A. 0.30, W.D. 15.2 mm). Time-lapse every 5–10 min on the phase-contrast setting.

3.30.4. Check for the successful isolation as characterized by the appearance of >10 self-organizing organoids per well after a 24–72 h period.

3.30.5. Allow cultures to continue for up to two weeks if organoid numbers are low (**Figure 3C**).

3.31. Top up the medium every 2–3 days using 50 µL of pre-warmed organoid medium to replenish depleted growth factors and overall volume.

3.32. Acquire images (as described in step 3.30) on days 1, 2, and 3 (time-lapse series), and on days 5, 7, and 10 before passaging (if applicable).

NOTE: Organoids (observed as generally round structures where you cannot see the edges of individual cells) are typically passaged 7-10 days following setup, depending on the success of the isolation**.** Before or following passaging, organoids can be treated with cytotoxics or therapeutic agents for up to 6 days and drug response measured using cell viability and cytotoxicity assays. Alternatively, organoids can be retrieved from BME (using 1 mg/mL dispase) and cryopreserved (bio banked), prepared for molecular testing, or embedded and assessed histologically (**Figure 4**).

REPRESENTATIVE RESULTS:

3D organoids were successfully established from human bladder cancer patient TURBT and cystectomy tissues. Briefly, this technique highlights a rapid formation of 3D multicellular structures that are both viable and suitable for other endpoint analyses such as histological evaluation, molecular characterization (by immunohistochemistry or quantitative real-time PCR), and drug screening. During the procedure (**Figure 1**), the various eluates during our filtration phases (**Figure 1**, **steps 1–6**) could be taken advantage of to cryopreserve single cells for isolation of tumor-infiltrating immune cells (TILS) and generation of a single cell biobank. The procedure allows for multiple aspects of a heterogeneous primary tumor to be biobanked appropriately and in a way that can be tracked across patients (**Figure 2A,B**), including histology (**Figure 2C,D**) and fresh frozen samples (**Figure 2E**).

A 2 h digestion with commercially available collagenase/hyaluronidase and DNase 1 allows for sufficient digestion of 0.5–1 mm^3 pieces of the more complex cystectomy biopsy tissue, including the neoplastic cells within the lamina propria and muscular layers of the bladder (**Figure 3A**). Larger post-digestion fragments (>100 microns) have proven suitable for culture in standard tissue culture plates of any size for the isolation of novel 2D cultures (**Figure 3B**). However, extensive molecular characterization is required to validate the cancer origin of such cell lines. Organoids which are successfully generated with this protocol undergo processes of dynamic

self-assembly, including phases of aggregation, compaction, and final formation, whereby they form into tight cellular structures (**Figure 3C**) that can be assessed for response to standard-of-care therapies. **Figure 3C** highlights the efficient self-assembly properties of the organoids. Histologically, these structures recapitulate the original patient tumor (**Figure 4**). Organoids generated in this procedure are suitable for a multitude of purposes, including further characterization, biobanking, and drug efficacy testing (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of organoid tissue culture from the collection of tumor samples from surgery to endpoint analyses. URN, unit record number; TIL, Tumor-infiltrating lymphocyte. (**1**) Tissue is mechanically minced in a 90 mm petri dish as finely as possible with a sterile scalpel blade (0.5–1 mm^3 pieces). (**2**) Tumor pieces are resuspended in an enzymatic dissociation medium consisting of the organoid medium, collagenase/hyaluronidase, and DNase 1. (**3**) Minced tumor tissue and enzyme solution are incubated for 1–2 h on a rotator incubator (150 rpm, 37 °C, 5% CO_2) and subsequently pelleted (**4**) to dissociate fragments into a finer cell suspension. The sample undergoes filtration through pre-wet reversible (**5**) 100 µm and (**6**) 37 µm strainers into new 50 mL tubes to remove large insoluble material and isolate 37–100 µm clusters. Following (**7**) centrifugation of the fraction isolated from the reverse of the 37 µm strainer, the cell clusters are suspended in (**8**) BME and organoid media before (**9**) live-cell imaging. Created with BioRender.com.

Figure 2: Sterile tumor tissue handling for organoid establishment. (**A**) Upon excision and transport to the laboratory, the specimen is placed in a sterile petri dish, weighed, and subsequently dissected. (**B**) A specimen dissection grid is used to mark the specimen for various processes. An example of dissection is shown in (**C**) where samples are taken from the tumor periphery as much as possible to avoid areas of central necrosis and annotated (inset) before (**D**) grossing the tissue prior to tissue fixation. Scale bar: 6 mm. Inset scale bar: 4 mm. (**E**) Small fresh fragments are taken for subsequent freezing and biobanking. Scale bar: 5 mm. Elements of this image were created with BioRender.com.

Figure 3: Enzymatic derivation and dynamic assembly of bladder tumor organoids. (**A**) Representative pictures of hematoxylin and eosin (H&E) stained parental bladder cancer tissue (pathology reviewed as T3bN0) on immediate fixation (left) and 2 h post-digestion with 1x collagenase/ hyaluronidase (right). Scale bar: 500 µm. Inset scale bar: 50 µm. (**B**) Representative images of the >100 µm, **37-1** and **70-1** (day 2) isolations. Scale bars are indicated within the image. (**C**) Representative images are shown for bladder cancer organoid isolation over 48 h and day 7 organoid H&E staining. Scale bar: 50 µm. Scale bar for the image in the inset: 20 µm. DIC: differential interference contrast. Elements of this image were created with BioRender.com

Figure 4: Bladder cancer-derived organoid cultures maintain the histological architecture of parental tumors. Representative H&E image of low-grade bladder cancer (Ta) and corresponding bright-field image of organoid at day 7 of culture. The far-right panel shows associated H&E staining of the day 7 organoid. Scale bars are indicated in the figure. LG: low grade, BC: bladder

cancer, H&E: haematoxylin and eosin, and DIC: differential interference contrast.

Figure 5: Downstream applications with bladder cancer patient-derived organoids. Created with BioRender.com.

Table 1: Supplements used for basal and complete organoid media

DISCUSSION:

While 3D organoid protocols derived from bladder cancer tissue are still in their infancy, they are an area of active research and clinical investigation. Here, an optimized protocol to successfully establish bladder cancer PDOs that are suitable for both NMIBC and MIBC specimens is detailed. This workflow integrates parallelly into hospital-based clinical trials and considers biobank sample accrual, including histological sample processing and fresh frozen tissue banking, which is an important consideration for clinical organoid pipelines. This protocol builds on existing methods and provides a consolidated methodology to build an organoid precision medicine pipeline.

The current success rate of this protocol - like others - is approximately 70%[25]. It is anticipated that this will improve as isolation techniques and characterization of the BC tumor microenvironment evolves. As expected, the quality of the initial specimen influences the success rate and is an important limiting step. Specimens obtained from chemotherapy naïve patients provide the highest success rate, while those from patients who have received neoadjuvant chemotherapy may have a reduced number of viable cells[26,27]. Typically, bladder cancer specimens (from both TURBT and cystectomies) provide an ample amount of samples with high tumor cellularity. This allows robust generation of organoids ranging between 30–100 µm within a 24–72 h growth period (given some <40 µm clusters are retained in the filtration steps). Importantly, the heterogeneity observed in organoid samples (i.e., cystic formations and more dense structures) indicates that this protocol provides conditions suitable to derive heterogeneous tumor cell populations.

Our optimized protocol can be performed on limited starting material (as is the case from some elective surgeries, including TURBT procedures or specimens acquired from patients on a clinical trial), enabling the incorporation of multiple analyses to maximize specimen value. This protocol includes methods to describe steps to grow 2D culture from extraneous tumor cells of interest that may reside in large insoluble tissues processed during the initial filtration step. Like other research groups which suggest processing specimens within 24 h post-surgery[28,29], it is observed that tissue hypoxia that occurs within this timeframe does not preclude generating viable organoids. Optimization is required to determine the density of cell (native clusters) seeding per well. In our experience, excessive numbers of clusters can result in poor growth of the cultures, while low visual density cultures can lead to cell death. This can be determined by the user empirically in the context of their volumes which can be diluted within a range as necessary. Additionally, TURBT specimens often contain burnt edges due to the resection process, which are apparent as prominent necrotic regions. A critical step is the careful isolation of macroscopic tumor tissue and subsequent dissection. This is paramount to deriving viable cultures and needs

to be communicated with the surgical team. In some instances, this may be an initial limitation to the procedure.

An additional limitation to the current technique is the absence of the tumor microenvironment parallel to the organoids. A necessary development will be future co-culture systems to increase cellular complexity and provide other components of the tumor microenvironment (i.e., stroma and immune cells). As organoids are defined as self-organizing structures with the ability to self-perpetuate their growth, further work will be required to elucidate the discrete cancer stem-cells (such as CD44 and CD49) which may allow for improved *in vitro* growth characteristics[31-33]. Establishing with certainty that the PDOs originate from tumor tissue is critical, as the contribution from normal urothelial cells in functional and molecular analyses will confound investigations into drug resistance. Sometimes the tumor origin of the urothelial cells in the PDOs is clear due to the use of metastatic tissue or papillary tumors (as is the case here; see **Figure 4**), but when the source tissue is excised from the bladder mucosa and thus contains margins of the benign urothelium, it is critical to confirm that the resultant PDOs are comprised of tumor cells using molecular techniques. Thus, immunohistochemical and genomic comparisons are required between the established organoids and the primary tumor from which they were derived[30].

Regarding the number and size distributions of the organoids per well, it is difficult to maintain uniform plating density of 3D organoids for cytotoxicity analysis. This is slightly mitigated by isolating defined ranges using the filters, but it is still a limitation if small clusters and larger organoids are isolated in the same well. Large particle sorters may mitigate this issue but are not widely available in medical research laboratories. Nonetheless, techniques that measure viability signals before and after treatment have been used successfully in bladder cancer organoids[34]. As this technology advances, this will enable researchers to develop a library of bladder cancer PDOs with varying genomic profiles, and pharmacodynamic responses that can be explored to investigate novel therapies. Additionally, the specimen collection procedure and dissociation method described herein allow concurrent preparations for tumor spatial profiling, multiparametric flow cytometry, and single-cell RNA sequencing. Taken together, this allows for a diverse range of powerful endpoints which can be brought together to explore a patient's tumor heterogeneity and applicability of drugs such as immunotherapies.

In summary, we describe a protocol to establish and evaluate cultures of 3D patient-derived bladder cancer organoids in the advent of precision medicine endpoints. It is important to note that the steps and notes described here are routine approaches used to isolate bladder cancer PDOs in our laboratory. The culture medium used in our procedure was originally described for prostate cancer cells and has now been proven to be suitable for the culture of bladder cancer. Importantly, this protocol is centralized, suitable for routine use in laboratories, and broadly applicable across urological cancer types. In combination with high fidelity molecular phenotyping and drug response endpoints, this technique will provide a useful tool to rapidly explore the precise therapeutic management of bladder cancer patients.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have no conflicts of interest.

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Figure

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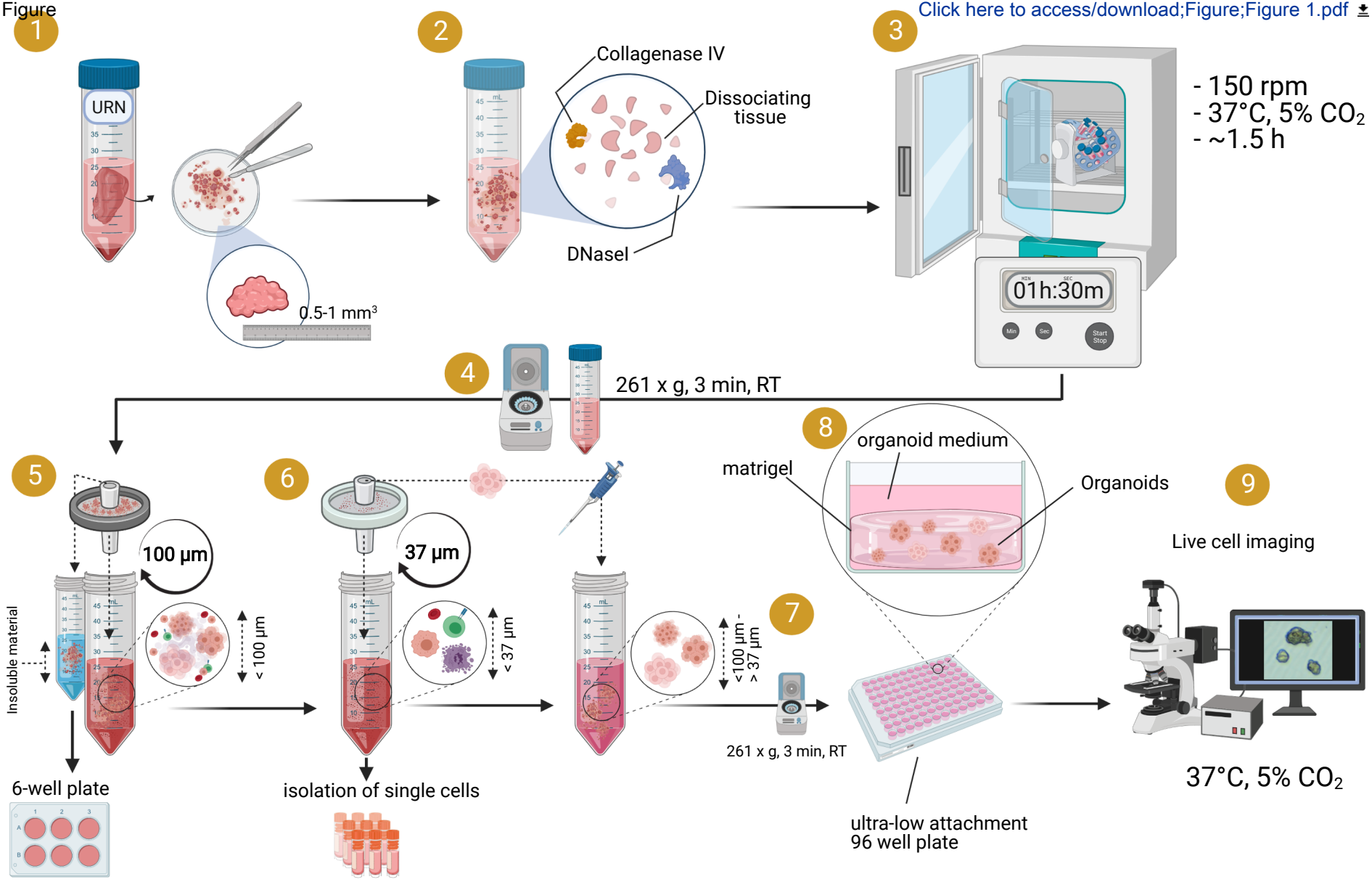
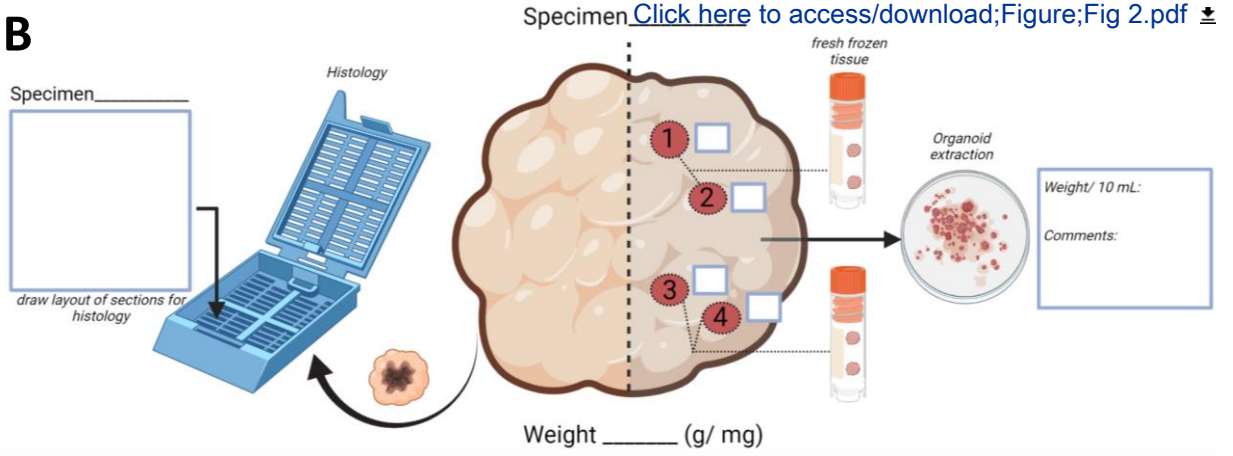
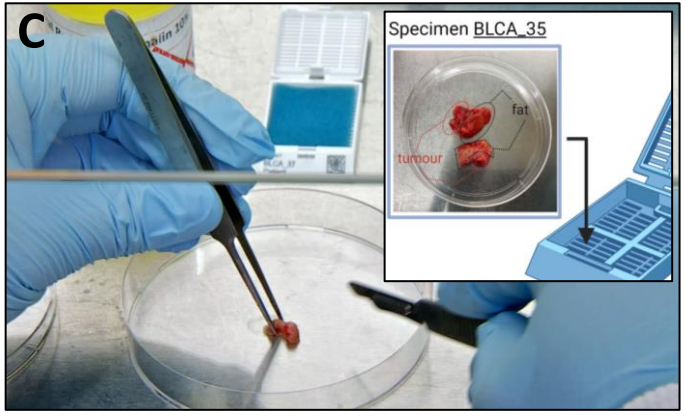
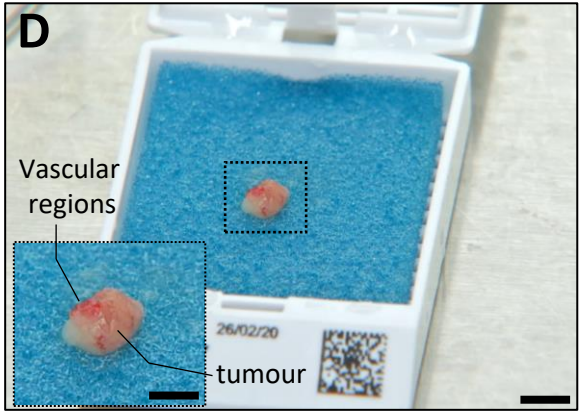


Figure 2


A
BLCA_35
Patient
marbig 975317B


B
Specimen
Click here to access/download;Figure;Fig 2.pdf
Specimen_____
draw layout of sections for histology
Histology
fresh frozen tissue
Organoid extraction
Weight/ 10 mL:
Comments:
1
2
3
4
Weight _____ (g/ mg)


C
Specimen BLCA_35
fat
tumour


D
Vascular regions
26/02/20
tumour

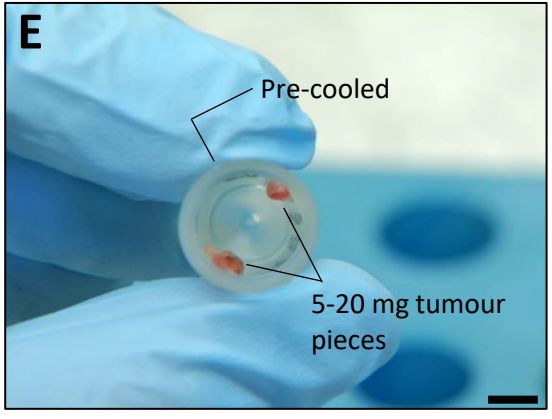

E
Pre-cooled
5-20 mg tumour pieces

Figure 3

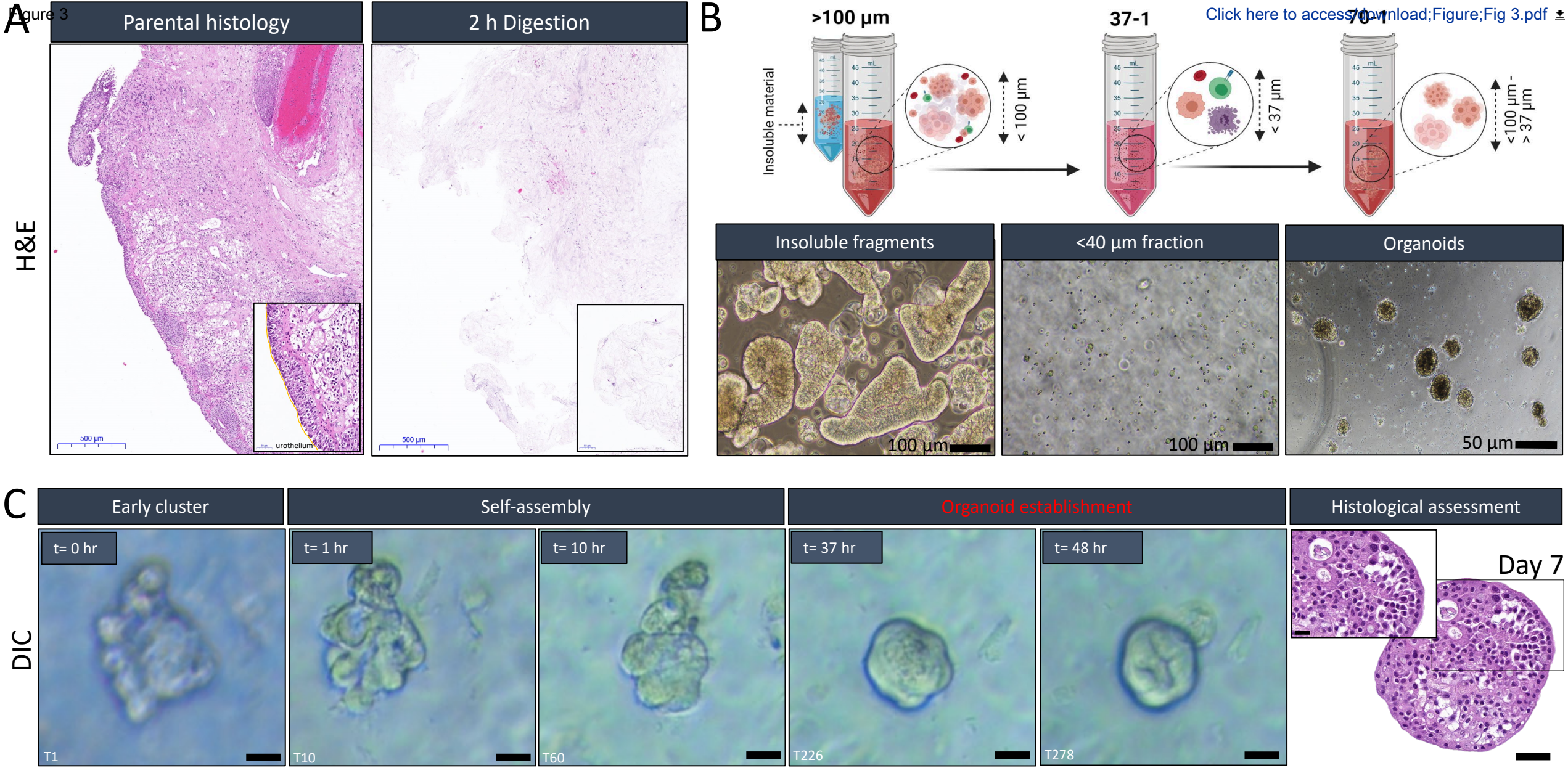


Figure 4

Click here to access/download;Figure;Fig 4.pdf

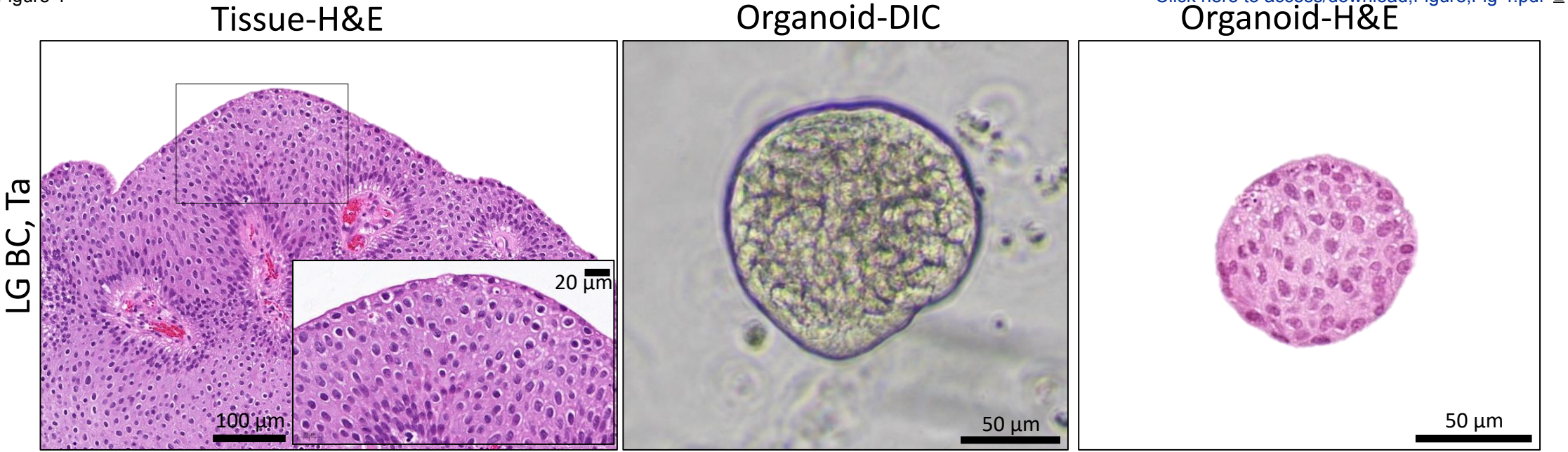
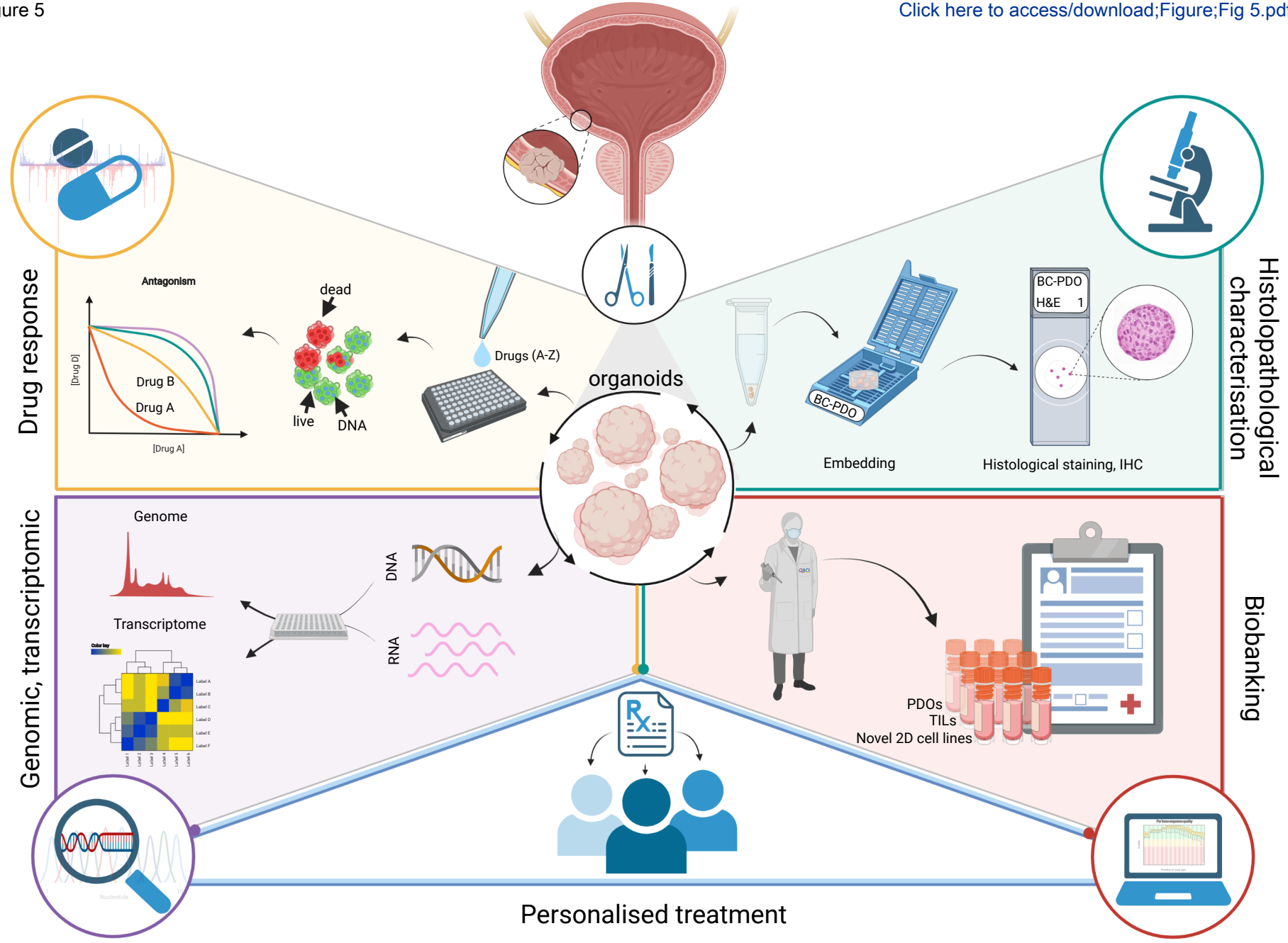


Figure 5



Table

Additive		Final Conc.	Stock Conc.
Basal Media	Glutamax	2 mM	1 M
	HEPES	10 mM	1M
Supplements for complete media	R-spondin 1 conditioned media	5% v/v	Conditioned Media
	Noggin conditioned media	5% v/v	Conditioned Media
	EGF	50 ng/mL	0.5 mg/mL
	FGF-10	20 ng/mL	100 µg/mL
	FGF-2	5 ng/mL	50 µg/mL
	Nicotinamide	10 mM	1 M
	N-acetyl-L-cysteine	1.25 mM	500 mM
	A 83-01	0.5 µM	50 mM
	SB202190	10 µM	50 mM
	Y27632	10 µM	100 mM
	B-27 additive	1X	50x
	Prostaglandin E2	1 µM	10 mM
	Primocin	100 µg/mL	50mg/mL

Solvent
NaCl
$NaCl/NaHPO_4$ buffered solution
Advanced DMEM/F-12
Advanced DMEM/F-12
PBS/0.1% BSA
PBS/0.1% BSA
PBS/0.1% BSA
1 g in 8.2 mL ddH_20
40 mL ddH_20
DMSO
DMSO
ddH_20
DMSO

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Firstly, we would like to thank the reviewers for their insightful and helpful comments and are grateful for the opportunity to revise our manuscript. We have addressed both the editorial and reviewers' comments below. Additions to the manuscript are highlighted in blue font in our Author's response below.

Editorial comments and changes to be made by the Author(s):

Author response
All requested editorial changes listed below have been made by the author in the submitted tracked version of the manuscript.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
2. Please revise the following lines to avoid previously published work: 73, 84-85, 241-247, 265-266, 273-275.
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4. Please use SI units as much as possible and abbreviate all units: L, mL, µL, cm, kg, etc. Use h, min, s, for hour, minute, second.
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6. Please specify the inclusion (and exclusion) criteria for your patients.
7. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you","our" etc.).
8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
    a. Line 303: How was the plate set up for imaging? How were the images acquired? Please elaborate the steps for acquiring images. Please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. Please keep in mind that software steps without a graphical user interface (GUI) cannot be filmed. If you don't think this is essential for the protocol, please cite a paper where you have described this in detail.
10. Please provide solution composition as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text. If you need to provide specific instructions on adding the components in a particular order or provide safety notes, please add those steps in the first section, citing this table.

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11. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video. To avoid exceeding the 3-page limit, avoid highlighting notes.

12. Please discuss all figures in the Representative Results. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.

13. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail with citations:

i. Critical steps within the protocol
ii. The significance with respect to existing methods

14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

Response to Reviewers comments:

Reviewer #1:

Comment
Even though the paper is mainly a protocol it would be good to acknowledge that even though heterogeneity is observed in the organoids morphologically, based on the information provided in the paper nothing is yet known about whether these organoids actually mimick the genetic and phenotypic make-up of the tumor. Especially, overgrowth of healthy cells has been a problem is multiple types of cancer organoids (i.e. NSCLC organoids) and at this point the organoids are not yet verified as being cancerous. Can the authors already say something about this based on the data that has been gathered to this point? Otherwise this could be more critically discussed in the Discussion as this is now only really shortly mentioned but is a major determinant in the efficacy of the protocol.

Response
We thank the reviewer for this suggestion. This method outlines the process to rapidly process clinical specimens in the effort to generate patient-derived organoids (PDOs) from primary bladder cancer tissue. Histopathological analyses show that the PDOs produced using this protocol are consistent with the histopathological appearance of the clinical tumour from which they are derived (Figure 3 and Figure 4). As part of a large urothelial carcinoma personalised medicine study, we are currently investigating the genetic and molecular phenotypes of PDOs isolated using this protocol in comparison to the parental primary tissues (Thomas, *unpublished*). We agree that every 3D cancer model exhibits intrinsic limitations for recapitulating patient-specific tumours and have thus added the following sentence into the discussion. (p15; Line 586):

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"Establishing with certainty that the PDOs originate from tumor tissue is a strong determinant of the procedure and serves to guide pre-clinical drug regimens that will exploit the oncogenic mutation of the tumor. Until the successful incorporation of a cellular niche, with high-fidelity mutational screening efforts, a contribution of normal urothelial cells will confound investigations into drug resistance. Thus, this platform cannot be used for those studies. Therefore, this protocol instead serves as a rapid, pre-clinical platform alongside validation studies and other screens such as prognostic marker expression."

Comment
The success rate is said to be around 70%, it would be good to know how many samples have been collected and how many samples actually were able to sustain long-term culture. This would help in assessing the efficacy of the protocol.

Response
We thank the reviewer for their suggestion. Tumour specimens from 26 BC patients have been collected to date. Given an unmet clinical need for rapid therapeutic testing we do not attempt to sustain long term culture and instead have prioritised patient drug testing and characterisation of these organoids.

Comment
The title talks about 'pharmacologic response', however the protocol does not say anything about assessing this in cancer organoids, nor whether it is actually successful in these organoids. Based on previous organoid research it will probably be possible (after the organoids have actually been verified to represent the parental tumor), but since it is not the focal point of the article perhaps the title should be slightly altered.

Response
We appreciate the reviewer's comment. The focal point of this protocol is to generate human primary bladder cancer organoids to enable evaluation of drug responses. In order to highlight the powerful use of bladder cancer organoids as a precision medicine tool, we think this title is appropriate.

Reviewer #2:

Comment
The engraftment of PDO from fresh tumors is the interest of the entire scientific community working on cancer. Regarding primary bladder cancer, the authors need to validate the model with clear read outs of the used protocol. Histological, genomic and molecular characterization of the model compared to origin tumor is mandatory. The authors must demonstrate that the PDO recapitulate the morphological architecture and genomic alterations of primary bladder cancer of origin according to others studies in the field. The authors might refer to Fusco et al., 2019 for the characterization of the model. In this paper, the authors followed a specific workflow to characterize the PDO model established from another tumor, Neuroblastoma. Without the experimental demonstration that PDO recapitulate the in vivo tumor the manuscript is weak. One of the major challenges in the field is the reliability with which a specific tumor can be used to generate an organoid.

Response
We thank the reviewer for raising this point. We agree that this in-depth characterisation – such as that performed by Fusco *et al* - would strengthen the overall manuscript, however, complete genomic and molecular characterisation of the model is outside the scope of the technique described in the JoVE methodology paper. We appreciate this is a vital aspect to organoid generation though and have included a histological image of parental patient tumour and matched organoid (see amended **Figure 3** and an additional **Figure 4**). Further (epi)genomic, immunohistochemical and molecular characterisation is

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being included as part of a large collaborative study assessing urothelial carcinoma personalised medicine (Thomas, *unpublished*). We have also included Fusco *et al*., 2019 as a reference in the main manuscript and would like to thank the reviewer for pointing us in the direction of this body of work.

Comment
In addition, the authors should show the PDOs stemness capability as peculiar feature for the successfully engraftment of model from fresh tumor biopsies.

Response
Again, we would like to thank the reviewer for this suggestion. While the presence of pluripotent stem cells or organ progenitors is a hallmark of the cellular diversity of organoids, no reliable urothelial stem cell markers are currently known. Our future work aims to isolate cells which express CD44 and CD49, which are described as putative urothelial stem cell markers. We have added the below comment to the manuscript: (p15; Line 583):

"As organoids are defined as self-organising structures with the ability to self-perpetuate their growth, further work will be required to elucidate these discrete cancer stem-cells (such as CD44 and CD49) which may allow for improved in vitro growth characteristics[31-33]"

Additionally, we have recently performed preliminary quantitative real-time PCR analysis to confirm the expression of *CD44* gene expression in bladder cancer biopsy tissues (**Figure 1)**. Future studies will explore immunohistochemical and flow cytometry expression of CD44 and other putative cancer stem cell markers such as CD49, OCT4 and SOX4.

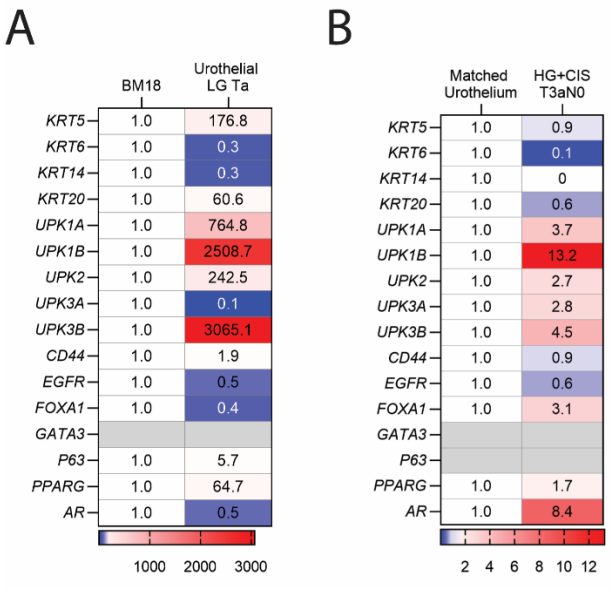

Figure 1. Tissues collected represent urological tissue. **(A)** Heat map of BLCA specific gene expression in BM18 prostate cancer patient-derived xenograft (PDX) compared against low-grade urothelial cancer (stage Ta). Each column shows the relative expression of a single gene. Fold-enrichment of each gene normalized to *RSP23* and compared to prostate cancer BM18 PDX. Fold-changes were log2 transformed and are displayed in the heat map as the relative expression. (n=1, performed with three technical replicates). **(B)** Heat map of BLCA specific gene expression in high-grade (HG) carcinoma in situ (CIS) urothelial carcinoma (stage T3aN0) compared against matched adjacent normal

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urothelium. Fold-changes were expressed as above and compared against normal urothelium. (n=1, performed with three technical replicates).

Comment
In the point 24 of Section 3: Generation of Bladder Tumor Organoids, the authors indicate in NOTE: "Seeding density must be high for successful organoid propagation". What is the minimum quantity to get efficient PDO engraftment? Have been the authors performed set up experiments to identify the ideal quantity of cells from fresh tumor samples? A limit for the PDO engraftment is often the small amount of tumor material derived from patients. To overcome the limit being able to engraft PDO from small quantity of material is sure the biggest challenge in this field.

Response
We thank the reviewer for this comment. We agree that a paramount challenge in the field is reproducible PDO generation from limited amounts of tissue. This is particularly evident in prostate cancer for example, where often only small cores can be provided surplus to pathology testing. By contrast, bladder cancer specimens are typically rather large and thus material is not rate limiting. We have explored the PDO generation success rate from as minimal input as 50 mg of tissue, we find that the most significant feature regarding the success of the organoid culture is the significant heterogeneity between samples and careful preparation of the specimen.

Comment
In the point 26 of Section 3: Generation of Bladder Tumor Organoids the authors indicate "Add 1:3 of 1X organoid medium media on top of Matrigel cell suspension". It is not clear the ratio 1:3 what it refers to! Please explain.

Response
We thank the reviewer for this comment. We have since amended the manuscript for clarity. (p10; Line 425, section 3.28)

"**3.28.** Add 1:3 ratio of 1X organoid medium media on top of BME cell suspension depending on the empirically assessed volume by the user. In the relevant starting point of 100 µL reconstituted cells/ BME mixture, add 50 µL of 1X organoid medium media on top.

Comment
The study would benefit from sharpening the conclusion on the powerful tool established considering the total absence of functional characterization of the system.

Response
We thank the reviewer for their comment and have revised the text to provide a more powerful rationale for this method. (p15; Line 601):

"Additionally, the specimen collection procedure and dissociation method described herein allow concurrent preparations for of tumor spatial profiling, multiparametric flow cytometry and single-cell RNA sequencing. Taken together, this allows for a diverse range of highly powerful endpoints which can be brought together to explore a patient's tumor heterogeneity and applicability of drugs such as immunotherapies.

And (p15; Line 607, 608, and Line 613):

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In summary, we describe a protocol to establish and evaluate cultures of 3D patient-derived bladder cancer organoids in the advent of precision medicine endpoints. It is important to note that the steps and notes described here are routine approaches used to isolate bladder cancer PDOs in our laboratory. Culture medium used in our procedure was originally described for prostate cancer cells and has now been proven to be suitable for the culture of bladder cancer. Importantly, this protocol is centralized, suitable for routine use in laboratories and broadly applicable across urological cancer types. In combination with high fidelity molecular phenotyping and drug response endpoints, this technique will provide a useful tool to rapidly explore precise therapeutic management of bladder cancer patients."

Yours, Sincerely

Elizabeth Williams

Associate Professor Elizabeth Williams
Group Leader – Queensland Bladder Cancer Initiative
Research Director, Centre of Personalised Analysis of Cancers (CPAC)

Clinical tissue processing

Date: _____

Processing by: _____

Codename: _____

Time collected: ____ ☐ am ☐ pm

Insert picture of specimen(s) here

Tissue descriptions

Tissue type	Weight	Sample description *(appearance, abnormalities, size, colour, necrosis)*
_____ TURBT: ☐ Cystectomy: ☐		

Gravity wash in 37°C basal media to remove dead cells. Tare empty 50 mL falcon and weigh (g or mg)

Other specimen descriptions

Specimen	# samples	Total volume	# Stored -80°C	Comments on sample
Blood (EDTA)			See PBMC isolation protocol	1mL Plasma taken ☐ _____
Serum (SST)				
Urine (whole)				

Whole urine	☐	
Urine sediment pellet	☐	
PreservCyte (cytology)	☐	Volume: mL urine: : Volume: mL PreservCyte (1: 3 ratio)
Urine cytospin	☐	
Urine agarose pellet	☐	

As per standard specimen processing protocols. Any modifications please outline in general comments

PBMC collection

Sample	Viability	Cell number	# of cryovials @ $1x10^7$	Comments
PBMC				
TILs				

Sample storage location: _____

Time: _____ ☐ am ☐ pm

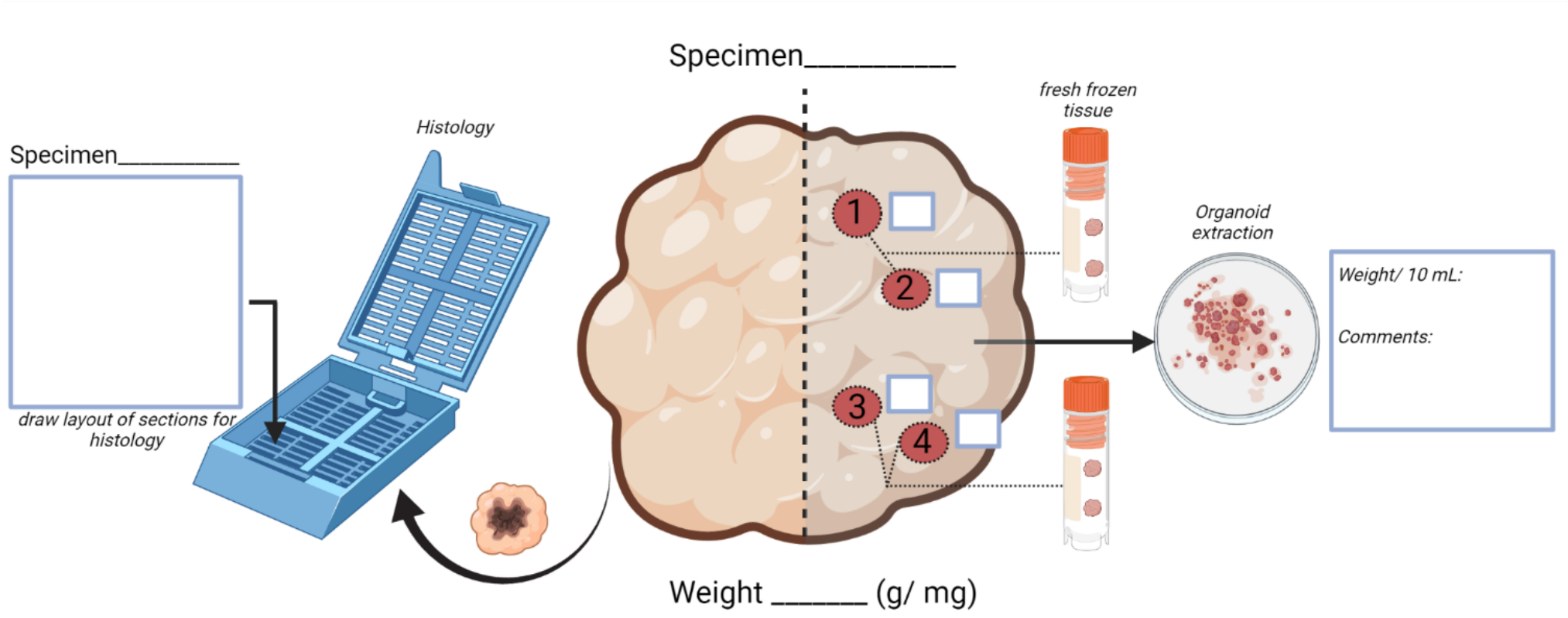
Cell clusters visible following extraction? Yes ☐ No ☐

Insert image of time = 0 of tumour organoids

General comments:

_____

_____


Specimen_____
Histology
Specimen_____
draw layout of sections for histology
fresh frozen tissue
1
2
3
4
Organoid extraction
Weight/ 10 mL:
Comments:
Weight _____ (g/ mg)