Queensland University of Technology

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Editorial Board
JoVE

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.
3. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: Matrigel, Eppendorf, Falcon, CryoStor CS10 Cell Freezing Medium, etc.
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.
5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.
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.

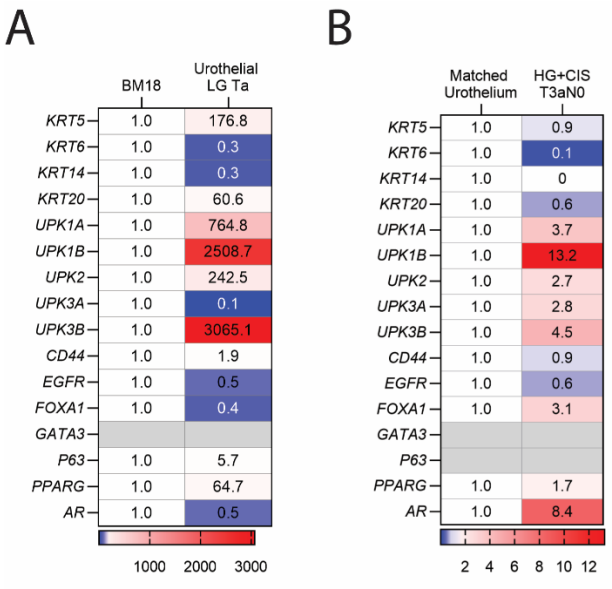

Figure 1. Tissues collected represent urological tissue. **(A)** Heat map of BLCA specific gene expression in BM18 prostate cancer patient-derived xenograft (PDX) compared against low-grade urothelial cancer (stage Ta). Each column shows the relative expression of a single gene. Fold-enrichment of each 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)