**MANUSCRIPT JoVE58569**

**“Establishment and analysis of tumor slice explants as a prerequisite for diagnostic testing”**

*Below, we address all editorial and reviewers’ comments. Text edits in the Manuscript Word document are indicated in track changes. Additional changes to the Manuscript text and Figures are summarized on page 10-11 of this Rebuttal Letter.*

**EDITORIAL COMMENTS:**

Changes to be made by the Author(s) regarding the written manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

*We have carefully gone through the written manuscript, and corrected minor spelling or grammatical errors as indicated in track changes.*

2. Please print and sign the attached Author License Agreement - UK. Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

*The editorial board’s e-mail pertinent to the revision of our manuscript did not contain an Author License Agreement document. However, we had uploaded a signed copy of the ALA during our first submission, and will enclose this with our revised Manuscript.*

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

*Although the originally submitted version of* ***Figure 1D*** *was adapted from Närhi et al., 2017, we have modified this figure by replacing ‘Histological sections’ with ‘FFPE sections’, ‘IHC detection of biomarkers’ with ‘IHC analyses’, and by changing the orange fillings to blue. In addition, we removed the following text in the* ***Figure Legend****, on* ***page 11****: ‘Panel (D) is adapted from our recent publication 23’. A copyright permission is therefore not required. Furthermore, in the revised Figure1, we added a new Figure panel, hence the Figure 1D is now represented as Figure 1E.*

4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

*We corrected the SI abbreviations as follows:* ***Protocol sections 1 and 4.2****, on* ***page 1 and page 7, respectively****: ml replaced with mL;* ***Protocol section 7.2.3****, on* ***page 10****: sec replaced with s.*

5. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

*We added spaces to separate the degree centigrade symbols at the following places:* ***Protocol sections 3.6.1,******5.1,*** *and* ***6.4*** *on* ***page 6, 7 and 9****.*

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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 and Reagents. For example: Leica VT1200 S, falcon, KOS, MathWorks, Inc., MATLAB, Pannoramic, Adobe Photoshop, CellProfilerTM, etc.

*To address this point, we made the following changes:*

* ***Introduction*** *paragraph 3 on* ***page 2****: replaced ‘ The Leica VT1200 S’, with ‘A Leica’*
* ***Protocol section 4.1*** *on* ***page 7****: NVP-BEZ235, and AZD6244 were removed*
* ***Protocol section 7.1.1*** *on* ***page 9****: ‘The MathWorks, Inc. USA’ was removed*
* ***Protocol sections 7.1. and 7.3,*** *and* ***Note*** *on* ***page 9,*** *and* ***10:*** *‘Pannoramic 250 3DHISTECH*’ *was removed*
* ***Protocol section 7.3*** *on* ***page 10:*** *‘Pannoramic Viewer 3DHISTECH*’ *was removed*
* ***Protocol section 7.3.1*** *on* ***page 10:*** *‘Pannoramic Viewer 3DHISTECH*’ *was removed*
* ***Protocol section 7.3.1*** *on* ***page 10****: replaced ‘ to CellProfilerTM (http://cellprofiler.org) 2.0.0’ with ‘for image analysis’*
* ***Protocol section 7.3.2*** *on* ***page 10:*** *replaced ‘CellProfiler’ with ‘image’*

***Table of Materials******and Reagent****:**company names and hyperlinks to the following software packages were added: MATLAB, 3D HISSTECH Pannoramic Viewer, Adobe Photoshop, Fiji-ImageJ and CellProfiler. Furthermore, updates to Leica VT1200 S and KOS are as follows: replaced ‘Vibratome’ with ‘Leica VT1200 S vibrating blade microtome’, ‘Leica’ with ‘Leica Biosystems’, and ‘KOS Microwave histo STATION’ with ‘KOS The microwave multifunctional tissue processor’, ‘Milestone’ with ‘Milestone SRL’. In addition, the company and catalog numbers for the 50 mL falcon tubes, Single-use needles for special applications, Sterican®, and PT module have been added, ‘F12 medium’ has been replace with ‘Ham’s F-12 medium’. Finally, ‘Life Technologies’ has been replaced with ‘Thermo Fischer Scientific’. All changes are indicated in red.*

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

*In* ***section 4.1*** *on* ***page 7,*** *“we tested” was removed**and the sentence was modified as follows: “In this case, 0.1-1 µM of the PI3K/mTOR inhibitor NVP-BEZ235 (dactolisib), and 0.05-0.5 µM of the MEK inhibitor AZD6244 (selumetinib) were tested on murine NSCLC slices. In addition, removed the following sentence from protocol* ***section 7.1*** *on* ***page 9****: “in our case, slides were scanned”*

8. Please revise the protocol to contain only action items that direct the reader to do something. 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.”

*We confirm that our protocol section is written in the imperative tense and descriptive/non-action verbs such as “may interfere”, “may alter”, or “can be” have been used only in the “Notes”.*

9. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

*We added more details to the protocol* ***section******3.5, Note of 3.4 and 5.1, 5.3****,* ***5.4,*** *and* ***6.3*** *on* ***page 6, page 7*** *and* ***page 8****. We believe that all other steps in the protocol are described as detailed as possible, and sufficiently supplement the actions to be depicted in the video.*

10. 1.2: Please mention what disinfectant is used.

*We replaced disinfectant with 70% EtOH on* ***page 4****, protocol* ***section 1.2****.*

11. 2.2: Please describe how to harvest the tumor-bearing lungs.

*This point has been addressed as follows: “Stretch the euthanized mouse onto a styrofoam lid by inserting 30 G needles in all four paws, so that the chest is exposed. Cut open the skin from the abdomen towards the chest, and up to the neck region. Cut open the rib cage, and then the diaphragm to expose the lung and heart. Keep the scissors in an angled position to avoid tissue damage”.*

12. 6.3: Please describe how H&E staining is done. Alternatively, add references that specify how to perform the protocol action.

*Details of the H&E staining have been described in the* ***protocol sections 6.4.1 - 6.4.3*** *of the revised Manuscript Text on* ***page 9****.*

13. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

*To address this point we divided some of the steps into several sub-steps as follows:*

* *The contents of section 3.6 is now divided between sections 3.6 and 3.6.1 on* ***page 6***
* *Section 7.1 has now been subdivided into sections 7.1.1 and 7.1.2 on* ***page 9***
* *Section 7.2 has now been subdivided into three sections 7.2.1, 7.2.2, and 7.2.3 on* ***page 10***
* *Section 7.3 has now been subdivided into two sections 7.3.1 and 7.3.2 on* ***page 10***

14. Please reference Figure 1 (workflow) in the Protocol.

*We now refer to* ***Figure 1*** *in the following sections of the protocol:*

***Figure 1A iii:*** *“Note” under section 3.2 on* ***page 5***

***Figure 1B ii:*** *section 3.5 on* ***page 6***

***Figure 1C i-ii:*** *section 3.6.1 on* ***page 6***

***Figure 1C i:*** *“Note” under section 3.6.1 on* ***page 6***

***Figure 1D:*** *section 5.1 on* ***page 7***

***Figure 1E:*** *section 6.3 on* ***page 8***

15. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

*We have highlighted the essential steps of the protocol for the video on* ***pages 4-8****.*

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

*We highlighted the essential steps and sub-steps on* ***page******4-8****.*

17. References: Please do not abbreviate journal titles.

*This has been addressed in the revised Manuscript Text.*

18. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.

*We added the lot numbers of used antibodies to the Table. We do not have RRIDs for the antibodies used in this protocol.*

**REVIEWERS' COMMENTS**  
  
**Reviewer #1:**Manuscript Summary:  
The manuscript provides a detailed and practical protocol for the culturing of tumour slice explants and their potential use for diagnostic testing. The tumour used in the protocol is murine non-small cell lung cancer. Of particular interest is the improvement of tumour viability by using a rotation incubation unit. Of further interest and relevance to any researchers wishing to use this culture system, is the testing of MAPK and mTOR pathway activity. The paper is well written and provides relevant and concise information regarding the potential use of this system as well as its development by other groups.

*We thank the reviewer for providing constructive feedback to improve our manuscript.*

Minor Concerns:

1. The authors may wish to comment on the use of the culture medium in their experiment.

*Tissue slice culture medium can vary depending on the tumor tissue as described in our previous study (Davies et al., 2015). In this protocol Ham’s-F12 medium was used for culturing murine NSCLC slices, as described in the protocol* ***section 1.4*** *on* ***page 4*** *and**in* ***Table of Materials and Reagents****. To address this point, we added a sentence in the* ***Note*** *under the* ***section 1.5*** *on* ***page 4****: “****Note****: Tumor slice culture medium and growth factor supplements can vary depending on the tumor tissue, as reported in (Davies et al., 2015)”.*

2. Figure 1: Photographs Aiii and Bi would be more informative, if taken at a higher magnification.

*To better capture the details, we have cropped previous* ***Figure 1A iii*** *and* ***B i*** *with the maximum resolution****.***

3. Tumour differentiation: did the grade of tumour differentiation or morphological phenotype of the tumour change during culturing?

*Quantitative IHC analysis of the NKX2-1 marker of well-differentiated lung adenocarcinoma (AC) showed that it was not significantly altered in cultured AC slices (up to 72 h) compared to 0 h uncultured slices (Figure 2A-B). This suggests that the differentiation status of AC histopathology tissue is not altered during this cultivation time period, as described in the* ***Representative Results*** *section on* ***page 11****:*  “*Results show that NKX2-1 expression is not significantly altered in cultured slices as compared to 0 h uncultured slices, suggesting that the process of tissue slicing and cultivation does not overtly affect the differentiation status of AC tumor tissue*”

*Furthermore, our recently published study on murine NSCLC tissue slices showed tumor histopathology subtype-dependent proliferative changes during 24-48 h cultivation, analyzed by Ki67 IHC (Närhi et al., 2018, Figure S3C). This may indicate that tissues undergo a wounding response that affects culture-induced proliferative changes, and this may affect accurate grading of the cultured samples. No gross morphological changes were visible in the cultured slices compared to 0 h samples.*

*To address this point we added a sentence in the fourth paragraph of the* ***Discussion*** *on* ***page 14*** *as follows: “Although gross morphological features of the murine NSCLC tumors were maintained during 72 h cultivation, culture-induced proliferative changes may affect accurate grading of the cultivated slices”*.

4. Figure 2B: there should be at least one picture at high magnification (e.g. insert) to allow the readers to view the quality of the immunostaining and appreciate the nuclear localisation of the staining.

*We have added higher magnification insets to each of the images shown in* ***Figure 2B****, and updated the corresponding Legend on* ***page 12****.*

5. Figure 2D: in the figure legend the scale bar is said to represent 1 micron - this must be incorrect, as the magnification is not much dissimilar from that in figure 2B.

*Snapshots of the low magnification images in Figures 2B and 2D are taken at slightly different magnifications, namely 3x or 2x using the 3DHISTECH Pannoramic Viewer. Although they may appear to be the same, the length of the scale bars are in fact slightly different, and the indicated scale bars are indeed correct.*

6. Testing with p4EBP1 and pERK1/2: how many different tumours were analysed? Where the findings quantified in any way?

*In the current manuscript, p4EBP1 and pERK1/2 analysis (****Figure 2D****) on dactolisib or selumetinib treated slices were done from one tumor. However, similar analyses were done on slices derived from multiple tumors in our published article Närhi et al, in Figure S8.*

**Reviewer #2:**   
  
Manuscript Summary:

The authors present a sophisticated method to cultivate tumor slices in vitro. This is an important aspect in precision medicine and preclinical drug testing and will even gain more importance in the near future. Thus the described protocol is timely and in my opinion will reach a broad readership.  
However the manuscript suffers from some inconsistencies and lack of critical explanations necessary for the complete understanding of the method.

*We thank the reviewer for the encouraging feedback and comments to improve our manuscript.*

Minor Concerns:

1.) Page 4, Chapter1:

2.) Numbering is wrong: 1.1 is wrongly labeled 1.

3.) 1.3 is missing. Please correct.

*Thanks for pointing this out; we have corrected this in the revised Manuscript Text.*

4.) 1.6: why is FBS omitted for the drug treatment? Please clarify? Is serum free medium used?

*To evaluate whether the addition of serum growth factors affects oncogenic signaling activities in cultured tumor slices, thus potentially compromising the ability of slices to mimic signaling networks active in the in situ tissue, we initially compared phosphoproteins marking signaling activities in murine NSCLC slices cultivated for 24 h in medium supplemented with 10% FBS, 10% autologous mouse serum, or without serum (Närhi et al., 2018, Figure S6B). This showed that pAKT and pERK1/2 levels were modestly increased and p4EBP1 and pSRC levels were significantly increased during cultivation, but this was independent on the inclusion or omission of serum. Since it cannot be ruled out that superfluous serum can affect pathway activities not analyzed, and perturbations were only done for 24h, we hence decided to perform short-term drug perturbations using serum free medium, to avoid potential serum effects on signaling pathway inhibitory responses.*

*To address the above, we added a sentence in the* ***Note*** *under the* ***section 1.5*** *on* ***page 4****: “Since growth factors in the serum may affect oncogenic signaling in cultured slices, serum-free medium is recommended for short-term drug perturbation studies on tumor slices. If longer-term slice cultures are analyzed, it is important to first evaluate the effect of serum-free medium on tissue viability and tumor-specific marker expression in untreated slice cultures”.*

5.) Page 5, chapter 3.2: why is the remaining lung tissue glued to the tumor? How? Please clarify the entire step. Optimal would be a small drawing of how to attach the tissue. The gluing of normal tissue also contradicts some statements made in the discussion. Please clarify and optimize.

*To clarify this point, we added following sentences:*

* *i)* ***Note*** *of* ***section 3.1*** *on* ***page 5****, “such that a cleared tumor region faces the vibratome blade.”*
* *ii)* ***Note*** *of* ***section 3.2*** *on* ***page 5***: “*The normal lung tissue glued to the specimen holder does not interfere with tumor tissue slicing, and slicing is stopped before the normal tissue is reached. The tumor tissue sometimes bends due to the spongy texture of the normal lung tissue glued to the specimen holder, compromising its upright position. If this happens, glue a piece of additional normal lung support tissue next to the pre-mounted normal lung tissue to retain the tumor in an upright position* ***(Figure 1A iii)****.*

*In addition, we added a cartoon in* ***Figure 1A iii*** *to describe the steps explained in* ***section 3****, and updated the corresponding Legend on* ***page 11****: “The red arrow points at an additional piece of normal lung support tissue to retain the tumor in an upright position”.*

6.) Page 6, chapter 3.6 note: please show the right positioning of the slices, and also indicate what a wrong positioning would look like

*To address this point, we indicated the incorrect and correct positioning of a slice as “X” and “✔”, respectively in* ***Figure 1C i,*** and updated the Legend on ***page 12*** as follows: “*X: indicates incorrect, and ✔: indicates correct positioning of the slice”.*

7.) 3.7: add a sentence that the 0h slices are immediately fixed and proceesed as described in 5.1

*Thanks for the suggestion; this sentence has been added to* ***section 3.7*** *on* ***page 7****.*

8.) Page 7, chapter 5.1: Is hematoxylin added undiluted? Please describe more precisely, add a photograph of how a stained slice would look like

*Hematoxylin added in* ***step 5.1*** *is diluted with deionized water (1:1). This point has been added in the* ***section 5.1*** *on* ***page 7****, as follows: “diluted Hematoxylin (1:1 in deionized water)”. In the revised* ***Figure 1D****, we added a photograph of a FFPE block of a hematoxylin stained tumor slice for visualization. Furthermore, we updated the* ***Figure Legend*** *on* ***page 12*** *as follows: (D) Photograph of the FFPE block of a tumor slice. Black arrow points at paraffin-embedded tissue slice stained with hematoxylin.*

9.) 5.3: What happens to the filter paper after tissue processing? Is the paper also embedded in the paraffin block? Please explain in more detail

*During tissue processing, the tissue slice is carefully lifted from the filter paper and transferred onto a mold for paraffin embedding, and the filter paper is discarded. To clarify this point we added a sentence to* ***section 5.4*** *on* ***page 8****: “For paraffin-embedding, open a histocassette and use a scalpel to carefully lift the slice from the filter paper. Discard the filter paper, and transfer the slice into a mold containing liquid paraffin”.*

10.) Page 8, chapter 7.1: H&E staining is not explained how its done. Please add a section before the IHC protocol; please rephrase the sentence "Necrosis may not always mark viability…." This does not make a lot of sense to me and should be improved. I am not sure what the authors intend to state. Also define better what "nearest" 0h slice means.

*We now describe the protocol for H&E staining on* ***page 9*** *in* ***section 6.4*** *as follows:*

*6.4.1) For H&E staining, deparaffinize and rehydrate the paraffin sections as follows: xylene 3 x 5 min, 100% EtOH 3 x 1 min, 96% EtOH 2 x 1 min, 70% EtOH 1x 1min, and deionized water 2 x 1 min.*

*6.4.2) Incubate the sections in freshly filtered hematoxylin solution for 10 min, and wash under running tap water for 5 min. Dip the sections in acid alcohol (1% HCl in 70% EtOH) for 2 times, and wash under running tap water for 5 min followed by incubation with 0.5% eosin for 2 min.*

*6.4.3) Following the eosin step, dehydrate the sections by immersing the slides in alcohol and xylene solutions, as follows: 96% EtOH 2 x 15 s, 100% EtOH 3 x 30 s, xylene 3 x 1 min. Finally, embed the sections in pertex, a xylene-based mounting medium.*

*We agree that the sentence "Necrosis may not always mark viability…." was unclear. This was to highlight that necrosis was the viability readout in murine NSCLC slices; slices derived from other tissue types may not show necrosis despite decreased viability, as shown in Davies et al. To address this, we modified the related sentence on* ***page 9*** *under the* ***Note*** *of* ***section 7.1.2*** *as follows: “While the mere cultivation of murine NSCLC slices induces necrotic cell death, biological responses to ex vivo culture conditions vary depending on the tumor tissue”.*

*The nearest 0 h slice is the one that is spatially the closest to the cultivated sample. If multiple treatments are done on the slices derived from a single tumor for example, vehicle control, single compound treatments and a combination treatment, this causes number of slices to be limited so that comparison between neighboring 0 h, and treated sample (200 µm apart) cannot necessarily be performed. In such cases, the slice which is 400-600 µm apart can be considered as nearest 0 h slice. This point has been clarified in* ***section 3.7*** *under the* ***Note*** *on* ***page 7****, as follows: “If the number of slices is limited, e.g. if multiple compound treatments or technical replicates are done, comparisons of each treated sample with its neighboring 0 h sample can be difficult. In such cases, use the nearest 0 h slice (at least 400-600 µm apart) to assess relative tissue viability or expression of relevant markers at culture onset”.*

11.) Ambient temperature: please indicate exact value in °C.

*The ambient temperature in our laboratory ranges from 21-23*°*C. This has been included in* ***section 7.2.1*** *on* ***page 10****.­­*

12.) Figure 1A. Please rewrite Figure legend, there are some errors. E.g. White arrowhead is pointing to a peace of lung tumor in a tissue culture dish , not glued to the specimen holder as stated. Correct.

*Thanks for pointing this out. We corrected this in the revised* ***Figure 1A*** *by pointing the white arrowhead to the tissue that is glued to the specimen holder.*

13.) Figure 1B. ii) the labeling of the plate is not easy visible in the picture. Add a white outline to the font. Moreover the rationale for the labeling is not intuitive for ma. What does the label really mean. Why are there time points (0, 24, 48) and C and T. Are the C and T treated the same time. 24 h or 48 hrs. What is the difference between the upper row and the lower C? is C vehicle control and the upper untreated. This should be better visualized and described.

*The labelling in the top and bottom row of the* ***Figure 1B ii*** *indicates that slices can either be utilized for time point analysis or for drug treatment, respectively. To address this point, we added the following sentence to the* ***Figure Legend*** *on* ***page 11****: “The slices can either be cultured for different time points (here, 24 - 72 h) to assess tumor-specific marker expression during cultivation (top row), or can be used to perform drug treatments. C: vehicle control, T: drug treatment”. In addition, we updated the* ***section 3.5*** *on* ***page 6*** *as follows: “Mark each well of the 24-well plate according to the experimental plan. For example, mark sequential wells of a 24-well plate as culture time points, or as 0 h, vehicle control (C), drug-treated (T) (****Figure 1B ii****)”.*

14.) Figure 2. A. What does T1, T2 mean? Make this more clear. What are the dark blue-grey areas? Please indicate.

*T1 and T2 in Figure 2A indicate biological replicates derived from two different tumors. We have added this to the* ***Figure Legend*** *on* ***page 12 of*** *our revised Manuscript. The dark blue-grey areas represent the tissue excluded from the analysis due to poor tissue quality or because of fibrotic stroma. This is indicated in the* ***Figure Legend*** *on* ***page 12:*** *“Dark blue represents H&E stained viable tissue, and pink indicates pseudocolored necrotic regions. Light blue indicates regions excluded from the analysis, either due to poor tissue quality or presence of fibrous stroma”.*

15.) Figure 2.D. Increase the size of the magnified insets at least by a factor of 2. Place it next to the overview. There is enough space. The histology is otherwise not nicely visible. Show also the 0h untreated controls.

*Thanks for suggesting this; Figure 2D has been updated by adding the 0 h samples and by increasing the size of the magnified insets. The corresponding* ***Figure Legend*** *has been updated on* ***page 12*** *as follows: “Representative IHC images of phosphorylated 4EBP1 or pERK1/2 expression in 0 h slices, or slices treated with DMSO or titrated amounts of dactolisib (dact, top row) or selumetinib (sel, bottom row). Black square boxes indicate areas shown in higher magnification. Scale bar 1 mm or 50 µm for low or high magnification, respectively”.*

*----------------------------------------------------------------------------------------------------------------------------*

**ADDITIONAL CHANGES MADE IN THE REVISED MANUSCRIPT:**

* The following changes were made in the **Abstract** on **page 1**:
  + Added “rotating” in the following sentence: “Our previous work showed that the use of **rotating** incubation units improves the….”.
  + replaced ‘useful with ‘valuable’
* Modified the following sentences in the **6th paragraph** of the **Introduction** on **page 3**: “Furthermore, immunohistochemistry (IHC) analyses on cultured slices revealed intra-slice viability gradients, ~~evidenced as viability gradients~~ detected as necrosis gradients in slices derived from murine non-small cell lung cancer (NSCLC), ~~and~~ estrogen receptor (ER), HIF1α and γH2AX gradients in breast cancer slices, or androgen receptor (AR) expression gradients in prostate cancer slices~~,~~ ~~or oxygenation gradients measured by HIF1α in breast cancer slices~~25”.
* In the **6th paragraph** of the **Introduction** on **page 3**, replaced “air-exposed” with “top” in the following sentence: “Particularly the ~~air-exposed~~ top side remained most viable…..”
* Added hyperlink to the vibratome manual in the protocol **section 1.2** on **page 4**.
* Updated the slicing speed to 0.1-0.14 ms in the protocol **section 3.4** on **page 6.**
* Added a hyperlink to user manual for the KOS microwave station in the protocol **section** **5.3** on **page** **8**.
* Modified some sentences in the protocol section as follows:
  + Section **7.1** on **page 9**: ~~in our case~~, slides were scanned using~~the Pannoramic 250 3DHISTECH~~ a scanner
  + Section **7.3** on **page 10**: Acquire whole slide scans of IHC-stained slides, ~~for example using a Pannoramic 250 3DHISTECH~~ ~~scanner~~, export them as TIFF images at a magnification ratio of 1:4 using an image viewer ~~the Pannoramic Viewer 3DHISTECH~~, and perform quantifications using ImageJ.
  + Section **7.2.2** on **page 10**: “or peroxidase goat anti-rat IgG (H+L)” has been removed.
* In the **Representative Results** section on **page 11** the following changes were made: “Results show that NKX2-1 expression is not significantly altered in cultured slices as compared to 0 h uncultured slices, suggesting that the process of ~~tissue slicing and~~ cultivation does not overtly affect the differentiation status of AC tumor tissue.
* In the 2nd paragraph of the **Discussion** on **page 13** we added “culture medium” and removed the word “culture” in the following sentence: “Furthermore, once sliced, it is critical that the slice is placed approximately in the middle of the grid, so to ensure accurate intermittent ~~culture~~ dipping in culture medium and oxygen exposure”.
* In the 3rd paragraph of the **Discussion** on **page 13,** the following changes were made: “We further showed that while proliferation or oncogenic phosphoprotein expression ~~is not affected~~ in freshly cut uncultured 0 h slices were similar ~~when compared to compared to~~ *in situ* tumors, ~~while~~ cultured slices showed altered expression of oncogenic phosphoprotein~~s~~ expression, specifically altered p4EBP1 and pSRC ~~expression~~,….”.
* In the 5th paragraph of the **Discussion** on **page 14,** the following changes were made: “in ~~the~~ murine NSCLC tissue slices, compromising accurate quantitation of spatial drug responses ~~so additional biomarkers should be identified for viability analyses.~~
* In **Figure 1A iii**, replaced “sample holder” with “specimen holder”.
* In **Figure 1C** replaced “rotator” with “rotating”.
* In **Figure 2D**, replaced dactolisib and selumetinib with dact and sel, respectively, and provided the abbreviation in the **Figure Legend** on **page 12**.
* A sentence in the **Figure Legend** on **page 11** has been modified as follows: “Placing the tissue slice for ~~rotator~~ cultivation using rotating incubation units”.
* A sentence in the **Figure Legend** on **page 12** has been modified as follows: “Schematics showing the sectioning order of ~~FFPE-fixed~~ the slices in FFPE blocks;…”.
* Added following names in the **Acknowledgements** on **page 15**: Meng Dong, Simon Barry, Wytske van Weerden and Hanneke van Zoggel, and Jouko Siro.
* Other minor corrections made to spelling and grammatical errors in the **Manuscript Text** are indicated in track changes.

**REFERENCES:**

Davies, E.J., Dong, M., Gutekunst, M., Narhi, K., van Zoggel, H.J., Blom, S., Nagaraj, A., Metsalu, T., Oswald, E., Erkens-Schulze, S.*, et al.* (2015). Capturing complex tumour biology in vitro: histological and molecular characterisation of precision cut slices. Sci Rep *5*, 17187.

Narhi, K., Nagaraj, A.S., Parri, E., Turkki, R., van Duijn, P.W., Hemmes, A., Lahtela, J., Uotinen, V., Mayranpaa, M.I., Salmenkivi, K.*, et al.* (2018). Spatial aspects of oncogenic signalling determine the response to combination therapy in slice explants from Kras-driven lung tumours. J Pathol *245*, 101-113.