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Corresponding Author:	Emmy Verschuren FINLAND
Corresponding Author's Institution:	
Corresponding Author E-Mail:	emmy.verschuren@helsinki.fi
Order of Authors:	Emmy W. Verschuren
	Ashwini S. Nagaraj
	Jie Bao
	Annabrita Hemmes
	Mafalda Machado
	Katja Närhi
Additional Information:	
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TITLE:

Establishment and Analysis of Tumor Slice Explants as a Prerequisite for Diagnostic Testing

AUTHORS AND AFFILIATIONS:

Ashwini S. Nagaraj[1], Jie Bao[1], Annabrita Hemmes[1], Mafalda Machado[1], Katja Närhi[1], and Emmy W. Verschuren[1]

[1] Institute for Molecular Medicine Finland (FIMM), HiLIFE, University of Helsinki, Helsinki, Finland.

Corresponding Author:
Emmy W. Verschuren
emmy.verschuren@helsinki.fi

Email Addresses of Co-authors**:**
Ashwini S. Nagaraj (ashwini.nagaraj@helsinki.fi)
Jie Bao (jie.bao@helsinki.fi)
Annabrita Hemmes (annabrita.schoonenberg@helsinki.fi)
Mafalda Machado (mafimac@hotmail.com)
Katja Närhi (katja.narhi@alumni.helsinki.fi)
Emmy W. Verschuren (emmy.verschuren@helsinki.fi)

KEYWORDS:

Organotypic explants; tissue slices; diagnostics; preclinical models; non-small cell lung cancer; rotating incubation unit; drug treatment; vibratome; KRAS; adenocarcinoma.

SUMMARY:

We provide a method for the generation, cultivation and systematic analysis of organotypic slices derived from murine lung tumors. We also describe how to optimize for slice thickness, and how to select drug concentrations to treat tumor slices.

ABSTRACT:

Organotypic primary tissue explant cultures, which include precision-cut slices, represent the three-dimensional (3-D) tissue architecture as well as the multicellular interactions of native tissue. Tissues slices immediately cut from freshly resected tumors preserve spatial aspects of intratumor heterogeneity, thus making them useful surrogates of *in vivo* biology. Careful optimization of tissue slice preparation and cultivation conditions is fundamental for the predictive diagnostic potential of tumor slice explants. In this regard, murine models are valuable, as these provide a consistent flow of tumor material to perform replicate and reproducible experiments. This protocol describes the culturing of murine lung tumor-derived tissue slices using a rotating incubation unit, a system that enables intermittent exposure of tissues to oxygen and nutrients. Our previous work showed that the use of rotating incubation units improves the viability of tissue compared to other culture methods, particularly floating slices and stagnant filter supports. Here, we further show that slice thickness influences the viability of cultured slices, suggesting that optimization of slice thickness should be done for

different tissue types. Pronounced ITH in relevant oncogenic functions, such as signaling activities, stromal cell infiltration or expression of differentiation markers, necessitates evaluation of adjacent tissue slices for the expression of markers altered by drug treatment or cultivation itself. In summary, this protocol describes the generation of murine lung tumor slices and their culture on a rotating incubation unit and demonstrates how slices should be systematically analyzed for the expression of heterogeneous tissue markers, as a prerequisite prior to drug response studies.

INTRODUCTION:

Solid tumor tissues, including lung cancer, exhibit genetic and phenotypic heterogeneity, and harbor complex microenvironments[1,2]. The interplay between tumor cells and their surrounding microenvironment influence on drug sensitivity and resistance mechanisms[3]. This highlights the need for preclinical models that can accurately model biological complexities and functions acting in native tumors. Precision-cut slices immediately derived from fresh tumors provide a unique resource, as they have a principal capacity to represent *in vivo* biology, at least for a short window of time, including phenotypes spatially distributed in an individual tumor. Resected clinical tumors are one of the few personalized specimens that can be obtained from a cancer patient, and their diagnostic use deserves scrutiny.

The history of organotypic cultures dates back to the early 19th century, when human intracranial tumors were hand-cut into tissue pieces and cultured using the so-called hanging drop method. Tissue fragments were attached to a coverslip and allowed to dip into heparinized human plasma, after which the coverslips were inverted, sealed and cultured for several weeks[4]. Manually cut tumor pieces have since been cultured using a variety of other methods, such as on plasma clots[5], in liquid media[6], or on 0.45 µm pore size filters[6]. The term "organotypic" was first used in 1954, in a study on retinal differentiation of the chick embryo eye[7]. This was followed by studies that used lung and heart tissue explants derived from chick embryos[8], and brain explants from adult rats[9].

Various slicing methods have been described, namely manual choppers[10,11], the Krumdieck tissue slicer[12,13], and vibratomes[11,14,15,16]. The Krumdieck tissue slicer generates cylindrical tissue cores, which are then sliced into circular tissue slices using a microtome. A vibratome, on the other hand, uses a vibrating blade microtome. In a study on liver slices, it was shown that the Leica vibratome generates more reproducible and consistent slices compared with the Krumdieck slicer[15]. Slice thicknesses ranging from 250 to 500 µm have been used, and studies report maintenance of viability and morphological features even until 16 days[14,10,17,18]. However, tumors have variable metabolic profiles that can affect nutrient requirements, and parameters such as tissue stiffness and matrix composition can influence on aeration and nutrient flow. It is therefore likely that each tissue type requires optimization of slicing and culture conditions.

Different cultivation methods have been used to support slice cultures: i) stationary interphase culture, also called stagnant support culture, in which slices are placed on top of a semi-porous membrane insert immersed in the culture medium. In this, the top of the slices is exposed to the air, while the bottom is supplemented with nutrients via the porous insert[14,19]; ii) the Trowel

method, originally developed to culture whole organs or embryonic tissue slices. In this, slices are placed on top of a cotton sheet or filter supported by a metal grid, and the filter is soaked in the culture medium. To keep the tissues moist, a thin layer of medium is added on top of the slices[20-22]. These first two are so called air-liquid interphase cultures; iii) roller tube cultures, in which slices are placed inside the flat side of a plastic tube containing medium, and slow tube rotation ensures that the tissue is covered with medium during the first part of a cycle, or aerated during the second[23]; iv) rotating incubation units, in which slices are intermittently exposed to medium with nutrients and aeration. Different from roller tubes, in this method slices are placed on top of porous titanium grids placed in 6-well plates with culture medium[24].

Tissue slices derived from resected solid tumors logically present an attractive *ex vivo* model in which to test the treatment response of anti-cancer agents, as they permit the evaluation of viability, targeted pathway activity, and molecular profiles of a specific tumor in the presence of its native tumor microenvironment. However, to evaluate whether the drug responses measured in tumor slices are predictive of *in situ* responses, it is important to assess to what extent tissue slices preserve tumor-specific biological functions such as cell proliferation, histopathology-specific cell differentiation or oncogenic signaling activities. The impact of mechanical stress elicited during slice preparation, slice handling, or culture-induced adaptions on both the quality and biological functions of tissue slices are fundamental questions, tightly linked to the ability to implement tumor-derived slices for functional diagnostics.

Our IMI-funded consortium project PREDECT (http://www.predect.eu) sets out to systematically address these fundamental questions, by studying slice explants from a variety of sources. Using slices derived from breast, prostate and lung cancer models, this joint effort utilized qualitative read-outs as well as quantitative hematoxylin and eosin (H&E) - based readouts to demonstrate a requirement for atmospheric oxygen and stagnant filter supports to sustain the viability of cultured slices until 72 h. Furthermore, immunohistochemistry (IHC) analyses on cultured slices revealed intra-slice viability gradients, evidenced as necrosis gradients in slices derived from murine non-small cell lung cancer (NSCLC), estrogen receptor (ER), HIF1α and γH2AX gradients in breast cancer slices, or androgen receptor (AR) expression gradients in prostate cancer slices[25]. Interestingly, intra-slice viability gradients in 24 h cultures of murine NSCLC were rescued by cultivation in a rotating incubation unit, and our recent study showed that viability was extended to 72 h[26]. Particularly the top side remained most viable[26], endorsing that drug response analyses on slices are best carried out on this side of the tissue.

Even though it remains a question as to how far tissue slices can recapitulate *in situ* tumor functions, they have been extensively used to test responses to anti-cancer agents, including targeted drugs, monoclonal antibodies and chemotherapy agents[10,11,13,14,18,27]. We recently showed that murine NSCLC slices show dynamic changes in proliferation and oncogenic signaling activities following cultivation when compared to freshly cut 0 h slices[26]. This indicates that it is important to investigate whether the targeted *in situ* biological functions are appropriately preserved during cultivation, prior to perturbation studies. Despite these findings, we showed that tumor slices can model spatial response to targeted therapies, midst drug treatments remain brief (24 h) and are initiated at the onset of culturing[26]. The following protocol describes

important validation aspects relevant to the establishment and analysis of tumor slice cultures, prior to their application in pharmacological drug testing.

PROTOCOL:

All mouse experiments described in this study were performed by following the guidelines from the Finnish National Board of Animal Experimentation, and were approved by the Experimental Animal Committee of the University of Helsinki and the State Provincial Office of Southern Finland (License number ESAVI/9752/04.10.07/2015).

1. Preparations Prior to Slicing

1.1. Keep the following materials ready: vibratome specimen holder, vibratome buffer tray, 10 cm culture plate, 24-well plate, 10 mL pipette, pipette boy, waste bag, 70% EtOH in a 50 mL tube to disinfect the instruments, and tissue glue.

1.2. Prepare the vibratome: wipe the blade holder with 70% EtOH, attach a new blade, and perform a vibrocheck according to instructions provided in the manual (http://photos.labwrench.com/equipmentManuals/10103-3895.pdf).

Note: It is important to perform the vibrocheck step, as it minimizes the vertical deflection of the blade and ensures good quality slices.

1.3. Fill each well of the 24-well plate with 1 mL of Hanks Balanced Salt Solution (HBSS) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (HBSS + P/S); keep the plate on ice.

1.4. Prepare F12 culture medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamax, 22 mM glucose, and 10% fetal bovine serum (FBS).

Note: Tumor slice culture medium and growth factor supplements can vary depending on the tumor tissue[25].

1.5. Prepare the required amount of F12 medium for drug treatment, using the same composition as described in step 1.4, but omitting the FBS.

Note: 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.

2. Collection of Tumor-Bearing Lungs

2.1. Euthanize a tumor-bearing mouse by cervical dislocation when it shows symptoms of

177 labored breathing and loss of body weight.
178
179 **Note:** CO_2-mediated euthanasia is known to induce hypoxic conditions in the lungs, which may
180 have an effect on slice viability or oncogenic activities via eliciting a hypoxic response.
181
182 2.2. Stretch the euthanized mouse onto a Styrofoam lid by inserting 30 G needles in all four
183 paws, so that the chest is exposed.
184
185 2.3. Cut open the skin from the abdomen towards the chest, and up to the neck region. Cut
186 open the rib cage, and then the diaphragm to expose the lung and heart. Keep the scissors in an
187 angled position to avoid tissue damage.
188
189 2.4. Dissect the tumor-bearing lungs together with the heart and place them into a 50 mL tube
190 containing 30 mL of ice-cold HBSS + P/S; keep the tube ice-cold and proceed to the next step as
191 quickly as possible.
192
193 **Note**: Delays in processing of lung tumors may alter oncogenic functions, for example signaling
194 pathway activities.
195
196 **3. Generation of Precision-Cut Lung Tumor Slices**
197
198 3.1. Transfer the tumor-bearing lungs in HBSS + P/S into a 10 cm tissue culture plate kept
199 inside a laminar hood. Separate the lung lobes using sterile scissors and forceps, and select lobes
200 with tumors on the surface for slicing.
201
202 **Note:** Tumors >3 mm in size are suitable for slicing. Since normal lung tissue surrounding the big
203 tumor compromises the slicing due to differences in tissue stiffness, tumor tissue undergoing
204 slicing should be separated away from the normal tissue, such that a cleared tumor region faces
205 the vibratome blade.
206
207 3.2. Generate a flat tissue piece surface by cutting part of the normal lung tissue that
208 surrounds the tumor away with a sterile scalpel, and dip the flat slide in a drop of cyanoacrylate
209 adhesive. Mount this side onto the vibratome's specimen holder so that the tumor faces the
210 blade in an upright position. Let the glue dry for 2-3 min.
211
212 **Note:** The normal lung tissue glued to the specimen holder does not interfere with tumor tissue
213 slicing, and slicing is stopped before the normal tissue is reached. The tumor tissue sometimes
214 bends due to the spongy texture of the normal lung tissue glued to the specimen holder,
215 compromising its upright position. If this happens, glue a piece of additional normal lung support
216 tissue next to the pre-mounted normal lung tissue to retain the tumor in an upright position
217 (**Figure 1A iii**).
218
219 3.3. Place the specimen holder into the metal buffer tray and fill it with cold HBSS + P/S until
220 the tissue is immersed in the buffer. Place the metal buffer tray onto the white ice bath and add

ice so to keep the tissue cool while slicing.

3.4. Attach the white ice bath to the vibratome. Select suitable slicing settings: amplitude ranging between 2.5-2.8, slicing speed between 0.10-0.14 ms, and a cutting thickness ranging between 160-250 µm.

Note: Slicing settings need to be adjusted according to the hardness of the tissue. Hard tissue is easier to slice than softer tissue, and softer tissues requires slicing with lower speed (0.1-0.12 ms) and higher amplitude (2.6-2.8). A 4-5 mm large tumor typically provides 15-20 slices of 200 µM thickness. For short-term cultivation, murine tumors can be sliced under semi-sterile conditions outside the laminar hood. However, clinical tumors should always be sliced inside a class II biosafety laminar hood to avoid exposure to possible infectious agents in the human tissue.

3.5. Using sterile forceps, collect the slices in 1 mL of HBSS in separate wells of a 24-well plate held on ice, closely keeping track of the order in which slices are sliced. 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**).

Note: Do not disturb or pull the tumor tissue while collecting a slice, as this will alter the tumor's orientation with respect to the angle of the blade, leading to inconsistencies in the thicknesses of the subsequent slices.

3.6. When all slices are collected, transfer them onto titanium grids (2-3 slices per grid) placed in a 6-well plate containing 2.5 mL of culture medium per well. Make sure that no air bubbles are formed between the titanium grid and the medium.

3.6.1. To load a slice on to the grid, keep the 6-well plate in an angled position so that a portion of the medium covers the grid, and place the slice in the medium on the grid; use forceps to spread the slice if it curls. Load the 6-well plates onto the rotating incubation unit placed inside a humidified incubator maintained at 37 °C with 95% air and 5% CO_2, and start the rotation cycle (**Figure 1C i-ii**).

Note: Metallic grids and the 6-well plates need to be accurately weight balanced before turning on the rotating unit. It is important to position the slices in the middle of the grid so that they fully alternate between the air and liquid phases during the rotation cycles. Slices that are placed too low or high are not appropriately exposed to oxygen or nutrients (**Figure 1C i**), which can compromise tissue viability. It is important to follow the position of the slice on the grid during cultivation, as the slice can occasionally slide down. If this happens within 1-2 h of culture onset, correct its position and note down that this sample may be damaged. Slices that are mispositioned for an extended period of time should be discarded, as tissue viability is significantly affected by improper oxygenation and nutrient supply.

3.7. Collect a tissue slice adjacent to the cultured slices as a 0 h, uncultured, reference. Collect

at least three reference slices to represent the top, middle and center of the tissue being sliced. Fix the 0 h slices immediately and process as described in 5.1.

Note: 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.

3.8. For long-term cultivation, replenish the culture medium every day. Lift the grid containing the tissue slices using sterile forceps, and place it in an empty well of the 6-well plate; replace 70% of the medium with fresh culture medium, and place the grid back in the medium. Continue the rotation cycle as explained in step 3.6.

4. Treatment of Tumor Slices with Small Molecule Inhibitors

4.1. Prepare the required concentrations of compounds in treatment medium. Typically, a 10-fold higher drug concentration compared to the IC50s measured in cell cultures is required to achieve target inhibition in tissue slices. To avoid unspecific cytotoxicity, test a range of concentrations to obtain minimally effective concentration for each compound. Here, we tested 0.1-1 µM of the PI3K/mTOR inhibitor dactolisib and 0.05-0.5 µM of the MEK inhibitor selumetinib on murine NSCLC slices.

4.2. Add 2.5 mL of media with the diluted drug or DMSO or other vehicle control into the 6-well plate. Place the titanium grids into the wells.

4.3. Place the tissue slices onto the grids as described in step 3.6.

4.4. Perform vehicle or drug treatments for 24 h. Proceed with tissue fixation and processing of the slices into paraffin blocks as described in section 5.

Note: Duration of the drug treatment can be optimized depending on objective of the experiment, taking into consideration the ability of tissue slices to retain *in situ* tissue functions analyzed during the culture period.

5. Fixation and Processing of the Tissue Slices

5.1. Carefully lift the uncultured 0 h reference or cultured slice onto a filter paper soaked in PBS.

5.1.1. To do this, add 2-3 mL of PBS in a 10 cm plate, let the slice float in PBS and 'fish' it out by lifting the slice onto the filter paper with a pair of forceps.

5.1.2. Transfer the filter paper into a histocassette, and add a drop of diluted hematoxylin (1:1 in deionized water) on top of the tissue slice to visibly mark the position of the slice during the

subsequent processing steps (**Figure 1D**).

5.1.3. Close the cassette, and transfer it into 4% neutral buffered formalin solution. Fix the tissues overnight at 4 °C.

Note: While placing the slice on top of the filter paper, make sure that the top section of the slice is facing upwards; this is necessary to follow the top, middle, and bottom section of a slice during sectioning and analysis as described in step 6.3.

5.2. The next day, transfer the cassettes into 70% EtOH, and immediately proceed with the paraffin-embedding tissue processing step.

5.3. Prior to tissue processing, wash the histocasettes in 100% EtOH 2x for 10 min each. In this case, use a microwave station for tissue processing. Select the program used for 1 mm tissue thickness and follow the instructions provided in the manual (https://www.totaltissuediagnostics.com/images/MM073-005_-_KOS__Operator_Manual.pdf).

Note: When using other tissue processing machines, use the program suitable for thin tissue samples.

5.4. 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.

5.4.1. Press the tissue against the bottom of the embedding mold, for instance with a flat weight, to ensure even sectioning. Place the bottom part of the histocasette on top of the mold, let the mold cool on a cold pate for 30 min, and separate the mold from the paraffin block.

Note: As an alternative to horizontal embedding, it is possible to embed the tumor slice vertically by positioning the slice in an upright position. Vertical sections readily permit analysis of gradients in viability or functional marker expression on a single section [25]. Gradient analysis with horizontally-embedded slices requires paraffin sectioning as described in step 6.2.

6. Processing and Analysis of Formalin-Fixed and Paraffin-Embedded (FFPE) Tissues

6.1. Prepare 4 µm thin sections of the FFPE tissue slice blocks using a microtome. When sectioning, adjust the angle of the block so that the surface of the block is horizontally oriented with respect to the blade; this is necessary to obtain even sections throughout the tissue.

6.2. To enable capture of a potential culture-induced viability gradient, cell migration across the slices, or gradients in biomarker expression across cultured slices, collect sections from the top, center and bottom layers of each of the tissue slice on object slides as explained below.

6.3. Collect sequential tissue sections of the paraffin-embedded tissue slice first on the upper

part of the glass slides. Continue collecting the sections of the deeper tissue layers to the middle followed by bottom part of the glass slides (**Figure 1E**).

Note: To accommodate three sections on a glass slide, trim away the excess of paraffin surrounding the embedded tissue slice.

6.4. Allow the sections to dry overnight at 37 °C, and proceed with H&E staining or immunohistochemistry as described below.

Note: Loss of antigenicity can occur when FFPE sections are stored at high temperatures or for extended periods of time. Paraffin sections are recommended to be stored at 4 °C, and IHC analyses should be carried out within 6 months after sectioning.

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 a xylene-based mounting medium.

7. Analysis of Tissue Viability and Biomarker Expression

7.1. Acquire high resolution images by generating whole slide scans of H&E-stained slides using a scanner. To assess tissue viability, take snapshots from the tissue scans representing the top, middle and bottom sections of the slices.

7.1.1. Using photo manipulator software, manually draw masks on the necrotic areas of the tissues, followed by quantification of necrotic regions using MATLAB.

7.1.2. Calculate the relative viability of the slices cultivated for different time points compared to the nearest 0 h slice as done in our previous study (Närhi *et al.,* **Figure S2B&C**)[26]. Similarly, assess potential intra-slice viability gradients by quantifying viability in the top, middle and bottom section of a cultured slice, and calculate the relative viability of each section to its closest 0 h slice.

Note: 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. For example, gradients in HIF1α, ER, and macrophages marked by F4/80 were detected in filter-supported slice cultures derived from a breast cancer model[25]. Therefore, H&E- as well as IHC-based analyses of

cultured slices need to be considered for each tissue type.

7.2. Perform IHC on the paraffin sections. The following IHC protocol is a starting point, and requires further optimization for other antibodies. Briefly, 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.

7.2.1. To expose the antigenic epitopes, perform heat-mediated antigen retrieval using 10 mM citric acid at pH 6 in a PT module, followed by blocking with 1% Bovine Serum Albumin (BSA) and 10% Normal Goat Serum (NGS) in 1 x PBS for 30 min at ambient temperature (21-23 °C).

7.2.2. Incubate the primary antibody diluted in 1% BSA and 5% NGS in 1x PBS, either for 1-2 h at ambient temperature. Incubate with anti-rabbit secondary antibody, for 30 min at ambient temperature, followed by detection using 3,3'-Diaminobenzidine (DAB).

7.2.3. Sections are counterstained with hematoxylin (diluted to 1:10 in deionized water) for 30 s, and wash under tap water for 5 min, followed by dehydration by immersing the slides in alcohol and xylene solutions, as follows: 70% EtOH 1 x, 96% EtOH 2 x, 100% EtOH 3 x, xylene 3 x (1 min each step). Finally, embed the sections in a xylene-based mounting medium.

7.3. Acquire whole slide scans of IHC-stained slides, export them as TIFF images at a magnification ratio of 1:4 using the using an image viewer, and perform the quantifications using ImageJ.

Note: As an alternative to the scanner, microscopic images at 20x or 40x magnification can be acquired for quantifications. Image magnification can be chosen depending on the marker to be quantified; high magnification images are recommended for nuclear staining, while cytoplasmic or membrane markers can be quantified using 20x images.

7.3.1. For quantification of nuclear markers, in this case NKX2-1 expression, convert each image to 16-bit images using Fiji-ImageJ, and upload images for image analysis.

7.3.2. Customize the image analysis pipeline depending on the analysis. In this protocol, use the following pipeline for the quantification of NKX2-1 positive nuclei: **Identify Primary Objects | Measuring Object Intensity | Filter Objects (minimal value=0.0025) | Calculate Math.** The results are represented as percentages of DAB-stained nuclei of the total number of nuclei.

REPRESENTATIVE RESULTS:

Figure 1 represents the workflow for the generation, cultivation and analysis of precision-cut tissue slices derived from murine NSCLC tumors. For this demonstration, we utilized tumors from a genetically engineered mouse model (GEMM) harboring conditional activation of *Kras*^{G12D} together with the loss of *Lkb1* (also known as Serine/Threonine Kinase 11), hereafter called KL. Mouse breeding and lung tumor initiation was performed as described in[26,28]. **Figure 2A** demonstrates the effect of tissue slice thickness on the viability of slices cultured for 24 h using

a rotating incubation unit. The results show that 160 µm thin slices contain large necrotic areas across the slice. In addition, 250 µm thin slices show a necrosis gradient across the slice compared to 200 µm thin slices. It is likely that the poor overall viability of the thinnest 160 µm is caused by technical handling during positioning of the slices on top of the grids, as these are fragile and tend to curl. On the other hand, when slices are too thick, they can become prone to deficiencies in oxygen or nutrient diffusion across the slices, which in murine NSCLC explants is evidenced as necrotic death gradient[25]. However, it should be noted that slices with variable thicknesses can be generated from one tumor, despite use of identical vibratome settings. It is therefore recommended to analyze multiple replicates from different tumor samples. Importantly, each tissue type requires slice thickness optimization to achieve maximum viability, as the tissue texture and hardness can affect oxygenation and nutrient flow. **Figures 2B-2C** illustrates quantitative IHC analyses of NKX2-1 expression, a marker of well-differentiated lung adenocarcinoma (AC) in samples cultivated up to 72 h and matched 0 h slices. 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 cultivation does not overtly affect the differentiation status of AC tumor tissue. **Figure 2D** demonstrates the utility of tumor tissue slices for assessing the effectiveness of targeted drugs. We recently showed that *Kras* mutant murine ACs exhibit high expression of phosphorylated ERK1/2 (marking increased MAPK pathway activity) when compared to adenosquamous (ASC) tumors, while expression of phosphorylated 4EBP1 (marking mTOR activity) is similarly expressed in both AC and ASC tumors. To test if these pathways can be effectively targeted on tissue slices, KL AC tissue slices were treated with DMSO or titrated amounts of compounds, namely 0.1 µM -1 µM dactolisib to target the mTOR pathway or 0.05 µM - 0.5 µM selumetinib to target the MAPK pathway. Results show that 1 µM dactolisib or 0.5 µM of selumetinib are effective in inhibiting the phosphorylation of 4EBP1 or ERK1/2, respectively. Furthermore, dose-dependent inhibition of the targeted phosphoproteins indicates that tissue slices can also be utilized to validate phosphorylation-specific antibodies.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of the workflow for establishment and analysis of murine NSCLC tumor-derived slice explants. (A) Schematic describing the collection and preparation of tumor-bearing lungs for slicing. Lung lobes are harvested from a mouse and tumor tissue is dissected away from normal tissue. The black arrowhead and asterisk indicate approximately 4 mm and 1 mm tumors, respectively. The white arrowhead indicates lung tissue glued to the surface of the specimen holder. The red arrow points at an additional piece of normal lung support tissue to retain the tumor in an upright position. (B) Vibratome slicing and collection of tissue slices. White arrow indicates the slicing direction. Collection of sequential slices into a 24-well plate containing cold HBSS + P/S. 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. (C) Placing the tissue slice for cultivation using rotating incubation units. Tilt the 6-well plate so that some medium covers the top of the grid, place the tissue slice in the middle of the grid on top of the medium, and spread the slice using forceps. Ensure that the 6-well plates are weight balanced for a smooth rotation cycle. X: indicates incorrect, and ✓: indicates correct positioning of the slice. (D) Photograph of the FFPE block of a tumor slice. Black arrow points at paraffin-embedded tissue

slice stained with hematoxylin. (E) Schematics showing the sectioning order of the slices in FFPE blocks; these sections can be processed to assess tissue viability and tumor-specific biomarker expression.

Figure 2. Assessment of viability and histotype-specific marker expression, and targeted drug treatment on NSCLC tissue slices. (A) Representative H&E images of AC NSCLC slices of the indicated thicknesses cultured for 24 h. 200 µm thin slices maintain better viability compared to 160 µm or 250 µm thin slices. 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. T1 and T2 represent biological replicates derived from two different tumors. Scale bar 500 µm. (B) Representative IHC images of NKX2-1 expression in AC slices cultured for the indicated time points. Arrow indicates the area shown in higher magnification. Results show that NKX2-1 expression is not altered in the cultured slices compared to 0 h slices. Scale bar 500 µm and 50 µm for low and high magnifications, respectively. (C) Quantification of the data shown in (B). (D) 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 phosphorylated pERK1/2 expression in 0 h slice, or slices treated with DMSO 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**.**

DISCUSSION:

Various complex *in vitro* tumor models, including 3D cultures and organoids, have been developed to recapitulate the architecture and oncogenic functions of *in vivo* tumor tissue[29,30]. However, the establishment of 3D cultures or organoids involves tissue dissociation and selective growth of a single cell type or co-culture of a select few cell types in an artificial environment. As a consequence, such models incompletely capture the intricacies of tumor heterogeneity and tumor–stroma interactions. Organotypic tumor slices, on the other hand, maintain the tissue architecture and biological complexities of the *in situ* tumor, without extensive manipulation. This ability of tissue slices to model tumor cells in their native microenvironment renders them particularly attractive for preclinical studies. We previously reported an optimized workflow for the establishment and analysis of precision-cut tumor slices, and showed that, compared to filter supports, a rotating incubation unit improve the viability of short-term murine NSCLC slice cultures[25,31]. However, cultivation on rotating units is technically challenging and requires constant monitoring. We here present a protocol for tumor tissue slice generation and practical use of a rotating incubation unit to culture them, as well as accompanying methods to monitor the ability of slices to capture *in situ* tumor biology, a prerequisite prior to drug response testing.

Several critical steps in the protocol ensure tissue integrity and viability of the tumor slices. If normal lung tissue surrounds the tumor, the vibratome can generate slices with inconsistent thickness or damage the slices, due to differences in texture and stiffness between normal and tumor tissues. It thus is important to remove the surrounding normal lung tissue prior to tumor slicing. Another critical step is the slicing thickness, which should be carefully optimized for each tissue type. Furthermore, once sliced, it is critical that the slice is placed approximately in the middle of the grid, so to ensure accurate intermittent dipping in culture medium and oxygen

exposure. Finally, it is important to follow the position of a slice during its rotation period, as a slice can drop down in to the medium; if this happens, further actions can be taken as explained in step 3.6 of the protocol.

In addition to tissue handling to assure integrity, there are also critical steps in the IHC analysis to interpret how the slice resembles the native tissue. Our previous study showed that murine NSCLC tumors exhibit pronounced intra-tumor spatial heterogeneity in oncogenic signaling activities[26]. This means that the use of spatially distinct tissue slices for controls or drug-treated samples can affect reliable experimental data interpretation, and hence closely adjacent slices should be used as controls and test samples. We further showed that while proliferation or oncogenic phosphoprotein expression in freshly cut uncultured 0 h slices were similar to *in situ* tumors, cultured slices showed altered oncogenic phosphoprotein expression, specifically altered p4EBP1 and pSRC, as well as altered proliferation analyzed by Ki67 IHC. Altered p4EBP1 expression was similarly detected in 24 h human NSCLC and prostate cancer slice cultures (Narhi *et al*., Supplementary Figure S3B-S3C, S5 and S7)[26]. These findings endorse that comparison of cultured slices with their nearest 0 h uncultured slice is critical to assess the preservation of *in situ* tumor functions in cultured slices.

Despite improving the viability of organotypic slices[25], there are limitations with the rotator system in terms of technicalities. Placing the tissue slices onto titanium grids is more challenging compared to filter inserts, and a rotating incubation unit may not be available. As an alternative, stagnant filter supports can be used, but in that case only the air-exposed side of the slices should be analyzed, as air-to-filter gradients in viability and hypoxia measured by HIF1α expression are rapidly formed in filter-supported slice cultures (Davies *et al*., Figure 5 and Figure 7A-7B)[25]. We have further shown that tumor slice cultures can exhibit altered proliferation and oncogenic signaling activities compared to their native tumors[26], possibly because of wound-healing responses or metabolic adaptation of the slices to *ex vivo* culture[32]. 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. Thus, tissue slices should only be utilized for short-term functional studies.

Use of a rotating incubation unit at least partially rescues intra-slice viability or biomarker expression gradients, particularly during the first 24 h of culture. Once validated for integrity and function, this provides tissue material for functional studies, such as drug treatment studies. In addition to drug response profiling, altered target expression following drug treatment can also benefit antibody validation. This is particularly relevant for the detection of murine epitopes with mouse monoclonal antibodies, as these tend to give high staining background. In addition, well-validated antibodies are required to achieve reliable and reproducible data in diagnostic and clinical settings. Thus, modulation of the abundance or phosphorylation of relevant epitopes following drug treatment in tissues slices provides a handy practical application in antibody validation. A major advantage of tumor slice cultures is the ability to model spatially-distributed functions, including oncogenic signaling activities or drug response in tumor or stromal cells, which makes them an attractive *ex vivo* model. However, slices rotate during cultivation, and the process of cultivation can further damage the tissue particularly at the edges. It is therefore

challenging to precisely overlay the biomarker-stained IHC images of 0 h slices with the necrotic regions detected in cultured slices, which compromises the ability to precisely link spatial biomarker activities to drug response. In addition, tumor-intrinsic, culture-induced and drug-induced necrotic responses are indistinguishable at least in murine NSCLC tissue slices, compromising accurate quantitation of spatial drug responses. Finally, the use of tumor slice cultures permits a researcher to the test multiple compounds on the same tumor, without a need to treat animals, thus refining, reducing, and replacing experiments on laboratory animals.

As a future application, the described protocol can be adopted to clinical solid tumor samples. Further tissue type-dependent modifications or optimizations are likely required, starting with adjustments to the vibratome settings including slice thickness and vibration speed to optimize these for tumor texture or stiffness. In addition, nutrient and growth factor requirement may vary for different tumor tissues. As an example, breast cancer slices have been cultured with insulin supplemented in the medium[13,18]. Given that limited tissue material is obtained during surgery or biopsy, optimization of patient-derived tumor slice cultures can be challenging due to difficulties in obtaining sufficient numbers of replicate samples. Furthermore, data reproducibility is also challenged by pronounced patient-to-patient sample heterogeneity, particularly in the percentage of tumor cells versus fibrotic regions or stromal infiltrates, as well as necrotic tissue components. Finally, application of tumor slices in diagnostic settings would require investigation of the extent to which drug responses in slice explants of pre-treatment biopsies matches to post-treatment *in vivo* responses.

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DISCLOSURES:

Authors declare no conflict of interest.

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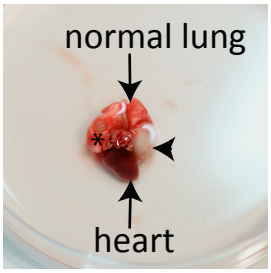
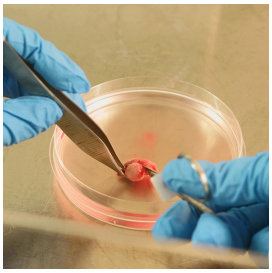
Figure 1

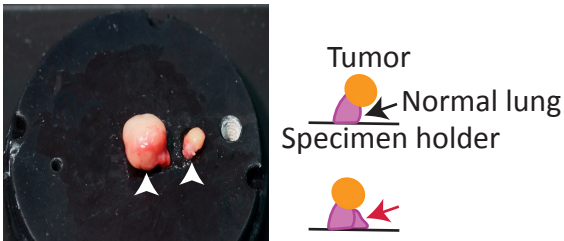
A

Preparation of lung tumor for slicing

i) Collect tumor-bearing lungs ii) Dissect tumor tissue iii) Mount on specimen holder



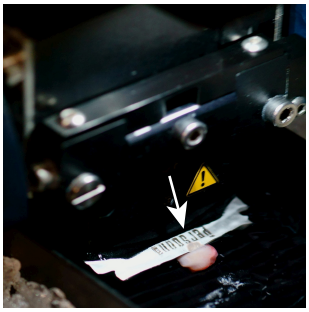


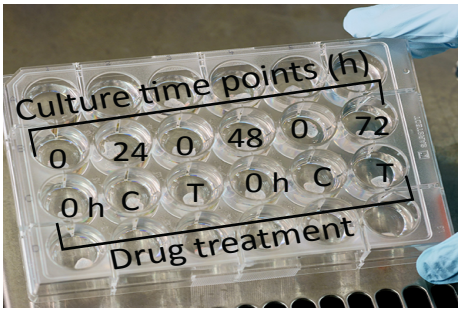


B

Vibratome sectioning and collection of tissue slices

i) Horizontal tissue slicing ii) Collect slices in order of slicing

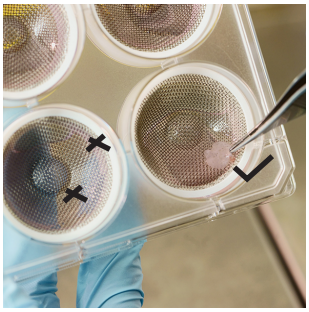


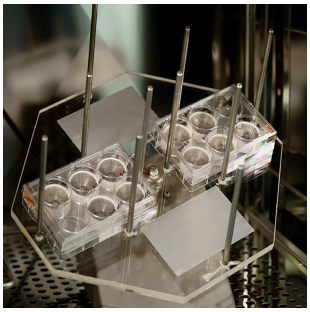


C

Placing the slices for cultivation on rotating incubation unit

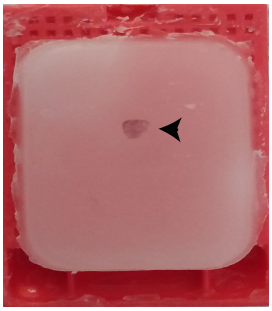
i) Place slice on titanium grid ii) Balance 6-well plates before cultivation





D

Generate FFPE blocks



E

Analysis of the tumor tissue slices

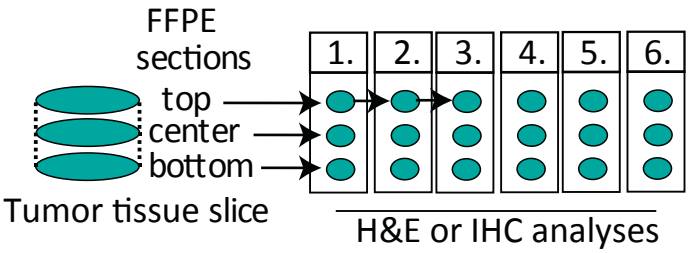
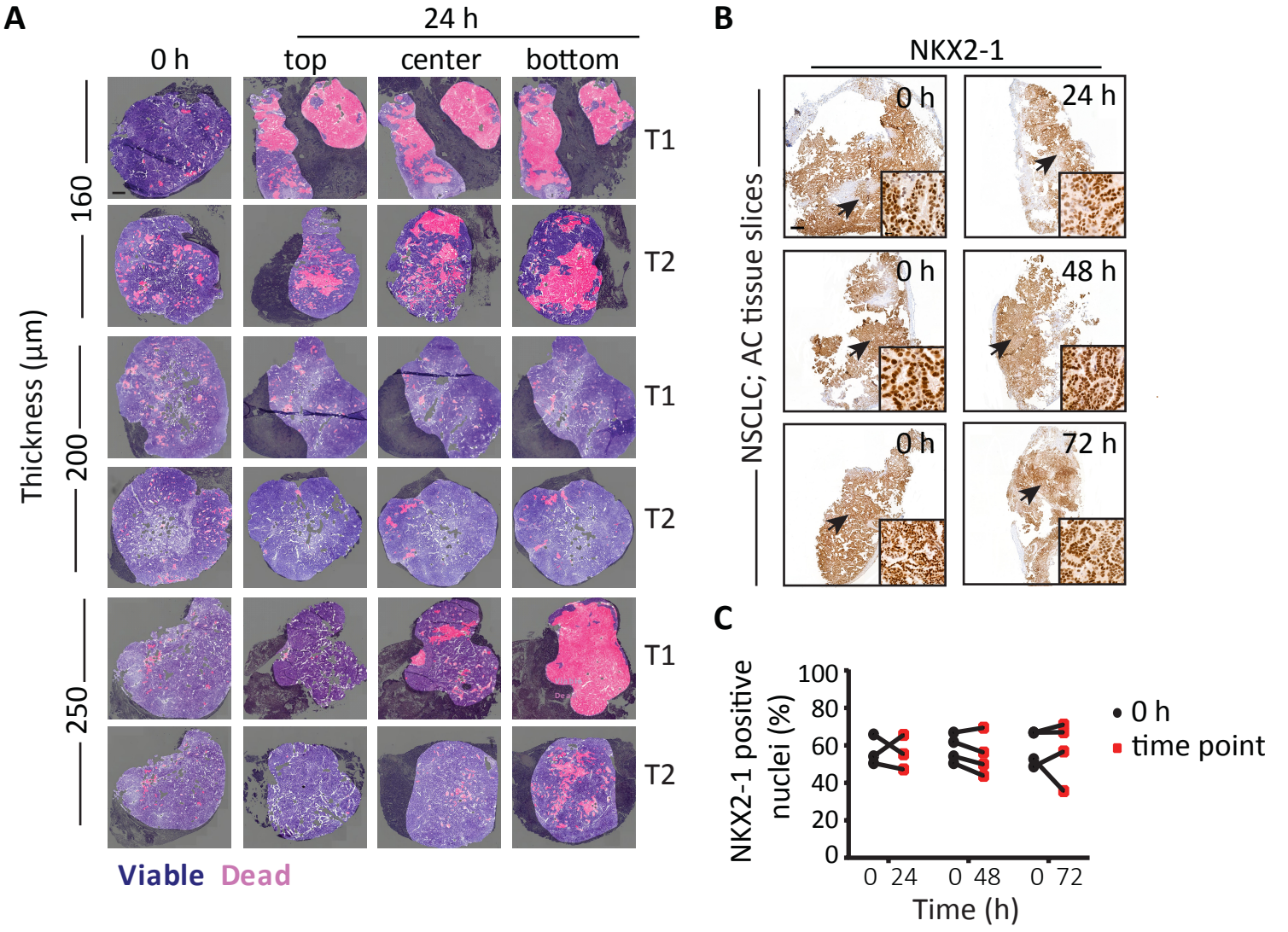


Figure 2



Name of the Reagent/Equipment	Company
Hank's Balanced salt solution (HBSS)	Sigma
Penicillin Streptomycin solution	Thermo Fischer Scientific
Ham's F-12 medium	Thermo Fischer Scientific
Glucose	VWR
FBS	Thermo Fischer Scientific
Glutamine 200mM	Thermo Fischer Scientific
Cyanoacrylate adhesive (GLUture)	Abbott
Leica VT1200 S vibrating blade microtome	Leica Biosystems
Slicing blade	VWR
Titanium grids	Albamma Research and D
Slice incubation unit	Albamma Research and D
10 cm tissue culture plate	Sarstedt
24-well plate	Sarstedt
6-wellsplate	Sarstedt
Single-use needles for special applications	VWR
50 mL falcon tube	Greiner
PBS	Lonza
Formaldehyde	Fisher
Trifold histo cassette paper	Cancer Diagnostics
Histo cassettes	VWR
KOS The microwave multifunctional tissue	Milestone SRL
Microtome	Thermo Fischer Scientific
Superfrost Ultra plus slides	VWR
BSA	Sigma
NGS	Thermo Fischer Scientific
Citric acid	Sigma
PT-Module	Thermo Fischer Scientific
Hematoxylin for H&E staining	Merck
Hematoxylin for counter staining	Dako
Eosin	Sigma
NKX2-1 antibody	Abcam
pERK 1/2 antibody	Cell Signaling Technologie
p4EBP1 antibody	Cell Signaling Technologie
BrightVision poly-HRP Goat anti-rabbit se	ImmunoLogic
MountING medicum pertex	VWR
DAB	ImmunoLogic
Dactolisib (NVP BEZ-235)	Selleckchem
Selumetinib (AZD264)	Selleckchem
Pannoramic 250 slide scaner	3DHISTECH

MATLAB	MathWorks
3DHISTECH PANNORAMIC VIEWER	3DHISTECH
Adobe Photoshop CS6	Adobe
Fiji-ImageJ	ImageJ
CellProfiler	CellProfiler cell image ana

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MD2500	
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4656300	
227261	
BE17-517Q	
F/1501/PB08	
DX26280	
720-2199	
CAT307EN-003	
HM355S	
631-0099	
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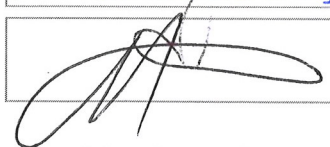
CORRESPONDING AUTHOR:

Name: Emmy W. Verschuren

Department: Institute for Molecular Medicine Finland (FIMM)

Institution: University of Helsinki

Article Title: Establishment and analysis of tumor slice explants as a prerequisite for diagnostic testing

Signature: 

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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.

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*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 CellProfiler™ (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.

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- 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.

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