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Organotypic Slice Cultures as Preclinical Models of Tumor Microenvironment in Primary Pancreatic Cancer and Metastasis

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TITLE:

Organotypic Slice Cultures as Preclinical Models of Tumor Microenvironment in Primary Pancreatic Cancer and Metastasis

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KEYWORDS:

pancreatic cancer, metastasis, organotypic slice culture, preclinical model, chemotherapy, treatment response

SUMMARY:
This protocol describes the preparation of organotypic slice cultures (OTSCs). This technique facilitates the *ex vivo* cultivation of intact multicellular tissue. OTSCs can be used immediately to test for their respective response to drugs in a multicellular environment.

ABSTRACT:
Realistic preclinical models of primary pancreatic cancer and metastasis are urgently needed to test the therapy response *ex vivo* and facilitate personalized patient treatment. However, the absence of tumor-specific microenvironment in currently used models, e.g., patient-derived cell lines and xenografts, only allows limited predictive insights. Organotypic slice cultures (OTSCs) comprise intact multicellular tissue, which can be rapidly used for the spatially resolved drug response testing.

This protocol describes the generation and cultivation of viable tumor slices of pancreatic cancer and its metastasis. Briefly, tissue is casted in low melt agarose and stored in cold isotonic buffer. Next, tissue slices of 300 µm thickness are generated with a vibratome. After preparation, slices are cultured at an air-liquid interface using cell culture inserts and an appropriate cultivation medium. During cultivation, changes in cell differentiation and viability can be monitored. Additionally, this technique enables the application of treatment to viable human tumor tissue *ex vivo* and subsequent downstream analyses, such as transcriptome and proteome profiling.

OTSCs provide a unique opportunity to test the individual treatment response *ex vivo* and identify individual transcriptomic and proteomic profiles associated with the respective response of distinct slices of a tumor. OTSCs can be further explored to identify therapeutic strategies to personalize treatment of primary pancreatic cancer and metastasis.

INTRODUCTION:
Existing preclinical models of pancreatic ductal adenocarcinoma (PDAC) and respective metastases are poor predictors of response to the treatment in patients which is a major drawback in drug development and the identification of predictive biomarkers[1]. Although models such as patient-derived organoids and patient-derived xenografts are promising, their use remains limited[2]. Major limitations of these *in vitro* models are the lack of the tumor microenvironment and xenografting in non-human immunocompromised species. Especially in PDAC and its metastases, the tumor microenvironment has considerably gained interest over the last years because of its crucial functions in tumor biology. It comprises cellular and acellular components, such as (myo-)fibroblasts, pancreatic stellate cells, immune cells, blood vessels, extracellular matrix, cytokines, and growth factors[3]. This microenvironment is not a non-functional tumor component, but this microenvironment induces tumor progression and metastasis and seems to contribute substantially to radio- and chemotherapy resistance[4]. The PDAC microenvironment not only mechanically compromises drug delivery, but also possesses immune and drug-scavenging activity[5–7]. Thus, preclinical models which reflect the complex interaction of tumor cells and the tumor microenvironment are urgently needed to adequately test patients' treatment response *ex vivo* and guide individualized clinical treatment.

Ex vivo cultures of fresh tumor samples represent a close approximation of the tumor *in situ*. Organotypic slice cultures (OTSCs) have been recently developed and studied for several tumors, such as head, neck, breast, prostate, lung, colon, and pancreatic cancers[8–12]. It has been shown that OTSCs maintain their baseline morphology, proliferative activity, and microenvironment during the cultivation for a defined, tissue-dependent period[11–13]. OTSCs of PDACs maintained their viability, morphology, and most components of their tumor microenvironment for 4–9 days in several *in vitro* studies[5,12,14]. Perspectively, this technique enables an immediate application of the treatment to viable human tumor tissue *ex vivo* and subsequent downstream analyses, such as profiling of the transcriptome and proteome.

The establishment of OTSCs provides a unique opportunity to test the treatment response *ex vivo* promptly after surgery. Thus, OTSCs will prospectively allow to identify therapeutic strategies to personalize treatment of metastatic disease. This protocol describes the generation and cultivation of viable OTSCs of pancreatic cancer.

PROTOCOL:

Tissue specimens were collected and processed after approval by the local ethics committee of the University of Lübeck (# 16–281).

1. Fresh tissue collection and handling

NOTE: Every unfixed human tissue specimen should be handled with caution to prevent the risk of infection from blood-borne pathogens. All patients should be tested to be negative for HIV, HBV, and HCV prior to tissue processing. Wear a protective coat and handle human tissue specimens with gloves.

1.1. Collect fresh, unfixed, and unfrozen PDAC tissue specimen with a minimum size of 0.4 x 0.4 cm immediately after surgery and transport the specimen to the laboratory in a tissue storage solution.

1.2. When possible, process the fresh tissue immediately.

1.3. Alternatively, store tissues in tissue storage solution on wet ice at 4 °C overnight. However, tissue storage might result in impaired viability and should generally be avoided.

2. Preparation

2.1. Low melting agarose preparation

2.1.1. Prepare 100 mL of low-melting agarose (8%) by dissolving 8 g of agarose in 100 mL of prewarmed Ringer's solution and store it at 4 °C until needed.

2.1.2. Upon the announcement of tumor resection, melt the agarose in a microwave.

2.1.3. Place the agarose in a pre-heated water bath (37 °C) allowing it to cool down to physiological temperatures prior to the preparation.

2.2. Vibratome setup

2.2.1. Place a razor blade into the holder of the vibratome and perform an automated angle adjustment according to the manufacturer's instructions, if applicable.

2.2.2. Cool down the jacket of the cutting chamber using a cooling unit or wet ice.

2.2.3. Fill the cutting chamber with approximately 100 mL of a physiological cutting solution (e.g., Ringer's solution).

2.2.4. Place the mounted razor blade into the pre-chilled cutting solution, allowing the razor blade to cool down.

3. Tissue embedding in low-melting agarose

3.1. Wash the tissue specimen with cooled (4 °C) PBS and place the tissue in PBS onto a large (~14 cm) Petri dish on ice.

3.2. Remove macroscopically visible excess connective tissue on ice using a scalpel since it might impede the cutting efficiency.

3.3. Place the tissue into a small Petri dish (~3 cm).

3.4. Adjust the tissue orientation so that remaining macroscopically visible connective tissue has the same orientation as the plane of the bottom of the Petri dish. The bottom of the Petri dish has the same orientation as the cutting plane.

3.5. Pour the prepared low-melting agarose into the small Petri dish.

3.6. Readjust the orientation of the tissue, if needed, using forceps.

3.7. Place the Petri dish on wet ice for faster hardening of the agarose.

3.8. Carefully cut the tissue using a scalpel, leaving at least 5 mm surrounding agarose on each side of the tissue.

3.9. Carefully transfer the embedded tissue and glue it on the sample holder using super glue.

3.10. After a few seconds, place the sample holder into the cutting chamber.

3.11. Adjust the orientation of the tissue toward the razor blade, if needed.

4. Slicing of the agarose-embedded tissue using a vibratome

4.1. Define the outer limits of the cutting range (y axis) according to the size of the tissue specimen.

4.2. Adjust the blade toward the top of the tissue block.

4.3. Set cutting speed to 0.04 mm/s, cutting amplitude to 1 mm and slice thickness to 300 µm.

4.4. Carefully cut the first slices and transfer the slices to a separate container with prechilled (4 °C) cutting solution on wet ice.

5. Culture of organotypic slice cultures

5.1. Prepare a 6-well plate with 1 mL of the appropriate cultivation medium per well.

5.1.1. Medium A: Advanced DMEM/F12, 10% FBS, 1% Penicillin/Streptomycin.

5.1.2. Medium B: RPMI 1640, 10% FBS, 1% Penicillin/Streptomycin, 4 µg/mL Insulin, 8 ng/mL EGF, 0.3 µg/mL hydrocortisone.

NOTE: The medium for optimal culture conditions might vary depending on the tissue and the patient. Two distinct tissue culture media were compared, one based on DMEM/F12 (medium A), the second based on RPMI (medium B). No substantial differences were detected with this tissue specimens. For all experiments shown in this protocol, medium A was used.

5.2. Place the 6-well plate with the medium into an incubator, allowing temperature and pH to adjust prior to cultivation.

5.3. Place slices onto cell culture inserts (e.g., hydrophilic PTFE inserts with 0.4 µm pore size) using a gaze filter.

5.4. Remove any excess cutting solution by placing the loaded filter onto a sterile cloth.

5.5. Place the loaded filter into the prepared 6-well plate. Do not add any additional medium to the insert.

5.6. Place the 6-well plate in an incubator (37 °C, 5% CO_2).

5.7. Change the medium every 2 days by repeating steps 5.1, 5.2, and 5.5 with a new 6-well plate.

NOTE: Organotypic slice cultures can be cultured for various periods depending on the individual

research question.

6. Resazurin viability assay

NOTE: The resazurin viability assay measures general metabolic activity of the organotypic slice cultures based on the reduction of non-fluorescent blue resazurin to red fluorescent resorufin in living cells[15]. The assay has no toxic effects on cells and can be applied to the cultures repeatedly depending on the individual research question. Viability was measured using the resazurin assay every 2–3 days.

6.1. Preparation of resazurin stock solution

6.1.1. Turn off the light of the sterile hood, since resazurin stock solution is light sensitive.

6.1.2. Prepare a stock solution with 10 mg/mL of resazurin sodium salt in 1x PBS.

6.1.3. Store the stock solution in light protected aliquots at 4 °C in the fridge until use.

6.2. Assessment of overall slice viability

6.2.1. Turn off the light of the sterile hood, since resazurin stock solution is light sensitive.

6.2.2. Dilute the resazurin stock solution 1:250 with an appropriate medium.

NOTE: The medium used for dilution should be the same as used for cultivation (medium A or medium B).

6.2.3. Prepare 1 mL of the final resazurin solution per slice and add an additional 1 mL for the blank control, e.g., for 6 slices dilute 28 µL of resazurin stock solution in 7 mL of medium.

6.2.4. Dispense the resazurin solution in 6-well plates, with 1 mL of the diluted resazurin solution per well.

6.2.5. Transfer the cultivation filters with the slices into the wells with the resazurin solution. One well with the resazurin solution is kept empty as blank control.

NOTE: To simplify the experimental procedure, this step can be done together with changing the medium (step 5.7). However, in case additional viability measurements are needed, the assay can be done anytime during the cultivation of the slice cultures.

6.2.6. Place the tissue slices in an incubator for 1 h at 37 °C and 5% CO_2.

6.2.7. Prepare new 6-well plates with the culture medium if slice culture is continued (see steps 5.1 and 5.2).

6.2.8. Remove the cultivation filters with the slices from the resazurin solution and remove excess solution by placing the loaded filter onto a sterile cloth.

6.2.9. Transfer the cultivation filters with the slices onto the previously prepared culture plate.

6.2.10. From each 6-well, take 100 µL of resazurin solution and transfer it onto a 96-well plate. From blank control, place three samples (3 x 100 µL) in separate wells of the 96-well plate.

6.2.11. Quantify the extinction with a plate photometer according to the manufacturer's instructions. Excitation wavelength is set to 545 nm and emission wavelength is set to 600 nm.

7. Formalin fixation and paraffin embedding of OTSCs

7.1. Cautiously transfer the cultivated tissue slices to a plastic embedding cassette. To do so, follow the steps below.

7.1.1. Place the cultivation filter with the mounted slice on a Petri dish.

7.1.2. With a scalpel, carefully cut out the filter membrane with the mounted tissue slice.

7.1.3. Carefully transfer the filter membrane with the mounted slice into a biopsy nylon bag and place it in an embedding cassette.

7.1.4. Subsequently transfer plastic embedding cassettes in a container with pre-chilled (4 °C) 4.5% formalin. Slices can be kept in formalin solution at 4 °C until further use.

NOTE: OTSCs need to be transferred with great caution as they tear apart easily.

7.2. Cautiously rinse the formalin-fixed slice culture with running tap water for 1.5 h.

7.3. Dehydrate the formalin-fixed tissue slice by incubation in 70% ethanol (2x for 3 h), 95% ethanol (1x for overnight, 1x for 3 h), followed by absolute ethanol (1x for 3 h, 1x for overnight).

7.4. Clear the formalin-fixed tissue slice by 3 h incubation in xylene twice.

7.5. Immerse the tissue with paraffin at 60 °C (1x for overnight, 1x for 2 h). Embed the tissue in a paraffin block in a tissue embedding mold.

7.6. Section the paraffin-embedded tissue block at 4 µm thickness with a microtome and float in a 40 °C water bath containing distilled water. Transfer the sections onto glass slides.

7.7. Incubate paraffin sections for 1 h at 60 °C to bond the tissue to the glass. Incubate the slides overnight at 37 °C.

8. Hematoxylin and Eosin (H&E) staining

8.1. Deparaffinize sections from step 7.7 by incubation in xylene (3x for 5 min).

8.2. Re-hydrate by incubation in absolute alcohol (2x for 5 min), 95% alcohol (2x for 5 min), and 70% alcohol (1x for 5 min). Rinse briefly with distilled water.

8.3. Stain in Mayer hematoxylin solution for 5 min. Rinse with running tap water for 10 min.

8.4. Counterstain in 0.5% Eosin solution for 40 s. Rinse with distilled water.

8.5. Dehydrate by incubation in 70% alcohol (maximal 1 min), 95% ethanol (2x for 3 min), and absolute alcohol (2x for 3 min), respectively.

8.6. Clear in three changes of xylene (few seconds each). Place a drop of mounting medium and cover slides with a coverslip.

9. Immunohistochemistry of OTSCs

9.1. Deparaffinize sections by incubation in xylene 2x for 10 min, followed by 1:1 ethanol/xylol for 10 min.

9.2. Transfer slides to 100% ethanol (2x for 3 min), 96% ethanol (2x for 3 min), 70% ethanol (1x for 3 min), and then to 50% ethanol (1x for 3 min).

9.3. Perform antigen retrieval to unmask the antigenic epitope. Heat the slides in a microwave in citrate buffer for 5 min at 900 W, followed by 2x for 8 min at 600 W. Allow the slides to cool to room temperature for 20 min.

9.4. Wash in PBS 3x for 3 min on a shaker.

9.5. Perform permeabilization of cell membranes by incubation in 0.1% Triton X-100 in PBS (200 µL per slide) in a humidified chamber at room temperature for 10 min.

9.6. Wash in PBS for three changes, 3 min each on the shaker.

9.7. Incubate sections with 3% H_2O_2 solution in methanol (200 µL per slide) in a humidified chamber at room temperature for 10 min to block endogenous peroxidase activity.

9.8. Wash in PBS 3x for 3 min on a shaker.

9.9. Add 200 µL of blocking buffer (1:50 horse serum in PBS) and incubate in a humidified chamber at room temperature for 25 min.

9.10. Drain off the blocking buffer from the slides by tilting the slide on a paper tissue.

9.11. Apply 200 µL of appropriately diluted primary antibody in antibody diluent on the slides and incubate in a humidified chamber at 4 °C overnight. As a negative control, use appropriate isoform of mouse immunoglobulins at the same dilution as the primary antibody.

9.12. Wash 3x for 3 min in PBS on a shaker.

9.13. Apply 200 µL of biotinylated secondary antibody (1:50 solution in PBS) on the slides and incubate in a humidified chamber at room temperature for 30 min.

9.14. Wash 3x for 3 min in PBS on a shaker.

9.15. Prepare the avidin/biotin-based peroxidase complex according to the manufacturer's instructions prior to application. Apply 200 µL of avidin-biotin-peroxidase complex on the slides and incubate in a humidified chamber at room temperature for 30 min.

9.16. Wash 3x for 3 min in PBS on a shaker.

9.17. Apply 200 µL of DAB substrate solution (freshly made directly before use: 1 drop of DAB in 1 mL of substrate), 200 µL per slide. Allow the color development 1–3 min until the desired color intensity is reached.

9.18. Rinse with running tap water for 10 min.

9.19. Counterstain the slides by immersing sides in Hematoxylin for 5 min.

9.20. Rinse with running tap water for 10 min.

9.21. Cover the slides using aqueous mounting medium and coverslips. The mounted slides can be stored at room temperature permanently.

REPRESENTATIVE RESULTS:

Figure 1 provides an overview of the workflow to culture OTSCs from fresh, unfrozen tumor tissue. Specimens of primary PDACs and metastases were collected directly after surgical resection and stored overnight on wet ice at 4 °C in the tissue storage solution. The specimens were processed, and slices were cultured as described in the protocol. The macroscopic morphology of each OTSC did not change grossly during cultivation. However, the size of the surface area of the OTSCs decreased over time as exemplarily shown in **Figure 2A** during cultivation for 6 days. The overall viability of OTSC was assessed by resazurin viability assay on days 0, 2/3, and 6 (**Figure 2B**). The resazurin viability assay measures general metabolic activity of the OTSC based on the reduction of non-fluorescent blue resazurin to red fluorescent resorufin in living cells[15]. **Figure 2B** shows the comparison of viability measured by resazurin reduction after

the cultivation of OTSCs from two representative primary tumors in two different media. OTSCs were cultivated either in medium A (advanced DMEM/F12, 10% FBS, 1% Penicillin/Streptomycin) or medium B (RPMI 1640, 10% FBS, 1% Penicillin/Streptomycin, 4 µg/mL Insulin, 8 ng/mL EGF, 0.3 µg/mL hydrocortisone), which resulted in similar overall viability independently of the culture medium. Of note, a decrease in viability after day 0 can be observed frequently and is expected due to the slicing procedure and adjustment to culture conditions. However, we also observed increased viability as shown exemplarily in the right panel (**Figure 2B**).

After defined periods of cultivation in medium A, OTSCs were fixed in formalin for further immunohistological characterization (IHC). After formalin fixation, OTSCs were paraffin embedded and sectioned. H&E stained sections showed that the overall structure of the tissue was preserved over the entire time of cultivation *ex vivo* (**Figure 3**). Tumor and stroma cells were discriminated by IHC for cytokeratin 7 and vimentin, respectively. No substantial changes in the tumor to stroma ratios were detected during cultivation.

Microscopic histopathologic evaluation of H&E sections did not reveal a substantial increase in necrosis of all cultivated tissues during cultivation (**Figures 3–5**). Additionally, Ki-67 and cleaved caspase 3 were stained for evaluation of proliferation and apoptosis, respectively. Again, we did not detect gross changes of proliferation and apoptosis during the culture period of 6 days (**Figure 3)**. However, the proportion of apoptotic cells increases over time during extended cultivation periods as measured by cleaved caspase 3 staining. **Figure 4** shows the increase in cleaved caspase 3 positive cells after cultivating a primary PDAC for 15 days. **Figure 5** shows the histopathology of a peritoneal metastasis of a PDAC. This experiment demonstrates a high intratumor heterogeneity between individual slices regarding the tumor/stroma content as well as naturally occurring apoptosis measured by cleaved caspase 3 staining. Hence, histopathological evaluation of cultivated OTSCs is important for the evaluation of the tumor/stroma architecture and viability of each cultivated tumor or its metastasis.

Figure 6 shows the histopathology of a PDAC metastasis of the abdominal wall. H&E staining as well as IHC for cytokeratin 7 showed that the derived OTSCs did not contain any tumor cells, but only consisted of connective tissue, which was partially necrotic. A drawback of the OTSC technology is that fresh, unfixed, and unfrozen tissue cannot be evaluated for its tumor cell content prior to cultivation.

Overall, these data demonstrate that the multicellular architecture of a tumor and its respective metastasis, comprising distinct cell types, are reflected in OTSCs. Particularly tumor-stroma interactions are preserved.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview over the workflow for cultivation of OTSCs from fresh, unfrozen tissue tumor/metastasis specimens. The fresh, unfrozen tissue specimen is sectioned using a vibratome and cultivated at air-liquid interface on PTFE cell culture inserts. Overall viability can be measured at defined time points by resazurin viability assay. This assay allows further cultivation after viability measurement. Slices are formalin-fixed at defined time points for

441 further histopathological examination.
442
443 **Figure 2: Macroscopic morphology and viability of OTSCs during cultivation.** A PDAC specimen
444 was cultivated as OTSCs for 6 days. (**A**) The macroscopic morphology of each OTSC did not change
445 grossly during cultivation. However, the size of the surface area of the OTSCs decreased over
446 time. (**B**) Overall viability was quantified by resazurin viability assay in medium A and B. A
447 decrease in viability can frequently be observed after day 0 due to the sectioning procedure and
448 adjustment to culture conditions. However, we also observed increases of viability as shown
449 exemplarily in the right panel. Each panel represents an individual tumor specimen with different
450 yields of derived slice cultures (left panel n = 10, right panel n = 10).
451
452 **Figure 3: Histopathological evaluation of OTSCs after a total cultivation time of 6 days.** OTSCs
453 (n = 6) were cultured in medium A. Tissue morphology was assessed by H&E staining. Tumor and
454 stroma cells were discriminated by IHC for cytokeratin 7 and vimentin, respectively. Ki-67 and
455 cleaved caspase 3 were stained for evaluation of proliferation and apoptosis, respectively. No
456 substantial increase in apoptosis was observed over a total cultivation period of 6 days. Each
457 scale bar represents 100 µm.
458
459 **Figure 4: Histopathological evaluation of OTSCs after a total cultivation time of 15 days.** OTSCs
460 (n = 8) were cultured in medium A. Tissue morphology was assessed by H&E staining. Tumor and
461 stroma cells were discriminated by IHC for cytokeratin 7 and vimentin, respectively. Ki-67 and
462 cleaved caspase 3 were stained for evaluation of proliferation and apoptosis, respectively.
463 Apoptosis substantially increased after 15 days of cultivation as exemplified by cleaved caspase-
464 3 staining. Each scale bar represents 100 µm.
465
466 **Figure 5: Histopathological evaluation of OTSCs of a peritoneal metastasis of a PDAC.** OTSCs (n
467 = 8) were cultured in medium A. Tissue morphology was assessed by H&E staining. Tumor and
468 stroma cells were discriminated by IHC for cytokeratin 7 and vimentin, respectively. Ki-67 and
469 cleaved caspase 3 were stained for evaluation of proliferation and apoptosis, respectively.
470 Histopathological evaluation revealed a high degree of intratumor heterogeneity between
471 individual slices regarding the tumor/stroma content as well as naturally occurring apoptosis
472 measured by cleaved caspase 3 staining. Each scale bar represents 100 µm.
473
474 **Figure 6: Histopathological evaluation of OTSCs of a PDAC metastasis of the abdominal wall.**
475 OTSCs (n = 5) were cultured in medium A. Tissue morphology was assessed by H&E staining.
476 Tumor and stroma cells were discriminated by IHC for cytokeratin 7 and vimentin, respectively.
477 Ki-67 and cleaved caspase 3 were stained for evaluation of proliferation and apoptosis,
478 respectively. Histopathological evaluation revealed the lack of tumor cells in the cultivated
479 metastasis and partial necrosis, which could not be determined prior to cultivation. Each scale
480 bar represents 100 µm.
481
482 **DISCUSSION:**
483 OTSCs of fresh tumor samples are a close approximation of the tumor *in situ*. They maintain their
484 baseline morphology, proliferative activity, and microenvironment during the cultivation for a

defined, tissue-dependent period[11–13]. This technique enables the immediate application of treatment to viable human tumor tissue *ex vivo* and subsequent downstream analyses, such as profiling of the transcriptome and proteome. A specific advantage of OTSCs is that spatially resolved downstream analyses such as MALDI-imaging can be applied to intact multicellular tumor tissue.

Here we describe a method for the generation, cultivation, and histopathological evaluation of OTSCs of PDAC and its metastases. The key issue for this technique is to cut fresh tissue without any freezing procedures and cultivation at an air-liquid interface. A limitation of this method is that fresh, unfixed, and unfrozen tissue cannot be evaluated for its tumor cell content prior to cultivation. As exemplified in our results, OTSCs may reveal to consist of tissue components other than expected from rapid sections of other specimens from the same tumor. Histopathological evaluation of the OTSCs after cultivation is imperative for data evaluation of individual slice cultures. The described viability assay only allows a general assessment of overall viability of each OTSC. In general, the described method can be implemented in any laboratory equipped with a vibratome and tissue culture unit. Due to the simplicity of this technique, it can be easily modified toward various tumors and research questions.

Multiple methodologies have been developed to culture human tumor tissue *ex vivo*, e.g., primary cell lines, patient-derived organoids, and xenografts. However, their use for drug development and the identification of predictive biomarkers remains limited mostly due to the loss of the tissue context, i.e., interaction of stroma and tumor cells[2].

Although the presented method of organotypic slice culture establishment preserves the multicellular tissue architecture, several limitations need to be considered. First, a considerable degree of (intra)tumor heterogeneity needs to be taken into account. Distinct OTSCs derived from a single tumor biopsy might show considerable variations in their proportions of tumor and stroma cells. Besides overall tissue quality, this might be one reason for different slice viability during culture. Secondly, the medium for optimal culture conditions might vary depending on the tissue and patient. We compared two distinct tissue culture media: the first based on DMEM/F12 (medium A) and the second based on RPMI (medium B). Each specimen of primary tumors or its metastases might require modifications. Thirdly, tumor necrosis might occur already *in situ*[16] and is not necessarily due to insufficient culture conditions of the OTSCs. Comparison with the histopathology of the primary tumor should be considered.

The establishment of OTSCs as described in this protocol provides an opportunity to test treatment response promptly after surgery in an *ex vivo* model system that preserves the tumor microenvironment. Prospectively, OTSCs will prospectively facilitate the development of individual therapeutic strategies to personalize treatment of metastatic disease.

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DISCLOSURES:

The authors disclosed no potential conflicts of interest.

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Figure 1

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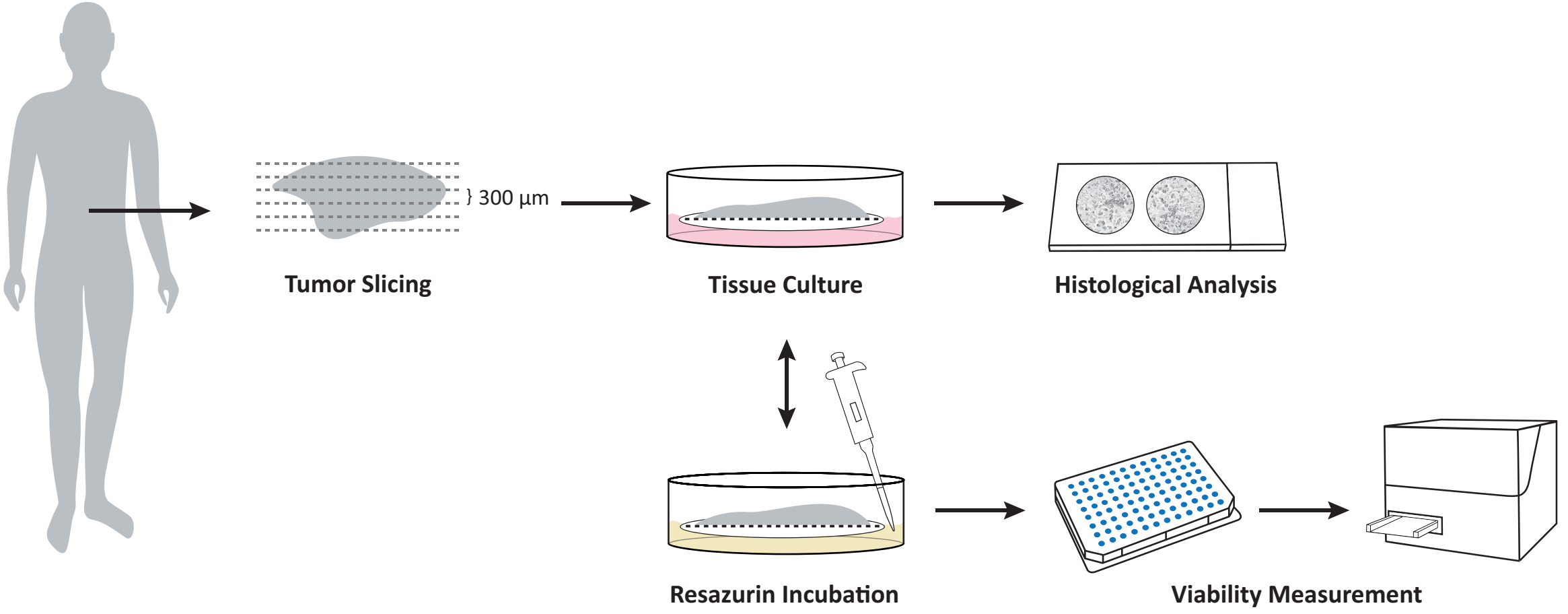

Figure 2

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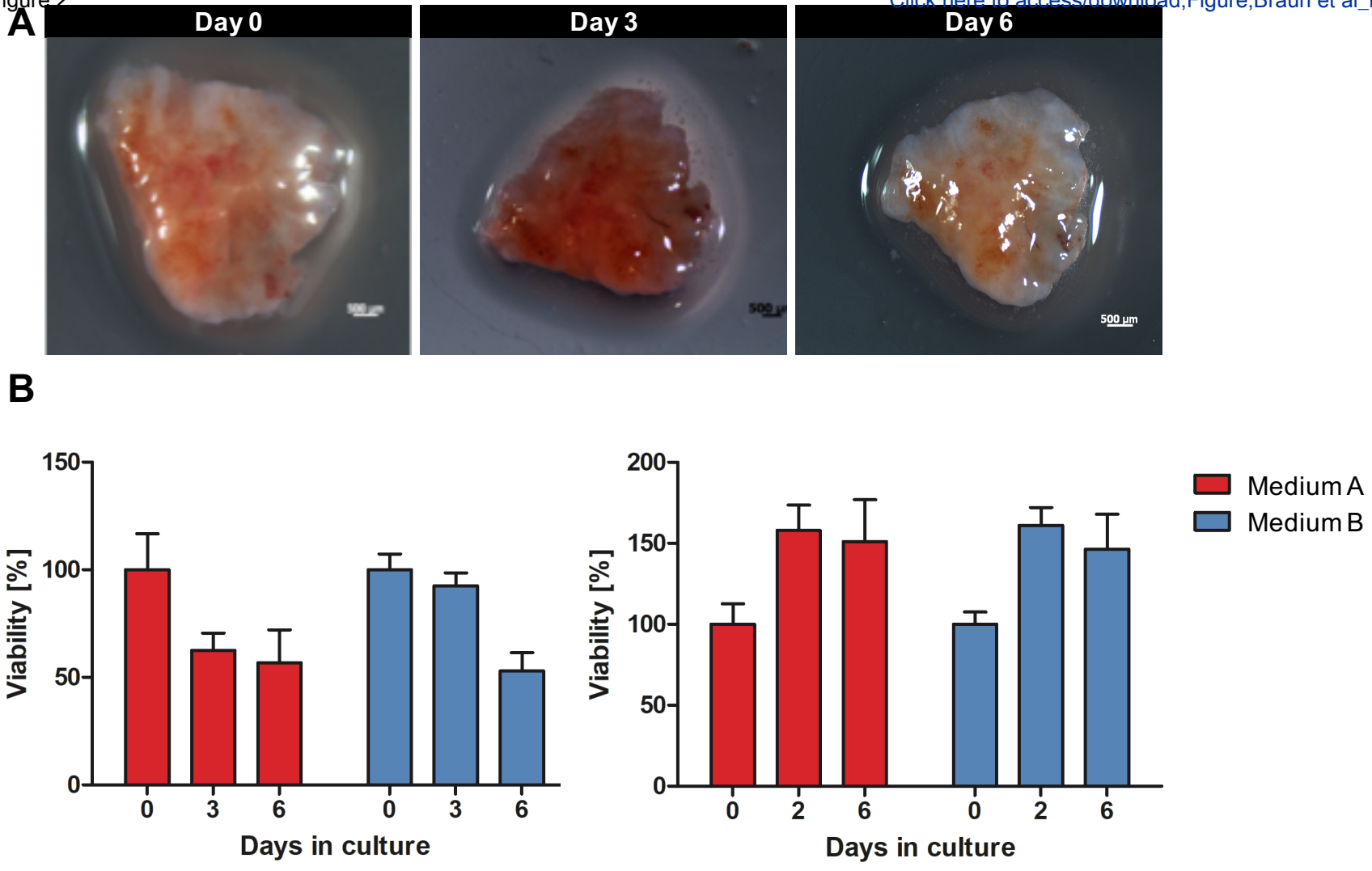

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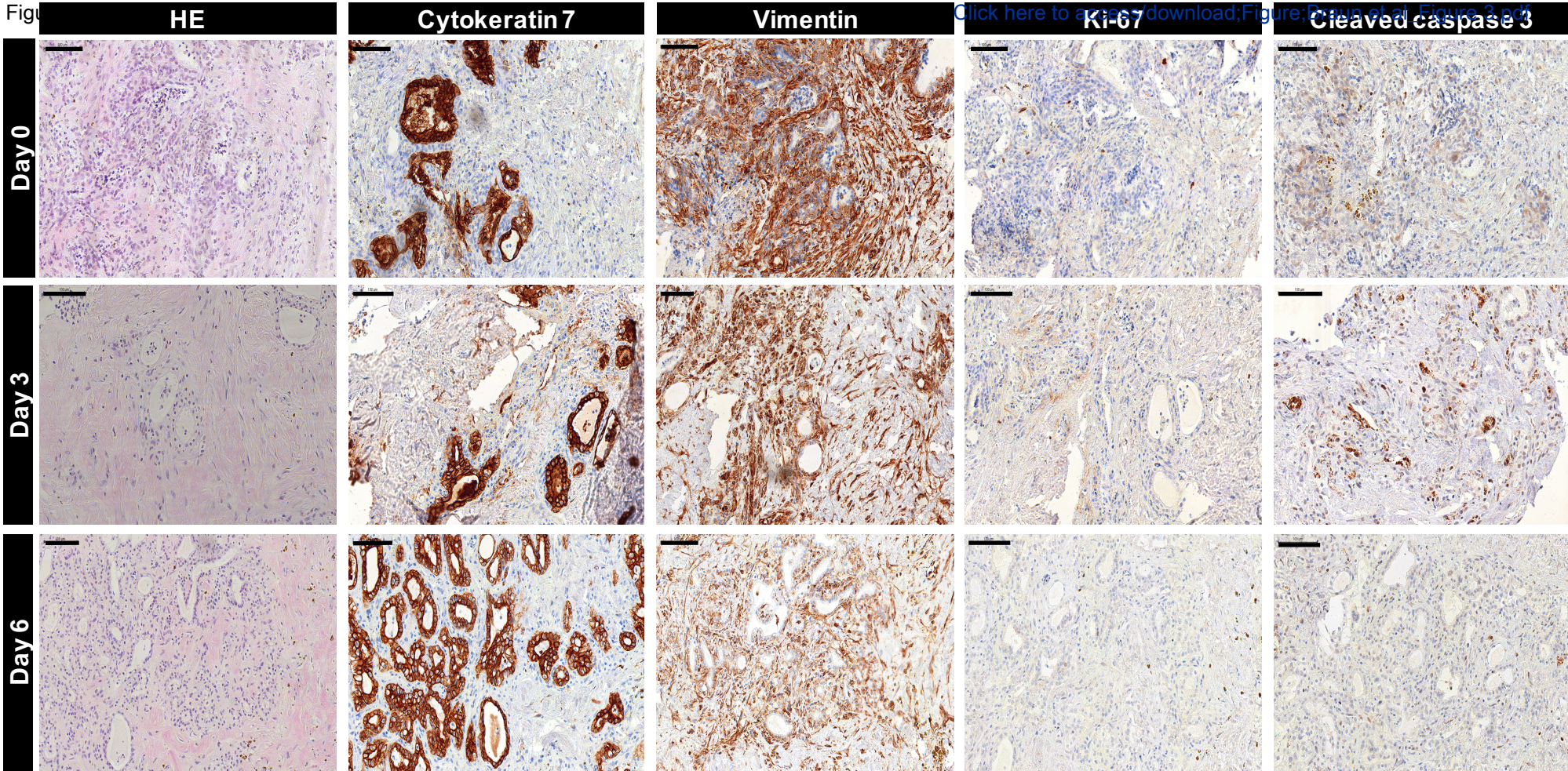

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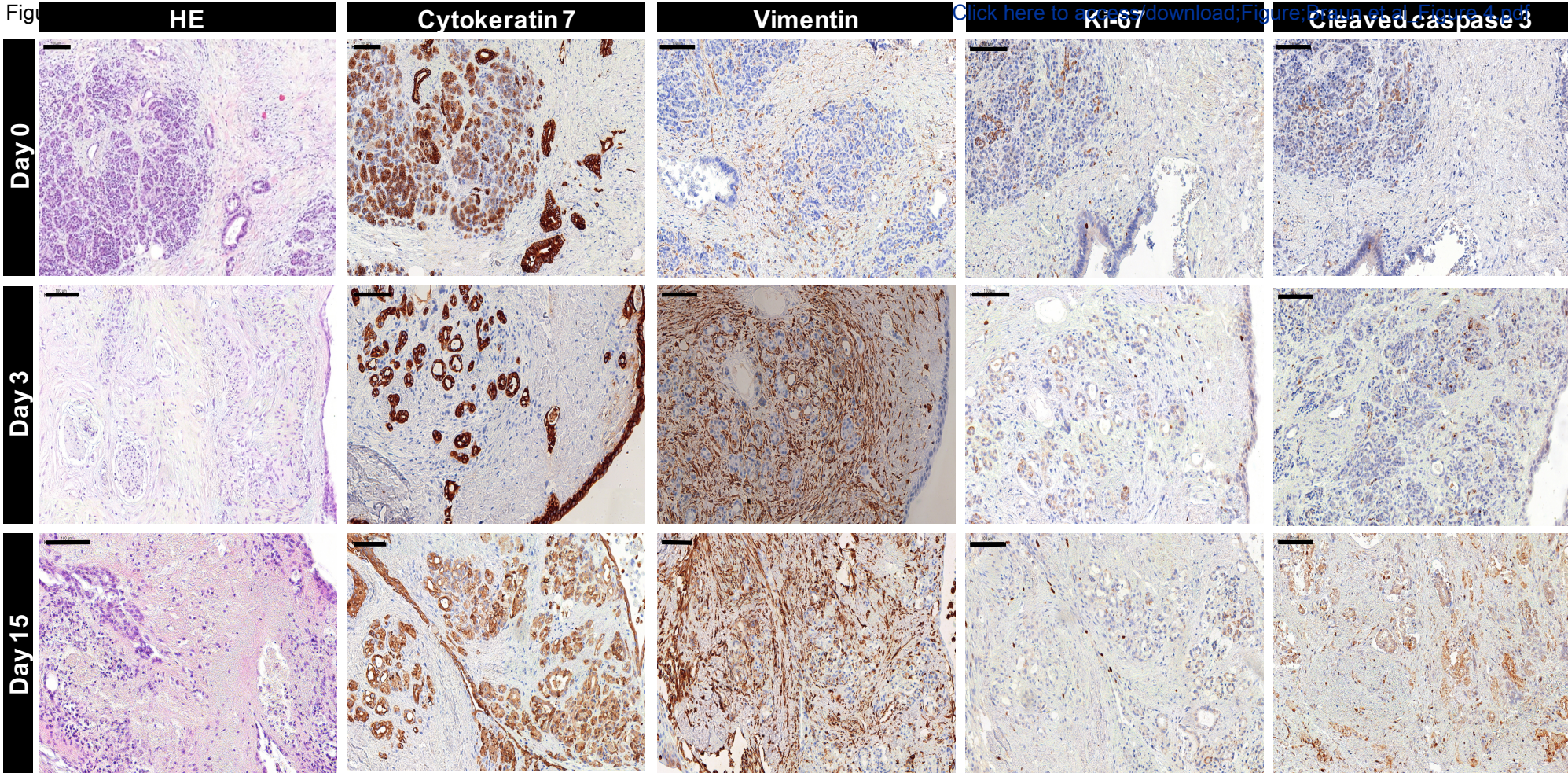

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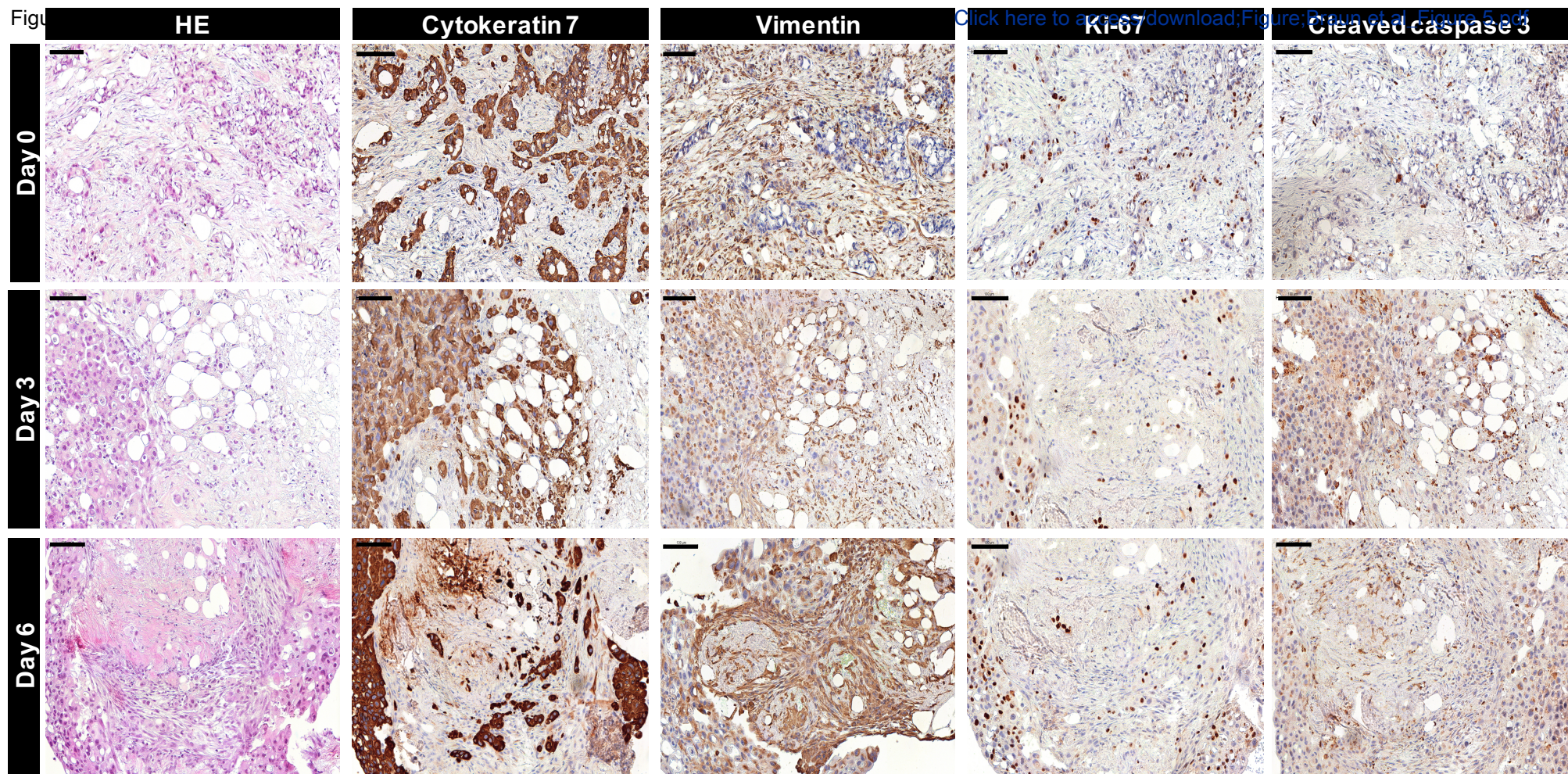

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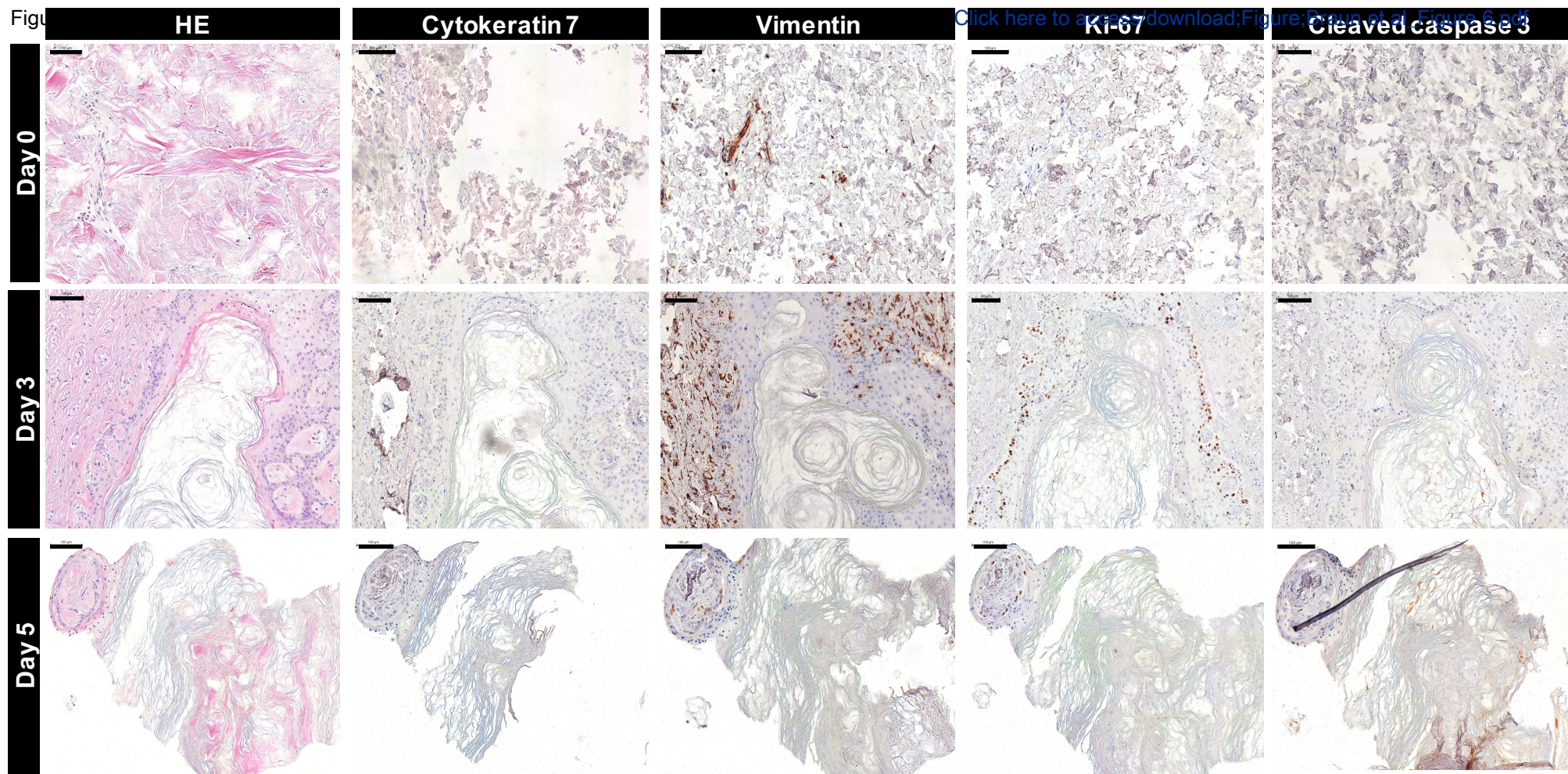

Click here to access/download;Table of Materials;Braun et al_Table of Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Advanced DMEM/F-12 Medium	Gibco	12634028	
Agarose Low Melt	Roth	6351.2	8% in Ringer solution
Antibody Diluent, Background Reducing	Dako	S3022	
AquaTex	Merck	108562	
Bioethanol (99%, denatured)	CHEMSolute	22,119,010	
Citric Acid monohydrate	Sigma Aldrich	C7129	
Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb	Cell Signalling Technology	9664	1:400 dilution
Derby Extra Double Edge Safety Razor Blades	Derby Tokai		
Embedding cassettes	Roth	H579.1	
Eosin Y-solution 0,5% aqueous	Merck	109,844,100	
Eukitt Quick hardening mounting medium	Sigma-Aldrich	3989	
Fetal bovine serum	Gibco	10270106	
Formaldehyde solution 4,5%, buffered	Büfa Chemikalien	B211101000	
Hem alum solution acid acc. to Mayer	Roth	T865	
Human EGF	Milteniy Biotec	130-097-794	
Hydrocortisone	Sigma Aldrich (Merck)	H0888	
Hydrogen peroxide 30%	Merck	1,085,971,000	
Insulin human	Sigma Aldrich (Merck)	12643	
Liquid DAB+ Substrate Chromogen System	Dako	K3468	
MACS Tissue Storage Solution	Milteniy Biotech	130-100-008	
Methanol	Merck	10,600,092,500	
Microscope Slides Superfrost Plus	Thermo Scientific	J1800AMNZ	
Millicell Cell Culture Insert, 30 mm, hydrophilic PTFE, 0.4 µm	Millipore (Merck)	PICM0RG50	
Monoclonal mouse anti-human Cytokeratin 7 (Clone OV-TL 12/3	Dako	M7018	1:200 dilution
Monoclonal mouse anti-human Ki67 Clone MIB-1	Dako	M7240	1:200 dilution
Monoclonal mouse Anti-vimentin (Clone V9)	Dako	M0725	1:200 dilution
Negative control Mouse IgG2a	Dako	X0943	1:200 dilution
Negative control Mouse IgG1	Dako	X093101-2	1:200 dilution
Paraffin (melting temperature 56°- 58°)	Merck	107,337,100	
Penicillin-Streptomycin (10.000 U/ml)	Gibco	15140122	
PBS pH 7,4 (1x) Flow Cytometry Grade	Gibco	A12860301	

Resazurin sodium salt; 10 mg/ml in PBS	Sigma Aldrich	R7017	1:250 dilution
Ringer's solution	Fresenius Kabi	2610813	
RPMI-1640 Medium	Sigma Aldrich (Merck)	R8758	
Tissue culture testplate 6	TPP	92006	
Triton X-100	Sigma Aldrich	9002-93-1	
VECTASTAIN Elite ABC-Peroxidase Kit	Vector Laboratories	PK-6200	
Xylene (extra pure)	J.T.Baker	81,185,000	

Equipment

ClarioStar Microplate Reader	BMG Labtech
Paraffin Embedding Center E61110	Leica
Rotary Microtome Microm HM355S	Thermo Scientific
Section Transfer System Microm STS	Thermo Scientific
VT 1200S Vibratom	Leica

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Dear Dr. Bajaj,

We thank you for your email dated April 07, 2021, and appreciate it that you will consider a revised version of our manuscript for publication in *JoVE*. We would also like to thank the reviewers for their constructive comments, which we addressed below point by point and in the revised version of the manuscript.

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

We used file *62541_R1_RE.docx* provided in the Editorial Manager.

2. Please address specific comments marked in the manuscript.

We addressed all specific comments marked in the provided version (62541_R1_RE) of the manuscript.

3. Please address reviewers' comments as well.

We addressed the reviewers' comments below.

4. Once done, please highlight 3 pages of the protocol section including headings and spacing to be used for filming purposes. Please ensure that the highlighted section forms a cohesive narration.

We highlighted 3 pages of the protocol section for filming purposes in yellow.

5. Please proofread the manuscript well before submission.

We proofread the manuscript again and did not find any errors.

Reviewer #1:

Accept manuscript Organotypic Slice Cultures as Preclinical Models of Tumor Microenvironment in Primary Pancreatic Cancer and Metastasis.

We appreciate that reviewer 1 suggests acceptance of our revised manuscript.

Reviewer #3:

Major point 1:
1. Measurement of cell viability.
The Resazurin assay actually measured mitochondria functions. Though in cell cultures, It has been used as readout for cell viability, the authors should acknowledge the limitations of this assay in their result section. In addition, they need provide complementary measurements. For example, in each tissue slice, they could measure total nuclei numbers/field of view, then calculate the average from five different fields.

We agree with reviewer 3 that viability measurement by the resazurin assy only gives a general assessment of overall viability based on the reduction of oxidized non-fluorescent blue resazurin to red fluorescent resorufin in living cells. We modified the respective sentence in the results section to acknowledge this limitation (page 10, lines 402 ff.: ”The resazurin viability assay measures general metabolic activity of the OTSC based on the reduction of non-fluorescent blue resazurin to red fluorescent resorufin in living cells.”). We realize that numerous complementary methods can be applied to additionally measure viability of OTSCs. As described in our manuscript, we additionally assessed the viability of specific cells in the tissue context by HE staining and immunohistochemistry for cleaved caspase 3 as a marker for apoptosis. As suggested by reviewer 3 numerous additional methods can be applied to the established OTSCs by the individual user depending on individual preferences.

Major point 2:
2. Evaluation of microenvironment changes
One major benefit of the tissue slices cultures is the maintenance of tissue structures and microenvironment. However, the changes of stromal cell types have not been investigated. I suggest the authors stain tissues for FAP, SMA, IL6 as markers for fibroblast activities, and CD3, CD4, CD8 for T cell functions, using the tissues studied in Fig3-6. This staining will provide valuable information to readers and help them determine whether and how the method could help their own researches.

The method of cultivation of tumors *ex vivo* as OTSCs as described in our manuscript can be applied to a wide range of research questions in the field of cancer research. We agree with reviewer 3 that a major benefit of OTSCs is the maintenance of tissue structure and microenvironment *ex vivo*. Analyzing fibroblast activity and T cell function are definitely highly interesting research questions for which the method described in our protocol can be used. However, analyzing fibroblast activity and T cell function within the submitted protocol is beyond our scope of describing the method of establishment and cultivation of OTSCs from fresh tumor tissue. We suggest both analyses to be part of subsequent work by users of the protocol.

Minor point 1:
Please indicate number of experiments repeated and what kind of medium was used in the Figure Legend.

We added the information on the medium used for cultivation and number of slices cultures derived from each individual tumor specimen, which represents the number of experimental repeats, to the figure legends (page 11, lines 454 ff.; changes marked in green).