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# Isolation of proximal fluids to investigate the tumor microenvironment of pancreatic adenocarcinoma

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| **Corresponding Author:** | Nina Cortese<br><br>ITALY |
| **Corresponding Author's Institution:** | |
| **Corresponding Author E-Mail:** | Nina.Cortese@humanitasresearch.it |
| **Order of Authors:** | Greta Donisi |
| | Marialuisa Barbagallo |
| | Giovanni Capretti |
| | Gennaro Nappo |
| | Panteleimon G. Takis |
| | Alessandro Zerbi |
| | Federica Marchesi |
| | Nina Cortese |
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**TITLE:**

# Isolation of Proximal Fluids to Investigate the Tumor Microenvironment of Pancreatic Adenocarcinoma

**AUTHORS AND AFFILIATIONS:**

Greta Donisi[1], Marialuisa Barbagallo[2], Giovanni Capretti[1,3], Gennaro Nappo[1,3], Panteleimon G. Takis[4,5,6], Alessandro Zerbi[1,3], Federica Marchesi[2,7], Nina Cortese[2]

[1]Section of Pancreatic Surgery, Humanitas Clinical and Research Center-IRCCS, Rozzano, Italy
[2]Department of Immunology and Inflammation, Humanitas Clinical and Research Center-IRCCS, Rozzano, Italy
[3]Department of Biomedical Sciences, Humanitas University, Rozzano, Italy
[4]Giotto Biotech S.R.L., Sesto Fiorentino, Florence, Italy
[5]Section of Bioanalytical Chemistry, Division of Systems Medicine, Department of Metabolism, Digestion and Reproduction, Imperial College London, London, UK
[6]National Phenome Centre, Department of Metabolism, Digestion and Reproduction, Faculty of Medicine, Imperial College London, London, UK
[7]Department of Medical Biotechnology and Translational Medicine, University of Milan, Milan, Italy

**EMAIL ADDRESSES OF CO-AUTHORS:**

| | |
|---|---|
| Greta Donisi | (greta.donisi@humanitas.it) |
| Marialuisa Barbagallo | (marialuisa.barbagallo@humanitasresearch.it) |
| Giovanni Capretti | (giovanni.capretti@humanitas.it) |
| Gennaro Nappo | (gennaro.nappo@humanitas.it) |
| Panteleimon G. Takis | (p.takis@imperial.ac.uk) |
| Alessandro Zerbi | (alessandro.zerbi@hunimed.eu) |
| Federica Marchesi | (federica.marchesi@humanitasresearch.it) |
| Nina Cortese | (nina.cortese@humanitasresearch.it) |

**CORRESPONDING AUTHOR:**

Federica Marchesi (federica.marchesi@humanitasresearch.it)
Nina Cortese (nina.cortese@humanitasresearch.it)

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pancreatic adenocarcinoma, pancreatic juice, tumor interstitial fluid, proximal fluid**,** liquid biopsy, biomarkers, proteomics

**SUMMARY:**

Pancreatic juice is a precious source of biomarkers for human pancreatic cancer. We describe here a method for intraoperative collection procedure. To overcome the challenge of adopting this procedure in murine models, we suggest an alternative sample, tumor interstitial fluid, and describe here two protocols for its isolation.

**ABSTRACT:**

Pancreatic adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death, and soon to become the second. There is an urgent need of variables associated to specific pancreatic pathologies to help preoperative differential diagnosis and patient profiling. Pancreatic juice is a relatively unexplored body fluid, which, due to its close proximity to the tumor site, reflects changes in the surrounding tissue. Here we describe in detail the intraoperative collection procedure. Unfortunately, translating pancreatic juice collection to murine models of PDAC, to perform mechanistic studies, is technically very challenging. Tumor interstitial fluid (TIF) is the extracellular fluid, outside blood and plasma, which bathes tumor and stromal cells. Similarly to pancreatic juice, for its property to collect and concentrate molecules that are found diluted in plasma, TIF can be exploited as an indicator of microenvironmental alterations and as a valuable source of disease-associated biomarkers. Since TIF is not readily accessible, various techniques have been proposed for its isolation. We describe here two simple and technically undemanding methods for its isolation: tissue centrifugation and tissue elution.

**INTRODUCTION:**

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive tumors, and soon to become the second leading cause of death[1–3]. It is well-known for its immunosuppressive microenvironment and for its unresponsiveness to immunotherapy protocols[4]. Currently, surgical resection is still the only curative option for PDAC, yet there is a high frequency of early relapses and postsurgical complications. The lack of specific symptoms until an advanced stage does not allow for an early diagnosis, contributing to the deadliness of the disease. Furthermore, the overlap of symptoms between PDAC and other benign pancreatic pathologies can hamper the achievement of a prompt and reliable diagnosis with the current diagnostic strategies. The identification of variables associated to specific pancreatic pathologies could facilitate the surgical decision-making process and improve patient profiling.

Promising results in biomarker discovery have been achieved using easily accessible body fluids, such as blood[5–7], urine[8], saliva[9] and pancreatic juice[10–12]. Many studies have exploited comprehensive "omics" approaches, such as genomic, proteomic and metabolomic techniques, to identify candidate molecules or signatures that could discriminate between PDAC and other benign pancreatic afflictions. We recently demonstrated that pancreatic juice, a relatively unexplored body fluid, can be used to identify metabolic signatures of patients with distinct clinical profiles[12]. Pancreatic juice is a protein-rich fluid, which accumulates the secretome of pancreatic ductal cells and flows to the main pancreatic duct, and then to the main common bile duct. Due to its proximity to the pancreas, it could be strongly affected by microenvironmental perturbations induced by the tumor mass (**Figure 1**), and therefore more informative than blood or urine, or tissue-based profiling. Several studies have explored the potential of pancreatic juice to identify novel biomarkers of disease using various approaches, including cytologic analysis[13], proteomic analysis performed by mass-spectrometry[14,15], assessment of genetic and epigenetic markers such as *K-ras* and *p53* mutations[16,17], alterations in DNA methylation[18], and miRNAs[19]. Technically, pancreatic juice can be collected intraoperatively or with minimally invasive procedures, such as endoscopic ultrasound, retrograde cholangio-pancreatography, or by endoscopic collection of duodenal juice secretion[20]. It is not yet clear to what extent pancreatic juice composition is affected by the collection technique

used. We describe here the intraoperative collection procedure and show that pancreatic juice can represent a precious source for PDAC biomarkers.

[Place **Figure 1** here]

The collection of pancreatic juice in genetic and orthotopic mouse models of PDAC would be appreciated in the perspective to exploit this biofluid in preclinical mechanistic studies; however, this procedure can be technically very challenging and is not feasible for simpler models such as subcutaneous tumors. For this reason, we identified tumor interstitial fluid (TIF) as an alternative source to pancreatic juice, for its similar characteristic of acting as an indicator of surrounding perturbations. Interstitial fluid (IF) is the extracellular liquid, found outside blood and lymphatic vessels, which bathes tissue cells[21]. IF composition is affected by both blood circulation to the organ and local secretion; in fact, surrounding cells actively produce and secrete proteins in the IF[21]. The interstitium reflects microenvironmental changes of surrounding tissues and could therefore represent a valuable source for biomarker discovery in several pathological contexts, such as tumors. The high concentration of locally secreted proteins in TIF can be used to identify candidate molecules to be tested as prognostic or diagnostic biomarkers in plasma[22–24]. Several studies have proven TIF to be a suitable sample for high-throughput proteomic approaches, such as mass spectrometry techniques[23–25], as well as multiplex ELISA approaches[26], and microRNA profiling[27].

Several approaches have been proposed for the isolation of IF in tumors, which can be broadly categorized as in vivo (capillary ultrafiltration[28–31] and microdialysis[32–35]) and ex vivo methods (tissue centrifugation[22,36–38] and tissue elution[39–42]). These techniques have been reviewed in extensive detail[43,44]. The choice of the appropriate method should take into account issues such as the downstream analyses and applications and the volume recovered. We recently used this approach as a proof of principle to demonstrate the different metabolic activity of tumors from two murine pancreatic adenocarcinoma cell lines[12]. Based on literature[24,38], we chose to use the low speed centrifugation method to avoid cell breakage and dilution from intracellular content. Both the amount of glucose and lactate in TIF reflected the different glycolytic characteristics of the two different cell lines. Here we describe in detail the protocol for the two most commonly used methods for the isolation of TIF: tissue centrifugation and tissue elution (**Figure 2**).

[Place **Figure 2** here]

**PROTOCOL:**

For all patients enrolled, peripheral blood, tumor tissue, and adjacent normal tissue were collected at the time of surgery according to protocols approved by the Ethical Committee of the Institution. All the patients were enrolled in the study after signed informed consent including collection of biological specimens and clinical data. The study was approved by the Ethical Committee of the Institution (protocol number ICH-595, approval issued on May 2009). Procedures involving mice and their care were conformed to EU and Institutional Guidelines (protocol ID 121/2016-PR).

**1. Isolation of pancreatic juice**

NOTE: The withdrawal of pancreatic juice is executed in the context of an open procedure of pancreatic resection (e.g., pancreaticoduodenectomy, total pancreatectomy, distal pancreatectomy) by an equipe of expert pancreatic surgeons.

1.1. Patient selection

1.1.1. Consider for the procedure any patient scheduled for an open pancreatic resection.

1.1.2. Confirm inclusion if main pancreatic duct size is deemed sufficient to allow pancreatic juice retrieval. The minimum limit in diameter of the main pancreatic duct is considered 2 mm at contrast-enhanced CT imaging.

1.2. Pre-operative study of pancreatic duct at contrast-enhanced CT imaging to help the planning of pancreatic juice retrieval

1.2.1. Tridimensionally localize the main pancreatic duct within the gland at the level of the pancreatic neck: measure the distance of the main pancreatic duct from the anterior, superior and inferior pancreatic margin on cross-sectional slides, and coronal and sagittal renderings. Once in the operating theatre, use these measurements to approximate the correct place where to puncture the pancreas to cannulate the Wirsung duct and to retrieve pancreatic juice.

1.3. Preparation of material

1.3.1. Sterile material: Open the sterile envelope of one 25 G needle and one 3 mL syringe, and position them in the sterile field with the cooperation of the scrub nurse.

1.3.2. Unsterile material: Keep a 3 mL $K_2$EDTA vacuum test tube ready at hand in the operating room for the storage of the fluid.

1.4. Preparation of the patient

1.4.1. Position the patient on the operating room bed. Induce mixed general anesthesia, using Remifentanil, Sevorane and Rocuronium, then intubate and start ventilating the patient. Position the patient in supine decubitus with the right arm tucked to the body and the left arm abducted to 90° degrees secured on an armboard.

1.4.2. Disinfect skin of the abdomen at the site of the incision. Create and maintain a sterile field on the abdomen draping the patient.

1.5. Surgical procedure

1.5.1. Perform a subcostal incision and gain access to the abdominal cavity. Position a Rochard abdominal retraction for organs exposure.

1.5.2. Expose and mobilize the pancreas through Kocher maneuver, opening of the gastrocolic ligament, incision of the retroperitoneal tissue along superior and inferior border of the pancreas creating a dissection plane between pancreatic neck and superior mesenteric vein located posteriorly.

1.5.3. Once the pancreas is mobilized and exposed, proceed to pancreatic juice withdrawal before sectioning of pancreatic neck.

1.6. Identification and localization of the pancreatic duct

1.6.1. Estimate the location of the pancreatic duct using the measurement taken at imaging and then palpate the anterior surface of the pancreas to identify its precise location.

1.7. Collection of pancreatic juice

1.7.1. Hold the pancreatic head and the duodenum from underneath and elevate it with the left hand, marking the location of the pancreatic duct with the first digit.

1.7.2. Take hold with the right hand of the 3 mL syringe with the 25 G needle mounted on.

1.7.3. Use the right hand to insert the needle in the pancreas just distal to the left thumb. Decide the depth of penetration and the degree of inclination of the needle based on preoperative measurements and on the perception of having penetrated the duct wall.

1.7.4. Withdraw the juice with the syringe. If it is not possible to retrieve the juice, relocate the needle in the four directions trying to incannulate the pancreatic duct.

1.7.5. Once the pancreatic juice is retrieved, move it outside the sterile field and transfer it to the 3 mL $K_2$EDTA vacuum test tube. Keep at 4 °C until the sample is transferred to the lab and proceed to further processing as early as possible.

NOTE: The volume of pancreatic juice that can be recovered with this procedure varies greatly, ranging approximately from 0.2 mL to 3 mL in our experience. The amount of juice retrieved is highly dependent on the patient: the dimension of the Wirsung duct and the functional status of the pancreas (functioning versus atrophic gland). In our experience there is no expedient that can be used to increase the amount of pancreatic juice retrieved.

**2. Processing of pancreatic juice**

2.1. Centrifuge pancreatic juice at 400 x *g* for 10 min at 4 °C to remove any cells or debris.

NOTE: Pancreatic juice should be clear and transparent in color before centrifugation. Blood contamination during surgery can sometimes occur, making the sample appear murkier and redder in color. Consider excluding such samples from further analyses.

2.2. Recover the supernatant, aliquot and store at -80 °C until further analyses.

**3. Induction of subcutaneous tumors**

NOTE: The murine Panc02 and DT6606 cell lines were obtained from Prof. Lorenzo Piemonti (San Raffaele Diabetes Institute, Milan, Italy) and Prof. Francesco Novelli (Center for Experimental Research and Medical Studies, Torino, Italy) respectively, as previously described[12].

3.1. Growth of tumor cells

3.1.1. Culture Panc02 and DT6606 cells in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS), 2mM L-Glutamine and 1% penicillin-streptomycin antibiotic.

3.1.2. Thaw frozen cells 1-2 weeks before tumor injection, according to the growth rate of the cell line.

3.1.3. Grow cells at 37 °C with 5% $CO_2$ and 95% humidity in sterile conditions.

3.1.4. Detach cells with 0.025% Trypsin/EDTA solution for 5 min at 37 °C when they reach 80% confluency and eliminate trypsin by centrifugation.

NOTE: DT6606 are primary, not immortalized, cells, derived from the LSL-KrasG12D-Pdx1-Cre mouse, and should not be passaged more than 3 times before injection in vivo in order to maintain their original characteristics. It is recommended to thaw DT6606 cells 7-10 days prior to injection.

3.2. Injection of tumor cells in vivo

3.2.1. Trypsinize cells (see step 3.1.4) and wash them once with Phosphate-Buffered Saline (PBS). Eliminate PBS by centrifugation and resuspend cells in fresh PBS before counting.

3.2.2. Count the cells and resuspend them in PBS at a concentration of 0.5-1 x $10^7$ cells/mL in order to have a final concentration of 0.5-1 x $10^6$ cells/100 mL to inject in each mouse. Prepare the cells in excess. Keep the cells at 4 °C or on ice until the end of the procedure.

3.2.3. Group the animals (8-week old female C57BL/6J mice) in different cages according to different cell lines or treatments.

3.2.4. Restrain the animals manually and anaesthetize them using a mixture of Ketamine (80 mg/kg) and Xylazine (10 mg/kg) or according to locally-approved procedures.

3.2.5. Shave the site of injection, usually a flank above a leg, with an electric shaver and carefully clean the site of injection with alcohol.

3.2.6. Pipette up and down the cell suspension with a 1 mL syringe, removing any air bubbles by moving the piston up and down. Attach a 25 G needle to the syringe and push the piston upwards until the cell suspension reaches the needle opening.

3.2.7. Pinch the skin of the flank with flat-tipped forceps and carefully insert the needle at the base of skin fold between the forceps without puncturing the peritoneal cavity or the musculature. To verify the correct position of the needle, gently try to move the tip of the needle sideways under the skin. The needle should move freely.

3.2.8. Slowly inject 100 mL of cell suspension (containing 0.5-1 x $10^6$ cells), clamp gently the site of injection for a few seconds and slowly withdraw the needle without any sideways movement.

3.2.9. Return the mouse back to its cage and monitor the recovery from anesthesia.

3.2.10. Check tumor growth using a caliper for 3-4 weeks. Euthanize the animals when tumors reach approximately 0.5-1 $cm^3$ using $CO_2$ or according to locally-approved procedures.

**4. Isolation of tumor interstitial fluid (TIF)**

4.1. Excision of subcutaneous tumors

4.1.1. Block the limbs of the animals with paper tape and clean the skin with alcohol. Cut the skin open on the abdomen in order to separate it from the peritoneum and go on up to the limbs. Excise the tumor grown under the skin of the flank helping with scissors, clamps and eventually a scalpel.

4.1.2. Weigh the tumor and keep it in a clean tube on ice until following isolation of TIF.

4.2. Isolation of TIF by centrifugation

4.2.1. Cut the tumor in half, rinse the two parts quickly in PBS and blot them gently on filter paper to remove excess of PBS. Carry out these steps as fast as possible to avoid evaporation from the tumor.

4.2.2. Immediately transfer the tumor into a 20 mm nylon cell strainer affixed atop a 50 mL conical tube.

4.2.3. Centrifuge the tube at 400 x *g* for 10 min at 4 °C.

NOTE: This low speed centrifugation preserves cell integrity avoiding contamination of TIF by intracellular compartment. Intracellular content leakage can be tested in downstream applications for example by assessing the presence of intracellular housekeeping proteins, such as ribosomal proteins[25].

4.2.4. Recover the TIF from the bottom of the tube, eventually aliquot it and immediately freeze it on dry ice and store at -80 °C until further analysis.

4.2.5. Optional: Based on the downstream proteomic analysis to be performed, dilute the sample in PBS with a protease inhibitor cocktail to avoid degradation of specific molecules.

NOTE: Depending on the composition of the tumor, in some cases very small tumors do not yield any fluid.

4.3. Isolation of TIF by elution

4.3.1. Cut the tumor into small pieces (≈1-3 $mm^3$) with scissors or a scalpel and rinse carefully with cold PBS.

NOTE: In this step it is very important to work fast and perform minimum manipulation to avoid cell damage.

4.3.2. Transfer the tumor pieces into a 1.5 mL tube and add 500 mL of PBS with a protease inhibitor cocktail to avoid degradation of analytes. Incubate for 1 hour at 37 °C and 5% $CO_2$.

4.3.3. Recover the supernatant and transfer it to a new 1.5 mL tube. Centrifuge at 1,000 x *g* for 5 min at 4 °C to remove any cells from the sample.

4.3.4. Transfer the supernatant to a new tube and centrifuge again at 2,000 x *g* for 8 min at 4 °C.

4.3.5. Transfer the supernatant to a new tube and centrifuge again at 20,000 x *g* for 30 min at 4 °C to remove any debris. Recover the supernatant. Immediately aliquot and freeze TIF sample on dry ice and store at -80 °C until further analysis.

**REPRESENTATIVE RESULTS:**

We followed the procedure described above to obtain pancreatic juice from patients with PDAC (n=31) and other benign pancreatic afflictions (non-PDAC, n=9), including pancreatitis (n=2), papillary-ampulla tumors (n=4), neuroendocrine tumors (n=2), intraductal papillary mucinous neoplasia (IPMN; n=1)[12]. The pancreatic juice samples were then subjected to metabolomic analysis using nuclear magnetic resonance ($^1$H-NMR)[12]. By filtering the broad NMR signals of macromolecules (e.g., lipoproteins, lipids, etc.) we were able to appreciate in detail small molecular weight metabolites, as shown in the 1D Carr-Purcell-Meiboom-Gill (CPMG) spectra (**Figure 3A**). Supervised OPLS-DA analysis showed that the metabolic profile detected in pancreatic juice was able to discriminate between PDAC and non-PDAC patients with an accuracy of 82.4% obtained by cross-

validation (**Figure 3B**). Interestingly, metabolomic analysis performed on plasma samples from the same patients did not yield the same discriminative power (accuracy of 75.2% obtained by cross-validation) (**Figure 3C**). These results indicate that pancreatic juice is a protein-rich sample and suitable for comprehensive "omics" analyses. Furthermore, the superior discriminative power of pancreatic juice compared to plasma suggests that moving "upstream" to samples more proximal to the tumor site could be a successful strategy to identify biomarkers that help singling out PDAC from other pancreatic diseases.

To demonstrate that TIF content reliably reflects changes in the tumor microenvironment, we injected subcutaneously two pancreatic cancer cell lines with opposite glycolytic rate, Panc02 (highly glycolytic) and DT6606 (lowly glycolytic), following the protocol described above. We then isolated TIF from the excised tumors using the low speed centrifugation method described above (see paragraph 4.2). From tumors of weight between 0.25-1 g, we recovered a range of 5-15 mL of TIF, which was then used to quantify the concentration of glucose and lactate. TIF from highly glycolytic tumors contained less glucose and more lactate compared to lowly glycolytic tumors (**Figure 4**). This data shows that TIF can be used as a source for tumor-derived metabolites and changes according to the tumor itself.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Schematic representation of pancreatic juice.** (**A**) Schematic representation depicting the secretion of pancreatic juice into the pancreatic duct and its collection during surgery. The inset shows a close-up of the tumor microenvironment: pancreatic juice collects molecules released by tumor and stromal cells in the pancreatic ducts.

**Figure 2: Schematic representation of tumor interstitial fluid isolation methods.** Schematic illustration of the techniques described in detail in the protocol, namely tissue centrifugation (**A**) and tissue elution (**B**).

**Figure 3: Metabolomic content in pancreatic juice and plasma.** (**A**) $^{1}$H-NMR CPMG spectra of PDAC (n=31, blue) and non PDAC samples (n=9, green) showing details on the small molecular weight metabolites contained in the pancreatic juices. ppm, parts per million. (**B**) Scores of supervised multivariate OPLS-DA analysis on CPMG spectra from pancreatic juice PDAC samples (blue circles) and non-PDAC samples (purple triangles). The analysis segregates the two groups (each one outlined by a spider plot), with an accuracy of 82.4% obtained by cross validation. (**C**) Scores of supervised multivariate OPLS-DA analysis on 1D-NOESY spectra from plasma PDAC samples (n=22, blue circles) and non-PDAC samples (n=7, purple triangles). Accuracy of 75.2% obtained by cross validation. This figure has been modified from Cortese et al.[12] with permission.

**Figure 4: Glucose and lactate content in TIF reflects intrinsic tumor features.** (**A**) Glucose and (**B**) lactate concentration in interstitial fluid, isolated using the tissue centrifugation method, from Panc02 (highly glycolytic, n=10 in **A**, n=9 in **B**) and DT6606 (lowly glycolytic, n=5 in **A**, n=4 in **B**) subcutaneously implanted tumors. Box plots give median, lower quartile, and upper quartile by the box, and minimum and maximum by the whiskers.

**DISCUSSION:**

In this study we have described the technique to intraoperatively collect pancreatic juice, a largely unexplored fluid biopsy. We have recently shown that pancreatic juice can be exploited as a source of metabolic markers of disease[12]. Metabolomic analysis on other liquid biopsies, such as blood[5–7], urine[8], and saliva[9], have shown promising results in discriminating between PDAC and healthy subjects or pancreatitis. Pancreatic juice, however, is directly secreted by pancreatic ductal cells, and due to its close proximity to the tumor site, it could be a more reliable indicator of the changes in the surrounding tissue, even in the very early stages of disease. In fact, whereas H-NMR spectral data of pancreatic juice achieved a discrimination between PDAC patients and other benign pancreatic diseases, plasma samples did not. Therefore, although less accessible than other fluid biopsies, pancreatic juice represents a precious source of clinical biomarkers, and could help in the identification of rapidly evolving diseases. We have described here the intraoperative technique for pancreatic juice collection; however, it could also be performed during minimally invasive procedures, in the perspective of identifying much needed early markers of disease. It remains to be ascertained whether the different collection procedures affect the proteomic composition of pancreatic juice.

Whereas other fluid biopsies are easily accessible in PDAC murine models, the collection of pancreatic juice in vivo can be very challenging if considering genetic or orthotopic models, and is not feasible at all in subcutaneous models. Therefore, in this paper we have suggested the use of tumor interstitial fluid as a valuable and widely applicable alternative. In fact, interstitial fluid composition, similarly to pancreatic juice, is directly affected by alterations in the local milieu. Proteomic profiling on TIF from different tumors, such as hepatocellular[45,46], renal cell[41], ovarian[22,47,48], and breast[42,49] carcinomas, have shown encouraging results in the research for candidate biomarkers. However, there is little overlap in the candidate proteins identified, suggesting that the approach used to isolate TIF, together with the downstream analysis performed and the data collection, might affect the detected analytes. A recent study on squamous cell carcinoma comparing the two TIF isolation methods described here, centrifugation and elution, observed a strong consistency in TIF composition independently of the method used, although centrifugation yielded a higher content of extracellular proteins[25].

Both methods described here for the isolation of TIF, tissue centrifugation and tissue elution, have the advantage of being technically undemanding, rapid, and only require basic lab equipment. Compared to other approaches, such as microdialysis and capillary ultrafiltration, the centrifugation and elution techniques present with the intrinsic limitation of only being feasible ex vivo. It is important to take into account several issues when choosing the appropriate method, such as the analytical purpose of the experiment, the volume recovered and cell breakage. The composition of the tumor should also be considered, as it can influence the volume of TIF recovered. Tissue centrifugation was originally developed for cell-poor and collagen-rich tissues such as cornea[38]; tumors on the other hand, are usually tissues characterized by high cellularity and rich vascularization, both features which increase hydraulic conductivity, and should therefore facilitate the isolation of TIF by centrifugation. However, some tumors, including PDAC, present with an abundant stromal or fibrotic component, rich in extracellular matrix proteins, such as collagens, which tend to retain macromolecules in the tissue[21].

Tissue elution on the other hand, is based on passive diffusion of proteins from the tissue to the eluate, and has been successfully exploited for a wide variety of tumors[43]. Elution grants the recovery of bigger volumes, however proteins will be diluted. For this reason, tissue elution might not be suitable for molecules with very low abundance. Moreover, elution requires a greater degree of manipulation of the tumor, possibly resulting in intracellular content leakage in TIF. This might be irrelevant for biomarker discovery but could introduce a bias if the aim is to determine local production of proteins. The amount of cell breakage should be tested in downstream applications in order to choose the most appropriate method according to the tumor type. This can be performed in several ways, for example by testing the presence of housekeeping proteins, such as ribosomal proteins, in TIF[25]. Another suggested approach is that of comparing the concentrations of selected extracellular substances, such as creatinine or $Na^+$, in TIF and plasma, which should be similar[22]. Intracellular content leakage should also be considered for the centrifugation method; in fact, there is still no general consensus as to what should be considered "low-speed" in order to avoid cell breakage, possibly due to the differences in tumor composition. We chose to use a 400 x *g* speed, since it has been shown that no dilution from intracellular content occurs for g forces <424[38].

Regardless of the approach, TIF represents a precious and widely applicable source to sample the tumor microenvironment. We have shown that it recapitulates tumor-intrinsic features and could be useful for the identification of biomarkers of disease or therapeutic targets.

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**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES:**

1 Costello, E., Greenhalf, W., Neoptolemos, J. P. New biomarkers and targets in pancreatic cancer and their application to treatment. *Nature Reviews Gastroenterology & Hepatology.* **9** (8), 435-444 (2012).

2 Siegel, R. L., Miller, K. D., Jemal, A. Cancer statistics, 2020. *CA: A Cancer Journal for Clinicians.* **70** (1), 7-30 (2020).

3 Neoptolemos, J. P. et al. Therapeutic developments in pancreatic cancer: current and future perspectives. *Nature Reviews Gastroenterology & Hepatology.* **15** (6), 333-348 (2018).

4 Sahin, I. H., Askan, G., Hu, Z. I., O'Reilly, E. M. Immunotherapy in pancreatic ductal adenocarcinoma: an emerging entity. *Annals of Oncology.* **28** (12), 2950-2961 (2017).

5 Mayerle, J. et al. Metabolic biomarker signature to differentiate pancreatic ductal adenocarcinoma from chronic pancreatitis. *Gut.* **67** (1), 128-137 (2018).

6 Bathe, O. F. et al. Feasibility of identifying pancreatic cancer based on serum metabolomics. *Cancer Epidemiology, Biomarkers & Prevention.* **20** (1), 140-147 (2011).

7 Mayers, J. R. et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. *Nature Medicine.* **20** (10), 1193-1198 (2014).

8 Napoli, C. et al. Urine metabolic signature of pancreatic ductal adenocarcinoma by (1)h nuclear magnetic resonance: identification, mapping, and evolution. *Journal of Proteome Research.* **11** (2), 1274-1283 (2012).

9 Sugimoto, M., Wong, D. T., Hirayama, A., Soga, T., Tomita, M. Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. *Metabolomics.* **6** (1), 78-95 (2010).

10 Chen, R. et al. Comparison of pancreas juice proteins from cancer versus pancreatitis using quantitative proteomic analysis. *Pancreas.* **34** (1), 70-79 (2007).

11 Mori, Y. et al. A minimally invasive and simple screening test for detection of pancreatic ductal adenocarcinoma using biomarkers in duodenal juice. *Pancreas.* **42** (2), 187-192 (2013).

12 Cortese, N. et al. Metabolome of Pancreatic Juice Delineates Distinct Clinical Profiles of Pancreatic Cancer and Reveals a Link between Glucose Metabolism and PD-1$^{+}$ Cells. *Cancer Immunology Research.* (2020).

13 Tanaka, M. et al. Cytologic Analysis of Pancreatic Juice Increases Specificity of Detection of Malignant IPMN-A Systematic Review. *Clinical Gastroenterology and Hepatology.* **17** (11), 2199-2211.e21 (2019).

14 Chen, K. T. et al. Potential prognostic biomarkers of pancreatic cancer. *Pancreas.* **43** (1), 22-27 (2014).

15 Tian, M. et al. Proteomic analysis identifies MMP-9, DJ-1 and A1BG as overexpressed proteins in pancreatic juice from pancreatic ductal adenocarcinoma patients. *BMC Cancer.* **8**, 241 (2008).

16 Shi, C. et al. Sensitive and quantitative detection of KRAS2 gene mutations in pancreatic duct juice differentiates patients with pancreatic cancer from chronic pancreatitis, potential for early detection. *Cancer Biology & Therapy.* **7** (3), 353-360 (2008).

17 Rogers, C. D. et al. Differentiating pancreatic lesions by microarray and QPCR analysis of pancreatic juice RNAs. *Cancer Biology & Therapy.* **5** (10), 1383-1389 (2006).

18 Matsubayashi, H. et al. DNA methylation alterations in the pancreatic juice of patients with suspected pancreatic disease. *Cancer Research.* **66** (2), 1208-1217 (2006).

19 Cote, G. A. et al. A pilot study to develop a diagnostic test for pancreatic ductal adenocarcinoma based on differential expression of select miRNA in plasma and bile. *The American Journal of Gastroenterology.* **109** (12), 1942-1952 (2014).

20 Yu, J. et al. Digital next-generation sequencing identifies low-abundance mutations in pancreatic juice samples collected from the duodenum of patients with pancreatic cancer and intraductal papillary mucinous neoplasms. *Gut.* (2016).

21 Wiig, H., Swartz, M. A. Interstitial fluid and lymph formation and transport: physiological regulation and roles in inflammation and cancer. *Physiological Reviews.* **92** (3), 1005-1060 (2012).

22 Haslene-Hox, H. et al. A new method for isolation of interstitial fluid from human solid tumors applied to proteomic analysis of ovarian carcinoma tissue. *PLoS One.* **6** (4), e19217 (2011).

23 Zhang, J. et al. In-depth proteomic analysis of tissue interstitial fluid for hepatocellular carcinoma serum biomarker discovery. *British Journal of Cancer.* **117** (11), 1676-1684 (2017).

24 Sullivan, M. R. et al. Quantification of microenvironmental metabolites in murine cancers reveals determinants of tumor nutrient availability. *Elife.* **8** (2019).

25 Matas-Nadal, C. et al. Evaluation of Tumor Interstitial Fluid-Extraction Methods for Proteome Analysis: Comparison of Biopsy Elution versus Centrifugation. *Journal of Proteome Research.* **19** (7), 2598-2605 (2020).

26 Espinoza, J. A. et al. Cytokine profiling of tumor interstitial fluid of the breast and its relationship with lymphocyte infiltration and clinicopathological characteristics. *Oncoimmunology.* **5** (12), e1248015 (2016).

27 Halvorsen, A. R. et al. Profiling of microRNAs in tumor interstitial fluid of breast tumors - a novel resource to identify biomarkers for prognostic classification and detection of cancer. *Molecular Oncology.* **11** (2), 220-234 (2017).

28 Yang, S., and Huang, C. M. Recent advances in protein profiling of tissues and tissue fluids. *Expert Review of Proteomics.* **4**, 515-529 (2007).

29 Huang, C. M. et al. Mass spectrometric proteomics profiles of in vivo tumor secretomes: capillary ultrafiltration sampling of regressive tumor masses. *Proteomics.* **6** (22), 6107-6116 (2006).

30 Leegsma-Vogt, G., Janle, E., Ash, S. R., Venema, K., Korf, J. Utilization of in vivo ultrafiltration in biomedical research and clinical applications. *Life Sciences.* **73** (16), 2005-2018 (2003).

31 Schneiderheinze, J. M., Hogan, B. L. Selective in vivo and in vitro sampling of proteins using miniature ultrafiltration sampling probes. *Analytical Chemistry.* **68** (21), 3758-3762 (1996).

32 Hardt, M., Lam, D. K., Dolan, J. C., Schmidt, B. L. Surveying proteolytic processes in human cancer microenvironments by microdialysis and activity-based mass spectrometry. *Proteomics Clinical Applications.* **5** (11-12), 636-643 (2011).

33 Xu, B. J. et al. Microdialysis combined with proteomics for protein identification in breast tumor microenvironment in vivo. *Cancer Microenvironment.* **4** (1), 61-71 (2010).

34 Bendrik, C., Dabrosin, C. Estradiol increases IL-8 secretion of normal human breast tissue and breast cancer in vivo. *The Journal of Immunology.* **182** (1), 371-378 (2009).

35 Ao, X., and Stenken, J. A. Microdialysis sampling of cytokines. *Methods.* **38** (4), 331-341 (2006).

36 Ho, P. C. et al. Phosphoenolpyruvate Is a Metabolic Checkpoint of Anti-tumor T Cell Responses. *Cell.* **162** (6), 1217-1228 (2015).

37 Choi, J. et al. Intraperitoneal immunotherapy for metastatic ovarian carcinoma: Resistance of intratumoral collagen to antibody penetration. *Clinical Cancer Research.* **12** (6), 1906-1912 (2006).

38 Wiig, H., Aukland, K., Tenstad, O. Isolation of interstitial fluid from rat mammary tumors by a centrifugation method. *The American Journal of Physiology-Heart and Circulatory Physiology.* **284** (1), H416-24 (2003).

39 Li, S., Wang, R., Zhang, M., Wang, L., Cheng, S. Proteomic analysis of non-small cell lung cancer tissue interstitial fluids. *World Journal of Surgical Oncology.* **11**, 173 (2013).

40 Fijneman, R. J. et al. Proximal fluid proteome profiling of mouse colon tumors reveals biomarkers for early diagnosis of human colorectal cancer. *Clinical Cancer Research.* **18** (9), 2613-2624 (2012).
41 Teng, P. N., Hood, B. L., Sun, M., Dhir, R., Conrads, T. P. Differential proteomic analysis of renal cell carcinoma tissue interstitial fluid. *Journal of Proteome Research.* **10** (3), 1333-1342 (2011).
42 Turtoi, A. et al. Novel comprehensive approach for accessible biomarker identification and absolute quantification from precious human tissues. *Journal of Proteome Research.* **10** (7), 3160-3182 (2011).
43 Wagner, M., Wiig, H. Tumor Interstitial Fluid Formation, Characterization, and Clinical Implications. *Frontiers in Oncology.* **5**, 115 (2015).
44 Haslene-Hox, H., Tenstad, O., Wiig, H. Interstitial fluid-a reflection of the tumor cell microenvironment and secretome. *Biochimica Biophysica Acta.* **1834** (11), 2336-2346 (2013).
45 Hsieh, S. Y. et al. Secreted ERBB3 isoforms are serum markers for early hepatoma in patients with chronic hepatitis and cirrhosis. *Journal of Proteome Research.* **10**, 4715-4724 (2011).
46 Sun, W. et al. Characterization of the liver tissue interstitial fluid (TIF) proteome indicates potential for application in liver disease biomarker discovery. *Journal of Proteome Research.* **9** (2), 1020-1031 (2010).
47 Haslene-Hox, H. et al. Increased WD-repeat containing protein 1 in interstitial fluid from ovarian carcinomas shown by comparative proteomic analysis of malignant and healthy gynecological tissue. *Biochimica Biophysica Acta.* **1834** (11), 2347-2359 (2013).
48 Wang, T. H. et al. Stress-induced phosphoprotein 1 as a secreted biomarker for human ovarian cancer promotes cancer cell proliferation. *Molecular & Cellular Proteomics.* **9**, 1873-1884 (2010).
49 Gromov, P. et al. Up-regulated proteins in the fluid bathing the tumour cell microenvironment as potential serological markers for early detection of cancer of the breast. *Molecular Oncology.* **4** (1), 65-89 (2010).

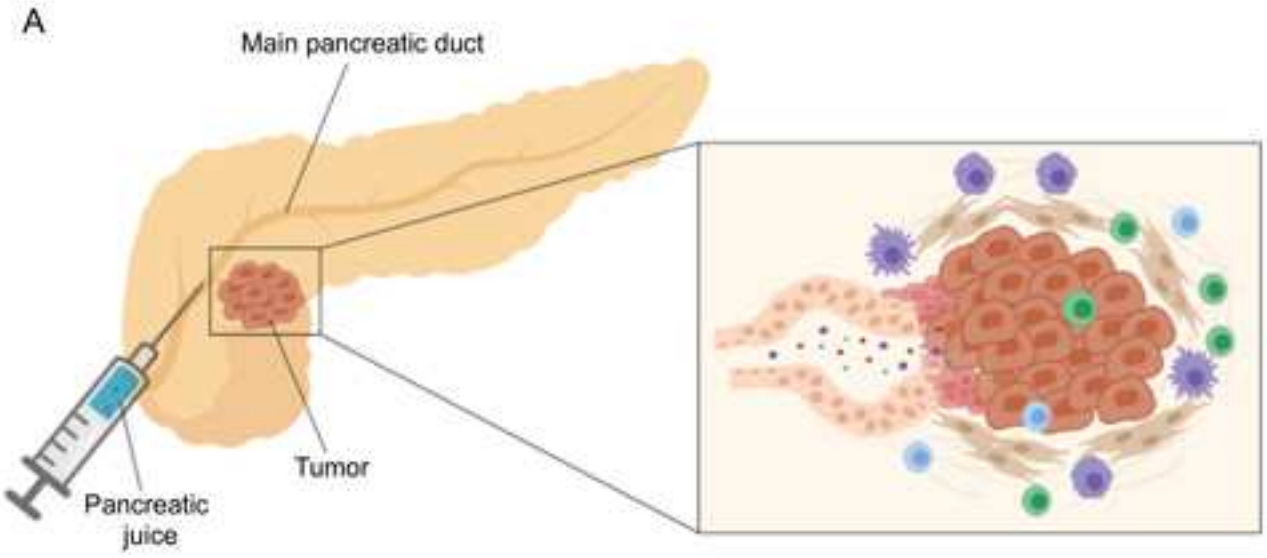


Figure 1

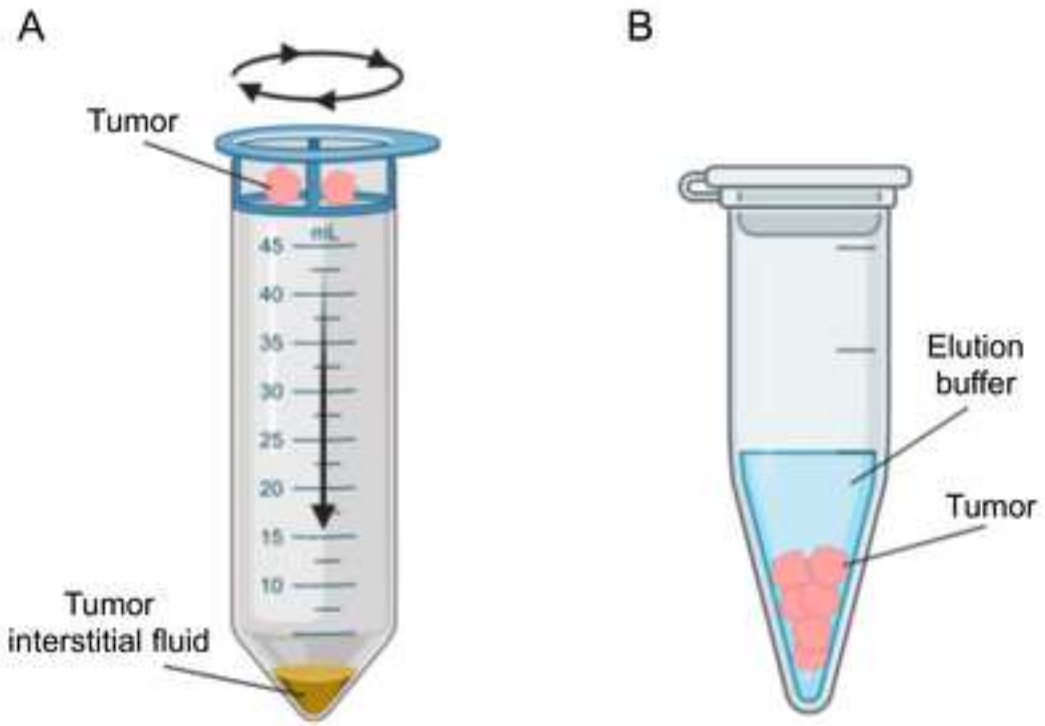


Figure 2

Figure 3

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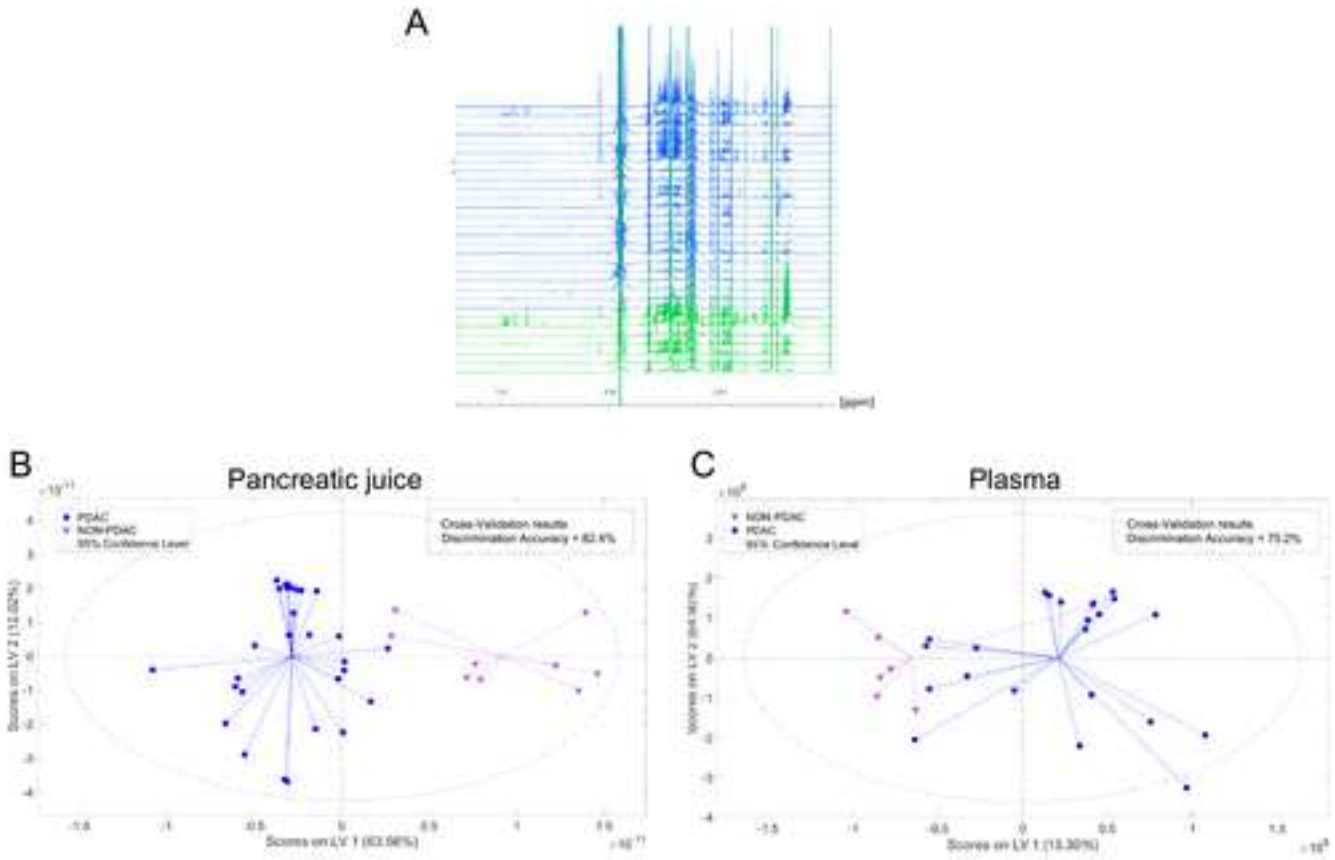


Figure 3

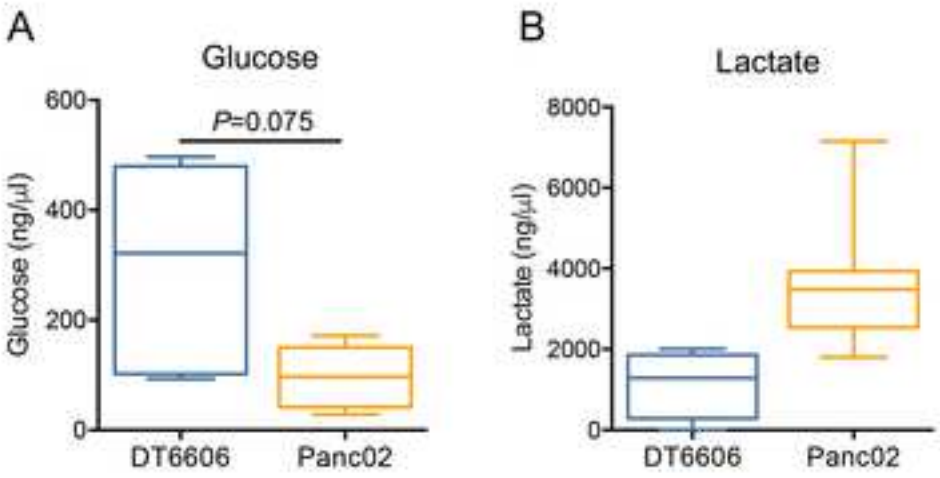


Figure 4

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 1 mL syringe | BD Biosciences | 309659 | |
| 1.5 mL Eppendorf tube | Greiner BioOne | GR616201 | |
| 20 µm nylon cell strainer | pluriSelect | 43-50020-03 | |
| 25G needle | BD Biosciences | 305122 | |
| 3 mL $K_2$EDTA vacutainer | BD Biosciences | 366473 | |
| 3 mL syringe | BD Biosciences | 309656 | |
| 50 mL Falcon tube | Corning | 352098 | |
| Clamps | Medicon | 06.20.12 | |
| Disposable scalpel | Medicom | 9000-10 | |
| Fetal bovine serum | Microtech | MG10432 | |
| Flat-tipped forceps | Medicon | 06.00.10 | |
| Penicillin-Streptomycin | Lonza | ECB3001D | |
| Phosphate-Buffered Saline (PBS) | Sigma-Aldrich | D8537 | |
| Protease inhibitor cocktail | Roche | 34044100 | |
| RPMI medium | Euroclone | ECB9006L | |
| Scissors | Medicon | 02.04.09 | |
| Trypsin/EDTA 1x | Lonza | BE17-161F | |
| Ultraglutamine 100x | Lonza | BE17-605E/U1 | |

HUMANITAS
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Milan, July 20th 2020

To the attention of the

Editorial Board of *Journal of Visualized Experiments*

we are grateful to this Editorial Board for the opportunity to revise our manuscript entitled “Isolation of proximal fluids to investigate the tumor microenvironment of pancreatic adenocarcinoma”, by Donisi et al.

We carefully read the Reviewers’ comments and found them helpful and valuable in order to ameliorate our manuscript. We agree with the criticisms raised by the two Reviewers, and we addressed their questions. Please find enclosed our point-by-point reply, indicating in which page and line we have modified the text to address the Reviewers’ comments. Page and line number refer to the revised version of the corrected manuscript, which includes the tracked changes. All changes in the revised text have been underlined.

Yours sincerely,

Nina Cortese, PhD
Department of Immunology and Inflammation
Humanitas Clinical and Research Center-IRCCS
Via Manzoni 56, 20089 Rozzano, Milano, Italy
Phone: +39 02 8224 2444, 45, 46

Federica Marchesi, PhD
Department of Immunology and Inflammation
Humanitas Clinical and Research Center-IRCCS
Assistant Professor Pathology and Immunology
Department of Medical Biotechnology and Translational Medicine
University of Milan
Via Manzoni 113, 20089 Rozzano, Italy
Phone. +39 0282245113

HUMANITAS
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***Editorial comments:***

*• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.*

We thank the Editor and the Reviewers for having provided us with a useful critical evaluation of our work. We carefully went through the manuscript and corrected all spelling mistakes.

*• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.*
*• Examples NOT in the imperative: 1.5.1-1.5.4, 3.1.2,*

We changed all protocol text to the imperative form.

*• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added 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.*
*1) 1.3: Mention patient prep steps briefly. Include positioning, draping, anesthesia, initial incisions and tools used.*

We included two new protocol steps (1.4-1.5, page 4) to include a brief description of patient preparation and initial surgery steps.

*2) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.*

We moved the ethics statement to the start of the protocol section as suggested (page 3).

*• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.*
*1) **Some implantation steps should be included to ensure continuity.***

We understand the Editor's concern, however it is very complicated for us to film inside the animal facility, and for this reason we had excluded this section of the protocol from highlighting. Considering that the procedure of implantation of subcutaneous tumors is a standard one, and not the focus of this manuscript, we would like to ask the editors if a cartoon of the procedure could be a suitable solution to ensure continuity in the video.

*2) The highlighting must include all relevant details that are required to perform the step. 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 included in the highlighting.*

*3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.*
*4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.*
*5) Notes cannot be filmed and should be excluded from highlighting*

We went through the text and modified the highlighting as suggested by the editors.

• ***Discussion:*** *JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.*

Following the Reviewers' suggestions, we have broadened the Discussion section to include critical issues of the protocol related to volume recovery (page 11 line 441) and contamination from intracellular content (page 11 line 457).

• ***References:*** *Please spell out journal names.*

We provided to modify the reference formatting.

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***Reviewers' comments:***

***Reviewer #1:***

*The manuscript by Donisi et al. is nicely written and provide an interesting procedure for isolation and use of proximal fluids from PDAC tissues. These fluids appear to be quite informative of the biological features of the tumor and might therefore be considered as an alternative to tissue-based profiling.*
*Overall, I think that the protocol will be very useful for the scientific community, especially for those working in the field of pancreatic cancer.*
*I have several suggestions to improve comprehensibility of the protocol (see specific points). As a general comment, the authors should keep in mind that the average reader has no experience with this type of procedures described and might not be familiar with pancreatic cancer. Accordingly, I think that the authors should provide more details when it comes to QC steps (e.g., how to control for cell preservation) of the procedure as well as with the regard to the amount of fluid that can be recovered from tissues (e.g., volume of fluid starting from x mg of tissues or what to expect from a necrotic vs non-necrotic tissue). I would also add more information on the type of downstream analyses that can be conducted on those type of materials.*

We received this comment as very positive, supporting the novelty of our approach and the potential clinical implications. We found the Reviewer's comments very helpful and did our best to address the issues raised in order to improve our manuscript.

*Specific points*

** Abstract: add cancer-related death to the first sentence that reads "the fourth leading cause of death".*

We inserted “cancer-related death” in the abstract (page 2 line 53)

** Introduction: I would provide more background to the utility of pancreatic juice as an alternative source of biomarkers as compared to tissue-based profiling*

We modified the Introduction section to mention possible downstream applications of pancreatic juice in order to give the reader an insight on the usefulness of this biopsy (page 2 line 88).

** Introduction, line 93: I think there is a misprint referring to Figure 1*

If the Reviewer refers to [Place Figure 1 here] at page 3 line 97, we followed the journal guidelines for inserting figures in the text.

** Protocol, 1.2.1.: This point is redundant as the same information is present in point 1.1.2.*

We agree with the Reviewer and have now removed this point from the protocol.

** Protocol, 1.2.2: please, explain better the reasons for measuring all those parameters and how this relates to the subsequent steps of the protocol.*

The measurements are important to identify the best point to puncture the pancreas in order to cannulate the Wirsung duct. We added a sentence to specify this in the revised manuscript (page 4 line 156).

** Protocol, 1.5.5.: please, provide a precise indication about the length of time that the pancreatic juice can be kept at 4 C before downstream processing. Is it possible to store at different temperatures (e.g., -20C, -80C) for long time without compromising the content of the pancreatic juice? Not sure this is the rate place, but I think it would be very useful for the reader to understand which type of analyses can be performed on pancreatic juice (proteomics, glycomics etc)*

We agree with the Reviewer that this is an important information. In our experience, leaving the sample at 4°C for 24h before processing does not alter sensibly the concentration of lactate, measured by commercially available colorimetric kit. However, we think this is highly dependent on the stability of the different molecules of interest, and on the sensitivity of the downstream technique, and should therefore be tested by the reader. We think it is generally good practice, to avoid compromising the sample, to process it as soon as possible, and we wrote this in the text (page 5 line 213).

Concerning the possible types of analyses, we only have experience of metabolomic analysis by NMR, however, as previously mentioned, we inserted in the introduction several examples of different published analyses performed on pancreatic juice (page 2 line 88).

** Note, lines 187-188: the range of volume of pancreatic juice that can be retrieved following the described procedure is pretty big. Any suggestions about the possible reason and measures to take to avoid retrieving small volumes?*

The amount of juice retrieved is highly dependent on the patient, e.g. the dimension of the Wirsung duct and the functional status of the pancreas (functioning versus atrophic gland). In our experience there is no expedient that can be used to increase the amount of pancreatic juice retrieved. We inserted this information in the text (page 5 line 217).

** Protocol 2.2.: I think an indication of the size of the aliquots will be very useful. I guess the size depends on the type of downstream analysis, which should be listed here*

We agree with the Reviewer's comment that the aliquot size depends on the type of downstream analysis, and we therefore feel it could be counterproductive to suggest one size. We have added several examples of downstream applications in the Introduction (page 2 line 88), where we think it is more appropriate.

** NOTE, lines 223-224: the authors should clarify why the PDAC cells DT6606 cannot be propagated for more than 3 continuous passages.*

DT6606 are primary, not immortalized, cells, derived from the LSL-KrasG12D-Pdx1-Cre mouse, which display features of early-stage murine PDAC. Therefore, this cell line should not be passaged more than 3 times before injection in vivo in order to maintain their original characteristics. We have modified the text to include this information (page 6 line 256).

** Protocol, 3.2.1: I think the numbering of the appropriate step is different than that reported here (step 2.4). Overall, the procedures describing cell trypsinization and collection should be revisited as it is rather confusing in the actual form.*

We agree with the Reviewer and apologize for the mistake in the text. We have now modified this step to make it clearer to the reader (page 6 line 263).

** Protocol, 3.2.4: I think the authors should acknowledge the possibility that mouse procedure are conducted differently in different countries/institutions. Therefore, I would add something that reads like: "or according to locally-approved procedures". The same comment applies to step 3.2.10*

We accept the Reviewer's criticism and have modified the manuscript as suggested (page 7 line 274 and page 7 line 294).

** Protocol, 4.1.1: describe how you block the limbs of the animal*

We have modified the text to make this step clearer (page 7 line 300).

** Protocol, 4.2.3: Centrifuge the "tube" not the tumor*

We thank the Reviewer for this correction and have modified the text accordingly (page 8 line 315).

** Note, line 280: Preservation of cell integrity is quite an important factor as this will affect interpretation of downstream analyses conducted on fluids. The authors should indicate how to measure preservation of cell integrity; a sort of QC step.*

We agree with the concern raised by the Reviewer, since this is one of the critical issues to consider when choosing the appropriate method and interpreting results. We have now mentioned one possible approach in the Note referred to by the Reviewer (page 8 line 318) and have better addressed this topic in the Discussion section (page 11 line 457).

** Protocol, 4.2.4: Any indication about the amount of fluid that can be recovered starting from a given amount (mg) of tissue or a particular composition of the tissue?*

We are aware that the amount of fluid recovered from a given amount of tissue is an important parameter to take into account, however, in our experience it is very difficult to make a prediction based on the size of the tissue. The amounts of fluid recovered are variable and do not correlate directly with the size or composition of the tumor. We have added the range of tumor weights and TIF volumes referred to our data in the Representative results section to give the reader an indication (page 9 line 373).

** Optional, line 286: please indicate the different type of proteomic analysis that can be conducted on this type of samples*

Following the Reviewer's suggestions, we have inserted examples of the possible downstream proteomic analyses performed on TIF in the Introduction section (page 3 line 110), since we do not think the Protocol section is appropriate for this digression.

** Note, line 289. Please, be more specific about the reasons for not retrieving any fluid. You mention composition but it is not clear what you mean by that; low neoplastic cellularity, fibrosis, necrosis?*

The Reviewer raises an important issue concerning the composition of the tumor. It is still not entirely clear to us why some samples do not yield any fluid. Sometimes this happens with tumors of very small size. This phenomenon has been documented previously by others (*Sullivan MR, Elife, 2019*) on murine PDAC. The composition of the tumor can certainly affect the volume of TIF recovered; for example, densely vascularized tumors should yield higher volumes of TIF, whereas highly fibrotic tumors tend to retain interstitial fluid (*Wiig and Swartz, Physiol Rev, 2012*). We have briefly mentioned in the Discussion section how tumor composition can affect TIF volume (page 11 line 441).

** Note, line 297. Same as in note line 280, the authors should provide indications about means to measure cell integrity/damage.*

As mentioned above, we have indicated one possible suggestion in the Note (page 8 line 318) and have addressed this issue in more detail in the Discussion section (page 11 line 457).

** Protocol, 4.3.2: I think in this step it is more appropriate to use "add" instead of "elute".*

We have modified the text accordingly (page 8 line 339).

** Figure 3C. I would add accuracy for the analysis conducted on plasma*

Following the Reviewer's suggestion, we have now added the accuracy obtained by cross-validation to Figure 3C, in the Representative results section (page 9 line 362) and in the Figure legends (page 10 line 395).

***Reviewer #2:***
*Manuscript Summary:*
*The authors present a protocol to isolate interstitial fluid as an alternative source to pancreatic juice. This protocol has recently been used by the authors in another publication to demonstrate different metabolic activity of tumours from two murine PDAC cell lines.*

*Major Concerns:*
*No major concerns observed.*

*Minor Concerns:*
*1) Within step 3.2.10 (line 257), please indicate the approximate size that the tumour should reach before proceeding to euthanise the animal.*

We agree with the Reviewer's comment and have added this information (page 7 line 293).

*2) Does co-culture of epithelial cancer cell with fibroblasts affect the yield of TIF?*

The Reviewer raises a very interesting point, concerning the yield of TIF and how to increase it. Unfortunately, we do not have experience in co-culturing fibroblasts with epithelial cancer cells prior to implantation *in vivo*, and therefore we are not certain how this can impact the yield of TIF. However, in the context of PDAC, fibroblasts are responsible for an excessive deposition of extracellular matrix proteins, known as desmoplastic reaction (*Hosein AN, Brekken RA & Maitra A, Nat Rev Gastroenterol Hepatol, 2020*). As previously mentioned in response to Reviewer #1, the composition of the tumor can affect the yield of TIF, and collagens in particular tend to retain interstitial fluid (*Wiig and Swartz, Physiol Rev, 2012*). We have briefly mentioned how composition of the tumor can affect the yield of TIF in the Discussion section (page 11 line 441). Based on these premises, it is reasonable to think that co-culture of epithelial cancer cells with fibroblasts, in the context of PDAC, could negatively impact the yield of TIF.

*3) Please name the non-PDAC pathologies that where used (line 314).*

We have provided to list the non-PDAC pathologies used in the study (page 9 line 353).

*4) Have the researcher done a side by side comparison to identify differences in the results obtained when using TIF obtained by centrifugation or elution?*

The Reviewer raises an important issue related to the differences in the results obtained between the two methods. We have not performed this comparison ourselves, and we agree with the Reviewer that this should be tested on PDAC, however a recent paper on squamous cell carcinoma focused on this question specifically (*Matas-Nadal C, J Proteome Res, 2020*); this study observed a strong consistency in TIF composition independently of the method used, although centrifugation yielded a higher content of extracellular proteins. We have mentioned this study in the Discussion section (page 10 line 430).