Application Note

Single Pancreatic Organoid Proteomics Study

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Abstract

In this application note, we focus on characterizing mouse pancreatic ductal organoids and comparing them to the original organ. To this end, we implement a systematic proteome comparison between native tissue and single organoid models. Our goal is to study the extent to which these models recapitulate the original cellular diversity and tissue organization, and the value of using pancreatic ductal organoids as models. To achieve this, we employed the spheroONE to isolate single pancreatic organoids before proceeding with downstream proteomics analyses.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the third deadliest cancer in the United States and Europe, with a poor survival rate below 10% (1). Translational pancreatic research and efficient drug screening are currently hindered by three major bottlenecks: limited access to human tissue for research, incomplete understanding of human pancreatic epithelial physiology, and a lack of

human-relevant disease models (2). Current pancreatic ductal adenocarcinoma models include 2D immortalized cell lines, patient-derived xenografts, and genetically engineered mouse models, which do not recapitulate key features of human disease and fail to predict therapeutic outcomes (3). Despite pancreatic ductal adenocarcinoma being one of the deadliest cancers, research on pancreatic secretory processes remains limited, lacking relevant model systems providing access to the apical membrane of ductal cells. Whereas adherent cell lines are widely used as research standards, they fail to mimic *in vivo* processes. Hence, the development of suitable models to accurately study PDAC biology and treatment response is essential.

Organoid culture (OC) models are known to be suitable alternatives for overcoming these limitations. Improved understanding and characterization of OC systems are crucial to truly validate their potential to replace animal models.

To this end, we combined automated cellular aggregate isolation using spheroONE with high-sensitivity mass spectrometry proteomics measurements. spheroONE is a nanoliter dispenser enabling sorting and isolation of spheroids and organoids. Here, we demonstrated the use of spheroONE for isolating a single or 10 pancreatic organoids. These pancreatic organoids were isolated prior to sample preparation and proteomics analysis by liquid chromatography–mass spectrometry (LC-MS).

Materials and methods

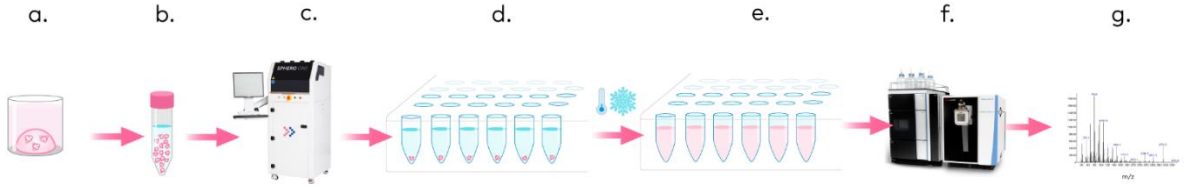

Figure 1: Workflow to prepare single pancreatic ductal organoids for proteomic analysis. Mouse pancreas organoids cultured in Matrigel dome were recovered from the extracellular matrix (a) and re-suspended in PBS (b)**.** The organoid suspension was then processed on a spheroONE instrument to automatically isolate and dispense single organoids (c) in assay-ready 384 well plates pre-filled with lysis buffer (d)**.** After freeze and thaw, samples were lysed and digested (e), then placed into an LC-MS/MS autosampler (f) prior to mass spectrometry analysis (g).

The process for preparing pancreatic ductal organoids for proteomic analysis is briefly described in **Figure 1** and more details are provided in the protocol below.

Generation of mouse pancreas organoid cultures

Mouse pancreatic organoids were obtained using tissue samples collected from sacrificed animals. The detailed procedure is described in the **Supplementary Information (SI 1)**.

Whole pancreas organoid preparation for sorting

Mouse pancreatic ductal organoids were recovered from Matrigel domes using a dedicated recovery procedure following the manufacturer's instructions (Cell Recovery Solution, Corning). Recovered organoids were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich) for 30 minutes and washed three times with PBS. Organoids suspension was stored at 4°C prior to isolation.

Single pancreas organoid sorting

The spheroONE's optical system, including a high-resolution camera, a dark-field illumination module, and a glass nano dispense capillary (NDC), enabled the detection, analysis, sorting, and isolation of particles ranging from 50 to 600 µm in diameter. In this study, a suspension of fixed mouse pancreatic ductal organoids in PBS (~500 spheroids/mL) was processed using spheroONE to isolate organoids with a size range between 50 and 300 µm. Detection and isolation parameters were set as shown in **Table 1** below. Single organoid or exactly ten organoids were isolated and dispensed into individual wells of a 384 well plate (V-bottom low-binding) pre-filled with 5 µl or 10 µL of 0.2% DDM (N-Dodecyl-β-D-maltoside, Sigma-Aldrich), 100mM TEAB (triethylammonium bicarbonate, Thermo Scientific) lysis buffer solution. The plate containing isolated organoids was then frozen prior to sample preparation for LC-MS analyses.

Table 1. spheroONE detection and isolation parameters used for pancreatic ductal organoids isolation

	Min Diameter (µm)	*Max Diameter (µm)*	*Max Elongation*
Detection parameters	*30*	*700*	*4*
Isolation parameters	*50*	*300*	*2.5*

Sample preparation for mass spectrometry

Sample preparation for pancreatic organoids sorted using spheroONE is described in the **Supplementary Information (SI 2)**. Briefly, organoids were lysed, proteins were digested and reduced into peptide fragments. Finally, samples were diluted and acidified before LC-MS injection.

LC-MS/MS measurements

Details of the LC-MS/MS measurements undertaken using an Exploris 480 MS equipped with FAIMS Pro (Thermo Fisher Scientific) are described in the **Supplementary information (SI 3)**.

LC-MS/MS Data Analysis

Raw data were searched against a mouse database (Uniprot reference, 2022-03-04, 21,962 sequences) and a contaminant database (in-house, 344 sequences). Data analysis was carried out using Proteome Discoverer 3.0.0.757, with CHIMERYS as search engine and a fragment mass tolerance of 20 ppm. Variable modifications were set to methionine oxidation. Quantification was performed using apQuant, allowing Match Between Runs. False discovery rate was controlled at 1% for PSM, peptides, and proteins.

Results and discussions

In recent years, *ex vivo* organoid cultures (OCs) derived from tissue-specific adult stem cells or induced pluripotent stem cells have emerged as an important translational bridge between *in vitro* and *in vivo* models of human organ development and disease (4). Although the capacity of pancreatic organoids derived from healthy tissue to recapitulate tissue differentiation and architecture is limited, pancreatic organoids offer an opportunity to develop regenerative medicine therapies for diabetes and disease modelling.

Tissue-specific leucine-rich repeat (LRR)-containing domain G protein-coupled receptor 5-positive (Lgr5+) adult stem cells and pancreatic organoids are models of organ development and disease. Organoids can be cultured long-term in 3D extracellular matrix-based hydrogels. Also, the epithelial cells in these organoids can maintain the cellular diversity and organization of the original organ. These two characteristics make organoid cultures excellent research alternatives to bypass the lack of suitable animal and cellular 2D models. However, despite its great potential, the system's reductionist nature currently limits the use of OCs for more complex research questions (e.g., the effect of stroma on tumor cell drug sensitivity), necessitating improved characterization of OCs. Improved comprehension and characterization of OCs is essential to truly validate their potential to replace animal models. Hence, by combining controlled spheroONE sorting with high-sensitivity mass spectrometry, we aimed to characterize the proteome of mouse pancreatic ductal cell OCs.

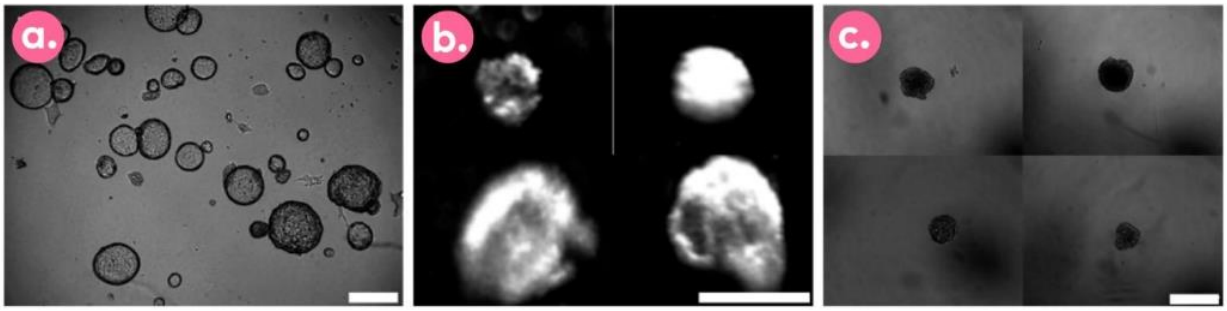

Figure 2. Visualization of pancreatic organoids using spheroONE. a. Micrographs of OCs. **b.** Images of isolated fixed organoids as detected and isolated by the spheroONE instrument. **c.** Micrographs of isolated fixed organoids using the spheroONE instrument. (Scale bar = 200 µm).

The workflow using the spheroONE yielded healthy, individually dispensed single organoids in lysis buffer for subsequent proteome analyses, as visualized in **Figure 2**.

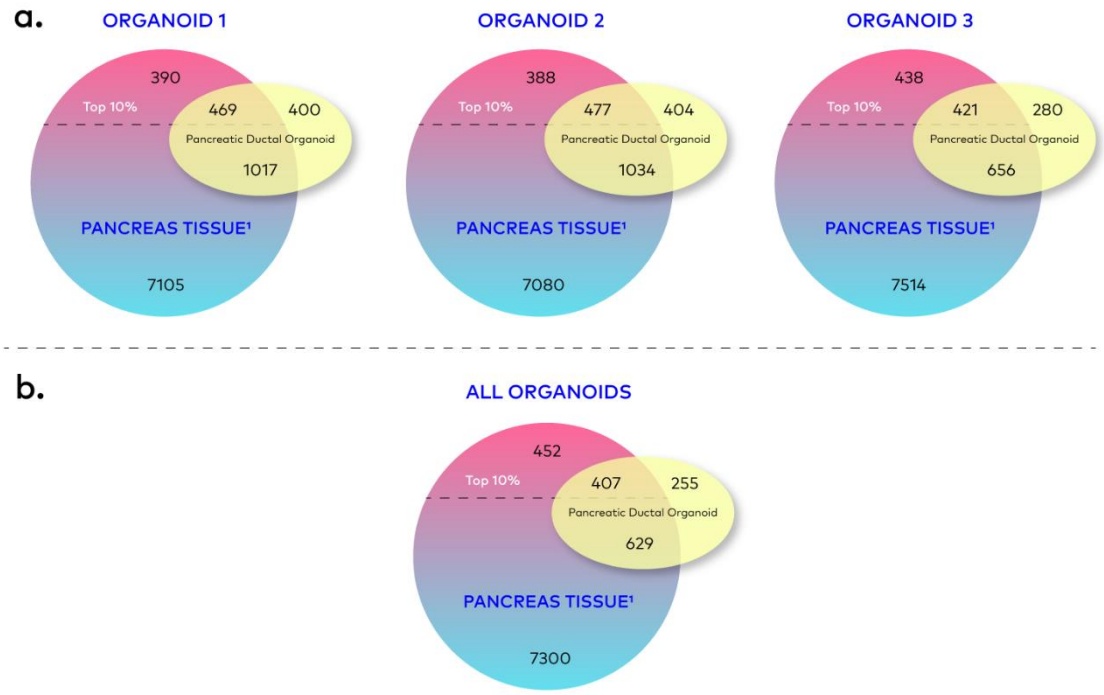

Figure 3. **Comparison of identified proteins in pancreatic ductal organoids compared to previously published primary pancreas tissue[1].** Venn diagrams illustrating the overlap proteins identified between each pancreatic ductal organoid (a., Organoids 1–3, yellow circles) or overlap of the three individual organoid proteomic datasets (b., yellow circle) with those in primary pancreas tissue[1] (a. and b. blue/pink circles). The top 10% of the most abundant proteins across primary pancreas tissue are indicated with a pink shade and a dashed line. Numbers indicate protein counts within each region of overlap or exclusivity.
[1] refers to the dataset published by P. Giansanti et al. (5).

Figure 3 summarizes the number of identified proteins in 3 individual pancreatic ductal organoids by mass spectrometry (Organoids 1–3; yellow circles). Those proteins were compared to a previously published mouse pancreatic tissue dataset by Giansanti, P., et al (5) (blue/pink circles). The three individual pancreatic ductal organoids revealed 1886, 1915, and 1357 proteins, respectively (**Figure 3a**). The overlap of the three datasets revealed that the individual organoids shared 1291 proteins (**Figure 2b**). Interestingly, 80% of the proteins identified in the organoids were also present in the mouse pancreatic tissue proteomic dataset (5). Of the top 10% most abundant proteins identified in pancreas tissue (indicated with pink shade and dashed line), around 50% were also present in the three individual pancreatic ductal organoids (469 of 859, 477 of 865, 421 of 859 in Organoids 1–3, 407 of 859 in the overlap of the three individual organoid proteomic datasets).

Next, cell markers in the isolated mouse pancreatic ductal organoids were assessed using cell fate analysis to confirm cellular identity and validate these organoids as models.

Figure 4 summarizes the results of the cell fate proteomic data analysis. The mouse pancreatic ductal organoids revealed high expression levels of ductal cell markers [epithelial cell adhesion molecule (Epcam), carbonic anhydrase-2 (Ca2), aquaporin-5 (Aqp5), and e-cadherin (cdh1)) as well as the absence of endothelial cell markers [pancreatic polypeptide (Ppy), somatostatin (Sst), chromogranin (Chga and Chgb)], acinar cell markers [chymotrypsin-like elastase (Cela2a and Cela3b), regenerating islet-derived 2 (Reg2), amylase (Amy1a, Amy1b, and Amy1c)], and vascular marker endothelial cadherin (cdh5). These data confirmed that the mouse pancreatic organoids contain exclusively ductal epithelial cells, with no contamination from other pancreatic lineages. This directly supports their relevance as ductal organoid models.

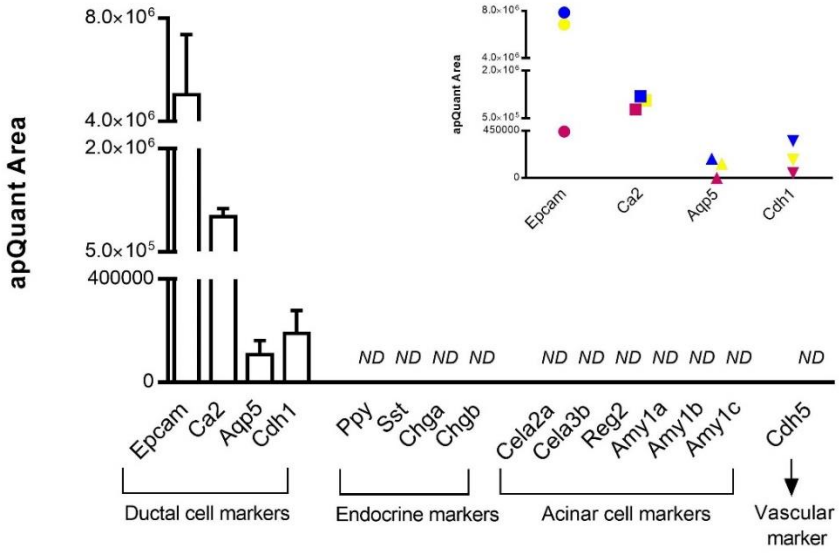

Figure 4. Proteomic cell marker analysis of mouse pancreatic ductal organoids. Quantitative apQuant Area values indicating the enrichment of key ductal, endocrine, acinar, and vascular cell markers detected in single pancreatic ductal organoids. Ductal markers (Epcam, Ca2, Aqp5, Cdh1) display strong protein abundance. Endocrine markers (Ppy, Sst, Chga, Chgb), acinar markers (Cela2a, Cela3b, Reg2, Amy1a, Amy1b, Amy1c), and the vascular endothelial marker (Cdh5) were not detected (ND. The inset in the top right shows individual organoid replicates plotted for the Epcam, Ca2, Aqp5, and Cdh1 ductal markers.

Conclusion and future direction

Here, we demonstrated a complete single organoid proteomic analysis workflow. Mouse pancreatic ductal organoids were readily processed by spheroONE, yielding isolated, individually dispensed organoids in lysis buffer for subsequent proteome analyses. Proteomes' comparison from isolated organoids showed that (i) organoids specifically expressed ductal cell markers, and (ii) high correlations with the reference dataset. This short study demonstrated the use of spheroONE for sorting and isolating single organoids before analysis, providing the basis for systematic proteome analyses of OCs to improve our understanding of these complex *in vitro* models.

References

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Supplementary Information

SI 1. Generation of mouse pancreas organoid cultures

Mouse pancreatic tissue samples were collected from a sacrificed animal. The collected tissue samples were placed in splitting media (Table 1), and enzymatic digestion was performed at 37 °C in digestion media (Table SI 1). The efficiency of tissue digestion was confirmed by stereomicroscopy every 10 minutes. The digested cell suspension was centrifuged (200 RCF, 10 min, 4°C, Rotor radius: 180 mm). The cell pellet was then washed 3 times in total in wash media (Table 1) and resuspended in Matrigel. Next, 10 µL of Matrigel was placed in each well of a 24-well cell culture plate and incubated for 10 minutes at 37°C to solidify, then 500 µL of feeding media was added to each well (Table 1). For culture splitting/subculturing, Matrigel removal and cell separation were performed simultaneously using a 25V/V% TrypLE™ Express Enzyme in DPBS at 37°C for 15-20 minutes in a vertical shaker, followed by two washes and a plating step as described above.

Component	*Manufacturer/Cat.No.*	*Final cc/volume*
Splitting media		
Adv. DMEM/F-12	*Gibco 12634-010*	*500 mL*
1M HEPES	*Gibco 15630080*	*5 mL (10 mM)*
GlutaMax (100x)	*Gibco 35050061*	*5 mL (1x)*
Primocin (400x)	*Invivogen ant-pm-2*	*1.25 mL (1x)*
Digestion media		
Splitting media	*-*	*20 mL*
Collagenase IV	*Worthington S004188*	*1250 U/mL*
Dispase	*Sigma-Aldrich D4693*	*0.5 U/mL*
Fetal Bovine Serum	*Gibco 10500064*	*0.5 mL (0.25% v/v)*
Trypsin inhibitor	*Sigma-Aldrich T9128*	*1 mg/mL*
Wash media		
Splitting media	*-*	*-*
Fetal Bovine Serum	*Gibco 10500064*	*2.5% v/v*
Antibiotic-Antimycotic solution (100x)	*Gibco 15240062*	*1x*
Kanamycin (100x)	*Gibco 15160047*	*1x*
Voriconazole	*TOCRIS 3760/10*	*2 µg/mL*
Feeding media		

Splitting media	*-*	*19 mL*
L-WRN conditioned media	*-*	*25 mL*
A-83	*TOCRIS 2939*	*500 nM*
mEGF	*Gibco PMG8041*	*50 ng/mL*
hFGF10	*Peprotech 100-26*	*100 ng/mL*
Gastrin I	*TOCRIS 3006*	*0.01 µM*
N-acetylcystein	*Sigma-Aldrich A9165*	*1.25 mM*
Nicotinamide	*Sigma-Aldrich N0636*	*10 mM*
B-27 supplement (50x)	*Gibco 17504001*	*1 mL (1x)*
Y-27632 Rho-Kinase inhibitor	*TOCRIS 1254*	*10.5 µM*
Prostaglandin E2 (PGE2)	*TOCRIS 2296*	*1 µM*
Antibiotic-Antimycotic solution (100x)	*Gibco 15240062*	*1% v/v*
Kanamycin (100x)	*Gibco 15160047*	*1x*
Voriconazole	*TOCRIS 3760/10*	*2 µg/mL*

Table SI 1. Media Formulation

SI 2. Sample preparation for mass spectrometry

5 µL and 10 µL 0.2% DDM (N-Dodecyl-β-D-maltoside, Sigma-Aldrich), 100 mM TEAB (triethylammonium bicarbonate, Thermo Scientific) were added prior to the organoid's isolation for the lysis of 1 or 10 organoid samples, respectively. After sorting and freezing of the plate, 5 freeze/thaw cycles (5 min dry ice, 5 min 95°C) were performed to facilitate organoid lysis. Cellular proteins were reduced at 57°C for 30 min by adding DTT (dithiothreitol) in 0.2% DDM/100mM TEAB to a final concentration of 10mM. Alkylation was achieved at 20 mM final IAA (iodoacetamide) concentration, with incubation at RT for 30 min. Alkylation was quenched by the addition of DTT to a final concentration of 5 mM and incubation for 30min at RT. Digestion was performed with 5 ng/uL trypsin and LysC each overnight at 37°C. Samples were finally acidified to 0.2% TFA with an end volume of 10 µL for 2-organoid samples and 20 µL for 10 organoid samples.

SI 3. LC-MS/MS measurements

Tryptic peptide samples were separated on a Vanquish Neo liquid chromatography system hyphenated to an Exploris 480 equipped with FAIMS Pro (Thermo Fisher Scientific). Peptide separation was achieved by enriching the sample peptides on a 0.5 cm trapping cartridge (Thermo Fisher Scientific, 174500) before separation at 250 nL/min on a 5.5 cm micropillar array prototype column employing a 20 min gradient with a run-to-run time of 40 min. Peptide ionization was performed using an EASY-Spray source (Thermo Fisher Scientific, ES081) equipped with a 10 µm ID-fused silica emitter (Bruker, 1893527) mounted via a spray adapter (MS Wil, PSS2).

Ionized peptides were analyzed using a data-dependent wide window acquisition (WWA) method with an isolation width of 4 m/z. At the MS level, the resolution was set to 120,000, the m/z range to 375-1200, and the normalized AGC target to 300%. A single FAIMS voltage of -50 V was used. Charge states 2-5 were chosen for fragmentation with a minimum intensity of 5000. Fragmented ions were set on an exclusion list with a tolerance of ±10 ppm for 120 s. On the MS/MS level, settings included a resolution of 60,000, 30% HCD collision energy, a maximum ion injection time of 118 ms, a 75% normalized AGC target, and centroid as the data type.

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