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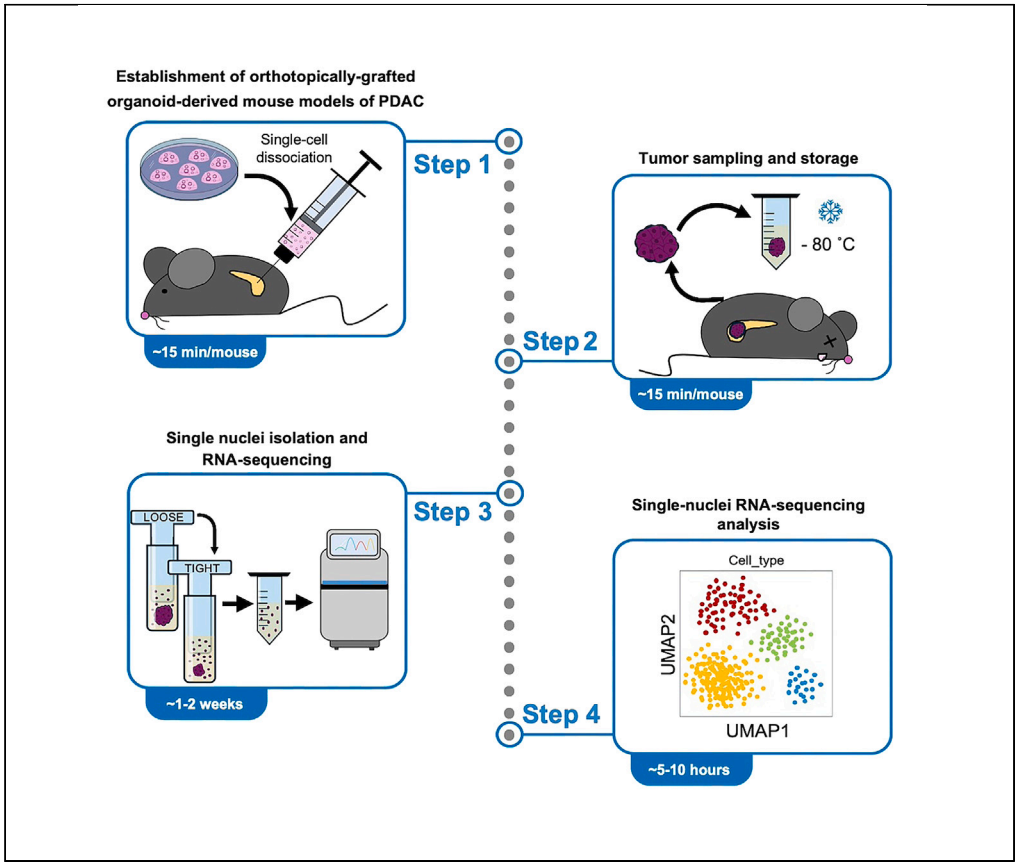
Protocol for the characterization of the pancreatic tumor microenvironment using organoid-derived mouse models and single-nuclei RNA sequencing



Single-nuclei RNA sequencing (snRNA-seq) allows for obtaining gene expression profiles from frozen or hard-to-dissociate tissues at the single-nuclei level. Here, we describe a protocol to obtain snRNA-seq data of pancreatic tumors from orthotopically grafted organoid-derived mouse models. We provide details on the establishment of these mouse models, the isolation of single nuclei from pancreatic tumors, and the analysis of the snRNA-seq datasets.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Organoid-derived mouse models of pancreatic cancer recapitulate its complex stroma

Single-nuclei processing isolates cancer and stromal cells from biobanked tissues

Single-nuclei RNA sequencing enables investigation of pancreatic cancer-stromal crosstalk

Jihad et al., STAR Protocols 5, 103203
September 20, 2024
https://doi.org/10.1016/j.xpro.2024.103203

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Protocol for the characterization of the pancreatic tumor microenvironment using organoid-derived mouse models and single-nuclei RNA sequencing

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https://doi.org/10.1016/j.xpro.2024.103203

SUMMARY

Single-nuclei RNA sequencing (snRNA-seq) allows for obtaining gene expression profiles from frozen or hard-to-dissociate tissues at the single-nuclei level. Here, we describe a protocol to obtain snRNA-seq data of pancreatic tumors from orthotopically grafted organoid-derived mouse models. We provide details on the establishment of these mouse models, the isolation of single nuclei from pancreatic tumors, and the analysis of the snRNA-seq datasets.

For complete details on the use and execution of this protocol, please refer to Mucciolo et al.[1]

BEFORE YOU BEGIN

The protocol below has been used to study the composition of cancer-associated fibroblasts (CAFs) in pancreatic tumors originated from orthotopically grafted organoid-derived mouse models.[1,2] However, we have also applied the second and third sections of this protocol, with minimal modifications, to generate single-nuclei RNA sequencing (snRNA-seq) datasets of autochthonous tumors from genetically engineered mouse models (GEMMs) of pancreatic ductal adenocarcinoma (PDAC) and of pancreas tissues from mouse models of chronic pancreatitis.

This protocol is divided in four main sections: (1) establishment of orthotopically grafted organoid-derived mouse models of PDAC, (2) tumor sampling and storage, (3) single nuclei isolation and RNA sequencing, and (4) single-nuclei RNA sequencing analysis. Prior to these sections, some initial steps are required, as described below.

Institutional permissions

This protocol involves experiments on live vertebrates - ensure you have all Institutional permissions required. All animal procedures described in this protocol were reviewed by the Cancer Research UK Cambridge Institute (CRUK-CI) Animal Welfare and Ethical Review Body, approved by the UK Home Office and conducted under PPL number PP4778090, in accordance with relevant institutional and national guidelines and regulations.

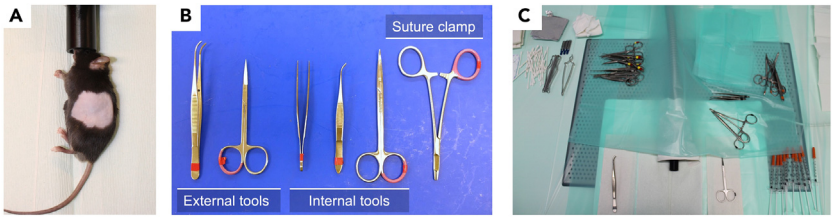

Figure 1. Pre-surgery preparation
(A) A mouse shaved for pancreatic surgery.
(B) A set of surgical instruments.
(C) Surgery table set up with sterile consumables and surgical instruments.

Mouse depilation

Timing: ~15 min per mouse

Shaving is required to ensure the sterility of the orthotopic injection of pancreatic cancer organoids and facilitate its execution. Proceeding with this step one day before the surgery will avoid spreading of fur residues in the operating room during surgery and will enable the completion of large batch surgeries in a few hours. However, this step can also be incorporated in the surgery workflow (see below), avoiding mice to be anesthetized twice. Total timing may vary based on the number of animals to be injected.

1. Anesthetize the animal according to the standard operating procedure (SOP) of your institution.
2. Shave the left flank of the animal with a hair clipper.

Note: Shave as parallel as possible to the skin to avoid lesions.

3. Apply a shaving cream to the same area.
   a. Leave the cream for a maximum of 60 s and then immediately wipe it by using a sterile gauze embedded with distilled water.
   b. Repeat this washing step until the cream has been completely removed.
   c. Dry the area with a sterile gauze (Figure 1A).

⚠ CRITICAL: It is important to remove the shaving cream within 1 min and wash the area with abundant distilled water to avoid any skin irritation.

4. Place the animal on a clean tissue into a recovery chamber pre-set at 37°C until the mouse has fully recovered from the anesthesia.

Note: Skip this last step if the shaving procedure is incorporated in the surgery workflow (see below).

Sterilization of surgical instruments and consumables

Timing: 1 h

Surgical instruments and consumables need to be prepared and sterilized, if not already sterile, before surgery.

Note: While we employ the surgery tools described below, others could also be appropriate.

5. Double-wrap and autoclave one ready-to-use pack per mouse, containing:
   a. Surgical instruments (Figure 1B).
      i. curved forceps (external tool);
      ii. straight surgical scissors (external tool);
      iii. small straight forceps;
      iv. small curved forceps;
      v. curved surgical scissors;
      vi. suture clamp (i.e., needle holder).
   b. Cotton swabs and gauzes to perform hemostasis, if needed.
   c. Tissues and foil for handling non-sterile equipment.

Note: It is important to use one set of sterile surgical instruments per mouse. If you do not have enough sets of surgical instruments to be autoclaved prior to surgery, use a Hot Bead Sterilizer during surgery to sterilize the tips of the instruments between each mouse.

Surgery room preparation

Timing: 15–20 min

This section gives suggestions about the surgery room setup on the day of surgery. Please note that this section may be mainly relevant to Institutions where the Laboratory Animal Science Association (LASA) guiding principles for aseptic techniques need to be followed (i.e., in the UK).

6. On a surgical table place a heat mat (to maintain the animal's body temperature during surgery) and a nose cone mask (for the administration of the anesthetic isoflurane).
7. Clean the table and mat with 70% ethanol and cover with large sterile drapes.
8. Open and carefully drop on the surgical table sterile gloves, sutures (one per mouse), insulin syringes (one per mouse) and the autoclaved packs containing the surgical tools, foil, tissues, gauzes and cotton swabs (Figure 1C).
9. Carefully open and drop on the surgical table also small sterile drapes (approximately 15 × 15 cm) that will be used to cover the mouse to avoid contact of the surgical instruments, gloves and sutures with the animal's fur.
10. With sterile scissors cut a hole (1–2 cm) in the sterile drapes to later expose the area of skin relevant for the injection.
11. Cover a separate bench, close to the surgical area, with a sterile drape and place on it a 200 μL pipette (pre-set at 30 μL and wrapped in autoclaved tin foil), a box of P200 tips and an ice bucket containing the cell aliquots (see 'cell preparation' section).
12. On a separate surgical table set up a pre-operative area with a heat mat and an isoflurane vaporizer machine.
13. Prepare a solution of Iodinated Povidone diluted with saline solution (1:2) and an analgesic solution according to your SOPs.

Note: In this protocol, mice are injected with 5 mg/kg of Carprofen (Rimadyl), but other analgesics could also be appropriate.

14. Turn on a recovery chamber, add clean tissues at the bottom and set the temperature at 37°C.
15. Prepare clean cages to host the animals after the surgery.
    a. Place some food pellets at the bottom of the cage.
    b. Place one Gel Recovery cup containing 0.06 mL of 50 mg/mL Rimadyl.

Note: Mice do not need to be singly housed after surgery.

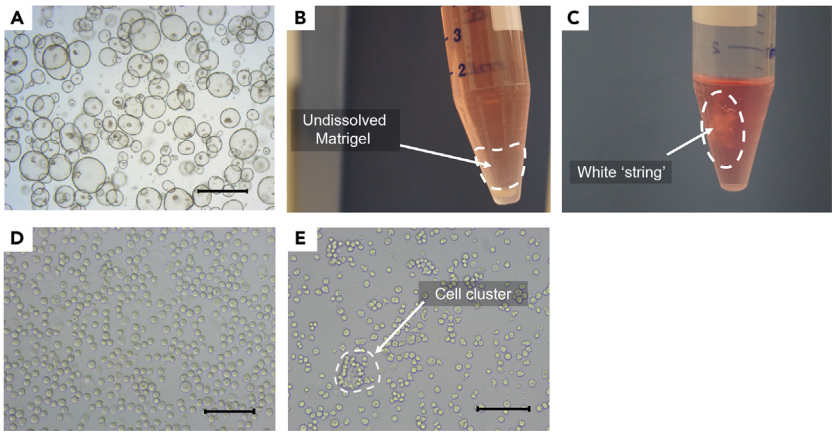

Figure 2. Cell preparation

(A) Bright field image of murine PDAC organoids ready to be harvested for single cell preparation. Scale bar = 200 μm.

(B) Following Step 17 in the "cell preparation" section, the Matrigel may not dissolve properly. In that case, a "cloud" of material is visible above the pellet after centrifugation.

(C) After 10 min incubation with TrypLE, a white "string" of floating material typically appears.

(D) Bright field image of murine PDAC organoids successfully dissociated into single cells. Scale bar = 50 μm.

(E) Bright field image of a low-quality cell preparation with several cell doublets and clusters (scale bar = 50 μm). Results from two different organoid lines are shown in (D) and (E).

Cell preparation

Timing: 2.5–3 h

This section describes all the steps necessary to dissociate PDAC organoids and prepare aliquots of single cells in Matrigel/PBS for orthotopic injection in 8 to 10-week-old C57BL/6J mice (females or males). We used PDAC organoid lines, named T6-LOH and T69A, derived from male (T6-LOH) and female (T69A) KPC ($Kras^{LSL\text{-}G12D/+}$; $Trp53^{LSL\text{-}R172H/+}$; Pdx-1-Cre) mice.[3]

Note: Age and strain of mice can differ. We have established tumors by orthotopically injecting PDAC organoids in C57BL/6J mice from 6 weeks through 80 weeks of age. In addition to C57BL/6J mice, we had equal success with 6-12-week-old Nod Scid Gamma, nu/nu and Rag2 KO mice.

Note: The PDAC organoid lines can also differ. We have orthotopically injected murine primary or metastatic KPC-derived PDAC organoids, CRISPR-modified PDAC organoids that carry additional mutations, and PDAC patient-derived organoids.

Note: To obtain enough cells for the orthotopic injection, we thawed murine organoids and cultured them in Organoid Culture Media for around 2 weeks before surgery. A confluent dome of organoids (Figure 2A) typically contains 50,000 to 100,000 cells.

Note: The overall timing of this section may vary depending on how many organoid lines need to be dissociated.

16. Prepare the Dissociation Media and add it to your organoid cultures to enzymatically digest the Matrigel.
    a. Prepare Splitting Media by adding 1% Penicillin/Streptomycin solution and 0.1% BSA Bovine Serum Albumin (BSA) in Dulbecco's Modified Eagle Medium (DMEM).
    b. Keep it on ice.

Note: For every 8 domes to be dissociated, 60 mL of Splitting Media will be required.

c. Calculate the total volume of Dissociation Media needed by using the following formula: Number of mL required = [(number of domes to dissociate × 0.625) + 2].

Note: Keep in mind that 7 mL is the minimum amount of Dissociation Media required to cover the domes in a 6 cm dish. If you are using a 10 cm dish, the minimum amount required will be 17 mL.

d. Prepare the Dissociation Media by adding the amount of Dispase required in the Splitting Media (i.e., 2 mg per mL of Dispase in Splitting Media).
e. Vortex to completely dissolve the Dispase.
f. Remove the Organoid Culture Media from the dish.
g. Add 500 μL of ice-cold Dissociation Media for each Matrigel dome of organoids cultured in a 6 or 10 cm dish.
h. Incubate at 37°C for 20 min (in a cell culture incubator).

Note: After this step, keep the Dissociation Media at 21°C–23°C.

17. Mechanically disrupt the Matrigel domes.
    a. Detach the domes from the dish using a cell lifter and transfer the domes and buffer into 15 mL low-binding tubes on ice.

Note: Avoid adding more than 8 Matrigel domes per 15 mL tube as this will lead to poor Matrigel digestion and a low-quality single cell preparation.

b. Wash the dish with an appropriate amount (e.g., 5 mL) of ice-cold Splitting Media to ensure maximum collection of organoids.
c. Add this into the same 15 mL low-binding tube(s).
d. Top up the 15 mL low-binding tube with ice-cold Splitting Media and gently pipette 15–20 times with a 5 mL pipette to dissolve Matrigel residues.

Note: If you started with 24 domes, split this solution in three tubes, top up with ice-cold Splitting Media and resuspend again.

e. Centrifuge at 260 *g* at 4°C for 5 min.
f. Carefully remove the supernatant.

⚠ CRITICAL: It is important to remove as much media as possible at this stage to get rid of the Dispase contained in the Dissociation Media, as this may affect the following steps. If, following centrifugation, a cloud of material is visible above the pellet, the Matrigel did not dissolve properly (Figure 2B). In this case, aspirate the media without disturbing the Matrigel, as it contains cells, add 5 mL of ice-cold Splitting Media and pipette gently with a 5 mL pipette to further disrupt any Matrigel residues. Top up the tube with ice-cold Splitting Media and centrifuge as above.

18. Dissociate the organoids into single cells.
    a. Add 1 mL of TrypLE to each 15 mL low-binding tube and gently resuspend your cells by pipetting up and down 15–20 times with a P1000 pipette.
    b. In a thermomixer, incubate the tubes for 10 min at 37°C with agitation at 750 rpm.
    c. Remove the tubes from the thermomixer and keep at 21°C–23°C.
    d. Add 10 μL of 10 mg/mL DNase I and 1 mL of Dissociation Media in each tube.
    e. Resuspend by pipetting up and down 15–20 times with a P1000 pipette.

f. In a thermomixer, incubate the tubes for 20 min at 37°C with agitation at 750 rpm.
g. Top-up the 15 mL low-binding tube(s) with Splitting Media and centrifuge for 5 min at 260 *g* at 4°C.
h. Carefully remove the supernatant.

⚠ CRITICAL: After 10 min of incubation with TrypLE a white "string" of floating material may appear (Figure 2C). As it contains cells, it is essential to completely dissolve it by gently pipetting up and down following the addition of DNase I and Dissociation Media rather than removing it. Moreover, after the centrifugation step, it is important to remove as much media as possible to get rid of the Dispase contained into the Dissociation Media, as this may affect the following steps.

Note: If you have multiple 15 mL tubes containing the same type of cells, you can merge them into one tube at this point by using 1 mL of Splitting Media, top-up with ice-cold Splitting Media and centrifuge again for 5 min at 260 *g* at 4°C.

19. Resuspend in 1 mL of ice-cold Splitting Media on ice and proceed with cell counting.
    a. Resuspend 19.8 μL of cells in an Eppendorf tube containing 2.2 μL of Acridine Orange/Propidium Iodide (AOPI).

Note: Trypan blue can be used as an alternative to AOPI (and should be used if, for example, the organoids express green fluorescent protein), although we find that AOPI enables more precise counting when debris is present.

    b. Pipette up and down before taking 10 μL of solution.
    c. Pipette 10 μL into each side of a counting slide and read both sides on a cell Counter (e.g., LUNA-FL cell counter).
    d. Take note of the viability and number of live cells.

Note: For optimal counting, we recommend the number of cells/mL to be less than 1×10^6.

    e. Calculate the average of the two repeats.

⚠ CRITICAL: While counting, it is important to confirm the presence of mostly single cells (Figure 2D) and no clusters (see troubleshooting problem 1 and Figure 2E), although a few doublets are typically present. It is also essential to have similar counting between the two repeats.

20. Aliquot preparation: for each mouse, prepare one aliquot of 35 μL of PBS:Matrigel (1:1) containing 10,000 cells.
    a. Calculate the total amount of cells required (plus 1 or 2 extra samples).
    b. Transfer the volume needed to a new ice-cold 1.5 mL low-binding tube.
    c. Centrifuge for 5 min at 260 *g* at 4°C.

Note: Place the 1.5 mL low-binding Eppendorf tube on top of an open 15 mL tube to perform the centrifugation in a tabletop centrifuge rather than a mini-centrifuge. This will ensure that the cells are at the bottom (not on the side) of the Eppendorf tube.

    d. After the centrifugation, aspirate as much media as you can without disturbing the pellet.
    e. Calculate the total amount of μL of PBS:Matrigel needed to make all aliquots (μL of PBS:Matrigel = (number of injections + 1 or 2 extra) × 35) and divide it by 2. This will be the total amount of Matrigel required.

Note: The amount of PBS will be the same amount of Matrigel minus 10 μL (to account for the media left over following the centrifugation step above).

f. Add the required quantity of ice-cold and sterile PBS without pipetting.
g. Add the quantity of sterile Matrigel required and pipette up and down with a P1000 to disrupt the pellet and slowly mix the suspension avoiding bubbles.
h. Aliquot 35 μL of solution in ice-cold 1.5 mL low-binding tubes.

⚠ CRITICAL: It is crucial to avoid bubble formation during aliquot preparation by slowly releasing the solution at the bottom of the low-binding tube. Also, always keep the 1.5 mL low-binding tubes on ice, even before the aliquoting, to avoid solidification of the Matrigel.

Note: The number of cells injected can be adjusted according to your experimental goal. For example, 10,000 cells from murine PDAC organoids are appropriate to recapitulate a CAF-rich tumor microenvironment suitable for our studies in 6–8 weeks. However, if you plan to use murine 2D PDAC cell lines, which have typically faster tumor kinetics compared to PDAC organoids, 1,000 cells or less should be injected. Similarly, if you use human PDAC organoids, which can have slower tumor kinetics compared to murine PDAC organoids, you may need to inject more cells (even > 500,000).

Note: The amount of PBS:Matrigel injected can also be reduced (down to 10 μL). However, because this would also reduce the accuracy in terms of number of cells injected between mice (e.g. the error will be greater if 1/10 μL instead of 1/35 μL is not injected). Thus, we do not recommend to make aliquots with a smaller PBS:Matrigel volume if you want to compare tumor kinetics across different cohorts.

Single nuclei isolation bench setup

Timing: 15 min

21. Set up the working bench/hood.
    a. Spray bench, pipets, racks and pipet tip boxes with 70% ethanol, and then RNAse Zap.
    b. Clean Dounce and crushers with 70% ethanol and RNAse Zap.
    c. Rinse them with nuclease free water and leave to dry for approximately 5 min (Figure 3A).

Note: Each sample requires a clean set of Dounce homogenizers. Prepare two sets of Dounce homogenizers if you need to isolate nuclei from more than two samples to save time with the cleaning.

    d. For each sample, place on ice a 50 mL falcon tube with a 40 μm cell strainer, a 5 mL polystyrene tube with a 35 μm cell strainer cap, 2 × 1.5 mL low-binding Eppendorf tubes, and 2 × 1.5 mL Eppendorf tubes for counting (Figure 3B).
22. Prepare lysis buffer and wash buffer (see 'materials and equipment' section) and keep them on ice (Figure 3B).

⚠ CRITICAL: Prepare the lysis buffer and wash buffer right before starting the single nuclei isolation and always keep them on ice.

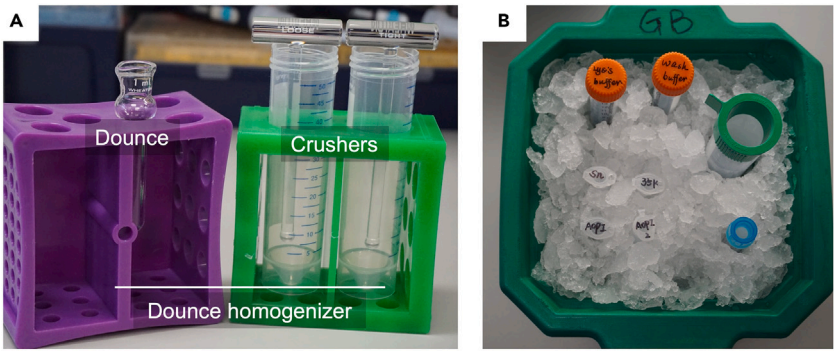

Figure 3. Single nuclei isolation bench/hood setup

(A) Leave the cleaned homogenizer Dounce and crushers with 70% ethanol, RNAse Zap and nuclease-free water to dry for around 5 min.

(B) For each sample, place on ice freshly prepared lysis buffer and wash buffer, a 50 mL falcon tube with a 40 μm strainer, a 5 mL polystyrene tube with a 35 μm cell strainer cap, 2 × 1.5 mL low-binding Eppendorf tubes, and 2 × 1.5 mL Eppendorf tubes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Rimadyl	Zoetis	5414736054155
Videne – iodinated povidone	Ecolab	3030440
Lubrithal – eye gel	Dechra	N/A
Diet gel recovery – 2 oz	Clear H_2O	72-06-5022
Hair remover	Nair	N/A
Advanced DMEM/F12	Thermo Fisher Scientific	12634028
Penicillin/Streptomycin solution	Gibco	15070-063
HEPES	Thermo Fisher Scientific	15630080
GlutaMAX	Thermo Fisher Scientific	35050038
A 83-01	Sigma-Aldrich	SML0788
Mouse EGF recombinant protein	Thermo Fisher Scientific	PMG8043
Recombinant human FGF-10	PeproTech	100-26
Gastrin I (human)	Bio-Techne	3006
Recombinant murine Noggin	PeproTech	250-38
N-Acetyl-L-cysteine (NAC)	Scientific Laboratory Supplies	A9165
Nicotinamide	Sigma-Aldrich	N0636
R-Spondin1-conditioned medium	Boj et al.[2]	N/A
B-27 supplement (50X)	Thermo Fisher Scientific	17504044
DMEM	Gibco	41966029
Bovine serum albumin	Sigma-Aldrich	A9876
Dispase	Gibco	17105-041
Cell lifter	Thermo Fisher Scientific	08-100-240
Protein LoBind tube 1.5 mL	Eppendorf	22431081
Protein LoBind conical tube 15 mL	Eppendorf	30122216
Matrigel (phenol red-free)	Corning	356231
Matrigel (phenol red)	Corning	354230
TrypLE	Thermo Fisher Scientific	12605010
DNase I	Sigma-Aldrich	D5025
PBS	Thermo Fisher Scientific	11593377
Acridine orange/propidium iodide (AOPI)	VWR	CS2-0106-5M
Trypan blue stain 0.4%	Thermo Fisher Scientific	T10282

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Advanced DMEM/F12	Thermo Fisher Scientific	12634028
HEPES	Thermo Fisher Scientific	15630080
GlutaMAX	Thermo Fisher Scientific	35050038
RNaseZAP	Sigma-Aldrich	R2020
IGEPAL	Sigma-Aldrich	I8896
5 M NaCl	Sigma-Aldrich	59222C
1 M Tris-HCl (Trizma hydrochloride solution pH 7.4)	Sigma-Aldrich	T2194
1 M $MgCl_2$	Sigma-Aldrich	M1028
Nuclease-free water	VWR	D092
RNasin plus (40 U/μL)	Promega	N2615
Critical commercial assays		
Chromium Next GEM Single Cell 3′ Kit v3.1	10× Genomics	PN-1000268
Chromium Next GEM Chip G Single Cell Kit	10× Genomics	PN-1000120
Dual Index Kit TT Set A	10× Genomics	PN-1000215
Chromium Next GEM Single Cell 3′ Reagent Kits v3.1 (Dual Index) user guide	10× Genomics	Manual Part CG000315 Rev E
Agilent High Sensitivity D5000 ScreenTape	Agilent Technologies	5067–5588
Agilent High Sensitivity D1000 ScreenTape	Agilent Technologies	5067–5584
Quant-iT dsDNA Assay Kit for high sensitivity	Invitrogen	Q33120
S4 Flow cell Illumina	Illumina	N/A
KAPA Library Quantification Kits – Complete Kit (ROX Low)	Roche	07960336001
Deposited data		
Raw and analyzed single-nuclei RNA sequencing data	Mucciolo et al.[1]	GEO: GSE244142
Mouse reference genome GRCm38 (release 102)	Genome Reference Consortium	http://nov2020.archive.ensembl.org/Mus_musculus/Info/Index
Experimental models: Cell lines		
Mouse: T69A PDAC organoids (5-month-old female KPC GEMM-derived)	Oni et al.[3]	N/A
Mouse: T6-LOH PDAC organoids (6-month-old male KPC GEMM-derived)	Oni et al.[3]	N/A
Experimental models: Organisms/strains		
Mouse: C57BL/6J (females and males)	Charles River Laboratory	Strain number 632
Software and algorithms		
Cell Ranger v7.0.1	10× Genomics	https://github.com/10XGenomics/cellranger
CellBender	Fleming et al.[4]	https://github.com/broadinstitute/CellBender
SOLO	Bernstein et al.[5]	https://docs.scvi-tools.org/en/stable/user_guide/models/solo.html
Scvi-tools	Gayoso et al.[6]	https://github.com/scverse/scvi-tools
Harmony	Korsunsky et al.[7]	https://github.com/immunogenomics/harmony
Harmony implementation in Scanpy		https://scanpy.readthedocs.io/en/stable/generated/scanpy.external.pp.harmony_integrate.html
Scanpy	Wolf et al.[8]	https://github.com/scverse/scanpy
DESeq2	Love et al.[9]	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
Decoupler-py	Badia-i-Mompel et al.[10]	https://github.com/saezlab/decoupler-py
PangloaDB	Franzén et al.[11]	https://panglaodb.se/
Celltypist	Domínguez Conde et al.[12]	https://github.com/Teichlab/celltypist
Python implementation of inferCNV of the https://github.com/broadinstitute/inferCNV Trinity CTAT Project		https://github.com/broadinstitute/inferCNV
infercnvpy		https://github.com/icbi-lab/infercnvpy
MAST R package	Finak et al.[13]	https://github.com/RGLab/MAST
Clusterprofiler R package (v 4.8.2)	Yu et al.[14]	https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Active scavenging unit	VetTech UK	AN005.VS
5-L oxygen concentrator	VetTech UK	AN037/5
Wall/bench anesthetic bracket unit	VetTech UK	AN009
Recovery chamber	VetTech UK	HE011R
PDS Plus antibacterial suture	Ethicon	PDP9102H
Micro-Fine+, U-100 insulin syringes – 0,3 mL, 30G × 8 mm	BD	324826
Surgical drape, 30 × 45 cm	CuraVet	320500
Surgical drape, 60 × 90 cm	CuraVet	320510
Extra narrow scissors 10.5 cm	Fine Science Tools	14088-10
Semken forceps – curved / 13 cm	Fine Science Tools	11009-13
Olsen-Hegar needle holder with scissors 12 cm	Fine Science Tools	12002-12
Graefe forceps – straight/serrated	Fine Science Tools	11050-10
Graefe forceps – curved/serrated	Fine Science Tools	11051-10
Shea scissors – curved/12 cm	Fine Science Tools	14105-12
Dounce homogenizer	Active Motif	40401
40 µm strainer	Greiner	542040
LUNA cell counter	Logos Biosystems	LUNA-FL
PhotonSlides	Logos Biosystems	L12005
5 mL polystyrene tube with 35 µm cell strainer cap	Corning	352235

MATERIALS AND EQUIPMENT

Analgesic stock solution

Reagent	Final concentration	Volume
Rimadyl (50 mg/mL)	1 mg/mL	200 µL
Saline solution	N/A	9.8 mL

Can be stored at 4°C for up to 1 week.

Advanced DMEM/F12 +++

Reagent	Final concentration	Volume
Advanced DMEM/F12	N/A	485 mL
Pen/Strep	1%	5 mL
HEPES	1%	5 mL
Glutamine	1%	5 mL

Can be stored at 4°C for up to 1 month.

Organoids culture media (20 mL)

Reagent	Final concentration	Volume
Advanced DMEM/F12 +++	N/A	17.25 mL
A 83-01	0.5 µM	20 µL
Mouse EGF Recombinant Protein	0.05 µg/mL	20 µL
Recombinant Human FGF-10	0.1 µg/mL	20 µL
Gastrin I (human)	0.01 µM	20 µL
Recombinant Murine Noggin	0.1 µg/mL	20 µL
N-Acetyl-L-cysteine (NAC)	1.25 mM	50 µL
Nicotinamide	10 mM	200 µL
R-Spondin1-Conditioned Medium	10%	2 mL
B-27 Supplement (50X)	2%	400 µL

Can be stored at 4°C for up to 2 weeks.

Splitting media for dissociation of organoids		
Reagent	Final concentration	Volume
DMEM	N/A	493.333 mL
Bovine Serum Albumin solution, 30% in DPBS	0.10%	1.667 mL
Pen/Strep	1%	5 mL
Can be stored at 4°C for up to 1 month.		

Dissociation media (for 5 organoid domes)		
Reagent	Final concentration	Volume
Dispase	2 mg/mL	10 mg
Splitting media	N/A	5 mL
Prepare this freshly on the day of cell preparation and surgery, and keep it on ice.		

Cryopreservation buffer for collecting tissues for nuclei isolation (1 sample)		
Reagent	Final concentration	Volume
Advanced DMEM/F12 +++	40%	200 µL
FBS	50%	250 µL
DMSO	10%	50 µL
Prepare this freshly on the day of tissue collection, and keep it on ice.		

Lysis buffer for single nuclei isolation (2–3 samples)		
Reagent	Final concentration	Volume
Nuclease free water	N/A	3.88 mL
10% IGEPAL	0.10%	40 µL
NaCl (5 M)	10 mM	8 µL
pH 7.5 Tris-HCL (1M)	10 mM	40 µL
MgCl2 (1M)	3 mM	12 µL
RNASein plus (40 U/µL)	0.2 U/µL	20 µL
Keep RNASein plus frozen and add just before processing the sample(s). Prepare this freshly on the day of single nuclei isolation, and keep it on ice.		

Wash buffer for single nuclei isolation (2–3 samples)		
Reagent	Final concentration	Volume
PBS	N/A	3.98 mL
RNASein plus (40 U/µL)	0.2 U/µL	20 µL
Keep RNASein plus frozen and add just before processing the sample(s). Prepare this freshly on the day of single nuclei isolation, and keep it on ice.		

STEP-BY-STEP METHOD DETAILS

Establishment of orthotopically grafted organoid-derived mouse models of PDAC

Timing: ~15 min per mouse

This section describes the orthotopic injection of PDAC organoids in male and female 8 to 10-week-old C57BL/6J mice. The pancreatic tumors formed following this procedure will be used for snRNA-seq.

1. Prepare the animals for surgery.

a. Weigh the animals and calculate the amount of analgesic required.

Note: In this protocol, we inject 5 mg/Kg of Carprofen (Rimadyl) per mouse, but the dose may vary if other analgesic solutions are used. To calculate the volume of analgesic solution required to administer 5 mg/Kg of Carprofen (see 'materials and equipment' section), use the following formula: µL of analgesic solution = animal weight (g) × 5.

b. In the pre-operative space, place the first animal into the anesthetic induction box. Observe the animal and monitor its breathing.
c. When adequately anesthetized, remove the animal from the induction box, lay it on its right side on a heated mat covered with paper towels, and place its face in the anesthetic nose cone.
d. Apply eye gel with a cotton swab on the animal's eyes to prevent them drying out during surgery (since they will remain open).
e. Ear punch the animal for identification purposes, if not done previously.
f. Administer analgesic solution (see 'materials and equipment' section) by subcutaneous injection according to the weight of the animal.

Note: If not done the day before, you can shave the animal at this stage.

g. Prepare the skin with Iodinated Povidone diluted 1:2 with saline solution (Figure 4A).
    i. Clean 3 times from the center of the shaved area outwards with a circular motion using a new sterile cotton swab each time.
    ii. Ensure that any excess of liquid is removed from the skin with a sterile gauze.
h. When ready, move the animal on the heated mat in the surgical area. Ensure that the animal's nose is entirely inside the nose cone and that the anesthesia unit is switched on.

Note: If no one else is assisting you during the animal preparation described above, change gloves at this point prior to proceed to the next steps.

i. Place a pre-cut sterile drape on the mouse to expose only the portion of the skin relevant for the injection (Figure 4B).

Note: Having the head of the mouse outside the drape will enable to monitor its position more effectively in the nose cone.

j. To ensure the mouse is sufficiently anesthetized, check the pedal reflex and monitor its breathing, which should be steady.

2. Cut the skin.
    a. Using the curved forceps (external tools), lift the skin in the middle of the shaved area and make a small incision (approximately 1 cm wide) beneath the lower rib using the straight surgical scissors (Figure 4C).
    b. Gently separate the skin from the body wall using the small forceps and the curved scissors, to better visualize the spleen below the peritoneum (Figure 4D).
3. Locate the spleen. Using the small straight forceps, lift the peritoneum and make a small incision (< 1 cm) above the spleen using the curved scissors (Figure 4E).
4. Gently grasp the spleen and exteriorize it. This procedure will also expose the pancreas, as it is attached to the spleen (Figure 4F).
5. Resuspend the cell aliquot and aspirate it with an insulin syringe.
    a. Gently resuspend the cells with the P200 pipette (pre-set at 30 µL to avoid bubbles formation and wrapped in sterile foil) while holding the 1.5 mL tube containing the cells with autoclaved foil (Figure 4G).
    b. Use a 30G insulin syringe to aspirate the cells, then gently place the syringe on the surgery table.

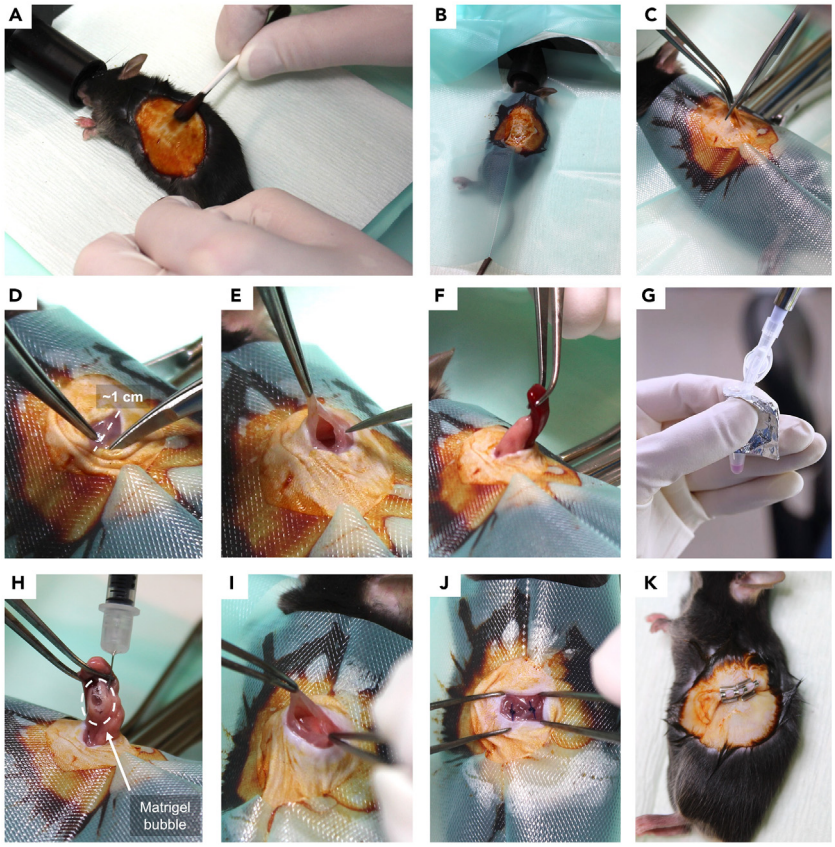


Figure 4. Orthotopic injection of pancreatic cancer organoids

(A) Prepare the skin with Iodinated Povidone diluted 1:2 with saline solution.
(B) Cover the animal with a pre-cut sterile surgery drape to expose the surgery area.
(C) Make a small skin incision (with the external tools) in the middle of the shaved area below the ribs.
(D) Visualize the spleen below the peritoneum.
(E) Change tools and make a small incision in the peritoneum.
(F) Gently grasp the spleen with the curved forceps and exteriorize it together with the pancreas.
(G) Resuspend the cells by touching the tube with a piece of sterile foil to avoid contaminations.
(H) Gently grab spleen and pancreas together with the small curved forceps. Inject 35 μL of cells into the tail of the pancreas.
(I) Reinsert both pancreas and spleen into the body cavity.
(J) Close the peritoneum using 2–3 sutures.
(K) Close the skin by applying 2–3 wound metallic clips.

6. Identify an adequate area of the pancreas for the injection.
   a. Whilst grabbing the spleen and pancreas with the small curved forceps in your non-dominant hand.
   b. Use the straight forceps with your dominant hand to better position the pancreas so to identify an adequate spot for the injection.
   c. Place the straight forceps on the surgery table and take the insulin syringe containing the cells.
7. Inject the cells into the tail of the pancreas while holding up the spleen and pancreas vertically (Figure 4H).

   ⚠ CRITICAL: It is important to see the formation of a "bubble" during the injection (see troubleshooting problem 2).

8. Hold the needle in place for 2–3 s before gently withdrawing it.

a. Gently let go of the pancreas and spleen.
b. Wait 20–30 s before reinserting them into the peritoneal cavity by holding the peritoneum and taking care not to touch the pancreas (Figure 4I).

9. Close the peritoneum with 2–3 absorbable sutures, depending on the size of the incision (Figure 4J).
10. Close the skin by applying 2–3 metallic clips, depending on the size of the incision (Figure 4K).

Note: Skin sutures can replace the use of clips. However, this will increase the time spent during the surgery for each mouse and, consequentially, the time required to complete this procedure for the entire cohort. This will negatively impact the viability of the cells that need to be injected, as they will remain on ice for longer.

11. Place the mouse in the recovery chamber and monitor its breathing. When the mouse has fully recovered from the anesthetic, place it in a clean cage, as detailed above.
12. Repeat for all other mice.

Note: After surgery, give oral analgesic to the mice (e.g. 0.06 mL of 50 mg/mL of Rimadyl dissolved in a Recovery Diet Gel cup) for 72 h. Monitor closely the wound healing and take any actions if required. Remove wound clips (or skin sutures) typically 7–10 days after surgery.

Pause point: 6–8 weeks. After surgery, monitor tumor growth by abdominal palpation and/or ultrasound-based imaging. The PDAC mouse model described in this protocol usually develops tumors in 6–8 weeks. However, by using murine PDAC organoids with different tumor kinetics you may need to sample mice between 4 and 12 weeks. This will typically be longer for human PDAC organoid-derived mouse models.

Note: We advise to monitor tumor growth by weekly palpation and ultrasound-based imaging to harvest tumors when they are around 10–13 mm diameter and avoid the presence of a necrotic/hypoxic core, which is typical of larger tumors and may affect your snRNA-seq results.

Tumor sampling and storage

Timing: ~15 min per mouse

This section describes how to collect and store PDAC tissues for snRNA-seq. These steps can also be followed to harvest and store other tissues (e.g., normal and inflamed pancreas) in the same buffer.

13. Prepare the cryopreservation buffer on the same day of taking the mice (see "materials and equipment" section).
    a. Prepare a cryovial with 500 μL cryopreservation buffer per sample.
    b. Keep on ice.
14. After culling the animal (according to the SOP provided by your institution), spray the mouse with 70% ethanol to avoid fur contamination on your tissues.

Note: We typically collect blood by exsanguination via cardiac puncture under anesthesia using isoflurane, and then perform cervical dislocation to confirm the death of the animal.

15. Using sterile straight scissors, cut the skin and then the peritoneum, exposing the bowels.
16. Carefully remove the tumor using a new set of sterile scissors and forceps.
17. Remove any normal pancreatic tissue attached to the tumor (Figure 5A).
18. By using a scalpel, cut the tumor longitudinally and in the middle to obtain a slice of the tumor core. Then cut the slice again to obtain a small piece (~3 mm^3) of tumor (Figure 5B).

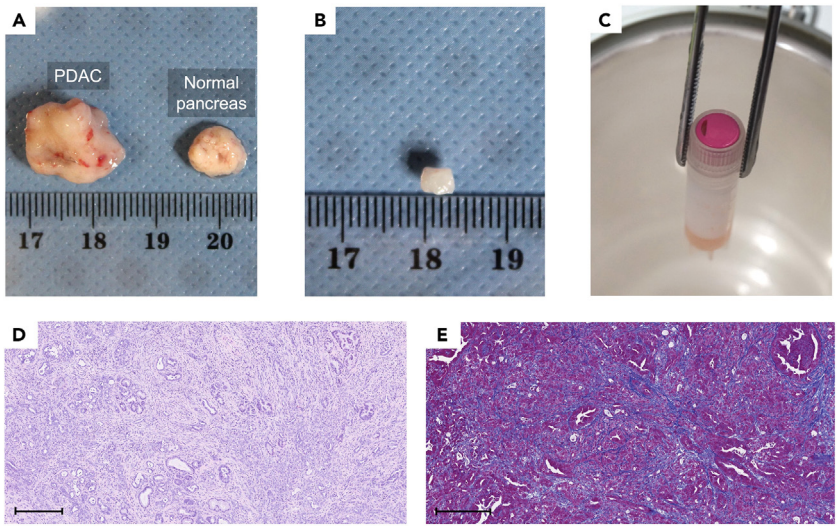


Figure 5. Sampling and histology of orthotopically grafted organoid-derived murine PDAC tissues
(A) Murine PDAC and adjacent normal pancreas samples.
(B) PDAC piece sampled for nuclei isolation.
(C) Image showing how to vertically hold the cryovial containing the PDAC piece in cryopreservation buffer upon initial snap freezing in liquid nitrogen.
(D and E) Representative pictures of Hematoxylin & Eosin (left) and Masson's trichrome stain (right) of formalin-fixed paraffin-embedded murine PDAC tissue sections. Scale bars = 300 μm.

19. Put the piece of tumor in a cryovial with 500 μL cryopreservation buffer and immediately snap-freeze the vial in liquid nitrogen.

⚠ CRITICAL: While snap-freezing, make sure that the tissue is covered by the liquid by holding the cryovial vertically with forceps in the liquid nitrogen (Figure 5C).

Note: We suggest collecting a couple of vials/samples per mouse, if possible, as the single nuclei isolation protocol and submission may need to be optimized for your specific tissue (see below).

20. Move the vial to a −80°C freezer.

Note: A second slice of tumor can be taken, formalin-fixed and paraffin-embedded. This also enables to check the tumor histology with Hematoxylin & Eosin stain (Figure 5D) and the collagen deposition with Masson's Trichrome stain (Figure 5E).

Pause point: You can store the tumor tissues at −80°C until the single nuclei isolation step.

Note: While single nuclei can be obtained from archival samples, such as patient tissues, that have been stored at −80°C for years before snRNA-seq, we suggest proceeding to nuclei isolation and snRNA-seq within 12 months after tissue harvest. This will avoid potential negative effects on the quality of RNA, and cell composition due to longer-term storage.

Single nuclei isolation and RNA sequencing

Single nuclei isolation

Timing: 20 min per sample

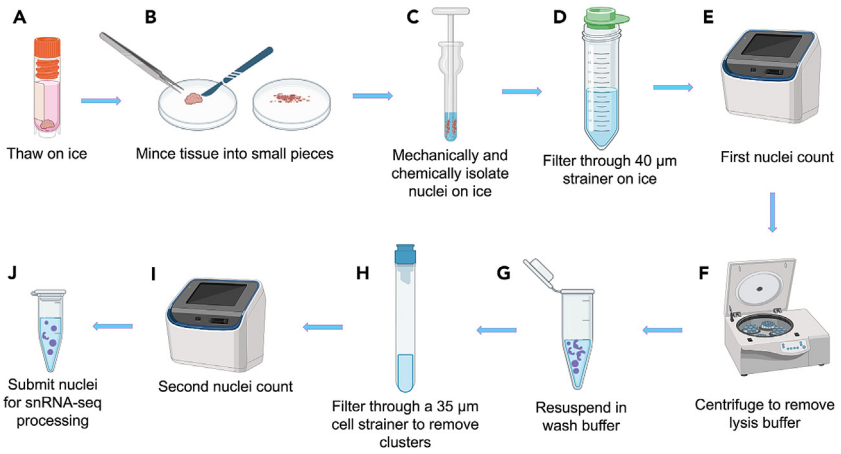


Figure 6. Single nuclei isolation procedure

(A) Thaw the sample on ice.
(B) Mince the tissue into small pieces using scalpel and forceps.
(C) Mechanically and chemically isolate the nuclei on ice using a Dounce homogenizer.
(D) Filter through a 40 µm cell strainer on ice.
(E) Count the nuclei.
(F) Centrifuge the sample to remove the lysis buffer.
(G) Resuspend the nuclei in an appropriate amount of wash buffer based on the first count.
(H) Filter (twice) through a 35 µm cell strainer to remove clusters.
(I) Re-count the nuclei.
(J) Prepare the required number of nuclei in wash buffer and submit for snRNA-seq processing.

This section provides instructions for isolating nuclei from PDAC tissues, which can be used for snRNA-seq. This approach can be applied, with minimal modification, for nuclei isolation from other tissues, such as normal pancreas or chronic pancreatitis tissues.

21. Isolate single nuclei from tissues (Figure 6).
    a. Thaw the frozen tissue on ice (Figure 6A).

    Note: We suggest thawing two samples at a time and process maximum 4–6 samples per sequencing submission to reduce the processing time and limit nuclei aggregation (see troubleshooting problem 3).

    b. Rinse the Dounce homogenizer and crushers with 500 µL ice-cold lysis buffer, and leave them on ice.
    c. Place the tissue on a 10 cm dish on the bench at 21°C–23°C.
    d. Quickly mince the sample into pieces smaller than 1 mm^3 using a scalpel (Figure 6B).
    e. Scoop the tissue into the Dounce homogenizer using the surgical forceps and scalpel.
    f. Use 500 µL of ice-cold lysis buffer to wash the 10 cm plate where the sample was minced, then add this buffer to the Dounce homogenizer.
    g. Incubate 2 min on ice.
    h. Stroke for 5 times with the loose crusher (Figure 6C).

    Note: You can stroke more times if big clumps are still visible.

    i. Incubate 2 min on ice.
    j. Stroke slowly for 10–15 times with the tight crusher.

Note: The number of strokes depends on how homogeneous the solution looks. Also, you would expect to feel some resistance when using the tight crusher but stroke slowly to avoid creating bubbles.

k. Incubate 2 min on ice.
l. During the above incubation step, use 250 μL of wash buffer to pre-wet a 40 μm strainer on top of a 50 mL tube kept on ice (Figure 3B).
m. Filter the sample through the 40 μm strainer by tipping the homogenizer's content on top of the strainer and gently crushing the tissue against the filter using the round end of the tight crusher (Figure 6D).
n. Wash the Dounce homogenizer and strainer with 250 μL ice-cold wash buffer.

Note: After this step, start with the nuclei isolation for the next sample while a second person proceeds with the next steps. Having a second person involved will speed up the process and is recommended to limit nuclei clustering (see troubleshooting problem 3). Clean the Dounce homogenizers between samples, as described in the single nuclei isolation bench setup section. If more than 3 samples need to be processed, having a third person dedicated to wash the Dounce homogenizers will further speed up the process and it is recommended.

22. Prepare single nuclei for RNA sequencing submission.
    a. Check that the tissue has been lysed properly by taking 19.8 μL and mixing with 2.2 μL of AOPI (or by taking 11 μL of nuclei and adding 11 μL Trypan blue).

Note: We find that AOPI is a more accurate and reliable method for nuclei counting compared to Trypan blue, which may give false positive signals due to non-nucleated cells or debris. However, when counting nuclei from tissues that have high autofluorescence, such as the normal or inflamed pancreas, Trypan blue is a better option.

    b. Count nuclei as described above (Figure 6E, and 'cell preparation' section - Step 19).
    c. Transfer the lysed sample into a 1.5 mL low-binding Eppendorf tube kept on ice (Figure 3B).

Note: This first counting step will guide the volume of wash buffer needed in step 22.e below.

    d. Spin down the 1.5 mL low-binding Eppendorf tube with the nuclei at 500 *g* for 5 min (Figure 6F) at 4°C. Remove the supernatant carefully using a P1000 pipette.

Note: Place the 1.5 mL low-binding Eppendorf tube on top of an open 15 mL tube to perform the centrifugation in a tabletop centrifuge rather than a mini-centrifuge. This will ensure that the nuclei are at the bottom (not on the side) of the Eppendorf tube.

    e. Gently resuspend the pellet with an appropriate amount of wash buffer (Figure 6G).

Note: The amount of wash buffer needed depends on the number of nuclei counted (see step 22.b above), and the number of nuclei and volume of wash buffer required for sequencing submission. For example, if 35,000 nuclei are required in 43 μL of wash buffer for RNA-seq submission, an appropriate amount of wash buffer should be added to have more than 814,000 nuclei/mL. This will ensure that the nuclei are not too diluted for the submission step. At the same time, enough wash buffer should be added to avoid the nuclei to be too concentrated (e.g. nuclei should be less than 3×10^6/mL for the example above). This will limit inaccurate counting.

Note: If you have 4–6 samples to process, you may leave the first 2–3 samples on ice for up to 30 min before carrying on with the next steps. This will limit nuclei aggregation after counting prior to snRNA-seq processing.

f. Filter the nuclei through a 35 μm cell strainer cap of a 5 mL polystyrene tube (Figure 6H).
g. Recount nuclei as above (Figure 6I).

⚠ CRITICAL: The concentration of nuclei at this stage should be higher or at least equal to that required for snRNA-seq submission (see above). If the concentration of nuclei is lower than expected, you can spin down again and resuspend the sample in a smaller amount of wash buffer.

h. Aliquot the required number of nuclei in a 1.5 mL low-bind tube, top up with wash buffer to the required volume (e.g., 43 μL) and keep on ice. Samples are ready for snRNA-seq processing with 10X Genomics (Figure 6J).

Note: Do not leave nuclei on ice for longer than 30 min before downstream snRNA-seq processing.

Note: After initial pilot experiments, we submitted 35,000 nuclei of each murine PDAC sample for snRNA-seq. However, the number of nuclei will likely need to be adjusted for other tissues to obtain optimal sequencing datasets.

Single-nuclei RNA sequencing

Timing: 1–2 weeks

This section provides instructions for how snRNA-seq libraries are prepared and sequenced. Please note that this protocol is specific to what is done in our Genomics Core Facility at the Cancer Research UK Cambridge Institute. Appropriate modifications may be applicable elsewhere. Overall, the Chromium Next GEM Single Cell 3′ Kit v3.1, the Chromium Next GEM Chip G Single Cell Kit, the Dual Index Kit TT Set A, and the Chromium Next GEM Single Cell 3′ Reagent Kits v3.1 (Dual Index) user guide (Manual Part CG000315 Rev E; 10X Genomics) are needed.

23. Add isolated nuclei in wash buffer to the 3′ V3.1 RT mastermix using a wide-bore pipette and mix.
24. Load the mixture of nuclei and RT mastermix into Chromium microfluidic chips with 10X Genomics 3′ v3.1 chemistry to generate single-nuclei Gel bead-in-emulsions (GEMs) using the Chromium controller (10X Genomics) according to the manufacturer's recommendations (https://cdn.10xgenomics.com/image/upload/v1660261285/support-documents/CG000204_ChromiumNextGEMSingleCell3_v3.1_Rev_D.pdf).

Note: While we used the Chromium X, which is the newest handler, 10X Genomics still supports previous iterations.

25. Post GEM-formation, the GEMs are visually inspected to confirm the presence of a uniform emulsion as dictated by 10X Genomics 3′ V3.1 user manual (see link above).

Note: Presence of non-uniform GEMs may indicate poor sample quality (i.e. the nuclei tend to clump resulting in fluidic clog on the chip).

26. Reversely transcribe RNA from the barcoded nuclei for each sample in a C1000 Touch Thermal cycler (Bio-Rad).
27. Generate amplified cDNA according to the manufacturer's protocol (see link above) with no modifications.

Note: We used 13 cycles for cDNA amplification in this protocol based on our initial pilot experiments. We suggest a pilot experiment is performed to find the optimal number of cycles for your tissues and conditions.

28. Measure the quality and quantity of cDNA on the Agilent TapeStation 4200 using the Agilent High Sensitivity D5000 ScreenTape.
29. Use 10 μL or 25% of the total cDNA generated for gene expression library preparation.

Note: The profile of the cDNA traces can be used as a quality check before library preparation (Figure 7). However, optimal cDNA concentration can be largely variable between single nuclei samples isolated from different tissues. Thus, we advise to perform pilot studies for your tissue samples.

Note: The total number of cycles for sample indexing is optimized based on the 25% of cDNA carried forward. The following table displays the recommended cycle numbers.

cDNA input (ng)	Cycle numbers
0.25–25	14–16
25–150	12–14
150–500	10–12
500–1,000	8–10
1,000–1,500	6–8
>1,500	5

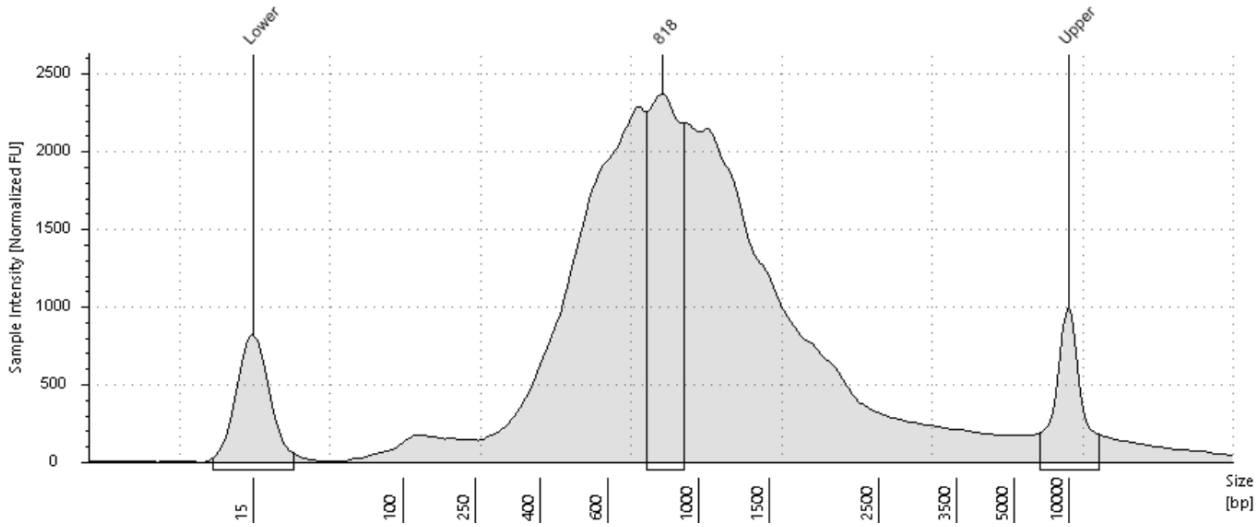
30. Confirm library quality and evaluate the library sizes as quality check prior to sequencing by using the Agilent High Sensitivity D1000 ScreenTape on the Agilent TapeStation 4200.
31. Use the BMG Labtech Clariostar Monochromator Microplate Reader with Quant-iT dsDNA Assay Kit for high sensitivity to evaluate dsDNA quantity.
32. Normalize each sample to equal molar concentration (10 nM) and pool the samples together to generate one sample taken forward for sequencing.
33. Measure the concentration using the KAPA qPCR Illumina kit targeting P5/P7 adapters to determine the optimal loading concentration of the pool.
34. Sequence the pool on an appropriate flow cell on the NovaSeqX or NovaSeq6000.

Note: Which flow cell type and how many lanes to use is determined by calculating the total number of reads required for the pool using the number of nuclei captured, and the number of reads per nuclei required (read depth). For example, we sequenced a pool of 8 samples on 1 lane of a S4 flow cell Illumina NovaSeq 6000 with the following parameters: 28 bp, read 1; 10 bp, i7 index; 10 bp, i5 index and 90 bp, read 2, aiming for 2B reads.[1]

Single-nuclei RNA sequencing analysis

Timing: 5–10 h

This section provides instructions for how snRNA-seq analysis can be performed. Additional steps and appropriate modifications may be applicable.

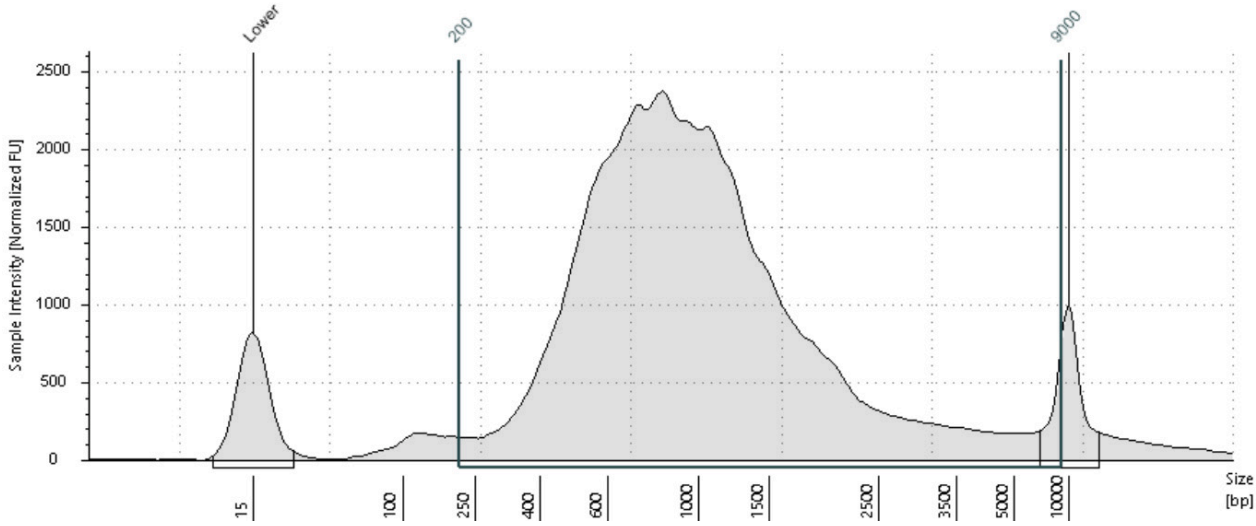


Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
C4	978	5928_80		

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
15	308	-	31600	-		Lower Marker
818	978	-	1840	100.00		
10000	180	180	27.7	-		Upper Marker



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
200	9000	1158	5960	11100	95.32		

Figure 7. Example of good-quality cDNA trace for snRNA-seq library preparation

cDNA profile of sample 80 from Mucciolo et al.[1] The cDNA was measured with the Agilent TapeStation 4200 using the Agilent High Sensitivity D5000 ScreenTape.

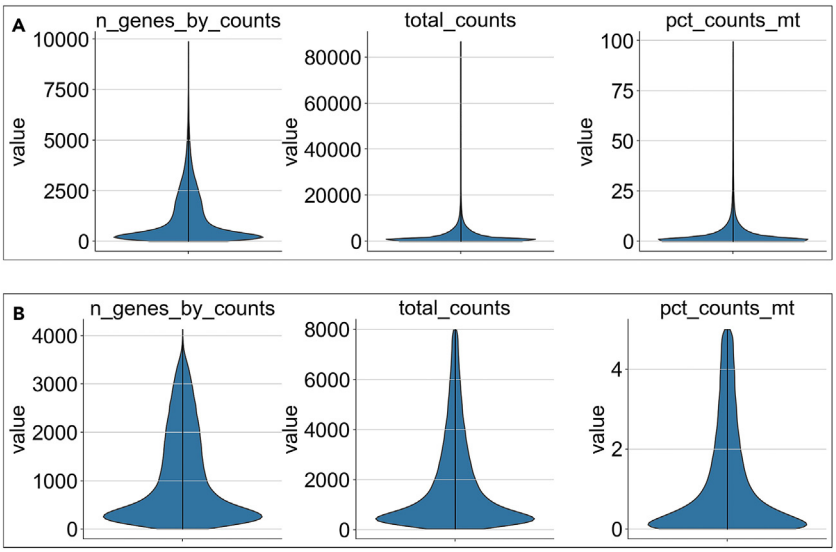


Figure 8. Quality metrics assessment of snRNA-seq per nuclei

(A and B) Violin plots showing the distribution of single nuclei based on: number of genes per nucleus (left), total number of counts per nucleus (middle) and percentage of mitochondrial genes per nucleus (right) before (A) and after (B) applying QC filtrations by removing low-quality nuclei, doublets and outlier nuclei.

35. Input FASTQ files into Cell Ranger (10× Genomics) to align raw reads to the Cell Ranger prebuilt mouse transcriptome reference (Mouse mm10 (GENCODE vM23/Ensembl98) 2020-A (Jul 7, 2020)) to generate the raw gene expression counts.
36. Use the ''remove-background'' function of CellBender[4] on the Cell Ranger output file ''raw_feature_bc_matrix'' to remove ambient and background RNA that resulted from technical artifacts.

⚠ CRITICAL: Different false positive rate (fpr) parameters should be examined (e.g. 0.1, 0.05 and 0.01). The lower fpr value is, the more conservative CellBender is when removing ambient RNA. Fpr = 0.01 is the default and recommended fpr value to begin with. The examination of the best fpr is uncertain and needs to be tested on the expression of different genes when they are wrongly expressed in a cell cluster that they should not be expressed in. After using a specific fpr value, if genes are still wrongly expressed, then more stringent fpr should be examined until these genes are dropped. In our case, fpr = 0.01 worked well.

37. Use Scanpy[8] package to read the raw counts using ''sc.read_10x_h5'' function into python as anndata object.
38. Calculate QC metrics (Figure 8).
    a. Filter out low-quality nuclei that have less than 200 genes.
    b. Filter out nuclei that have a percentage of mitochondrial genes higher than 5%.
    c. Filter out nuclei that have more than 8000 counts per nucleus.
39. Use SOLO[5] tool from scvi-tools[6] to estimate doublets (and multiplets) that would affect the quality of the downstream analyses.
    a. From the output of SOLO model add the 'prediction' column to the observations of anndata as an annotation for each nucleus.
    b. Save the anndata object as h5ad file.

Note: All previous steps should be applied on each sample separately.

40. Read all h5ad files using Scanpy ''sc.read_h5ad'' function into a list.
41. Use Scanpy ''sc.concat'' function to concatenate all samples into one anndata.

42. Save raw counts in a new layer and call it "counts".
43. Normalize to the total count and apply a Log10 transformation to the data.
44. Perform dimensionality reduction on the top 2000 highly variable genes.
45. Compute the neighborhood graph of cells on the top 30 PCs.
46. For batch correction use Harmony[7] tool that is implemented in scanpy using (scanpy.external.pp.harmony_integrate).
47. A variable called "X_pca_harmony" will be added to (adata.obsm): replace the "X_pca" with the "X_pca_harmony".
48. For clustering, apply the Leiden graph-clustering method with a starting resolution = 0.8.
49. For visualization apply Uniform Manifold Approximation and Projection (UMAP).

Note: Other clustering methods can also be applied.

50. Define the markers of each cluster by applying "rank_genes_groups" function from Scanpy.
51. To score and visualize gene signature (group of genes) use "score_genes" function from Scanpy.
52. Use decouplerpy[10] to annotate nuclei to their cell types from their markers based on Panglaodb[11] and celltypist.[12]

Note: Due to the nature of snRNA-seq data, the expression of certain markers, which would be highly expressed in scRNA-seq data (as it captures also cytoplasmic transcripts), is missing. This makes annotating nuclei clusters with automated cell type annotator tools more challenging as they are typically dependent on these genes. Defining the identity of each nuclei cluster may thus require prior knowledge of the biology of your sample(s) and model(s).

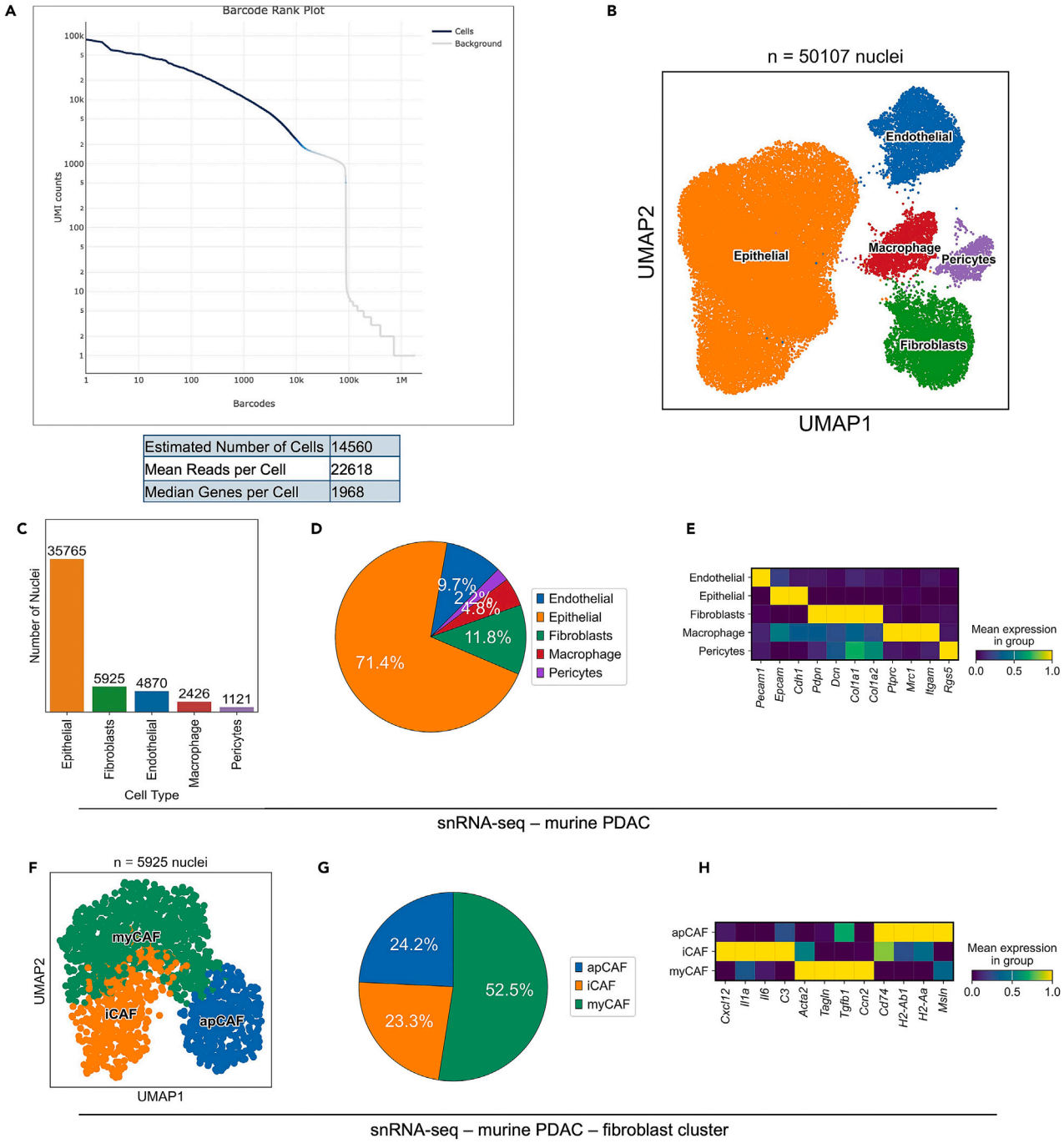
53. To confirm the malignant cell cluster, perform copy number variation (CNV) analysis using a python implementation of inferCNV of the Trinity CTAT Project (https://github.com/broadinstitute/inferCNV).
    a. Use a normal cell cluster (in our case, we used the fibroblasts) as a reference key and the epithelial cell cluster(s) as query key(s).
    b. Set 250-genes as a window size.

⚠ CRITICAL: The python implementation of inferCNV is a fast, easy-to-visualize, and memory-efficient tool, but it might not always give the most optimized results. Thus, it is recommended to double check its results with the original R based inferCNV tool (i.e. the same cluster(s) should have many DNA rearrangements as expected for the injected PDAC malignant cells).

Note: Due to the nature of the snRNA-seq data, we only use the CNV profile to identify the malignant cell cluster, rather than performing in-depth genotype analyses or comparisons between the two tools described above.

54. Apply differential expression analysis (DEA) by performing pseudo-bulk method on the raw counts that are kept in the "counts" layer.
    a. Pool all cells within a specific cell type by summing the gene expression of each gene to create a pseudo-bulk expression profile of each sample.
    b. Use the python implementation of decoupler[10] R package with the default options to generate the pseudo-bulk profile.
    c. Use DESeq2[9] to perform DEA on the pseudo-bulk data to define differentially expressed genes (DEG) between two sample groups.
    d. Consider genes with adjusted p-value < 0.05 as significantly differentially expressed.

Note: For DEA of snRNA-seq data, the number of samples is an important determinant to choose the method. Pseudo-bulk performs better when there is a sufficient number of samples



Estimated Number of Cells	14560
Mean Reads per Cell	22618
Median Genes per Cell	1968

Figure 9. Expected results after snRNA-seq analysis of murine PDAC using Cell Ranger and Scanpy

(A) Top: barcode rank plot showing the distribution of barcode counts and the barcodes associated with the nuclei for one sample (Cell Ranger). The upper blue curve shows the nuclei reads and the gray part shows the background noise of potentially degraded nuclei or empty partitions. y axis, number of unique molecular identifier (UMI) counts mapped to each barcode; x axis, number of barcodes. Bottom: table provided by Cell Ranger showing the expected number of nuclei, Mean reads per nucleus, and Median Genes per nucleus.

(B) Uniform Manifold Approximation and Projection (UMAP) plot showing the number and type of cell clusters found by snRNA-seq of 8 murine PDAC samples.

(C) Bar plot showing the number of nuclei of each cell type from (B).

(D) Pie chart showing the percentage of each cell type from (B).

(E) Matrix plot showing the scaled expression of cell type-defining markers in each cell type from (B).

Figure 9. *Continued*
(F) UMAP plot showing the CAF subtypes identified by snRNA-seq data from the fibroblast cluster in (B). iCAF, inflammatory CAF; myCAF, myofibroblastic CAF; apCAF, antigen-presenting CAF.
(G) Pie chart showing the percentage of each CAF subtype in the fibroblast cluster from (F).
(H) Matrix plot showing the expression of CAF subtype-defining markers in each CAF subtype from (F). Data shown are from Mucciolo et al.[1]

per condition.[15] Alternatively, other methods, such as t-test, Wilcoxon (implemented in Scanpy) and MAST,[13] could be used.

55. Perform gene set enrichment analysis (GSEA) on pre-ranked genes based on the negative logarithmic p-value and the sign of the Log_2 Fold change.
    a. Run GSEA using clusterprofiler[14] against the Hallmark, Reactome, and C2 canonical pathway collection (C2.cp.v5.1) downloaded from the Molecular Signatures Database (MSigDB).[16]
    b. Consider pathways with adjusted p-value < 0.25 and NES > 1.5 or < −1.5 as significantly differentially expressed.

EXPECTED OUTCOMES

For the generation of orthotopically grafted organoid-derived mouse models for snRNA-seq in Mucciolo et al.,[1] we dissociated confluent T69A PDAC organoids (Figure 2A) derived from a female *Kras*$^{LSL\text{-}G12D/+}$; *Trp53*$^{LSL\text{-}R172H/+}$; *Pdx1*-Cre (KPC) mouse model. We started with 8 domes and obtained approximately 700,000 live cells. However, remember that the number of single cells obtained at the end of the cell preparation step can vary according to the confluency of the organoids, number of initial Matrigel domes, as well as the organoid line.

For Mucciolo et al.,[1] we injected 10,000 cells per mouse in 8-week-old female C57BL/6J mice. We obtained tumors with around 10 mm diameter after approximately 8 weeks post-surgery (Figure 5). However, you may adjust the number of cells injected according to your experimental goal. Consequently, the tumor kinetics and time to harvest tumor for snRNA-seq may vary in your experiments. Thus, ultrasound-based imaging would help to determine the optimal experimental endpoint for your study.

For Mucciolo et al.,[1] we isolated single nuclei from tumor pieces stored for 1–2 months at −80°C. We typically isolated > 500,000 nuclei from one piece of PDAC tissue > 3 mm^3. We used AOPI for counting and most nuclei were stained positive, typically showing 0.1%–1% viability across the samples. If your single nuclei isolation has been successful, you can expect less than 1% viability at the last cell count before snRNA-seq submission.

As mentioned above, the profile of cDNA traces can help assess the quality of the cDNA. An example of a cDNA trace obtained by following this protocol is provided in Figure 7.

The expected number of nuclei detected by the sequencer (i.e., from the Cell Ranger output) varied from sample to sample. Moreover, this did not perfectly match the number of nuclei counted during the nuclei isolation step. This led to some differences in the number of input nuclei. On average (based on the 8 samples analyzed[1]), the Cell Ranger output showed that about 18,000 nuclei were obtained out of 35,000 nuclei submitted (~48% of yielding rate, one sample is shown in Figure 9A). The number of genes with at least 1 count per nucleus (n_genes_by_counts) was on average 16,000 and the number of counts per nucleus (total_counts) was on average 1,500.

We pre-processed the data to filter out poor-quality nuclei based on the total number of genes (n_genes_by_counts), total number of counts, and the percentage of mitochondrial genes (pct_counts_mt) (Figure 8A). The final QC metrices after removing doublets and pre-processing data are shown in Figure 8B. By comparing the initial number of nuclei to the final number of nuclei of each sample we observed that we filtered out 45%–50% of the nuclei (including low-quality nuclei, doublets, and outliers).

In Mucciolo et al.,[1] we performed snRNA-seq analysis on 8 tumor samples and obtained 50107 nuclei in total after filtering, from which we identified 5 cell types: epithelial cells (71.4%), fibroblasts (11.8%), endothelial cells (9.7%), macrophages (4.8%) and pericytes (2.2%) (Figure 9B). The proportion and number of nuclei of each cell type are shown in Figures 9C and 9D. Additionally, the fibroblast cluster was further investigated and subclustered. We were able to detect the three main populations of PDAC CAFs: myofibroblastic CAFs (myCAFs), inflammatory CAFs (iCAFs) and antigen-presenting CAFs (apCAFs) (Figures 9F–9H).

LIMITATIONS

In this protocol, we describe the establishment of orthotopically grafted organoid-derived mouse models of PDAC to study the tumor microenvironment. Compared to GEMMs, these organoid-derived transplantation models do not enable to evaluate the early stages of tumor progression. Nonetheless, organoid-derived models are preferable to 2D cell line-derived models, as the latter typically lead to highly cellular tumors with undifferentiated histology and limited stroma abundance. Our analysis failed to identify lymphoid cells and granulocytes as distinct cell clusters. Whether this is due to sample storage, processing and/or sequencing depth remains unclear. Certainly, due to the nature of the approach, low abundance transcripts (e.g., cytokines and chemokines) are more difficult to detect by snRNA-seq compared to, for example, scRNA-seq techniques. Regardless, this technique is not recommended for analysis of lymphoid cells and granulocytes.

TROUBLESHOOTING

Problem 1

Doublets or cell clusters (Figure 2E) during cell preparation (Related to the "cell preparation" section, Step 19).

During the cell preparation step, it is important to obtain single cells (Figure 2D) and an accurate cell count. This is mainly critical to be able to compare different experimental cohorts in terms of tumor growth, metastasis formation and survival.

Potential solution

After the second incubation step with Dispase (step 18.d), check the cells at the microscope to confirm their singlet status by positioning the 15 mL falcon tube underneath and observing with a 10X Objective. If you see many clusters, add 1 mL of TrypLE and repeat step 18.b.

Problem 2

Unsuccessful orthotopic injection of pancreatic cancer organoids (Related to the "establishment of orthotopically grafted organoid-derived mouse models of PDAC" section, Step 7).

If after the orthotopic injection you do not see the formation of a "bubble" (Figure 4H) or you see partial liquid leakage at the injection site, it means that the injection was unsuccessful. This may lead to the formation of non-pancreatic tumors in the abdominal cavity and/or peritoneum. These mice can deteriorate more quickly following tumor formation, compared to properly injected ones. Thus, take note of which mouse was "leaked" at surgery and monitor them closely. Additionally, you cannot use these mice for tumor growth kinetic and metastasis formation comparisons (even if re-injected).

Potential solution

To make sure you obtain a tumor, absorb the liquid around the injection area with a sterile cotton swab to remove as much leaked liquid as you can. Re-check the pancreas for a better injection site. Repeat the injection and take note if it is successful or not.

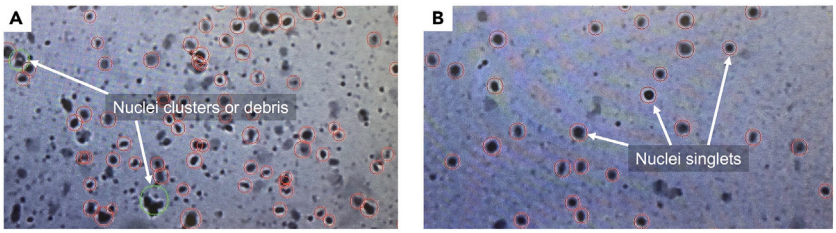


Figure 10. Nuclei counting

Representative images of isolated nuclei counted on a LUNA cell counter using trypan blue.

(A) Nuclei clustered together (with debris) are wrongly counted.

(B) Nuclei from (A) after filtering for a second time through a 35 μm cell strainer cap of a 5 mL polystyrene tube. Individual nuclei tagged with a red circle are correctly counted as "dead cells."

Problem 3

Clusters after nuclei isolation (Related to the "single nuclei isolation and RNA sequencing" section, Step 22.g). When performing the second nuclei count, you may find some nuclei clustered together with possible debris (Figure 10A). This will result in inaccurate counting.

Potential solution

It is important not to leave the nuclei on ice for longer than 30 min after completing the first and second nuclei count. If nuclei aggregation is observed, filter the nuclei through a 35 μm cell strainer cap of a 5 mL polystyrene tube for a second time. Isolated nuclei should appear as singlets (Figure 10B) with < 1% viability.

Problem 4

Low number of estimated nuclei after Cell Ranger analysis and insufficient number of nuclei after data pre-processing (Related to the "single-nuclei RNA sequencing analysis" section, Step 35 and Steps 38–39).

Potential solution

To limit this issue, the number of input nuclei needs to be optimized for each new tissue type in a pilot experiment. The range of single nuclei required for sequencing may vary between 35,000 and 70,000. The appropriate input should yield enough nuclei without a high percentage of doublets.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Giulia Biffi (Giulia.Biffi@cruk.cam.ac.uk).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contacts: Gianluca Mucciolo (Gianluca.Mucciolo@cruk.cam.ac.uk) for the sections "establishment of orthotopically grafted organoid-derived mouse models of PDAC" and "tumor sampling and storage," Wenlong Li (Wenlong.Li@cruk.cam.ac.uk) for the section "single nuclei isolation and RNA sequencing," and Muntadher Jihad (Muntadher.Jihad@cruk.cam.ac.uk) for the section "single-nuclei RNA sequencing analysis."

Materials availability

All unique/stable reagents generated in this study will be made available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

- The accession number for the snRNA-seq datasets reported in this paper is GEO: GSE244142.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

The authors would like to thank the BRU, Genomics, Bioinformatics, RICS, and Histology core facilities at the Cancer Research UK Cambridge Institute (CRUK-CI), University of Cambridge. Additionally, the authors would like to thank Alecia-Jane Twigger (Cambridge Stem Cell Institute) and Kui Hua (CRUK-CI) for sharing their optimized single-nuclei isolation protocol. This work was mainly supported by a Cancer Research UK (CRUK) institutional grant (A27463) and a UK Research and Innovation (UKRI) Future Leaders Fellowship that also supported G.B., S.P.T., W. Li, S.A., and J.S.M. G.B. is also the recipient of a Pancreatic Cancer Research Fund grant and a US Department of Defense PCARP grant that supported G.M. and J.S.M., an NCI-CRUK Cancer Grand Challenge grant that supported M.J. and W. Luo, and a Pancreatic Cancer UK Future Leaders Academy grant that supported P.S.W.C. Moreover, J.A.H. was supported by a Harding Distinguished Postgraduate Programme PhD studentship (Cambridge Trust), and E.G.L. was supported by an MRC Doctoral Training Grant.

AUTHOR CONTRIBUTIONS

M.J. and W. Li conducted the experiments and wrote the protocol. G.M. designed the experiments, conducted the experiments, and wrote the protocol. J.A.H. designed the experiments and conducted the experiments. S.P.T., J.S.M., E.G.L., S.A., W. Luo, and P.S.W.C. conducted the experiments. A.S. and A.P. helped with designing the snRNA-seq experiments. A.A. helped with single-nuclei isolation. G.B. designed the experiments, supervised the study, conducted the experiments, and wrote the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Mucciolo, G., Araos Henríquez, J., Jihad, M., Pinto Teles, S., Manansala, J.S., Li, W., Ashworth, S., Lloyd, E.G., Cheng, P.S.W., Luo, W., et al. (2024). EGFR-activated myofibroblasts promote metastasis of pancreatic cancer. Cancer Cell *42*, 101–118.e11. https://doi.org/10.1016/j.ccell.2023.12.002.

2. Boj, S.F., Hwang, C.-I., Baker, L.A., Chio, I.I.C., Engle, D.D., Corbo, V., Jager, M., Ponz-Sarvise, M., Tiriac, H., Spector, M.S., et al. (2015). Organoid Models of Human and Mouse Ductal Pancreatic Cancer. Cell *160*, 324–338. https://doi.org/10.1016/j.cell.2014.12.021.

3. Oni, T.E., Biffi, G., Baker, L.A., Hao, Y., Tonelli, C., Somerville, T.D.D., Deschênes, A., Belleau, P., Hwang, C.-I., Sánchez-Rivera, F.J., et al. (2020). SOAT1 promotes mevalonate pathway dependency in pancreatic cancer. J. Exp. Med. *217*, e20192389. https://doi.org/10.1084/jem.20192389.

4. Fleming, S.J., Chaffin, M.D., Arduini, A., Akkad, A.-D., Banks, E., Marioni, J.C., Philippakis, A.A., Ellinor, P.T., and Babadi, M. (2023). Unsupervised removal of systematic background noise from droplet-based single-cell experiments using CellBender. Nat. Methods *20*, 1323–1335. https://doi.org/10.1038/s41592-023-01943-7.

5. Bernstein, N.J., Fong, N.L., Lam, I., Roy, M.A., Hendrickson, D.G., and Kelley, D.R. (2020). Solo: Doublet Identification in Single-Cell RNA-Seq via Semi-Supervised Deep Learning. Cell Syst. *11*, 95–101.e5. https://doi.org/10.1016/j.cels.2020.05.010.

6. Gayoso, A., Lopez, R., Xing, G., Boyeau, P., Valiollah Pour Amiri, V., Hong, J., Wu, K., Jayasuriya, M., Mehlman, E., Langevin, M., et al. (2022). A Python library for probabilistic analysis of single-cell omics data. Nat. Biotechnol. *40*, 163–166. https://doi.org/10.1038/s41587-021-01206-w.

7. Korsunsky, I., Millard, N., Fan, J., Slowikowski, K., Zhang, F., Wei, K., Baglaenko, Y., Brenner, M., Loh, P., and Raychaudhuri, S. (2019). Fast, sensitive and accurate integration of single-cell data with Harmony. Nat. Methods *16*, 1289–1296. https://doi.org/10.1038/s41592-019-0619-0.

8. Wolf, F.A., Angerer, P., and Theis, F.J. (2018). SCANPY: large-scale single-cell gene expression data analysis. Genome Biol. *19*, 15. https://doi.org/10.1186/s13059-017-1382-0.

9. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550. https://doi.org/10.1186/s13059-014-0550-8.

10. Badia-i-Mompel, P., Vélez Santiago, J., Braunger, J., Geiss, C., Dimitrov, D., Müller-Dott, S., Taus, P., Dugourd, A., Holland, C.H., Ramirez Flores, R.O., and Saez-Rodriguez, J. (2022). decoupleR: ensemble of computational methods to infer biological activities from omics data. Bioinform. Adv. *2*, vbac016. https://doi.org/10.1093/bioadv/vbac016.

11. Franzén, O., Gan, L.-M., and Björkegren, J.L.M. (2019). PanglaoDB: a web server for exploration of mouse and human single-cell RNA sequencing data. Database 1–9. https://doi.org/10.1093/database/baz046.

12. Domínguez Conde, C., Xu, C., Jarvis, L.B., Rainbow, D.B., Wells, S.B., Gomes, T., Howlett, S.K., Suchanek, O., Polanski, K., King, H.W., et al. (2022). Cross-tissue immune cell analysis reveals tissue-specific features in humans. Science *376*, eabl5197. https://doi.org/10.1126/science.abl5197.

13. Finak, G., McDavid, A., Yajima, M., Deng, J., Gersuk, V., Shalek, A.K., Slichter, C.K., Miller, H.W., McElrath, M.J., Prlic, M., et al. (2015). MAST: a flexible statistical framework for assessing transcriptional changes and characterizing heterogeneity in single-cell RNA sequencing data. Genome Biol. *16*, 278. https://doi.org/10.1186/s13059-015-0844-5.

14. Yu, G., Wang, L.-G., Han, Y., and He, Q.-Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS *16*, 284–287. https://doi.org/10.1089/omi.2011.0118.

15. Junttila, S., Smolander, J., and Elo, L.L. (2022). Benchmarking methods for detecting differential states between conditions from multi-subject single-cell RNA-seq data. Brief. Bioinform. *23*, bbac286. https://doi.org/10.1093/bib/bbac286.

16. Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., and Mesirov, J.P. (2005). Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. USA *102*, 15545–15550. https://doi.org/10.1073/pnas.0506580102.