

Video Article

# Immunophenotyping of Orthotopic Homograft (Syngeneic) of Murine Primary KPC Pancreatic Ductal Adenocarcinoma by Flow Cytometry

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## Abstract

Homograft (syngeneic) tumors are the workhorse of today's immuno-oncology (I/O) preclinical research. The tumor microenvironment (TME), particularly its immune-components, is vital to the prognosis and prediction of treatment outcomes, especially those of immunotherapy. TME immune-components are composed of different subsets of tumor-infiltrating immune cells assessable by multi-color FACS. Pancreatic ductal adenocarcinoma (PDAC) is among the deadliest malignancies lacking good treatment options, thus an urgent and unmet medical need. One important reason for its non-responsiveness to various therapies (chemo-, targeted, I/O) has been its abundant TME, consisting of fibroblasts and leukocytes that protect tumor cells from these therapies. Orthotopically implanted PDAC is believed to more accurately recapture the TME of human pancreatic cancers than conventional subcutaneous (SC) models.

Homograft tumors (KPC) are transplants of mouse spontaneous PDAC originating from genetically engineered KPC-mice ( $Kras^{G12D/+}/P53^{-/-}/Pdx1-Cre$ ) (KPC-GEMM). The primary tumor tissue is cut into small fragments (~2 mm<sup>3</sup>) and transplanted subcutaneously (SC) to the syngeneic recipients (C57BL/6, 7–9 weeks old). The homografts were then surgically orthotopically transplanted onto the pancreas of new C57BL/6 mice, along with SC-implantation, which reached tumor volumes of 300–1,000 mm<sup>3</sup> by 17 days. Only tumors of 400–600 mm<sup>3</sup> were harvested per approved autopsy procedure and cleaned to remove the adjacent non-tumor tissues. They were dissociated per protocol using a tissue dissociator into single-cell suspensions, followed by staining with designated panels of fluorescently-labeled antibodies for various markers of different immune cells (lymphoid, myeloid and NK, DCs). The stained samples were analyzed using multi-color FACS to determine numbers of immune cells of different lineages, as well as their relative percentage within tumors. The immune profiles of orthotopic tumors were then compared to those of SC tumors. The preliminary data demonstrated significantly elevated infiltrating TILs/TAMs in tumors over the pancreas, and higher B-cell infiltration into orthotopic rather than SC tumors.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/57460/>

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) causes nearly half a million mortalities world-wide annually, one of the top 5 cancer killers. There are few effective treatment options and no approved immunotherapies; therefore, new treatments are desperately needed. Cancers are increasingly being recognized as immunological diseases, including PDAC, in addition to the genetic diseases as known today. Immunological and genetic factors would likely determine disease prognosis as well as treatment outcomes. Tumors evade host immune surveillance and eventually advance to cause death. Many of these immune processes occur within the tumor microenvironment (TME)<sup>1,2,3,4</sup> where different types of immune cells interact with tumor cells, with each other and with other tumor stromal components, directly or indirectly via cytokines which ultimately determine disease outcome. Therefore, characterization of the tumor immune components of the TME, or tumor immunophenotyping, including subtyping, numeration and localization of different lineages of immune cells, is critical to understanding anti-tumor immunity. In the case of PDAC, it has been proposed that elevated tumor-infiltrating suppressive macrophages (TAM) and B-cells have led to prevention of T-cell infiltration and/or activation and high levels of fibrosis<sup>5,6</sup>.

The common approach to investigating immune TMEs experimentally would be using surrogate tumor preclinical animal models, mainly relevant mouse tumor models<sup>7</sup>, particularly mouse syngeneic (homograft) or genetically engineered mouse models (GEMM) of cancers, on the assumed similarity of mouse and human for tumors and immunity<sup>8,9</sup>. It is understood in reality that there are inherent differences between the two species<sup>10,11</sup>.

Transplanted mouse tumors have significant operational advantages over spontaneous tumors<sup>7</sup>, namely synchronized tumor development, in contrast to the parental GEMM spontaneous tumor development. Homografts of spontaneous murine tumors are considered primary tumors

having never been manipulated *in vitro*, and mirroring original mouse tumor histo-/molecular pathology<sup>7</sup>, as well as possible immune profiles. These murine homografts are often considered to be "a mouse version of patient-derived xenografts (PDXs)". They therefore likely have a better translatability than conventional syngeneic cell line-derived mouse tumors<sup>12</sup>. In particular, many homografts are derived from specific GEMM where specific human disease mechanisms, e.g. oncogenic driver mutations, are engineered, and these homografts should therefore have advantages to their clinical relevance. In particular, KPC GEMM develop mouse PDAC within 15–20 weeks of age, which morphologically recapitulates human disease with predominately well- to moderately-differentiated glandular architecture and highly enriched stroma. This model also recapitulates the most common genetic features of human PDAC, namely Kras activating mutation and P53 loss-of-function, which occur in 90% and 75% of human PDAC, respectively<sup>5,6</sup>.

Sites of transplantation have also been suggested to play a role in model translatability. The specific surrounding tissue environment, such as a corresponding orthotopic environment, could be a niche for specific tumors to progress, as opposed to the uniform subcutaneous (SC) environments for commonly transplanted tumors. It would be of particular interest if, and/or, what difference exists between the two transplantation sites, in terms of immune-microenvironment, and the relevance to human cancer, e.g. in the case of PDAC.

One of the most important aspects of immune profiling, or immunophenotyping, is to determine tumor-infiltrating immune cells of different lineages, the numbers, relative percentage within tumors, as well as their activation states, and locations. This includes tumor-infiltrating lymphocytes (TILs, both T- and B-), tumor-infiltrating macrophages (TAMs), tumor-infiltrating natural killer cells (NKs) and tumor-resident dendritic cells<sup>3,13,14,15,16,17</sup>, and the subcellular localization of certain cells<sup>18,19,20</sup>, etc. Fluorescence Activated Cell Sorting (FACS) or flow cytometry is a single-cell detection technology that is commonly used to measure the specific parameters of a cell. Multi-color flow cytometry measures multiple markers on a single cell<sup>3,4,21</sup> and is the most commonly used method to determine the numbers and relative percentage of different subsets of immune cells, including those within tumors.

This report describes procedures for profiling tumor-infiltrating immune cells: 1) Implantation of orthotopic PDAC mouse tumor homografts, along with SC implantation; 2) tumor tissue harvest and single cell preparation via tumor dissociation; 3) flow cytometry analysis of all of the cells derived from tumors as a baseline; 4) comparison of baseline profiles of both transplantation approaches.

## Protocol

All the protocols and amendment(s) or procedures involving the care and use of animals will be reviewed and approved by the Crown Bioscience Institutional Animal Care and Use Committee (IACUC) prior to the conduct of studies. The care and use of animals will usually be conducted in accordance with AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International guidelines as reported in the Guide for the Care and Use of Laboratory Animals, National Research Council (2011). All animal experimental procedures will be under sterile conditions at SPF (specific pathogen-free) facilities and conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals from different government institutions (e.g. The National Institutes of Health). The protocol will need to be approved by the Committee on the Ethics of Animal Experiments at the facility institution (e.g. institutional IACUC Committee).

## 1. Preparation for Tumor Transplantation

### 1. Animal Housing

1. Obtain generic C57BL/6 mice from a commercial breeding vendor.
2. House mice (5) in individual ventilated cages at the following conditions: Temperature: 20–26 °C; humidity 30–70%; lighting cycle: 12 h light and 12 h dark.
3. Use corn cob bedding that is changed weekly.
4. For diet, provide irradiation sterilized dry granule food during the entire study period.
5. For water, provide animals free access to sterile drinking water.

### 2. Donor tumor fragment preparation

1. Begin by monitoring body weight (BW), via weighing using a balance, and tumor volume (TV), by caliper measurement, of tumor-bearing donor mice.
2. When TV reaches 500–1,200 mm<sup>3</sup>, euthanize the animal in a biohazard hood as per protocol.
3. Sterilize the skin around the tumor using iodophor swabs.
4. Surgically remove the tumor (details described in section 3: Necropsy and tumor harvest) and place the tumor in a Petri dish containing 20 mL of PBS (pre-chill the media or buffer to 4 °C prior to euthanizing animals).
5. If there is contaminating blood, transfer the tumor into another Petri dish and wash the tumor with PBS.
6. Cut the tumor in half, removing any extra skin, vessels, calcification and/or necrosis.
7. Choose only intact pieces of tumor and place them into a sterile 50 mL centrifuge tube and add 20 mL of PBS then transport the tube to a separate animal room for pharmacology studies.

## 2. Orthotopic and Subcutaneous (SC) Engraftment

### 1. SC Inoculation

1. Cut tumors into 2 mm diameter pieces using a scalpel, putting 1 chunk into each trocar, for SC implantation or for later orthotopic implantation (see below).
2. Anesthetize the recipient animal with 5% isoflurane, which is maintained by a nose cone at 1%. The animal will begin to relax, losing their righting reflex and eventually become immobile. At this depth of anesthesia they can easily be roused by painful stimuli; allow anesthesia to deepen until such responses to pain are absent.

3. Once anesthetized, fix the mice on an experiment board in the right lateral position. Sterilize the mouse using iodophor swabs, particularly the areas surrounding the tumors.
4. With a scalpel, make a 0.5 to 1.0 cm skin incision on the left flank, just cranial to the hip.
5. Tunnel under the skin towards the forelimb, two to three centimeters, with blunt forceps.
6. Aseptically transfer one cube of tumor from the medium and place deep inside the subcutaneous tunnel.
7. Visually confirm that the tumor is deep in the tunnel (and not at the skin incision).
8. Close the wound with wound clips.
9. Monitor the animals post inoculation until they regain sufficient consciousness to maintain sternal recumbence. Return animals back to their cage only after their full recovery from anesthesia. Complete the Anesthesia Log, and initiate the Animal Health Chart. Weigh recipient animals daily for three days
10. For a standard procedure, inoculate 5-10 mice per group for tumor growth monitoring.

## 2. Pancreatic orthotopic implantation

1. Anesthesia and Analgesia
  1. Use a 2 mL ketamine injection and 0.42 mL xylazine injection (20 mg/mL) mixed in 5.91 sterile injection water or saline at a dose volume of 0.06–0.1 mL/20-25 g body weight.  
NOTE: According to animal welfare, analgesia is necessary both pre and post operation. 0.05–0.1 mg buprenorphine /kg, SC. The first dose is pre operation and then dosed 3 times every 4 hours post operation continually.
2. Surgical operation for orthotopic implantation
  1. Anesthetize mice via intramuscular injection (IM) per Step 2.2.1.
  2. After the animals are fully anesthetized, fix the mice on an experiment board in the right lateral position.
  3. Keep the mice in the right lateral position. Disinfect the skin around the spleen with iodine then de-iodinate with 75% ethyl alcohol.
  4. Find the medium point of the spleen and make a 1 cm vertical incision on abdomen to expose the spleen.
  5. Draw out a part of pancreas tissue under the spleen gently with flat-tip tweezers, and suture a mouse homograft tumor piece from seed mouse on the pancreas of recipient mouse by 9-0 Absorbable surgical suture.
  6. Close the abdomen with a 6-0 silk suture by double seam. Achieve homeostasis by compression.
  7. After finishing tumor implantation, if neither bleeding nor tumor tissue leakage occurs, keep the animals in a warm cage.
  8. Monitor the animal until it regains sufficient consciousness to maintain sternal recumbency; return the animal to the animal room after full recovery from the anesthesia. Monitor the tumor bearing mice by palpating the abdomen near the spleen and select out the mice bearing orthotopic tumors.

## 3. Tumor-Bearing Mice Health Monitoring

1. Check the water and food consumption daily.
2. Examine the mouse appearance for an ungroomed hair coat, lumps, thinness, abnormal breath or ascites.
3. Palpate the abdomen to check if there are spontaneous tumors on the liver or spleen.
4. Weigh mice weekly using a balance.
5. If any of the following clinical signs are observed, the mice are sacrificed for sample collection and necropsy: BW loss >20%; impaired mobility (not able to eat or drink); unable to move normally due to significant ascites and enlarged abdomen; effort in respiration.

## 3. Necropsy and Tumor Harvest

1. Inspect visually and by palpation for the presence of palpable tumors before termination.
2. For termination, first euthanize mice as per approved protocol, and open the abdomen and visually examine the pancreas for tumors.
3. Cut off the tumor by post mortal surgery and add it to cold PBS. Place all animals in a clean, uncharged, translucent euthanasia chamber with a special lid with a port for delivery of the carbon dioxide to be used.
4. Discharge gas into the chamber at a flow rate that produces rapid unconsciousness with minimal distress to the animal. The optimum flow rate for CO<sub>2</sub> should be around 2–2.5 L/min.
5. Visually observe each animal during the euthanasia procedure to assure all animals receive adequate gas concentrations and do not regain consciousness during the euthanize procedure.
6. Maintain gas flow for at least 1 min after apparent clinical death to minimize the possibility that an animal may recover (if apneic but not dead).
7. Remove adjacent non-tumor tissues. Cleaned tumor can be photographed quickly.  
Put the tumor tissues into RPMI-1640 and keep on ice before dissociation.  
NOTE: Animal carcasses are bagged and stored in the appropriate freezer to await removal.

## 4. Tumor Tissue Dissociation and Single Cell Preparation

### 1. Reagent preparation

NOTE: The Tumor Dissociation Kit contains 2 vials of Enzyme D (lyophilized powder), 1 vial of Enzyme R (lyophilized powder), 1 vial of Enzyme A (lyophilized powder) and 1 mL of Buffer A.

1. Prepare Enzyme D by reconstituting the lyophilized powder in each vial with 3 mL of RPMI-1640. Prepare aliquots of an appropriate volume to avoid repeated freeze-thaw-cycles.
2. Prepare Enzyme R by reconstituting the lyophilized powder in the vial with 2.7 mL of RPMI 1640. Prepare aliquots of an appropriate volume to avoid repeated freeze-thaw-cycles.

3. Prepare Enzyme A by reconstituting the lyophilized powder in the vial with 1 mL of Buffer A supplied with the kit. Do not vortex. Prepare aliquots of an appropriate volume to avoid repeated free-thaw-cycles.
4. Prepare the Enzyme mix by adding 2.35 mL of RPMI-1640, 100  $\mu$ L of Enzyme D, 50  $\mu$ L of Enzyme R and 12.5  $\mu$ L of Enzyme A into each gentleMACS C-tube.

## 2. Tumor Dissociation

1. For each tumor prepare one C-tube with tumor digestion mix, please refer to section 4.1 for digestion buffer dilution and preparation.
2. Use 3 mL digestion buffer for tumor fragments <0.8 g, and ensure the tumor is fully digested.
3. Label with the study code, tumor, mouse ID, treatment group, and tumor weight.
4. Collect the tumor from the mouse, wash the tumor in cold PBS and clean out the tissue attached on the tumor (such as blood vessels, fat, fascia, etc.).
5. Put the tumor in digestion media in one well of a sterile 6 well plate.
6. Hold the tumor in place with sterile tweezers/forceps and slice with a scalpel. Slice the tumor well enough to break into smaller pieces (~1 mm<sup>3</sup>).
7. Place the tumor pieces back into the C-tube and use the remaining digestion buffer to wash the plate and then transfer the fluid into the C-tube that is placed on ice until digestion.
8. Switch on the dissociator with heaters.
9. Place tumor dissociation C-tubes upside down into sleeves of the vacant positions and adjust the status of tube positions from Free to Selected. Choose a dissociation program (37\_c\_m\_TDK\_1), followed by the selection of the required folder, where the list of Programs is displayed.
10. After termination of the program, take C-tubes off the dissociator and spin briefly (300 x g, 4 °C) to pellet the sample.
11. Re-suspend samples and put into a cell strainer above a 50 mL tube. Wash the cells through the cell strainer with 10 mL of wash buffer to provide a single-cell suspension.
12. Centrifuge the tubes at 300 x g for 5 min, discard the supernatant and re-suspend the cells with 5 mL of wash buffer, count viable cells using trypan blue and/or by cell counter and adjust the cell concentration to 1 x 10<sup>6</sup> cells per tube or per sample. Include the correct isotype control antibodies to ensure staining is specific

## 5. Immune Panel Design and Flow Data Acquisition

### 1. Panel design

1. Please see **Table 1**.

### 2. Immunostaining

1. Fc-Block sample cells: re-suspend the cells in 200  $\mu$ L of staining buffer with 1  $\mu$ g/mL Fc-Block, followed by incubation on ice or 4 °C refrigeration for 15 min in the dark.
2. Stain cells using the desired antibody/fluorescence panels (e.g. T-cell panel, macrophage panel, etc.): Add the antibody mixture diluted in Fc blocking buffer to each sample, stain for at least 30 min on ice in the dark.
3. Add 1 mL of ice cold PBS to each tube and re-suspend the cells gently, followed by centrifugation at 300 x g for 5 min. Discard the resulting supernatant.
  1. Repeat to wash the cells twice.
4. Stain for intracellular markers if needed, following steps 6-10, otherwise jump to step 10.
5. Re-suspend the cell pellet by pulse vortex and add 200  $\mu$ L of prepared Fixation/Permeabilization working solution for each sample. Pulse vortex again, and then incubate at 4 °C overnight (preferred) or 30 min at room temperature in the dark.
6. Spin down the cells and remove the supernatant.
7. Wash twice by adding 1 mL of 1x Permeabilization Buffer (made from 10x Permeabilization Buffer, diluted with distilled H<sub>2</sub>O) followed by centrifugation and decanting of supernatant.
8. Add intracellular marker antibody in 1x Permeabilization Buffer and incubate at room temperature for 30 min in the dark.
9. Wash cells twice with 1 mL of 1x Permeabilization Buffer. Centrifuge and decant supernatant.
10. Resuspend cells in 150  $\mu$ L of Staining Buffer and analyze on a cytometer. Due to the fixation and permeabilization procedure, the FSC (forward-light scatter)/SSC (sidelight scatter) distribution of the cell population will be different to live cells. Therefore, the gate and voltages will need to be modified.

### 3. FMO Controls (Fluorescence Minus One Control)

NOTE: Multi-color flow analysis is particularly important for the analysis of tumor-infiltrating immune cells. Therefore, there is a need to find a way to identify and gate cells in the context of data spread due to the multiple fluorochromes in a given panel. FMO (Fluorescence Minus One) Control is an important approach for this purpose.

1. To this end, include additional mice in each group for FMO controls (at least 2 per Rx) and process individually for each tissue. After dissociation, pool tissues. For example, in a study with 4 Rx groups, 8 additional tumors should be processed individually and then pooled into one sample for FMOs.

### 4. Flow Instrument Setup

1. Make compensation beads while the machine is warming up (at least 20 min).
2. Use CS&T beads to check performance.
3. Voltage and compensation settings: use UltraComp beads, vortex the Comp beads thoroughly before use.
4. Label a separate 12 x 75 mm<sup>2</sup> sample tube for each fluorochrome-conjugated antibody.
5. Add 100  $\mu$ L of staining buffer to each tube. Add 1 full drop (approximately 60  $\mu$ L) of the beads to each tube.
6. Add antibodies and perform the staining procedure exactly as the sample process stated in section 4.
7. Add 0.5 mL of staining buffer each, to completely re-suspend bead pellets via vortex.

8. Set flow cytometer PMT voltage per target tissue for the given experiment.
9. Run through flow cytometer for data acquisition, by gating on the singlet bead population per FSC and SSC readings.
10. Set Flow rate around 200–300 events/s.
11. Set appropriate compensation for a given fluorescein [FITC]-conjugated antibody, use an FL1 vs. FL2 dot plot.
12. Place a quadrant gate so that negative beads are within lower left quadrant and the positive beads are in the upper or lower right quadrant. Adjust the compensation values until the median fluorescence intensity (MFI) of each population (as shown in the quadrant stats window) is approximately equal (*i.e.* for FL2-%FL1, the FL2 MFI of both beads should be similar).
13. Repeat steps 5.4.11 and 5.4.12 for all tubes.
14. Proceed to acquiring the actual stained samples. Run the compensation wizard and save the settings with the format "date experiment your initials".

## 6. Flow Data Analysis and Presentation

1. Analyze data by Flowjo and/or Kaluza.

### Representative Results

Orthotopic implantation of PDAC resulted in rapid tumor growth similar to that seen for SC implantation. After the donor tumor fragments were implanted into recipient mice, both subcutaneously and orthotopically according to the protocols described in Steps 2.1 and 2.2, the implanted KPC homograft tumors demonstrated similar rapid growth as shown in **Figure 1A**. KPC homograft tumors harvested at different time points are shown in **Figure 1B** and representative H&E images are shown **Figure 1C**. Our data demonstrated similar growth of SC and orthotopic implants.

Viable tumor cells and cells in the TME, including tumor-infiltrating immune cells, originating from either orthotopic or SC implantation, can be efficiently recovered. Tumors were harvested and digested to prepare single cell suspensions for subsequent FACS analysis using a commercial dissociator (**Figure 2A**) according to the protocol described in Step 4. We usually obtained reasonably high viable cell yields from the tumor samples of both types of implantation (~80% viability based on Trypan Blue); the representative FACS plot shows viable cells from tumor, separated from the dead cells/cell debris (**Figure 2B**).

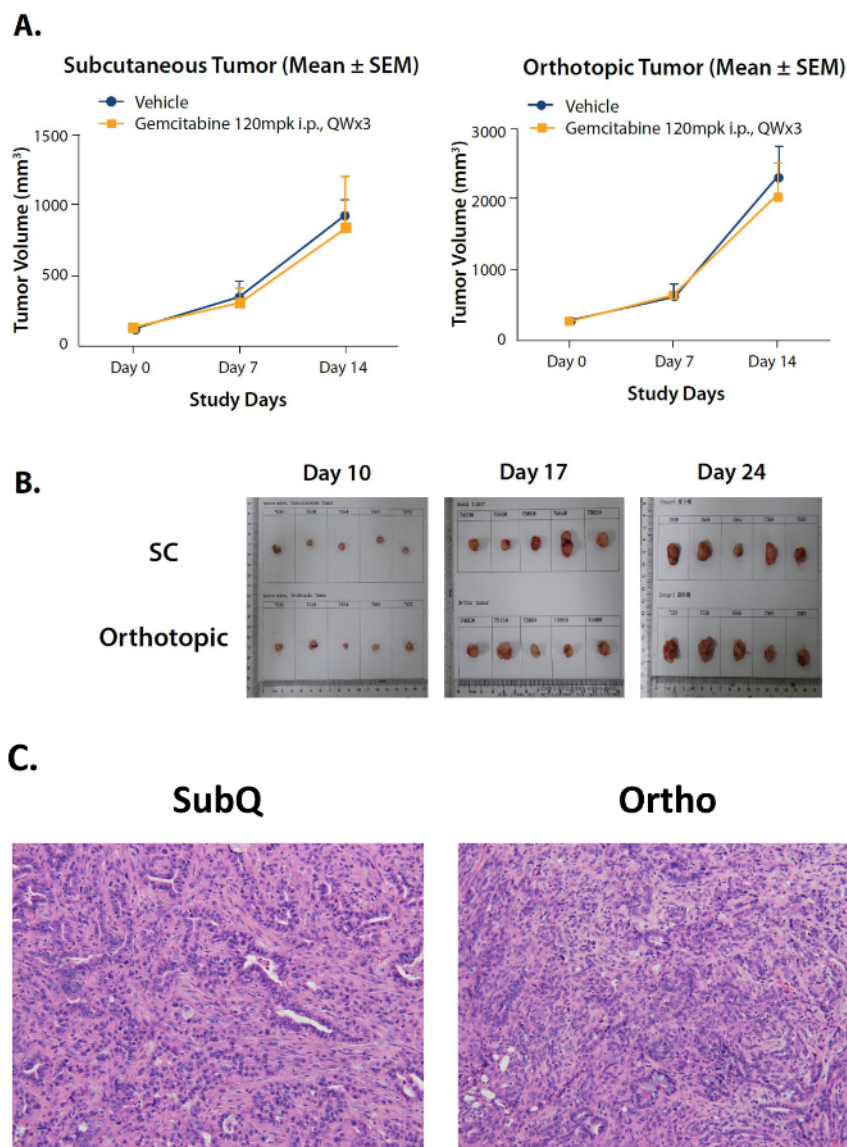
Tumor-infiltrating immune cells of different subsets have been identified in both orthotopic and SC implanted tumors, while their profiles have differences. The single cell suspension prepared from tumor tissues by using the method described in step 4.2 were subjected to FACS analysis after staining with a 16 color panel of markers shown in **Table 1**, which covers different lineages of important immune cells as well as tumor cells. The gating strategy or immune lineage hierarchy along with representative flow plots are shown in **Figure 3A**. CD45, a marker for all mature immune cells, was used for distinguishing tumor cells and tumor-infiltrating immune cells. All immune lineages were subsequently analyzed from CD45<sup>+</sup> populations as displayed by the panel (**Table 1**).

Beside markers, cell size is also used to differentiate different subpopulations (**Figure 3B left**) to quantify cell subsets, including T, B lymphocytes, macrophages and MDSCs, etc. Tumor infiltrating immune profile comparison of SC vs. orthotopic homografts of pancreatic cancers. The major enumerated cell populations of several key subsets of tumor infiltrating immune cells are shown **Figure 3C**. The data clearly shows that the tumor has significantly increased immune cell infiltration compared with the pancreas of healthy mice. In addition, different percentages of subset of tumor-infiltrating immune cells were found in orthotopic vs. SC homografts, *e.g.* significantly more B-cells in orthotopic than in SC.

Marker	Immune Cell Population
CD45	Total leukocytes
CD3	Total T cells
CD4	CD4+ T helper cells
CD8	CD8+ cytotoxic T cells
CD44/CD62L*	Naïve, memory and effector T cells
CD69/CD44/OX40/CD25 <i>etc</i> *	Activation markers
CD4+CD25+FoxP3+	Regulatory T cells
CD11b+Ly6c/Ly6g	G-MDSC and M-MDSC
CD11b+ F4/80	Macrophages
IA/IE/CD206	M1 and M2 macrophages
CD3-CD335+	NK cells
CD3+CD335+	NKT cells
CD19	B cells
TNF- $\alpha$ /IFN- $\gamma$ /IL-7/IL-3 <i>etc</i> *	Cytokines
PD-1/PD-L1/CTLA-4/TIM-3 <i>etc</i> *	Checkpoint inhibitors
Granzym B <i>etc</i> *	Commonly requested markers
KI67/Brd U/PNCA <i>etc</i>	Proliferation
Live/dead (fixable)	Live/dead
*Note: Further markers can be added as needed	

**Table 1: 16-color flow panels designed for analysis of tumor-infiltrating mouse immune cells.**



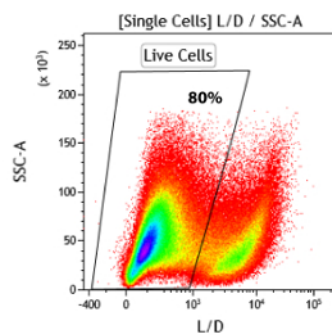


**Figure 1: Both orthotopic and SC (subQ) implanted PDAC homograft tumors in C57BL/6 mice demonstrated similar growth with or without gemcitabine treatment. (A)** growth curve: SC-left, and orthotopic-right. Blue: Vehicle; Gold: Gemcitabine (initiated on Day 10 post inoculation when average SC tumor volume reached 200 mm<sup>3</sup>). **(B)** tumor tissues at different time points. **(C)** Representative H&E staining of both types of homografts (40 x 10). [Please click here to view a larger version of this figure.](#)

**A.**

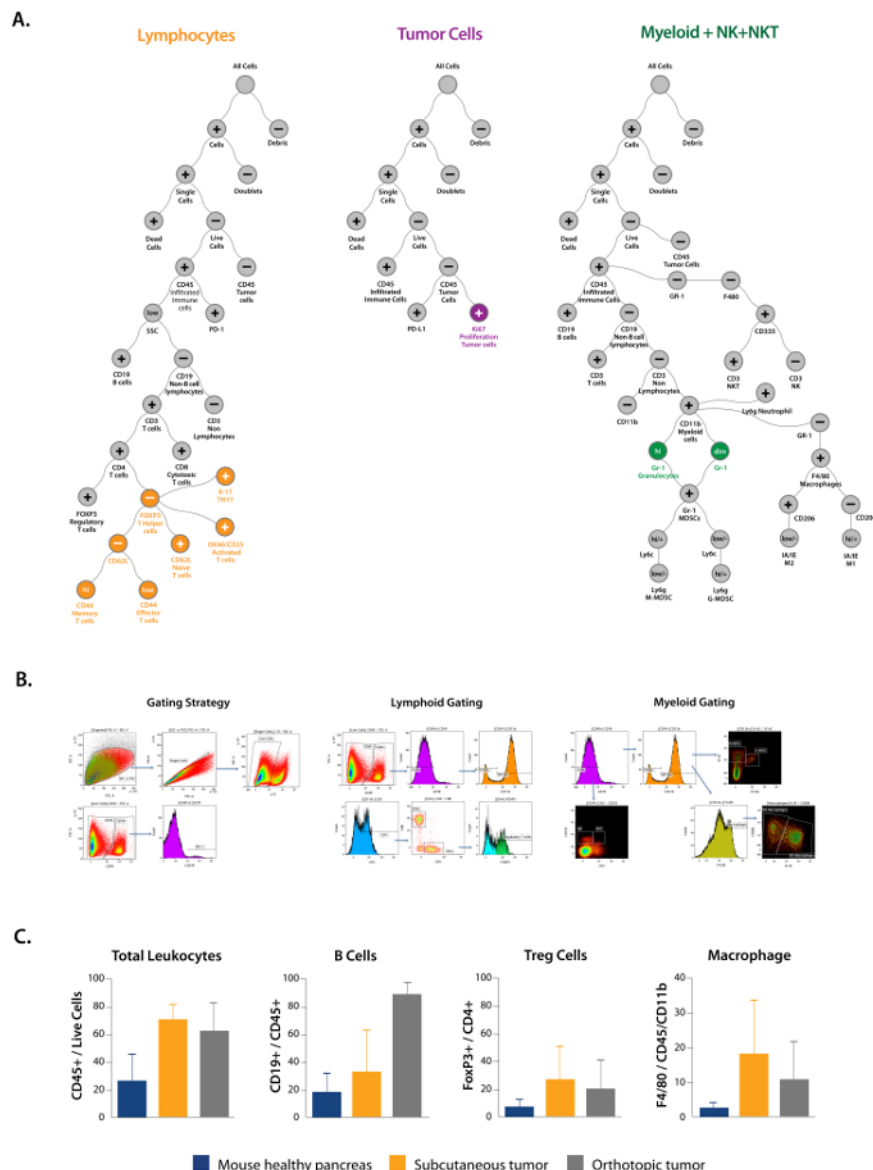


**B.**



**Figure 2: Tumor tissue dissociation to prepare viable single cell suspension. (A)** the use of the disassociator; **(B)** the viable cells, separated from dead cells, as shown by flow analysis. [Please click here to view a larger version of this figure.](#)





**Figure 3: Multi-color flow analysis of tumor-infiltrating immune cells of both orthotopic and SC PDAC homografts.** The raw data from each tumor sample were acquired from the flow cytometry instrument, followed by analysis using Flowjo FACS analysis software. **(A)** The standard gating strategy for the analysis, is displayed as an example; **(B)** Representative flow gating and analysis data using the standard gating strategy; **(C)** Representative tumor-infiltrating immune cells are displayed to include B-cells, Treg and macrophages. [Please click here to view a larger version of this figure.](#)

## Discussion

Although studies using SC tumors are more readily conducted, orthotopically implanted tumors models can potentially be more relevant for preclinical pharmacology studies (particularly I/O investigations) to provide enhanced translatability. This report aims at helping the interested readers/audience to be able to directly visualize the technical procedures that can be used in their respective research. Our protocols demonstrate that orthotopic implantation of PDAC can result in efficient tumor growth, similar to SC implantation. Our observations also seem to suggest the presence of different immune profiles of TMEs from the different implantations. The major challenges to adapt orthotopic, as compared to SC implantations, are that complex skills are required, the process is time consuming, the surgical procedures for implantation are labor intensive and also the difficulty in monitoring orthotopic tumor growth in life.

There are four critical steps to ensure that orthotopic pancreatic tumor experiments are successfully performed: 1) the surgical procedure of implantation; 2) the careful and timely monitoring of tumor development; 3) the importance of performing pre-experiment tests first to familiarize with the procedure and assess the take rate and tumor growth rate; 4) using single cell suspensions of dissociated tumors as an alternative engraftment method. This report procedure is helpful to readers for performing research using this specific homograft, as well as other pancreatic orthotopic models, and even other orthotopic models involving abdomen-opening surgery.

Flow cytometry or FACS is currently the most important tool to perform immunoprofiling. Immunophenotyping of tumors by FACS significantly differs from that of cells from different organs, such as peripheral blood, spleen, lymph node and bone marrow in the following ways. Generally, there is a very small percent of immune cells present in tumors (small sample size). The extreme heterogeneity of tumors and the small number of immune cells present make recovering viable rare immune cells technically challenging, requiring custom-developed tumor tissue dissociation involving machines. Both previous points make simultaneous multi-parameter measurement using multi-color flow cytometry essential. Multi-color flow requires complex marker panel design, compensation, and gating strategies, due to fluorescence spectral overlap. This report also attempted to demonstrate to the interested readers/audience the process of tumor immune profiling via defined tumor tissue dissociation and multi-color flow cytometry analysis.

Three critical steps could be particularly important to yield productive TIL analysis: first, a high yield of viable cells recovered from dissociated tumors using customized tumor dissociation procedures; second, the optimized design of large multi-color staining panels based on available reagents; third, an optimized gating strategy in the analysis. The authors would like to emphasize the training and experience of the operators of both data acquisition and analysis are essential for the successful flow cytometry analysis of TIL.

## Disclosures

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