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TITLE:

An Orthotopic Resectional Mouse Model of Pancreatic Cancer

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SUMMARY:

In the clinical context, patients with localized pancreatic cancer will undergo pancreatectomy followed by adjuvant treatment. This protocol reported here aims to establish a safe and effective method of modelling this clinical scenario in nude mice, through orthotopic implantation of pancreatic cancer followed by distal pancreatectomy and splenectomy.

ABSTRACT:

There is a lack of satisfactory animal models to study adjuvant and/or neoadjuvant therapy in patients being considered for surgery of pancreatic cancer (PC). To address this deficiency, we describe a mouse model involving orthotopic implantation of PC followed by distal pancreatectomy and splenectomy. The model has been demonstrated to be safe and suitably flexible for the study of various therapeutic approaches in adjuvant and neo adjuvant settings.

In this model, a pancreatic tumor is first generated by implanting a mixture of human pancreatic cancer cells (luciferase-tagged AsPC-1) and human cancer associated pancreatic stellate cells into the distal pancreas of Balb/c athymic nude mice. After three weeks, the cancer is resected by re-laparotomy, distal pancreatectomy and splenectomy. In this model, bioluminescence imaging can be used to follow the progress of cancer development and effects of resection/treatments. Following resection, adjuvant therapy can be given. Alternatively, neoadjuvant treatment can be given prior to resection.

Representative data from 45 mice are presented. All mice underwent successful distal pancreatectomy/splenectomy with no issues of hemostasis. A macroscopic proximal pancreatic margin greater than 5 mm was achieved in 43 (96%) mice. The technical success rate of pancreatic resection was 100%, with 0% early mortality and morbidity. None of the animals died during the week after resection.

In summary, we describe a robust and reproducible technique for a surgical resection model of pancreatic cancer in mice which mimics the clinical scenario. The model may be useful for the testing of both adjuvant and neoadjuvant treatments.

INTRODUCTION:

Pancreatic ductal adenocarcinoma (pancreatic cancer [PC]) is associated with a poor prognosis[1]. Surgical resection remains the only potentially curative treatment for PC and should be considered for patients presenting with early stage disease. Unfortunately, even with R0 resection (i.e., resection margins free of tumor), the recurrence rate (local or from undetected metastatic disease) is high[2,3]. Therefore, systemic adjuvant therapy is indicated in almost all patients who undergo resection[4]. Furthermore, while neoadjuvant therapy is now recommended only for borderline-resectable cancers, its indications are expanding such that its routine use is the focus of much clinical research[5-8]. In order to develop novel therapeutic approaches for PC involving resection, these approaches need to be first assessed in pre-clinical models that accurately recapitulate clinical settings.

Orthotopic mouse models of PC have been frequently used in the past to test drug treatments[9,10]. Many of these were produced by injection of cancer cells alone into mouse pancreas, resulting in tumors that lacked the prominent stroma that is characteristic of PC. More recently, orthotopic models, such as the one we first developed by injecting a mixture of human PC and human pancreatic stellate cells (PSCs, the primary producers of the collagenous stroma in PC), have come into regular use[11,12]. The tumors produced by such co-injection of cancer and stromal cells exhibit (i) both the cancer elements and the characteristic stromal (desmoplastic) component of PC, and (ii) enhanced cancer cell proliferation and metastasis[11]. Thus, this model closely resembles

human PC. While a number of resectional models of orthotopic PC have been described[13-16], none have reflected the clinical realities of pancreatic resection in humans as accurate as this model, and therefore have been suboptimal for testing adjuvant or neoadjuvant treatments.

The aim of the mouse model presented was to demonstrate how to: (i) successfully implant orthotopic pancreatic cancer while minimizing inadvertent peritoneal dissemination and (ii) subsequently completely resect the cancer. The paper highlight tips and potential pitfalls of this technique.

PROTOCOL:

All procedures were approved by the Animal Care and Ethics Committee of the University of New South Wales (17/109A). Female athymic Balb/c nude mice, aged 8-10 weeks weighing 16-18 g, were used for this protocol. Mice were housed in microisolator cages and fed commercially available pelleted food and water *ad libitum*.

1. Orthotopic pancreatic cancer implantation

1.1 Prepare the cells for implantation. First, calculate the number of cells required for the procedure (1×10^6 luciferase-tagged AsPC-1 cells and 1×10^6 cancer-associated human pancreatic stellate cells [CAhPSCs] are required for each animal).

1.1.1 Maintain these cells in a humidified temperature-controlled CO_2 incubator and perform routine mycoplasma testing. Culture medium used for AsPC-1 and CAhPSCs are RPMI 1640 (with 300 mg/L L-glutamine, 20% v/v foetal bovine serum, 1% v/v penicillin/streptomycin) and IMDM (with 4 mM L-glutamine, 10% v/v foetal bovine serum, 1% v/v penicillin/streptomycin).

1.1.2 Use standard cell culture techniques to trypsinize the cells into a cell suspension. Neutralize the trypsin using the respective complete culture medium at a volume twice that of the trypsin solution used.

1.1.3 Wash these cells twice with phosphate buffered saline (PBS) and resuspend into a mixture containing 1×10^6 AsPC-1 cells and 1×10^6 CAhPSCs in a 50 µL cell suspension.

1.1.4 Keep this suspension on ice until use.

1.2 Prepare a class II biosafety cabinet for the procedure. Use a heating mat overlaid by a sterile plastic drape. For magnification during the procedure, use a pair of 2.5x to 3.5x magnification surgical loupes.

1.3 Prepare purse-string swabs by cutting a hole, 1 cm in diameter, into a gauze swab. Secure this hole with a purse-string suture. Any fine braided suture can be used for this (e.g., 5/0 polyglycolic acid suture). Braided suture material is recommended as it allows the loose knot to stay in place after tightening. This is illustrated in **Figure 1a**.

1.4 Anaesthetize the mouse with 80 mg/kg of ketamine and 10 mg/kg of xylazine by intraperitoneal injection. Once anaesthetized, place the mouse on the sterile field in a supine position and apply povidone-iodine followed by 70% ethanol for skin preparation.

1.5 Make a longitudinal incision in the skin of the left cranial quadrant of the abdomen, and then enter the abdomen by incising the muscular layer between forceps.

1.6 Load a 29 G insulin syringe with 50 µL of cell suspension – this equates to 1 x 10^6 CAhPSCs and 1 x 10^6 luciferase-tagged AsPC-1 cells per injectate. Mount it on the injection device. The design and function of this injection device is explained in detail in **Figure 1b** and its legend.

1.7 Place the purse-string swab over the laparotomy incision and then exteriorize the spleen and pancreatic tail through the opening of this swab. Tighten the purse-string to gently encircle the body of the pancreas, exposing the pancreatic tail for injection. It is important to be tight enough that the gauze contacts the pancreas circumferentially while at the same time not constricting it.

1.8 Using a pair of forceps, grasp the tail of the pancreas and gently place lateral tension on it. Puncture the ventral peritoneal surface with the needle at a shallow angle and then inject the cell suspension into the pancreas in a slow and controlled fashion (over 10–15 s) with the injection device.

1.9 During the injection process, carefully observe for leakage—both around the injection site (from reflux) and on the other side of the pancreatic lobule (in case of through-and-through penetration). If visible leakage occurs, stop the injection and note the volume of leakage by checking the volume of remaining injectate in the syringe. If the leakage is of small volume (<10 µL), and then absorb any leakage with gauze and reposition the needle into a different pancreatic lobule to complete the injection.

1.10 After injection, hold the needle in place for a few seconds before withdrawing to minimize leakage. Use a povidone-iodine-soaked swab to carefully dab the site to absorb any inadvertently leaked cell suspension.

1.11 Replace the spleen and pancreas and close the abdominal wall with 5/0 polyglycolic acid suture in a continuous fashion. Close the skin with clips.

1.12 Administer 5 mg/kg enrofloxacin antibiotic prophylaxis, 2.5 mg/kg flunixin analgesia and 1 mL of 0.9% saline subcutaneously.

1.13 Monitor the mouse in a warmed cage until recovered from the anaesthetic. Once awake and alert, move the mouse back to its cage.

2 Cancer resection surgery: Distal pancreatectomy and splenectomy

2.1 The timing of resection in relation to implantation can vary depending on the experimental protocol. In general, allow the tumors to grow at least for 3 weeks prior to resection, but optimize this empirically for the particular implanted cancer cell line.

2.2 On the day prior to the resection surgery, perform bioluminescence imaging on the animals to confirm the presence of a localized primary tumor. Note that this imaging study is simply used to exclude mice with obvious extra-pancreatic disease from resection. Neither size nor radiant flux were used as thresholds for determining eligibility for resection.

2.2.1 Weigh mice and inject with D-luciferin intraperitoneally (150 mg/kg).

2.2.2 Determine the timing of the imaging step in relation to luciferin injection for each experiment by the performance of a luciferin kinetic curve. The period of time where the radiant flux is above 90% of its maximum represents the optimal time for bioluminescence imaging (in this experiment, 18 to 26 minutes post-injection)

2.2.3 Induce anaesthesia and maintain using isoflurane (4% and 3% with oxygen, respectively) and perform imaging using a bioluminescent imaging device (e.g., IVIS Lumina II). Use automatic exposure and binning settings (this can, however, be optimized for the expected radiant flux).

2.3 Prepare the class II biosafety cabinet for procedure. Use a heating mat overlaid by a sterile plastic drape. For magnification during dissection, use a pair of 2.5x to 3.5x magnification surgical loupes.

2.4 Anaesthetize the mouse with 80 mg/kg of ketamine and 10 mg/kg of xylazine by intraperitoneal injection.

2.5 Place the mouse on the sterile field in a supine position and apply povidone-iodine followed by 70% ethanol for skin preparation.

2.6 Make a longitudinal incision in the skin of the left cranial quadrant of the abdomen, preferably through the previous incision site.

2.7 Bluntly dissect the skin off the underlying muscular abdominal wall, and then place an Alm self-retaining retractor to hold the skin wound open.

2.8 Incise the muscular layer between forceps just to one side of the suture line of the previous operation, and then extend the incision to excise the entire previous suture line.

2.9 Exteriorize the spleen and distal pancreas and retract it cranially. At the caudal aspect of the pancreas, the colon may be found attached by filmy adhesions. If this is found, bluntly dissect the colon off.

2.10 Carefully pass a pair of forceps dorsal to the body of the pancreas and splenic vessels and open this space. This frees up a segment of pancreas for subsequent ligation.

2.11 Ligate the body of the pancreas proximal to the tumor with a titanium ligation clip, and then transect the pancreas distal to this with cautery. An alternative way to control the pancreatic stump is to ligate it in continuity with 5/0 polyglycolic acid suture before transection.

2.12 Retract the pancreas caudally and cauterize the gastrosplenic vessels between the cranial pole of the spleen and the stomach.

2.13 Remove the specimen and confirm haemostasis.

2.14 Close the abdominal wall with 5/0 polyglycolic acid suture in a continuous fashion. Close the skin with clips.

2.15 Administer 5 mg/kg enrofloxacin antibiotic prophylaxis, 2.5 mg/kg flunixin analgesia and 1 mL of 0.9% saline subcutaneously.

3 Postoperative management

3.1 In the immediate post anaesthetic period (for both of the above procedures), monitor the mouse in a warmed cage until recovered from the anaesthetic. Once awake and alert, move the mouse back to its cage.

3.2 Subsequently, monitor mice daily for weight, food intake and activity. Examine incision sites and palpate for tumor size. Remove skin clips on the seventh postoperative day.

3.3 Euthanize the mouse if humane endpoints are reached. These humane endpoints include: loss of body weight >20%, features of untreatable distress (including hunched posture, lack of movement or grooming) and tumor size greater than 1 cm^3 as estimated by external palpation.

REPRESENTATIVE RESULTS:

Fifty-nine consecutive mice underwent implantation surgery. Gross leakage occurred in eight (14%) mice. The degree of leakage at the time of injection is estimated as described above in the protocol section. After three weeks to allow these implanted tumors to grow, pre-resection bioluminescence imaging was performed to exclude mice with gross metastatic disease prior to resection. Forty-five (76%) mice underwent surgical resection.

All 45 (100%) mice underwent successful distal pancreatectomy/splenectomy with no issues of haemostasis. A macroscopic proximal pancreatic margin greater than 5 mm was achieved in 43 (96%) mice.

At the time of resection, local metastasis was found in 9/45 (20%) mice – mostly in the suture line (discontinuous with the primary tumor) with three of the nine showing additional isolated

nodules on the greater curve of the stomach and one showing a subcapsular nodule on the liver. The primary pancreatic tumor was adherent to the suture line in five (11%) mice and to the liver in one (2%) mouse. These adherent structures were excised *en bloc*.

The mean (SEM) surgery time (induction to closure) was 22 (0.9) minutes. None of the animals died within 1 week after resection.

One-week post resection, mice underwent bioluminescence imaging to detect residual disease. The ratio of the maximum radiance over the ventral surface of the mouse was compared to that of the background. Thirty-two (71%) mice had a maximum radiance ratio (mouse:background) of <10, indicating minimal or no residual disease.

FIGURE AND TABLE LEGENDS:

Figure 1: Custom-made devices to facilitate tumor implantation. (**a**) Purse-string gauze swab: (i) Central hole, approximately 1 cm in diameter, through which the pancreatic tail will be placed at the time of injection; (ii) Purse-string suture around the hole; (iii) Double-layered gauze; (iv) Single throw knot; (v) One limb of the suture material is secured to the gauze with sterilising indicator tape; (vi) A handle, made from indicator tape, is fashioned on the other end of the suture material. (**b**) Injection device: (I) Actuating syringe. Slots cut through the body of this syringe allows the injection syringe (with the cell suspension injectate; not shown) to be mounted on this syringe body; (II) Controller syringe. This is filled with water. Depression of the plunger on the smaller controller syringe by the surgical assistant causes displacement of the larger actuating syringe plunger. The displacement of the actuating plunger is smaller, but with a mechanical advantage which allows the injection to overcome the resistance associated with the injection syringe mechanism as well as the tissue's resistance to expansion by the injectate. This allows for precise and smooth injection of 50 µL over 10–15 seconds; (III) Polytetrafluoroethylene (PTFE) connection tubing with internal diameter of 0.5 mm.

DISCUSSION:

A resectional orthotopic mouse model of pancreatic cancer is important because it allows for the testing of adjuvant and neoadjuvant treatments. This is particularly important in pancreatic cancer where surgery remains the most effective treatment but is associated with high risk of recurrence. This paper describes a method which will reliably produce a pancreatic cancer which is potentially curable with resection, replicating the clinical scenario where neoadjuvant/ adjuvant therapy is required.

Significance with respect to existing methods

Despite the importance of adjuvant and neoadjuvant therapies in pancreatic cancer, there are few well-described orthotopic resectional mouse models in the literature. These described resectional models varied in their fidelity of replication of the clinical situation in humans. These previous models can be broadly classified into: (i) tumor excision only, with fluorescence guidance; (ii) subtotal pancreatic resection with no splenectomy; (iii) distal pancreatectomy/splenectomy.

Tumor excision with fluorescence guidance has been described in the greatest number of reports[15,17-21]. Many of these papers originated from the same research group. Unfortunately, in humans, local excision of the tumor alone (enucleation) is not performed for pancreatic adenocarcinoma (PC) due to the high likelihood of local recurrence, as well as the inability to assess lymph node status[22,23]. Therefore, the use of a non-clinically relevant comparator group (non-fluorescence guided enucleation) clouds the reporting of the oncological outcomes in papers describing this technique. Not surprisingly, the non-fluorescence enucleation groups invariably had excessive rates of local recurrence[15,20,21]. In contrast, Torgenson et al.[14] described a similar fluorescence-guided resection technique, and reported a reasonably low recurrence rate of 58% (at eight weeks post-resection). Overall, these studies appear to demonstrate the utility of fluorescence guidance for visualization of residual disease during surgery. However, this is not yet the standard of care in humans, which is a limitation in terms of its use in a mouse model aiming to replicate the clinical scenario. Of course, this may change if fluorescence-guided surgery were to be widely adopted in clinical practice.

Another resection model was based on subtotal pancreatectomy without splenectomy for a tumor implanted into the *body* of the pancreas[13,24]. The clinical relevance of this is also called into question as the operation described was neither a pancreaticoduodenectomy nor distal pancreatectomy as performed in humans. Not surprisingly, these mice also suffered from high rates of tumor recurrence, both distant and local. Of particular note is that splenic recurrence was common, suggesting either inadequate resection or possible peritoneal tumor seeding at implantation[24].

Ni et al.[16] described a distal pancreatectomy/splenectomy model performed with fluorescence imaging guidance. Disappointingly, despite the use of a clinically relevant operation (with fluorescence guidance), the survival was very short (mean survival of 18 days), even in the distal pancreatectomy group. This degree of progressive disease appears to be even worse than palliative treatment models[25-27], suggesting the possible presence of gross residual disease after resection. Most recently, Giri et al.[28] reported a distal pancreatectomy and partial splenectomy mouse model. This study is notable in that it represents an immunocompetent mouse model of cancer. However, this study reported almost universal local and other intraperitoneal tumor recurrence, possibly indicating occult iatrogenic metastasis at implantation.

The use of mouse models where there is gross residual disease post resection for testing adjuvant treatments may be inappropriate. The issue is that treatment for gross residual disease cannot truly be classified as adjuvant treatment but rather should be considered to be treatment with palliative intent. In that case, such mouse models offer no advantage compared to non-resectional models with low volume disease.

Tips and pitfalls of critical steps

Tumor implantation procedure

In order to replicate the clinical scenario, there are distinct challenges in this model which relate to the implantation and resection procedures. For the implantation procedure, the major challenges which need to be overcome are successful implantation and prevention of leakage.

These two issues are interrelated as failure of injection would result in gross leakage of the tumor cell suspension into the abdominal cavity. This would produce a mouse model with peritoneal metastasis, which will progress regardless of pancreatic resection. This reflects the well-known clinical scenario in humans where pancreatic resection in metastatic PC does not affect the patient outcome. This is the basis of the staging laparoscopy in humans[29].

The success of implantation of the tumor can be seen intraoperatively as the successful generation of a "bubble" of cell suspension without obvious leakage. Of most importance in achieving a good result is the accurate placement of the needle within the pancreatic parenchyma. This could only be achieved by "stretching out" the pancreas so that the peritoneal surface is taut. Puncture should occur with the needle bevel facing upwards (ventrally). Once the needle punctures the peritoneal surface, it should be advanced while the needle tip is slightly lifted-up so that the beveled surface glides just beneath the peritoneum. This will prevent inadvertent through-and-through puncture of the pancreas, a common pitfall due to the small dimensions of mouse pancreatic lobules. Once the entire bevel is within the substance of the pancreas, the cell suspension is injected. Magnification of vision with surgical loupes is highly desirable to visualize accurately the depth of the needle penetration.

A number of techniques can be used to further minimize the risk of inadvertent leakage.

Selection of a large lobule for injection. Small lobules require higher pressures to inflate (following Laplace's law), thereby increasing the risk of leakage around the needle at the puncture site.

Optimization of the speed of injection. The use of an injection device (**Figure 1b**) which allows the cell suspension to be injected over 10-15 seconds serves three purposes. First, it decreases the rate of change of pressure in the pancreas, giving the tissues time to deform and reduces the risk of reflux of the suspension. Second, it allows the injection process to be monitored and, if necessary, stopped and needle repositioned. Any leakage can be mopped up by a povidone-iodine-soaked gauze. Third, it frees the operator from needing to depress the plunger, allowing the operator to focus on keeping the needle tip within the pancreas while the assistant injects the cell suspension.

Use of a double-layered purse-string gauze. This gauze forms a collar around the pancreatic tail which will absorb any leakage of the cell suspension and therefore minimize contamination in the abdominal cavity.

Some studies in the literature have used an extracellular matrix mixture (Matrigel) which solidifies with time after injection[13,15,24]. This may reduce the risk of leakage post-injection. However, a potential disadvantage of this strategy is that Matrigel or other similar extracellular matrix solutions may exert non-physiological effects on PSCs[30]. For instance, Matrigel has been shown to render PSCs quiescent thereby potentially negating the effects of PSCs in the model[31,32]. An alternative to injection of cancer cells is the orthotopic implantation of tumor tissue (either directly from patients or from subcutaneous mouse models). However, these approaches have their own disadvantages. First, heterogeneity may arise from sampling error or from variations in the volume of tissue implanted. Such heterogeneity may reduce the power of subsequent treatment comparisons. Second, passaging of tumor tissue with a subcutaneous mouse model

may lead to selection of sub-clones which have different biological behaviours to the original patient tumor.

Tumor resection procedure

In this model, we have utilized a distal pancreatectomy/splenectomy procedure akin to that performed in humans. The challenges relating to the resectional surgery depend on pathological and anatomical factors.

The key pathological factor is tumor dissemination. Low volume local spread can be resected at the time of pancreatic resection, although it may indicate the possibility of more distant peritoneal and other metastasis. We routinely excise the suture line from the first operation as it is a possible area of local recurrence. If the tumor is attached to surrounding structures, such as the abdominal wall or the left lobe of the liver, these can be resected *en bloc*. Anatomically, the key step is dissecting the plane dorsal to the body of the pancreas. The splenic vein can often be visualized behind the pancreas once the pancreas is exteriorized. This is a key landmark, as the embryological bloodless plane is immediately dorsal to this.

There are two other potential anatomical pitfalls in the model described here. The colon may be adherent to the caudal aspect of the pancreatic body. Failure to mobilize this structure away could lead to inadvertent colonic injury at the time of pancreatic division or ligation. The gastrosplenic vessels are small and may easily bleed if avulsed or inadequately cauterized. Furthermore, once avulsed, the bleeding point often retracts deep into the abdomen behind the greater curve of the stomach, making subsequent control of bleeding more challenging. Therefore, careful retraction of the spleen and cautery of the gastrosplenic vessels are required. One approach for successful hemostasis is to cauterize these vessels on the hilar aspect of the spleen which minimizes the risk of inadvertent thermal injury to surrounding hollow viscera.

We have found that using a titanium ligation clip, widely used in human surgery for ligation of vessels, is a rapid and effective way of controlling the pancreatic stump, with consequent reduction in total operative time compared to the use of ligatures. This was also used by Giri et al.[28].

Limitations of the technique

There are limitations to this resectional model of the pancreas. One limitation relates to the time allowed to produce recurrence/metastasis. On the one hand, one needs to maximize the development of metastatic disease, but on the other hand, one needs to resect the tumor before it became locally advanced. The period between implantation and resection may therefore need to be adjusted for the particular clinical scenario one wishes to replicate. Another limitation relates to the inadvertent spillage and subsequent peritoneal metastasis of cancer cells which is discussed above.

A major challenge of adjuvant treatment models is dissecting the adjuvant treatment effect from the surgical treatment effect. Clearly, a well-designed study which is randomized, with a control group undergoing resection surgery is required. To further improve the assessment of the

relative treatment effects, we suggest assessing tumor burden *in vivo* (for example, by using luciferase-tagged cancer cells and performing in vivo bioluminescence imaging). Despite the semi quantitative nature of this assessment in orthotopic models (as the bioluminescence signal is attenuated by passage through the overlying tissues), this approach allows longitudinal assessment of tumor burden, including the assessment of the post-surgical residual disease.

Modifications and future applications

The implanted cell line and/or cell numbers with or without pancreatic stellate cells could be modified to reflect the target clinical scenario[12]. The duration between implantation and resection could also be modified to change the risk of metastasis formation. Other variations could include implantation of patient- or mice-derived xenografts or organoids[33].

Neoadjuvant therapy can also be tested within the basic features of the model described here. It would simply require commencement of drug treatment prior to surgical resection[34]. Similarly, both neoadjuvant and adjuvant therapy could be studied in the same mice.

Finally, while we have described the use of athymic Balb/c nude mice which represents an immunodeficient model, an alternative immunocompetent model may involve KPC tumor cells implanted into C57B6 mice[28]. This may be a useful alternative for the testing of adjuvant/neoadjuvant immune therapies.

In summary, we describe a robust and reproducible technique for a surgical resection model of pancreatic cancer in mice which mimics the clinical scenario and does not require specialized equipment. This model may be useful for the testing of both adjuvant and neoadjuvant treatments.

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DISCLOSURES:

The authors have nothing to disclose with respect to this project.

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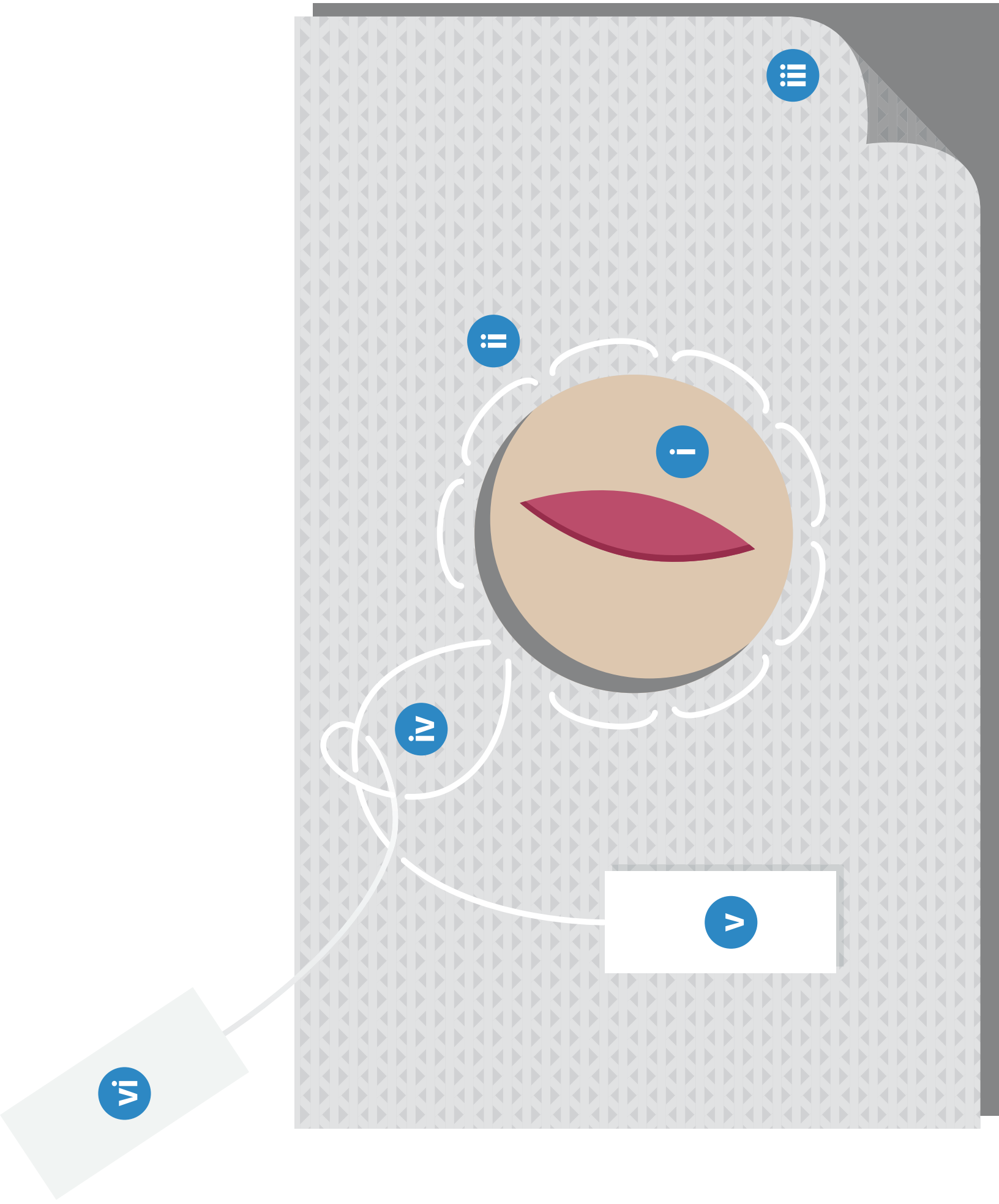
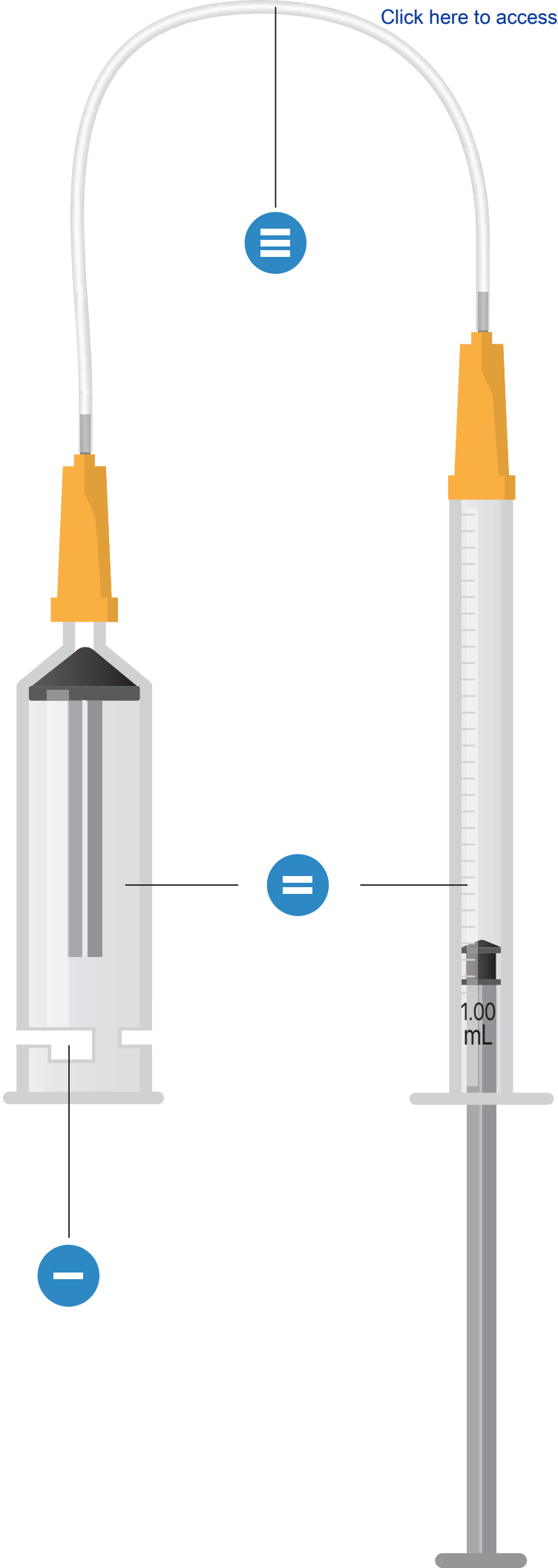
Figure

Click here to access/download;Figure;Figure 1a.eps



Figure

Click here to access/download;Figure;Figure 1b.eps



Name of Material/Equipment

Animals, Materials and Equipment for Implantation Procedure

AsPC-1 human pancreatic cancer cell line, luciferase tagged (luc+ gene from Promega PGL3 Basic plasmid)
Autoclip wound clips, 9 mm
Basic Dressing Pack
Cancer associated human pancreatic stellate cells

Cryogenic tubes, 1.0 mL
Disposable stainless-steel scalpel blade with handle, size 15
Foetal bovine serum (FBS)
Gilles fine tooth forceps 12 cm
Heated mats to maintain body temperature during surgery and postoperative recovery
Homozygous athymic nude mice: Strain BALB/c-Fox1nu/Ausb, female
Iscove's modified Dulbecco's medium (IMDM) with 4mM L-glutamine and no phenol red
Jewellers forceps 11.5 cm
Micro needle holder (round handle) 15 cm straight
Micro scissors (round handle) 15 cm straight
Penicillin 10,000 U/mL, streptomycin 10,000 µg/mL
Polyglycolic acid suture, size USP 5/0 on 13mm half-circle round-bodied needle
Portable weighing scale
Reflex clip applier and clip remover
Roswell Park Memorial Institute (RPMI) 1640 with phenol red and 300 mg/L Lglutamine
Round bodied vessel dilator 15 cm, 0.1 mm tip
Trypsin 0.05%, EDTA 0.02%
Trypsin 0.25%, EDTA 0.02%
U-100 insulin syringes, 0.5 mL with 29 G (0.33 mm) × 13 mm needle

Equipment for Resection Procedure
Alm self-retaining retractor
Autoclip wound clips 9 mm

Basic Dressing Pack
Disposable stainless-steel scalpel blade with handle, size 15
Gilles fine tooth forceps 12 cm
Hand-held high temperature fine tip cautery
Heated mats to maintain body temperature during surgery and postoperative recovery
IVIS Lumina II Bioluminescent Imaging Device
Jewellers forceps 11.5 cm
Micro needle holder (round handle) 15 cm straight
Micro scissors (round handle) 15 cm straight
Polyglycolic acid suture, size USP 5/0 on 13mm half-circle round-bodied needle
Portable weighing scale
Reflex wound clip applier and clip remover
Round bodied vessel dilator 15 cm, 0.1 mm tip
Titanium “Weck style” Ligaclip, small
Titanium Ligaclip applier for open surgery, small
Volatile anaesthetic machine, including vapouriser and induction chamber

Drugs for Procedures
70% w/w ethanol solution
Buprenorphine 0.3 mg/mL
D-Luciferin (1 U/g)
Enrofloxacin 50 mg/mL
Flunixin 50 mg/mL
Isoflurane
Ketamine 100 mg/mL
Povidone-Iodine 10% w/v solution
Refresh eye ointment (liquid paraffin 42.5% w/w, soft white paraffin 57.3% w/w)
Sodium chloride 0.9% w/v
Water for injections BP
Xylazine 20 mg/mL

Company	Catalog Number	**Comments/Description**
American Type Culture Collection, Manassas, VA, USA		supplied by Professor Takas
Becton Dickson Pty Ltd, North Ryde, NSW, Australia	500346	
Multigate Medical Products Pty Ltd, Villawood, NSW, Australia		
Pancreatic Research Group cell bank		In house cell bank
Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia	366656	
Livingstone International, Mascot, NSW,	SCP15	
Life Technologies Corporation, Tullamarine, VIC, Australia	16000044	
		Generic stainless steel micr
Generic		
Australian Bioresources, Moss Vale, NSW, Australia		
Life Technologies Corporation, Tullamarine, VIC, Australia	21056023	
		Generic stainless steel micr
		Generic stainless steel micr
		Generic stainless steel micr
Life Technologies Corporation, Tullamarine, VIC, Australia	15140122	
Braun Australia Pty Ltd, Bella Vista, NSW, Australia	C1049407	
Precision balances, Bradford, MA, USA		
World Precision Instruments, Sarasota, FL, USA	500345	
Life Technologies Corporation, Tullamarine, VIC, Australia	11875085	
		Generic stainless steel micr
Life Technologies Corporation, Tullamarine, VIC, Australia	25300054	For pancreatic stellate cells
Life Technologies Corporation, Tullamarine, VIC, Australia	25200056	For ASPC-1 cells
Terumo Medical Corporation, Elkton, MD, USA		
		Generic stainless steel micr
Becton Dickson Pty Ltd, North Ryde, NSW	500346	

Multigate Medical Products Pty Ltd, Villawood, NSW, Australia	08-559NP		
Livingstone International, Mascot, NSW,	SCP15		
			Generic stainless steel micr
Bovie Medical Corporation, Melville, NY, USA	AA01		
Generic			
Caliper Life Sciences, Hopkinton, MA, USA			
			Generic stainless steel micr
			Generic stainless steel micr
			Generic stainless steel micr
Braun Australia Pty Ltd, Bella Vista, NSW, Australia	C1049407		
Precision balances, Bradford, MA, USA			
World Precision Instruments, Sarasota, FL, USA		500345	
			Generic stainless steel micr
HZMIM, Hangzhou, China			
HZMIM, Hangzhou, China			
Generic			Generic vapouriser and ind
Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia			Applied topically as surgical
Troy Laboratories Pty Ltd, Glendenning, NSW, Australia			Dose: 0.05 mg/kg s.c.
PerkinElmer, Inc., Waltham, MA, USA		122799	diluted in PBS to 15 mg/mL
Troy Laboratories Pty Ltd, Glendenning, NSW, Australia			Dose: 5 mg/kg s.c.
Norbrook Laboratories Australia, Tullamarine, VIC, Australia			Dose: 2.5 mg/kg s.c.
Zoetis Australia Pty Ltd., Rhodes, NSW, Australia			Dose (vapourised with oxyg
Maylab, Slacks Creek, QLD, Australia			Dose: 80 mg/kg i.p.
Perrigo Australia, Balcatta, WA, Australia	RIO00802F		Applied topically to the ant
Allergan Australia Pty Ltd, Gordon, NSW, Australia			Applied to both eyes
Braun Australia Pty Ltd, Bella Vista, NSW, Australia	9481P		Dose: 900 µL s.c.
Pfizer Australia, Sydney, NSW, Australia			For dilution of drugs
Troy Laboratories Pty Ltd, Glendenning, NSW, Australia			Dose: 10 mg/kg i.p.

shi Murakami, Saitama Medical University, Saitama, Japan

osurgical instrument set

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osurgical instrument set

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osurgical instrument set
osurgical instrument set
osurgical instrument set

osurgical instrument set

uction chamber

l skin preparation

. Dose: 150 mg/kg i.p

gen): 4% induction, 3% maintenance

erior abdomen as surgical skin preparation

Responses to editorial and reviewers comments

Our responses to comments are in blue.

Editorial Comments:

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

• Please include at least 6 keywords/phrases.

An additional keyword has been added.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
1) 1.1: briefly mention the culturing steps including media used, environmental conditions, trysinization steps, trypsin neutralization, etc.

These steps have been added.

2) 1.12, 2.15: mention dosages.

Dosages added.

3) 2.2: unclear what is done here. Mention luciferin preparation, injection dosage and timing, anesthesia, imaging settings etc.

Details added.

4) 2.5 is fur shaved?

No fur shaving is required as these are nude (hairless) mice.

5) Add a one-line space after each step.

Additional line space added.

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

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from

one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Highlights added

• **Results:** Please provide some visual results in the form of a table or figure so that they can be easily shown in the video.

The results presented in this manuscript are not amenable for presentation as Tables or Figures. We would be happy to provide a one slide summary of the results for the video if required.

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

All these points had been addressed in the discussion, albeit with slightly different subheadings. We have now modified the headings to improve compliance with reader and editor expectations: (1) Modifications and future applications (replacing *Potential modifications)*; (2) Limitations of the technique (heading updated); (3) Significance with respect to existing methods (replacing *Pancreatic resectional models in the literature)*; (4) Future applications (covered in *Modifications and future applications* section); (5) Tips and pitfalls of critical steps (replacing *Tips and pitfalls)*.

2) Minimize subheadings in this section.

Beyond the subheadings suggested above, there is only one further level of sub-subheadings (for *Tips and pitfalls* section) of which there are only two instances. As the procedure involves two parts, we feel that it would be easiest for the reader to follow if we maintained these sub-subheadings. If this is not possible, we could split the *Tips and pitfalls of critical steps* subsection into two sections (*Tips and pitfalls for tumour implantation* and *Tips and pitfalls for tumour resection*). We prefer the current wording, but would be happy to defer to the Editor's discretion.

• **Figures:** Please provide each figure (if multiple panels are present per figure, keep them within 1 file) as an individual SVG, EPS, AI, TIFF, or PNG file.

Figure 1a and 1b have been uploaded as two separate EPS files.

• **Figure/Table Legends:** Provide a common title for the figure.

Title added.

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

Amended as per request.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Ligaclip
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial

products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.

Ligaclip replaced with generic term “ligation clip”.

• **Table of Materials:**
1) Please sort in alphabetical order.

Materials sorted in alphabetical order

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

These are original figures not previously published

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
A sophisticated and improved protocol for a clinically/surgically relevant in vivo model of PDAC treatment modalities is provided. The data built upon previous work of the authors´ group and a couple of other groups.The suggested procedure is highly relevant since there are still many publications in the field with totally unacceptable (misleading) s.c. tutor models.

Major Concerns:
- the manuscript come with many claims ("for the first time..."; the "only clinically relevant" model, which are not justified. As (mostly) cited by the authors, there are many - even decades ago -previous pioneering publications in the field of resection models of PDAC, monitoring and treatment modalities. Details below.

- All pancreatic tumour resection models xeno- or syngeneic and what ever kind of modality are an artefact per definition and do never ever replicate the clinical situation in humans. The discussion should be more modest and appreciate previous findings which were really pioneering.

We thank the Reviewer for his comments. The text has been adjusted accordingly for the two issues raised by the Reviewer.

- l. 336-337: Changed the description “none replicate the clinical situation in humans” to “These described resectional models varied in their fidelity of replication of the clinical situation in humans.”
- l.501: Deleted “for the first time”.

- Matrigel comes in different formulations and the finding of Ralph Jesenowsky does not challenge the use in combination with stellate cell inoculation.

We agree with the Reviewer’s point. Matrigel’s physical property (of solidifying at body temperature) makes it a useful agent for tumour implantation. However, we did not use Matrigel in our studies, because it is well established that its components such as basement membrane proteins induce quiescence in pancreatic stellate cells which would interfere with the facilitatory effects of PSCs on cancer growth. To clarify this, the sentences discussing the use of Matrigel have been rephrased to emphasise that there are “potential disadvantages” of using Matrigel when using a mixture of PSCs and cancer cells. Furthermore, the Jesenowsky paper is now presented as an example of such a disadvantage, rather than presenting as a definitive argument against Matrigel use.

l. 423-427: “This may reduce the risk of leakage post-injection. However, a potential disadvantage of this strategy is that Matrigel or other similar extracellular matrix solutions may exert non-physiological effects on PSCs.[30] For instance, Matrigel has been shown to render PSCs quiescent thereby potentially negating the effects of PSCs in the model.[31,32]”

- Luciferase activity measurements is not really quantitative in an o.t. model

The Reviewer makes a valid point, since the radiance is affected by the deep location of the tumour. We have clarified this by stating that this provides a “semi-quantitative method” of assessing total tumour burden.

l. 480-484: Despite the semi quantitative nature of this assessment in orthotopic models (as the bioluminescence signal is attenuated by passage through the overlying tissues), this approach allows longitudinal assessment of tumour burden, including the assessment of the post-surgical residual disease.

Minor Concerns:
- Lane 90: the fact that a couple of manuscripts come from the "same group" shouldn´t be used as a kind of negative argument

We agree with the Reviewer. It was not our intention to imply that it is negative for multiple manuscripts to come from the same group. We have now revised the text as follows:

l. 343-344: “Many of these papers originated from the same research group.”

- lane 270: the fact, that fluorescence-guided surgery is still not widely used isn't an argument AGAINST appropriate animal models which built the basis for clinical utility (surgeons sometimes need time... - and appropriate equipment)

We thank the Reviewer for this comment. The text has now been slightly modified as follows:

l. 355-356: Of course, this may change, if fluorescence-guided surgery were to be widely adopted in clinical practice.

- lane 340: heterogeneity is important to be addressed > THIS is clinically relevant - and remains a challenge for those of us working with such models. It must not be put aside because of complicated feasibility.

We agree with the reviewer that this is an important point. However, as this paper focusses on a technique, we believe that a detailed discussion of this issue is beyond the scope of this study.

- Primary inoculation under s.c bears a risk for specific selection of sub clones which like a better angiogenesis. This is not a clinically relevant suggestion.

We agree with the reviewer regarding this assertion and it was not our intention to recommend this. However, we do believe that the reader should be presented with the possibility of this occurring in the experimental setting.. We have thus modified the text as follows.

l. 429-434: However, these approaches have their own disadvantages. First, heterogeneity may arise from sampling error or from variations in the volume of tissue implanted. Such heterogeneity may reduce the power of subsequent treatment comparisons. Second, passaging of tumour tissue with a subcutaneous mouse model may lead to selection of sub-clones which have different biological behaviours to the original patient tumour.

. lane 382: has been done before ; Egberts et al Cancer Res

We believe the comments relating to line 382 refers to the challenges of dissecting adjuvant treatment effect from the surgical treatment effect (line 382 refers to a blank line in our version). We have raised this point as a generic “challenge” for any adjuvant treatment model, rather than claiming that this is a novel point. Egberts et al, and many other papers have used the tumour burden discovered at necropsy to infer treatment effects. The disadvantage of this approach is that only the “end state” of (adjuvant) treatment is assessed without information on the “initial state” (post-resection but pre-treatment = post-resection residual tumour burden). While a randomised controlled experiment allows for the initial state to be randomly distributed, the variability of the “initial state” reduces the statistical power of such experiments. To overcome this limitation to some extent, we have recommended the use of in vivo bioluminescence imaging.

- lane 398: has been done before: Egberts and Tepel et al.

We apologise for this omission. The Egberts and Tepel citation has now been added (l. 493).

- lane 405: "for the first time...": this claim is not adequate, the authors provide a very important improvement - that's good

We have removed the phrase “for the first time” (l. 501).

Reviewer #2:
Manuscript Summary:
This is a well written and thorough manuscript regarding the technique of orthotropic implantation and resection of pancreatic cancer in nude mouse models. The authors are to be commended for their work. They have done a nice literature review and also comment on the potential preclinical applications and limitations for their model.

Major Concerns:
None

Minor Concerns:
None

Reviewer #3:
I would like to congratulate the authors on an excellent paper detailing a mouse model of pancreatic cancer resection. The text reads well, the description is easy to follow and the discussion is very insightful. I have a few questions and observations:

1. Would the authors be able to show if tumor kinetics or resectability of tumors change when pancreatic cancer cells and stellate cells are used at a different ratio during implantation?

It is well established that the rate of tumours growth differs significantly depending on the ratio of stromal cells to cancer cells implanted in the pancreas (Hwang et al., 2008 - doi:10.1158/0008-5472.CAN-07-5714), which would then be expected to influence the timing of resection after cell implantation. Our experience with using a 1:1 ratio of cancer cells:PSCs (the subject of several previous publications by our Group), has enabled us to gain a good estimate of the expected rate of tumour growth in our orthotopic model. We therefore elected to use the same ratio in this study and were able to reproducibly show that tumours could be fully resected at the 3 week time point after cell implantation, an important feature for a model aimed at replicating the clinical setting of surgical resection in patients with pancreatic cancer.

2. It would be worthwhile to see use of either an adjuvant or a neo-adjuvant treatment algorithm that was followed using bioluminescence to see the effect of resection and subsequent therapy.

The current manuscript was intended as a short methodological paper focussing on the technique of resection rather than testing therapeutic approaches. We agree with the Reviewer that treatment algorithms would be useful to assess in such a model, and indeed are in the process of submitting a significantly larger study of adjuvant therapy with treatment effects assessed using bioluminescence imaging as well as necropsy findings.

3. What was the bioluminescence criteria used to determine eligibility for resection in these mice? Was size taken into consideration or was it just the extent of disease?

The exclusion criterion for resection was the presence of obvious extrapancreatic disease (e.g., a deposit in the right abdomen due to leakage at implantation). Neither size nor radiant flux were used as thresholds for determining eligibility for resection. This has been clarified in the text.

l. 199-202: Note that this imaging study is simply used to exclude mice with obvious extra-pancreatic disease from resection. Neither size nor radiant flux were used as thresholds for determining eligibility for resection.

4. Why do the authors think there is such high incidence of metastasis in the suture line?

One may speculate that this is related to the proximity of the distal pancreas to the suture line or due to the interaction of cancer cells with the wound myofibroblasts. As this is speculative, we have not included these explanations in the discussion.

5. In the mice that show minimal or no residual disease, how many mice eventually succumb to recurrent disease and in how many days?

In this paper, since the focus is on the technique of pancreatic implantation and resection, we only highlighted the early outcomes of these mice (within 7 days of resection). The reason for this is that the outcomes beyond this time (when adjuvant treatment is commenced) depends not only on disease progression but also treatment effects and side-effects. Longer term studies wth adjuvant treatment are the subject of another manuscript as noted above.

6. The following manuscript should be referenced describing a very similar model. An Immunocompetent Model of Pancreatic Cancer Resection and Recurrence. Giri B, Ferrantella A, Sharma P, Jain T, Jacob HKC, Modi S, Kurtom S, Roy P, Sethi V, Banerjee S, Merchant N, Ramakrishnan S, Saluja A, Dudeja V. J Gastrointest Surg. 2020 Jun 15. doi: 10.1007/s11605-020-04681-9. Online ahead of print.
PMID: 32542554

Thank you for this suggested citation. At the time of manuscript submission, this article was not yet published which explains why it was not included in the original manuscript. This has now been added as detailed below.

l. 371-375: “Morest recently, Giri et al.[28] reported a distal pancreatectomy and partial splenectomy mouse model. This study is notable in that it represents an immunocompetent mouse model of cancer. However, this study reported almost universal local and other intraperitoneal tumour recurrence, possibly indicating occult iatrogenic metastasis at implantation.”

l. 464-465: “This was also used by Giri et al.[28]”

Editorial revision – notes

Request from editorial team:

Before we can formally accept your manuscript, please provide a visual figure or a summary figure of the results to be included in the submission. This is essential as we need some visual to show during the representative results section of the video.

Response from authors:

Thank you. We have summarised the representative results into a single powerpoint slide which may be shown during the video. We have not created a figure as our results are not in a form conducive for a single visual representation. We hope this is acceptable.

Additional comments from authors:

Regarding grant support for this manuscript, we had forgotten to state that the authors have received grant support from the Avner Pancreatic Cancer Foundation.

Representative results

45 mice underwent distal pancreatectomy:

- 100% successfully performed
- 96% macroscopic *pancreatic* proximal resection margin >5 mm
- 20% local metastasis (mainly suture line)
- 71% no/minimal residual disease at 1 week post-resection on bioluminescence imaging

Surgery time (anaesthesia induction to wound closure):

- 22 ± 0.9 minutes