Journal of Visualized Experiments

GENERATION OF AN ORTHOTOPIC XENOGRAFT OF PANCREATIC CANCER CELLS BY ULTRASOUND-GUIDED INJECTION

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63123R1
Full Title:	GENERATION OF AN ORTHOTOPIC XENOGRAFT OF PANCREATIC CANCER CELLS BY ULTRASOUND-GUIDED INJECTION
Corresponding Author:	Annarosa Arcangeli University of Florence: Universita degli Studi di Firenze Florence, Italy ITALY
Corresponding Author's Institution:	University of Florence: Universita degli Studi di Firenze
Corresponding Author E-Mail:	annarosa.arcangeli@unifi.it
Order of Authors:	Tiziano Lottini
	Matteo Buonamici
	Claudia Duranti
	Annarosa Arcangeli
Additional Information:	
Question	**Response**
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TITLE:

Generation of an Orthotopic Xenograft of Pancreatic Cancer Cells by Ultrasound-Guided Injection

AUTHORS AND AFFILIATIONS:

Tiziano Lottini [1], Matteo Buonamici[1], Claudia Duranti[1], Annarosa Arcangeli[1]

[1]Department of Experimental and Clinical Medicine, Section of Internal Medicine, University of Florence, Florence, Italy

E-mail addresses of co-authors:

Tiziano Lottini (tiziano.lottini@unifi.it)
Matteo Buonamici (matteo.buonamici@outlook.com)
Claudia Duranti (claudia.duranti@unifi.it)
Annarosa Arcangeli (annarosa.arcangeli@unifi.it)

Corresponding author:

Annarosa Arcangeli (annarosa.arcangeli@unifi.it)

SUMMARY:

We present a protocol to generate a minimally invasive orthotopic pancreatic cancer model by ultrasound-guided injection of human pancreatic cancer cells and the subsequent monitoring of tumor growth *in vivo* by ultrasound imaging.

ABSTRACT:

Pancreatic cancer (PCa) represents one of the deadliest cancer types worldwide. The reasons for PCa malignancy mainly rely on its intrinsic malignant behavior and high resistance to therapeutic treatments. Indeed, despite many efforts, both standard chemotherapy and innovative target therapies have substantially failed when moved from preclinical evaluation to the clinical setting. In this scenario, the development of preclinical mouse models better mimicking *in vivo* characteristics of PCa is urgently needed to test newly developed drugs. The present protocol describes a method to generate a mouse model of PCa, represented by an orthotopic xenograft obtained by ultrasound-guided injection of human pancreatic tumor cells. Using such a reliable and minimally invasive protocol, we also provide evidence of *in vivo* engraftment and development of tumor masses, which can be monitored by ultrasound (US) imaging. A noteworthy aspect of the PCa model described here is the slow development of the tumor masses over time, which allows precise identification of the starting point for pharmacological treatments and better monitoring of the effects of therapeutic interventions. Moreover, the technique described here is an example of implementation of the 3Rs principles since it minimizes pain and suffering and directly improves the welfare of animals in research.

INTRODUCTION:

PCa, and its most common form, the Pancreatic Ductal Adeno Carcinoma (PDAC), is one of the most common causes of cancer-related death with a 1-year survival rate lower than 20% and a 5-year survival rate of 8%, regardless of the stage[1,2]. The disease is almost always fatal, and its

incidence is forecasted to continuously grow in the next years, unlike other cancer types, whose incidence is declining[3]. Factors such as late cancer detection, the tendency of rapid progression, and lack of specific therapies lead to a poor prognosis of PCa[4]. Great advances in cancer research have been obtained, thanks to the development of more accurate preclinical mouse models. The models have provided appropriate insights to the understanding of the molecular mechanism underlying cancer and to the development of new treatments[5]. These advances poorly apply to PCa, which, despite great recent efforts, remains resistant to current chemotherapeutic therapies[1]. For these reasons, the development of novel approaches to improve patients' prospects is mandatory.

Over the years, many PCa mouse models have been developed, including xenograft models, which are the most widely used models nowadays[5]. Xenograft models are classified as subcutaneous heterotopic and orthotopic, depending on the location of the implanted tumor cells. Subcutaneous heterotopic xenografts are easier and cheaper to accomplish but miss certain characteristic features of PCa (i.e., the peculiar tumor microenvironment, characterized by the accumulation of fibrotic tissue, hypoxia, acidity, and angiogenesis)[6,7]. This explains why subcutaneous xenografts often fail to provide robust data for therapeutic treatments leading to failures when translated to the clinical setting[8]. On the other hand, orthotopic xenografts resemble the tumor microenvironment more closely, leading to better mimicking of the natural development of the disease. In addition, orthotopic xenografts are more suitable for studying the metastatic process and the invasive features of PCa, which almost do not occur in subcutaneous models[9]. Overall, orthotopic xenograft mouse models are nowadays preferred to perform preclinical drug testing[9,10]. Orthotopic xenografts usually rely on surgical procedures to implant either cells or very small tumor tissue pieces into the pancreas. Indeed, several papers based on surgical models of PCa have been published in the last few decades[11]. However, the quality and the outcome of the surgical procedure for the establishment of an orthotopic tumor model strongly depend on the technical skill of the operator. Another key point for a successful orthotopic PCa xenograft for a translational clinical approach is the possibility to establish localized disease with predictable growth kinetics.

To address these, here we describe an innovative procedure to produce an orthotopic PCa xenograft, exploiting ultrasound (US)-guided injection of human PCa cells into the tail of the pancreas in immunodeficient mice. This procedure generates a reliable PCa mouse model. The tumor growth is followed *in vivo* by US imaging.

PROTOCOL:

The present protocol received approval from the Italian Ministry of Health with the authorization number 843/2020-PR. In order to ensure aseptic conditions, the animals were maintained inside the sterile room of the animal house (Ce.S.A.L.) of the University of Florence. All procedures were performed in the same space where the mice were housed at the LIGeMA facility of the University of Florence (Italy).

1. Cell preparation

1.1. Culture PCa cells from the PCa cell line in a 100 mm Petri dish containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% L-glutamine and 10% Fetal Bovine Serum (FBS).

1.2. Incubate the cells in normoxia at 37 °C with 5% CO_2.

1.3. Detach the cells with trypsin. Count, and resuspend 1 x 10^6 cells in 20 µL of PBS, 1 h before inoculation.

2. Mice preparation for ultrasound-guided injection (US-GI)

NOTE: Following steps were performed under sterile conditions. The entire procedure of US-guided injection, from the beginning of the anesthesia until the mouse is removed from the animal platform, takes around 10–12 min plus 5 min for complete mouse recovery.

2.1. Just before the intervention, administer carprofen (NSAID) subcutaneously (s.c.) at a final dose of 5 mg/kg using a tuberculin syringe with a 27 G needle.

NOTE: For the present protocol, 20 Athymic Nude-Foxn^{1nu} female mice were used. The mice were 8 weeks old and weighed 20–22 g.

2.2. Turn on the imager and select **Mouse (Small) Abdominal** on the application menu from the transducer panel. Ensure that the B-Mode (Brightness Mode) imaging window appears, and the system is ready to acquire B-Mode data.

NOTE: B-Mode is the system's default imaging mode. The system displays echoes in a two-dimensional (2D) view by assigning a brightness level based on the echo signal amplitude. B-Mode is the most effective mode for locating anatomical structures.

2.2.1. Go to the **Study Browser**.

2.2.2. Select **New Study** and type the study name and information, i.e., date of the study, etc.

2.2.3. Fill in all the necessary information in the Series Name, i.e., animal strain, ID, date of birth, etc.

2.2.4. Tap **Done**; the program is ready for B-mode imaging.

2.3. Anesthetize the mouse in the gas chamber using 4% isoflurane with a gas flow of 2 L/min of O_2.

NOTE: Approximately 4 min are sufficient for proper anesthesia (breathing around 50–60 breaths per min).

2.4. Once the mouse is anesthetized, change the connection of the anesthetic machine to direct the isoflurane toward the mouse handling table.

2.5. Place the anesthetized mouse on its right flank onto the handling table (heated at 37 °C) with its snout in a nose cone to ensure that the mouse is anesthetized with a continuous flow of 2% isoflurane (**Figure 1A**).

2.6. Apply a drop of vet ointment on the mouse's eyes to prevent dryness while under anesthesia.

2.7. Tape the right hand, right foot, and tail firmly onto the electrode pads on the animal platform with adhesive gauze.

NOTE: The mouse's respiration rate and electrocardiogram (ECG) are recorded through the electrode pads.

3. Injection of PANC1 cells in the pancreas by US-GI method

3.1. Sanitize the mouse skin with 70% ethanol and keep the skin of the left flank stretched using adhesive gauze.

NOTE: Keeping the skin stretched is important to reduce resistance to the insertion of the needle and to prevent needle deformations.

3.2. Apply ultrasound gel on the abdomen and left flank of the mouse using a 20 mL syringe (without the needle).

3.3. Using the height control knob of the US transducer, lower the transducer to touch the left flank of the mouse and place it transversely to the body of the animal.

3.4. Move the transducer to visualize the pancreas on the transducer display using B-Mode imaging (**Figure 1B**).

3.5. Prepare a 50 µL Hamilton syringe, containing 1 x 10^6 PANC1 cells suspended in 20 µL of PBS, with a 30 mm 28 G needle and place the syringe on the appropriate holder (**Figure 1C**).

NOTE: Before using, sanitize the syringe needle with 70% ethanol.

3.6. Using the holder micromanipulator, lower the syringe to the mouse skin, with the needle bevel face up and in the same plane as the ultrasound transducer, forming an angle of 45° with the transducer (**Figure 1D**).

NOTE: From this step onward, proceed by monitoring the US image on the display.

3.7. Using the micromanipulator, perforate the skin and insert the syringe needle into the pancreas and observe the US image on the display, to follow its trajectory (**Figure 1E**).

NOTE: Before injection, take the tail of the pancreas as a reference which is located behind the spleen and close to the left kidney.

3.8. Once the needle is inserted into the pancreas, inject a 20 µL bolus containing the cells directly into the pancreas by applying constant pressure on the syringe plunger (**Figure 1F**).

NOTE: The correct injection procedure is checked by the presence of a small bubble in the pancreas and the flow of hypoechogenic fluid, which is barely visible from the needle tip.

3.9. Leave the needle in place for 5–10 s after the whole bolus is injected, and then slowly retract it.

3.10. Remove the US transducer, clean the gel from the flank, and place the mouse alone in a new cage. Observe the animal until it has regained sufficient consciousness to maintain sternal recumbency.

4. 3D US imaging for monitoring pancreatic tumors in mice

NOTE: The evaluation of tumor development was performed starting 8 days after the cell injection, using the same instrument used for the US-guided injection (listed in **Table of Materials**). Hence, some procedures, such as the system ignition (step 2.2.), anesthesia (steps 2.3. – 2.6.), and mouse placement on the animal platform (step 2.7.), fully match what was described above in the protocol.

4.1. Before starting the US imaging, set up the workstation as shown in **Figure 2A**.

4.2. Fix the transducer on the 3D motor system (**Figure 2A**).

4.3. Turn on the imager and select **New Study** on the **Study Browser**.

4.4. Place the mouse in the anesthesia induction chamber (4% isoflurane).

4.5. Place the anesthetized mouse on its right flank onto the handling table heated at 37 °C with its snout in the anesthetic tube and reduce isoflurane to 2% (**Figure 2B**).

NOTE: It is important that the mouse is placed in the same position as the US-guided injection, to maintain the same anatomical references.

4.6. Apply a drop of vet ointment on the mouse's eyes.

4.7. Apply a layer of ultrasound gel on the mouse's abdomen and left flank.

4.8. Position the 55 MHz transducer in the transverse orientation to touch the left flank skin such that the pancreas is approximately centered (**Figure 2C**).

4.9. Use the 3D motor to acquire images of the whole pancreas in the transverse orientation, ideally gathering 90–100 frames per acquisition (number of frames may vary depending on personal choice).

4.9.1. Select the **3D Motor Position** on the imager touchpad.

4.9.2. Indicate the scanning distance by moving the cursor to acquire images of the whole pancreas from both extremities.

4.9.3. Select **Scan Frames** and begin 3D acquisition.

4.10. Once 3D imaging is done, remove the transducer, clean the gel from the skin, and place the mouse alone in a new cage for recovery. Observe the animal until it has regained sufficient consciousness to maintain sternal recumbency.

REPRESENTATIVE RESULTS:

Following the protocol described above, mice were first anesthetized in an isoflurane chamber, and placed on the animal platform (**Figure 1A**). The pancreas was visualized with ultrasound imaging (**Figure 1B**). A 50 µL Hamilton syringe was loaded with 1 x 10^6 PANC1 cells suspended in 20 µL of PBS and placed on the needle holder (**Figure 1C**). The optimal angle between the syringe and the US transducer was 45° (**Figure 1D**). Using the micromanipulator, the syringe needle was inserted into the pancreas and its trajectory was observed on the US imager display (**Figure 1E**). A 20 µL bolus of tumor cells was then injected into the pancreas (**Figure 1F**) and after 10 s the needle was retracted. Relevant actions to prevent the recoil of cells and their subsequent leakage into the peritoneal cavity were pursued, such as applying a constant pressure during the inoculation, pausing after cell injection, and using a thin needle with a 30° bevel angle. After the US-guided injection, mice's weight was monitored every day to assess any signs of stress due to the procedure. No significant change in the weight of injected mice was observed.

3D US imaging was then applied to monitor tumor cell engraftment and tumor development. The first image acquisition was performed on day 8 after cell injection, followed by weekly acquisitions (for a total of 6 acquisitions). During imaging, animals were placed on the right flank onto the warmed (37 °C) platform and anesthetized by inhalation of isoflurane gas (induction dose at 4% and maintenance dose at 2%) (**Figure 2**). The 3D acquisition was performed in B-mode using the 3D motor that allows the transducer to scan the abdomen in various sections, perpendicular to its axis. A series of 2D images were obtained, which were then assembled by the analysis software, reconstructing the 3D anatomical image of the organ (3D re-rendering). To perform 3D imaging, it was necessary to synchronize the mouse breath phases with the acquisition of the 3D scan. The reproducibility of the protocol was confirmed by the presence of

the tumor mass, a hypo-echogenic structure (represented with dark colors) in the tail of the pancreas, starting at day 8, in 16 out of 20 (80%) animals (**Figure 3A**). In four mice (20% of animals), the development of tumor mass was observed 2 weeks after the injection. This latency could be traced back to a sub-optimal procedure of needle insertion into the pancreas, which caused leakage of cells into the peritoneum.

Figure 3B shows the mean tumor volume (n = 16) evaluated by US imaging of the US-guided injection mouse model. The day after the last US acquisition, mice were euthanized by cervical dislocation. The abdominal cavity was examined, and the tumor masses were explanted for histologic analysis (**Figure 3C–E**).

FIGURE LEGENDS:

Figure 1: US-guided method for the establishment of an orthotopic PCa mouse model. (**A**) The mouse is placed on the right flank on a special support heated at 37 °C. (**B**) The US transducer is located transversely to the animal body at the level of the spleen and the pancreas is visualized in B-Mode. The flank view of the mouse abdomen shows the pancreas (1) between the spleen (2) and the left kidney (3). Scale bar: 2 mm. (**C**) The Hamilton syringe is placed on the appropriate holder. (**D**) In this situation, the transducer and the syringe are positioned forming an angle of 45°. (**E**) The needle of the syringe (1) is inserted in the tail of the pancreas, just below the spleen. Scale bar: 2 mm. (**F**) A bolus containing 1 x 10^6 PANC1 cells in 20 µL of PBS is injected into the tail of the pancreas, using a Hamilton syringe with a 28 G needle. Scale bar: 2 mm.

Figure 2: Imaging workstation used for monitoring pancreatic tumors in mice. (**A**) Workplace for US imaging. (1) 55 MHz transducer; (2) Mouse handling table; (3) 3D Motor; (4) Height control knob of US transducer. (**B**) The mouse is placed on its right flank onto the handling table with its snout in the anesthetic tube (1), the skin is stretched, and the US gel is applied on the right flank. (**C**) The transducer is lowered to touch the mouse skin and positioned transversely to the animal body.

Figure 3: Development of orthotopic PCa xenograft in the mouse pancreas. (**A**) 2D US image acquisition of the tumor mass developed after echo-guided cell injection. The development of the tumor is monitored with US imaging after 8 days of cell injection, followed by once-a-week acquisition until day 44, for a total of 6 acquisitions. The tumor is represented by a hypo-echogenic structure in the pancreatic parenchyma. The hypo-echogenic structure is echographically represented by an area with echoes of reduced intensity, compared to the surrounding parenchyma or a neighboring structure. Scale bar: 2 mm. (**B**) Time course of the tumor volume of orthotopic PDAC derived from PANC1 cells. (**C**) US imaging acquisition of a tumor mass at day 43 after cell injection. Scale bar: 2 mm. 3D re-rendering of the tumor mass is shown in the inset. (**D**) After imaging, the animals are euthanized, and necropsy examination is performed. (1) Tumor mass; (2) A small portion of a healthy pancreas; (3) Spleen; (4) Stomach; (5) Liver. Scale bar: 5 mm. (**E**) Hematoxylin and eosin staining of the tumor mass at 4x magnification. Scale bar: 200 µm. On the right upper side of the panel, inset of the tumor mass in which pancreatic acinar cells are still visible; 40x magnification. Scale bar: 100 µm.

DISCUSSION:

Although the use of US imaging is widespread in the clinic, tumor development in many preclinical mouse models is usually described using bioluminescent imaging[11]. The latter is an indirect way to evaluate tumor engraftment and expansion and it also does not provide a reliable tumor growth kinetics. In the present study, we have applied US imaging for performing cell injection as well as for monitoring tumor development. The protocol we have described and the results we have provided represent a relevant breakthrough in PCa research.

The US-guided method described here for the injection of pancreatic tumor cells is minimally invasive and allows to overcome many drawbacks caused by the surgical procedure, which is usually applied to establish an orthotopic PCa mouse model. Although indications on mortality rate or side effects due to the surgical procedure are missing in the literature, our experience suggests that the surgical method produces higher postoperative mortality in a low percentage of cases (around 10%), with frequent animal suffering, requiring a certain amount of recovery time[12]. In addition, the stitches applied after surgery prevent the correct application of imaging procedures. This results in the acquisition of US images with very poor quality, leading to inaccurate data extrapolation caused by artifacts, such as reverberation and shadows regions. Taking all these facts into consideration, a method that does not need a surgical procedure for the establishment of an orthotopic mouse model is preferred and strongly recommended for preclinical research purposes. Furthermore, being minimally invasive, the recovery time from cell inoculation is faster and the animal suffering is minimal in the US-guided method. A great advantage is that the US-guided method is compatible with the use of isoflurane anesthesia which allows faster induction and recovery, relatively less sparing effect on cardiovascular function and cerebral blood flow autoregulation, compared to other methods of anesthesia (i.e., ketamine/xylazine solution). Overall, the negligible metabolism of isoflurane makes it particularly useful in anesthetic management[13]. Indeed, roughly 5 min after the end of the procedure, animals regain sufficient consciousness to maintain sternal recumbency. In addition, mice are pre-medicated with an analgesic (carprofen) to minimize pain, and no post-operative treatments are necessary due to the rapid and complete recovery.

Nevertheless, there are some crucial troubleshooting steps in the procedure that must be considered. The number of cells to be inoculated must not exceed 1 x 10^6 cells and the volume of cell inoculum must not be greater than 20 µL to avoid the risk of cell leakage out of the pancreas. Cell injection should be performed immediately after their detachment, to avoid a decrease in cell vitality. After cell injection, it is necessary to wait for at least 10 s before removing the needle to avoid the leakage of cells out of the pancreas.

One of the most critical aspects of the present protocol concerns the positioning of the mouse on the animal platform. The mouse must be lying on its right side onto the animal handling table and the skin must be stretched. If the skin is not properly stretched during the injection, the needle will struggle to pierce and enter the pancreas, with the risk of cells spilling out of the pancreas. The ultrasound-guided injection to produce a PCa mouse model was first described by Huynh et al. in 2011[14]. Here, we focused on the methodology suitable to produce such a model, describing in detail all the steps to make it reproducible. Furthermore, compared to Huynh et

al.[14], we have introduced some innovations mainly related to the advancements in US technology that occurred in the last 5 years. In the present protocol, the use of a Hamilton syringe with a stiff needle and a bevel angle of 30° is strongly recommended to obtain better accuracy during cell injection and to minimize cell leakage. Finally, during the US-guided injection, mice were secured on its right side onto the animal platform, while in Huynh et al.[14] mice were placed in dorsal recumbency. The side position of the mouse allows to visualize the tail of the pancreas, just below the spleen, which acts as a visual reference (**Figure 1B**) during the cell injection, ensuring the reproducibility of the technique.

As with other techniques, there are also some limitations with the US-guided procedure. The greatest limitation is that the injection can be performed only in the tail of the pancreas because the body and the head are covered by other organs and are difficult to reach with the needle. Furthermore, another limitation associated with the use of US imaging to monitor tumor growth is the inability to visualize the whole tumor mass when it becomes too large, after 6 weeks of inoculation (**Figure 3C-E**). Although, such large volumes do not mimic the clinical course of the disease.

Another advantage of the PCa model obtained with the protocol described here is the slow cell engraftment and growth of the tumor masses (**Figure 3B**). This is ideal for precise identification of the starting point for pharmacological intervention and better monitoring of the effects of therapeutic interventions over time.

Finally, the technique described is an example of implementation of the 3Rs principles involving the refinement of technique and development of procedure which minimizes pain, suffering, distress, or lasting harm, directly improving the welfare of the animals in research.

Future applications of the PCa model obtained by the US-guided injection include studies aimed at the development of appropriate therapeutic treatments or treatment combinations. In fact, the use of such innovative *in vivo* techniques could be coupled with the use of small molecules, e.g., antibodies, antibody fragments, and antibody-drug conjugates (ADC), with a theragnostic approach. The latter could be used alone, for therapeutic purposes, as our group has demonstrated in our recent work[15] or after a proper fluorophore conjugation, with the goal of monitoring tumor growth.

ACKNOWLEDGMENTS:
This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC, grant no. 15627, IG 21510, and IG 19766) to AA, PRIN Italian Ministry of University and Research (MIUR). Leveraging basic knowledge of ion channel network in cancer for innovative therapeutic strategies (LIONESS) 20174TB8KW to AA, pHioniC: European Union's Horizon 2020 grant No 813834 to AA.

DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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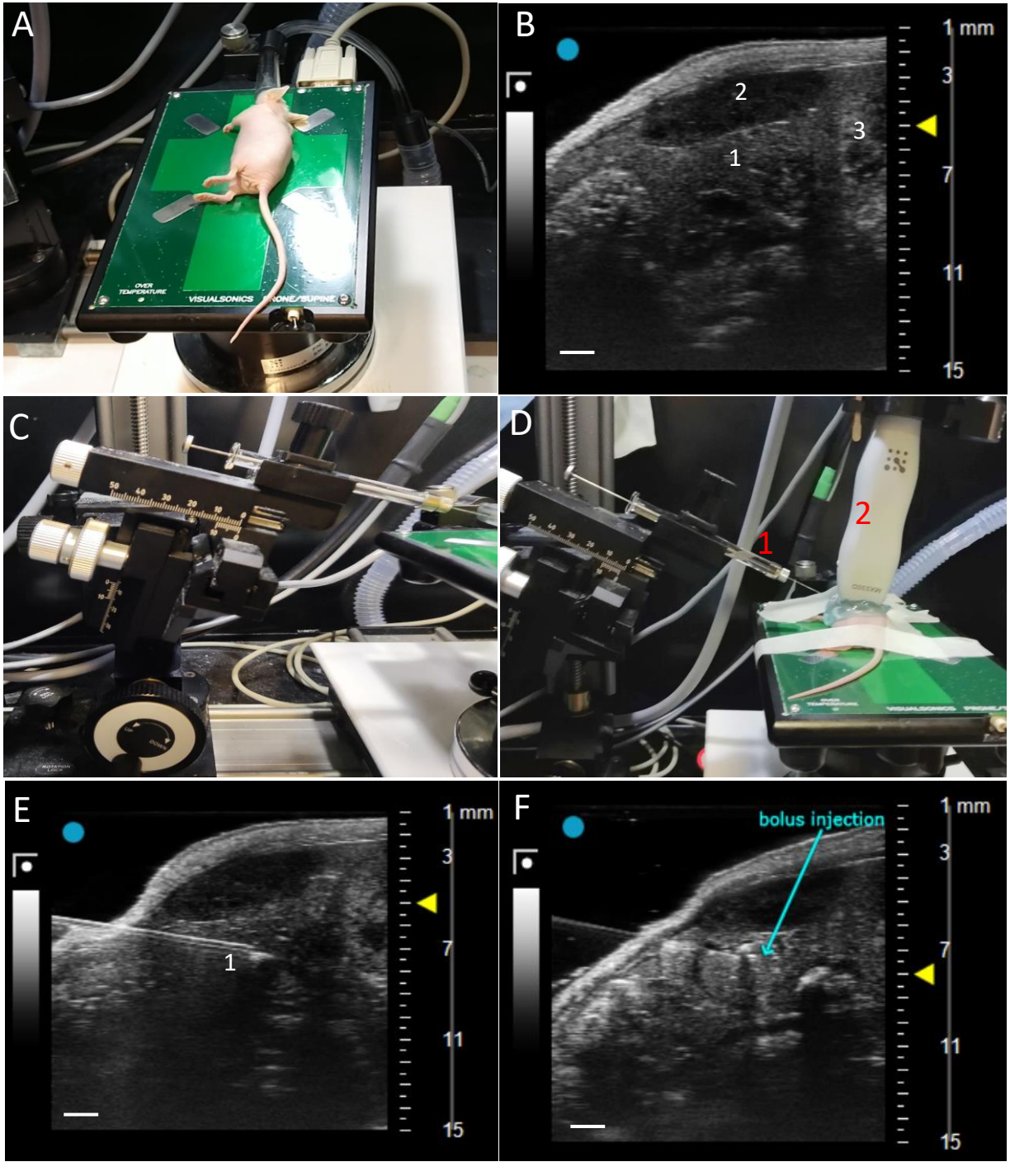

Figure 2

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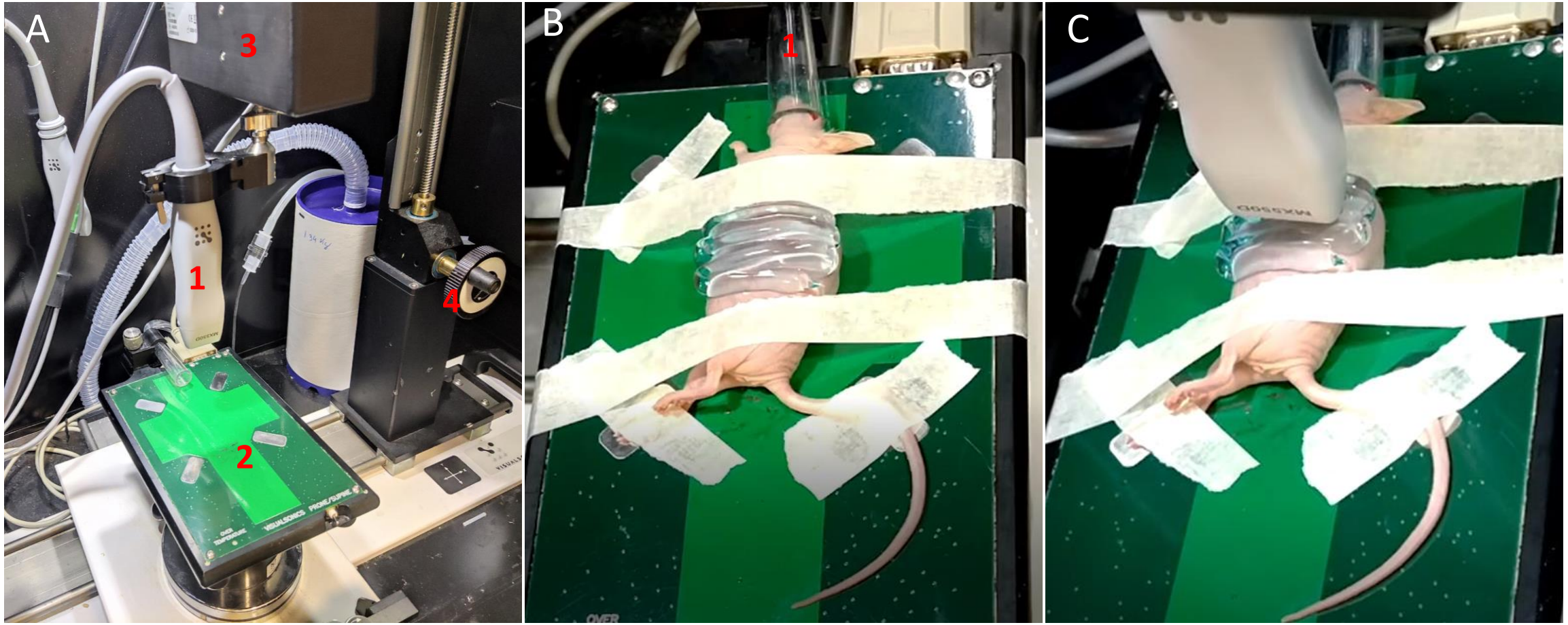
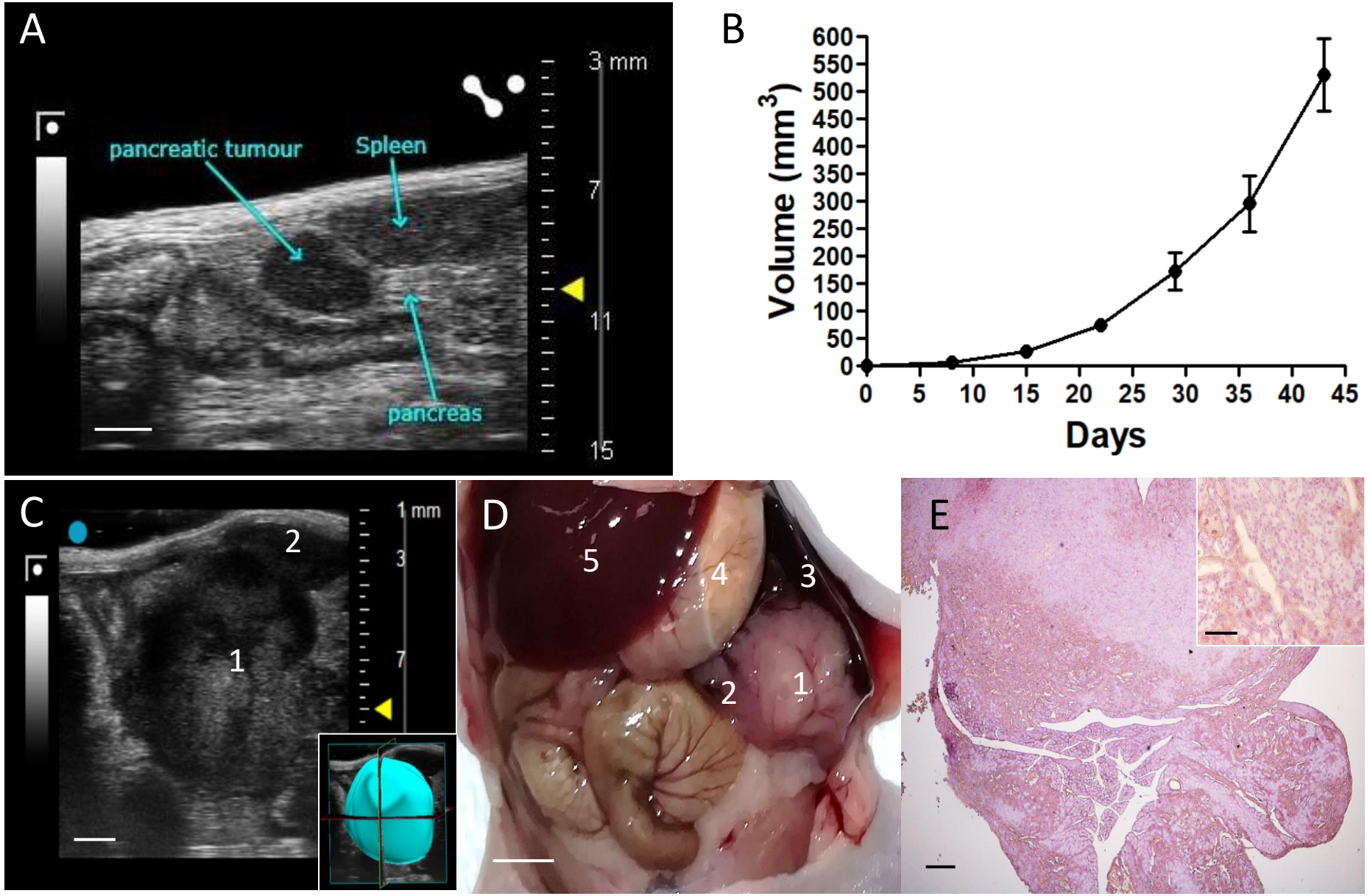


Figure 3



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Dear Editor, Dear Reviewers,

I would like to thank you for giving us the opportunity to revise the manuscript, which has led to a significant improvement of our work.

The manuscript has been thoroughly revised to address Editor and Reviewers criticisms. In particular:

1) We have thoroughly revised the manuscript to correct spelling and grammar and tenses issues. We have also implemented the number of references.

2) Claudia Duranti has been included among the authors because she cooperated to work on the revision.

3) A brief Summary has been added before the Abstract section (page 1).

4) We have increased the word count of the abstract.

5) The Representative Results have been rewritten, explaining the results we have obtained with US-guided technique (see Representative results, page 5). All the figures have been cited both in the Protocol and in the Representative Results section.

6) We have added a Protocol step in which we explained in detail the US imaging used to evaluate the cells engraftment and tumor development (see Protocol step: 3D US imaging for monitoring pancreatic tumors in mice; Pages 4-5, lines 162-189).

7) A **new Figure 2** has been added in which the is illustrated the workstation set up for the US imaging, as reported in the Protocol step 4 (3D US imaging for monitoring pancreatic tumors in mice). The old Figure 2 has become the new Figure 3.

8) We added new **panels C-F** in **Figure 3** in which we presented the comparison between the echography of a tumor mass at the experimental end-point and its image at the necropsy examination. Panel E and F of the Figure 3 show Hematoxylin and Eosin staining of the same tumor.

9) The scale bars have been added to all figures.

10) The Discussion has been rewritten to better address all the points suggested by the Reviewers.

All the changes we made in the manuscript are highlighted in red.

Attached please find the specific answers point by point to the requests.

Editorial comments

1) Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have accurately checked the manuscript and edited the English and spelling errors.

All the abbreviations have been defined at first use.

2) Please provide an email address for each author.

We added all the e-mail address below the Author's affiliation.

3) Before the abstract, please add a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words.

We added a Summary section in the manuscript (page 1).

4) Please increase the word count of your abstract to 150-300 words.

We have increased the word count of the abstract.

5) For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Following the Editor's suggestions and JoVE Journal guidelines, the references have been entered as numbered superscripts.

6) JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (this includes the figures

and the legends) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents, e.g., Sarstedt, Germany; CO2 Incubator Function Line, Heraeus Instruments, Germany; Visualsonics Fujifilm; MX550; VEVO; VevoLAZR-X photoacoustic ultrasound system; VevoLab software etc

All the trademark and registered symbols and company names have been removed from the manuscript (included figure and legends). The names of all the commercial products have been added in the Table of Materials and Reagent.

7) Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Following the Editor's suggestion, we avoided the use of personal pronouns in the protocol section.

8) Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Please provide details about the animals (number, sex, age, weight, strain etc) in the protocol and Table of Materials.

All the detail concerning the number of animals, sex, weight, strain, and age have been reported in the Table of Materials and Reagent and in the Protocol section (lines 103-104)

Regarding animal treatment in the protocol, please add the following information to the text:

a) Please specify the euthanasia method without highlighting it.

Mice were sacrificed by cervical dislocation. We added this information at page 6, lines 221-222

b) Please mention how proper anesthetization is confirmed.

Proper anesthetization is confirmed by unconsciousness of the mouse, and slower respiratory rate of around 50-60 breath per minute. We added this information in the Protocol section (lines 116-117).

c) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

We have applied lubricant on the eyes of the mouse. We added this information in the present version of the manuscript (line 123).

d) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

Being the US-guided method not invasive, the recovery time is fast and the animal pain is minimal. A great advantage of the US-guided method is compatibility with the use of isoflurane, which compared to other method of anesthesia (i.e. ketamine/xylazine solution) allows faster induction and recovery. After around 5 minutes from the end of the procedure the animals regain sufficient consciousness and normal breathing.

Moreover, mice are pre-medicated with an analgesic (carprofen) to minimize animal pain, while no post-operative treatments were needed due to the rapid and completely recover. These data are discussed in Discussion (lines 244-253).

e) Discuss maintenance of sterile conditions during survival surgery.

We reported in the Protocol section (lines 81-84, lines 98-99, line 130 and line 141) that all the procedures performed in mice were carried out while maintaining the sterile conditions of all surfaces and equipment.

f) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency. g) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

The animals are held alone, not returned to the company of other animals, and it is observed until it has regained sufficient consciousness and recovered the sternal recumbency. We added this information in protocol section (lines 158-160).

9) 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 details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please ensure the inclusion of 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.

Following the Editor's suggestion, we added as much details as possible regarding the procedure and trying to answer the question "how" and to supplement the actions seen in the video, in order to make the protocol as understandable and replicable as possible.

10) Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

As suggested by the Editor we have formatted the manuscript, following the Editorial guidelines. We have also highlighted in yellow the pages of the protocol to be included in the protocol section of the video (pages 3 and 4).

11) Please discuss all figures in the Representative Results. However, for figures showing the experimental setup, please reference them in the Protocol.

The figures have been discussed in the Representative Results section, pages 5-6. Moreover, we referenced all the figure in the Protocol section.

12) Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

The Representative Results have been rewrite better explaining the results we have obtained in the context of the US-guided technique (see Representative Results, page 5, lines 193-223). Furthermore, in the Results section we have referred to all the figures.

13) Evaluation of tumor development, performing the US are key procedures in your protocol. Please include these sections in the protocol to help users of different levels of experience and expertise follow your protocol. If you have already described these procedures in detail elsewhere, please cite those references but describe the steps briefly here. Please ensure that the paper and the video bring your main message (indicated by your title) to the readers and viewers.

Following the Editor's suggestion, we added a section in the Protocol in which we detailed explain the method to perform US imaging for pancreatic tumor evaluation. (pages 4-5, lines 162-189).

14) All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).

As suggested, all the scale bars have been added to all figures.

15) As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

The Discussion section has been rewritten to address Editor criticisms:

a) Critical steps have been discussed at lines 258-265.

b) A paragraph with the troubleshooting of the technique have been added (lines 254-259).

c) Limitations associated with the US-guided technique have been discussed at lines 276-282.

d) We discussed the significance and the innovations introduced with respect the existing methods at lines 233-251, lines 265-275 and lines 287-289.

e) Future applications of the technique described in the manuscript are reported at lines 290-296.

16) Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

We have inserted the References as recommended.

17) Please add all items (plastic and glassware, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.

Following the suggestion, we have added all the items sorting the Materials Tables alphabetically (see Table of Materials).

Response to Reviewer's comment

Reviewer 1

Minor comments:

2) INTRODUCTION:

- page 2, line 22: unlike is preferable instead of "at difference from"

As suggested by the reviewer we have corrected (lines 45).

- page 2, line 23 to 25 : two repetitive phrases have to be corrected "Late cancer detection, lack of a specific therapy and the tendency to a rapid progression, determine a poor prognosis of PCa. Factors like low rate of early detection, rapid progression, and lack of proper therapy led to a poor prognosis of PCa." A reference should be added.

We addressed this point at line 46-47. The reverence has been added.

- page 2, line 25 : I would suggest : Advances in research have greatly "improved" instead of "greatly advanced"

As suggested, we modified advanced with improved (line 47).

- page 2, line 38 : I would suggest : very small "tumor" tissue pieces.

The word tumor has been added (line 67).

3) PROTOCOL:

- page 3, line 59: add C for centigrade after 37 °

We have inserted C (line 92).

-page 3, line 62: 20µl instead of 20 ul

We have made the correction (line 94).

- page 4, line 67: dose of 4% isoflurane gas is in oxygen , why is indicated as solution ? please specify

In the sentence “4% isoflurane gas is in oxygen”, we made a mistake. We mean that isoflurane is mixed with medical air and the percentage of isoflurane used for anesthesia was 4%, with a gas flow of 2 L/min in gas chamber.

- page 4, line 81: Please revise the phrase "Place the syringe needle perpendicular to the animal body, on the same plane as the ultrasound transducer, forming an angle of 40 degrees with it (Figure 1D)." Looking at the figure the syringe needle is not perpendicular. The trasducer is nearly perpendicular and the syringe is forming an angle of 45 degrees (see legend of Figure 1 D) with the trasducer. The correct description is at page 7.

We agree with the reviewer that the step was not clearly described. In this version of the manuscript we have better described the (line 142-144).

- page 5, line 92: "then" slowly retract it.

We have corrected the mistake (line 156).

4) REPRESENTATIVE RESULTS :

- page 5, line 97: 6 weeks or 6 acquisition ? please specify referring also to the legend of figure 2A.

We meant 6 acquisitions. We have specified in the text, line 207.

page 5, line 98: isoflurane gas 2% ? please specify

We have specified in the text that the isoflurane was administrated initially with an induction dose at 4% and then maintenance dose at 2% (line 208-209).

5) DISCUSSION:

- page 6, line 122: I would suggest obtain instead of derive

We have made the correction (line 229).

- page 6, line 126 to 127: "we have established are the slow establishment" , I would suggest cell engraftment instead of estabilishment

We are agree with the Reviewer and we have replaced the word “establishment” with “engraftment” (line 284).

11) Name of Material : add the analgesic drug

Following the suggestion, we have added in the Material Table the analgesic drug used for the procedure (Rimadyl (Carprofen)).

Reviewer 2.

Minor Concerns

1- Line 33-35 "Subcutaneous heterotopic xenografts are easier and cheaper to be accomplished, but often lack to provide robust data for therapeutic treatments, which hence fail once translated to the clinical setting" - please could you elaborate by adding a sentence on how subcutaneous tumours do not accurately represent the natural tumour microenvironment and why this is important.

As suggested by the reviewer we better elaborate the sentence also adding a citation (Killion JJ. et al). The tumor microenvironment of heterotopic xenograft is different from that to the original tumor, the new angiogenesis is minimal, whereas the orthotopic mouse models are considered to resemble the natural tumorigenesis more closely. Orthotopic xenografts are more suitable also for metastasis studies because subcutaneous models rarely develop metastasis. The new elaboration has been inserted at lines 55-65.

2- How long from start to finish does the whole procedure take?

The entire procedure of US-guided injection, from the beginning of the anesthesia until the mouse is removed from the animal platform, takes around 10-12 minutes. Plus 5 minutes for the mouse completely recover.

3- Please describe what happens after the cells have been injected. How is the mouse recovered? Was there any post-op care? Where mice weighed daily to monitor weight loss?

The correct injection procedure it will be checked by the presence of a small bubble on the pancreas and the flow of hypoechogenic fluid it will be barely visible from the needle tip. We added this information at lines 153-155.

Moreover, mice completely recovered after around 5 minutes and no post-operative care were needed. The treatment with analgesic drug was done just before the injection to prevent post-operative pain. This point has been discussed on Discussion section, lines 244-253.

In addition, the mice were weighted daily for the first days after US-guided procedure and no significant weight-loss or sign of stress was observed (Representative Results section, lines 203-204).

4- I would be useful to show an end-point tumour by ultrasound and the corresponding histology H&E section to indicate that the tumour was indeed growing in the intended position. What percentage of tumours were growing in the pancreas?

We added in figure 3 the panels C-F in which we presented the echography of a tumor mass at the experimental end-point, its image at the necropsy examination and the corresponding Hematoxylin and Eosin staining of the tumor mass. At the end point healthy portion of the pancreas is minimal, as we can observe in Figure 3D. Moreover, we observed that the 80% of mice developed tumor mass in the pancreas, confirmed since day 8 post cell injection by US imaging. We reported this data at page 5, lines 216-219.

Reviewer 3.

Major Concerns:

1- The introduction and discussion are not very strong. Particularly the background section does not indicate that this protocol is an improvement of an existing orthotopic model. The advantages are mainly described, not shown or presented in a comparative way. Indicative for the superficiality is the number of references in the discussion.

Following the reviewer's suggestion, we have implemented Introduction and we have rewritten the Discussion section, better discussing the background and the advantages with the existing orthotopic PDAC models (see Discussion section, pages 6-7).

Minor Concerns:

1- Next to the rather superficial content of the main text, also the overall presentation, i.e. spelling, grammar, tenses, adequate description of sources and equipment seems somewhat careless or indifferent(?)

A typical example, out of many more, is the use of the word 'weakly' in line 96.

As suggested by the reviewer we took the opportunity to revise the manuscript also for thoroughly proofread the manuscript to correct spelling and grammar issues. We also better described the equipment and we implementer the number of references.

Reviewer 4

Major Concerns:

A highly similar method was published in 2011 by Huynh et al., PLoS ONE, Volume 6, Issue 5, e20330. This article should be cited, and mentions of the novelty of the presented method de-emphasized.

As suggested by the reviewer, we have cited the article published in 2011 and we discussed the main differences with the method described by Huynh (lines 265-275). The method has been described for the first time by Huynh et all. and become a milestone of the echo-guided method. Compared to the Huynh's paper we have introduced some innovations and changes mainly due to technological advancement and the need to optimize the technique. For the injection Huynh used the Vevo 2100 imaging station, while in the present work we used the VevoLAZR-X, that is the most recent and technological advanced platform on market. Moreover, in the present work the mouse has been positioned in a different way on the animal platform respect to the paper published by Huynh. In our opinion, the method we have described represents an improvement and refinement of what has been described by Huynh et al. in 2011.

We thank the Editorial Board for the consideration given to our manuscript and we truly hope that this revised version of the manuscript will be suitable for publication in JoVE Journal.

Yours sincerely,

Prof. Annarosa Arcangeli, M.D., Ph.D