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Pancreatic Duct Infusion: A Effective and Selective Method of Drug and Viral Delivery

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Abstract:	The pancreas is a bifunctional organ with both endocrine and exocrine components. A number of pathologies can afflict the pancreas, including diabetes, pancreatitis, and pancreatic cancer. All three of these diseases are active areas of study, not only for immediate therapy, but also to better understand their pathophysiology. There are few tools to further these areas of study. Pancreatic duct infusion is an important technique that can allow for lineage tracing, gene introduction, and cell line specific targeting. Although the technique is, at firs, technically challenging, the applications are myriad. Ambiguity in the specifics of the procedure among other groups stimulated the need for a standard protocol. Here we show expression of a green fluorescent protein (GFP) within the pancreas after pancreatic duct infusion of a viral vector expressing GFP versus a sham surgery. The infusion and therefore expression is specific to the pancreas without expression present in any other tissue type.
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Date: July 31st, 2016

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Dear Editor:

Please find the enclosed manuscript entitled: "Pancreatic Duct Infusion: An Effective and Selective Method of Drug and Viral Delivery" which I am submitting for exclusive consideration of publication as an article in the *Journal of Visualized Experiments.*

This paper demonstrates a targeted therapeutic delivery method that is both effective and selective to the pancreas. Pancreatic duct infusion is a useful tool in exploring the pathophysiology of diabetes, pancreatitis, and pancreatic cancer. As such this paper should be of interest to a broad readership including all clinicians as well as researchers.

Thank you for the consideration of our work. Please address all correspondence concerning this manuscript to me and feel free to correspond with me by e-mail (joseph.fusco@chp.edu).

Sincerely,

Joseph Fusco

TITLE:
Pancreatic Duct Infusion: An Effective and Selective Method of Drug and Viral Delivery

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KEYWORDS:
Pancreas; pancreatitis; pancreatic cancer; diabetes; pancreatic duct; viral therapy; lineage tracing; gene introduction

SHORT ABSTRACT:
Pancreatic duct infusion is an important technique that can allow for lineage tracing, gene introduction, and cell line-specific targeting. A pancreatic duct infusion technique for drug and viral delivery to pancreatic cells is presented here.

LONG ABSTRACT
The pancreas is a bifunctional organ with both endocrine and exocrine components. A number of pathologies can afflict the pancreas, including diabetes, pancreatitis, and pancreatic cancer. All three of these diseases mark active areas of study, not only to develop immediate therapy, but also to better understand their pathophysiology. There are few tools to further these areas of study. Pancreatic duct infusion is an important technique that can allow for lineage tracing, gene introduction, and cell line-specific targeting. The technique requires the intricate dissection of the second portion of the duodenum and ampulla, followed by the occlusion of the bile duct and the cannulation of the pancreatic duct. Although the technique is technically challenging at first, the applications are myriad. Ambiguity in the specifics of the procedure between groups highlighted the need for a standard protocol. This work describes the expression of a green fluorescent protein (GFP) within the pancreas after the pancreatic duct infusion of a viral vector expressing

GFP versus a sham surgery. The infusion and therefore expression is specific to the pancreas, without expression present in any other tissue type.

INTRODUCTION:

The pancreas is a bifunctional organ, with both endocrine and exocrine components. A number of pathologies can afflict the pancreas, including diabetes, pancreatitis, and pancreatic cancer[1]. All three of these diseases mark active areas of study, not only to develop immediate therapy, but also to better understand their pathophysiology.

A targeted therapeutic delivery method would be beneficial. We have developed a pancreatic duct infusion technique that is an effective and selective method for drug and viral delivery to pancreatic cells. In this model, the pancreatic duct is selectively cannulated, and the common bile duct is occluded, allowing for delivery exclusively to the pancreas.

This technique can allow for the lineage tracing of specific cell types to further elucidate the developmental pathways important to pancreatic development and pathology[2,3]. Different promoters in viral vectors allow for the specific targeting of cell lines and for the introduction of genes into these cell lines[4,5]. This work demonstrates the expression of a green fluorescent protein (GFP) within the pancreas after the pancreatic duct infusion of a viral vector expressing GFP versus a sham surgery.

PROTOCOL:

All animal experiments were approved by the Animal Research and Care Committee at the Children's Hospital of Pittsburgh and the University of Pittsburgh Institutional Animal Care and Use Committee.

1. Preoperative preparation.

1.1 Administer inhaled isoflurane (1-3% for maintenance and up to 5% for induction) via a nose cone to an appropriate anesthetic level. Monitor the adequacy of anesthesia by toe pinch with forceps. The lack of a response is considered adequate, and be sure not to overmedicate, as respiratory depression may occur.

1.2 Place the animal on its back on the dissecting microscope stage, with the abdomen centered in view of the objective. Immobilize the animal with surgical tape. Apply ophthalmic ointment to the eyes of the animal to prevent dryness while under anesthesia.

1.3 Apply a smooth, thick layer of hair removal cream and leave it in place for 3 min. Check a small area for hair removal. Gently wipe off the cream and hair with 70% ethanol-soaked gauze.

1.4 Cleanse and disinfect the abdomen of the animal by wiping it with a 70% ethanol-soaked gauze followed by a betadine-soaked gauze. Maintain sterile conditions throughout the surgical procedure.

2. Proper exposure.

2.1 Make a midline incision in the skin from the xyphoid process to the umbilicus using a scalpel. Lift the peritoneum with Adson forceps and make a small hole in the midline using scissors.

2.2 Extend the peritoneal incision to the length of the skin incision, taking care to remain on the midline (linea alba).

Note: This will produce an upper midline abdominal laparotomy.

2.3 Elevate the liver with blunt retractors to expose the common bile duct. Clamp the common bile duct with a curved bulldog vascular clamp.

Note: This maneuver prevents undesired infusion into the liver.

3. Identification of the pancreatic papilla and duodenotomy.

3.1 Identify the duodenum and, with Arruga forceps, retract it caudally to display the papilla.

Note: The papilla is a white spot on the anterior surface of the second portion of the duodenum.

3.2 Retract the duodenum caudally, allowing for the identification of the course of the pancreatic duct. With a 30½-gauge needle, puncture the wall of the duodenum tangentially, directly opposite the papilla.

Note: The needle should follow the same angle as the duct as it enters the duodenum.

4. Infusion.

4.1 Pass the catheter through the duodenotomy created in step 3.2 until the catheter is visible within the duct. Advance the catheter into the duct no more than 1 cm to prevent the bypass of minor ductal tributaries.

4.2 Clamp the catheter in place with a second curved bulldog vascular clamp. Turn on the pump and infuse the selected volume. The volume infused may range from 25-250 μL, with an ideal volume of approximately 150 μL.

4.3 Administer an intraperitoneal injection of approximately 1.5 mL of saline to offset losses. Cover the exposed bowel with a moist gauze to prevent desiccation.

4.4 At the conclusion of the infusion, remove the bulldog clamp that was holding the catheter in place. Use the bulldog clamp to gently remove the catheter from the pancreatic duct. Release the clamp from the common bile duct.

4.5 Close the peritoneum and the skin in one or two layers using a running suture.

Note: There is no need to close the duodenotomy, as it will seal on its own.

4.6 As postoperative analgesia, administer a dose of ketofen at 5 mg/kg during the procedure and one on the following day.

4.7 Return the mouse to its cage under a heat lamp until it recovers. Provide the mouse with food and water ad libitum.

4.8 Do not leave the animal unattended until it has regained sufficient consciousness to maintain sternal recumbency. Do not return an animal that has undergone surgery to the company of other animals until it has fully recovered.

5. Sample preparation.

5.1 Place the animal in a carbon dioxide chamber or perform cervical dislocation.

5.2 Harvest the pancreas, with the spleen, liver, and duodenum as controls. Fix the tissues in paraformaldehyde overnight at 4 °C. Cryoprotect the sample in 30% sucrose overnight, as previously described[6].

5.3 Snap-freeze the samples. Section the tissue at a thickness of 6 μm using a microtome. Perform imaging on a microscope with fluorescence imaging, as previously described[7].

REPRESENTATIVE RESULTS:

With practice and careful surgical technique, the survival rate of these mice should be greater than 95%. One week after infusion, the desired effect should be evident in the pancreas. Mice at 10 to 12 weeks of age were utilized for pancreatic duct infusions. Here we use an adeno-associated virus serotype 8 (AAV8) with a CMV promoter to express green fluorescent protein (GFP), as compared to a sham surgery. Mice pancreases were harvested 7 days after the infusions. Sections of pancreas, spleen, and duodenum were stained with insulin antibody and Hoescht. Figure 1 shows that there is extensive expression of GFP throughout the pancreas in the mice that underwent pancreatic duct infusion with AAV8, as opposed to those that underwent sham surgery. There is no expression in the liver, spleen, or duodenum in these mice, thus documenting the selective nature of the infusion. These data can be confirmed with several other methods, including DNA and RNA expression profiles.

FIGURE LEGEND:

Figure 1. Expression of GFP after pancreatic duct infusion. (a) Sham surgery as compared to (b) pancreatic duct infusion with AAV8 expressing GFP. The scale bar represents 50 μm.

DISCUSSION:

This work describes in detail the methodology behind pancreatic duct infusion, an effective mouse model for the delivery of genes and other molecules specifically and effectively to the pancreas. Ambiguity in the specifics of the procedure between various groups highlighted the need for a standardized protocol[8-10].

There are several critical steps of the procedure, beginning with the selection of young, healthy mice. Perforation of the duodenum, however small and controlled, is a traumatic event in the mouse that undoubtedly leads to the recruitment of inflammatory cytokines and other factors that have not been completely characterized. Therefore, a delicate dissection is crucial, as is creating the smallest duodenotomy possible for the introduction of the catheter. The most important step of the entire procedure is the cannulation of the pancreatic duct with the delivery system.

Difficulties can arise with excessive disruption of the pancreatic papilla, leading to an overwhelming systemic inflammatory response. The pancreas is a delicate organ; in the surgical community, it is colloquially described as an organ not to "mess" with. Success rates will vary only slightly with practitioner skill level. As long as bile duct is properly occluded, the infusion will be 100% specific to the pancreas alone.

The learning curve for the procedure is quite steep, and the main limitation is overcome with practice. As previously stated, excessive manipulation of the duodenum and pancreas can lead to an overwhelming inflammatory response that must be avoided.

The procedure has broad applicability across many different disciplines. It can be utilized as a method for targeted gene delivery through the use of specific viral vectors[11]. The procedure can also allow for the specific infusion of molecules that aid in the elucidation of pancreatic cell function. The specific delivery of certain molecules is an appealing method for modifying the signaling pathways within the islets to produce proliferation[12]. The system can also obviate the need for transgenic breeding by directly producing a desired genotype and phenotype within the pancreas. The applications are myriad.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure

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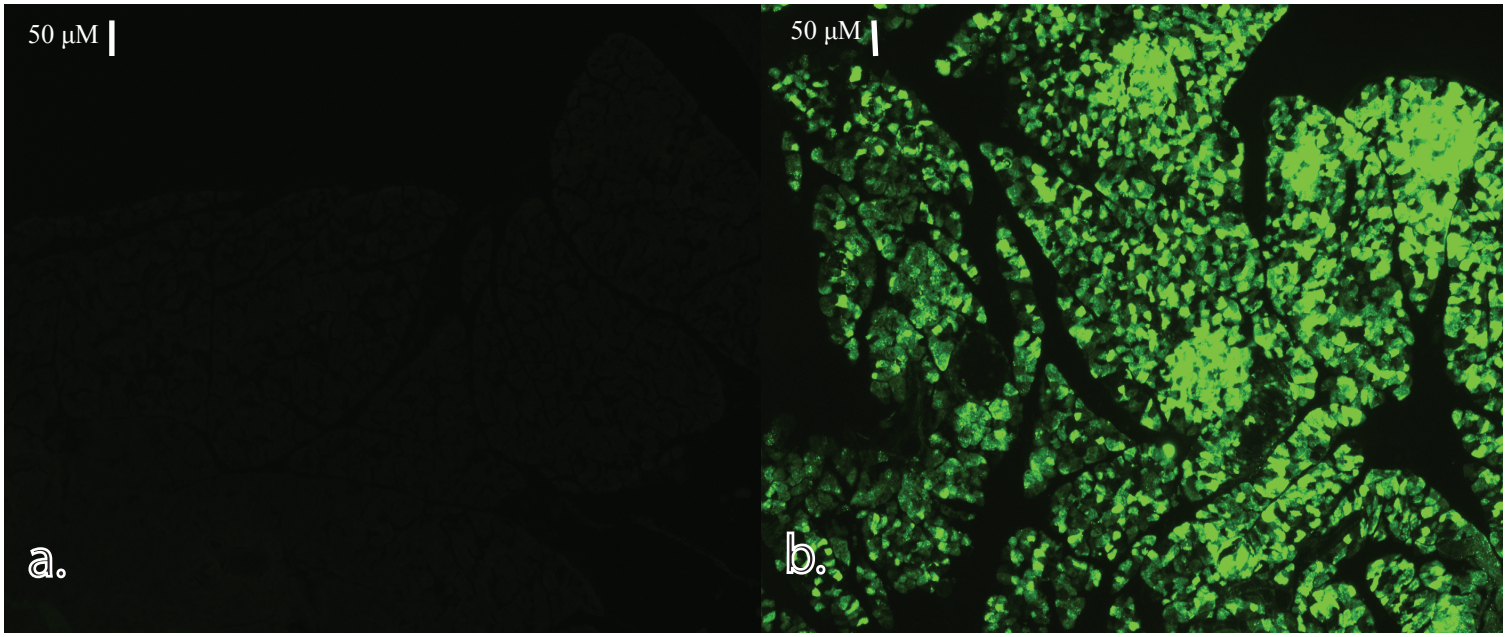

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Mice (25-30g, 8-12 weeks old)	Jackson Laboratory		
Ketofen	Henry Schein Inc.	5487	
Ethanol (70%)	Sigma-Aldrich	34923	
Sterile Saline Solution	Baxter Healthcare Corp.	2B-13-00	
Protective Equipment			
Hair Removal Product			Nair or trimmer
Gauze Pads 2in x 2in	Fisher Healthcare	22-362-178	
Needles (30.5G and 25G)	Becton Dicksinson and Co.	305106 and 305122	
Syringe for Ketofen and Saline	Becton Dicksinson and Co.	309657	
Syringe for Pump	Henke Sass Wolf	4010.200V0	
Infusion Pump	Pump Systems Inc.	NE-1000	
Infusion Catheter	World Precision Instruments	CMF31G	
Curved Bulldog Clamps (2)	Roboz	RS-7439	
Arruga Forceps	Roboz	RS-5163	
Adson Forcep	Roboz	RS-5234	

Needle Holder	Roboz	RS-7882
Scissor	Roboz	RS-5982
Cotton Tip Applicators	Physician Sales and Services	22-9988
Dissecting Microscope		
Suture	Owens and Minor	SXMD1B402

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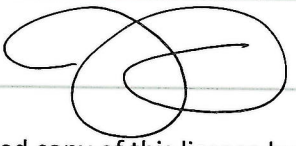
Article Title:	Pancreatic Duct Infusion: An Effective and Selective Method of Drug and Viral Delivery
Signature:	
Date:	7/31/16

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Response to Comments

•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.
Thank you for the excellent comments as we attempt to perfect the manuscript.
•The Short Abstract should clearly summarize the nature and aims of the protocol.
The short abstract has been edited to better summarize the aims and nature of the protocol.
•Scattered grammar issues should be addressed: -Line 33: "at firs" -1.3: "Apply a smooth thick layer of hair removal and leave in place for 3 minutes." Missing a word, probably hair removal cream? -The first sentence of step 3.2 is not written in imperative tense; it could become a Note after 3.1, or could be re-written to use imperative tense as part of 3.2. -Line 102: should be a space between 1 and cm. -In the Note following 4.1, units should not be crashed. -Step 8.1 and 9.2: Should be "perform" rather than "preform." -Step 9.1: Degree symbol is incorrectly crashed with the temperature number.
The various grammatical errors have been corrected.
•Regarding formatting, a protocol section should have at least three steps. For instance, it is not necessary to create a separate section for euthanasia, when this "section" consists of a single step. We recommend combining all operative sections under one heading or, at the least, combining pre-infusing and post-infusion sections.
All protocol sections have been edited to streamline the protocol.
•Additional detail is required: -1.1: Please specify means of administration (one assumes a nose cone?). -1.2: Please specify type of microscope (one assumes a dissecting microscope). -9.2: Section using what?
These details have been added.
•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.
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The DOIs have been included when available.
•IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional

copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.
•NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.
Reviewers' comments:
Reviewer #1: *Manuscript Summary:* This is a great paper from a lab with top expertise in this technology. *Major Concerns:* None. *Minor Concerns:* None. *Additional Comments to Authors:* N/A
Thank you for the excellent feedback.
Reviewer #2: *Manuscript Summary:* In this manuscript entitled "Pancreatic Duct Infusion: A Effective and Selective Method of Drug and Viral Delivery" by Joseph Fusco et al., the authors describe in detail a standardized protocol to infuse solutions to the pancreas through the pancreatic duct. The paper is clearly written, providing the necessary level of detail for the readers to replicate it. Critical steps and limitations are identified and described. Some points could be clarified:
Major Concerns:
1. In the Protocol Section, step 4. Infusion, 4.1- it would be beneficial to provide a range of adequate infusion volumes. What is the minimum and maximal volume that should/can be infused into the pancreas to balance maximum efficiency with the least tissue disturbance?
This range has been added to the step.
2. Protocol section, step 9. Sample Preparation 9.1- should the liver be added to the list of collected control tissues? It is mentioned in line 153 as a control but not mentioned during the collection step. Also, the liver is one of the organs most at risk of unspecific delivery and should be consistently evaluated for specificity of the procedure.
The liver was added as an additional control tissue.
Minor Concerns:
1. The title should read: Pancreatic Duct Infusion: AN effective method…. vs. A effective method…..
The title has been changed.
2. Line 146- the word "realized" in "the desired effect should be realized in the pancreas" should be replaced by a more adequate and clear concept. Perhaps "evident", "clear"?
The wording has been edited.
Additional Comments to Authors: N/A
Thank you for the thoughtful feedback.
Reviewer #3: *Manuscript Summary:* A surgical procedure for the delivery of agents to the mouse pancreas is described. The agent used in this example is an adeno-associated viral vector expressing GFP. The procedure is described simply and effectively with attention to suitable methods to prevent infusion into the liver and other non-targeted organs. Key points are appropriately emphasized, especially the technical challenge of the surgical

procedure and the likelihood of eliciting variable degrees of inflammation from the procedure.
Major Concerns: No major concerns other than suggesting that the authors provide an estimate of the success rate (measured as exclusive delivery to the pancreas) that might be expected for the novice, well-trained and expert practitioner.
The success rate has been included.
Minor Concerns: Typographic issues identified by line and some general minor concerns: 33 - "firs" should be "first" (Also in front page abstract) 134, 141 - "preform" should be "perform" 201 - "The have" should be "They have" or preferably, "The authors have" 70 - The reference to "vet ointment" can be understood by context but should be replaced with a more specific compound. The compound should be added to the list of materials. In many places, a space is needed between the value and unit of measure (e.g, 10uL, 5mg/kg, ...)
These typographical errors have been edited.
Additional Comments to Authors: N/A
Thank you for the thoughtful feedback.

Editorial comments:

1. Thank you so much for submitting your revised manuscript. All of your previous revisions have been incorporated into the most recent version of the manuscript.
Thanks so much for the thoughtful feedback.

2. NOTE: Please download this version of the Microsoft word document (File name: 55332) for any subsequent changes.
This version was edited.

3. In step 1.1 please provide the percentage of isoflurane used?
The percentages were added to the manuscript (1-3% for maintenance up to 5% for induction).

4. Please try to avoid usage of phrases such as “should be”, “could be”, “would be” and write in the active/imperative style. See step 1.1.
The grammar was modifies to the imperative style.

5. In step 1.4, how is the abdomen area disinfected and cleaned? What strength ethanol is used?
Betadine and 70% ethanol were added to this step.

6. In step 2.1 what tool is used to make the incision?
A scalpel is used for the primary incision.

7. Animal care note – To ensure passage of vet review, initial incision for recovery surgeries should be made with a scalpel instead of scissors.
A scalpel is used for the primary incision.

8. What is used to lift the peritoneum? And how is the small hole made in the midline in step 2.1?
The peritoneum is lifted with Adson forceps and a scissor is used to made the incision in the midline.

9. Please check step 2.2. for grammar. What is the white line?
The white line or “linea alba” is the midline.

10. In step 3.1 how is the duodenum retracted?
The duodenum is retracted with Arruga forceps.

11. In step 3.2 how is the wall of the duodenum punctured?
The duodenum is punctured tangentially with a 30 and ½ gauge needle.

12. In step 4.1 please provide a step-citation for “previously created duodenotomy”.

The citation was added.

13. Please provide a citation for step 9.1 “Harvest the pancreas with the spleen and duodenum as controls”.
The citation was added.

14. Similarly please provide a citation for step 9.2 in lieu of the details for fluorescence imaging.
The citation was added.

15. Please expand your representative results in the context of the technique you describe; i.e. how do these results show the technique, suggestions about how to analyze the outcome etc. This text should be written in paragraph form under a "Representative Results" heading and should refer to all of the results figures. You may include the figure captions under this heading but the captions and figure text must be separate entities.
An additional line was added detailing other methods to analyze and confirm the outcome.

16. Please make sure that the “Discussion” is written under the following sections.
a. Critical steps within the protocol.
b. Modifications and troubleshooting.
c. Limitations of the technique.
d. Significance of the technique with respect to existing/alternative methods.
e. Future applications or directions after mastering this technique.
The discussion section was broken down into these headings.

17. IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.
The manuscript has been copy-edited.

18. NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.
Thanks so much for the opportunity to share our work and publish in your journal.