Journal of Visualized Experiments

Intraductal Delivery to Rabbit Mammary Gland

--Manuscript Draft--

Manuscript Number:	JoVE55209R2
Full Title:	Intraductal Delivery to Rabbit Mammary Gland
Article Type:	Methods Article - JoVE Produced Video
Keywords:	Rabbit; Intraductal Delivery; Mammary Gland; ultrasound; localized delivery, breast; Drug Administration
Manuscript Classifications:	1.1.236: Breast; 5.2.319: Drug Therapy
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Abstract:	Localized intraductal treatments for breast cancer offer potential advantages, including reduced systemic toxicity and adverse effects, as well as improved efficiency of drug delivery to the tumor. However, several challenges remain before these can be applied more widely. Development and validation of intraductal therapeutics in an appropriate animal model facilitates the development of intraductal therapeutic strategies for patients. While the mouse mammary gland has been widely used as a model system of mammary development and tumorigenesis, the anatomy is distinct from the human gland. A larger animal model such as rabbit may serve as a better model for mammary gland structure and intraductal therapeutic development. In contrast to mice, in which ten ductal trees are spatially distributed along the body axis, each terminating in a separate teat, the rabbit mammary gland more closely resembles the human gland with multiple overlapping ductal systems that exit through separate openings in one teat. Here we present a minimally invasive method for the delivery of reagents directly into the rabbit mammary duct and for visualization of the delivery itself by high resolution ultrasound imaging.
Author Comments:	
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Question	**Response**
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Cover Letter

The University of Texas at Austin
107 W. Dean Keeton St
Austin, TX 78712

June 28, 2016

Dear Editors,

We are writing to submit a research manuscript entitled *Intraductal Delivery of Ultrasound Contrast Agents to the Rabbit Mammary Gland* for consideration in the Journal of Visualized Experiments.

Intraductal administration of therapeutics to patients with breast cancer has been explored in Phase 1 studies. The majority of relevant animal studies have been in rodent mammary gland. However, rabbit mammary gland provides a model that more closely reflects human anatomy and may also be more tractable for some studies as the size of the ducts is larger. Fewer researchers are experienced in working with rabbit as an experimental model and thus we believe a video submission will be an ideal way to communicate this work to the scientific community.

Author contributions are summarized below.
Amelia Clark: performed animal procedures, including intraductal administration and anesthesia, and wrote the manuscript
Nora Bird, DVD: assisted with animal imaging and monitoring of anesthesia
Amy Brock, PhD: conceived and led the study, assisted in animal monitoring, and wrote the manuscript.

Contact information for 6 potential reviewers with expertise in intraductal breast biology and technologies has been uploaded.

Thank you for your consideration. Please do not hesitate to contact me with any additional questions.

Best regards,
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TITLE:
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KEYWORDS:
Rabbit, intraductal delivery, mammary gland, ultrasound, localized delivery, developmental biology, drug administration

SHORT ABSTRACT:
Here, we describe a technique for the localized delivery of reagents to the rabbit mammary gland via an intraductal injection. In addition, we describe a protocol for visualization and the confirmation of delivery by high-resolution ultrasound imaging of contrast agents.

LONG ABSTRACT:
Localized intraductal treatments for breast cancer offer potential advantages, including efficient delivery to the tumor and reduced systemic toxicity and adverse effects[1-7]. However, several challenges remain before these treatments can be applied more widely. The development and validation of intraductal therapeutics in an appropriate animal model facilitate the development of intraductal therapeutic strategies for patients. While the mouse mammary gland has been widely used as a model system of mammary development and tumorigenesis, the anatomy is distinct from the human gland. A larger animal model, such as the rabbit, may serve as a better model for mammary gland structure and intraductal therapeutic development. In contrast to mice, in which ten ductal trees are spatially distributed along the body axis, each terminating

in a separate teat, the rabbit mammary gland more closely resembles the human gland, with multiple overlapping ductal systems that exit through separate openings in one teat. Here, we present minimally invasive methods for the delivery of reagents directly into the rabbit mammary duct and for visualization of the delivery itself with high-resolution ultrasound imaging.

INTRODUCTION:

The intraductal delivery of therapeutic agents has been studied in rodent models and in early stage human trials[3-6,11-12]. A recent Phase I study demonstrated the safety and feasibility of intraductal carboplatin or intraductal pegylated liposomal doxorubicin in women awaiting mastectomy for the treatment of invasive cancer[2].

Previous protocols for intraductal delivery have been developed for mouse and rat mammary glands[6-9]. For research purposes, intraductal tumor cell injections and the lentiviral vector delivery of oncogenes have also been performed in rodent models[13-16]. However, an ideal *in vivo* model of the intraductal delivery process should permit the development of novel classes of therapeutic compounds and facilitate preclinical assessment. Anatomical differences between rodents and humans have complicated the translation of these studies.

Unlike mice, in which each duct ends at a separate teat, the human breast consists of 5 to 9 independent ductal systems, each with a separate opening ending at the teat. Rabbit mammary glands harbor four independent ductal systems, each separately accessible through one of four orifices in a single teat. A rabbit model more closely matches the human anatomy and permits the study of intraductal drug delivery in a more relevant context.

Here, we use two techniques to assess intraductal delivery. The co-administration of a vital dye permits visualization through the skin and provides a simple and rapid confirmation of the method. For some applications, higher resolution mapping of the ducts may be preferred. We present here a protocol for ultrasound imaging of the ducts through the intraductal delivery of a non-targeted contrast reagent.

PROTOCOL:

Procedures using animal subjects have been approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

1. Preoperative Preparation

1.1) Record the body weight of each rabbit. As with all preclinical studies, monitor animal weights regularly to assess potential toxicity.

1.2) Prior to anesthetizing the rabbit, fill a 50-mL conical tube with commercially available ultrasound gel and spin at 500 x g for 30 s; there should be no visible bubbles in the gel upon completion of the centrifugation.

1.3) Administer glycopyrrolate subcutaneously at a dose of 0.1 mg/kg and acepromazine intramuscularly at a dose of 0.75 mg/kg. Wait 15-20 min for the sedative to take effect while monitoring vital signs and behavior.

NOTE: Glycopyrrolate is an anti-cholinergic agent that prevents bradycardia and reduces respiratory and GI secretions. Acepromazine is a sedative that serves as a premedication for anesthesia.

1.4) Administer 35 mg/kg of ketamine and 5 mg/kg of xylazine subcutaneously as an anesthetic. However, as the operator becomes more experienced and can accomplish the intraductal delivery more rapidly, decrease the drug dosages to 15 mg/kg of ketamine and 3 mg/kg of xylazine subcutaneously; this will shorten the anesthesia time and the time required for the animal to recover from anesthesia.

1.5) Check and document the heart rate, SPO_2, temperature, respiratory rate, and mucus membrane color every 15 min. Apply eye lubricant to both eyes.

NOTE: Only personnel who have undergone appropriate training and have been approved by their institution's IACUC should administer or monitor anesthesia. The use of a veterinary anesthesia monitor can aid in acquiring vital signs and is recommended. The veterinary anesthesia monitor, however, does not replace the need to manually check the animal every 15 min. See the manufacturer's instructions for the proper use of a veterinary monitor.

1.6) Verify the onset of anesthesia by a gentle toe pinch; the rabbit should be non-responsive before continuing.

1.7) Carefully shave the caudal abdomen of the rabbit in the area around the third and fourth pairs of inguinal teats.

1.8) With the majority of the hair removed, apply an over-the-counter hair removal cream to the shaved area. Remove the cream 10 min after application using damp paper towels wetted with warm water.

1.9) Wipe the area with alcohol-soaked gauze pads to clean the injection site.

1.10) Place the rabbit on its dorsum in a v-shaped trough lined with a recirculating warm water blanket and an absorbent pad.

2. Preparation of the Contrast Agent

2.1) Reconstitute the non-targeted contrast reagent according to the manufacturer's instructions. Pipette up and down gently to mix.

NOTE: The contrast agent used in this protocol is stable at room temperature for 4-6 h after reconstitution. Gently rock the vial between each retrieval.

NOTE: The volume of solution required will depend on the number of ducts to be injected. The rabbit has 4 ductal openings per teat, and 0.2 mL of solution is sufficient to fill one ductal tree of an adult New Zealand White Rabbit (*Oryctolagus cuniculus*). Thus, a total volume of 0.8 mL may be delivered to one mammary gland.

3. Intraductal Delivery

3.1) Locate the appropriate teats to be injected; the 3rd and 4th pairs of inguinal teats are recommended, as they are easily visualized when the animal is positioned on its dorsum.

3.2) Load 0.2 mL of sterile 0.9% saline into a 1-mL Luer-lock tuberculin syringe with a 22-gauge needle. Properly dispose of the 22-gauge needle once the saline is in the syringe and replace it with a sterile 25-gauge needle. Gently wipe the area with 85% isopropyl alcohol on a gauze pad.

3.3) With the bevel of the needle up and the syringe parallel to the body of the animal, insert the bevel of the needle into the side of the teat and slowly inject 0.1-0.2 mL of saline; this will allow for better visualization of the ductal openings.

3.4) Load 0.2 mL of injection solution into a 1-mL Luer-lock tuberculin syringe.

3.5) Hold the teat gently with the thumb and index finger and lift it slightly to position it for the intraductal injection; a wearable loupe may aid in visualizing the ductal openings.

3.6) While maintaining the lifted position of the teat, carefully cannulate the duct of interest using a 25-gauge blunt-tip needle.

3.7) After cannulation, gently twist the Luer-lock syringe on the hub of the blunt-tip infusion needle until it is locked in place.

3.8) Inject the solution slowly to minimize potential damage caused by rapidly moving fluid within the duct; at no time should there be resistance when injecting the solution.

4. Ultrasound Imaging

4.1) Apply a liberal amount of centrifuged ultrasound gel to the skin of the area of interest. Ensure that there are no bubbles in the gel, as these will compromise the image quality.

4.2) Set the imaging depth to 6 mm. Place the 21-MHz transducer in contact with the gel and scan the area of interest in B-mode. Observe the contrast medium in the scanned region including the ductal opening and throughout the duct.

NOTE: These settings have been developed for use with a specific photoacoustic ultrasound machine. Refer to the Table of Materials for more details. It may be necessary to adjust the transmit power and imaging depth of other imaging systems to optimize mammary gland visualization.

4.3) Remove the ultrasound gel from the animal's skin with a paper towel wetted with warm water.

5. Postoperative Care

5.1) Observe the injection site: there should be no signs of trauma to the teat region or to the surrounding tissue, and swelling in the area surrounding the teat likely indicates a mammary fat pad injection rather than a successful intraductal injection.

5.2) Place the rabbit in a sternal position. If needed, give 0.2 mg/kg of yohimbine intravenously to the marginal ear vein; this will reverse the effect of the xylazine and allow the animal to recover more quickly from anesthesia.

5.3) Monitor the rabbit every 15 min throughout the recovery period until the animal is alert, responsive, and maintains a sternal position.

NOTE: This procedure should not result in tissue damage or swelling. If redness or swelling are observed, administer a dose of meloxicam 0.1-0.2 mg/kg PO once the animal is alert and able to take medication orally. Contact the institution's veterinary staff for further guidance.

REPRESENTATIVE RESULTS:

Here, we show that the intraductal delivery of contrast reagents to the mammary ducts of a rabbit can be achieved without trauma to the tissue **(Figure 2)**. In rabbits, four separate ductal systems converge at one teat and thus may be accessed and imaged individually using this method. Individual ductal openings are easily visualized; note the arrowhead marking a second ductal opening adjacent to the cannulated duct in **Figure 2B**.

High-resolution ultrasound imaging with untargeted contrast reagent in linear imaging mode can provide a real-time readout of the intraductal delivery. Representative images show detection of the reagent up to 45 min post-delivery **(Figure 3)**. This technique may also be useful for monitoring the kinetics of therapeutic delivery through the ducts.

The robustness of this injection method is highly dependent upon the operator. To master the injection technique, it is recommended to inject a solution of 0.2% Evans Blue dye and monitor the integrity of the gland. This provides the operator with an additional readout of success and also aids in the determination of the appropriate volumes to be injected into each gland. Simple visual assessment can be used to determine whether the dye reaches the entire ductal system **(Figure 4)** and whether any ducts are damaged during the delivery.

FIGURE LEGENDS:

Figure 1. Schematic of rabbit mammary glands. The two lower pairs of dots represent the teats of the inguinal glands.

Figure 2. Preparation and cannulation of the inguinal mammary gland for

intraductal delivery. A) The teat of the right inguinal mammary gland is shown here immediately after the delivery of 0.2 mL of 0.9% sterile saline. Upon injection, the ductal openings are visualized more clearly. **B)** A ductal opening in the same teat is then cannulated with a 25-gauge blunt-tip infusion needle. The arrow shows a ductal opening without a cannula.

Figure 3. Non-targeted contrast reagent visualized within the mammary duct by ultrasound imaging. A) The contrast reagent is localized immediately after delivery and is visualized **B)** 30 min post-delivery and **C)** 45 min post-delivery. The persistence of the reagent inside the mammary duct allows visualization throughout the duration of this protocol.

Figure 4. An inguinal mammary gland injected through the teat with Evans Blue saline solution. A) The external appearance after the intraductal injection of 0.2 mL of Evans Blue solution. **B)** Upon opening the skin, the Evans Blue permits the visualization of the entire mammary ductal tree and confirms the intact ductal structure. **C)** Whole-mount specimen of a region of inguinal mammary gland after fixation and staining with carmine alum.

DISCUSSION:

This method of intraductal delivery to the rabbit mammary gland may be used for ultrasound contrast reagents and many other aqueous solutions, including vital dyes and therapeutics. Previous studies have demonstrated the intraductal delivery of hormones[17-19]. In rodent models, the intraductal delivery of nucleic acids[8], chemotherapeutics[6,7], and nanoparticle carriers[8,20] have been performed. The protocol described here could be adapted for these applications as well.

For some applications, the confirmation of the intraductal delivery by Evans Blue vital dye, as visualized through the skin, may be sufficient. However, visualization through the skin is diffuse, and individual ducts are not well demarcated. In endpoint studies, Evans Blue vital dye does provide a clear map of the entire ductal tree, but this requires the isolation of the mammary tissue. Therefore, contrast-enhanced ultrasound provides an alternative approach for visualizing the intraductal delivery to individual ducts in live animal studies. We note that Evans Blue dye maps the entire ductal tree, including the smallest-diameter terminal ducts, while contrast reagent and ultrasound maps only include the larger ducts. Another distinction is the possibility to monitor temporal dynamics in ultrasound, whereas Evans Blue provides only a single snapshot measurement.

As in the method for intraductal delivery to the rodent mammary duct[9], the most significant challenge and limitation to this technique is likely to be the reliance on operator expertise. However, the larger size of the ductal openings in a rabbit model simplifies the procedure, eliminates the need for performing the technique with the aid of a stereomicroscope, and shortens the time required for new operators to develop proficiency. In our experience, the injection of 0.1-0.2 mL of saline to the side of the teat prior to the intraductal delivery is a critical step that enables the clear visualization of the

ductal openings (step 3.2, above). Accurate positioning and lifting of the delivery site is also essential; this ensures that the solution flows into the duct (steps 3.5 and 3.6, above). We note that the co-administration of the contrast reagent will necessarily reduce the available volume for testing other reagents or therapeutics. However, intraductal delivery can also be performed without imaging or with simple inspection by Evans Blue to confirm the delivery.

The most common noninvasive lesion of the breast is ductal carcinoma *in situ* (DCIS), in which abnormal ductal epithelial cells proliferate inside the mammary duct but do not penetrate through the basement membrane to the adjacent tissue. With advances in mammographic imaging, the detection rates of DCIS have increased dramatically. In the United States, approximately 25% of newly diagnosed breast lesions are classified as DCIS, and by 2020, more than 1 million women will be living with DCIS in the United States alone[22-25]. However, many DCIS lesions remain dormant, and most estimates find that only 15-40%[21-25] of DCIS lesions will ever progress to invasive cancer. However, there are currently no predictive biomarkers to aid in the identification of which tumors will become invasive.

As more women are diagnosed with this pre-cancerous lesion, serious questions regarding over-diagnosis and overtreatment have emerged. The treatment of premalignant disease is typically aggressive. Most patients with DCIS will undergo surgery (lumpectomy or mastectomy), and many also receive radiation[25]. Some patients with hormone receptor-positive DCIS will also receive 5 or more years of endocrine therapy, which has been shown to reduce recurrence. Side effects of this treatment may include stroke, blood clots, bone loss, and elevated risks of uterine and endometrial cancers. All of these options have serious systemic side effects and impact patient quality of life. There is a significant need for less invasive therapeutic strategies[25].

The intraductal delivery of chemotherapeutic agents in both mouse models and in breast cancer patients has previously been shown to be effective, with no evidence of systemic toxicity or long-term histopathological changes[3-6]. The intraductal administration of therapeutics could one day offer new options for women diagnosed with DCIS that has not yet progressed to a locally invasive lesion. The potential for halting tumorigenesis while also preserving ductal structure makes this an attractive therapeutic strategy[10]. Importantly, the localized delivery approach ensures that the treatment reaches the relevant abnormal cells while potentially minimizing collateral damage to other tissues. While rabbit tumor models are not available, the normal mammary gland of rabbits may provide a relevant model to test the localized delivery, safety, transport, and kinetics of therapeutics uptake inside the mammary duct. These *in vivo* studies will enable the testing and validation of candidate diagnostics and therapeutics within a relevant tissue environment.

A similar approach enabled the intraductal administration of therapeutics to live mice and allowed for minimally invasive and localized drug delivery to the mammary ductal system[8-9]. However, the anatomy of the mouse mammary gland differs from the human breast in a number of important ways, including the tissue composition and the number

of ducts that end at each teat. Here, we extend this technique to a larger animal model in which the mammary epithelial structure more closely represents the anatomy of the human breast[12,14]. This opens up the possibility for extended monitoring by imaging and for assaying the concomitant intraductal delivery of various reagents to the rabbit mammary ductal epithelium. Advances in localized delivery to an appropriate animal model, with ductal anatomy similar to the human gland, should accelerate the application of non-invasive, targeted therapeutic strategies in humans.

ACKNOWLEDGMENTS:
The authors acknowledge support from a Translational Breast Cancer Research Grant (14-60-26-BROC to AB) from the Breast Cancer Research Foundation and the American Association for Cancer Research.

DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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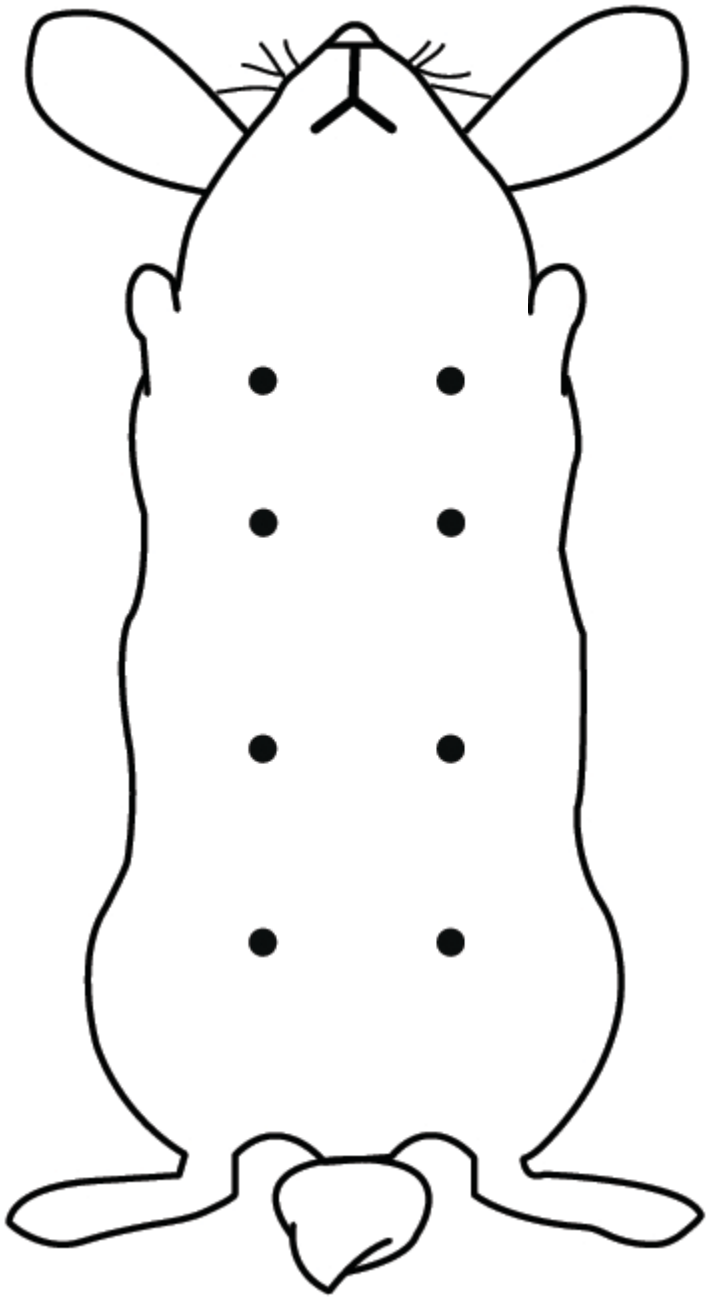

Figure 2

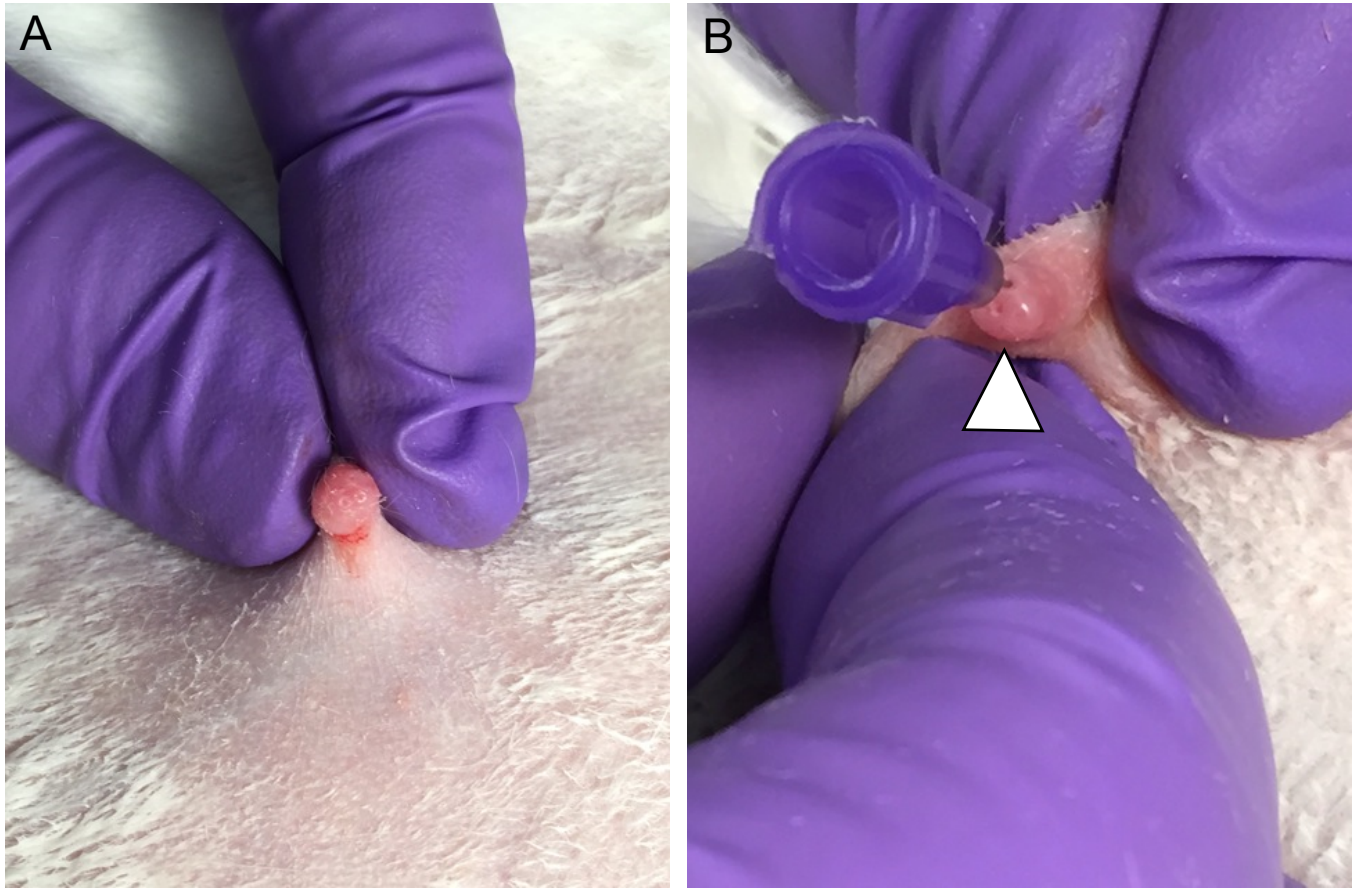

Figure 3

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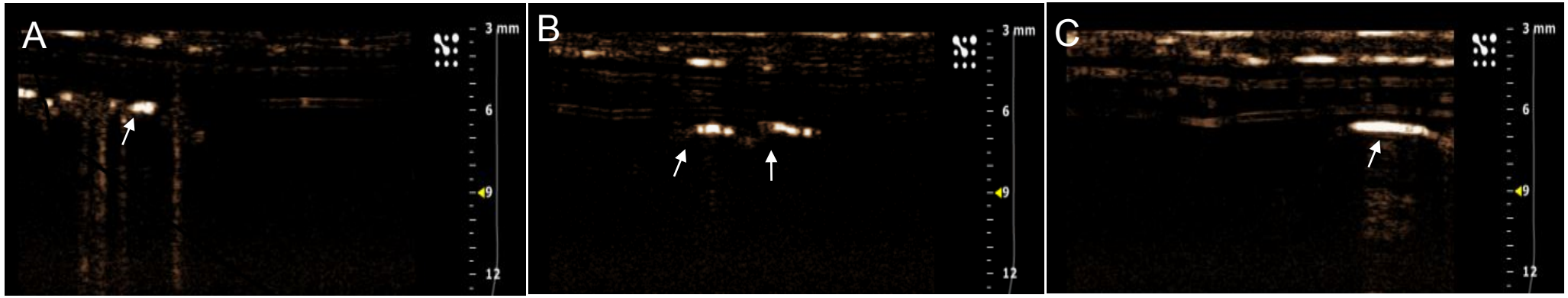
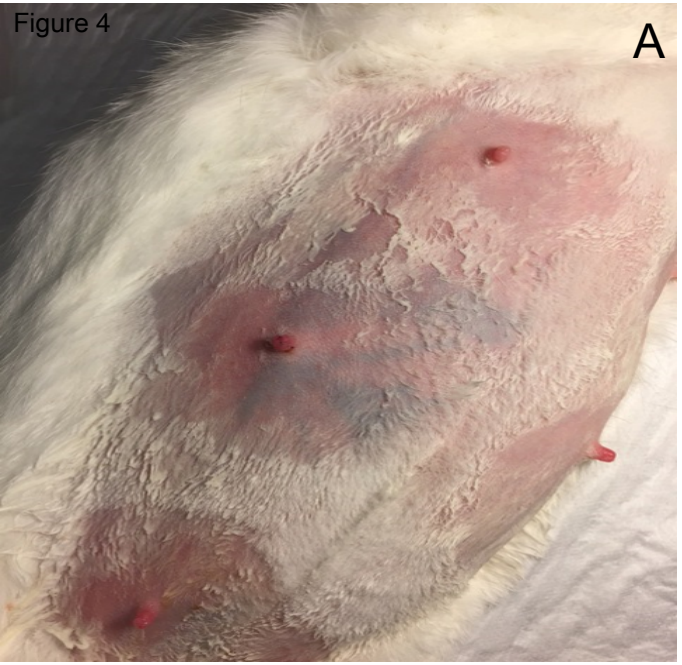
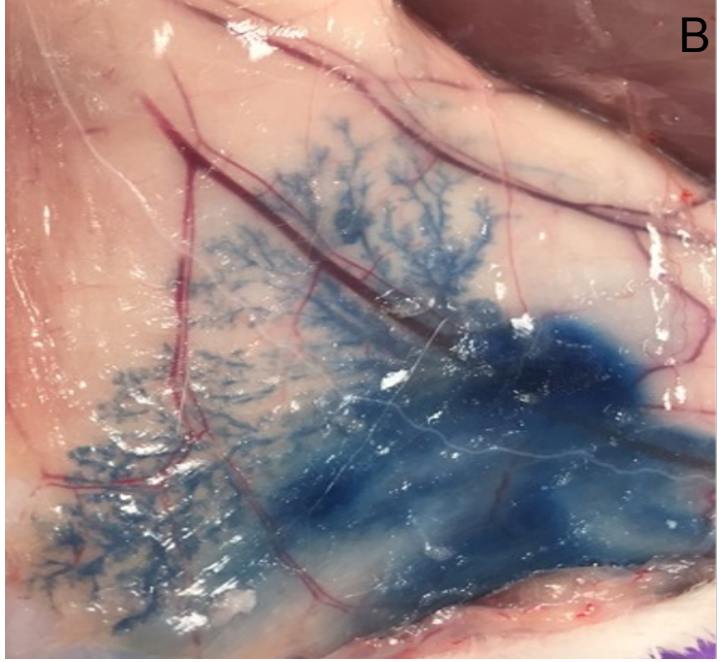
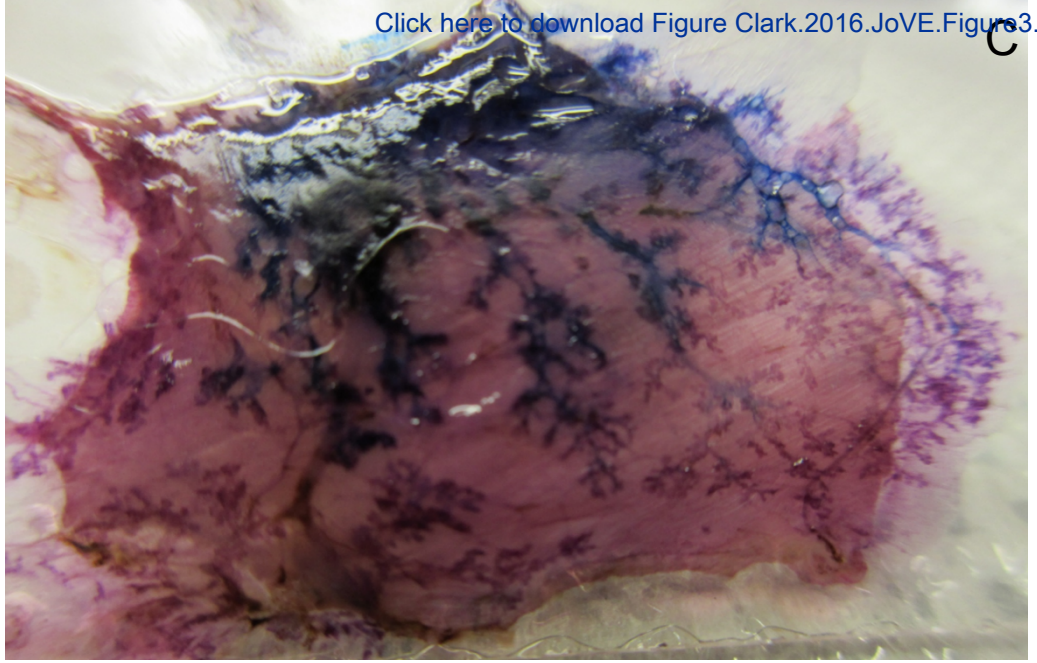


Figure 4







Name of the Material/Equipment	Company
MicroMarker non-targeted contrast reag	VisualSonics
Luer Lock 1mL Syringes	BD
Glycopyrrolate 0.2mg/mL	Wedgewood Compounding Pharmacy
Atropine Sulfate 0.5 mg/mL	Animal Health International
Ketamine HCL 100mg/mL	Animal Health International
Acepromazine 10mg/mL	Animal Health International
Xylazine 20mg/mL	Animal Health International
Yohimbine 0.2mg/mL	Animal Health International
Hair Removing Cream	Veet
Blunt tip infusion needles	Sai Infusion Technology
Veterinary Pulse Oximeter	EdanUSA
Warm Water Pump	Gaymar
Warm Water Blanket	Animal Health International
Ultrasound system	VisualSonics

Catalog Number	Comments/Description
VS-11694	
309628	
GLYCOP-INJ013VC	6 month shelf life, supply may be limited.
15320764	If glycopyrrolate is unavailable. Not to be combined with glycopyrrola
21250699	http://www.animalhealthinternational.com/
17640541	
20101547	
14588965	
	Sensitive skin solution. Available through local retailers.
B14-50	http://www.sai-infusion.com/collections/blunt-needles
VE-H100B	http://www.edanusa.com/Product/VE-H100B-Veterinary-Pulse-Oximeter.
TP700	
21232696	Maxi-Therm Lite Warming Pads
Vevo 2100	

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Title of Article: Intraductal Delivery to Rabbit Mammary Gland

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Response to Reviewers Suggestions

Dear Dr. Upponi,

We thank the reviewers of our manuscript 55209-R1-072716 for their thoughtful and considered comments. We have addressed each request in the revised manuscript and detail these changes below (black text). These changes have strengthened the manuscript and clarified the message; we look forward to working with JoVE to bring the manuscript and accompanying video protocol to publication.

Please do not hesitate to contact me if you have any questions or concerns.

With best regards,
Amy

Amy Brock, Ph.D.
Asst. Professor
The University of Texas at Austin
Department of Biomedical Engineering

•Formatting: -Please use italics for all Latin phrases such as in vivo.
-Please use “x g” rather than RCF for centrifuge speeds.
-Step 2.0 should be 1.10 (line 105).
5.3 – Should this be “min” rather than “15 m?” As written, this means 15 meters.

Author response: These editorial points have been changed.

•Additional detail is required: 4.2 – What should be seen during imaging?

Author response: An additional sentence has been added to 4.2.

•Branding: 3.6, Figure 1 legend – Sai Infusion Needle

Author response: Brand name has been removed (note this is relabeled as Figure 2 in the revised manuscript).

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.

Author response: All figures are original.

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Author response: We have confirmed that the DOI is included for all references for which they are available.

Reviewer #1:

Manuscript Summary: The manuscript describes an intraductal injection technique that was developed for the rabbit mammary gland. A major difference between the presented technique and intraductal injection methods developed for rodent mammary glands is that the rabbit gland contains 4 separate ductal trees, each with its own orifice, located within a single teat. Explanation of how to approach intraductal injection of this type of gland as well as evidence of successful delivery is presented. Regarding the rationale for this study, I recommend the authors revise the introduction of the paper. First, comparisons are drawn between the rabbit and rodent mammary glands - while I agree there are important anatomical differences in the ductal trees, this does not necessarily mean there are "physiological" differences. And on top of this, the authors use inguinal glands in the study, which are not the best model of human breasts (thoracic glands are the best model). Thus for accuracy and clarification, I suggest sticking with anatomical/structural differences and deleting "physiology". Secondly, it is unwarranted to equate the differences in ductal trees with differences in "tissue microenvironment" - there is no supporting evidence and thus this too should be deleted.

Author Response: We have made these recommended deletions.

Method section: -1.4) The rationale for lowering the dose of xylazine and ketamine "as expertise in this technique is gained" is not clear - presumably, the authors mean that the injections will be achieved in shorter time with experience. If so, please state.

Author Response: This is correct and we have clarified the point in the text for 1.4.

1.7) A diagram of the location of inguinal teats on the rabbit (relative to the other pairs) would be useful for investigators unfamiliar with this species.

Author Response: We have prepared a new figure (new Figure 1).

-Starting here and in the remainder of the paper (e.g. 3.1), there is a switch from using "teat" to "nipple". For accuracy, refer to "teat" when describing an animal mammary gland, and "nipple" when describing human breast.

Author Response: This terminology is now applied consistently.

-4. Ultrasound imaging The purpose of performing the ultrasound imaging is unclear. The distribution pattern of the injected solution in Figure 3 doesn't in any way resemble the pattern of the Evan's Blue dye, and the Evan's blue dye seems adequately visible through the skin of the shaved gland region. Thus how is the ultrasound imaging useful?

Author Response: This point was also raised by Reviewer 3 and in response, we have added a new paragraph to the Discussion section (paragraph 2) detailing how these two methods (Evan's blue dye and ultrasound imaging) provide different information.

In the discussion, the paper would benefit from mention of how the rabbit might become a useful model of breast cancer. As the rodent is the prevailing breast cancer model, how would the rabbit be used? E.g., can tumor cells be injected into the rabbit gland? If the only advantage of the rabbit is that its glands contain multiple independent ductal trees, what value will this model contribute to breast cancer research?

Author response: We have focused the subfinal paragraph of the Discussion on this question. While rabbit tumor models are not currently available, we propose that the normal mammary gland of rabbit may provide a useful model to aid in development of localized therapies. In particular, the model may be useful to assess the distribution, transport and kinetics of therapy uptake inside the mammary duct as well as considerations such as long-term safety and tissue changes.

Author Response:

Major Concerns: N/A *Minor Concerns:* N/A *Additional Comments to Authors:* N/A

Reviewer #2:

Manuscript Summary: This manuscript presents a valuable protocol for using intra-ductal injection of rabbit mammary glands as a means to test drugs or therapeutic approaches which might target early-stage or pre-invasive ductal carcinoma in situ (DCIS). Rational approaches to treat DCIS at source would certainly have valuable extrapolations into human clinical settings.

Major Concerns: - The authors refer to the clear structural differences between mouse and human mammary ductal structure. However they do not adequately acknowledge the fact that, although rabbit more closely resembles human than does mouse, there are still some key structural differences which separate the species. - If this technique is to be used to test therapeutic approaches targeting DCIS, the authors need to provide evidence that viable models of DCIS in the rabbit mammary gland actually exist. To my knowledge they do not, in which case the authors really need to justify whether or not this approach is valuable. Is there any evidence from the literature that rabbits can even tolerate implantation of tumor cells (intraductal or otherwise)? Accordingly, could those implanted cells survive and form tumors? These points need to be addressed and justified. -

Author response: This point was also requested by Reviewer 1. We have focused the subfinal paragraph of the Discussion on this question. While rabbit tumor models are not currently available, we propose that the normal mammary gland of rabbit may provide a useful model to aid in development of localized therapies. In particular, the model may be useful to assess the distribution, transport and kinetics of therapy uptake inside the mammary duct as well as considerations such as long-term safety and tissue changes.

The authors have not discussed the fact that the need for prior injection of contrast reagent, plus saline to distend the structure, reduces the volume of therapeutic reagent which could feasibly be delivered.

Author response: Saline is not delivered into the duct itself but rather into the side of the teat (as noted in protocol step 3.3). Visualization in the video will make this more clear. We have now added a caveat to the Discussion which notes that co-delivery of the contrast reagent would reduce the delivery volume of therapeutic reagent. We also mention that imaging would likely be part of a pilot study or a specific application in which localization was important.

Minor Concerns: - The title isn't very strong or descriptive - perhaps it could also incorporate the concepts of therapeutic delivery or drug administration. -

Author response: Since we did not deliver a therapy in this protocol, we kept the title rather general. We are open to changing the title if the editor believes this is appropriate.

In protocol step 1.3 - it would be advantageous to indicate what glycopyrrolate and acepromazine are, for clarity, as in section 1.4 the indication for administering ketamine and xylazine was noted. -

Author response: We have added additional test to step 1.3 to clarify the roles of each of these pre-medications.

Protocol step 1.8 and 4.3 - perhaps a more scientific term than 'wetted' could be used.

Author response: Word choice has been edited in both instances.

Point 2.0 should be labelled

Point 1.9. - Point 2 - would be useful to include exact contrast medium used (it doesn't appear on list of materials).

Author response: The specific name was removed in the editorial phase to comply with the journal's policy (no trademark or specific brand names.)

Figure legend for Fig 2 lacks detail.

Author response: This legend has been expanded and additional explanation has also been added to the relevant description in the Results section. (Note that this is labeled "Figure 3" in the revised manuscript.)

Additional Comments to Authors: N/A

Reviewer #3: *Manuscript Summary:* N/A

Major Concerns: The rationale for use of ultrasound is not clear. That the ducts have been injected properly is ascertained by lack of resistance and easy flow of fluid. US does not confirm that either. Perhaps the author can explain that further

Author Response: This point was also raised by Reviewer 1 and is addressed through the addition of a new paragraph (Paragraph 2) in the Discussion section.

Dear JoVE Editors,

Thank you for providing feedback on our submission. We have made the following revisions to the manuscript entitled "Intraductal Delivery to Rabbit Mammary Gland" (JoVE55209) and address each change in the points below.

1. Editor modified the formatting of the manuscript and made minor copy-edits. In keeping with the JoVE format, a few of your protocol steps may have been revised to the imperative tense and any step that could not be written in the imperative tense may have been added as a "Note". Please maintain the current formatting throughout the manuscript. You can find the updated manuscript attached to this e-mail.

2. Step 1.5 of the protocol – how is each parameter monitored? Please provide stepwise instructions or reference(s).

We have added detail about the monitoring of heart and respiration rate, animal coloring and respiratory rate during administration of anesthesia. We were not sure if it was appropriate to include very basic instruction (use of stethoscope, etc). Please let us know if this requires additional expansion. Our veterinary staff felt that this level of instruction should be a core component of the institutional training for any personnel administering or monitoring veterinary anesthesia. A concern was that this protocol does not attempt to substitute for that training. In light of this feedback, we have also added a note to this effect.

3. Step 2.1 Note: Please revise the grammar used in the first sentence.
This step has been revised.

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Although the protocol is less than 3 pages, we suggest that the steps describing administration and recovery from anesthesia are not central to the new procedure and could be left out of the filmable content. We have used Comment boxes to mark the beginning and end of the most pertinent filmable content (steps 1.7-5.1).

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We are mindful of this important issue and note that the veterinarian Director of the University of Texas at Austin Animal Resource Center has read and provided feedback on this submission.

a) Please mention how proper anesthetization is confirmed.

This has been added in 1.6.

b) Discuss maintenance of sterile conditions.

Steps 1.9 and 3.2 now address this point. (Please note that this procedure is minimally invasive; there is no surgical incision that would present a high risk of infection.)

c) Discuss treatment for post-procedural pain, if any.

This has been added in step 5.4.

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We have removed the specific brand name.

7. Please expand your discussion to cover the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods (please cite comparable methods), 4) critical steps in the protocol.

These points have been covered in the discussion through the addition of new paragraphs at the beginning of the Discussion.

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We have checked that the citations conform to the JoVE format.