**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

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•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.

•JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

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.