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A new best practice for validating tail vein injections in rat with near infrared labeled agents

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To the JoVE editorial team:

I am pleased to submit an original methods article entitled "Validating tail vein injections in rat with near infrared labelled agents; a new best practice" for consideration for publication in JoVE Medicine. We previously demonstrated theranostic utility of a nanoemulsion in a rat model of chronic constriction injury – one that is packaged with drug and near infrared dye (Janjic, Jelena M. et al. Journal of Neuroimmunology, 2018). Whole body imaging in a fluorescence scanner reveals neuroinflammation. This manuscript builds on our prior study to demonstrate a method of validating the quality of agent administration by the tail vein. A procedure that can be difficult to perform and may be critical to experimental outcome. Partial or incomplete injection into the vein may go un-noticed, leaving much of the biological agent sequestered in the tail.

In this manuscript, we show that by imaging the tail of rodents before and after agent administration, and quantifying resulting fluorescence, a determination of injection success can be made.

We believe that this manuscript is appropriate for publication by JoVE because it addresses quality control for a method of agent administration that is notoriously difficult to validate.

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

Thank you for your consideration!

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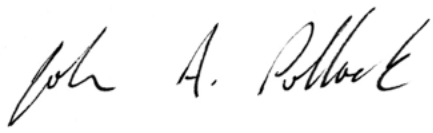

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TITLE:

A New Best Practice for Validating Tail Vein Injections in Rat with Near-infrared-labeled Agents

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KEYWORDS:

tail vein injection, tail vein, injection, rat vein, murine, imaging agents, nanoemulsion, lateral tail vein, NIRF, fluorescence imager, theranostics

SUMMARY:

Here we present a method to validate tail vein injections in rats by utilizing near-infrared fluorescence imaging data from dyes incorporated into agents or biological probes. The tail is imaged before and after the injection, the fluorescent signal is quantified, and an assessment of the injection quality is made.

ABSTRACT:

Intravenous (IV) administration of agents into the tail vein of rats can be both difficult and inconsistent. Optimizing tail vein injections is a key part of many experimental procedures where reagents need to be introduced directly into the bloodstream. Unwittingly, the injection can be subcutaneous, possibly altering the scientific outcomes. Utilizing a nanoemulsion-based biological probe with an incorporated near-infrared fluorescent (NIRF) dye, this method offers the capability of imaging a successful tail vein injection in vivo. With the use of a NIRF imager, images are taken before and after the injection of the agent. An acceptable IV injection is then qualitatively or quantitatively determined based on the intensity of the NIRF signal at the site of injection.

INTRODUCTION:

The route of administration of agents into small animals serves as a critical point of many experiments. It determines where the agent is to be delivered and, subsequently, what will happen to the agent thereafter. Although other routes can be used for agent administration[1], the intravenous route of delivery is a preferred route for certain agents. IV injection allows agents to be directly injected into the bloodstream, bypassing first-pass tissue effects and the need for extraneous solute absorption[1]. This also allows for targeting cells in the bloodstream[2,3] and direct delivery to all tissues within the circulatory system. In rodents, several veins can be considered, including the jugular, the saphenous, and the tail vein.

In this method, a NIRF dye containing a biological probe—in this case, a nanoemulsion (**Figure 1A**)[3,4,5,6]—is injected into the lateral tail vein of rats. This particular NIRF-containing nanoemulsion has been used previously to image and track neuroinflammation in vivo and ex vivo[7,8] in a rat model[9] of neuropathic pain[3,4,5,10,11,12]. Imaging is conducted before and after the injection with a preclinical NIR fluorescence imager (see **Table of Materials**). This serves as a tool to validate the quality of the agent administration. Imaging prior to the tail vein injection serves as a basis for obtaining a baseline image.

Increasingly in animal studies, intravenously administered nanoemulsions are being utilized as biological probes and targeting agents[13,14,15,16]. It is a proven challenge to administer an agent via the tail vein[17,18]—be it a drug, a viral vector, or another probe—and to ensure that the entire contents of the injection have successfully entered the bloodstream and not the surrounding tissues[18]. Therefore, a method of visualizing and evaluating the quality of a successful injection is beneficial.

Typically, a heat lamp or warm water is used to warm the tail, which causes dilation of the vein, permitting its visualization prior to injection. While this ensures easier entry into the vein, there is not a quantitative way to discern whether the compound has entered the bloodstream in its entirety[19,20,21,22]. This becomes more difficult still in strains of animals where the vein contrasts faintly with the skin, such as in black mice. Typically, the investigator can gauge a failed injection by experiencing resistance during the injection and, in some cases, visualizing a bulge on the tail, indicating a subcutaneous leakage of the agent[23,24].

In this study, NIRF imaging of the nanoemulsion injected into the lateral tail vein of live rats is performed on a small-animal NIRF imaging system (see **Table of Materials**). Rats are fed a special purified diet (see **Table of Materials**) to reduce nonspecific gut fluorescence. Simultaneous image acquisition of white light and 800 nm fluorescence is captured using the NIRF imager and associated software. The relative fluorescence intensity is measured on the tail at the preinjection and postinjection states. The fluorescence intensity for the region of interest (ROI) at the site of injection is recorded and divided by the area of the ROI. Qualitative assessments can be made on which injections are acceptable. Optionally, further quantitative analysis can be performed by setting thresholds for acceptable injections and assigning ROI measurements into groups, at which point statistical significance can be calculated.

By utilizing this validation strategy following tail vein injections, the standard of a research study improves due to increased consistency of agent administration. This method of assessing the quality of tail vein injection can be easily customized for different injectable agents to include infra-red fluorescent probes provided commercially by several companies.

PROTOCOL:

All protocols were performed in accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and Institutional Animal Care and Use Committee (IACUC) at Duquesne University.

1. Preparation and anesthesia

NOTE: Aseptic techniques are used for the entirety of the procedure. Only new sterile materials and autoclaved sterile instruments are to be used. Personal protective equipment (sterile gloves, hair bonnet, surgical mask, scrubs) needs to be worn to avoid contamination.

1.1. Use adult male Sprague-Dawley rats weighing 250–300 g. Acclimate the rats to standard living conditions, keep them on a 12 h light/12 h dark cycle, and provide food and water ad libitum. House the animal socially, keep them on paper bedding, and provide a special diet (see **Table of Materials**) to avoid autofluorescence during imaging.

1.2. With the use of a properly placed heating pad, anesthetize the animal under an initial 5% isoflurane in 20% oxygen, followed by a maintenance level of not less than 1.5% isoflurane and not more than 3%, unless the animal wakes up or retains feeling.

1.3. Confirm proper anesthesia via a lack of response to tail pinches. Monitor the blood flow as well via vital signs throughout the procedure.

2. Preinjection

2.1. Image the animal in a preclinical NIR fluorescence imager by positioning the animal laterally to expose the injection site on the lateral tail to establish a baseline of fluorescence in the tail (**Figure 1C,E**).

2.2. Following imaging, move the animal back to the surgical table, and place it under anesthesia for the tail vein injection.

NOTE: Continue monitoring the rat's vital signs and recheck proper anesthetization via tail pinch.

3. Tail vein injection with NIRF-containing agent

3.1. With the animal in the prone position, orient the tail with the dorsal side facing up. Dilate the tail vasculature in warm water for a minimum of 1 min. Orient the tail vein so the lateral side (either right or left) is turned 30° (clockwise or counterclockwise) to expose the right or left tail

vein (**Figure 1B**).

3.1.1. Once a lateral tail vein has been located (which appears dark-colored upon dilation), sterilize the entire tail with alcohol pads, repeating 2x.

3.1.2. At an appropriate dosage based on the study design, begin injections in the distal coccygeal vertebrae region of the tail and moving more proximal if proper needle placement fails.

3.2. Insert a 25–27 G sterile needle, bevel up, into the lateral tail vein, with the tail at a 180° angle, inserting the needle parallel to the lifted tail. Observe blood flashback in the rim of the needle to ensure correct placement. If no flashback is apparent, slowly move the needle tip (without removing it from the tail) to find vein insertion. If placed subcutaneously, no blood flashback will occur.

3.3. Insert the syringe with the injectable materials into the rim of the needle. When proper placement is achieved, the injectable fluid will not incur resistance upon injection. The injection will advance smoothly and easily. Once injected, remove the needle and the syringe, apply pressure with sterile gauze for at least 1 min to ensure clotting, and mark the spot of injection with a pen on the tail, ensuring it is visible on the white light image.

NOTE: No hematoma or lesion will be visible at the site of injection.

3.4. If the needle tip moves during the syringe insertion, remove the needle and retry the needle entry procedure more proximal on the ipsilateral tail vein. Do not reuse the same needle if a different reentry point is tried.

NOTE: Alternatively, the injection can be performed with an IV catheter (see **Table of Materials**). This has the benefit of visual confirmation of the catheter during venipuncture. Insert the catheter, bevel side up, at the angle previously described. Observe prompt flashback in the entire length of the needle and the catheter to ensure correct placement. Slight back pressure can be used to pull blood into the syringe to confirm proper placement in the vessel before injecting. Again, no resistance will be felt.

4. Postinjection

4.1. Perform quality assessment after the tail vein injection in a preclinical NIR fluorescence imager in the same orientation as the preinjection image. Increase the isoflurane anesthesia to 3% for several minutes and move the animal to the imager. Ensure the animal is still properly anesthetized.

4.2. Quickly, orient the animal on its lateral side to expose the injection site (as marked) on the lateral tail. Check to see if a NIRF signal is present only at the site of injection as this is the most optimal injection, indicating a successful tail vein injection (**Figure 1D**).

NOTE: If the signal is sparse but still within the proximal vicinity of the tail vein injection, the injection is acceptable and can be considered as a successful tail vein injection. If the signal is dispersed throughout the entire tail, it is considered to be subcutaneous and, thus, unsuccessful (**Figure 1F**). **Figure 2** shows additional examples of failed injections.

5. Image quantification

NOTE: Image quantification can be performed in the imaging software that accompanies the NIR imager, if this is a function of the software. Alternatively, other commercially available imaging software may be used[25].

5.1. In the postinjection image, draw an ROI around the area of fluorescence at the injection site and clone it in all animals in order to compare[2,6]. Perform a simultaneous image acquisition of white light (body view) and 785 nm excitation for 820 nm emission using the NIRF imager and associated software, with linked lookup tables (LUT). Perform a one-way analysis of variance (ANOVA; see **Table of Materials**) as a statistical analysis for the entire set of conditions revealing a treatment effect with a statistically significant *p*-value of 0.0024.

5.2. Measure the area and relative fluorescence intensity and record the measurement of the area/intensity.

NOTE: The researcher can decide on thresholds that discriminate good from bad injections or assign a percentage of quality to the injection.

REPRESENTATIVE RESULTS:

Rats were injected with NIRF-containing nanoemulsion into the lateral tail vein, and pre- and postinjection images were taken with the small-animal imager (**Table of Materials**) as described in the protocol. Postinjection images are qualitatively assessed for injection quality and placed into 'good injection' (n = 7) and 'bad injection' (n = 4) groups. Qualitative assessment was carried out by observing the postinjection area fluorescence intensity. In an optimal injection, the NIRF signal is confined to the site of injection. No signal will be seen if the injection is successful because the agent has been fully displaced into the bloodstream. A bad-quality injection displays a NIRF signal that is dispersed along the length of the tail.

Images were analyzed with the accompanying NIRF imager software. ROIs were drawn at the site of preinjection images (**Figure 1C, E**) and around the area of fluorescence in postinjection images (**Figure 1D, F**). Images where fluorescence was visible throughout the length of the tail were deemed unacceptable and removed from the analysis (**Figure 2**). Measurements of the area and fluorescence intensity were recorded. Values for area/fluorescence intensity were calculated and plotted (**Figure 1G**). A significant difference in fluorescence intensity between pre- and postinjection images was observed in the 'bad injection' group (**Figure 1G**) (p = 0.0024).

FIGURE AND TABLE LEGENDS:

Figure 1: NIRF based nanoemulsion and images of tail vein. (**A**) A nanoemulsion-based biological probe containing NIRF dye was injected into (**B**) the lateral tail vein and imaged in a NIRF imager. (**C** and **D**) Pre- and postinjection images of a good injection. (**E** and **F**) Pre- and postinjection images of a bad injection. White arrows indicate the point of injection. It is possible to qualitatively assess the success of a good injection compared to a bad injection by assessing the extent of the NIRF signal at the site of injection. Unacceptable injections display fluorescence throughout the length of the tail and were removed from the analysis (**Figure 2**). (**G**) The images can also be analyzed to reveal a quantitative measure of fluorescence intensity, with thresholds for injection quality assigned by the investigator. The error bars on the graph reflect the SEM. For the 'good injection' group, n = 7. For the 'bad injection' group, n = 4. There is a statistical difference in fluorescence intensity in the 'bad injection' group when comparing pre- and postinjection images (unpaired t-test; p = 0.0024).

Figure 2: Examples of bad injections. (**A**) Fluorescent signal seen in part of the tail. (**B**) Fluorescent signal seen over the full length of the tail. (**C**) Fluorescent signal dispersed heavily in the entire tail and caudal area of the animal's body.

DISCUSSION:

Research laboratories incur significant costs as a result of the misadministration of testing agents. Tail vein injections are a difficult technique to master to attain consistent success rate, with the most experienced of technologists often incurring misadministration errors. There is no reliable way to confirm a successful injection. This protocol offers a solution to this problem by giving researchers a qualitative and quantitative method to validate the success of a murine tail vein injection. Here, a NIRF-labelled nanoemulsion[7,8,26] incorporates the agent of choice (in this case, a drug) and is imaged at the site of injection in a NIRF small-animal imager. There is also the option to develop a non-nanoemulsion-based agent and use the same principle of NIRF imaging by incorporating commercially available infra-red dyes. Additionally, ready-to-use imaging agents with a variety of applications, such as tumor imaging, metabolic imaging, cell trafficking, and apoptosis are also commercially available. An injection is performed either by using a sterile needle or, alternatively, an IV catheter; this depends on the preference of the researcher. In addition, automated tail vein injectors[27] have been used to assist in this process and are compatible with this methodology. However, this technology has not yet become commercially available.

There are important steps in the tail vein injection method that ensure a higher rate of correct agent administration. First, the tail should be cleaned with ethanol to remove any dirt or debris, allowing researchers to better visualize the vein. Dilating the vein by submerging the tail in warm water is also a very important step in the method, as it allows a greater surface area for injection. Injecting at a more distal point on the tail vein allows for some error, in the event that multiple attempts are required. Injection should be attempted at a more proximal position in the tail as the tail vein increases in size as the caudal aspect of the animal's body is approached. In addition, the contralateral tail vein can be used if needle placement fails in more than three to five sites on the ipsilateral tail vein.

A successful administration of a test agent results in little to no NIRF signal at the point of injection. If no resistance is felt during the administration of the injection and there is little to no fluorescence at the tail, then the injection can be recorded as successful. If resistance is felt during injection and there is a trail of NIRF signal along some length of the tail, then the injection is recorded as unsuccessful and is likely partly subcutaneous. Fluorescence images are taken pre- and postinjection, and the quality of the injection is assessed by observing qualitatively or analyzing quantitatively the fluorescence signal at the site of injection. The software accompanying the NIR fluorescence imager is often capable of performing this analysis.

The method can be adapted in several ways. It is applicable to tail vein injection in both mice and rats. Most small-animal NIR fluorescence imagers will be capable of accommodating murine rodents. Levels of anesthesia need to be adjusted depending on the weight of the animal, in accordance with the research laboratory's IACUC protocol. Another possible modification is the preparation of a non-nanoemulsion-based probe either by incorporating an infrared dye into the researcher's formulated agent or by purchasing a ready-to-use imaging agent, tailored to a specific biological application.

If a rat is relatively large, it can often be difficult to position it in the small-animal imager. It is thus recommended that a test image is taken with the animal in the drawer before injecting, and a field of view ascertained where the tail is visible. It is helpful to tape the tail to the drawer of the imager, to ensure it does not move during imaging.

Alternative methods seeking to assess the quality of tail vein injections in small animals are limited to the utilization of labeling reagents that do not interfere with concurrent experimental procedures and require euthanasia of the animals postinjection[13,14]. Some reagents may impact study outcomes and the therapeutic assessment of the animals involved, so care in experimental design is recommended.

This method can, in the future, be refined with advances in small-animal imaging technology, as well as improvements in infrared fluorescent probes. Biological probes with an incorporated infrared dye, designed for a variety of different applications, can be used at the agent administration stage of a study design to validate the quality of an injection, as outlined in this method[10,3,28,29,30,31,32,33].

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J.A.P. and J.M.J. jointly designed the experimental approach for evaluating Nanoemulsions in the Chronic Constriction Injury rat model for effects on neuropathic pain. J.M.J. conceived and designed the overall macrophage-targeted drug delivery approach with nanoemulsions, the nanoemulsion composition, and processes for fabrication. J.M.J. produced the nanoemulsion, which was further fabricated by L.L. under the guidance of J.M.J. The stability of the nanoemulsion was assessed by J.M.J., L.L., and S.P. Animal care, surgery, behavior, tail vein injections, and NIRF imaging were carried out jointly by M.S. and A.M.S. under the guidance of J.A.P. The manuscript was written and prepared by M.S., and the protocol was written by A.M.S.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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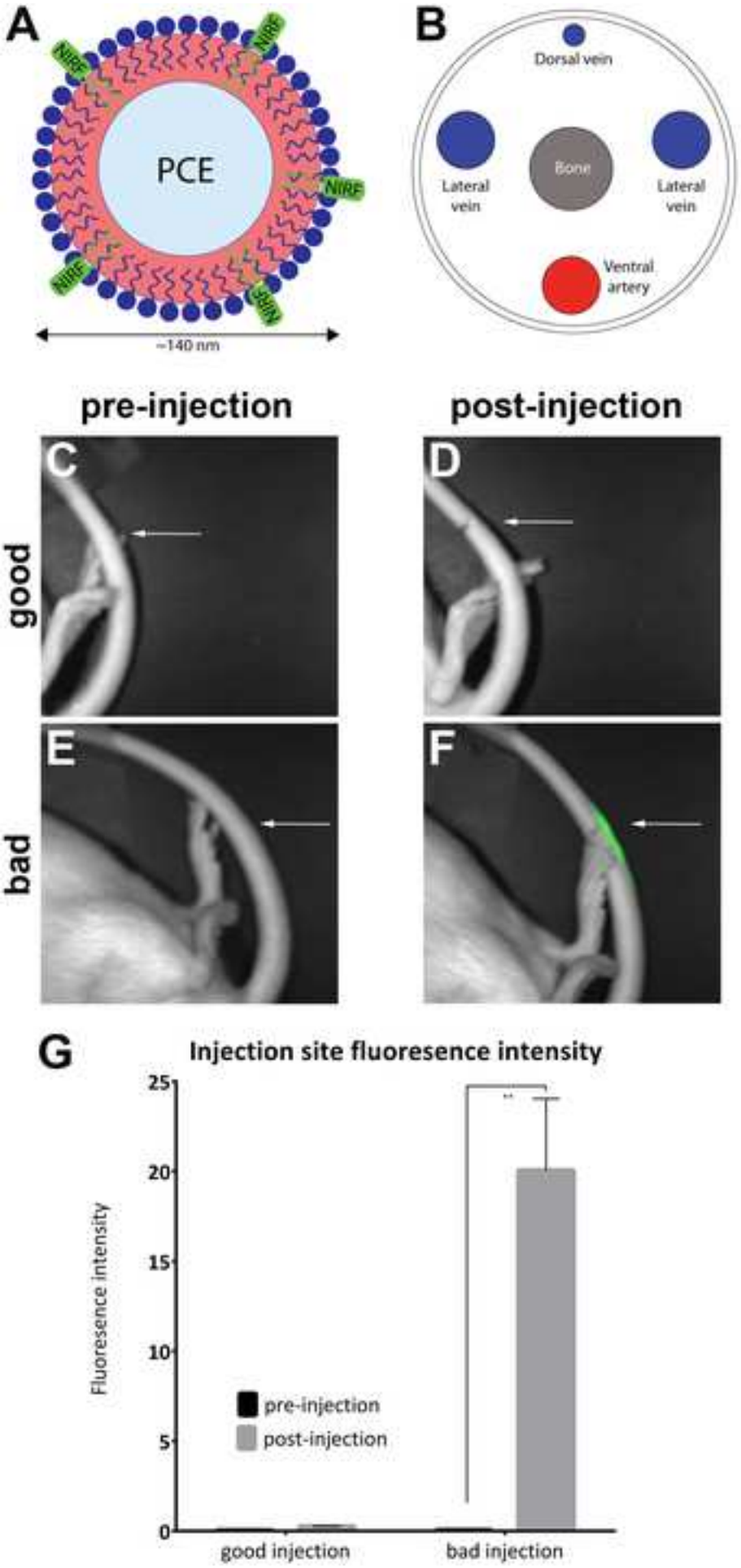

Figure

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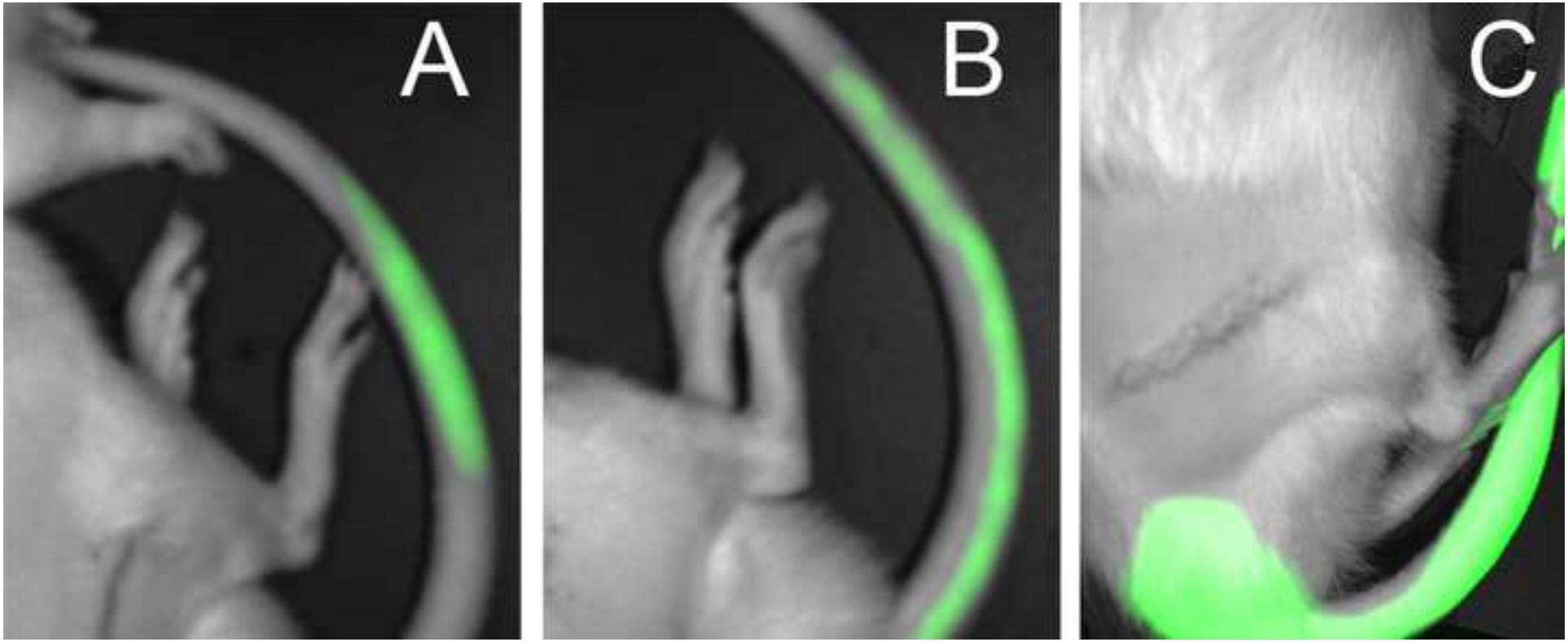

Table of Materials

Click here to access/download;Table of Materials;table of materials (2).xls

Name	Company	Catalog Number
100% Oxygen air tank	AirGas Heathcare	n/a
70% Ethanol	Multiple sources	n/a
Alcohol Pads	Henry Schein	112-6131
Artificial Tears	Henry Schein	100-2634
Beaker	Multiple sources	n/a
Distilled water	Multiple sources	n/a
Exhaust Fans	Hazard Technologies	n/a
Face Mask	Multiple sources	n/a
Gas Chamber with tubing and face mask	Multiple sources	n/a
Gauze Pads	Henry Schein	100-2634
Hair Bonnet	Multiple sources	n/a
Heating Lamp	Multiple sources	n/a
Heating Pad	Multiple sources	n/a
Isoflurane	Southmedic Inc.	ND66794-013-25
Padded Bench Cloth	Box Board Products Inc.	026755100I
Pearl Small Animal Imager	Li-COR Biosciences	
Pearl Trilogy Small Animal Imaging System	LI-COR Biosciences	n/a
Scrubs, lab coat, shoe covers	Multiple sources	n/a
Sharps container	Multiple sources	n/a
special diet	Research Diets, Inc, New Brunswick, NJ	
Sprague-Dawley rats	Hilltop Animals, Springdale, PA	
Sterile injection cap	Multiple sources	n/a
Sterile needle, 27G	Multiple sources	n/a
SURFLO IV Catheter, 24G, yellow	TERUMO	SR+OX2419C1
Surgical gloves	Multiple sources	n/a
Surgical Tape	Multiple sources	n/a

Comments
For ventilation of animal.

This protects the rats eyes while it is anesthetized.
This holds warm water to dilate the tail veins.

For ventilation of lab, if it is not built in.

Quote available via manufacturers web site. Other manufacturers such as Perkin Elmer (VisEn Medical I

This is an alternative to using a sterile needle. It provides additional indication of correct venous inserti

FMT) offer preclinical NIR fluoresence imagers.

on.

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ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Validating tail vein injections in rat with near infrared labelled agents; a new best practice

Author(s): Andrea M Stevens*, Muzamil Saleem*, Lu Liu, Jelena M Janjic, John A Pollock
*equal contribution

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CORRESPONDING AUTHOR:

Name:	John A Pollock
Department:	Biological Sciences
Institution:	Duquesne University
Article Title:	Validating tail vein injections in rat with near infrared labelled agents; a new best practice
Signature:	 Date: 10/24/2018

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December 20, 2018

Dear Dr. Vineeta Bajaj,

Thank you for your email of December 13, 2018 that requested corrections and revisions to our manuscript JoVE59295 "Validating tail vein injections in rat with near infrared labelled agents; a new best practice."

We have made all the requested corrections, which I will list below. Also, Figures 1 and 2 are provided as .tif (flattened) at 300 dpi. If you need, I will be happy to provide the original assembly of the figures as layered .psd.

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Authors Response: All authors have again proofed the manuscript.

2. Please note that Standard Access is checked in the uploaded ALA, while in the Questionnaire Responses Open Access is selected. Please be consistent.

Authors Response: We will be open access and will make the documentation consistent.

3. Please revise the title to avoid the use of semicolon.

Authors Response: The title has been revised to "A new best practice for validating tail vein injections in rat with near infrared labelled agents."

4. Abstract: Please do not include references here.

Authors Response: References have been removed from the abstract section and the text has been revised

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to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: LI-COR Pearl®, LI-COR Biosciences, Caliper, Xenogen IVIS, Perkin Elmer, VisEn Medical FMT, D100012G Research Diet, Inc., BrightSite™ IRDye®, SAIVI™, Hilltop Animals, SURFLO®, etc.

Authors Response: Commercial language has been excluded from the manuscript; in it's place, generic terms such as "small animal imager" and "see table of materials" have been used.

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

Authors Response: All personal pronouns have been removed from the manuscript.

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Authors Response:

- The Protocol contains only action items that direct the reader to do some specific action. These actions are described in the imperative tense in complete sentences. Any text this is not imperative has been added as a "Note." Notes have been used sparingly.
- Phrases such as "could be," "should be," and "would be," have been removed.
- All safety procedures have been included.
- Discussion about the protocol has been moved to the Discussion.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

Authors Response: The Protocol has been simplified to contain only 2-3 actions per step with a maximum of 4 sentences per step. Sub-steps have been used when necessary.

9. 1.3: Please mention how proper anesthetization is confirmed.

Authors Response: The text has been edited to indicate that proper anesthetization is confirmed via tail pinch.

10. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be

visualized to tell the most cohesive story of the Protocol.

Authors Response: Aspects of the Protocol (including headings and spacing) have been highlighting in order to identify essential steps for video production.

11. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Authors Response: Highlighted regions of the protocol are complete sentences, and include at least one action that is written in imperative tense. As requested, no steps describing anesthetization and euthanasia are included.

12. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Authors Response: All relevant sub-steps are included in highlighted sections where they are required for video production.

13. Discussion: Please discuss critical steps within the protocol.

Authors Response: The Discussion section has been edited to provide more depth on critical steps in the Protocol.

14. Please remain neutral in tone when discussing commercial products. The accompanying video cannot become an advertisement.

Authors Response: We have adjusted the text to remain neutral when discussing commercial products; all commercial products will be addressed with a generic term to avoid any sense of promotion.

15. References: Please do not abbreviate journal titles.

Authors Response: The reference list has been edited to included full journal titles.

16. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Authors Response: As requested, items have been sorted in alphabetic order according to the name of the material/equipment.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript is very useful and will help to avoid common and widespread problems with tail vein injections. A few points might be addressed and added to strengthen the manuscript.

Authors Response: We appreciate the positive comments from this reviewer.

Major Concerns:
None

Minor Concerns:

1. Please explain why tail injection is a preferred way for the delivery of the probes. Are there any other points of entries can be used?

Authors Response: There are other point of entry for venous injection, which we describe in the opening of the introduction.

2. What is the typical time when the mouse or rat with a subcutaneous tail injection can be reused for imaging?

Authors Response: This question goes beyond the scope of this work and will not be discussed in the manuscript. There are a number of variables that can influence this, which would be contingent on the nature and design of an individual experiment. That being said, in the case of a successful injection, the animal could likely be re-injected (in the contralateral vein) within hours. An unsuccessful injection that leave a subcutaneous bolus should be removed from the study and should not be re-used.

3. Some companies offer devices to facilitate tail vein injections, i.e Rotating Tail Injector: https://www.braintreesci.com/prodinfo.asp?number=RTI, or Tailveiner: https://www.medilumine.com/mouse-and-rat-restrainers-for-tail-vein-injections/ . Could you comment on these?

Authors Response:

4. There are also publications of using automatic tail vein injectors, not sure if there are commercially available. This information might also be added to the discussion

Authors Response: Automatic tail vein injectors have been commented on in the discussion section.

Reviewer #2:

Manuscript Summary:
Optimizing tail vein injections is very important for many experimental procedures. In this study, the authors describe a method of tail vein injections in rat with near infrared labelled agents. And they summarized the detailed procedures and notes in every step of tail vein

injections. It is helpful for the researchers to deal with similar experimental procedures.

Authors Response: The authors appreciate these supportive observations.

Major Concerns:
Just minor concerns, that is, in the discussion section, the author should give more discussion about their improvements and notes about experimental procedures rather than statements of experiment.

Authors Response: The authors have added to the discussion commentary on how this method offers improvement.

Some more papers can be referenced for improving, for examples,
Fluorescent chemical probes for accurate tumor ……, CHEMICAL SOCIETY REVIEWS, 2017, Volume: 46, Issue: 8, Pages: 2237-2271;
Ratiometric Near-Infrared Fluorescent Probe for ……, ANALYTICAL CHEMISTRY, 2018, Volume: 90, Issue: 6, Pages: 4054-4061;
A Ratiometric Near-Infrared Fluorescent Probe for ……, ADVANCED FUNCTIONAL MATERIALS, 2017, Volume: 27, Issue: 28, Article Number: UNSP 1700769

Authors Response: We appreciate these recommendations and have read these papers and find them useful and appropriate; and they are now cited in the discussion.

Sincerely,

John A. Pollock, Ph.D.
Professor of Biological Sciences
Director of the Partnership in Education
Co-Director of the Chronic Pain Research Consortium
Presidential Award for Excellence in Science, Mathematics and Engineering Mentoring (White House OSTP/NSF)
AAAS Fellow

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