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Intravenous and Intra-amniotic in Utero Transplantation in the Murine Model

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Dear Editor,

Please find enclosed our manuscript entitled *"Intravenous and Intra-amniotic in Utero Transplantation in the Murine Model"* that we would like to be considered for publication in Journal of Visualized Experiments. This paper describes a detailed protocol for performing in utero transplantation in the murine fetus through intravenous and intra-amniotic routes. We consider of value publishing these data in Journal of Visualized Experiments, as they describe a unique *in vivo* approach to studying stem cell biology, developmental immunology, tolerance induction, and prenatal gene therapy/genome editing. The techniques presented in this paper and demonstrated in video format will be highly useful for researchers working to test new ideas and develop new therapies for devastating congenital genetic, hematologic, immune and metabolic disorders.

John Stratigis and Nicholas Ahn performed the experiments, analyzed the data, and wrote the manuscript. During the preparation and submission of this manuscript, Indrani Mukherjee has kindly assisted us.

Thank you for your consideration of this manuscript. We look forward to hearing from you.

Sincerely,

William H. Peranteau, MD

TITLE:

Intravenous and Intra-amniotic *In Utero* Transplantation in the Murine Model

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KEYWORDS:

In utero transplantation, murine, vitelline vein, intravenous, intra-amniotic, injections

SHORT ABSTRACT:

We describe a protocol for performing an *in utero* transplantation (IUT) through intravenous and intra-amniotic routes of injection in the murine model. This protocol can be used to introduce cells, viral vectors, and other substances into the unique immune-tolerant fetal environment.

LONG ABSTRACT:

In utero transplantation (IUT) is a unique and versatile mode of therapy that can be used to introduce stem cells, viral vectors, or any other substances early in the gestation. The rationale behind IUT for therapeutic purposes is based on the small size of the fetus, the fetal immunologic immaturity, the accessibility and proliferative nature of the fetal stem or progenitor cells, and the potential to treat a disease or the onset of symptoms prior to birth. Taking advantage of these normal developmental properties of the fetus, the delivery of hematopoietic stem cells (HSC) *via* an IUT has the potential to treat congenital hematologic disorders such as sickle cell disease, without the required myeloablative or immunosuppressive conditioning required for postnatal HSC transplants. Similarly, the accessibility of progenitor cells in multiple organs during development potentially allows for a more efficient targeting of stem/progenitor cells following an IUT of viral vectors for gene therapy or genome editing. Additionally, IUT can be used to study

normal developmental processes including, but not limited to, the development of immunologic tolerance. The murine model provides a valuable and affordable means to understanding the potential and limitations of IUT prior to pre-clinical large animal studies and an eventual clinical application. Here, we describe a protocol for performing an IUT in the murine fetus through intravenous and intra-amniotic routes. This protocol has been used successfully to elucidate the necessary conditions and mechanisms behind *in utero* hematopoietic stem cell transplantation, tolerance induction, and *in utero* gene therapy.

INTRODUCTION:

Recent advances in antenatal screening and diagnosis have brought to light the possibility of treating the fetus for a number of congenital disorders which do not have adequate postnatal treatment options and result in significant morbidity and mortality. Specifically, *in utero* hematopoietic stem cell transplantation (IUHCT) and gene therapy/genome editing have the potential to take advantage of normal developmental properties of the fetus to treat congenital hematologic, immune, and genetic disorders more efficiently than postnatal HSC transplantation and gene therapy/genome editing can do[1,2]. Specifically, due to the small size of the fetus, the donor cell or viral vector dose can be maximized per the weight of the recipient. Additionally, the immunologic immaturity of the fetus allows donor HSCs to be injected without the myeloablative and immunosuppressive conditioning that is required in postnatal transplant protocols. Similarly, viral vectors carrying a therapeutic transgene or genome editing technology can be injected without a limiting immune response to either the transgene product or the viral vector. Finally, the accessibility and proliferative nature of fetal stem/progenitor cells afford the possibility of a more efficient transduction of target progenitor cells, as well as certain modes of genome editing (homology-directed repair) which require cycling cells to occur efficiently. The murine model serves as an insightful and affordable means to address important questions in stem cell biology and immunology prior to experimenting in pre-clinical large animal models and, as such, has served as the primary model in which IUHCT and *in utero* gene therapy have been explored [1-3].

Although many variables play an important role in the success of IUHCT and *in utero* gene therapy/genome editing in murine and large animal models, a key variable is the method of delivery of the HSCs or viral vector. The delivery of large doses of donor HSCs with a first-pass effect occurring in the fetal liver, the hematopoietic organ at the time of the IUHCT, has been shown to be instrumental in achieving macrochimeric levels of engraftment in mouse and large animal models[4,5]. This was achieved *via* an injection of donor cells *via* the vitelline vein in the mouse model and *via* an intra-cardiac injection in the canine model. The route of injection also plays a fundamental role in targeting progenitor cells of different organs during development. For example, an intravenous injection *via* the vitelline vein has been shown to efficiently transduce cardiomyocytes and hepatocytes following a late gestation injection[6,7]. Alternatively, an intra-amniotic injection of viral vectors allows the targeting of organs that are physically exposed based on the embryonic folding/development at the time of the injection[8]. This is best exemplified by the targeting of respiratory epithelium *via* an intra-amniotic injection late in the gestation to take advantage of normal fetal “breathing” movements, which exposes the respiratory tract to the viral vector in the amniotic fluid[9]. These two modes of IUT, intravenous *via* the vitelline vein and intra-amniotic, have been the basis for multiple past and ongoing

experiments in our laboratory. In this protocol, we describe in detail the methods for performing intravenous and intra-amniotic IUT in the murine model.

PROTOCOL:

The experimental protocols were approved by the Institutional Animal Care and Use Committee at The Children's Hospital of Philadelphia.

1. Creation of Injection Pipettes

1.1. Using a vertical micropipette puller, pull a 100 µL microcapillary pipette (**Figures 1A** - **1C**). Calibrate the micropipette puller so that the tapered end is > 1 cm long.

Note: Initially, the settings of the puller should be adjusted for an optimum length. A higher heat setting will make the tip longer, and a higher pull setting will make the diameter of the tip narrower.

1.2. Cut the tapered end so that it is ≥ 1 cm long. Ensure that the internal diameter at the tip of the needle is between 70 µm and 100 µm and that it is inversely proportional to the length of the tapered end.

Note: The internal diameter also depends on the calibration of the micropipette puller. See the instructions from the manufacturer of the vertical micropipette puller or any preferred micropipette puller type.

1.3. After ensuring that the needle has the correct internal diameter, create the bevel of 15 - 20 degrees by sharpening the tip using a micropipette beveller with a diamond sharpening wheel (**Figures 2A** - **2C**). Make sure to rest the tip gently on the wheel without too much pressure, to decrease chances of breaking or cracking the tip.

Note: A paintbrush can be used to wipe away any debris that builds up on the needle tip.

1.4. Evaluate the tip under a microscope and ensure that the tip is round without any chips or cracks. Reevaluate the internal diameter to make sure it is between 70 µm and 100 µm (**Figures 2D** - **2H**).

1.5. Draw lines on the rest of the needle to designate 5 µL of volume between them (*e.g.*, needles with an internal diameter of 1.3 mm should have lines drawn at 3.77 mm increments).

1.6. Place the needles under UV light for at least 1 h for sterilization.

2. *In utero* Injections

2.1. Prepare the necessary instruments by autoclaving them ahead of time. Include essential instruments such as microinjector needle holder, a surgical needle driver, a pair of Adson forceps,

a pair of curved regular tissue scissors, a 1 mL insulin syringe, a couple of cotton-tip applicators, a transfer pipette, a 50 mL conical tube, and a pack of 4-0 polyglactin 910 sutures.

2.2. Using a sterile technique, attach the needle to the needle holder and plug it into the microinjector.

Note: The settings of the compressed nitrogen used are as followed: inject 4 - 6 psi, balance 0 psi. Depending on what is being injected, specifically, the viscosity of the injectate, as well as the size of the micropipette, the injection times vary between 0.3 - 1.5 s.

2.3. Clean out the needle tip of any possible debris by drawing up 5 - 10 µL of sterile 1x phosphate-buffered saline (PBS) and then clearing it out. Repeat this 2 - 3x.

2.4. Prepare pregnant 2- to 6-month-old female mice for surgery by shaving their abdomens with a clipper. Be careful not to damage the nipples. If needed, administer oral pain medication (*e.g.*, 100 µL of 1.5 mg/mL meloxicam oral suspension per mouse).

2.5. Start filling the needle with the desired material (cells/vector/drug) at the desired volume. Be careful not to break the needle tip while filling the needle.

Note: The volume of injection per fetus varies depending on the specific experimental design. 20 µL works well for injecting a large number of cells (*i.e.*, up to 10^7 cells into the vitelline vein. For instance, we delivered 1 x 10^7 whole bone marrow cells isolated from C57BL/6TgN(act-EGFP)OsbY01 ["B6 Green Fluorescent Protein (GFP)"] mice *via* the vitelline vein into gestational day-14 Balb/c fetuses. For viral vector injections, a single injection of 10 µL of a 1:1 diluted vector with PBS works well.

2.6. To calibrate the injection time, take the following steps.

2.6.1. Push the **Mode** button 3x on the microinjector to get to the injection calibration screen. Adjust the injection time by adding intervals of 10 or 100 ms and push the **Mode** button 2x.

2.6.2. Push the **Balance** button and push the foot pedal once. Now push the pedal again and assess how much volume is emptied out of the needle per push. If not calibrated to the desired volume of 5 - 20 µL per pedal push, repeat steps 2.6.1 and 2.6.2.

Note: Generally, it is good to calibrate each push to deliver half the total target volume at a time. While it is possible to inject more than 30 µL or even 40 µL of the total volume, we do not generally go over 20 µL per fetus, intravenous or intra-amniotic.

2.7. Fill the needle up to the desired level.

2.8. Start delivering anesthesia to the mouse by adjusting the oxygen flowmeter to 1 L/min and the isoflurane vaporizer to 3%.

2.9. Confirm whether the mouse is anesthetized by checking for the absence of the pedal reflex. Transfer the mouse to a heating pad in a supine position.

2.10. Apply lubricant eye gel to avoid corneal desiccation. Secure the mouse in place by taping the upper and lower limbs to the pad.

2.11. Prep the abdomen with chlorhexidine or alcohol wipes and inject a local anesthetic (*e.g.*, 100 µL of 0.25% bupivacaine) subcutaneously (**Figure 3A**).

2.12. With scissors, make a 1 - 2 cm skin incision so that the lower border is no closer than 1 cm to the introitus; the fascia beneath is very thin and translucent.

2.13. Identify the midline of the fascia which is more transparent than the surrounding area. Be careful not to injure the epigastric vessels which lie on either side of the midline. Should the epigastric vessels get injured, hold pressure with cotton-tip applicators to stop the bleeding.

2.14. Using Adson forceps, pinch the fascia without grabbing any of the underlying organs such as the intestines, bladder, or fetuses. Open the fascia with scissors, being careful not to damage any of the organs. Once safely in the abdomen, extend the fascial incision. Make it no longer than the skin incision.

2.15. Use cotton-tip applicators to move the intestines into the upper part of the abdomen, thus exposing the gravid uterus. Deliver the uterus out of the incision, carefully identifying the right and left ovaries to ensure all fetuses are counted (**Figure 3B**).

2.16. Place the left uterus back into the abdomen so that only the right uterus is exposed; this prevents the desiccation of the uterus and keeps the non-injected fetuses warm.

2.17. Hold the most lateral fetus/amniotic sac between the thumb and index fingers of the operator's non-dominant hand (**Figure 3C**). Always be gentle as to not cause any damage to the fetuses.

2.18. Position the dissection microscope (a 10X magnification is ideal) and adjust the focus so that the fetus is in view. Adjust the lighting for a better visualization.

2.19. Identify the part that will be injected (vitelline vein, amnion). For intravenous injections, visualize both the vitelline veins and their anastomosis first. For intra-amniotic injections, orient the fetus with the right side in view.

2.20. Reach the target space with the needle as described below.

2.20.1. For an **intravenous injection**, do as follows.

2.20.1.1. Rotate the uterus so that the vitelline vein that is being injected is parallel to the tip of the needle; keep in mind that the injections must be made towards the anastomosis of the two veins.

2.20.1.2. Lay the needle on the uterus at a 5° angle and pierce the uterine wall. Now that the tip is between the uterine wall and the amniotic sac, place the tip directly atop the vitelline vein.

2.20.1.3. At a nearly tangential angle, glide the needle over the vein until the bevel pierces and advances into the vessel; this is evident by a flash of blood seen in the needle tip (**Figure 3D**).

Note: Accessing the vein may take a few tries as the needle may not pierce the vein with the first glide over the vein.

2.20.2. For an **intra-amniotic injection**, do as follows.

2.20.2.1. Rotate the amniotic sac and find a location devoid of vessels to pierce.

2.20.2.2. Point the needle perpendicular to the uterine wall and pierce through the uterus, the yolk sac, and then the amniotic sac. Be careful not to pierce through any fetal tissue. Make sure the needle has passed between the limbs as this confirms that the needle is in the amniotic sac. Then proceed with the injection.

2.21. Inject the appropriate volume of material desired (usually 10 - 20 µL) by pushing the foot pedal.

Note: Because the injector must maintain the visualization of the needle tip through the microscope at all times, a second person must read the markings on the needle to quantify the injected amount and inform the injector how much volume is left to inject. A discussion prior to the injection between the injector and assistant is important to avoid any confusion and delay. This is especially important for intravenous injections because a delayed removal of the needle will allow a backflow of venous blood into the needle and result in an inaccurate dosing.

2.22. Withdraw the needle from the injection site once the desired volume is delivered. As there may be some bleeding from the vessel puncture site with intravenous injections, hold pressure with the side of the needle for 10 - 15 s to stop the bleeding.

2.23. Proceed to the next fetus. Continue until all the fetuses of the right uterine horn have been injected.

2.24. Remove the left uterine horn from the abdomen and replace the right uterine horn back inside the peritoneal cavity.

Note: Occasionally, the needle needs to be refilled with the injectant.

2.25. Once all fetuses have been injected, replace the uterus into the abdomen (**Figure 3E**). Make sure to avoid a uterine or intestinal volvulus.

2.26. With a disposable transfer pipette, place roughly 2 mL of 1x PBS into the abdomen to replace any insensible losses.

2.27. Close the fascia and abdomen in one continuous layer using 4-0 polyglactin 910 sutures to avoid injuring the underlying organs during the closure (**Figures 3F** - **3G**).

2.28. Remove the tape and transfer the mouse to a cage underneath a heat lamp. Be careful not to place the heat lamp too close to the mouse. Make sure the cage has bedding, food, and water.

Note: A thermostatically controlled warm chamber may also be used. The mouse is awake when it is upright and walking.

2.29. Observe the mouse daily and give pain medication as needed.

Note: We routinely give meloxicam on postoperative days 1 and 2, and sometimes on day 3 if the mouse shows signs of pain.

2.30. If doing a batch surgery with the same injection material, clean out the injection needle with sterile PBS. If injecting with a different material, dispose of the needle in a sharps container and use a new needle.

Note: We recommend fostering the pups with surrogate dams immediately after birth in case the dam mounts an immune response to the injectant and transfers antibodies to the pups *via* breast milk.

REPRESENTATIVE RESULTS:

Survival and engraftment are important measures of success for IUHCT experiments. Depending on the specific endpoints of an experiment, fetuses that received an IUHCT may be analyzed prenatally by a C-section or postnatally. On average, the survival rates after intravenous injections range from 75 - 100%. The survival rates after intra-amniotic injections tend to fair better than intravenous injections, at around 85 - 100%.

In our laboratory, the training process to attain proficiency in these techniques takes approximately 8 - 12 months. To assess the acquisition of the skills required to perform these injections in a reproducible fashion, trainees are monitored for fetal survival and donor cell engraftment at short time points following the IUT. This is demonstrated by the following quality control experiments. Specifically, 1 x 10^7 whole bone marrow cells are isolated from C57BL/6TgN(act-EGFP)OsbY01 ("B6 GFP") mice as previously described[5] and are injected *via* the vitelline vein into gestational day-14 Balb/c fetuses. In one group, the IUT was performed by an experienced instructor, and in the other group by a trainee who has been practicing the technique for ~ 4 months. Representative fluorescent microscopy images of the harvested fetal

livers 24 h after the IUT are shown in **Figure 4A**. The livers of the fetuses who received the IUT by the trainee fluoresce less due to a lower engraftment of the transplanted GFP cells. These livers were then analyzed for GFP^+ donor cells by flow cytometry to quantify the engraftment levels. The difference in mean engraftment levels between the two injectors correlates with the images seen under fluorescent microscopy (**Figure 4B**).

The ability of viral vectors delivered *via* the intra-amniotic route to transduce cells in contact with the amniotic fluid is exemplified by the transduction of epithelial cells 48 h following an intra-amniotic injection of Ad-GFP (an adenovirus vector carrying GFP transgene[10]) in gestational day-12.5 fetuses (**Figures 5A** and **5B**).

FIGURE LEGENDS:

Figure 1. The process of making a glass pipette needle with the micropipette puller. (**A**) Mount the glass pipette and secure it by tightening the dials on either end of the puller. (**B**) Once secured, the pulling switch activates the heat. The settings shown are Heat #1: 985 and Pull: 27. The dark cover glass should be closed during this process for safety. It was opened for photoshoot purposes. (**C**) The micropipette is separated. Discard the bottom portion and take the top portion of the separated micropipette for the next steps.

Figure 2. The process of grinding the micropipette to shape the tip of the needle. (**A**) This panel shows the general setup of the grinding. A light source is needed to visualize the tip under the microscope. (**B** and **C**) Mount the micropipette at a 15 - 20° angle. (**D**) A buildup of debris is expected throughout the grinding process. Use a paintbrush to clear the tip to get a better visualization of the grinding process. (**E**) A well-ground needle tip without any identifiable chips or jagged edges is shown under a 4X magnification. (**F**, **G**, and **H**) A re-inspection of the ground needle under a 10X magnification shows a well-ground needle with a sharp tip and smooth edges around the tip. Make sure to check the needle at different angles.

Figure 3. Laparotomy and *in utero* transplantation. (**A**) Shave, anesthetize, and tape a pregnant dam and prep her abdomen. (**B**) After the laparotomy, deliver the entirety of the uterus outside the abdomen for an identification of all fetuses. (**C**) Position the fetus with the surgeon's non-dominant index finger and thumb while maintaining tension with the third finger. Identify the tip of the needle under the microscope in relation to the fetus. (**D**) A flash of blood back-flowing into the micropipette needle must be seen upon the cannulation of the vitelline vein. (**E**) After a completion of all injections, place all the fetuses back into the abdomen. (**F**) Close the abdomen with a single-layer running stitch using 4-0 polygalactin 910 sutures. (**G**) Once the abdomen is fully closed, let the dam recover under a heat lamp.

Figure 4. Engraftment of donor whole bone marrow mononuclear cells after a vitelline vein injection. (**A**) The difference in the degree of engraftment with (trainee) and without (instructor) the leakage of cells is shown clearly under fluorescent microscopy. (**B**) Percent chimerism also reflected the same finding shown by the flow cytometry analysis. Each data point represents the

liver from a different injected fetus. The experiment was performed by one trainee and one instructor. The error bars represent a standard deviation (SD).

Figure 5. The expression pattern of green fluorescent protein in a fetal embryo 48 h after an intra-amniotic injection of Ad-GFP. (**A**) This panel shows the cornea (red arrow) and skin stained with GFP at E14.5 after an intra-amniotic injection of Ad-GFP at E12.5 (E12.5/E14.5). (**B**) A cryosection of the back of the embryo (indicated with a light blue box in panel **A**) at a higher magnification shows that the viral transduction is limited to the superficial peridermal cell layer (red arrows) and not epidermis (epi).

DISCUSSION:

In utero transplantation is a potential therapy for many congenital disorders that can be diagnosed early in gestation. The murine model for IUT allows researchers to explore the fetal environment or to experiment with different therapies. Depending on what is being injected and what is being targeted, intravenous or intra-amniotic *in utero* transplantation can provide a reliable delivery of an injectant into the desired space.

When targeting specific organs, it is important to pick the appropriate embryological age of the fetus as well as the injection technique. While the intravenous injection of cells at E14 is ideal for targeting the hematologic niche, and the intra-amniotic injection at E16 is ideal for lung targeting, these are not the only options available. For example, intra-amniotic injections can be performed for fetuses as early as E8 with ultrasound guidance[8]. Systemic delivery is also possible before E14 with ultrasound-guided intra-cardiac injections at E9 - E10[11]. The feasibility of performing injections at various stages of the fetus' development offers great potential for experiments investigating the safety and efficiency of gene transfers and cell transplants as well as investigating basic questions of developmental biology.

Furthermore, in addition to an intravenous and intra-amniotic delivery, other sites are also available for targeting depending on the purpose of the therapy or the scientific question being pursued. The *in utero* intramuscular approach has been used for a gene transfer for muscular dystrophies[12], an intraspinal approach for the transduction of spinal cord motor neurons[13], and an intracranial approach for gene transfers to target central nervous system diseases[14]. For *in utero* hematopoietic cell transplantation, intrahepatic and intraperitoneal routes are additional viable options as each route of delivery ultimately targets the hematopoietic niche[15]. However, the intravenous transplantation route allows for a more efficient homing of the donor cells into the hematopoietic niche and a larger dosing of the donor cells, thus resulting in overall higher levels of stable long-term donor cell engraftment without added fetal mortality[1].

The protocols we have detailed above for performing IUT are powerful and versatile tools that allow for a unique *in vivo* approach to studying stem cell biology, developmental immunology and immunologic tolerance induction, developmental biology, and prenatal gene therapy/genome editing. These delivery methods also have relevant clinical implications and have been the basis for studies of IUHCT and *in utero* gene therapy in pre-clinical large animal models such as the canine and the ovine model[4,16]. They will continue to serve as a valuable tool

to test new ideas in developmental biology and explore new therapies for devastating congenital genetic, hematologic, immune, and metabolic disorders.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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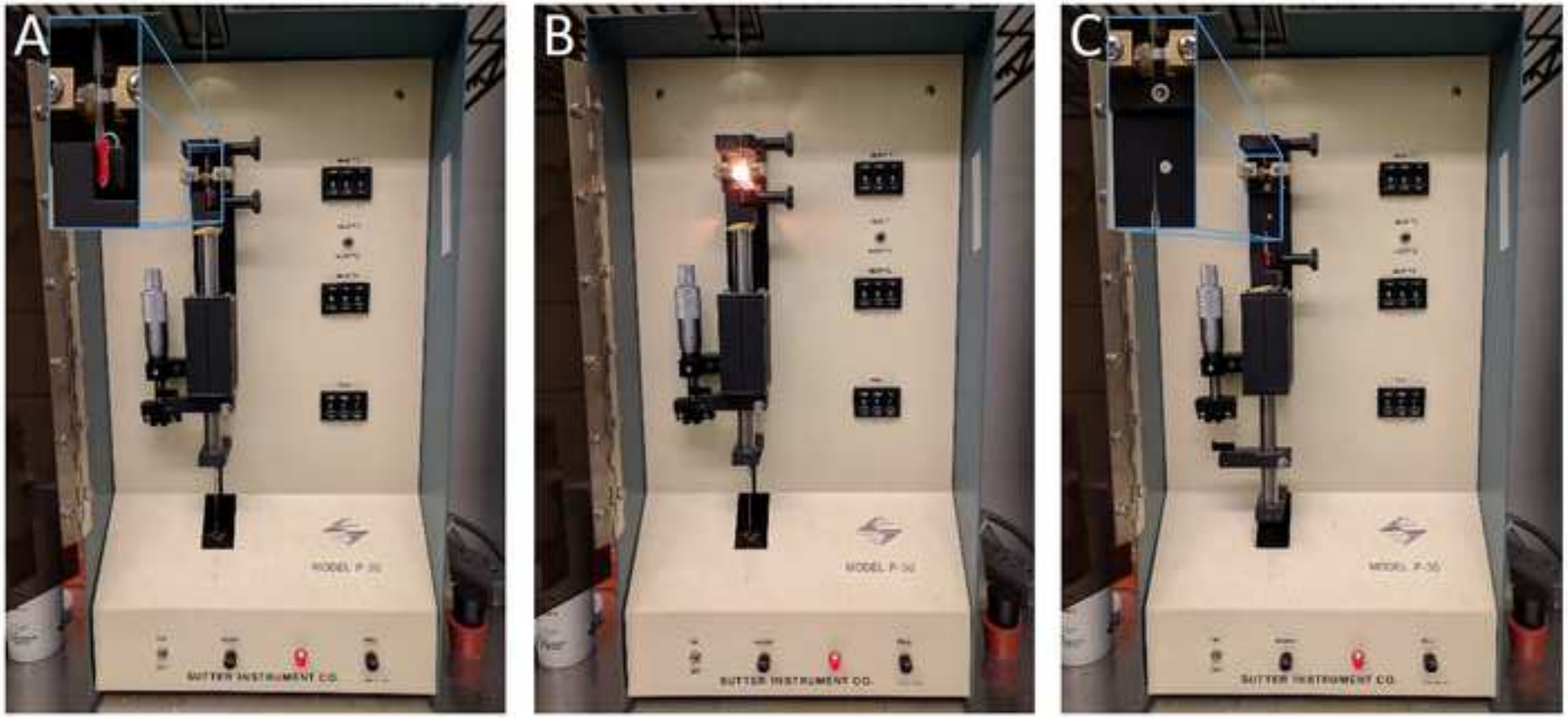

Figure 2

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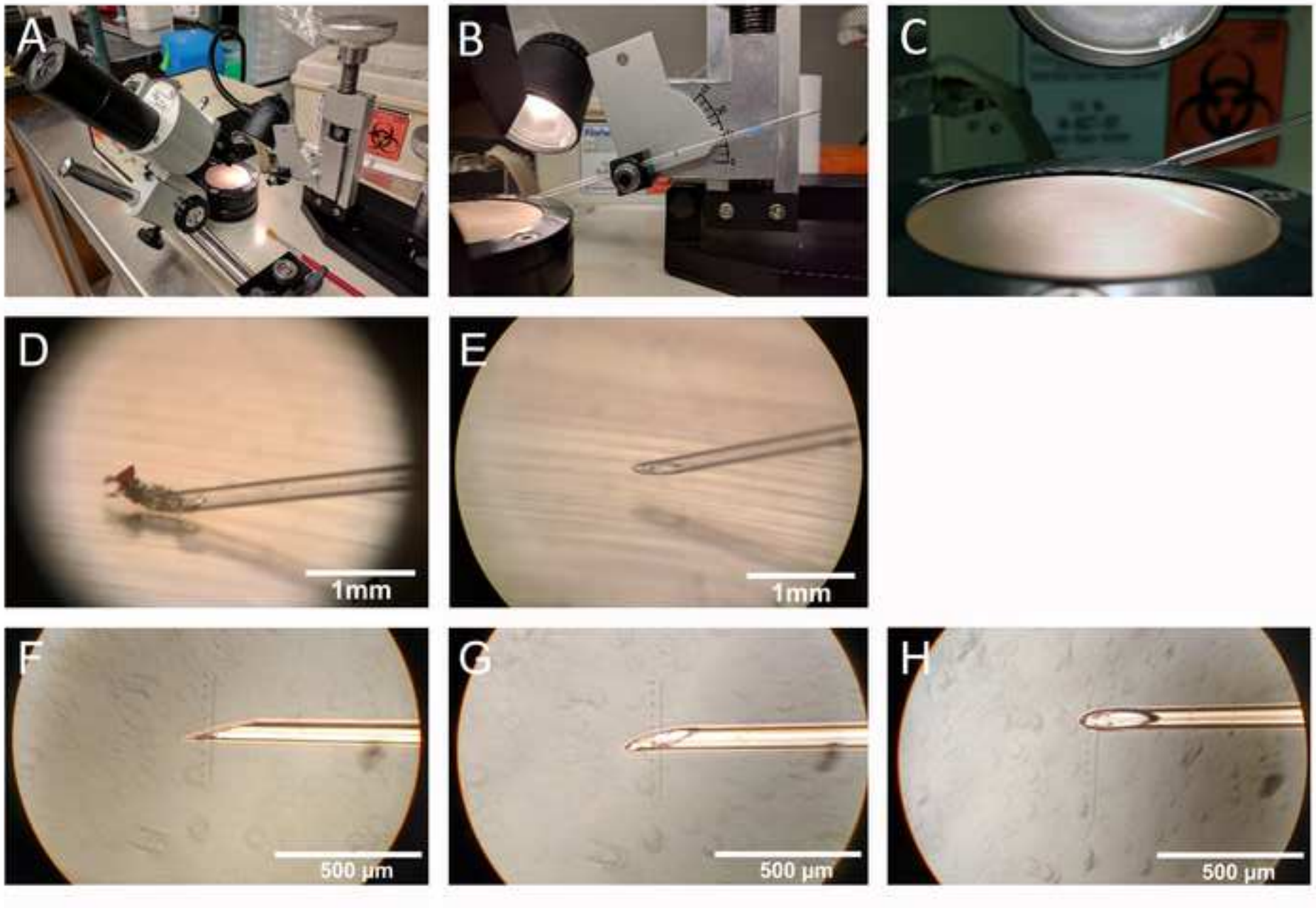

Figure 3

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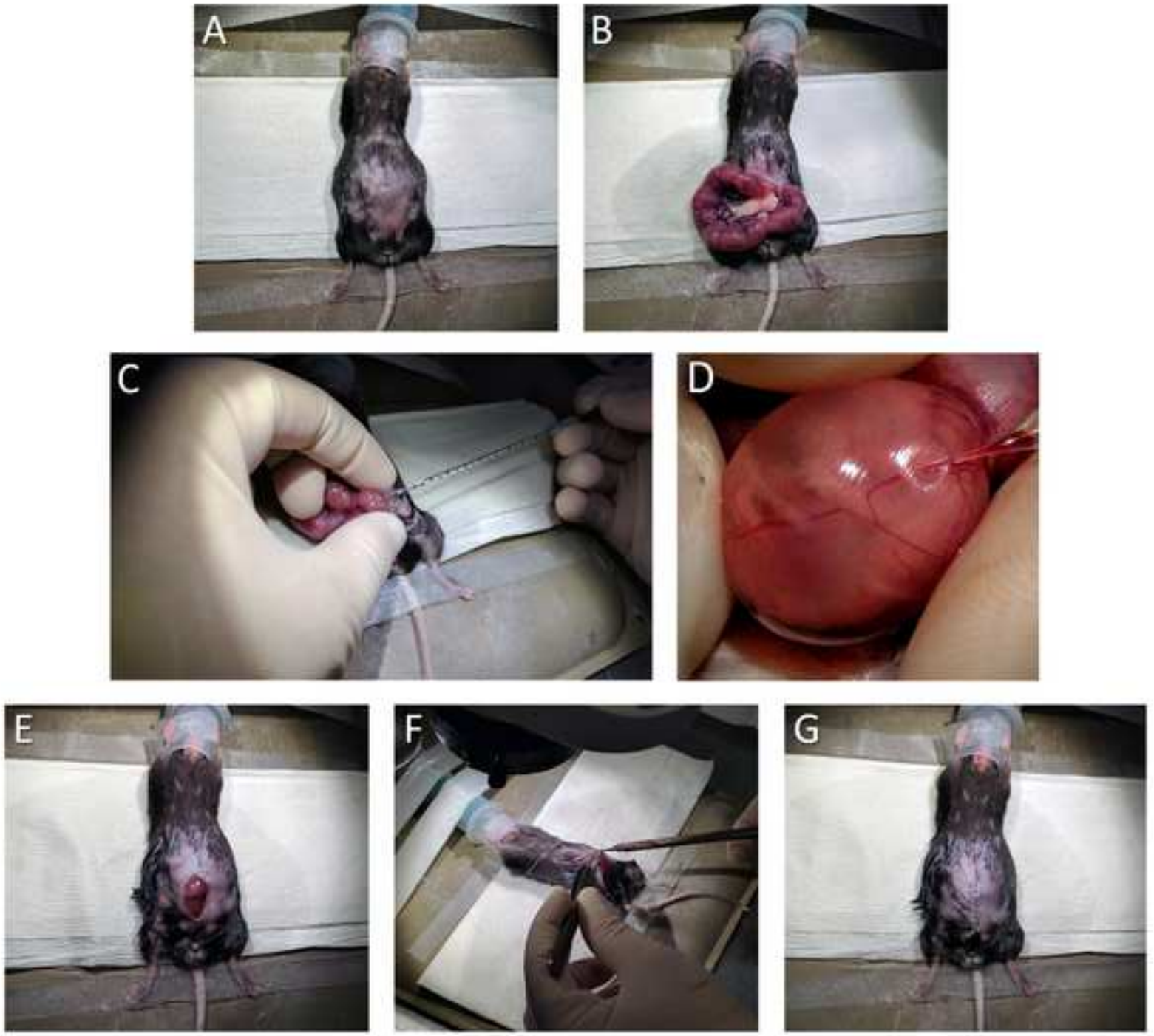

Figure 4

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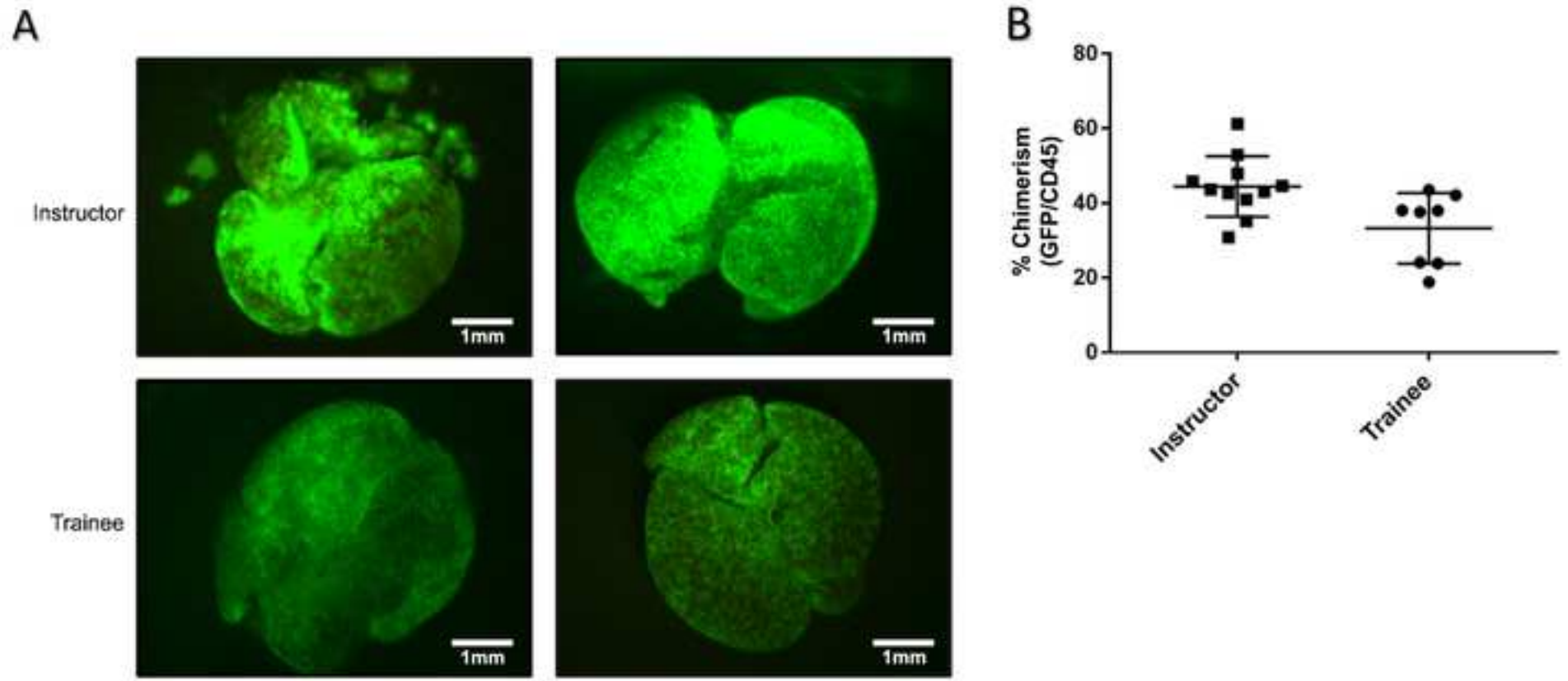

Figure 5

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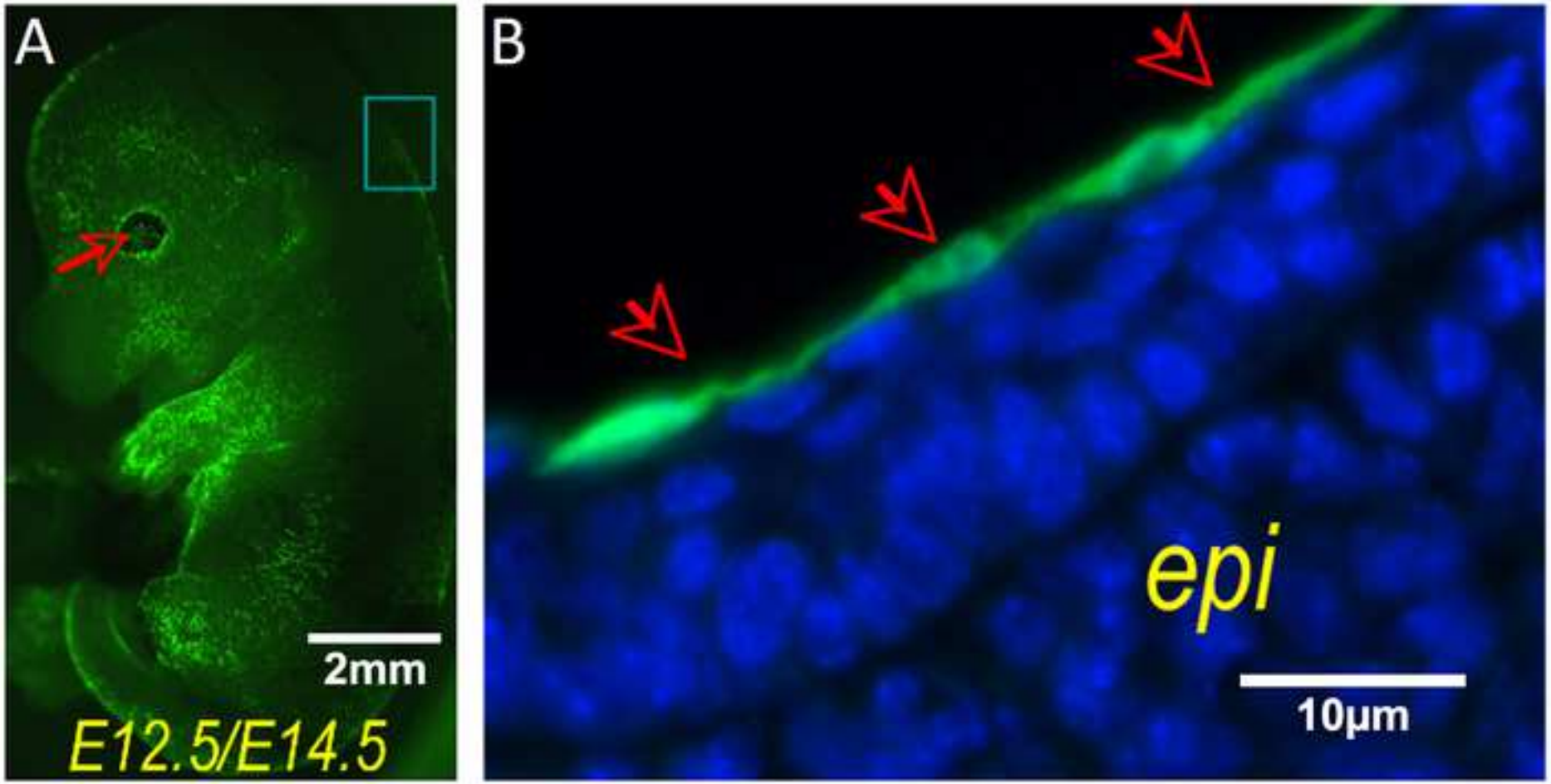

Materials Table

Click here to download Materials Table JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Gloves	Cardinal Health	2D73DP65	
Adson Forceps w/ teeth	Fine Science Tools	11027-12	
Adson Forceps w/o teeth	Fine Science Tools	11006-12	
Curved scissors	Fine Science Tools	14075-11	
Heavy Scissors	Fine Science Tools	14002-13	
Needle Driver	Fine Science Tools	12005-15	
Vicryl 2.0	Ethicon	JB945	
Transfer Pipette	Medline	GSI135010	
Cotton Tipped Applicators	Medline	MDS202000	
50 mL Conical tube	Fischer Scientific	14-432-22	
Tape	3M	1527-1	
Eye lubricant	Major LubriFresh	0904-6488	
Heating Pad	K&H	3060	
Stereomicroscope	Leica	MZ16	
Injector	Narishige	HI01PK01	
Glass Capillary tubes	Kimble	71900-100	
Vertical Micropipette Puller	Sutter Instruments	P-30	
Microelectrode Beveler	Sutter Instruments	BV-10	
IM-300 Pneumatic Microinjector	Narishige	IM-300	
Insulin Syringe	BD	305935	
Filter	Genesee Scientific	25-244	
Compac5 Anesthesia Machine	VetEquip Compac5	901812	
Isoflurane	Piramal Critical Care	NDC 66794-017-25	
N2 gas	Airgas	NI 125	
O2 gas	Airgas	OX 125	
Ad-GFP viral vector	Penn Vector Core	H5'.040.CMV.eGFP	

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Author(s): Nicholas J Ahn, John D Stratigis, Hyun-Duck Nah-Cederquist, William H Peranteau

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Article Title:	Intravenous and Intra-amniotic in Utero Transplantation in the Murine Model
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4/19/2018

Dear Editor,

Thank you for the additional comments in our manuscript, *"Intravenous and Intra-amniotic in Utero Transplantation in the Murine Model"*. Below please find a point-by-point response to the comments and an attached revised manuscript with the changes highlighted using the "track changes" function. We are grateful that these comments gave us another chance to further improve our manuscript.

Sincerely,

William H. Peranteau, MD

- 2.1. We added additional essential instruments that we normally open during this step that were not mentioned in the previous drafts.
- 2.3. Needle rinsing can be performed two or three times. We added “or three.”
- In the note after 2.5, we spelled out GFP, as that was never done anywhere in the manuscript and it is the first place in the manuscript where GFP is mentioned.
- 2.6. It was unclear what specific actions were to be performed. We described in more detail what actions are performed to calibrate the injection time in steps 2.6.1-2.6.5. Also, there was a question about if there is an upper limit to the volume to be injected. We addressed this in the note under 2.6.5.
- 2.7. We provided more details for our anesthesia protocol by adding the oxygen flow rate and
- 2.21. An example of the “appropriate volume” was requested, and we added 10-20 µL in parentheses.
- In the note under 2.30, a typo was found by the editor. We corrected this.
- In Results first paragraph, we were asked to give a specific number for the survival of intra-amniotic injections. We provided 85-100% as a survival rate range.
- In Results second paragraph, we were asked to provide a reference for the isolation of bone marrow cells. We provided a reference and added the citation to the References section.
- In Results third paragraph, we were asked to mention the vector, cite a reference for its use, and add the vector to the table of materials. We now state that the vector is Ad-GFP and explain that it is an adenovirus vector carrying GFP transgene in parentheses and cite a reference. We also added the vector to the table of materials.
- We were asked to remove the words “Figure 1”, “Figure 2” etc from all figures. They are now removed.
- Under Figure Legends 4, we were asked to :
    - Add “(%)” to the y axis label. We have done this.
    - Define how “chimerism” is defined with respect to “percentage of engraftment”: for the most part, we use chimerism and engraftment interchangeably in our lab. For 4B) we changed the Y axis label to % chimerism in the graph and in the manuscript for consistency.
    - Define what the various data points are. Each point in the graph represents chimerism from the liver of a separate injected fetus, and we have added this explanation in the legends. We also added that there was one trainee and one instructor who performed the injections.
- For Figure Legends 5, we were asked to:
    - Provide a figure title. We have done this.
    - Provide scale bars for both panels and define “epi”. We have done this and added more details to the figure legend.
- Some of the reference #s were changed in the Discussion.
- References section has been edited to follow the format suggested by the editor.