Journal of Visualized Experiments

Taking the Next Step: a Neural Coaptation Orthotopic Hind Limb Transplant Model to Maximize Functional Recovery in Rat

--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE60777R5
Full Title:	Taking the Next Step: a Neural Coaptation Orthotopic Hind Limb Transplant Model to Maximize Functional Recovery in Rat
Section/Category:	JoVE Medicine
Keywords:	Animal models, Limb transplantation, rat, rehabilitation, composite tissue allotransplantation (CTA), vascularized composite allotransplantation (VCA), microsurgery, immunology, peripheral nerve injury
Corresponding Author:	Kyle Koss, PH.D. Northwestern University Feinberg School of Medicine Chicago, IL UNITED STATES
Corresponding Author's Institution:	Northwestern University Feinberg School of Medicine
Corresponding Author E-Mail:	kyle.koss@northwestern.edu
Order of Authors:	Feibo Zheng
	Andy Tully
	Kyle Koss
	Xiaomin Zhang
	Longhui Qiu
	Jiao-Jing Wang
	Bilal Naved
	David Z Ivancic
	James Mathew
	Jason Alberta Wertheim
	Zheng Jenny Zhang
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)

TITLE:

Taking the Next Step: a Neural Coaptation Orthotopic Hind Limb Transplant Model to Maximize Functional Recovery in Rat

AUTHORSANDAFFILIATIONS:

Feibo Zheng[1,4], Andy Tully[1,3], Kyle M Koss[1], Xiaomin Zhang[1], Longhui Qiu[1], Jiao-Jing Wang[1], Bilal Naved[1], David Z Ivancic[1], James Mathew[1], Jason A Wertheim[1], Zheng Jenny Zhang[1]

[1]Comprehensive Transplant Center and Department of Surgery, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA
[2]Department of Radiology, Northwestern University, Chicago, IL, USA
[3]Department of Surgery, University of Illinois at Chicago, Chicago, IL, USA
[4]Department of Surgery, Tianjin Occupational Diseases Precaution and Therapeutic Hospital, Tianjin, China

Corresponding Author:
Zheng Jenny Zhang
zjzhang@northwestern.edu

Email Addresses of Co-authors:
Feibo Zheng: feibo.zheng@northwestern.edu
Andy Tully: andrew.tully@northwestern.edu
Kyle Michael Koss: kyle.koss@northwestern.edu
Xiaomin Zhang: x-zhang@northwestern.edu
Jiao-Jing Wang: jiao-jing.wang@northwestern.edu
LonghuiQiu: longhui.qiu@northwestern.edu
Bilal Naved: Bilal bilal.naved@northwestern.edu
David Ivancic: d-ivancic@northwestern.edu
James Mathew: james-mathew@northwestern.edu
Jason Wertheim: jason.wertheim@northwestern.edu

KEYWORDS:

Animal models, Limb transplantation, rat, rehabilitation, composite tissue allotransplantation (CTA), vascularized composite allotransplantation (VCA), microsurgery, immunology, peripheral nerve injury

SUMMARY:

This protocol presents a robust, reproducible model of vascularized composite allotransplant (VCA) geared toward simultaneous study of immunology and functional recovery. The time invested in meticulous technique in a right mid-thigh hind limb orthotopic transplant with hand sewn vascular anastomoses and neural coaptation yields the ability to study functional recovery.

ABSTRACT:

Limb transplant in particular and vascularized composite allotransplant (VCA) in general have wide therapeutic promise that have been stymied by current limitations in immunosuppression and functional neuromotor recovery. Many animal models have been developed for studying unique features of VCA, but here we present a robust reproducible model of orthotopic hind limb transplant in rats designed to simultaneously investigate both aspects of current VCA limitation: immunosuppression strategies and functional neuromotor recovery. At the core of the model rests a commitment to meticulous, time-tested microsurgical techniques such as hand sewn vascular anastomoses and hand sewn neural coaptation of the femoral nerve and the sciatic nerve. This approach yields durable limb reconstructions that allow for longer lived animals capable of rehabilitation, resumption of daily activities, and functional testing. With short-term treatment of conventional immunosuppressive agents, allotransplanted animals achieve morality up to 70 days post-operatively, and isotransplanted animals provide long lived controls beyond 200 days post-operatively. Evidence of neurologic functional recovery is present by 30 days post operatively. This model not only provides a useful platform for interrogating immunological questions unique to VCA and nerve regeneration, but also allows for in vivo testing of new therapeutic strategies specifically tailored for VCA.

INTRODUCTION:

Limb transplant under the broader category of vascularized-composite allotransplant (VCA) or composite tissue allotransplant (CTA) has yet to fulfill its therapeutic promise. Since the first successful human hand transplants in Lyon, France and Louisville, Kentucky in 1998 and 1999, over 100 upper extremity transplants have been performed worldwide in carefully selected patients[1]. Wider applicability has been stymied by substantial immunosuppression and limited functional neuromotor recovery. Current immunosuppression strategies result in 85% incidence of acute rejection in the face of 77% incidence of opportunistic infection[2]. On the other hand, functional recovery after hand transplant occurs; mean Disability of Arm Shoulder and Hand (DASH) scores improve from 71 to 43, but that level of function may still qualify as a disability[2]. Given the nonlife saving nature of limb transplant, current techniques must be refined in animal models to take the next step in VCA.

Since the first rat model of limb transplant in 1978[3], many innovative animal models have been developed to advance the field of VCA[4], incorporating vascular cuffed anastomoses to minimize operative time[5,6], heterotopic osteomyocutaneous transplants to minimize physiologic insult to the recipient animal[7-11], and novel immunologic approaches[7, 12-14]. The rat model of orthotopic right hind limb mid-thigh transplant presented here emphasizes meticulous, time-tested microsurgical techniques such as hand sewn vascular anastomoses and neural coaptation as an upfront investment in a robust, reproducible model platform to simultaneously investigate both aspects of current VCA limitation: immunosuppression strategies and functional neuromotor recovery.

PROTOCOL:

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) and were approved by the

Northwestern University Animal Care and Use Committee. The specific procedures were performed under protocol IS00001663.

NOTE: Two strains of rats were used, Lewis rats and August Copenhagen x Irish (ACI) rats. Animals were divided into three treatment groups: allotransplant without immune suppression (ACI to Lewis), allotransplant with conventional immune suppression (ACI to Lewis), and isotransplant (Lewis to Lewis or ACI to ACI). Lewis is an inbred strain, while ACI rats represent an out-bred wild-type, therefore this combination was chosen to model the worse-case rejection response. Conventional immunosuppression was administered subcutaneously either as rapamycin 1 mg/kg from post-operative day (POD) minus 1 to POD 28 or as FK506 3 mg/kg from POD 0 to POD 14, and then once weekly thereafter. Both male and female rats were eligible recipients from 8 to 16 weeks old, weighing between 250 and 400 grams at the time of surgery.

1. Donor right hind limb harvest

1.1. Induce general anesthesia with 5% isoflurane in pure oxygen through a vaporizer with an appropriate scavenging system.

1.2. Confirm adequate depth of anesthesia with toe pinch, and then use hair clippers to trim the fur off of the right hind limb and right groin surgical site

1.3. Down-titrate the isoflurane through a rodent nose cone to 2-2.5%.

1.4. Position the rat supine with spread limbs taped out to the sides on an operating board with a heating pad underneath. Disinfect the hairless skin with 70% rubbing alcohol and protect the surgical field with sterile gauze.

1.5. Using an appropriate microsurgical microscope, microsurgical instruments, and with easy access to bipolar and monopolar electrocautery, begin the dissection.

1.6. Use scissors to make a circumferential skin/subcutaneous tissue incision around the right hindlimb. Start in the inguinal crease medially at roughly the same level as the inguinal ligament and extend dorsal-laterally to complete the circumferential incision.

1.7. Having exposed the muscular layer directly beneath the incision, dissect and cauterize the superficial epigastric vessels that lead from the muscular layer to the proximal skin/subcutaneous flap just created.

1.8. Reflect the proximal flap superomedially to the inguinal ligament and the distal skin/subcutaneous flap inferolaterally to the knee.

1.9. Use a wire retractor or rolled gauze to help expose the field.

1.10. Observe that the inguinal anatomy of the rat is similar to humans; from lateral to medial lie the nerve, artery, and vein.

1.11. Dissect out the femoral nerve, divide it sharply at the inguinal ligament, proximal to the bifurcation if possible. Retract the divided nerve inferiorly, keeping it safely out of the way, covered beneath moist gauze.

1.12. Turning attention to the femoral artery and vein, use 4 cm 7-0 silk ties to atraumatically retract the vessels instead of handling them directly.

1.13. Ligate all branches of the femoral vessels as they arise with 7-0 silk ties; divide the branches between the ties. For very small branches, bipolar cautery may be used instead of ties.

NOTE: Arterial and venous branches which require division include the superficial circumflex iliac and the muscular vessels. The superficial circumflex iliac is usually largest and appears to dive deep as would the profunda femoral in humans, but the profunda is absent in the rat[15]. More distal branches of the femoral vessels such as the highest genicular and the saphenous branch do not usually require division.

1.14. Systemically inject 500 international units of heparin through the penile vein in a male rat donor. Use the superficial epigastric vein if the donor rat is female.

1.15. Allow the heparin to circulate systemically for 2 min before proceeding with the next steps.

1.16. Ligate the femoral artery with 7-0 silk ties as proximal to the inguinal ligament as possible and divide between the ties.

1.17. Similar to the artery, ligate and divide the femoral vein.

1.18. Reflect both artery and vein inferiorly, safely out of the way, covered beneath moist gauze together with the femoral nerve covered previously. Dissect the ventral muscle groups, taking care to cauterize any visible vessel that arises. Attention to hemostasis here will minimize recipient blood loss after reperfusion.

1.19. Deep to the ventral muscle groups, identify and sharply divide the sciatic nerve proximal to its branches. Three sciatic branches are usually visible: tibial, peroneal and sural. All three should all be preserved in the donor limb. A fourth cutaneous branch is not typically seen in this dissection[15,16].

1.20. Finish dividing the remaining ventral and dorsal muscle groups at mid-thigh level with meticulous hemostasis. It may be necessary to retract the limb medially to complete dividing the muscles.

1.21. Transect the femur bone at midshaft using a hand-held cordless rotary saw.

1.22. Having removed the limb graft from the donor, cut the silk tied ends from the graft side femoral artery and vein stumps, thereby re-opening the vessels.

1.23. Insert a 24-gauge angiocatheter into the graft artery stump and flush the graft with 250 international units of heparin diluted in 5 mL of ice-cold normal saline, watching it flow out clear through the opened vein.

1.24. Slowly, gently flush the graft for around 3 min. Excess forceful flushing may damage the endothelium.

1.25. Place the graft in a chilled saline dish nested in an ice bucket until transplantation.

1.26. Euthanize the donor rat with bilateral thoracotomy.

1.27. Clean all surgical instruments appropriately.

2. Recipient native right hind limb amputation

2.1. Induce anesthesia with isoflurane at 5%, confirm depth, trim the fur, position the animal, and disinfect the skin with alcohol as described for the donor rat.

2.2. Down-titrate isoflurane to 2-2.5% and inject subcutaneous preoperative analgesia with buprenorphine 1.2 mg/kg, and preoperative prophylaxis with enrofloxacin 7.5 mg/kg.

2.3. Same as for the donor, make a circumferential incision in the inguinal crease, reflect skin flaps assuring hemostasis, and dissect out the femoral nerve, artery and vein, ligating the same branch vessels as above.

2.4. Divide the femoral nerve more distally than for the donor, but proximally to the bifurcation if possible.

2.5. Dissect out the femoral artery and vein with enough space to clamp each separately at the level of the inguinal ligament. Clamp the vein and artery with microsurgical bulldog clamps. Once clamped, divide each vessel sharply with scissors.

2.6. Divide the ventral and dorsal muscles of the thigh at mid-thigh level with meticulous hemostasis, retracting the limb medially as necessary.

2.7. Identify and divide the sciatic nerves proximal to their branch points as above.

2.8. Transect the femur at midshaft using the saw.

2.9. Remove the recipient native right hind limb and dispose appropriately.

2.10. Down-titrate the isoflurane to 1-1.5% through the nose cone.

3. Donor to recipient limb implantation

3.1. Using the hand-held power saw, shave off any irregularities from both donor and recipient femur cut ends.

3.2. Using the saw, cut off the hub end of an 18-gauge needle, which will become the femur intramedullary rod.

3.3. Before manipulating the bone, apply a small amount of bone wax to the recipient cut end of femur bone to reduce marrow bleeding during the reaming process.

3.4. Coapt the donor and recipient femoral bones using the 18-gauge needle as an intramedullary rod. Some force is necessary, but do not ream either bone so far as to fracture the cortex.

3.5. As needed, remove the needle and trim it to an appropriate length so that both bones fit smoothly over the needle with no needle showing in between the bone.

3.6. Place a small support such as a pad of gauze or a small rock or modelling clay underneath the donor limb to keep it off tension.

3.7. Reapproximate the ventral muscle groups with eight to ten simple interrupted 5-0 polyglactin sutures so that the graft does not rotate around the femur needle. This gives the limb stability for the anastomoses.

3.8. Periodically irrigate the graft and surgical field with ice-cold saline for better visualization and to reduce warm ischemic reperfusion injury.

3.9. Align the donor and recipient femoral arteries and anastomose them in end to end fashion using simple interrupted 10-0 nylon suture, avoiding both tension and looping. The artery requires an average of six sutures.

3.10. Similar to the artery, anastomose the donor and recipient femoral veins in end to end fashion. The vein requires six to eight sutures.

NOTE: Generous cold saline irrigation, atraumatic vessel handling technique, and leaving long tails to serve as stay sutures for vessel retraction are important tools for effective microsurgical anastomoses.

3.11. Place a small amount of hemostatic cellulose powder around both anastomoses, and then remove the proximal microsurgical bulldog clamps on the vein and the artery.

3.12. Inspect both anastomoses for good patency and flow. Use cotton swab sticks to gently prod the vein and assure good hemostasis of both anastomoses. Hold pressure over bleeding sites and place more hemostatic cellulose powder if needed. Another suture may be placed through a bleeding hole at the risk of “back-walling" the needle only as a last resort.

3.13. When both anastomoses are confirmed satisfactory, trim any remaining long stay suture tails short to match the others.

3.14. Reposition the rat to the left lateral decubitus position, use liberal electrocautery to attain meticulous hemostasis of any reperfusion muscle bleeding.

3.15. Turn attention to the nerve anastomoses once muscle hemostasis is assured. Trim back any nerve cut ends that appear ragged.

3.16. Reapproximate the dorsal muscle groups under sciatic nerve with simple interrupted 5-0 polyglactin sutures.

3.17. Reapproximate the sciatic nerve. Eight to ten 10-0 nylon neural simple interrupted sutures will usually suffice.

3.18. Reapproximate the reminding dorsal muscle groups and then close the dorsal skin with 4-0 polyglactin continuous suture.

3.19. Reposition the rat back to supine position and reapproximate the femoral nerve. Two to three 10-0 nylon neural simple interrupted sutures will usually suffice.

3.20. Close the ventral skin with 4-0 polyglactin continuous suture. Avoid excess suture tail, which can be irritating to the rat once awake.

4. Post-operative care

4.1. Recover animals in their cages with a heating pad under the cage and ready access to food and water, monitoring for early complications daily for the first week.

4.2. Provide post-operative analgesia with subcutaneous meloxicam 1 mg/kg daily injection through POD 2. Provide post-operative antibiotic prophylaxis dilute enrofloxacin spray. Provide disincentive for autotomy (self-mutilation) with Bitter Safe Mist sprayed twice daily to the graft through POD 7.

4.3. Maintain transplanted rats in cages with other rats, to stimulate return to daily activities and rehabilitate the transplanted limb.

5. Post-operative sensation testing

5.1. Apply the Hargreaves testing of thermal sensation protocol, also described elsewhere[17,18].

5.2. Place the rat in the testing container and allowed it to acclimatize for 20 minutes. The apparatus glass is confirmed clean, and the heat source confirmed to be working with the investigator's finger.

5.3. Before testing, confirm that the rat is awake and the tested paw is positioned over the infrared motion detector.

5.4. Transmit thermal energy at intensity level 90. Time delay in the animal moving its paw away from the heat source is recorded. If no movement occurs within 20 seconds, the test is aborted to prevent injury.

5.5. Obtain five trials per tested limb, excluding the highest and lowest value before calculating the mean withdrawal latency time for each animal.

6. Post-operative motor testing

6.1. Using a gait analysis treadmill and integrated software analysis platform, select candidates for treadmill testing at four to six weeks post-surgery.

6.2. Trim all rat toenails one or two days before testing.

6.3. Acclimatize animals to the testing room for one hour before testing, and allow for one minute of pre-test petting to calm anxiety.

6.4. Placing the rat inside the treadmill, run the treadmill at trials of increasing speed, from 10 cm/s, to 14 cm/s, to the goal 18 cm/s. If the rat is reticent and cannot be coaxed to walk, abort the testing that day to avoid negative conditioning. Allow high performers to walk up to 24 cm/s.

6.5. Rinse the treadmill apparatus with 70% ethanol in between tested animals.

6.6. Gait parameters are output from the analysis platform's proprietary software.

REPRESENTATIVE RESULTS:

Survival and recovery depend on meticulous surgical technique. Attention to the vascular anastomoses and the neural anastomoses, as well as the bone coaptation as described above is crucial maximizing the success of this model. Operative design and representative anastomotic results are shown in **Figure 1**.

Overall mortality was dependent on immunosuppression strategy, with the majority of isotransplanted animals attaining the study endpoint of 100-200 post-operative days as seen in **Figure 2**. Once out of the acute post-operative window, treated allotransplanted animals could experience survival up to 58 post-operative days. Isografted rats lived indefinitely over the course of study while allograft transplanted rats had variable mortalities from rapamycin and FK506. Out of the treatments FK506 promoted the longest viability (day 57), while rapamycin was second best (day 20) over the untreated control (day 10).

Sensory and motor recovery can be shown in **Figure 3**. Animals were shown to have recovered sensory nerve function of the transplanted paw using the Hargreaves apparatus by day 30. Animals displayed significant recovery by four weeks after surgery (Aii). Animals shown marked improvements in motor function of the transplanted limb using a gait analysis treadmill and integrated software analysis platform. Example gait parameter based on specific limbs are shown (Bii) and a Sciatic Function Index (SFI) are also presented (Biii).

Figure 1: **Operative design is depicted in cartoon format.** (**A**) The rat is shown with (**B**) right hind leg cross-section depicting (i) the femoral bundle (nerve, artery, and vein) (ii) the sciatic nerve, and (iii) the bone. (**C**) Representative micrographs from the operating microscope (donor left and recipient right) were taken of the (i) sciatic nerve anastomosis (ii) the femoral nerve artery, and vein anastomoses (shown from top to bottom), and (iii) the 18-gauge needle intramedullary rod-femur bone coaptation. Note the donor structures appear to the left in each photo. Also note the femur is shown before full coaptation when both bones are opposed and the needle is concealed within.

Figure 2: Percent survival of animals as presented days post-surgery (POD). Groups shown include isograft, allografts with no treatment, rapamycin, and FK506 immunosuppressant drugs.

Figure 3: Sensory nerve recovery is demonstrated in (A) Hargreaves testing transplanted animals each at six post-operative time points and in (B) still shot of treadmill testing using DigiGait. (i) Representative pictures are shown with (ii) respective paw data. Respective color-coded images of paws are also in 0.025 ms frames. Digigate models (iii) are also shown. Significance was determined using a one-way ANOVA with a Bonferroni's multiple comparison test and SEM, where n=7 and p< 0.05. These particular DigiGait data were taken from an isogeneic animal tested at post-operative day 28.

DISCUSSION:

Limb transplant, under the broader category of vascularized component allotransplantation (VCA), has widely applicable therapeutic promise as yet unfulfilled. The main roadblocks lie in unsolved immunological issues unique to VCA and neuromotor recovery techniques used currently. Development of new techniques will depend on animal modeling that is flexible, robust, and reproducible.

Many animal models have been established in VCA, each with specific advantages[4]. Non-human

primate models offer attractive translatability to human patients, but have been hampered by cost concerns and toxic levels of immunosuppression required[4]. Canine models have been seen as advantageous for specific similarities of muscular structure as humans as well as a more experienced immune system[19,20]. Porcine models offer the benefits of a large animal model where the immune system is increasingly well studied[21,22]. Mouse model systems present the most advanced techniques to study immunology, but despite important advances in cuffed vascular microsurgical anastomosis[23], mouse limb transplant remains technically challenging and has some limitations in functional recovery assessment[5,24-26].

Rat models in VCA have been utilized since 1978[3], providing a mature platform to investigate both immunological and neuromotor hypotheses[6,9,13,14,17,27,28,38]. The model here combines the advantages of hindlimb orthotopic approach, suture anastomosis, nerve re-approximation, and potential for gait analysis. Hindlimb orthotopic as opposed to forelimb transplant is less of an encumbrance to the rat during the recovery process and allows for continued normal grooming and feeding behaviors post operatively. Suture anastomosis although painstaking may potentially offer less technical confounding for long term studies. Nerve re-approximation allows for future investigation[17,18] and gait analysis. This protocol relies on meticulous, time-tested microsurgical techniques well-described elsewhere[29], requiring constant attention to avoid the immediate pitfalls of anesthetic overdose, anastomotic failure, anastomotic thrombosis, and excessive surgical blood loss. Although multiple microsurgeons can improve the workflow, we have described a method by which a single operating microsurgeon can achieve sufficient experimental output.

Autotomy or self-mutilation has been a phenomenon noted in several microsurgical models, and it has been hypothesized to inversely correlate with nerve healing[30,31]. Autotomy was overall controlled in this model, possibly related meticulous neural anastomotic technique. Autotomy also decreased farther into the learning curve. Bitter Safe Mist was a valuable adjunct in controlling this phenomenon.

Gait analysis in rats has been studied for multiple models of injury[32-34], most relevantly for sciatic nerve injury[35,36]. Rats even when not limb transplant recipients are known to be heterogenous subjects for gait analysis, and investigators still debate which analysis parameters describe recovery[37]. In this model we have described several methods to obtaining the best data from transplanted recipients who are willing and able to walk. Preselection of adequate walkers was not predictive of post-operative cooperation. Although animals are able to move about their housing as soon as several hours after surgery, they are not ready for treadmill ambulation until at least four to six weeks after surgery.

A protocol's ability to measure nerve recovery in VCA is dependent on its strategy for rehabilitation. This protocol explicitly promotes transplant recipients interacting with other rats as inducement to function. This strategy is cognizant of the importance of modeling rehabilitation, yet is simple, economical, and is largely standard. Future strategies may include more active rehabilitation such as treadmill training.

The immunologic techniques applicable to this model are beyond the scope of this discussion, but in particular, comparing isotransplant versus allotransplanted animals provides a useful control to differentiate allograft immunologic phenomena and rejection from the ischemic reperfusion injury, inflammation, revascularization, and post-surgical infection processes inherent in the transplant surgery itself. Isotransplants provide a similar control for nerve function studies for the same reason.

Using this platform, investigators may be able to advance both VCA immunology and neuromotor recovery.

ACKNOWLEDGMENTS:

This work was funded by the Frankel Foundation and the Northwestern Memorial Hospital McCormick Grant (Operation RESTORE).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Elliott, R.M., Tintle, S.M., Levin, L.S. Upper extremity transplantation: current concepts and challenges in an emerging field. *Current Reviews in Musculoskeletal Medicine.* **7** (1), 83-88 (2014).
2. Petruzzo, P. et al. The International Registry on Hand and Composite Tissue Transplantation. *Transplantation.* **90** (12), 1590-1594 (2010).
3. Shapiro, R.I., Cerra, F.B. A model for reimplantation and transplantation of a complex organ: the rat hind limb. *Journal of Surgical Research.* **24** (6), 501-506 (1978).
4. Brandacher, G., Grahammer, J., Sucher, R., Lee, W.P. Animal models for basic and translational research in reconstructive transplantation. *Birth Defects Research Part C: Embryo Today.* **96** (1), 39-50 (2012).
5. Furtmuller, G.J. et al. Orthotopic Hind Limb Transplantation in the Mouse. *Journal of Visualized Experiments.* (108), e53483 (2016).
6. Sucher, R. et al. Orthotopic hind-limb transplantation in rats. *Journal of Visualized Experiments.* (41), e2022 (2010).
7. Horner, B.M. et al. In vivo observations of cell trafficking in allotransplanted vascularized skin flaps and conventional skin grafts. *Journal of Plastic, Reconstructive & Aesthetic Surgery.* **63** (4), 711-719 (2010).
8. Fleissig, Y. et al. Modified Heterotopic Hindlimb Osteomyocutaneous Flap Model in the Rat for Translational Vascularized Composite Allotransplantation Research. *Journal of Visualized Experiments.* (146), e59458 (2019).
9. Zhou, X. et al. A series of rat segmental forelimb ectopic implantation models. *Scientific Reports.* **7** (1) (2017).
10. Adamson, L.A. et al. A modified model of hindlimb osteomyocutaneous flap for the study of tolerance to composite tissue allografts. *Microsurgery.* **27** (7), 630-636 (2007).
11. Ulusal, A.E., Ulusal, B.G., Hung, L.M., Wei, F.C. Heterotopic hindlimb allotransplantation in rats: an alternative model for immunological research in composite-tissue allotransplantation.

Microsurgery. **25** (5), 410-414 (2005).
12. Fries, C.A. et al. Graft-implanted, enzyme responsive, tacrolimus-eluting hydrogel enables long-term survival of orthotopic porcine limb vascularized composite allografts: A proof of concept study. *PLoS One.* **14** (1), e0210914 (2019).
13. Cottrell, B.L. et al. Neuroregeneration in composite tissue allografts: effect of low-dose FK506 and mycophenolate mofetil immunotherapy. *Plastic and Reconstructive Surgery.* **118** (3), 5 (2006).
14. Benhaim, P., Anthony, J.P., Lin, L.Y., McCalmont, T.H., Mathes, S.J. A long-term study of allogeneic rat hindlimb transplants immunosuppressed with RS-61443. *Transplantation.* **56** (4), 911-917 (1993).
15. Greene, E.C. *Anatomy of the rat.* Volume N.S., 27, American Philos. Soc, Philadelphia, Pa (1935).
16. Schmalbruch, H. Fiber composition of the rat sciatic nerve. *Anatomical Record.* **215** (1), 71-81 (1986).
17. Cheah, M., Fawcett, J.W., Andrews, M.R. Assessment of Thermal Pain Sensation in Rats and Mice Using the Hargreaves Test. *Bio-protocol.* **7** (16), 10.21769/BioProtoc.2506 (2017).
18. Hargreaves, K., Dubner, R., Brown, F., Flores, C., Joris, J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain.* **32** (1), 77-88 (1988).
19. Hong, S.H., Eun, S.C. Experimental Forelimb Allotransplantation in Canine Model. *BioMed Research International.* **2016**, 1495710 (2016).
20. Mathes, D.W. et al. A preclinical canine model for composite tissue transplantation. *Journal of Reconstructive Microsurgery.* **26** (3), 201-207 (2010).
21. Ibrahim, Z. et al. A modified heterotopic swine hind limb transplant model for translational vascularized composite allotransplantation (VCA) research. *Journal of Visualized Experiments.* (80), e50475 (2013).
22. Fries, C.A. et al. A Porcine Orthotopic Forelimb Vascularized Composite Allotransplantation Model: Technical Considerations and Translational Implications. *Plastic and Reconstructive Surgery.* **138** (3), 71e (2016).
23. Kim, M., Fisher, D.T., Powers, C.A., Repasky, E.A., Skitzki, J.J. Improved Cuff Technique and Intraoperative Detection of Vascular Complications for Hind Limb Transplantation in Mice. *Transplantation Direct.* **4** (2), e345 (2018).
24. Chong, A.S., Alegre, M.L., Miller, M.L., Fairchild, R.L. Lessons and limits of mouse models. *Cold Spring Harbor Perspectives in Medicine.* **3** (12), a015495 (2013).
25. Sucher, R. et al. Mouse hind limb transplantation: a new composite tissue allotransplantation model using nonsuture supermicrosurgery. *Transplantation.* **90** (12), 1374-1380 (2010).
26. Tung, T.H., Mohanakumar, T., Mackinnon, S.E. Development of a mouse model for heterotopic limb and composite-tissue transplantation. *Journal of Reconstructive Microsurgery.* **17** (4), 267-273 (2001).
27. Tang, J. et al. A vascularized elbow allotransplantation model in the rat. *Journal of Shoulder and Elbow Surgery.* **24** (5), 779-786 (2015).
28. Yan, Y. et al. Nerve regeneration in rat limb allografts: evaluation of acute rejection rescue. *Plastic and Reconstructive Surgery.* **131** (4), 511e (2013).
29. Georgiade, N.G., Serafin, D. A Laboratory Manual of Microsurgery. *Fourth Printing.* (1986).

30. Tseng, S.H. Suppression of autotomy by N-methyl-D-aspartate receptor antagonist (MK-801) in the rat. *Neuroscience Letters.* **240** (1), 17-20 (1998).
31. Haselbach, D. et al. Regeneration patterns influence hindlimb automutilation after sciatic nerve repair using stem cells in rats. *Neuroscience Letters.* **634**, 153-159 (2016).
32. Kloos, A.D., Fisher, L.C., Detloff, M.R., Hassenzahl, D.L., Basso, D.M. Stepwise motor and all-or-none sensory recovery is associated with nonlinear sparing after incremental spinal cord injury in rats. *Experimental Neurology.* **191** (2), 251-265 (2005).
33. Berryman, E.R., Harris, R.L., Moalli, M., Bagi, C.M. Digigait quantitation of gait dynamics in rat rheumatoid arthritis model. *Journal of Musculoskeletal and Neuronal Interactions.* **9** (2), 89-98 (2009).
34. Hamers, F.P., Lankhorst, A.J., van Laar, T.J., Veldhuis, W.B., Gispen, W.H. Automated quantitative gait analysis during overground locomotion in the rat: its application to spinal cord contusion and transection injuries. *Journal of Neurotrauma.* **18** (2), 187-201 (2001).
35. Deumens, R., Marinangeli, C., Bozkurt, A., Brook, G.A. Assessing motor outcome and functional recovery following nerve injury. *Methods in Molecular Biology.* **1162**, 179-188 (2014).
36. Bozkurt, A. et al. Aspects of static and dynamic motor function in peripheral nerve regeneration: SSI and CatWalk gait analysis. *Behavioural Brain Research.* **219** (1), 55-62 (2011).
37. Neckel, N.D. Methods to quantify the velocity dependence of common gait measurements from automated rodent gait analysis devices. *Journal of Neuroscience Methods.* **253**, 244-253 (2015).
38. Yeh, L.S., Gregory, C.R., Theriault, B.R., Hou, S.M., Lecouter, R.A. A functional model for whole limb transplantation in the rat. *Plastic and Reconstructive Surgery*. **105** (5), 1704-11 (2000).

Figure 1

Click here to access/download;Figure;Figure 1l.psd

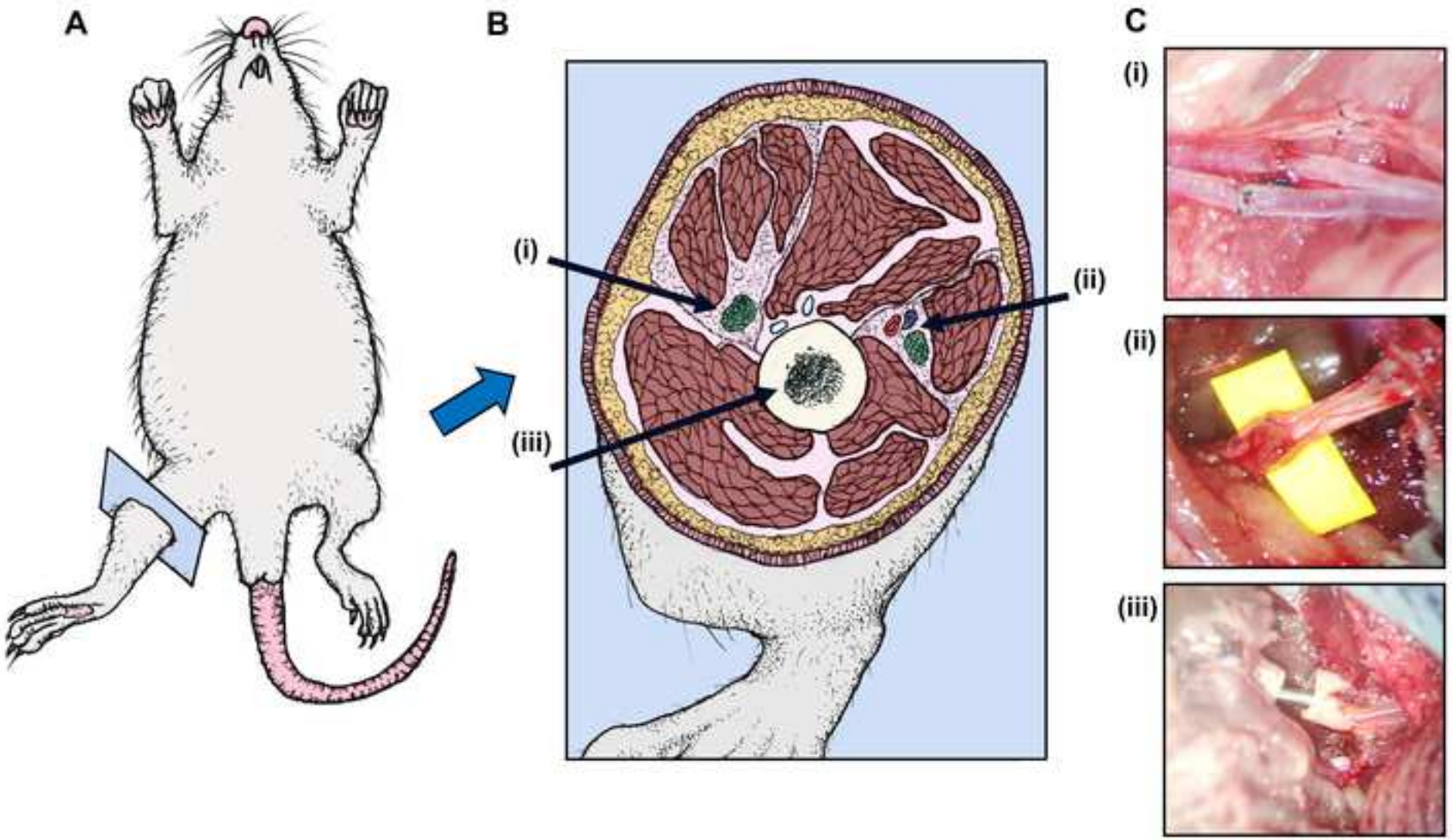


Figure 2

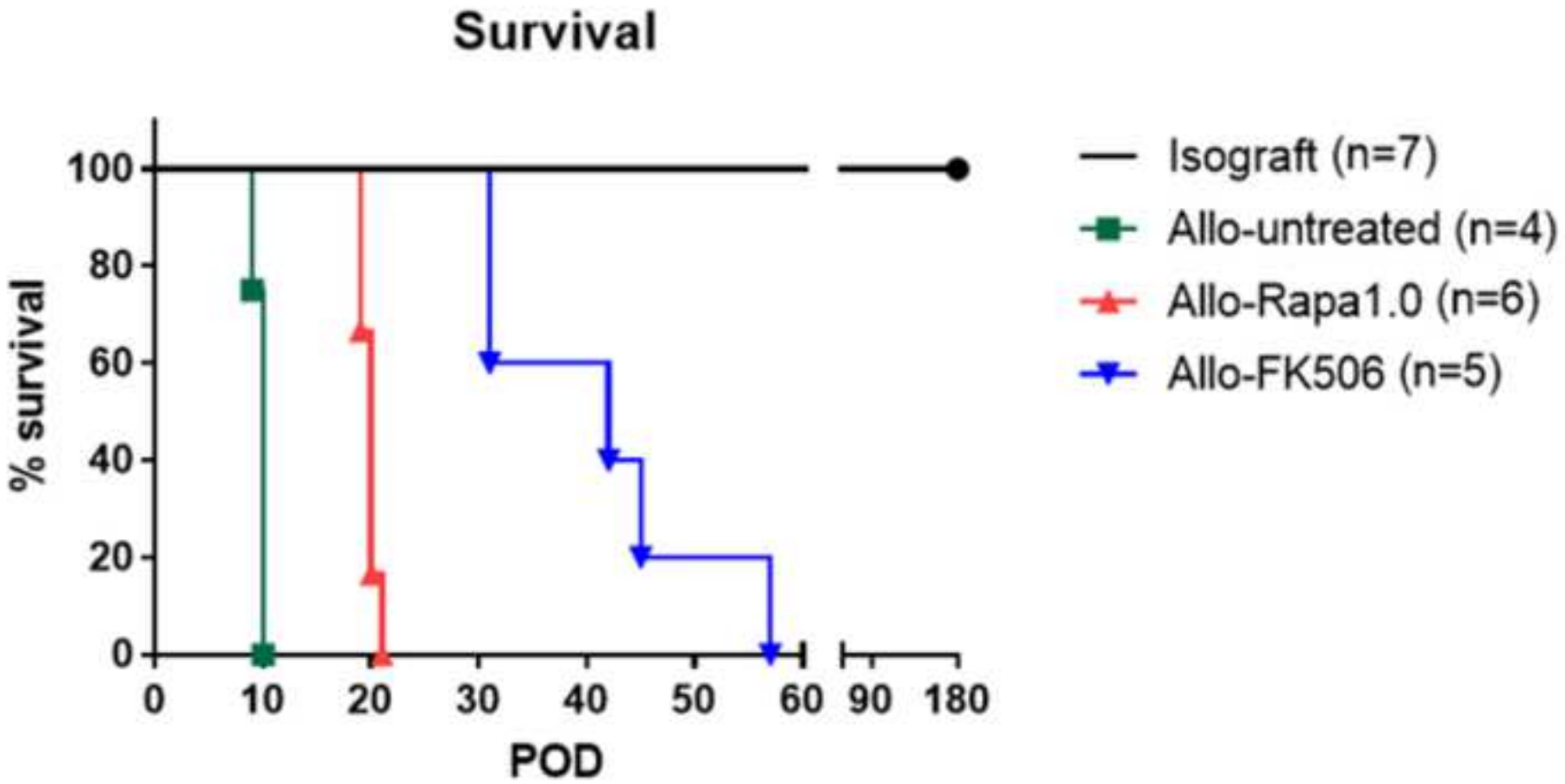


Figure 3

Click here to access/download;Figure;Figure 3e.psd

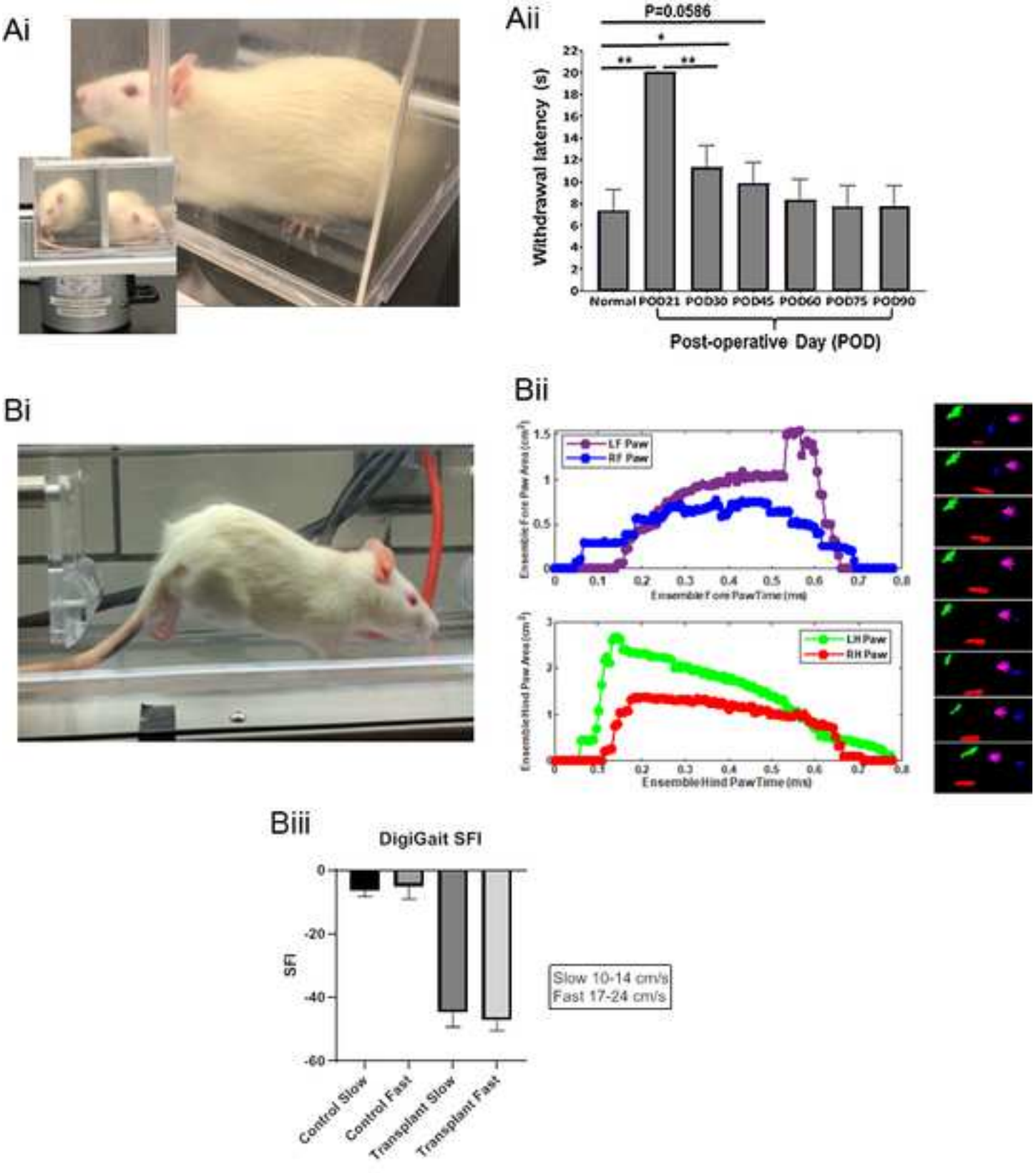


Table of Materials

Click here to access/download;Table of Materials;JOVE materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Anesthesia machine	Vet Equip	911103¤	
0.5cc syringe	Exel	26018	
18-gauge needle	BD	305196	
1cc syringe	BD	309659	
22-gauge needle	BD	305156	
24-gauge angiocatheter	Sur-Vet	SROX2419V	
25-gauge needle	Exel	26403	
3 cc syringe	BD	309657	
5cc syringe	Exel	26230	
Alcohol	Fisher Scientific	HC-600-1GAL	
Anesthesia induction chamber	Vet Equip	941443	
Anesthetic gas scavenger syste	Vet Equip	931401	
Bipolar electrocautery	Aura	26-500	
Bitter Spray Mist	Henry Schein	5553	
Bone wax	CP Medical	CPB31A	
Breathing circuit	Vet Equip	921413¤	
Buprenophine	Reckitt Benckiser	12496075705	
Castro-Viejos needle drivers	Roboz	RS-6416	
Cordless rotary saw	Dremel	8050-N/18	
Cotton swab stick	Fisher Scientific	23-400-101	For hemostasis
DigiGait Appparatus and Softw	Mouse Specifics	MSI-DIG, DIG-SOFT	
Dumont forceps (#4)	Roboz	RS-4972	
Dumont forceps (#5)	Roboz	RS-5035	
Enrofloxacin	Norbrook	ANADA 200-495	
FK-506	Astellas	301601	
Gauze	Kendall	1903	
Gauze	Covidien	8044	
Gloves	Microflex	DGP-350-M	
Hair clippers	Oster	078005-010-003	
Handheld monopolar electroc	Bovie	AA00	
Hargreaves Apparatus	Ugo Basile S.R.L. Gemonio, I	37370	
Heating pad	Walgreens	126987	
Heparin	Fresenius Kabi	42592K	
Hot plate	Corning	PC-351	For warming resusscitatio
Isoflurane	Henry Schein	29405	
Lactated ringers	Baxter	2B2074	
Large petri dish	Fisher Scientific	FB0875713	For donor graft while in cl
Meloxicam	Henry Schein	49755	
micro Collin Hartmann retractor			
Micro dissecting scissors	Roboz	RS-5841	
Microfibrillar collagen powder	BD	1010590	For hemostasis
Microvascular clips	Roboz	RS-5420	

Normal saline	Baxter	2F7124	
Opthalmic lube	Dechra	IS4398	
Rapmycin	MedChem Express	HY-10219	
Small petri dish	Fisher Scientific	FB0875713A	For warmed resusscitatio
Sterile drapes	ProAdvantage	N207100	
Surgical gown	Cardinal Health	9511	
Surgical mask	3M	1805	
Surgical microscope, optic mo	Zeiss	169756	
Surgical microscope, Universal	Zeiss	243188	
Suture 10-0 nylon	Covidien	N2512	
Suture 5-0 vicryl	Ethicon	J213H	
Suture 7-0 silk tie	Teleflex	103-S	
Tape	3M	1530-1	
Ultrasonic instrument cleaner	Roboz	RS-9911	
Vessel dilation forceps	Roboz	RS-5047	

n fluid

hilled saline

n fluid

Dear Jove Reviewers,

The following revisions changes were made:

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The objective of this study was to describe the procedural aspects of orthotopic hind limb transplantation in a rat model. Authors have performed allogeneic and syngeneic transplants, and studied neurologic functional recovery following transplantation.

General Comments/Concerns:
The methodology described in this manuscript in regard to orthotopic hind limb transplantation in rats has been published by many investigators in the past. Furthermore, the immunosuppression strategies used and sensory/motor functional recoveries studied are pretty standard. The authors have not highlighted the new knowledge/methods learned from this study as it appears there is none. However, what is different perhaps is the presentation of the procedural aspects through a video. There is no explanation why evidence of neurologic function recovery was present or reported only for 30 days when animals survived for 70 days (allotransplants) and beyond 200 days (iso/syngeneic transplants); was it because the surgical methods were not optimal for sustained functional recovery? Authors have not presented any data or discussed foot flexion contractures, a common occurrence with sciatic nerve repair and limb transplants. It is not clear sensory and motor function from which group/s (Isograft, Allo-untreated, Allo-Rapa, Allo-FK504) is presented in Figure 3. The figure legend is incomplete i.e. not clear what day the motor function was determined. Describing surgical methods in itself in the absence of optimal outcomes may not constitute complete/appropriate/successful methodology.

Response: *We appreciate the criticisms which raise important questions that this model is designed to answer. However, for the purpose of this methods paper, our goal contribution to the literature was to present a robust, reproducible model platform in vivid detailed technique through our video, as the reviewer points out. While other videos have been performed in related models, this video clearly highlights stepwise important aspects which novices to the model can readily appreciate and adapt. It also pointedly suggests future directions investigators can take this model, specifically immunologic and neuromotor recovery, and we show a general baseline of what results investigators can expect from standard well-known protocols before they move on to investigational protocols.*

Specific Comments/Concerns: Here are some of the specific deficiencies in the manuscript in addition to the ones mentioned under general comments:
1. Physiotherapy following limb transplantation is one of the critical post-operative care components that is not addressed in this manuscript.

Response: *Yes we agree that physiotherapy is important; we specifically comment on this point in point 4 of the post-operative care section. Our subjects responded best to the stimulation of other rats as an effective form of holistic physiotherapy. Further investigation of physiotherapy*

is a broader topic not suited to this methods paper, but this model is hoped to whet the appetite of investigators who wish to proceed in that direction. We included the following statement on page 11:
" Future strategies may include more active rehabilitation such as treadmill training."

2. It is not clear how authors confirm anastomosis of vasculature with no leak/hemorrhage post-operatively after closing the skin? If there was a leak, in how many animals? Was laser Doppler or other technology used to confirm leaky vasculature.

Response: *Using the techniques presented in this paper, we did not encounter that anastomotic difficulty. Using the cellulose and cotton swab methods described, taking extra time to assure hemostasis, once the skin was closed, we did not have post-operative hemorrhage. The animals were closely monitored clinically, especially regarding their surgical site, and any hematoma would have been clearly recognizable clinically while giving the animal its daily check-up and analgesic, immunosuppressive, and anti-autotomy spray as described.*

3. Not clear how donor and recipient bone fusion/repair was confirmed post-operatively following closure of the skin?

Response: *Bone healing is another excellent example of the many investigational directions in which this model can be taken. In the baseline protocol presented here, animals were allowed to run on the treadmill at 4 to 6 weeks post operatively, with good performance. This is functional clinical evidence of bone fusion. If any animal were to have demonstrated gait instability, it would not have been allowed to walk the treadmill, but there was no such animal at 4 to 6 weeks post operatively.*

4. Not explained why both isografts and allografts were used to in this study; furthermore, it was methodology demonstration.

Response: *As the reviewer rightly points out, this is a methodology paper, and allograft and isograft animals may be used to answer different sorts of questions. Because this model is primed to address both neuromotor and immunologic questions, we wanted to demonstrate to prospective investigators the sort of baseline experimental results they could obtain with standard experimental protocols on both sets of animals.*

5. There is no adequate explanation for DigiGait SFI (sciatic function) calculation; just referring to software use is not sufficient. Why paw prints are not shown to indicate animals were able to bear weight (physical proof)? Did flexion contractures interfere with SFI assessment - need some explanation/discussion.

Response: *The reviewer is right to point out a much deeper science behind gait analysis, which very quickly moves beyond the scope of this methods paper. DigiGait does provide pawprints we can include a sampling of, and it calculates these pawprint areas through proprietary algorithms unavailable to investigators and turns these areas into several recognizable metrics from the literature, including the SFI. A fuller explanation of DigiGait is available from the manufacturer and likely not of immediate interest to the reader to include here. Despite this, we*

included an additional figure (3Bii) to show paw print morphology and its related paw areas over time.

6. Figures 3Ai and 3Bi do not add much information; furthermore, right hind limb foot is not even in contact with the surface. Legend does not state on what day post-operatively Fig 3Bii measurements were recorded.

Response: *Thank you. The photos of the animals engaged in neuromotor testing are meant to give added perspective of research inputs and outputs for readers of a methods paper who wish to copy the methods. The figure 3Bi is a still shot from a digigait movie clip wherein the rat is walking; hence the lifted foot not in contact with the surface. We added a frame where the foot contacts the sensor. Figure 3Bii provides a representative comparison of potential DigiGait outputs, which number over 30 parameters, a full treatment of which is beyond the scope of this paper. Again, this figure's purpose is to provide an idea to the reader of the sorts of data obtainable through this model. These particular data were obtained on post-operative day 28, which will be included.*

7. Figure 1C is not labeled to show donor and recipient structures.

Response: *Thank you, the donor structures are to the left in the surgical microscopic images; the figure legend has been updated to clarify this information. The text was updated:* "Representative micrographs from the operating microscope (donor left and recipient right) were taken of the (i) sciatic nerve anastomosis, (ii) the femoral nerve artery, and vein anastomoses (shown from top to bottom), and (iii) the 18-gauge needle intramedullary rod-femur bone coaptation."

8. The manuscript can't stand by itself for previously well described orthotopic hind limb transplantation procedure; a better explanation and presentation with details of methodologies used to assess functional recovery might support it.

Response: *As the reviewer points out in the general comments, it is the video and the clear presentation of technique combined with the forward thinking perspective on protocols that can be added onto this model which is this paper's contribution to the literature and can be of benefit to new investigators in the field.*

9. What about muscle atrophy in animals that lived for 200 days? There is no mention of it. Post-operative structural features/histology and functional assessment are reflective of surgical methodology; details are lacking in the manuscript.

Response: *Again, these are excellent points and offspring quite nicely from the model platform we are describing here. The detailed characterization described has been underway;however, presenting it here would quickly distract from the operative technique itself which is the focus of this manuscript.*

10. Some of the key publications are not cited e.g. Yeh LS, Gregory CR, Theriault BR, Hou SM, Lecouter RA. A functional model for whole limb transplantation in the rat. Plast Reconstr Surg.

2000 Apr; 105(5):1704-11.

Response: *Yes, we are familiar with this paper and would be happy to include it in the references as well for the readers' benefit. The text was changed to:*
"Rat models in VCA have been utilized since 1978 [3], providing a mature platform to investigate both immunological and neuromotor hypotheses [6, 9, 13, 14, 17, 27, 28, 38]."
"38. Yeh, L.S., Gregory, C.R., Theriault, B.R., Hou, S.M.&Lecouter, R.A. A functional model for whole limb transplantation in the rat. *PlastReconstr Surg.* **105** (5), 1704-11, (2000)."

Reviewer #2:
Manuscript Summary:
Authors describe the procedure for experimental rat hindlimb allotransplantation, allowing a robust model for further immunosuppressive regimens testings and nerve (sensory and motor) recovery monitoring.

Major Concerns:
There are no major concerns.

Response: *Thank you*.

Minor Concerns:
The claim of novelty, even though not a critical criteria in such paper type, can not be used here as nothing differs from what is usually taught in serious microsurgery courses.
The video quality is very poor - should be checked prior to publication.

Response: *Two videos were uploaded as requested, the high quality and the simple version. We hope the reviewer was able to see the high-quality version. We believe this paper could be of benefit to new investigators trying a new model and to veteran microsurgeons sharing variation in technique. The value of the model lies in its potential as a platform to new improvements in both immunological manipulation and functional recovery. We did not and do not intend a broader claim to novelty and will clarify.*

Here are detailed comments:
Keywords
Line 38 : CTA is the former name for VCA. Just use VCA standing for "Vascularized Composite Allotransplantation".

Response: *We do hope to be searchable across multiple eras. We will clarify.*

Introduction
Line 69: not necessary to mention CTA
Response: *Thank you.*
Line 70: "Lyon, France and Louisville, USA"
Response: *Thank you.*
Line 75: would be useful, here or in the discussion, to quickly provide DASH score comparison with prosthetic devices in upper limb VCA.

Response: *Indeed, in some revisions we had incorporated this information, but had received feedback that it distracted from the focus of the methods paper.*

Protocol
Why these two strains of rats? please comment and justify.
Response: *The justification is more particular to our institution and the breeding colonies we have established for other separate immunologic studies. Moreover, the in-bred Lewis recipient, in combination with ACI out-bred donor were the optimal choice for appropriate rejection and the modeling of patient rejection in the clinical setting. We updated the manuscript (page 2)*
" Lewis is an inbred strain, while ACI rats represent an out-bred wild-type, therefore this combination was chosen to model the 'worst-case' rejection response."

Line 107: Please provide the injection protocol as not every can perform gaz anesthesia at the lab; moreover, such procedure can easily be performed with injection anesthesia (contrary to heart-lung transplantation i.e.).

Response: *Our lab also has had success with injection technique in other models. However, in this model we valued flexibility afforded by isoflurane anesthesia. Injection protocols are readily available online, and while we could comment on them, such a comment would not reflect our experience with it on this model.*

Line 140: Please provide volume and concentration - change with weight? Why not use superficial epigastric vein in both male and female rats? Put this step prior to point 13 (ligation of branches) in order to provide max diffusion of LMWH in transplant.

Response: *Our rats were at comparable weights at transplant, 250 to 400grams, so a standard 500 international unit dose was used.*
The male penile vein in our experience is more efficiently cannulated when available.
Our group values minimizing blood loss to avoid priming the innate immune system, so we here present our method of completing more dissection first before anticoagulant dosing.

Line 144: Maybe using a vascular clamp on the distal vessels stumps is less traumatizing than a ligature, leading to a new cut and thus vessels shortening at time of transplantation and vascular anastomosis. Clamp should be place as distal as possible on femoral vessels pedicle.

Response: *Yes, thank you; we had considered this, the point is well taken. In our model we found success with ligation, so we are presenting that here.*

Line 161, point 23: Why using heparin again? Injected twice, if once is enough then select one.

Response: *Thank you; it has been our protocol to flush the freshly amputated graft with ice cold dilute heparin solution, similar to widespread back benching practice in human clinical medicine.*

Line 168, point 27: Maybe more efficient to just prepare another different set of instruments for transplantation.

Response: *Yes that could be a viable alternative. Not every institution has the resources.*

Line 178: same as for line 144.

Response: *Thank you. Please refer to the our previous response on this.*

Line 217: "bulldog clamp" - actually its placement was not mentioned before.

Response: *Thank you; it appears they were referenced in step 5 of the recipient amputation; we will clarify.*

Line 228: 8 to 10 10/0 is a lot for nerve anastomosis - please justify.
Response*: Our surgeons value the close neural reapproximation afforded by this technique. We thought it useful to share and there makeup the novelty of this technique.*

Line 234: important feature is missing: a 3/0 nylon in an interrupted suture is usually advocated. Continuous suture should be avoided. Please discuss.
Response: *Yes, the drawbacks of continuous running suture are well known, but in the interest of completing the long surgery and restoring homeostasis as quickly as possible in a transplant subject, we found the trade-off to be beneficial, and so have presented such.*

Figure 1: A schematic drawing of femoral vessels branches should be of great interest to the reader, in order to picture and map correctly this step.

Response: *Thank you. We thought the video would be able to illustrate this with greater satisfaction.*

Discussion

Line 322: Vascularized "Composite Allotransplantation" - VCA acronym was already defined.

Response: *Thank you. We thought it helpful to refresh the reader's memory without having to scroll the entire paper, especially if they are new to the subject.*

Line 324: authors didn't define anything very new in the experimental rat hindlimb transplantation: this statement is a bit overrated.

Response: *Thank you; the advantage of this model is that it is a platform for new discoveries; we will clarify.*

Reviewer #3:
Manuscript Summary:
The authors describe the well established rat hindlimb transplant model in addition of techniques to evaluate nerve regeneration. Overall, the manuscript will help junior scientists who are

interested in transplant research.

Major Concerns:
There are no major concerns related to the manuscript.

Response: *Thank you.*

Minor Concerns:
I have some minor concerns as well as some recommendations for the manuscript.
1. In the abstract, please revise or delete the statement regarding survival of the VCA. There is no survival for isogenic VCA and they survive indefinitely. The survival of the allogeneic VCA depends on the immunosuppression protocol and if the immunosuppression is not lowered or weaned the rat limb survives more that 300 days.

Response*: When considering putting their research animals through a major surgery, new investigators to this field may well wonder how successfully the animals survive. They may even be thinking about this problem before choosing to read the article, which is why we include it in the abstract. We can revise to make it more acceptable with appropriate terminology.* For example on page 1:
" With short-term treatment of conventional immunosuppressive agents, allotransplanted animals achieve morality up to 70 days post-operatively, and isotransplanted animals provide long lived controls beyond 200 days post-operatively."

2. Page 1 line 77-78, the authors mention development of new techniques, however, the current manuscript refines an existing technique. Thus, the sentence needs correction.

Response: *Yes, we present a refinement of an existing platform on which researchers can develop and test new techniques. We added the following sentence on page 1:*
" Given the non-life saving nature of limb transplant, current techniques need to be seriously refined in animal models to take the next step in VCA."

3. Auto-mutilation is an important morbidity and mortality in limb transplantation. If the authors discuss the auto-mutilation and the effects of ketamine usage, postoperative pain management and phantom pain, the manuscript will be more useful for the readers.

Response: *Yes, we agree; even a cursory review of the literature demonstrates autotony remains an intractable problem. We debated including more about this, but it seemed 1) the topic would venture too far afield for a methods paper on surgical technique 2) most labs prefer their own anecdotal prophylaxis techniques 3) some of these techniques may venture into the new technologies discussed above.*

4. I personally recommend usage of opioid analgesics (such as buprenorphine) for the first 2 days, which is also recommended in many centers. The authors may add this option in the manuscript.

Response: *Our lab also has had success with this technique in other models. However, in this model we valued flexibility afforded by isoflurane anesthesia.*

5. Page 4, line 181-182; please rephrase the sentence indicating putting the clamp first, then dividing the vessels.

Response: *Thank you; we will clarify on page 4:*
"Dissect out the femoral artery and vein with enough space to clamp each separately at the level of the inguinal ligament. Clamp the vein and artery with microsurgical bulldog clamps. Once clamped, each vessel is then divided sharply with scissors."

6. Page 5, line 218; Please indicate that additional sutures can be used if there is blood leakage at the anastomosis site.

Response: *Thank you. This seemed intuitive to us from a surgical perspective, but we will clarify. We added the following sentence to page 5:*
" Another suture may be placed through a bleeding hole at the risk of "back-walling" the needle only as a last resort.*"*

7. Please discuss the importance of hand sewn vessel anastomosis and the nerve repair.

Response: *Yes, thank you, we do feel these are important, and we wanted to share this. However, there are several other competing techniques in the literature, and it seems at this juncture data has yet to accrue in favor of one versus another, and we thought it prudent to focus on the method itself rather than delve into data driven arguments. We are currently investigating other forms of nerve repair strategies that are beyond the scope of thismanuscript (cuffs with integrated electro-stimulation/3D printed cell transplants), however we did offer the explanation:*
"Suture anastomosis although painstaking may potentially offer less technical confounding for long term studies. Nerve re-approximation allows for future investigation [17, 18] and gait analysis."

Reviewer #4:
This is a video presentation of a time-tested rat hind-limb transplant model. The authors present their technique of performing the transplantation though understandably there many ways to skin a rat …a cat. A good description in the article is supplemented by the video which mirrors the text written. This is particularly helpful to read and follow along in the video.

1. At 3.13 using 7 0 suture to retract is not necessary. Someone with microsurgical skills should be able to hold the vessels without damaging them. The audio should be modified to include this. The vessels are similar to digital vessels in small finger or a teenage child.

Response: *While we agree experienced microsurgeons are of course capable of low trauma tissue handling, we feel the technique may benefit investigators on the other side of the learning curve who are using this video to get started. Also, it may well be worth reminding veteran investigators of another technique for use in trouble spots.*

2. Ligating branches with a silk tie is time consuming. A bipolar cautery or heat coagulation is efficient and effective.

Response: *We have found this as well and can add into the text; however, on the whole we prefer the hemostasis without thermal injury spread afforded by ties.*

3. Ligating the external iliac on proximal side only is enough. This will allow maximum length of the donor pedicle without having to revise the donor vessels if they have a tie on it.

Response: *The reviewer makes a good point; maximizing the pedicle length aids in efficiency of the recipient surgery. But we feel there is still an argument to be made for dividing between ties to minimize blood loss and the immune priming effects of donor hemorrhage.*

4. At 13.15: The technique of transplantation is purely a model. It allows testing of different immunosuppressive protocols but it does not, by itself, contribute to long term survival. The survival is dependent on the immunosuppressant protocol.

Response: *Although the long-term survival success is indeed dependent on the immunosuppressive strategy, as surgeons, we strongly submit that good technique does matter, and if the operation is shoddy, immune suppression will not be able to salvage the case. Hence the importance of investigators sharing good technique in a journal dedicated to such.*

The authors should comment on how microsurgically savy the operator ideally needs to be to achieve an optimum survival rate with such small diameter vessels. What is the post-operative monitoring / analgesia protocol? The authors need to comment on the complications to look out for. What is the rate of these complications occurring?

Response: *We are trying to strike a balance between providing useful information to new investigators to this field to show them how feasible it would be for their particular situation and providing a different technical perspective for veteran micro surgical colleagues who already have a good grasp on the questions above. For the interested reader, we do comment on these issues in the text, but we strive to keep the video more succinct.*

Thanks kindly for you revision advice. We look forward to hearing from you in the near future.

Kyle M. Koss

Response to Reviewers:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript was proof read.

2. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg).

The figures were submitted as .psd

3. Figure 2: Please include a space before parentheses: Isograft (n = 7).

Spaces were included in the specified areas.

4. Figure 3: Please use SI abbreviations for time: s instead of sec

The units were all converted to s.

Changes to be made by the Author(s) regarding the video:

1. Please remove the JoVE logo from the title card. Please repeat the title card at the end of the video as well.

The Jove logo was removed and the title card was repeated at the end.

2. Please increase the video resolution. Most of the video is very low resolution. The video is formatted at 1920x1080 pixels, but most of the video in the protocol section is far below that resolution (likely at 480i resolution).

The entire video was redone at a the best resolution available. Note that the interviews do not compare the quality of the other sections. We are unable to retake these due to the lockdown and Dr. F. Zheng is no longer in the USA. If this is unacceptable, we would be will to remove this section.

3. Video Issues:

• 06:22 There's a flash frame in the background here during the fade out. It should be removed.

The figures were submitted as .psd

• 11:15 There's a jump cut here of the finished sutures. Consider dissolving in similarly to the preceeding shots.

A smooth transition was added.

• Please add three seconds of white or black to the end of the video.

A three second black screen was added at the end of the video

4. Audio Issues:

• 01:39 There is a bump on the microphone here

This sound was removed.

• 02:20 "Dissect and cauterize the -" bit of a jumbled performance here. Consider re-recording this line.

This line was edited to remove the awkward jumble.

• 08:50 We can hear a computer mouse click here.

This sound was also removed.

5. Animal Use Standards:

• 01:06 For the animal use approval notification, we require that a written card be placed here indicating/stating the names of the approving institutions. Essentially, what is said out loud here currently should appear on a card. It can be in the similar style to the proceeding "Protocol" black-on-white chapter title card.

A title card was added with the committee and protocol number during this audio.

• 01:26-01:33 Shot of rat in isoflurane chamber. Shots showing use of anesthesia or euthanasia are not permitted.

This shot was edited out.

• 06:25 Please remove this brief shot of the anesthesia as well.

This shot was also edited out.

Please upload a revised high-resolution video here:

https://www.dropbox.com/request/MiwtKVHtghpwqlrl8jPn?oref=e

The video is being uploaded with these figs and response.

Response to Reviewers:

Changes to be made by the Author(s) regarding the written manuscript:

1. No changes are requested.

Changes to be made by the Author(s) regarding the video:

1. Video & Image Framing:

• 00:28, 00:44 There is a black triangle in the lower left of the frame. Is it obscuring something? Can this be removed?

The black triangle was removed by resizing. The footage was taken off angle, so I have to tilt it to restore it.

• 01:07 Please adjust the web site image so that the edges of the site fill the sides of the video frame. There should be no black gaps or footage "behind" the image.

The image was resized to remove black background

• 05:13 Please fade out the bottom layer behind the microscope shot. It pops out as-is

A faded change was added. This may be what you wanted. Perhaps you could clarify.

• 12:51 For the outgoing video layer, fade into a white background so there is no black border behind the figures in the Results section. Make sure the background layer color matches the figure image background color.

A white background was added.

2. Editing:

• 04:18-04:22 There is series of jump cuts here that should be converted to dissolves.

Transitions were added.

• 11:17 The "reapproximate" here is a little cut-off at the beginning.

The full word was added.

Response to Reviewers:

Changes to be made by the Author(s) regarding the written manuscript:

1. No changes are requested.

Changes to be made by the Author(s) regarding the video:

• In the opening and closing Title Cards, there are a couple stray pixels above the words "Step" and "Neural" that should be covered with a matching white block or other erasing technique.

The pixels were removed.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Taking the Next Step: a Neural Coaptation Orthotopic Hind Limb Transplant Model to Maximize Functional Recovery in Rat
Author(s):	F. Zheng, A. Tully, K. Koss, X. Zhang, L. Qiu, J. Wang, B. Naved, D. Ivancic, J. Mathew, J. Wertheim, Z. J. Zhang1

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Zheng Jenny Zhang
Department:	Department of Surgery
Institution:	Feinberg School of Medicine, Northwestern University
Title:	Professor

Signature:  Date: 09/09/2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

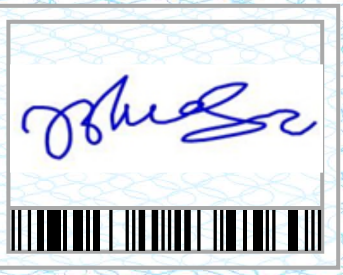
Signature Certificate

Document Ref.: PFVUN-N3HTP-N2U36-3CQVX

Document signed by:

Kyle Koss

Verified E-mail:
kyle.koss@northwestern.edu



IP: 165.124.144.97 | Date: 09 Sep 2019 18:06:05 UTC

Document completed by all parties on:
09 Sep 2019 18:06:05 UTC