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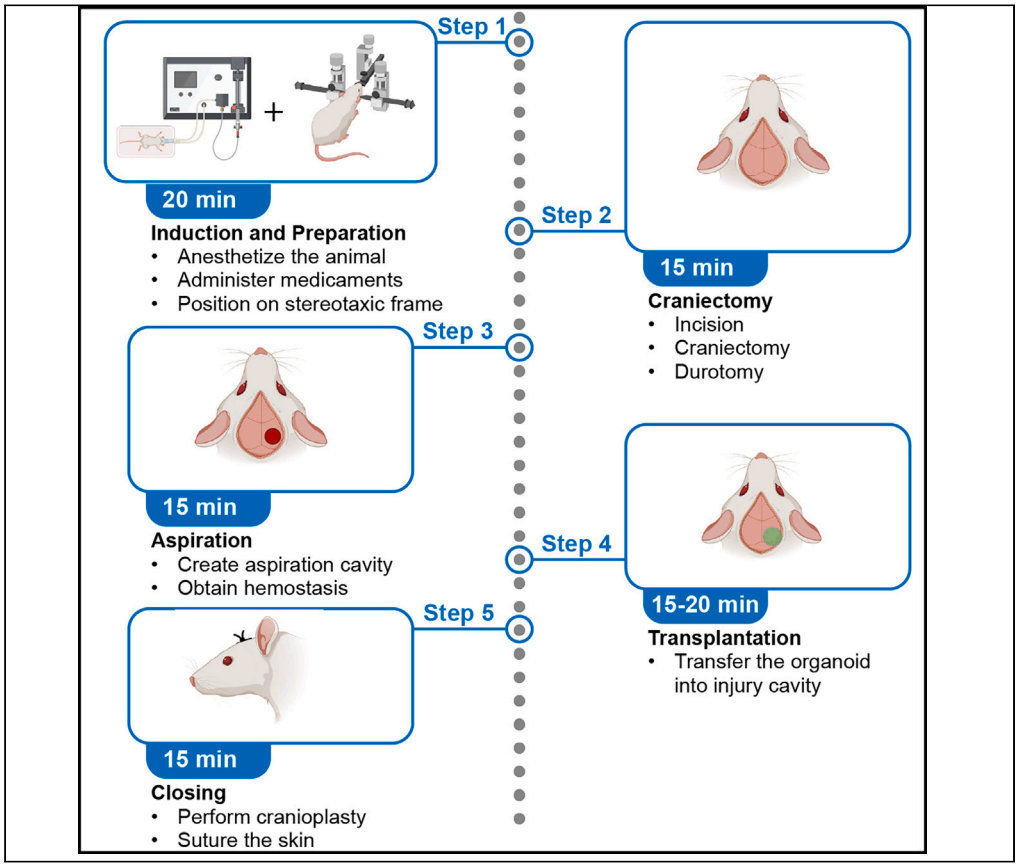
Protocol for human brain organoid transplantation into a rat visual cortex to model neural repair



Human stem-cell-derived organoids represent a promising substrate for transplantation-based neural repair. Here, we describe a protocol for transplanting forebrain organoids into an injured adult rat visual cortex. This protocol includes surgical details for craniectomy, aspiration injury, organoid transplantation, and cranioplasty. This platform represents a valuable tool for investigating the efficacy of organoids as structured grafts for neural repair.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Preparation of brain organoids for transplantation

Optimized protocol for creating a reproducible aspiration cavity in an adult rat cortex

Step-by-step guide for transplanting human forebrain organoids into an injury cavity

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Protocol for human brain organoid transplantation into a rat visual cortex to model neural repair

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SUMMARY

Human stem-cell-derived organoids represent a promising substrate for transplantation-based neural repair. Here, we describe a protocol for transplanting forebrain organoids into an injured adult rat visual cortex. This protocol includes surgical details for craniectomy, aspiration injury, organoid transplantation, and cranioplasty. This platform represents a valuable tool for investigating the efficacy of organoids as structured grafts for neural repair.

For complete details on the use and execution of this protocol, please refer to Jgamadze et al.[1]

BEFORE YOU BEGIN

The following protocol outlines the specific steps for transplanting human forebrain organoids into the visual cortex of young adult male Long Evans rats. The forebrain organoids described in this protocol were generated using a previously published protocol.[2] This method has been successfully implemented with organoids derived from both induced pluripotent stem cells and embryonic stem cell lines. For more detailed information on cell lines tried with this protocol, please refer to our previous study.[1,3] We intend for this protocol to be used primarily for the transplantation of cortical organoids into the rodent cortex, but it could be adapted for the transplantation of organoids of other region identities into the cortex. This injury repair model offers enhanced translational significance, enabling the examination of a brain organoid's structural and functional attributes in a living organism. Furthermore, it can serve as the foundation for extending neural transplantation efforts to brain areas beyond the visual system.

The aspiration method for creating a cortical cavity in this protocol provides a platform for investigating the basic principles of organoid graft integration with the neural circuitry of the host brain. It is also a reasonable model for repairing cavities created after neurosurgical resection of a lesion such as a brain tumor or vascular malformation. Study of neural repair for other conditions, including traumatic brain injury (TBI) and stroke, should adapt this protocol to specific models of those diseases (e.g., controlled cortical impact injury for TBI and middle cerebral artery occlusion for stroke).

Please note that the surgical procedures described in this manuscript involve advanced techniques and require specialized neurosurgical instruments. Furthermore, these procedures should only be attempted by individuals with a high level of experience in aseptic rodent surgery.[4]

Institutional permissions

All animal experiments described in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania and were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. Animals were housed in pairs under standard conditions and kept on a 12-h light/dark cycle (lights on at 6:00 a.m.) with ad libitum access to food and water. The transplantation of previously generated human pluripotent stem cell-derived brain organoids into animal hosts was reviewed and approved by the Stem Cell Research Oversight Committee at the University of Pennsylvania. Others who plan to replicate this protocol should obtain appropriate approvals from their institution.

Polydimethylsiloxane (PDMS) cranioplasty preparation

Timing: 2 days

1. Using the Sylgard silicone elastomer kit, combine 9 parts elastomer base to 1 part elastomer curing agent (both included in the kit) in a 100 mm dish.
2. Mix the base and curing agent thoroughly using a spatula for 3–4 min. The resulting mixture should be viscous and have a translucent appearance.

   Note: A total of 10 g of elastomer base and a curing agent will result in a piece of PDMS that is 1.5–2 mm thick.

   Optional: Place the dish in a vacuum chamber to eliminate any bubbles that may have formed.

3. To cure the PDMS, place the dish in an oven at 65°C–70°C for approximately 1 h or at 25°C for 48 h.

   Note: Sterile conditions are not required for this step.

4. Rinse the cured PDMS by briefly submerging it in isopropanol.
5. Let the PDMS dry.
6. To improve the biocompatibility of the PDMS and remove any silicone residue, submerge the PDMS in boiling water for 20 min using a 250 mL Pyrex beaker and a hot plate.
7. Let the PDMS dry.
8. Transfer the PDMS to an autoclave pouch.
9. Sterilize the PDMS using an autoclave.
   a. Suggested autoclave settings are 1 h at 121°C with 15 min of pre-vacuum at a pressure of 27 inches of mercury.
   b. After autoclaving, the PDMS is stored at 25°C and used within 48 h.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
Human WT iPSC line "C1.2" derived from healthy fibroblasts (male) and modified by lentiviral transduction to express GFP	ATCC fibroblasts (CRL-2097).[5]	C1–2-GFP
Experimental models: Organisms/strains		
Long-Evans rat (male)	Charles River Laboratories	006

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
32G needle	Thermo Fisher	NC9356425
Bulldog Serrefines X4	Fine Science Tools, Inc.(FST)	18051-35
Dumont #5 forceps X2	FST	11251-20
Dumont #7 fine forceps	FST	11274-20
Dumont #7b Forceps standard serrated Inox	FST	11270-20
Friedman Rongeurs bone cutter	WPI	14089
Hair clipper	Wahl	CL9990
Heat therapy water pump HTP-1500	Adroit Medical System	D8100518
Industrial Q-tips AP-5	As One	1-8584-05
Latex exam gloves: sterile, powder-free	DermAssist	104350
Micro curette	FST	10082-15
Micromotor drill	Stoelting	NC9278958
Needle holders with suture cutters	FST	12502-12
Scalpel handle #3	FST	10003-12
Standardization pipet tips, 0.1–200 µL	Thermo Fisher	02-681-142
Sterile disposable biopsy punch, 2.5 mm	IntegraMiltex	MFID: 33-32
Sterile disposable biopsy punch, 5 mm	IntegraMiltex	MFID: 33-35
Sure-Seal induction chamber mouse/rat 4″ x 9″ x 4″	Braintree Scientific	EZ-17-8
Surgical caliper	FST	18000-35
Surgical stainless steel sterile blades #10	Royaltek	22-079-683s
Ultra-Bright LED Longwave UV Flashlight	VWR	470154-572
Charcoal filter canister	EZ systems	EZ-258
Preparation area nose cone	EZ systems	EZ-103A
Induction chamber	EZ systems	EZ-178
Anesthesia machine	EZ systems	EZ-190F
Artificial tears ophthalmic ointment, 3.5 gm	Bausch+Lomb	IWM701013
Bupivacaine	APP Pharmaceuticals	CAS Number: 38396-39-3
Cyclosporine A (100 mg)	BioVision, Inc.	CAS Number: 59865-13-3
Dexamethasone sodium phosphate (4 mg/mL)	Mylan	CAS Number: 2392-39-4
Isoflurane, USP	Piramal Critical Care	CAS Number: 26675-46-7
Meloxicam	Midwest Vet Supply	CAS Number: 71125-38-7
Necrostatin-1	Enzo Life Sciences	CAS Number: 4311-88-0
Adhesive resin cement	3M ESPE	56892
Betadine swabs	Purdue	CAS Number: 25655-41-8
Bovie cautery	Bovie Medical	AA25
Conical centrifuge tubes 50 mL	Falcon	352070
Gelfoam	Medline	09-0353-01
Instant sealing sterilization pouches	Fisherbrand	01-812-54
Isopropanol, 99.5%	Thermo Fisher	CAS Number: 67-63-0
Paraformaldehyde solution 4%	ChemCruz	SC-281692
Petri dish 100 mm	Corning	BP93B-102
Sylgard silicone elastomer kit - polydimethylsiloxane (PDMS)	Dow Silicones Corporation	CAS Number: 68037-59-2
Silk suture 4-0	Ethicon	K871H
Sodium chloride 0.9% (normal saline), USP	VWR	CAS Number: 7647-14-5
Stereotaxic instrument, ultra precise small animal	Kopf	Model 963

Note: Alternative tools, drugs, and reagents could potentially be used but were not tested as a part of this protocol.

STEP-BY-STEP METHOD DETAILS

Pre-treatment of animals with cyclosporine

Timing: 5 min

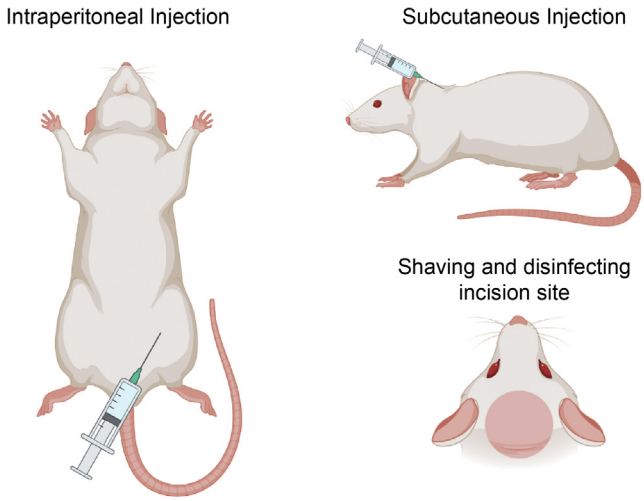

Figure 1. Diagrams illustrating pre-procedural steps such as intraperitoneal and subcutaneous injections as well as shaving and preparation of the cranial surgical site

Immunosuppression is vital for xenograft transplantation in immunocompetent animals, as they suppress the host's immune response and prevent graft rejection. Cyclosporine A enhances transplant success by inhibiting T-cell activation and interleukin-2 production.[6]

Note: An alternative to Cyclosporine A immunosuppression of immunocompetent host animals is the use of immunodeficient animals such as nude rats.

⚠ CRITICAL: Daily Cyclosporine injection must begin at least 2 days prior to transplantation surgery.

1. Administer intraperitoneal injections of Cyclosporine A (10 mg/kg).
   a. Identify the right or left lower quadrant of the rat's abdomen to avoid puncturing internal organs such as the cecum and bladder. (Figure 1).
   b. Clean the injection site with a 70% isopropyl alcohol pad.
   c. Hold the syringe at a 20°–30° angle to the rat's body (supine position), with the bevel of the needle facing up.
      i. Quickly and smoothly insert the needle into the peritoneal cavity, taking care not to insert it too deeply.
      ii. Aspirate gently to ensure that no blood, urine, or fecal matter is drawn into the syringe, which would indicate that an organ or blood vessel has been punctured.
      iii. Slowly inject the solution and withdraw the needle.
2. Continue performing cyclosporine A injections daily until the designated experimental time point for animal sacrifice.

Note: Cyclosporine A is stored at room temperature at a stock concentration of 50 mg/mL, which is diluted 1:10 to achieve a working concentration of 5 mg/mL immediately before use.

Organoid incubation with Necrostatin-1

Timing: 24 h

To mitigate post-transplantation cell death due to necroptosis, incubate the organoids in Necrostatin-1 (Nec-1) for 24 h immediately before transplantation. Nec-1 is a specific inhibitor of the kinase domain of receptor-interacting protein kinase-1 (RIPK1).[7]

We advise transplanting only organoids that have sharp borders suggesting a healthy neuroepithelium and no apparent cysts. We have successfully transplanted organoids at the ages of 20–80 days. For more details on organoid generation, please refer to our previously published study.[2]

3. Prepare Nec-1 solution.
   a. Prepare 5 mL of Nec-1 working solution (20 μg/mL), dilute 5 μL of 20 mg/mL Nec-1 stock solution in 5 mL of organoid maturation medium.[1]
   b. Vortex the solution briefly.
4. Transfer the organoid into the Nec-1 solution.
   a. Cut the tip off a P200 pipette tip using a sterile scalpel blade so that an organoid can fit within the tip without any physical contact with the tip itself.
   b. Use this cut pipette tip to transfer organoids to a 6-well plate (1 organoid per well).

   Note: This process is performed under sterile conditions.

   c. Add 5 mL of Nec-1 working solution per well,
   d. Place the plate on an orbital shaker until the organoids are ready for transplantation.
5. The next day, move the plate with organoids to an intermediary incubator in the surgical suite.

   Note: This step minimizes traffic between the surgical suite and the cell culture room and decreases the risk of culture contamination.

Animal preparation

This step encompasses anesthesia induction, all necessary pre-procedural medications, animal positioning in the stereotaxic frame, and sterilization of the surgical site. Anesthesia setup for major rodent surgeries typically involves a multi-stage process to ensure the comfort and safety of the animal. After the animal is initially anesthestized, a stable level of anesthesia is maintained for all subsequent steps of the surgical procedure.

Anesthesia induction and maintenance

Timing: 7–9 min

Note: Anesthesia procedures are performed in three main areas: the induction chamber, animal preparation area, and stereotaxic frame. The induction chamber and stereotaxic frame are connected to an Isoflurane vaporizer machine (e.g., EZ-190 Auto Flow Anesthesia Machine).

6. Determine the weight of the animal.
7. Place the animal into an anesthesia induction chamber.
   a. Initiate isoflurane flow at 5% and turn the oxygen flow meter to 1 L/min.
   b. Wait 5 min for anesthesia induction.
   c. All medications to be administered can be prepared at this stage.

   Note: The induction chamber is used to introduce the rodent to an anesthetic agent, commonly an inhalant anesthetic such as isoflurane, in a controlled environment. The chamber is a sealed, transparent container connected to an anesthesia machine, which delivers the anesthetic gas mixed with oxygen.

8. Transfer the animal to the surgical preparation area.
   a. Place the anesthesia device nose cone over the animal's nose to keep the animal anesthetized for the duration of this procedure.
   b. Decrease isoflurane concentration to 4%.

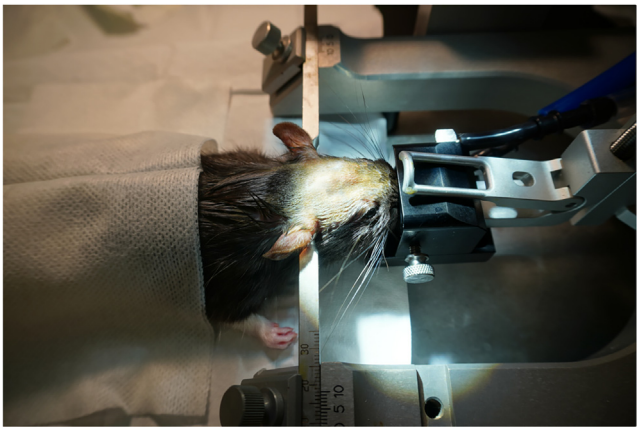

Figure 2. Rat head fixation in a stereotaxic frame
The animal is fixed in place using ear bars and a bite bar with the nose securely positioned within the nose cone to maintain stable anesthesia.

9. Apply ointment to both eyes.

Pre-procedural medication administration and surgical site preparation

Timing: 5 min

10. Administer intraperitoneal injections of Dexamethasone (1 mg/kg) and Cyclosporine A (10 mg/kg) as described in Step 1.
11. Administer Meloxicam subcutaneously (SQ, 2 mg/kg). The injection site is on the animal's upper back, near its scapula (Figure 1).
12. Remove fur from the cranial surgical site using a hair clipper. It is advisable to shave the majority of the scalp surface.
13. Initiate isoflurane and oxygen flow to the stereotaxic frame nose cone and wait 20–30 s for the gas to reach the nose cone before proceeding to the next step.

Positioning the animal in the stereotaxic frame

Timing: 5 min

⚠ CRITICAL: After placement in stereotactic frame, anesthesia is maintained via a nose cone connected to the anesthesia machine, with continuous monitoring of vital signs, such as heart rate, respiratory rate, and body temperature.

14. Carefully place the anesthetized rat in the prone position on the stereotaxic frame's platform (Figure 2).
    a. Ensure the rat's incisors engage with the bite bar to support the head.
    b. Use a cotton swab to ensure the bottom teeth and tongue are below the bite bar and do not obstruct the airway.
    c. Slide the nose cone over the rat's mouth, ensuring the nose cone covers the jaw.
    d. Position the left ear bar into the rat's ear canal. Avoid penetration of the eardrum.
    e. Position the right ear bar inside the other ear canal, following the same principles as the previous step.
    f. Ensure the ear bars are centered.
    g. Decrease the Isoflurane concentration to 2.5% by adjusting the flow rate on the isoflurane regulator.

15. Sterilize the incision area.
    a. Clean the area using a 70% isopropyl alcohol pad, followed by a betadine pad. Repeat the process three times using a fresh pad each time.

Note: Clean from the center of the incision site outwards in a circular motion

Incision and skull exposure

Timing: 10 min

16. Put on sterile gloves.
17. Check the animal's toe-pinch reflex.

Note: Use sterile gauze to touch non-sterile surfaces

18. Perform a midline scalp incision using a scalpel.
    a. Gently push connective tissue laterally on both sides of the skull using sterile cotton swabs.
    b. Retract the scalp with Bulldog Serrefines.
    c. Cut the full perimeter of the exposed connective tissue using a scalpel.
19. Use a Bovie cautery device to stop any bleeding.
20. Clean the exposed bone by rinsing it with saline. Remove the saline and dry the area using vacuum suction and sterile cotton Q-tips.
21. Gently scrape the exposed bone with a scalpel to improve the adhesion of bone cement in later steps.
    a. Gentle abrasion of the outer cortex of the skull should be performed using a scalpel blade with care to apply only light pressure.

Craniectomy

Timing: 15 min

Craniectomy is a surgical procedure in which a portion of the skull is removed to expose the underlying brain.

22. Mark the points of reference using a hand drill.

Note: The drill is used to make marks on the bone to preserve the sterility of the area. A newly opened sterile skin marker can be used as well.

    a. Use a drill to mark bregma on the bone surface lightly.

Note: Bregma is the point where the coronal suture intersects with the sagittal suture (Figure 3A).

    b. Identify and mark with the drill the point along the midline corresponding to the craniectomy's anterior-posterior center (5 mm posterior to bregma to target visual cortex).

Note: This mark and bregma will serve as guide points for the craniectomy.

    c. Mark the center of the circular craniectomy with the drill (2.5 mm lateral to the midline, 5 mm posterior to bregma).
    d. Etch the craniectomy circumference using the sharp edge of a sterile caliper (2.5 mm in radius).
    e. Mark the circumference of the craniectomy with the drill, ensuring the previously etched mark remains the outermost boundary of the craniectomy.

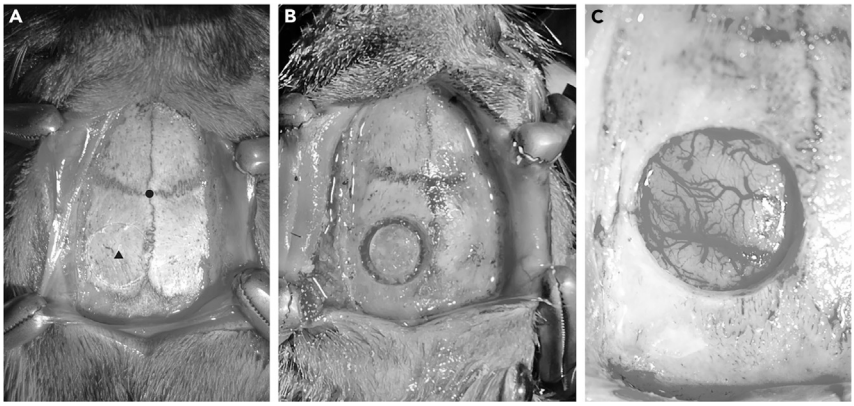

Figure 3. Skull exposure

To expose the skull before craniectomy, adipose tissue, and the underlying periosteum are removed.
(A) The craniectomy site is marked using a caliper by scratching the surface of the skull. The figure shows the skull with an etched circle that is 5 mm in diameter. Black dot: bregma. The black triangle is the center of the circular craniectomy (5 mm posterior to bregma, 2.5 mm lateral from midline).
(B) A drill has been used to create a trough along the etched circle in A. Only a very thin layer of flexible bone remains, which allows the bone flap to be peeled off. (C) The bone is carefully removed with curved tweezers revealing the underlying dura and brain.

23. Drill the craniectomy perimeter.
    a. Remove the bone uniformly along the trough made by the drill and avoid excessive force that may cause the drill to plunge through the skull and damage the underlying brain.

Note: As drilling proceeds, the remaining bone will become softer as it thins.

    b. Do not drill completely through the skull; thin the bone at the craniectomy perimeter until the bone flap moves vertically as you apply gentle pressure.

Note: Minimize drill usage when the bone flap is mobile to reduce potential trauma to the underlying brain.

24. Carefully remove the circular bone fragment.
    a. When the bone flap is adequately thinned, use fine forceps to remove it, exposing the dura and brain beneath.
    b. The use of two pairs of curved, serrated tweezers can facilitate the lifting of the flap in a uniform manner.

Note: Ensure that the bone flap edges do not sink into the brain when it is being removed, which can cause brain swelling.

25. Clean the craniectomy defect edge.
    a. Use a bone curette to remove any residual bone on the edge of the craniectomy defect.
    b. Ensure no residual bone is visible before proceeding to the next step.

⚠ CRITICAL: Removal of the bone flap in a manner that minimizes swelling of the underlying brain can improve transplantation outcomes.

Durotomy

Timing: 10–20 min

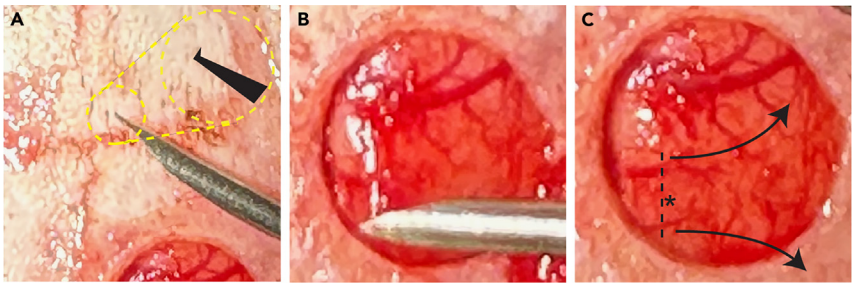

Figure 4. Durotomy

(A) To create a hook for opening the dura, the tip of a 32G needle has been bent slightly in the opposite direction of the bevel. The round inset shows a graphical representation of the bent needle tip.

(B) After the initial durotomy, the needle is cautiously inserted between the brain and the dura, with the bevel facing the brain. The needle is then moved horizontally to expand the opening of the durotomy in a linear fashion.

(C) Graphical representation of the durotomy procedure. The asterisk represents the initial tear of the dura with the bent needle. The dashed line shows the linear durotomy made by the needle, and the arrows show how tweezers are used to open the dura further.

26. Modify a 32G needle by creating a right-angle bend at the tip of a 32G needle.

Note: The needle can be gently tapped against a hard, **sterile** surface to create the bend (Figure 4A).

27. Use the modified needle to create a small opening in the dura by brushing the bent tip delicately against the dural surface.
    a. The tip should catch against the dura, and gentle pulling of the needle should result in a small tear in the dura. A position where the initial tear is made is shown in Figure 4B by an asterisk.
    b. Widen the dural opening (Figure 4B dashed line) by carefully inserting the right-angle bend of the needle between the brain and dura through the previously made dural tear.
    c. The bend of the needle should tent up the dura, and the bevel of the needle should face the brain.
    d. Once the needle tip is in position, slide it against the dura horizontally to cut the dura and further enlarge the durotomy. This should result in a long, linear dural opening.
    e. Further expand the dural opening to widely expose the brain using straight tweezers to tear the dura to the edges of the craniectomy defect. The arrows show the direction of tears using tweezers in Figure 4B.
    f. Throughout the procedure, intermittently apply saline to the exposed brain to prevent desiccation.

Note: Initial tear can be started anywhere at the craniectomy site. It is safest to initiate it away from the blood vessels. Do not open the dura medially; blood vessels tend to stick to the dura in that area; opening the dura will inadvertently lead to profuse bleeding in the area, which will be difficult to stop.

Aspiration

Timing: 15 min

28. Use a 2.5 mm sterile disposable biopsy punch to punch out 4–6 pieces of Gelfoam.
29. Identify an area close to the center of the craniectomy that avoids disruption of large blood vessels.
30. Attach a sterile 200 μL pipette tip to tubing that is hooked up to a vacuum system. The pipette tip will act as the aspiration instrument to remove brain tissue.

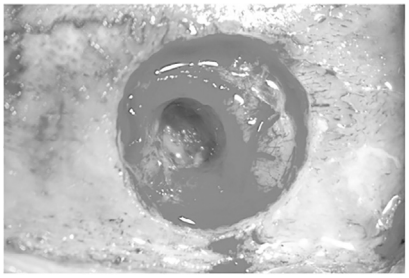

Figure 5. Aspiration cavity
A cortical cavity is created using vacuum aspiration.

31. Start aspirating brain tissue to create a 2–2.5 mm cavity in diameter, depending on the size of the organoid (Figure 5).

Note: The cavity should be just larger than the organoid so that manipulation of the brain and/or organoid is not needed for subsequent transplantation, and there is no excess space in the cavity.

a. Starting at the perimeter of the intended aspiration cavity, move the pipette tip in a spiral motion toward the center of the cavity.
b. Remove the tissue at a uniform depth within the cavity to improve visualization.
   i. Perform multiple "outside-in" spiral motions at progressively deeper depths.
c. Aspirate the entire thickness of the cortex until the corpus callosum is identified.

Note: Typically, the corpus callosum has a whiter appearance than cortical tissue.

d. After the cavity is made, irrigate it with saline for a few seconds before placing a piece of Gelfoam in the cavity to promote hemostasis.
e. Remove the Gelfoam after 1–2 min and reassess the state of hemostasis.
f. Exchange pieces of Gelfoam in the cavity with a 1–2 min wait after each fresh piece of Gelfoam until complete hemostasis is achieved.

Note: Gentle pressure on the Gelfoam with a cotton swab can help promote hemostasis. Patience is key to obtaining adequate hemostasis.

g. Once hemostasis has been achieved, fill the cavity with saline.

Transplantation

Timing: 15–20 min

32. Take the well plate with organoid out of the incubator.
33. Add sterile saline to an empty well. Add 1–2 mL of saline, depending on the well plate used.
34. Use a cut P200 pipette tip (see above) to transfer the organoid into a well with saline to wash off the media.
35. Transfer the organoid into the cortical aspiration cavity using the same cut pipette tip (Figure 6A).

⚠ CRITICAL: Make sure there is no evidence of any bleeding within the aspiration cavity before transferring the organoids. Hemostasis should be obtained using the steps described above if new bleeding is identified.

36. Gently remove excess fluid from the brain surface and skull. This step is necessary to increase organoid-to-host tissue adhesion.

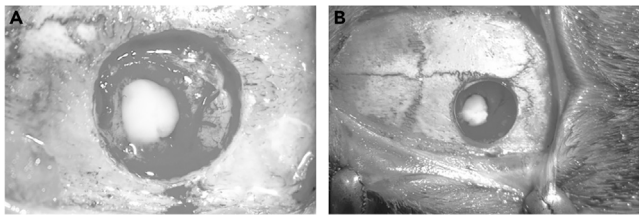

Figure 6. Insertion of forebrain organoid intro injury cavity
(A) Close-up view of an organoid graft after it has been placed in the cortical aspiration cavity.
(B) Lower magnification view of the transplanted organoid with the PDMS cranioplasty inserted.

a. Do not bring the vacuum aspirator near the transplanted organoid to prevent inadvertent damage to the organoid.
b. Place a piece of Gelfoam (pre-soaked but relatively dry) near the organoid to remove fluid near the organoid graft. Repeat this step as necessary to remove as much excess fluid as possible.

⚠ CRITICAL: Do not touch Gelfoam directly to the organoid as it might stick to the organoid and will be impossible to separate.

c. Reapply saline very slowly not to displace the organoid from the injury cavity.

Cranioplasty

Timing: 20–30 min

37. Punch out a 5 mm diameter piece of PDMS from the PDMS prepared earlier using a sterile tissue punch.
38. Transfer the PDMS piece into the craniectomy defect (Figure 6B).

Note: It will act as a replacement for the piece of skull that was previously removed. Careful attention to creating a circular craniectomy previously should ensure that the PDMS fits well and does not pop out.

a. Once the PDMS cranioplasty is in place, apply a layer of bone cement on top of the PDMS as well as the surrounding skull (Figure 7A).
b. Allow the bone cement to dry for 10–15 min.
c. Once the bone cement is dry, irrigate the surgical site with saline.
d. Close the incision with a silk suture by making 7–8 simple interrupted knots (Figure 7B).[8]

Postoperative care

Timing: Varies depending on the length of the study

After the surgery, proper care and monitoring of the animals are crucial to ensure their recovery and maintain experimental integrity. The following steps provide an outline for postoperative care and analysis of the brain samples.

39. Place the animal in a recovery chamber with a heating pad set to 37°C.
40. Monitor the animal's breathing and responsiveness. Ensure that the animal is recovering from anesthesia and regaining consciousness.
41. Return the animal to the facility only after the animal is fully recovered from anesthesia.

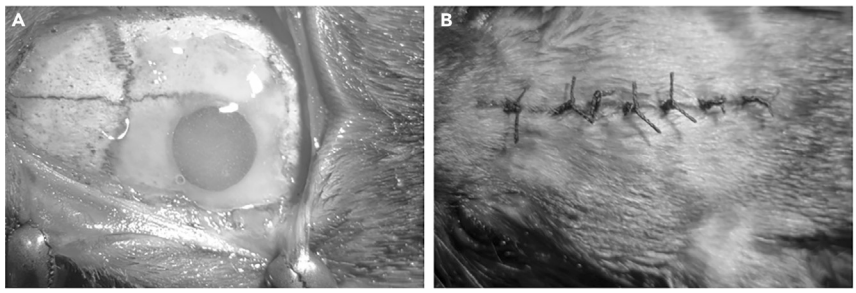

Figure 7. Bone cement application and incision closure
(A) Bone cement is applied over the PDMS cranioplasty.
(B) Using interrupted knots, the skin is approximated evenly.

42. Monitor the animal daily for signs of discomfort or infection. Check the surgical site for redness, swelling, or discharge.
43. Inject Meloxicam SQ (2 mg/kg) daily for two days after the procedure.
44. Remove the sutures 7–10 days after the procedure.

Brain extraction and gross organoid visualization

Timing: 20–30 min

Animal euthanasia and transcardiac perfusion[9] are performed using standard, widely-available protocols. However, a more specific aspect of our tissue processing protocol is making sure the organoid is not caught in connective tissue during extraction, which might cause the organoid to be inadvertently removed from the host brain. Additionally, we use a UV light to visualize GFP^+ organoid survival on gross inspection. Details of this process are provided below.

45. After standard transcardiac perfusion of the animal with 4% paraformaldehyde, open the prior incision with a scalpel and expose the skull and bone cement as detailed above.
    a. Use a scalpel to carefully remove the connective tissue layer without removing the PDMS or organoid.
    b. Visualize and remove the PDMS from the craniectomy site.
    c. If there is scar tissue, cut around it and leave it on the brain as it might be attached to the organoid.
    d. Confirm the presence of a GFP^+ organoid graft using UV light (Figure 8).
46. Make a small hole in the skull anterior to the brain using scissors.
    a. Using that hole, crack open the skull with the rongeur's bone cutters.
    b. Take off the skull piece-by-piece without damaging the brain, starting contralateral to the organoid.
    c. Take extra precaution when removing bone around the organoid, as dura may be attached to the organoid.
47. Extract the brain gently.
48. Place the brain into a 50 mL conical tube filled with 4% paraformaldehyde (PFA) and place it in a cold room (+4°C) for 24 h.
49. Transfer the brain to 30% sucrose solution in a 50 mL conical tube until the brain is quenched and sinks to the bottom of the conical tube.
50. Proceed with standard freezing and staining protocols.

EXPECTED OUTCOMES

Upon successful completion of the protocol without complications, we anticipate that the human forebrain organoid will effectively integrate structurally and functionally with the host brain. Our

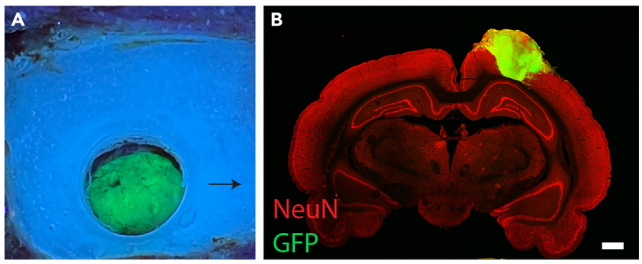

Figure 8. Organoid visualization after animal sacrifice

(A) The GFP-expressing organoid graft can be visualized *in situ* before brain extraction via illumination with ultraviolet light. Anterior is designated with the arrow.

(B) This micrograph shows a coronal brain section with the GFP-positive organoid (scale = 1 mm).

previous study document extensive projections emanating from the organoid with evidence of synaptic connectivity with the host brain using virus-based trans-synaptic tracing methods. Moreover, visual stimulation of the host animal resulted in an evoked single unit and local field potential responses, as well as orientation selectivity in response to drifting grating stimulation.[1]

Animals were maintained for up to three months following the procedure, as the efficacy of cyclosporine immunosuppression diminishes with long-term use. It is important to note that this protocol has not yet been tested in genetically immunosuppressed host animals.

LIMITATIONS

The use of cyclosporine for post-operative immunosuppression limits the time frame for graft survival to roughly 3 months post-transplantation. The use of immunodeficient rat models is an alternative that circumvents this limitation.

Standard forebrain organoids exhibit an occipital cortex phenotype, which potentially limits the generalizability of this organoid generation and transplantation protocol to non-visual cortex regions. Alternative organoid protocols may be required for organoid transplantation in other regions.

TROUBLESHOOTING

Problem 1

Bleeding at the edge of the craniotomy.

Potential solution

Such bleeding is usually caused by a remnant of a sharp bone fragment left behind after the bone flap is removed. Make sure to clear the edges of the craniectomy defect of any residual pieces of bone thoroughly before proceeding with durotomy. Gelfoam is effective for stopping any bleeding that is encountered.

Problem 2

Bleeding after the organoid is transferred.

Potential solution

If the bleeding occurs after the organoid is transferred, the organoid must be removed, the surgical cavity rinsed with saline, and hemostasis achieved before the organoid is replaced into the injury cavity. If the organoid gets damaged in the process, use a new organoid.

Problem 3

Craniectomy is bigger than 5 mm PDMS.

Potential solution

Have a bigger (6 mm) biopsy punch ready, punch out a bigger PDMS piece, and shape the PDMS as necessary to fit into the craniotomy.

Problem 4

Wound dehiscence.

Potential solution

Resuture the incision after removing any granulation tissue. Make sure to tighten the knots just enough to close the incision but not so tight that blood flow to the skin at the incision edges becomes overly restricted. Administer Meloxicam for two days to minimize local incision irritation that would cause the animal to scratch at the incision.

Problem 5

No organoid survival.

Potential solution

Many factors, including missed cyclosporine injections, mycoplasma contamination of the organoid before transplantation, and infection at the incision site can cause this problem.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Han-Chiao Isaac Chen (isaac.chen@pennmedicine.upenn.edu).

Materials availability

This paper does not generate new materials.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code.

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AUTHOR CONTRIBUTIONS

D.J. developed and modified the protocol described here. D.J., P.M.H., and H.-C.I.C. wrote the manuscript with help from all co-authors. All authors performed and analyzed the experimental part corresponding to this protocol. H.-C.I.C. supervised the study. All authors commented on and edited the manuscript.

DECLARATION OF INTERESTS

G.-l.M. is on the editorial board of *Cell Stem Cell*.

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