Protocol

Cell Transplantation as a Method to Investigate Spinal Cord Regeneration in Regenerative and Nonregenerative *Xenopus* Stages

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Mammals are not capable of regenerating their central nervous system (CNS); anamniotes, however, can regenerate in response to injury. The mechanisms that explain the different regenerative capabilities include: (i) extrinsic mechanisms that consider the cellular environment and extracellular matrix composition, (ii) intrinsic factors implicating the presence or absence of genetic programs that promote axon regeneration, and (iii) the presence or absence of neural stem and progenitor cells (NSPCs) that allow neurogenesis. *Xenopus laevis* is able to regenerate its CNS during larval stages (i.e., the regenerative stage [R-stage]). However, concomitant with metamorphosis this capacity decreases and is lost completely in juvenile froglets (i.e., nonregenerative stages [NR-stages]). The loss of the regenerative ability correlates with a reduction in the percentage of Sox2⁺ cells, which are putative NSPCs. This protocol shows the effect of transplantation of spinal cord cells from R-stage *Xenopus* larvae into NR-stage froglets. Using this procedure, it is possible to study axon regeneration and stem cell biology in vivo.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Bovine serum albumin (BSA; 0.5 mg/mL in L-15 Medium)

Ethyl 3-aminobenzoate methanesulfonate salt (0.02% [w/v] in 0.1× MBS [MS-222; Sigma-Aldrich A5040])

The solution can be stored for 1 mo at 4°C.

Fibrinogen (100 mg/mL in phosphate-buffered saline [PBS]; Sigma-Aldrich F6755)

Final medium <R>

L-15 Medium (Sigma-Aldrich L4386)

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From the *Xenopus* collection, edited by Hazel L. Sive.

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Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot101006

Modified Barth's solution (MBS) (1×) <R>

Penicillin

StemPro Accutase cell dissociation reagent (Gibco A1110501)

This enzyme has protease and collagenase activity to allow tissue and cell dissociation.

Streptomycin

Thrombin (Sigma-Aldrich T5772; 100 U/mL in PBS)

Thrombin cleaves fibrinogen into fibrin to form a clot.

Trypan Blue solution (Sigma-Aldrich T8154)

Trypan Blue is a vital stain that allows discrimination between dead and living cells because it is only incorporated by cells with a damaged membrane.

Xenopus laevis animals (stages NF50 and NF66; Nieuwkoop and Faber 1994)

Transgenic animals make the identification of donor and host cells easier.

Equipment

Forceps (e.g., FST, Student Dumont #5)

Hemocytometer

Light source (e.g., Fiber-Lite Mi-150 Illuminators [Dolan-Jenner])

Microcentrifuge

Microscissors, straight (e.g., Vannas-Tübingen spring scissors [FST 15003-08])

Needle (23-g) (optional; see Step 13)

Parafilm M (Sigma-Aldrich P7793)

Petri dish (glass, 100-mm)

Stereomicroscope (e.g., Nikon, SMZ745T)

Tissue paper (e.g., Kimwipes)

Tubes (microcentrifuge, 1.5-mL)

Vortex mixer (e.g., V1 Plus [Boeco])

METHOD

Spinal Cord Isolation and Tissue Dissociation

Use three to four tadpoles to obtain sufficient cells per each froglet to be transplanted.

1. Place a larva at NF Stage 50 into a Petri dish with 50 mL of MS-222. After 3 min, check for an escape reflex.

The absence of a response indicates that the animal is anesthetized.

2. Transfer the larva to a dissection surface under the stereomicroscope.
3. Remove and discard the head and the posterior third of the tail.
4. Holding the remaining part of the trunk, use tweezers to pull the spinal cord out of the body.

See Troubleshooting.

5. Transfer the spinal cord to a 1.5-mL microcentrifuge tube containing 500 μ L of Accutase. Seal the tube with Parafilm.

- i. Incubate at room temperature with agitation (e.g., using a vortex mixer) for 1 h.

- ii. To avoid having the spinal cords adhere to the walls of the tube, mix the tubes by manual inversion every 15 min.

6. Add 1 mL of 0.5 mg/mL BSA in L-15 Medium to inactivate the Accutase.

7. Pellet the cells by centrifugation at 200g for 5 min. Resuspend the pellet in 1 mL of final medium.

8. Stain a 10- μ L aliquot with trypan blue. Count the cells using a hemocytometer.
See Troubleshooting.
9. Pellet the cells again by centrifugation at 200g for 5 min. Carefully remove and discard the supernatant.
10. Resuspend the cells in final medium to attain a concentration of 1×10^5 cells/ μ L.
There is no need for further supplementation with growth factors.

Spinal Cord Resection

11. Place a froglet at NF Stage 66 into a Petri dish containing 50 mL MS-222 for 10 min. Check for the absence of an escape reflex.
See Troubleshooting.
12. Place the froglet under a stereomicroscope.
13. Using microscissors (or a 23-g needle) as a scalpel, open the dorsal skin with a longitudinal cut above the spinal cord between the fifth and sixth vertebrae.
14. Carefully lift the dorsal muscles to expose the vertebrae.
15. To expose the spinal cord, use a pair of tweezers to perform a dorsal laminectomy and remove the fifth and sixth vertebrae.
16. Using microscissors, perform a 1-mm resection of the spinal cord.
Make sure that no uninjured nerve tracts are left.
17. Blot the area using tissue paper until bleeding stops. Leave a cavity between both stumps to place the cells.

Transplantation Procedure

18. Mix 1 μ L of the cell suspension (1×10^5 cells/ μ L) obtained from the NF Stage 50 spinal cord (Step 10) with 0.5 μ L of fibrinogen and 0.5 μ L of thrombin.
Clot formation will begin as soon as the thrombin and fibrinogen mix. The growth factors in the suspension are diluted to a final concentration of 10 ng/mL.
19. Immediately transfer the mixture to the resected site using a micropipette.
See Troubleshooting.
20. Two minutes after cell transplantation, reappose the dorsal muscle and skin. Hold closed with tweezers for a few seconds.
Avoid disturbing the clot in the spinal cord.
21. Carefully transfer the froglet to a recipient Petri dish containing $0.1 \times$ MBS supplemented with penicillin (100 μ g/mL) and streptomycin (100 μ g/mL). Use a volume just sufficient to cover the animal.
See Troubleshooting.

TROUBLESHOOTING

Problem (Step 4): The spinal cord breaks during the dissection.

Solution: Try to remove the surrounding muscle to expose the spinal cord before pulling it out.

Problem (Step 8): Low amount of living cells or clusters of cells

Solution: We use 60 spinal cords per mL of the enzyme. With higher numbers, there is no guarantee of a good yield.

Problem (Step 11): Froglets are still awake after 10 min.

Solution: Froglets weighing 1 g or less should be anesthetized in 10 min. Larger animals take longer.

Problem (Step 19): The quantity of liquid overflows the injury site.

Solution: The cavity should hold up to 2 μ L. If the liquid overflows the site, check the size of resection. Larger volumes require larger pieces to be resected.

Problem (Step 21): The froglets die soon after the surgery.

Solution: Allow the animals to recover in a volume just barely sufficient to cover, as they will be unable to swim to reach the surface to breathe.

DISCUSSION

This procedure was adapted from transplantation experiments performed in rats (Lu et al. 2012). Here we use froglets at NF Stage 66 (Nieuwkoop and Faber 1994) as host, but we have obtained very similar results using NF Stage 56. It takes twenty to thirty days for the transplanted cells to populate the lesion site; their progression can be studied using classic hematoxylin and eosin staining of paraffin or cryosections. Although the histological organization is sufficiently different between donor and host tissue, the use of transgenic animals expressing fluorescent proteins in a tissue-specific manner makes the distinction between the two easier. Transplanted animals can be maintained up to 60 d without problems. The efficiency of this procedure can be improved by testing other growth factors (as was demonstrated on rodents [Lu et al. 2012]), performing transplantations at different days after resection, making the lesion in an anterior part of the spinal cord, or using animals at different stages as donors or hosts. The effects of immunosuppressing the host froglets can also be tested by thymectomy, as previously performed in limb transplantation experiments (Lin et al. 2013).

This procedure is compatible with anterograde tracing to study axon regeneration, gain- and loss-of-function experiments, pharmacological studies, and functional recovery analysis. Given that there is no regeneration after transection in froglets (Beattie et al. 1990; Gaete et al. 2012; Muñoz et al. 2015), this procedure provides an opportunity to understand the mechanisms that allow NSPCs to improve spinal cord regeneration, a process that has been poorly studied (Assinck et al. 2017).

RECIPES

Final Medium

Reagent	Final concentration
BDNF (R&D Systems 248-BD-025/CF)	20 ng/mL
Bovine serum albumin (Sigma-Aldrich)	0.5 mg/mL
EGF (R&D Systems 2028-EG-200)	20 ng/mL
FGF (R&D Systems 3139-FB-025/CF)	20 ng/mL
Prepare fresh in L-15 Medium (Sigma-Aldrich L4386).	

Modified Barth's Solution (MBS) (1×)

Reagent	Final concentration (1×)
CaCl ₂	0.41 mM
Ca(NO ₃) ₂	0.33 mM
HEPES	10 mM
KCl	1.0 mM
MgSO ₄	0.82 mM
NaCl	88 mM
NaHCO ₃	2.4 mM

Adjust the pH to 7.4. Prepare as a 10× solution and then autoclave. Store at 4°C for up to 1 mo. Dilute to 0.1× and add antibiotics before use, and store for up to 3 d.

ACKNOWLEDGMENTS

This work was supported by funds from CARE Chile UC-Centro de Envejecimiento y Regeneración (PFB 12/2007), Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) (1141162) to J.L. E.E.M.O. is a Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) Ph.D. fellow 21120400.

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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot101006 originally published online May 16, 2018

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