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Dissociating and culturing human dorsal root ganglia neurons

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Muhammad Saad Yousuf¹, Juliet Mwirigi¹, Marisol Mancilla Moreno¹, Morgan Schackmuth¹, Theodore Price¹

¹Department of Neuroscience, Center for Advanced Pain Studies, The University of Texas at Dallas

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University of Texas at Dallas

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We use this protocol and it's working

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Abstract

A protocol for culturing and maintaining human dorsal root ganglia (DRG) neurons.



Materials



protocol materials.docx 29.8KB

Materials:

1. 60 × 15mm petri dish (Fisher Scientific, 35002)
2. 100% Ethanol (Decon, 07-678-005)
3. Sterile Water
4. 12 mm coverslips (Chemglass Life Sciences, CLS-1760-012)
5. 15 mm coverslips (Chemglass Life Sciences, CLS-1760-015)
6. 18 mm coverslips (NEST Scientific, 801011)
7. 24 well plate (Fisher Scientific, 09-761-146)
8. 12 well plate (Fisher Scientific, 07-200-82)
9. 15 ml conical tube (Falcon, ThermoFisher, 14-959-53A)
10. 50 ml conical tube (Falcon, ThermoFisher, 14-959-49A)
11. Forceps (Dumont #5, Fine Science Tools, 11254-20)
12. Biosafety cabinet (ThermoFisher, 1300 Series II)
13. Parafilm (Fisher Scientific, 13-374-10)
14. Poly-D-Lysine (mol wt >300,000, Sigma Aldrich, P7405-5MG)
15. HBSS (Hank's Balanced Salt Solution, ThermoFisher, 14170161)
16. STEMxyme I (Worthington, LS004106)
17. DNase I (Worthington, LS002193)
18. Hibernate A (BrainBits, NC1787837)
19. Bovine serum albumin (BSA, Biopharm, 71-015-025)
20. N2 supplement (STEMCELL, 07152)
21. SM1 supplement (STEMCELL, 05711)
22. Penicillin-Streptomycin (Pen-Strep, ThermoFisher, 15070063)
23. GlutaMAX (ThermoFisher, 35050061)
24. Sodium Pyruvate (ThermoFisher, 11360070)
25. Nerve growth factor (NGF, R&D Systems, 256-GF-100)
26. Glial cell derived neurotrophic factor (GDNF, STEMCELL, 78058.1)
27. Fetal bovine serum (FBS, Cytiva, SH30088)
28. 10X Phosphate buffer solution (10X PBS, ThermoFisher, BP3994)
29. Steriflip-Vacuum filter (50 ml, FisherScientific, SCGP00525)
30. 0.22 µm EZFlow PES Filter (Foxy Life Sciences, 371-2215-OEM)
31. 10 ml syringe (BD, 302995)
32. 100 µm cell strainer (Corning, 431752)
33. Rocking shaker (ThermoFisher, 88882002)
34. Shaking water bath (ThermoFisher, TSSWB15)
35. Centrifuge (ThermoFisher, ST16R)

Stock Solutions

Notes: Use HBSS without Ca²⁺ and Mg²⁺ unless stated otherwise

Poly-D-lysine Stock (1000 µg/ml)

1. In a sterile biosafety cabinet, dissolve 5 mg of poly-D-lysine (PDL, molecular weight > 300,000) in 5 ml of sterile water to a final concentration of 1000 µg/ml. PDL can be dissolved in its glass vial and then aliquoted out.
2. Sterile filter the solution using a 10 ml syringe and a 0.22µm EZFlow filter
3. The stock PDL solution can be stored at -20°C in smaller aliquots, typically 100 µl.

STEMxyme I Stock (10 mg/ml)

1. In a sterile biosafety cabinet, dissolve 50 mg of STEMxyme I in 5 ml of sterile HBSS to a final concentration of 10 mg/ml. STEMxyme I can be dissolved in its glass vial and then aliquoted out.
2. The stock STEMxyme I solution can be stored at -20°C in small aliquots, typically 500 µl.

DNase I Stock (4 mg/ml)

1. In a sterile biosafety cabinet, dissolve 100 mg of STEMxyme I in 25 ml of sterile HBSS to a final concentration of 4 mg/ml. DNase I can be dissolved in its glass vial and then aliquoted out. Alternatively, a smaller amount of DNase I can be weighed out, diluted in HBSS, and sterile filtered prior to storage.
2. The stock DNase I solution can be stored at -20°C in small aliquots, typically 250 µl.

Human β-Nerve Growth Factor (NGF) Stock (100 µg/ml)

1. Reconstitute 100 µg of NGF in 1 ml of sterile 0.1%BSA-PBS to a final concentration of 100 µg/ml. Ex/ To make 0.1%BSA-PBS, dilute 10 mg BSA in 10 ml of 1X PBS and sterile filter the solution using a 10 ml syringe and a 0.22µm EZFlow filter.

Enzyme Solution

1. Combine the following components in a volume of 5 ml in a 15 ml conical tube based on same-day (Table 1) or overnight (Table 2) preparations
2. Sterile filter the solution using a 10 ml syringe and a 0.22µm EZFlow filter
3. Parafilm the lid and warm the enzyme solution in a water bath set to 37°C.

	Component	Stock Concentration	Final Concentration	Volume (µl)
	DNase I	4 mg/ml	0.1 mg/ml	125
	STEMxyme I	10 mg/ml	2 mg/ml	1000
	HBSS	-	-	3875
	Total			5000

Same-Day Enzyme Solution (step 24.1)



	Component	Stock Concentration	Final Concentration	Volume (μl)
	DNase I	4 mg/ml	0.1 mg/ml	125
	STEMxyme I	10 mg/ml	1 mg/ml	500
	HBSS	-	-	4375
	Total			5000

Overnight Enzyme Solution (step 24.2)

NMDG-aCSF Stock

See [Shiers et al., 2024](#) for material and instructions on preparation of NMDG-aCSF stock and working solutions.

Hibernate A Solution

1. Combine the following components in a volume of 10 ml in a 15 ml conical tube.
2. Sterile filter the solution using a 10 ml syringe and a 0.22μm EZFlow filter
3. Store at 4°C until ready to use.

	Component	Stock Concentration	Final Concentration	Amount (mg)	Volume (μl)
	Hibernate A	-	-	-	9200
	BSA	-	0.1%	100	-
	N2	100%	1%	-	100
	SM1	100%	2%	-	200
	Pen-Strep	100%	1%	-	100
	GlutaMAX	100%	1%	-	100
	Sodium Pyruvate	100% (100 mM)	2%	-	200
	Total				10000

Hibernate A Solution

Media

1. Combine the following components in a volume of 50 ml in a 50 ml conical tube.
2. Sterile filter using a 50 ml Steriflip-Vacuum filtration system.
3. Warm the media in a water bath set to 37°C until ready to use.

Note: NGF supplement in the media is assay dependent and varies from 2-25 ng/ml. 10 ng/ml is routinely used.

Other growth factor supplements like glial cell derived neurotrophic factor (GDNF, 10-100 ng/ml) and fetal bovine serum (1-2%) can be added in the media.

	Component	Stock Concentration	Final Concentration	Volume (μl)
	Pen-Strep	100%	1%	500
	N2	100%	1%	500
	SM1	100%	2%	1000
	GlutaMAX	100%	1%	500
	NGF	100 μg/ml	10 ng/ml	5
	BrainPhys	-	-	47495
	Total			50000

Media preparation

Safety warnings

 Safety training courses on Bloodborne Pathogens, Biological Safety, Chemical Hygiene, Compressed Gases, Biological Waste Management, Personal Protective Equipment, Autoclave and Media Kitchens, General Laboratory Safety, and Cryogen Safety are required by UTD and can be taken through its BioRAFT system.

Safety Training related to Biomedical Research with Human Subjects is required by UTD and can be taken through its CITI program.

Ethics statement

All human tissue recovery, research and data handling is performed in accordance with the University of Texas at Dallas Institutional Review Board (IRB) regulations.

Before start

Sterile technique is essential for maintaining viability and limiting contamination in cultures.

Coating the coverslip

- 1 Disinfect coverslips in a 60 mm petri dish containing 5 ml of 70% ethanol
 - 1.1 Completely immerse the coverslip in 70% ethanol
 - 1.2 Using sterile forceps, pick up and move the coverslip to a well plate
 - 1.3 Lean the cover slip on the side of the well
 - 1.4 Airdry and expose to UV lamp for a few hours to overnight to ensure all of the 70% ethanol has completely evaporated.
 - 1.5 Alternatively, a coverslip rack housing multiple coverslips can be immersed in 70% ethanol in a beaker and left to airdry.
 - 1.6 Sterile coverslips can be stored in a parafilm plate at room temperature until ready to coat.
- 2 Prepare a working solution of poly-D-lysine (PDL) by diluting stock PDL (1000 µg/ml) in sterile water to a final concentration of 10 µg/ml (1:100).
- 3 Add PDL as a droplet to each coverslip (50 µL for 12 mm, 100 µL for 15 mm, 150 µL for 18mm coverslip)
 - 3.1 Pipette in the center of the coverslip in a circular motion to cover roughly 70% of the surface area of the coverslip
 - 3.2 Place the lid over the plate containing coverslips and remove from the biosafety cabinet.
 - 3.3 Tightly parafilm the lid over the plate, ensuring that the PDL droplets do not fall off their coverslips.
- 4 Incubate at 4°C overnight.

- 5 Next day, aspirate PDL and wash 3 times with sterile water (50 – 150 μ L) and let it airdry in biosafety cabinet under UV light
- 5.1 Add sterile water to the center of the coverslip, roughly covering the same area as the PDL
- 5.2 Aspirate the water and then wash again for a total of 3 times to remove excess PDL, which can be toxic to cells.
- 5.3 Let the coverslips airdry in the culture hood under UV light without the plate cover
- 6 Cover the plate with its lid and wrap in parafilm. These coverslips can be stored at 4°C and used within a month.

Preparing digestion enzymes and media

- 7 Warm up a shaking water bath to 37°C and start the shaking mechanism.
- 7.1 If a shaking water bath is not available, a small tabletop water bath on an orbital shaker (80 RPM) can be used.
- 8 Prepare the following reagents and filter sterilize:
 - 8.1 10% bovine serum albumin (BSA) (300 mg in 3 mL in a 15 ml conical tube)
 - i. Use one 3 mL 10% BSA tube for half to one DRG depending on size of the tissue
 - ii. Do not vortex to mix. Instead, parafilm the lid and leave on a rocking shaker to dissolve. Avoid creating bubbles.
 - iii. Using a 10 ml syringe and a 0.22 μ m EZFilter, sterilize the 10% BSA and keep at room temperature until ready to use
This can be stored at 4°C for a month, but should be brought to room temperature before use.
 - 8.2 Digestion Enzymes (STEMxyme I (Worthington Biochemical,LS004106): 1-2 mg/ml, DNase I (Worthington Biochemical, LS002139): 0.1 mg/ml) in a 5ml solution.
 - i. Dilute stock enzyme solutions in HBSS as laid out in the methods and filter sterile the solution using a 0.22 μ m EZFilter and a 10 ml syringe
 - ii. Warm up the enzymes in a water bath at 37°C

- iii. Use one 5 ml enzyme solution for 1 small DRG or half of a large DRG
- iv. There are two protocols for differing timings:
Same day protocol: roughly 4-6 hours of enzyme treatment
Overnight protocol: approximately 12-16 hours of enzyme treatment

8.3 Culture Media

- i. Should be prepared enough for 1-2 ml per well for flooding
- ii. For 24 well plate, use 1 ml of media per well
- iii. For 12 well plate, use 2 ml of media per well
- iv. Sterile filter the culture media and warm to 37°C in a water bath

Preparing whole DRGs

- 9 Steps 10-12 can be performed in a non-sterile environment.
Once you have received the DRG from the organ collection team as per Shiers et al., 2024, place the DRG in a 35 mm dish and wash in sterile NMDG-aCSF (preparation from Shiers et al., 2024) twice to remove any blood and other contaminants.
- 10 Using sterile forceps and scissors, clean DRG by removing excess fat, connective tissue, meninges, epineurium, and nerve/roots (Fig 1). Ensure the DRG is immersed in sterile NMDG-aCSF throughout this process.
- 10.1 A clean DRG bulb will reduce debris and detritus in the final product

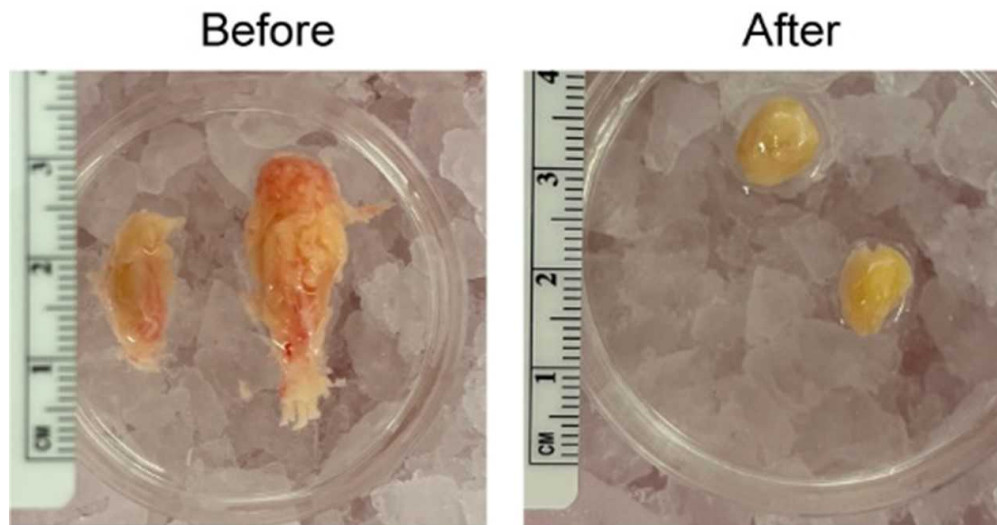


Fig 1.

Human DRGs immersed in artificial cerebrospinal fluid before and after the removal of fat, nerve, and connective tissue.

- 11 Alternatively, whole DRGs can be received and stored in ice-cold Hibernate A solution for 24 hours prior to digestion and dissociation

Culturing DRG neurons

- 12 Once a clean bulb is obtained, cut the DRG into small 2 mm pieces using scissors or a scalpel.
- 13 Transfer DRG pieces to the pre-warmed enzyme solution using sterile forceps (Fig 2)

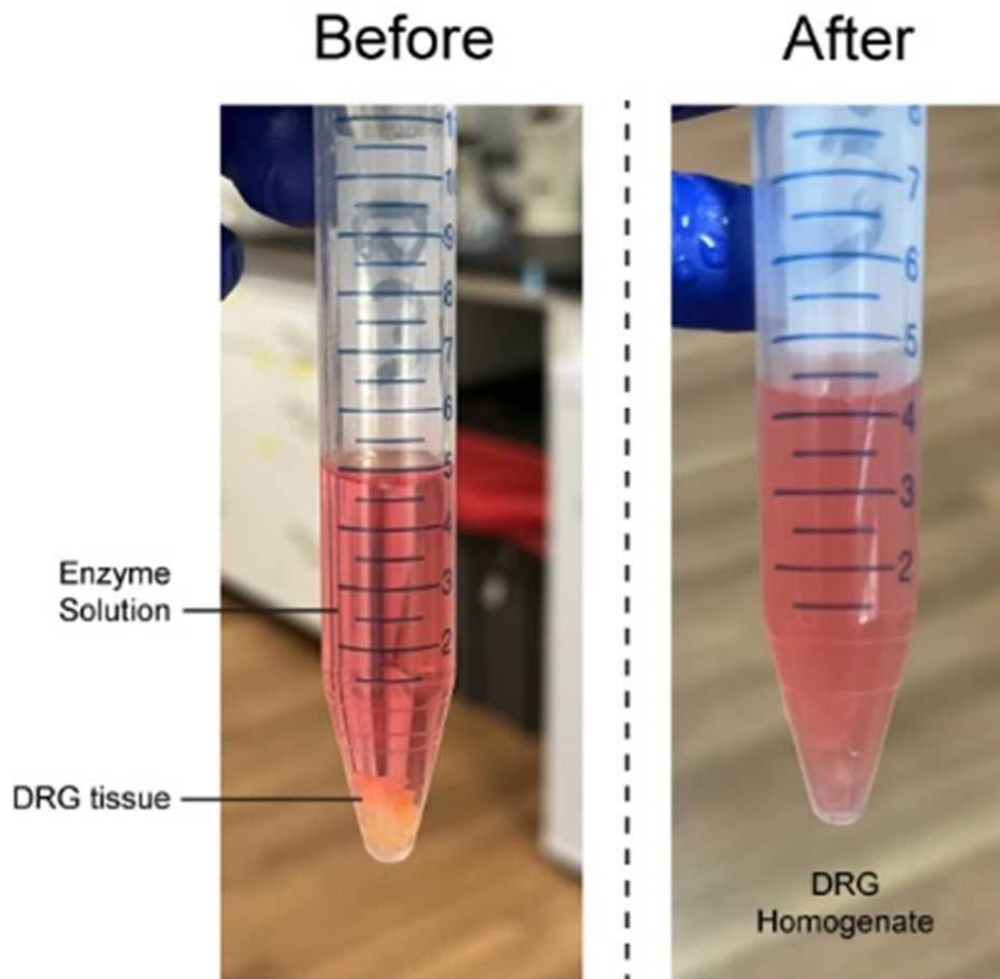


Fig 2.

Human DRGs are cut into 2 mm pieces that are homogenized in digestion enzymes containing STEMxyme I and DNase I.

- 14 Parafilm the lid and place in shaking water bath at 37°C for 4-6h for same day protocol or 12-16h for overnight protocol

- a. A 15 ml tube rack can be placed sideways and tied to the bottom of the shaker.
- b. Place the tube in the rack on its side as it shakes
- c. Ensure that the tissue is completely immersed in the enzyme solution and that no tissue is trapped at the bottom or the lid of the tube. The tissue should rock back and forth in the tube.

14.1 Same-day protocol:

- i. Keep the tube shaking in the water bath for 1.5 hour and then triturate using fire-polished glass pipettes
- ii. Repeat this process 3 times with a smaller glass pipette each trituration step

14.2 Overnight protocol:

- i. After 12-16h in the shaking water bath, remove the tube and triturate with a fire-polished glass pipette
- ii. If needed, the tube can be immersed again in the shaking water bath for 1-2 hours and trituration can be repeated.

15 Keep triturating until there is little to no resistance. The tissue should be digested at the end. Some small pieces of nerve and connective tissue might not digest completely. The aim is to digest the bulb of the DRG to release the neurons.

15.1 A brightfield upright microscope can be used to see if the neurons have been released from the bulb. Human DRG neurons have lipofuscin which is a dark pigmented waste accumulation in lysosomes.

16 Pass the mixture through 100 μ m cell strainer over a 50 ml conical tube

16.1 If the cell strainer gets clogged with tissue, replace with a new one

17 Layer filtered cell homogenate over 10% BSA prepared in a 15 ml conical tube (Fig 3)

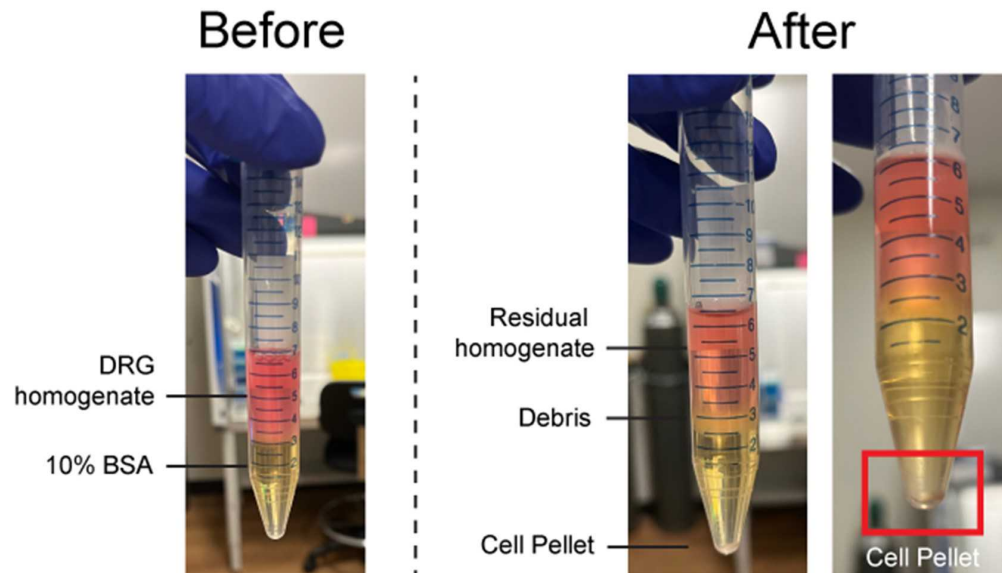


Fig 3.

Human DRG homogenate is gently layered onto 10% BSA solution. After centrifugation, a layer of debris is trapped between residual homogenate and 10% BSA while the cells are pelleted out of solution.

- 17.1 Tilt the tube and then gently add the cell homogenate to the side of the tube in order to not mix the two solutions
- 18 Centrifuge the homogenate/BSA mixture (900g for 5 min at room temperature, 9 for acceleration, 7 for deceleration)
 - 18.1 You should see a pellet at the bottom and a layer of debris trapped between the enzyme and the BSA solutions (Fig 3)
 - 18.2 Discard the supernatant and the debris layers, leaving only the cell pellet behind
- 19 Resuspend in warm 100 μ l of culture media and pipette 1 μ L of this onto a sterile 60mm dish
 - 19.1 Count the number of cells under a microscope
 - 19.2 Multiple the number of cells by 100 to get an estimate of how many cells are in the resuspension

- 19.3 Neurons can easily be identified by the presence of lipofuscin
- 20 Dilute the resuspended cells in warm media to desired concentration. For instance, 100-200 neurons are sufficient on a 12 mm coverslip.
- 21 Add 50 μ l-150 μ l of cell solution to the coverslips, depending on the diameter of the coverslip, as a droplet in the same area as previously coated with PDL.
- 22 Incubate for 3-4 hours at 37°C, 5% CO₂ for cells to adhere
- 23 Add 1-2ml of media per well by gently pipetting on the walls of the well
- 24 Incubate human DRG cells at 37°C, 5% CO₂ for the duration of the experiment (Fig 4)

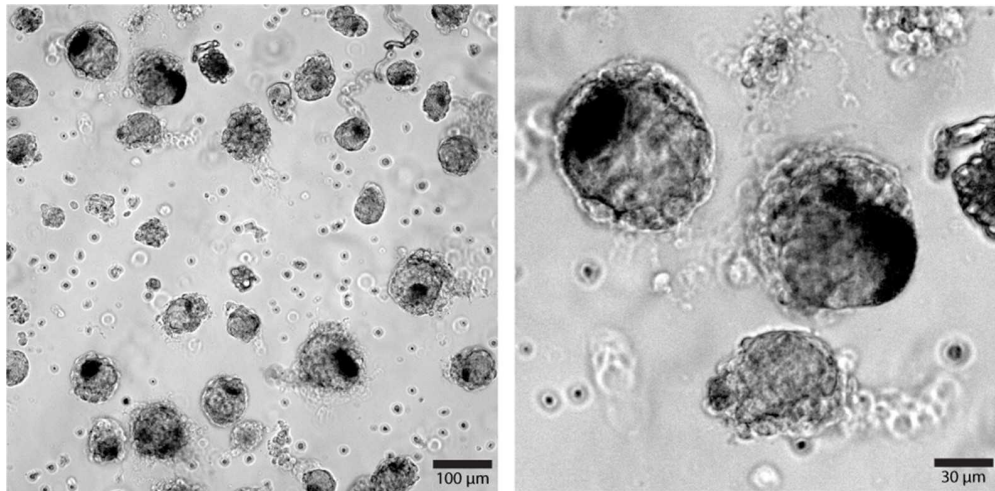


Fig 4.

Human DRG cells are plated onto glass coverslips. Neurons can readily be identified by the presence of lipofuscin, a dark-pigmented byproduct stored in lysosomes.

- 25 Perform half-media changes every other day.