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Neurostore: A Novel Cryopreserving Medium for Primary Neurons

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[Abstract] Primary neuronal culture from rodents is a key tool in neurobiology. However, the preparation of primary cultures requires precise planning, starting from animal mating. Furthermore, each preparation generates a high amount of cells that eventually go wasted. The possibility to cryopreserve primary neural cells represents a resource for *in vitro* studies and significantly reduces the sacrifice of animals. Here we describe that Neurostore buffer supports the cryopreservation of primary neurons.
Keywords: Neurons, Cryopreservation, Long-term storage, Reproducibility, Specimen sharing

[Background] Primary rodent cultures are a primary tool in any neurobiology laboratory. Primary cultures last 2-3 weeks. Therefore cells are normally prepared when necessary and employed in experiments immediately upon isolation. Cryopreservation is a routine procedure to allow long-term storage of mammalian cells. A protocol to achieve cryopreservation of neuronal cells would spare animals sacrifice and avoid the waste of cells. Furthermore, cell storage might permit the creation of cultures archive, support the reproducibility of experiments, and improve specimen sharing among different laboratories. Several studies have proposed a protocol to achieve the cryopreservation of neurons (Ichikawa *et al.*, 2007; Paynter, 2008; Ma *et al.*, 2010). Here we describe Neurostore, a novel buffer that promotes the long-term storage of primary mouse neurons and assures high viability upon thawing.

Materials and Reagents

1. P1000 tips (VWR, catalog number: GREI740280_60)
2. 15 ml Falcon tubes (EuroClone, catalog number: ET5050B)
3. Petri dishes 100 mm (EuroClone, catalog number: ET2100)
4. Cryovials (Corning, catalog number: 430659)
5. E 15.5 mouse embryos (Charles River, C57BL/6 Mouse, Strain Code: 027)
6. Fetal Bovine Serum EU Approved (FBS) (Euro Clone, catalog number: ECS0180L), store at -20 °C
7. Trypsin 2.5% in HBSS (Euro Clone, catalog number: ECB3051D), store at -20 °C
8. Gentamicin (Euro Clone, catalog number: ECM0011B0), store at 4 °C
9. HBBS w/o Calcium & Magnesium (Euro Clone, catalog number: ECB4007L), store at 4 °C
10. Magnesium sulfate ($MgSO_4$) (Sigma-Aldrich, catalog number: M7506), store at RT
11. HEPES (Sigma-Aldrich, catalog number: H3375), store at RT

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12. L-Glutamine 100x (200 mM) (Euro Clone, catalog number: ECB3000D-20), store at -20 °C
13. Neurobasal™ Medium (Thermo Fisher Scientific, catalog number: 21103049), store at 4 °C
14. Poly-D-lysine hydrobromide (Sigma-Aldrich, catalog number: P6407), store at -20 °C
15. B-27™ Supplement (50x), serum-free (Thermo Fisher Scientific, catalog number: 17504044)
16. Liquid nitrogen storage system (Midsci, catalog number: 38K-CS200)
17. Ultrapure water system Milli-Q® Direct (Merck Millipore, catalog number: C85358)
18. Dissection medium (see Recipes)
19. Disruption medium (see Recipes)
20. Neutralization medium (see Recipes)
21. Neuronal complete medium (see Recipes)
22. Neurostore medium (see Recipes)
23. Poly-D-Lysine coating solution (see Recipes)

Equipment

1. 10 ml pipettes (EuroClone, catalog number: EPS10N)
2. Cell cooler (Thermo Fisher Scientific, catalog number: 5100-0001)
3. Water bath (Thermo Fisher Scientific, catalog number: TSGP10)
4. Carl Zeiss™ Stemi™ DV4 Series Stereomicroscope with LED Illumination (Zeiss, catalog number: 0000001036143)
5. SL 8 Small Benchtop Centrifuge (Thermo Fisher Scientific, catalog number: 75007220)
6. CO_2 incubator (Thermo Fisher Scientific, catalog number: 51030992)
7. Automatic CO_2 Delivery Unit (Vet-Tech.co.uk, catalog number: AN044)
8. CO_2 Chamber (Vet-Tech.co.uk, catalog number: AN045BR)
9. Dissecting scissors, straight 112 mm (VWR, catalog number: 233-0011)
10. Dissecting scissors, straight 150 mm (VWR, catalog number: 233-0013)
11. Dissecting Forceps, Superfine Tip, Straight (VWR, catalog number: 82027-402)
12. Forceps Straight, blunt 105 mm (VWR, catalog number: 232-2111)
13. Bürker Counting chamber, 0.1 mm (VWR, catalog number: HECH40423001)

Procedure

A. Neuronal cells preparation and freezing procedure

1. Euthanize the pregnant mice with Carbon dioxide (CO_2) overdose, remove uterus with dissection scissors and free individual fetuses from the embryonic sack. Place embryos into 10 ml sterile Dissection medium (see Recipe 1) in a 100 mm Petri dish and keep it on ice.
   Note: Clean the abdomen with ethanol 70% before cutting the skin with scissors to avoid embryo contamination.

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2. Decapitate mouse embryos with dissection scissors, remove skin and skull and place the brain in a sterile 100 mm petri dish containing 10 ml cold Dissection medium.
   Note: Remove skin and skull with sharp superfine tip dissecting forceps.
3. Under a stereomicroscope discard the olfactory bulb, cerebellum, and midbrain from the mouse brain using the superfine tip forceps. Then remove meninges and blood vessels.
   Note: Be careful during the meninges removal procedure. Since the hippocampus is surrounded by meninges, you could inadvertently remove and discard it.
4. Dissect out the hippocampus and the cortex from each hemisphere with the superfine tip forceps.
   Note: Hippocampus can be easily identified as a "banana" shaped tissue. You can also find images and the procedure to dissect hippocampus in Salazar et al., 2017.
5. Collect the specimens in 12 ml cold Dissection medium in a 15 ml Falcon tube placed on ice.
   Note: Store the isolated specimens on ice during the whole procedure. Move the dissected tissues from the Petri dish to the 15 ml Falcon tube using the superfine forceps immediately after tissue isolation.
6. At the end of the dissection procedure let the tissues settle at the bottom of the Falcon tube by gravity. Wash tissues three times each with 10 ml fresh Dissection medium previously warmed at 37 °C in a water bath.
   Notes:
   a. *Add the dissection medium from the top of the Falcon and let the tissues settle at the bottom of the Falcon tube by gravity*
   b. *Remove as much medium as possible between each wash but keep the tissues covered by the medium during each step. Remove the medium gently using a 10 ml pipette. Avoid vacuum.*
7. Discard the medium and then add 10 ml disruption medium (see Recipe 2) to the tissue specimen in a 15 ml Falcon tube. Warm at 37 °C for 15 min and mix by inverting the Falcon every 2 min.
8. Let tissues settle at the bottom of the Falcon tube by gravity. Then discard the disruption medium and wash tissues with 10 ml Neutralization medium (see Recipe 3).
9. Let tissues settle at the bottom of the Falcon tube by gravity. Then wash tissues three times each with 10 ml fresh Dissection medium previously warmed at 37 °C.
10. Let tissues settle at the bottom of the Falcon tube by gravity. Discard the medium and then add to the tissue 5 ml Neuronal complete medium (see Recipe 4) previously warmed at 37 °C.
11. Mechanically disrupt the tissue with a 10 ml pipette and resuspend the tissue 15-20 times or until almost tissue clumps are dissolved.
12. Spin the cells at 600 *x g* for 10 min at room temperature in a 15 ml Falcon bench centrifuge.
13. Discard all the medium with a 10 ml pipette and add 1 ml Neuronal complete medium. Resuspend cells 10-20 times with a P1000 pipette.

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Note: Avoid cell clumps formation! If some tissue debris remains, let it settle at the bottom of the Falcon by gravity. Then collect the medium that contains all dissociated cells and transfer it to a new 15 ml Falcon tube. Discard the old 15 ml tube with the tissue debris.

14. Add 4 ml Neuronal complete medium.
15. Count neuronal cells. Average yield is about 8 x 10^6 cell/cortices, 2.5 x 10^5 cells/hippocampi obtained from E15.5 mice embryos. For cell counting dilute a small amount of cell suspension (20 µl) 1:4 in Neuronal complete medium and count cells in a Bürker Counting chamber.
    Note: Diluting cells facilitate cell counting. Include the round shaped and bright cells and exclude irregular shaped and dark cells in the counting. Upon counting, remember to apply the dilution factor.
16. Warm Neurostore medium (see Recipe 5) and cell cooler at 37 °C in a water bath.
17. Upon counting, spin cells at 600 *x g* for 10 min.
18. Discard all the Neuronal complete medium with a 10 ml pipette.
19. Gently resuspend the cell pellet in 1 ml of warm Neurostore medium with a P1000 pipette (about ten times). Once all cell clumps are removed, add warm Neurostore medium to reach the desired cellular concentration.
    Note: Suggested concentration is 5 x 10^6 cells/ml Neurostore medium.
20. Store 1 ml of Neurostore medium containing neurons in each cryovial.
    Note: Aliquot cells with a P1000 tip, 5 x 10^6 cells/cryovial.
21. Move cell cooler to -80 °C immediately. For long-term storage (above one month and up to one year) store cells in a liquid nitrogen tank.

B. Thawing procedure

1. Warm bath, Dissection medium, and neuronal complete culture medium at 37 °C.
2. Put 9 ml warm Dissection medium in a 15 ml Falcon tube.
3. Move cryovials to the 37 °C bath. Usually, complete thawing takes 2-3 min.
4. Add 1 ml Neurostore containing cells to the 15 ml Falcon tube with warm Dissection medium using a P1000 pipette.
5. Pellet cells at 600 *x g* for 10 min.
6. Discard the medium.
7. Gently resuspend cells in 1 ml warm Neuronal complete culture medium with a P1000 pipette (about ten times).
    Note: Resuspend until cell clumps disappear.
8. Add 4 ml warm neuronal complete culture medium.
9. Count the cells (as reported in Procedure A–Neuronal cells preparation and freezing procedure, Step A15).
    Note: Be fast in cell counting to reduce cellular stress. We suggest evaluating cellular viability by Trypan Blue staining.
10. Seed the desired number of cells on proper culture support in Neuronal complete medium.

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Notes:

a. *We suggest coating culture support with Poly-D-Lysine coating solution (see Recipe 6). Incubate o/n at room temperature or 2 h at 37 °C followed by 3 x ultra-pure water washes. For 12-well plates, we suggest adding a volume of 500 µl/ well of Poly-D-Lysine coating solution. We suggest scaling up or down the volume of the poly-D-Lysine coating according to the diameter of the plate wells.*
b. *We suggest seeding 750-1,000 cells/mm^2. Mix the proper amount of cells and Neuron complete medium to reach the desired number of cell. Add the correct volume of the mix into each well.*

Data analysis

Note: We refer to results and figures reported in our original publication (Pischedda et al., 2018. doi: 10.3389/fncel.2018.00081).

1. To verify the possibility to cryopreserve cortical neurons, we characterized E15.5 mouse cortical cells upon freezing and thawing. We thawed and seeded cultures after one week at -80 °C. After 14-days in culture, we noticed that cultures frozen in Neurostore generated a complex network of processes (Figure 1A).
2. Using MTT assay, we noticed comparable viability among fresh and cultures stored in Neurostore at -80 °C for 2 or 4 weeks (Figure 1B). Physiologically, neuronal cultures respond to pharmacological stimulation by triggering intracellular pathways. Cryopreserved cultures and acutely dissociated ones answered comparably in terms of AKT (Protein Kinase B) or ERK1/2 (Extracellular signal-regulated kinases 1/2) phosphorylation upon FGFb (Basic fibroblast growth factor) or EGF (Epidermal growth factor) stimulation (Figures 1F-1H). Cryopreservation procedure may induce oxidative stress. However, ROS (Reactive oxygen species) production resulted similar between fresh and cryopreserved cultures (Figures 2C and 2F).
3. Next, we infected cell with GFP expressing virus and analyzed the morphology of the culture at DIV14 (Figure 3A). The analysis of neurite number, total length, and average length did not reveal any significant difference among acutely dissociated and cryopreserved cultures (Figures 3B-3D).
4. To appreciate synaptic architecture, we stained acutely dissociated and 2- and 4-week cryopreserved cultures for synapsin and PSD-95 at DIV14 (Figure 4A). We could not detect any major differences among the experimental groups under analysis (Figures 4B-4D). To complement this analysis, we investigated by Western-blotting the expression of pre- and postsynaptic proteins in acutely dissociated and 2- and 4-week cryopreserved cortical cultures at DIV14 (Figures 5A-5C).
5. Lastly, we analyzed the electrical features of acutely dissociated and 2- and 4-week cryopreserved cortical cultures by whole-cell patch clamp recording. The quantification of all parameters demonstrated no significant differences between frozen cultures and fresh neurons

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(Figure 6 and Table 2).

Notes

Neuronal mortality in Neurostore medium is estimated in 20%-30% for both cortical and hippocampal neurons if cells are stored at the concentration of 5 x 10^6 cells/ml Neurostore medium. It is extremely important to count cells after thawing to reduce variability between different neuronal preparations.

Recipes

1. Dissection medium
   HBSS 1x
   6 mM $MgSO_4$
   10 mM HEPES, pH 7.4
   10 µg/ml gentamicin
2. Disruption medium
   HBSS 1x
   6 mM $MgSO_4$
   10 mM HEPES pH 7.4
   10 µg/ml gentamicin
   0.25% Trypsin
3. Neutralization medium
   HBSS 1x
   6 mM $MgSO_4$
   10 mM HEPES, pH 7.4
   10 µg/ml gentamicin
   10% FBS
4. Neuronal complete medium
   Neurobasal 1x
   B-27 supplement 1x
   0.5 mM L-glutamine
   10 µg/ml gentamicin
5. Neurostore medium (under patent consideration, available upon request. Please contact Prof. Giovanni Piccoli giovanni.piccoli@unitn.ita)
6. Poly-D-Lysine coating solution
   Resuspended poly-D-Lysine powder in ultra-pure water at final concentration 50 µg/ml

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Competing interests

Neurostore recipe is under patent consideration.

Ethics

All procedures involving animals were approved by Body for the Protection of Animals at the University of Trento and National Agency (autorizzazione 793/2016-PR).

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