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Nuclei Isolation from human cortical tissue for enrichment of endothelial and microglial cells

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Bruno A. Benitez[1]

[1]Beth Israel Deaconess Center

Bruno A. Benitez
Beth Israel Deaconess Center, Harvard Medical School

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We use this protocol and it's working

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Abstract

This protocol of nuclei Isolation from human cortical tissue is enriched for isolation of endothelial & microglial cells

Image Attribution

Modified by Dr. Richard Roberts

Guidelines

- Use standard BSL-2 precautions when working with human brain samples.
- Fresh frozen brain tissue could be a potential source of infectious agents. Wear proper PPE, including gloves, mask,
  and lab coat if outside hood. Disposable gown, goggles, or face shield if possible.
- Decontaminate every reusable equipment (homogenizers) with 25% bleach. Apply RNASe Zap to all surfaces in use.
- All discarded tissue remnants should go into 10-20% bleach for at least one hour before disposal.
- All waste is to be collected and disposed. Discarded tubes and tips go into a biohazard waste bag/box.

Materials

1. Prepare the following: (note that these volumes are optimized for 4 mL centrifuge tubes, * Stock solutions should be prepared the day before and not used for longer than a week)

	***Diluent Buffer (50mL)** 900 mM KCL; 30 mM MgCl2; 120 mM Tricine-KOH (pH 7.8)	***Homogenization Medium (50mL)**
2M KCl	22.5 mL	3.75 mL
1M MgCl2	1.5 mL	.25 mL
1M Tricine-KOH	6 mL	1 mL
Sucrose		4.28 g
Water	20 mL	Up to 50 mL

SHB: Supplemented Homogenization Buffer (prepare right before use)		
Homogenization buffer	**35 mL**	**50 mL**
Spermine	3.5 ul	5.0 ul
Spermidine	3.5 ul	5.0 ul
RNASIN	35 ul	50 ul

EDTA free protease inhibitor tablet	1	2

-

***50% Iodixanol (8 samples)**	
60% Iodixanol	35 mL
Diluent Buffer	7 mL

-

35% Iodixanol (8 samples)	
50% Iodixanol	14 mL
Supplemented Homogenization Buffer	6 mL

-

Nuclei Wash Buffer 1x PBS; 1.5% BSA; .2U/ul RNAsin	
10x PBS	5 mL
BSA	1.5 g
RNAsin	50 uL

Appendix. Solutions:

1. Stock
   Optiprep 60% Iodixanol [Sigma-Aldrich D1556-250mL]

1. Dilutant:

1. Final
   Concentrations: 900 mM KCL; 30 mM $MgCl_2$; 120 mM Tricine-KOH (pH 7.8)

1. Recipe
   for 50 mls

1) 22.5 ml of 2M KCL, +
2) 1.5 ml of 1M MgCl2, +
3) 6 ml of 1 M Tricine-KOH
4) brought up to 50 ml with molecular biology grade water.

1. Components

1) Tricine-KOH is 17.92 grams Tricine (Sigma T0377) and 100 mL RNAse free water for 1 M solution (pH to 7.8 with KOH as needed. Usually takes about 3-4 mL of 3M KOH).
2) 1 M MgCl2 (AM9530G) and 2M KCl (AM9460G) are commercial RNAse free stocks from Ambion.

1. Homogenization
   medium (need 6 ml per sample + ~ 2ml extra for 30% Iodixanol):

1. Recipe
(stable at 4C) for 100 mls / 50 mls

1) .25 M Sucrose (8.56 g / 4.28g)
2) 150 mM KCL (7.5 ml / 3.75of 2M)
3) 5 mM $MgCl_2$ (.5 ml / .25 ml of 1M)
4) 20 mM Tricine-KOH (2 ml / 1 ml of 1M)
5) ddH_2O up to 100 ml /50 ml in RNAse free H20

1. Supplementation (right before use):

1)
For 4 samples make 35 mls of homogenization buffer and supplement with:
1.
3.5 ul spermine,
2.
3.5 ul spermidine,
3.
35 ul RNASIN
4.
EDTA free protease inhibitor tablet.
-

1. Iodixanol Dilutions:

1. 50% Iodixanol

1)
Recipe:
5 volumes stock (60%) optiprep + 1 volume of dilutant.

2)
Scale:
need 2 ml per sample tube + 3 ml per sample for 35% Iodixanol
3)
Example:
4 samples add 17.5 ml stock to 3.5 ml dilutant.

1. 35% Iodixanol

1)
Recipe:
7 ml 50% Iodixanol + 3 ml supplemented homogenization medium.
2)
Scale:
need 4 ml per sample
3)
For
4 samples add 5.14 mL supplemented homogenization buffer to 12 mL of 50% Iodixanol.

1. Resuspension Buffer for Nuclei Wash (new from 10x Genomics recipe)

i.
1x PBS with
1.0% BSA and 0.2 U/uL
1)
Recipe:_____
commercial PBS (46-013CM 10x PBS from Corning)
2)
Recipe:
_____ BSA (001000162 from Jackson Immunoresearch)
3)
Recipe:
___1:1000_____ RNAsin

Troubleshooting

1 Protocol:

1. Turn on the ultracentrifuge, set it to 4oC and ensure that decelerations are set to no brake. Get the Beckman SW 60 Ti Rotor (Swinging Bucket) and from the cold room. Beckman centrifuge tubes Ref#=328874 for 4 mLs.
2. Keep all the solutions and materials on wet ice. Prepare of rest of the working solutions. Keep the tubes with the brain samples on dry ice until use. 100-500 mg of brain cortical tissue could yield 1-2 million nuclei.
3. Add pre-chilled 2 mL supplemented homogenization buffer (SHB) to the brain sample and keep it on wet ice. Transfer the brain tissue in 2ml of SHB to a 7 mL Dounce homogenizer. All the samples should be handled equally at all the steps, as different cell types' nuclei might be more or less vulnerable to physical dissociation. All pipetting and resuspension steps should be gentle and consistent to avoid nuclei shearing and clumping.
4. Homogenize the brain tissue.

   i. Transfer the brain tissue into a pre-chilled 7 ml Dounce glass homogenizer

   ii. Homogenize with 15 strokes on ice with pre-chilled 'B' pestle and 15 with 'A' grinder.

   iii. Transfer the brain lysate into 5 ml microcentrifuge tubes.

   iv. Add another 2mL SHB to the glass homogenizer and transfer the remaining lysate to the same microcentrifuge tubes.
5. Spin 5 ml microcentrifuge at 500 RCF for 5 minutes at 4o in a swing bucket centrifuge.
6. Pour off the supernatant
7. (optional) Resuspend in 3 mL of SHB. Repeat steps 7 & 8.
8. While spinning, you can add 2 mL of 35% Iodixanol to a centrifuge tube (4 mL, open-top thin-wall polypropylene tube) for each sample. Make sure the Iodixanol is in solution.
9. Resuspend each pellet to a final volume of 1 mL in SHB.
10. Add 1mL of 50% Iodixanol to the samples. Mix 50% Iodixanol right before usage. This step will create 25% Iodixanol with the samples.
11. Pipette the samples up and down with low retention pipette tips (Biotix M-1000-9FC) and carefully layer the sample on top of the 35% Iodixanol solution by slowly into the wall of the tube slightly above the 35 % Iodixanol and making sure the layers do not mix.
12. Centrifuge the tubes at 16,000 RFC for 30 minutes at 4 C. *Check decelerations with no brakes again* Note: we found that 16,000 RFC brings down endothelial cells from

the “myelin layer” more successfully. This brings down vasculature as well and may need to be filtered through a 40um mesh strainer.

13. Carefully remove the centrifuge tubes. The nuclei should be visible as a cloudy layer in the interface of the 25:35% Iodixanol. With a 3 mL plastic Pasteur pipette, remove the top brown layer (myelin/cell debris). Check addendum for parallel work.
14. With a low-retention P1000 tip remove the nuclei from the interface, then place them into a with nonstick, RNase-free microfuge Tubes, 1.5 mL (Cat#: AM12450-Ambion). 750 uLs for 1.5 mL. At this point, a swing bucket rotor is preferable but a fixed angle rotor when using a 1.5mL tube also works.
15. Add Nuclei Wash buffer to fill the tubes & mix by inverting or gently pipetting.
16. Centrifuge at 500 RCF at 4 C.
17. Nuclei should be pelleted at the bottom. Most of the time, the nuclei get stuck to the wall of the 1.5 mL. You could find the nuclei if you pay attention to the orientation of the tube before and after you place it in the microcentrifuge.
18. Remove & discard the supernatant
19. Repeat wash with Nuclei Wash buffer
20. Resuspend nuclei in 1,000 uLs of Nuclei Wash buffer and pass them through a 40 um (70 um also worked) Nylon mesh cell strainer.
21. Take out 10 uLs, mix 1:1 with a 1:2000 DAPI solution, and count on hemacytometer using a fluorescent microscope or Countess® II FL Automated Cell Counter. Check the morphology, size, and absence of nuclei clumps or tissue debris. 10x Genomics recommends an average of four separate readings.
22. Adjust aliquot of nuclei to desired dilution (10x Genomics recommends 1,600 cells per ul for loading onto the controller. If multiplexing, increase concentrations accordingly). Count again to confirm dilution.
23. The rest may be stored as follows: Pellet and resuspend in 10% DMSO in FBS or FCS.

Addendum from Step 13.

1. The myelin fraction contains many nuclei and endothelial cells (vasculature). Transfer the myelin/cell fraction to 2ml of SHB to a 7 mL Dounce homogenizer and homogenize with 15 strokes on ice with pre-chilled ‘B’ pestle and 15 with ‘A’ grinder.
2. Transfer the myelin fraction lysate into 5 ml microcentrifuge tubes.
3. Add another 2mL SHB to the glass homogenizer and transfer the remaining lysate to the same microcentrifuge tubes
4. Spin 5 ml microcentrifuge at 500 RCF for 5 minutes at 4o in a swing bucket centrifuge.
5. Pour off the supernatant. Resuspend each pellet to a final volume of 1 mL in SHB.
6. Add 1mL of 50% Iodixanol to the samples. Mix 50% Iodixanol right before usage.
7. Add 2 mL of 35% Iodixanol to a centrifuge tube.
8. Pipette the samples up and down with low retention pipette tips (Biotix M-1000-9FC) and carefully layer the sample on top of the 35% Iodixanol solution and making sure the layers do not mix.
9. Centrifuge the tubes at 16,000 RFC for 30 minutes at 4 C with no brakes.

10. With a low-retention P1000 tip remove the nuclei from the interface (750 uLs), then place them into a 1.5mL nonstick, RNase-free microfuge Tubes, 1.5 mL (Cat#: AM12450-Ambion) and add Nuclei Wash buffer to fill the tubes & mix by inverting or gently pipetting.
11. Centrifuge at 500 RCF at 4 C. Nuclei should be pelleted at the bottom. Most of the time, the nuclei get stuck to the wall of the 1.5 mL.
12. Remove & discard the supernatant
13. Repeat wash with Nuclei Wash buffer
14. Resuspend nuclei in 1,000 uLs of Nuclei Wash buffer and pass them through a 70 um Nylon mesh cell strainer.
15. Take out 10 uLs, mix 1:1 with a 1:2000 DAPI solution, and count them.
16. Adjust aliquot of nuclei to desired dilution. Count again to confirm dilution.
17. The rest may be stored as follows: Pellet and resuspend in 10% DMSO in FBS or FCS.

Protocol references

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