Video Article

Neuronal Nuclei Isolation from Human Postmortem Brain Tissue

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Abstract

Neurons in the human brain become postmitotic largely during prenatal development, and thus maintain their nuclei throughout the full lifespan. However, little is known about changes in neuronal chromatin and nuclear organization during the course of development and aging, or in chronic neuropsychiatric disease. However, to date most chromatin and DNA based assays (other than FISH) lack single cell resolution. To this end, the considerable cellular heterogeneity of brain tissue poses a significant limitation, because typically various subpopulations of neurons are intermingled with different types of glia and other non-neuronal cells. One possible solution would be to grow cell-type specific cultures, but most CNS cells, including neurons, are ex vivo sustainable, at best, for only a few weeks and thus would provide an incomplete model for epigenetic mechanisms potentially operating across the full lifespan. Here, we provide a protocol to extract and purify nuclei from frozen (never fixed) human postmortem brain. The method involves extraction of nuclei in hypotonic lysis buffer, followed by ultracentrifugation and immunotagging with anti-NeuN antibody. Labeled neuronal nuclei are then collected separately using fluorescence-activated sorting. This method should be applicable to any brain region in a wide range of species and suitable for chromatin immunoprecipitation studies with site- and modification-specific anti-histone antibodies, and for DNA methylation and other assays.

Video Link

The video component of this article can be found at https://www.jove.com/video/914/

Protocol

Nuclei Extraction

1. Put 250 mg of frozen postmortem brain tissue in 5 mL Lysis Buffer[1] in a douncer and place it on ice. To receive about 5 million nuclei after FACS sorting, we usually use 1000 mg of tissue per sample.
2. Once the tissue has thawed, dounce the tissue for 1 minute while on ice.
3. Take the homogenized tissue and place it into a 15 mL clear ultracentrifuge tube. Put the tube on ice.
4. Take 9 mL of Sucrose Solution[2] with a pipette, put the pipette at the bottom of the clear ultracentrifuge tube, and release the Sucrose Solution[2] to create a concentration gradient with the homogenized tissue solution on top of the Sucrose Solution[2].
5. Weigh the ultracentrifuge tubes to make sure that they will all be balanced while rotating within the ultracentrifuge. Make any adjustments to the weight by adding Lysis Buffer[1] to the top layer.
6. After the tubes have been weighed, place them into the rotor's buckets.
7. Place all buckets into a SW28 rotor and carefully place the rotor into the ultracentrifuge.
8. Ultracentrifuge the samples at 107163.6 RCF for 2.5 hours at 4°C.
9. Once the centrifugation is complete, remove the supernatant, along with the layer of debris found at the concentration gradient, with the use of a vacuum, while being careful not to disturb the pellet containing the nuclei at the bottom of the tube.
10. Add 500 µL of 1X PBS to each pellet and let sit on ice for 20 minutes. This allows the pellet to dissolve a little on its own, making it easier to later mechanically dissolve it by pipetting up and down, which lessens the amount of stress the nuclei experience.
11. After the nuclei have incubated on ice for 20 minutes, mechanically pipette up and down to completely dissolve the nuclei within 1XPBS.
12. Remove the nuclei-containing solution from the ultracentrifuge tubes and combine the contents of two tubes into a single 2 mL microcentrifuge tube. Again, place the samples on ice.

Immunostaining for FACS

1. At this point, make an immunostaining mixture composed of primary and secondary antibody, and Blocking Mix. For each sample, combine 300 µL of 1XPBS containing 1.2 µL of NeuN antibody, 100 µL of Blocking Mix, which contains 0.5% BSA and 10% Normal Goat Serum in 1XPBS, and 1 µL of Alexa Fluor 488. For the control samples, exclude the primary antibody NeuN and, instead, just add more 1XPBS.
2. Vortex the immunostaining mixture and incubate for 5 minutes at room temperature in the dark.
3. While the staining mixture is incubating, make the controls for the samples. For FACS sorting, a sample, containing nuclei alone, called the "unstained control" is needed; a second sample, a negative control, containing the nuclei with the secondary antibody Alexa Fluor 488 without the primary antibody NeuN is also needed.

4. Aliquot 20 µL of the nuclei containing solution into a 2 mL microcentrifuge tube containing 1XPBS for the unstained control, and another 20 µL into another 2 mL tube containing 980 uL of 1XPBS for the negative control. Raise the volume of the nuclei-containing sample back up to 1 mL with 1XPBS. All samples are placed back on ice.
5. Now that the immunostaining mixture has finished incubating, add its contents of 401 µL into the appropriate samples. The nuclei sample will receive the mixture containing both NeuN and Alexa Fluor 488, while the negative sample will receive the mixture containing only the secondary antibody Alexa Fluor 488.
6. Take the samples into the cold room to rotate in the dark for 45 minutes.
7. After the samples have incubated in the dark for 45 minutes, put them on ice and bring them to the FACS facility. During the transport to the FACS facility, the samples should be kept on ice and in the dark.

FACS

1. At the FACS facility, filter the samples through a 40 um filter. Then, run them through the instrument for a few minutes, while being kept cold, in order to get some preliminary data before sorting.
2. At this point, it is necessary to set the appropriate gates required for sorting. Several different gates are used to sort samples. The first gate gives an idea of the size of the different nuclei found within the sample, and allows separatation of debris from actual nuclei. The second gate allows us to sort nuclei individually. Discard any aggregates are discarded at this point. Finally, the third gate is set to a specific fluorescence wavelength, matching that of the fluorochrome found in our secondary antibody. Placing this gate allows us to sort and separate NeuN +, hence neuronal nuclei from NeuN - nuclei.
3. After all the gates are chosen, the nuclei can be sorted into 1XPBS.
4. Once the whole sample has gone through, check the purity of the sort by running an aliquot of the sorted sample through the instrument. Make sure that the sample's fluorescence is not spread out, but rather is contained within a single quadrant. It is best to choose samples which are over 90% pure.

After FACS

1. Once the sample has been sorted, processing of the nuclei can begin. Take 10 mL of sorted sample and add to it 2 mL of Sucrose Solution[2], 50 uL of 1M $CaCl_2$ and 30 ul of 1m $Mg(Ace)_2$. As a reminder, always be sure to keep the sample on ice since the nuclei are unfixed.
2. If there is less than 10 mL of sorted sample, raise the volume up to 10 mL by adding 1XPBS, or adjust the additional materials accordingly.
3. Invert the sample several times and place on ice for 15 minutes.
4. After the samples have incubated on ice for 15 minutes, centrifuge them in a swing bucket rotor at 1786 RCF for 15 minutes at 4°C.
5. Discard the supernatant with the use of a vacuum, without disturbing the white pellet found at the bottom of the tube.
6. Dissolve the pellet in 200 µl of whatever solution you require for the proceeding experiments, and pipette up and down.
7. Transfer the solution into a smaller tube and place it into the -80°C freezer until further use.

Discussion

The protocol presented here should be particularly useful for studies focused on epigenetic changes of neuronal chromatin and, more generally, on the molecular phenotype of the neuronal nucleus, during normal developing and aging, or in neurological or psychiatric disease. The method is of particular advantage when the cellular heterogeneity of brain tissue, including potential shifts in neuron-to-glia ration during the course of aging or due to disease are a concern [1]. For example, by combining the present sorting protocol with chromatin immunoprecipitation techniques, we recently described neuron-specific histone methylation patterns at the brain derived neurotrophic factor (BDNF) gene promoter, and for additional neuronal genes [2]. A similar method had been used previously to collect neurons from adult cerebral cortex for retrospective birth dating [3]. The sorting technique was also employed in mouse brain [2], and because of the stability of nuclei in frozen-then-thawed tissue, we anticipate that the approach presented here is applicable to a wide range of species. Because chromatin immunoprecipitation from as little as 10,000 cells for is now feasible even for a more comprehensive genome coverage [4], it may be possible to use much less than the 1000 mg of starting material that we recommended above.

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