APPLICATION NOTE

Isolation of Intact Nuclei from Rattus norvegicus Brain Tissue Using a Dounce

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Introduction

Comprehensive bottom up profiling of tissue proteomes is limited by the large dynamic range of protein expression and the ability of modern LC-MS/MS instrumentation to adequately separate and detect peptides in such a complex mixture. Tissue proteomes are diverse and a common method for segregating the proteome is subcellular fractionation; isolating organelles which contain smaller subsets of proteins that can be more readily detected. Isolating tissue organelles is commonly performed manually using a dounce or Potter-Elvehjem homogenizer.

In this study, we demonstrate a process for isolating intact nuclei from rat brain tissue using a dounce and compare nuclei purity and nuclear proteome coverage. Following the extraction, nuclei were isolated by centrifugation. Purity was then assessed by both microscopy and western blotting for known nuclear and cytoplasmic protein markers.

Materials & Methods

Equipment: 7 mL Dounce with tight fitting pestle (Cat # 07-357542)

Subcellular Fractionation: *Rattus norvegicus* brain tissue was cut into 40-60mg and placed into 7 mL Dounce.500 uL of ice cold 1X Phosphate buffered saline (PBS) was added to wash tissue then removed. 2 mL of ice cold S1 buffer (0.25M Sucrose, 10mM MgCl2) was added to Dounce and tissue was homogenized with 10 strokes using the tight fitting pestle. The sample was then put on ice for 5 min and was transferred to an ice cold 15 mL conical tube. 1 mL of ice cold S1 buffer was added to tube and was spun at 1000rpm (~104 x g) for 5 min at 4° C. During this time, 3 mL of ice cold S2 buffer (0.35M Sucrose, 0.5mM MgCl2) was placed into a new 15 mL conical tube and put on ice. After centrifugation, the supernatant (cytoplasmic fraction) was carefully removed, transferred to an ice cold 5 mL tube and placed in the -80o C freezer until needed. The pellet was resuspended with 3 mL of S1 buffer and then layered on top of the tube containing S2 buffer. The tube was then spun at 3500rpm (~1274 x g) for 10 min at 4° C. After centrifugation, the supernatant was discarded and the pellet was retained.

Nuclei Staining and Lysis: A small amount of the pellet (nuclear fraction) was affixed to a microscope slide via heating and stained per the Hematoxylin/Eosin protocol (1) with a few exceptions: Five drops of acidic alcohol total were added to the slide with rinsing in between each drop after the addition of the Hematoxylin stain. Eosin solution was added to slide for 45 sec, rinsed, and then dried. Nuclei were visualized via light microscopy (Figure 1). The remaining pellet was resuspended in 400 µL of a nuclear lysis buffer (50mM Tris, 150 mM NaCl, 1% Triton X-100) and passed through a 21 gauge needle 10 times then centrifuged at 10,000 rpm for 1 min. This process was repeated three times. Proteins were precipitated from nuclear and cytoplasmic fraction samples using a TCA/Acetone protein precipitation protocol (2). The pellet was dried and resuspended in 50 µL of a 32.9 mM Tris, 1% SDS solution. Protein concentrations were determined by BCA on a NanoDrop spectrophotometer (Thermo Scientific).

Immunoblotting: 2.5 µg (10 µg for western blot) of protein from each fraction was added to a microcentrifuge tube along with 5 µL of Laemmli buffer and placed on a heating block at 95°C for 10 minutes. Samples and 5 µL of Precision Plus Protein ladder (Biorad) were added to two seperate 4-20% Mini-Protean TGX gels (Biorad) andelectrophoresed at 150V for 45 minutes. One gel was silver stained using the ProteoSilverTM Silver Stain kit (Sigma-Aldrich) as per manufactures' instructions and visualized (Figure 2) on a Gel-Doc EZ system (Biorad). Proteins from the second gel were transferred to a nitrocellulose membrane at 100V for 1 hour. The membrane was blocked for 1 hr in 5% milk TBS-T 0.1% then incubated overnight in 1:1000 Lamin B1 (Nuclear Target) and 1:1000 alpha tubulin (Loading Control) rabbit polyclonal antibodies (Proteintech). The membrane was washed 3 x 5 minutes in TBS-T 0.1% and incubated in 1:10,000 IRDye® 680RD Goat anti-Rabbit IgG (H + L)(Li-Cor) at 4°C overnight. Protein detection was performed on a Li-Cor Odyssey Imaging system.

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Results

Herein, we evaluated the ability to isolate intact nuclei through mechanical disruption using a dounce and differntial centrifugation. Ten strokes with the pestle was the ideal amount that produced a homogenate while still keeping the most amount of nuclei intact. Through differential centrifugation, a pellet was produced containing the intact nuclei (Figure 1) that resembled purple spheres under magnification. Minimal cytoplasmic debris (pink) was observed. Herein, we evaluated the ability to isolate intact nuclei through mechanical disruption using a dounce and differntial centrifugation. Ten strokes with the pestle was the ideal amount that produced a homogenate while still keeping the most amount of nuclei intact. Through differential centrifugation, a pellet was produced containing the intact nuclei (Figure 1) that resembled purple spheres under magnification. Minimal cytoplasmic debris (pink) was observed.

Western blotting was performed to confirm protein abundance for both lamin and the loading control alpha tubulin for each fraction. Figure 3 shows the Lamin B1 marker (66 kD) for both fractions. The nuclear fraction showed a band whereas the cytoplasmic fraction did not. Figure 4 shows the alpha tubulin marker (50 kD) for both fractions. Two intense bands were observed for each fraction.

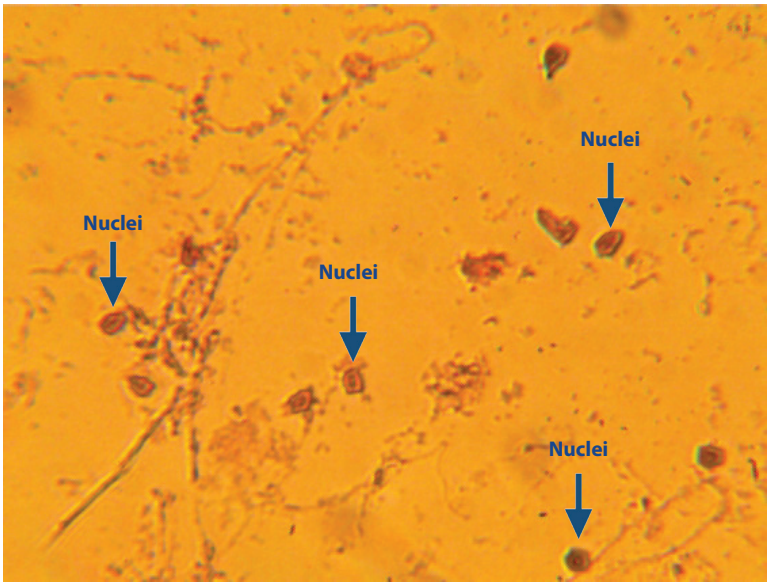
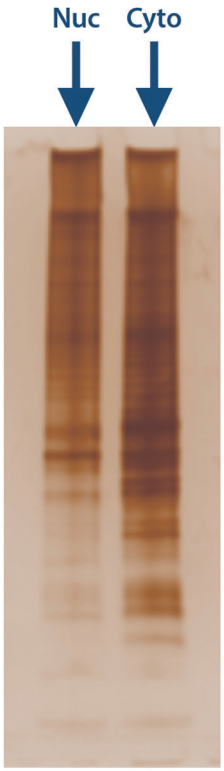


Figure 1. Microscopy of Nuclvear pellet from Dounce Homogenization(400X)

Dounce Tissue Grinder 7mL Cat# 07-357542



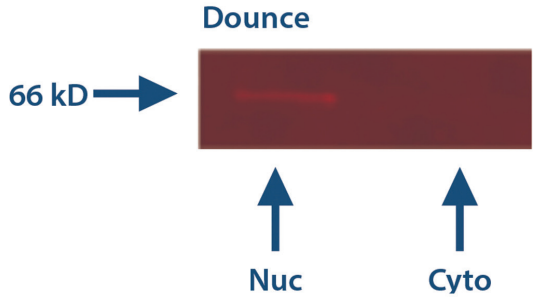


Figure 3. Western Blot for Detection of Lamin B

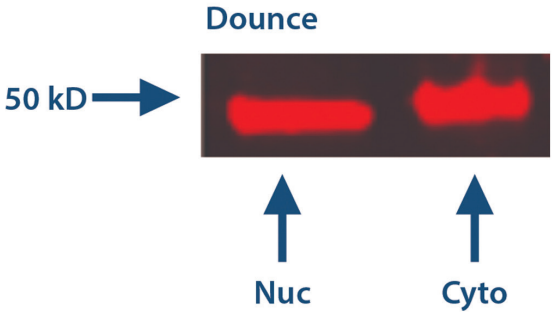


Figure 4. Western Blot for Detection of Alpha Tubulin

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Conclusion

The dounce homogenizer was successfully able to disrupt rat brain tissue to isolate intact nuclei. After centrifugation, the intact nuclei were visable via microscopy. Western blot confirmed that nuclear protein Lamin B1 was detected in nuclear fraction and was not detected in the cytoplasmic fraction.

References

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