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Development of a High Throughput Organelle Extraction Procedure from Rat Tissues

Brandon Easparro[1], Shari Garrett[1], James Atwood III[1]
Omni International Inc, 935-C Cobb Place Blvd, Kennesaw, GA 30144, USA

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The Homogenizer Company™

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 tissues organelles is commonly performed manually using either a dounce or rotor stator homogenizer. However, this process is not amendable to high throughput quantitative proteomic studies as the reproducibility is low and the processing time is significant.

In this study, we evaluate the potential for automated bead mill disruption of tissues for isolation of nuclei from rat tissues and compare nuclei purity, nuclear proteome coverage and protein extraction reproducibility to the extracts isolated by dounce and rotor stator homogenization. Following each extraction, nuclei were isolated by centrifugation. Purity was then assessed by both microscopy and western blotting for known nuclear and cytoplasmic protein markers. Proteins from each extraction method were then analyzed by LC-MS/MS and protein extraction yields were quantified by spectral counting

Shear-Flow™ Concept

Mechanical disruption methods, including bead beating, are routinely used for organelle isolation. However, all current homogenization technologies require that an impact or shearing device come in contact with the sample and for organelle isolation care must be taken to limit the force imparted to the sample.

To reduce the forces generated during the bead milling process a new tube was designed that contains an insert to restrict sample flow within the tube during the milling process. The Shear-Flow™ tube allows dissociation of tissues and cells on a bead mill without the use of beads (Figure 1). While shear forces are sufficient enough to dissociate tissues it was hypothesized that the imparted forces are gentle enough to maintain organelle integrity.

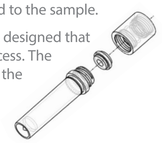


Figure 1: Shear-Flow™ tube design

Methods

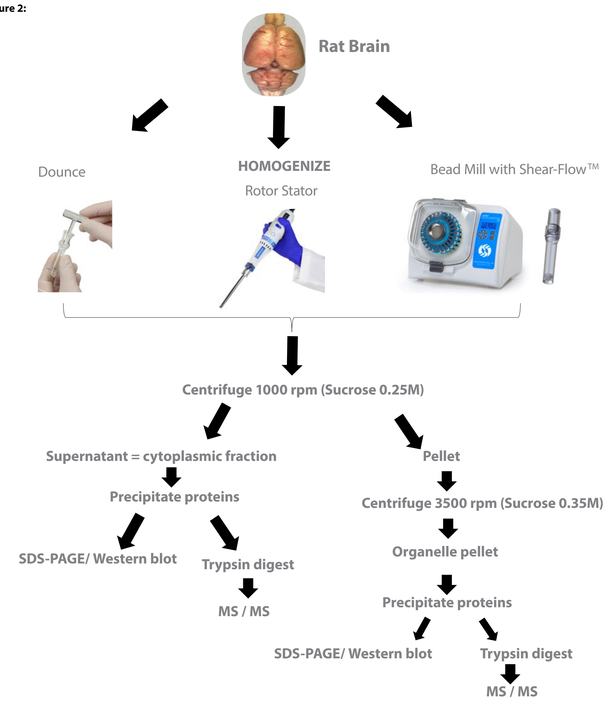
Figure 2 describes the experimental workflow. Rattus norvegicus brain tissue was cut into 40-60 mg sections and washed twice with 0.5 mL of PBS. A organelle fractionation protocol was followed as previously described with minor modifications (1). Buffer 1 was 0.25 M sucrose, 10 mM MgCL2. Buffer 2 was 0.35 M sucrose, 0.5 mM MgCL2. Organelle Lysis Buffer (OLB) was 50 mM Tris, 150 mM NaCL, 1% Triton X-100.

Samples were resuspend in ice cold Buffer 1 and mechanically disrupted using three different methods as described in Table 1. Following disruption samples were centrifuged at 1000 rpm for 5 minutes at 4C. Supernatant was extracted as the cytoplasmic fraction (CF). The pellet was resuspened in ice cold Buffer 1 and layered onto 3 mL of ice cold Buffer 2 and centrifuged at 3,500 rpm for 10 min at 4C.

A small amount of pellet (organelle fraction (OF)) was affixed to a microscope slide via heating and stained per the

Methods Flow Chart

Figure 2:



Methods (cont)

Hematoxylin/Eosin protocol with a few exceptions (2). Five drops of acidic alcohol were added to slide then rinsed in between each drop after addition of Hematoxylin stain. Eosin solution was added to slide for 45 sec, rinsed, then dried. Nuclei were visualized via microscopy (Figure 3-5).

Remaining pellet was resuspended in 400 µL OLB 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 (3). The pellet was dried and resuspended in 50 µL of a 32.9 mM Tris and 1% SDS. Protein concentrations were determined by BCA on a NanoDrop spectrophotometer (Thermo Scientific).

Protein Separation and Detection: 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 a 4-20% Mini-Protean TGX gel (Biorad) and electrophoresed 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 6) 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 4oC overnight. Protein detection was performed on a Li-Cor Odyssey Imaging system (Figure 7-8).

Protein Digestion, LC-MS/MS and Database Search: All precipitated protein samples were treated with 1 mM dithiothreitol at 25°C for 30 minutes, followed by 5 mM iodoacetimide at 25°C for 30 minutes in the dark. Proteins were digested with 1:100 (w/w) lysyl endopeptidase (Wako) at room temperature for 2 hours followed by overnight incubation with 1:50 (w/w) trypsin (Promega) at 37°C. Peptides were desalted with a Sep-Pak C18 column (Waters) and dried under vacuum.

The dried peptides were resuspended in 10uL of loading buffer (0.1% formic acid, 0.03% trifluoroacetic acid, 1% acetonitrile). Peptide mixtures (2 uL) were separated on a self-packed C18 (1.9 um Dr. Maisch, Germany) fused silica column (15 cm x 75 uM internal diameter (ID); New Objective, Woburn, MA) by a Agilent 1100 dual split LC system and monitored on a LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific , San Jose, CA). Elution was performed over a 80 minute gradient at a rate of 300nl/min at the column tip with buffer B ranging from 1% to 80% (buffer A: 0.1% formic acid in water, buffer B: 0.1 % formic in acetonitrile). The mass spectrometer cycle was programmed to collect 1 survey MS scan followed by 10 tandem MS/MS scans. The MS scans (400-1600 m/z range, 1,000,000 AGC, 500 ms maximum ion time) were collected at a resolution of 60,000 at m/z 200 in profile mode and the CID MS/MS spectra (2 m/z isolation width, 35% collision energy, 5,000 AGC target, 150ms maximum ion time) were detected in the ion trap in centroid mode. Dynamic exclusion was set to exclude previous sequenced precursor ions for 30 seconds within a 10 ppm window. Precursor ions with a charge stateof +1 were excluded from sequencing. Spectra were searched using Proteome Discoverer 2.0 against a rat uniprot database (retrieved April 20, 2015; 29370 target sequences). Methionine oxidation (+15.9949 Da), asparagine and glutamine deamidation (+0.9840 Da), and protein N-terminal acetylation (+42.0106 Da) were variable modifications (up to 3 allowed per peptide); cysteine was assigned a fixed carbamidomethyl modification (+57.0215 Da). Only fully tryptic peptides were considered with up to 2 miscleavages in the database search. A precursor mass tolerance of ±50 ppm was applied. Spectra matches were filtered by Percolator to a psm fdr of less than 1 percent.

Table 1: Homogenization of Rat Brain Samples

Mass of tissue (mg)	Volume of Buffer 1 used	Homogenization Type	Process/Settings
54mg	2 mL	Dounce (Cat# 07-357542)	10 strokes
44mg	2 mL	Rotor Stator (Omni TH, Cat# TH115) with Soft Tissue Disposable Probe (Cat# 32750)	23,750 rpm for 2 x 5 sec bursts
47mg	2 mL	Bead Mill (Bead Ruptor 24 (Cat# 19-040) with Shear Flow™ tube /cap design	4 m/s for 8 sec

Table 2:

SF Cyto		SF Nuc		Dounce Cyto		Dounce Nuc		RS cyto		RS Nuc	
Proteins	#Peptides (Unique)	Proteins	#Peptides (Unique)	Proteins	#Peptides (Unique)	Proteins	#Peptides (Unique)	Proteins	#Peptides (Unique)	Proteins	#Peptides (Unique)
307	1625 (1240)	332	1745 (1341)	335	1766 (1279)	336	1554 (1197)	370	1912 (1392)	346	1797 (1377)

Table 3

Description	Sub-cellular Localization	SF Cyto		SF Nuc		Dounce Cyto		Dounce Nuc		RS cyto		RS Nuc	
		#Peptide	#PSMs	#Peptide	#PSMs	#Peptide	#PSMs	#Peptide	#PSMs	#Peptide	#PSMs	#Peptide	#PSMs
Tubulin alpha-1A chain	Cytoskeleton	17	178	18	151	18	157	17	132	17	157	17	128
Glyceraldehyde-3-phosphate dehydrogenase	Cytoplasm	10	101	8	24	13	187	9	29	11	178	8	25
Dihydropyrimidinase-related protein 2	Cytoplasm	20	92	15	35	24	148	17	36	24	138	12	25
Creatine kinase B-type	Cytoplasm	12	69	7	23	13	105	10	46	13	89	10	40
L-lactate dehydrogenase B chain	Cytoplasm	10	28	2	2	12	67	6	13	13	60	4	5
Microtubule-associated protein	Cytoplasm	16	25	7	12	8	13	3	6	7	11	3	4
ATP synthase subunit beta, mitochondrial	Nucleus\Mitochondrion	23	76	23	149	23	99	24	152	23	102	23	186
ATP synthase subunit alpha, mitochondrial	Mitochondrion	15	39	20	81	16	41	21	81	20	58	22	89
Cytochrome b-c1 complex subunit 2, mitochondrial	Mitochondrion	4	5	10	21	1	1	9	22	4	7	10	29
Cytochrome b-c1 complex subunit 1, mitochondrial	Mitochondrion	1	2	5	14	1	4	5	16	3	7	7	18
Cytochrome c oxidase subunit 2	Mitochondrion	2	3	4	14	3	3	4	12	3	6	3	9
Histone H1.0	Nucleus	1	1	3	9	0	0	1	2	1	2	3	6
Nucleolin	Nucleus	0	0	7	11	0	0	0	0	2	2	2	2
Lamin-B1	Nucleus	0	0	8	15	0	0	1	2	0	0	1	1
Lamin A, isoform CRA_b	Nucleus	0	0	12	20	0	0	4	6	0	0	1	1
Histone H1.5	Nucleus	0	0	3	7	0	0	1	1	1	2	2	4
Inositol 1,4,5-trisphosphate receptor type 1	ER	1	1	2	3	0	0	15	24	1	1	22	37
Protein disulfide-isomerase A3	ER	0	0	0	0	0	0	1	1	1	1	4	4

Results

Herein, we evaluated the potential for a modified bead mill tube (Shear-Flow™) to allow gentle tissue disruption for the downstream isolation of intact organelles. The Shear-Flow™ approach was compared to dounce and rotor-stator homogenization.

It was determined that all methods, including Shear-Flow™ bead mill based disruption produced pellets containing intact nuclei as observed as purple spheres under magnification. Minimal cytoplasmic debris was observed as the absence of pink debris (Figures 3-5).

Tandem mass spectrometry was performed to quantify protein abundance based on homogenization method and fraction. Table 3 displays the number of peptides (#Peptides) and the spectral counts (#PSMs) for each protein as a function of fraction and homogenization method. Increased PSMs are observed for proteins known to localize to the cytoplasm in the cytoplasmic fractions for all three homogenization methods. PSMs for known mitochondrial and nuclear protein markers were increased by an average of 4X in the organelle fraction as compared to the cytoplasmic. While all homogenization methods were compatible with organelle enrichment, the Shear-Flow™ and Dounce disruption methods were shown to produce cytoplasmic fractions containing lower PSM values for organelle marker proteins.

Western blotting was performed to further confirm protein abundance for both lamin and the loading control alpha tubulin for each homogenization method and fraction. Figure 7 shows the Lamin B marker (66 kD) for all the fractions of Dounce, rotor stator and bead mill. The nuclear fraction of Dounce shows a band whereas the cytoplasmic fraction does not. The nuclear and cytoplasmic fractions of rotor stator show bands with relative amounts of intensity. For Shear-Flow™, the nuclear fraction shows a more intense bavnd than in the cytoplasmic fraction.

Figure 3: Microscopy of Nuclear pellet from Dounce Homogenization (400X)

Figure 4: Microscopy of Nuclear pellet from Rotor Stator Homogenization (400X)

Figure 5: Microscopy of Nuclear pellet from Bead Mill Homogenization (400X)

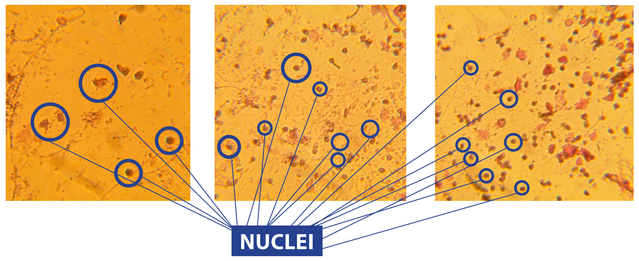


Figure 6: Silver Stained Gel

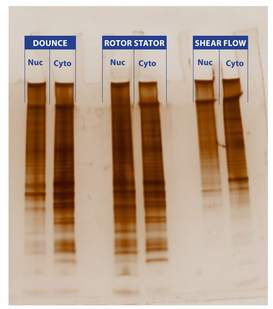


Figure 7: Western Blot for Detection of Lamin B

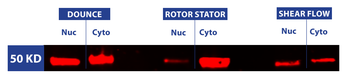
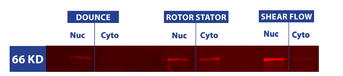


Figure 8: Western blot for Detection of Alpha Tubulin



Acknowledgements and References

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Conclusions

- Bead mill has the potential to be a standard for isolating organelles
- Bead mill is able to process multiple samples at once reducing time and cross contamination
- More research needs to be done to obtain a "clean" nuclear pellet without cytoplasmic protein contamination using a bead mill.