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TITLE:

Isolation of Adult Spinal Cord Nuclei for Massively Parallel Single-Nucleus RNA Sequencing

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Nuclei, RNA sequencing, snRNA-Seq, massively parallel, spinal cord, sucrose gradient

SUMMARY:

Here, we present a protocol to rapidly isolate high-quality nuclei from the fresh or frozen tissue for downstream massively parallel RNA sequencing. We include detergent-mechanical and hypotonic-mechanical tissue disruption and cell lysis options, both of which can be used for isolation of nuclei.

ABSTRACT:

Probing an individual cell's gene expression enables the identification of cell type and cell state. Single-cell RNA sequencing has emerged as a powerful tool for studying transcriptional profiles of cells, particularly in heterogeneous tissues such as the central nervous system. However, dissociation methods required for single cell sequencing can lead to experimental changes in the gene expression and cell death. Furthermore, these methods are generally restricted to fresh tissue, thus limiting studies on archival and bio-bank material. Single nucleus RNA sequencing (snRNA-Seq) is an appealing alternative for transcriptional studies, given that it accurately

identifies cell types, permits the study of tissue that is frozen or difficult to dissociate, and reduces dissociation-induced transcription. Here, we present a high-throughput protocol for rapid isolation of nuclei for downstream snRNA-Seq. This method enables isolation of nuclei from fresh or frozen spinal cord samples and can be combined with two massively parallel droplet encapsulation platforms.

INTRODUCTION:

The nervous system is comprised of heterogeneous groups of cells that display a diverse array of morphological, biochemical, and electrophysiological properties. While the bulk RNA sequencing has been useful for determining tissue-wide changes in the gene expression under different conditions, it precludes the detection of transcriptional changes at the single-cell level. Recent advances in the single-cell transcriptional analysis have enabled the classification of heterogeneous cells into functional groups based on their molecular repertoire and can even be leveraged to detect sets of neurons that had been recently active.¹⁻⁴ Over the last ten years, the development of single cell RNA sequencing (scRNA-Seq) has enabled the study of gene expression in individual cells, providing a view into cell-type diversity.⁵

The emergence of scalable approaches such as massively parallel scRNA-Seq, has provided platforms to sequence heterogeneous tissues, including many regions of the central nervous system.⁶⁻¹⁵ However, single cell dissociation methods can lead to the cell death as well as experimental changes in gene expression.¹⁶ Recent work has adapted single cell sequencing methods to enable preservation of endogenous transcriptional profiles.^{1,3,4,17-19} These strategies have been particularly suitable for detecting immediate early gene (IEG) expression following sensory stimulus or behavior.^{3,4} In the future, this strategy could also be used to study dynamic changes in tissues in disease states or in response to stress. Of these methods, single nucleus RNA sequencing (snRNA-Seq) is a promising approach that does not involve stress-inducing cell dissociation and can be used on difficult to dissociate tissue (such as the spinal cord), as well as frozen tissue.^{4,17-19} Adapted from previous nuclei isolation methods,^{20-23,25} snRNA-Seq typically utilizes rapid tissue disruption and cell lysis under cold conditions, centrifugation, and separation of nuclei from cellular debris.⁴ Nuclei can be isolated for the downstream next-generation sequencing on multiple microfluidic droplet encapsulation platforms.^{4,7,24,25} This method allows for a snapshot of the transcriptional activity of thousands of cells at a moment in time.

There are multiple strategies for releasing nuclei from cells before isolation and sequencing, each with their own advantages and disadvantages. Here, we describe and compare two protocols to enable isolation of nuclei from the adult spinal cord for the downstream massively parallel snRNA-Seq: detergent-mechanical lysis and hypotonic-mechanical lysis. Detergent-mechanical lysis provides complete tissue disruption and a higher final yield of nuclei. Hypotonic mechanical-lysis includes a controllable degree of tissue disruption, providing an opportunity for selecting a balance between the quantity and purity of the final nuclear yield. These approaches provide comparable RNA yield, detected numbers of genes per nucleus, and cell-type profiling and also can both be used successfully for snRNA-Seq.

PROTOCOL:

All animal work was performed in accordance with a protocol approved by the National Institute of Neurological Disorders and Stroke Animal Care and Use Committee. Balanced samples of male and female ICR/CD-1 wild-type mice, between 8 and 12 weeks old, were used for all experiments. Mice should be handled in accordance with local Institutional Animal Care and Use Committee guidelines.

1. Preparation of Materials and Buffers

1.1 Prepare all buffers the day of use and pre-chill on ice (see **Table 1**).

1.1.1. If using detergent-mechanical lysis, prepare the detergent lysis buffer (> 500 μ L per sample), low sucrose buffer (> 6 mL per sample), sucrose density buffer (> 12.5 mL per sample), and the resuspension solution (> 1 mL).

1.1.2. If using hypotonic-mechanical lysis, prepare the hypotonic lysis buffer (> 5 mL per sample), HEB medium (> 5 mL per sample), low sucrose buffer (> 3 mL per sample), sucrose density buffer (>12.5 mL per sample), and the resuspension solution (> 1 mL).

1.1.3. Add 25 μ L of dithiothreitol (DTT) to 25 mL of the low sucrose buffer and another 25 μ L of DTT to 25 mL of the sucrose density gradient buffer just before starting the protocol.

1.2 Cover the dissecting surface with aluminum-foil to minimize contamination of the sample with fibers from paper towels or bench protectors, which can clog microfluidic channels used for capturing single nuclei.

1.3 Spray dissecting tools and bench space with an RNase decontamination solution. Additionally, spray the inside of the Dounce homogenizer tube (if using detergent-mechanical cell lysis) and Oak Ridge tube with an RNase decontamination solution. Rinse out the Dounce and Oak Ridge tube with ultrapure, RNase-free water.

1.4 Pre-chill all collection tubes (50 mL conical, Oak Ridge) and Dounce homogenizer tubes on ice.

1.5 Fire polish a series of Pasteur pipettes (if using hypotonic-mechanical cell lysis).

2. Preparation of the Spinal Cord

2.1 If using fresh tissue, euthanize the mouse by CO₂ inhalation. Following euthanasia, spray the coat of the mouse with 70% ethanol to minimize hair contamination in the sample.

2.2 Decapitate the mouse with sharp, RNase-free surgical scissors. Next, gently lifting the abdominal skin with forceps and make an incision along the length of the body to expose the inner organs.

2.3 Eviscerate the mouse by pulling the inner organs from the body cavity using forceps. Do not use paper towels to clean the area or to remove organs as this may introduce contaminants. Using spring scissors, cut the vertebral column between the L2 and L3 spinal vertebrae.

Note: With practice, this step can be achieved in less than 30 seconds.

2.3.1 To eject the spinal cord, fit a 3 mL syringe containing ice-cold PBS with a 25 G $\frac{1}{4}$ inch needle. Place the tip of the needle into the sacral end of the vertebral column. Use two fingers to pinch the vertebrae to create a tight seal around the tip of the needle and press down on the plunger to eject the spinal cord rostrally. Place the spinal cord in a petri dish with ice-cold PBS.

2.3.2 At this point, freeze the tissue and store at -80 °C or use immediately for either detergent-mechanical (Step 3) or hypotonic-mechanical (Step 4) lysis.

2.4 If using frozen tissue, maintain the tissue on dry ice, proceed to detergent-mechanical (Step 3) or hypotonic-mechanical (Step 4) lysis.

3. Detergent-Mechanical Cell Lysis

3.1 Place the lumbar spinal cord in a pre-chilled Dounce homogenizer and add 500 μ L pre-chilled detergent lysis buffer.

Note: A mouse lumbar spinal cord is 325.5 mg \pm 63.9 mg standard error of the mean (SEM, N = 4). 50 mg – 1.5 g of tissue can be successfully used.

3.2 Dounce with 5 strokes of pestle A ('loose' pestle), then 5-10 strokes of pestle B ('tight' pestle). Avoid lifting the homogenizer out of the lysis solution in between strokes and avoid introducing bubbles.

3.3 Place a 40 μ m strainer over a pre-chilled 50 mL conical tube and prewet with 1 mL of low sucrose buffer.

3.4 Add 1 mL of low sucrose buffer to the Dounce homogenizer containing the crude nuclei in the lysis buffer and mix gently by pipetting 2-3 times.

3.5 Pass the crude nuclei prep over the 40 μ m strainer into the pre-chilled 50 mL conical tube.

3.6 Pass an additional 1 mL low sucrose buffer over the 40 μ m strainer, bringing the final volume to 3 mL of the low sucrose buffer and 500 μ L of the lysis buffer.

3.7 Repeat steps 3.1—3.6 if combining multiple cords, pooling in the same conical tube.

3.8 Centrifuge the sample at 3200 x g for 10 min at 4 °C. Once the centrifugation is complete, decant the supernatant. Proceed to Step 5.

4. Hypotonic-Mechanical Cell Lysis

4.1 Place the lumbar spinal cord in 5 mL of the hypotonic lysis buffer in a tissue culture dish. Use the blunt end of spring scissors to bisect the spinal cord, then use spring scissors to cut the cord into 3-4 mm pieces, but do not mince.

Note: 50 mg – 1.5 g of tissue can be successfully used.

4.2 Incubate on the ice for 15 min, swirling 2-3 times.

4.3 Add 5 mL of HEB medium to dilute the hypotonic lysis buffer.

4.4 Triturate the tissue 10 times with a 5 mL serological pipette, or until all of the pieces of the tissue move smoothly through the opening of the pipette.

4.5 Triturate with a series of three fire-polished Pasteur pipettes with progressively narrower diameters (~900 – 600 µm).

4.5.1 For each pipette, triturate 5-15 times, allow tissue to settle, remove 1-2 mL of supernatant containing dissociated nuclei and pass over a 40 µm strainer into a pre-chilled 50 mL conical tube.

4.5.2 After trituration with the smallest-sized Pasteur pipette, ensure that the homogenate flows smoothly through the pipette tip. Pass the remaining solution over the 40 µm strainer into the 50 mL conical tube.

Note: The total number of triturations can be adjusted as desired. The meninges of the mouse spinal cord will remain, but it is important to triturate any visible chunks of spinal cord. Pass the remaining homogenate over the 40 µm strainer. Avoid introducing bubbles during trituration.

4.6 Centrifuge the filtered sample at 1000 x g for 10 min at 4 °C. Once the centrifugation is complete, decant and discard the supernatant. Proceed to Step 5.

5. Homogenization and Sucrose Density Gradient

5.1 After either Step 3 or 4, resuspend the pellet using 3 mL of low sucrose buffer. Gently swirl to remove the pellet from the wall to facilitate the resuspension. Let the sample sit on ice for 2 min and transfer the suspension to an Oak Ridge tube.

5.2 Using the homogenizer at setting 1, homogenize the nuclei in low sucrose buffer for 15-30 s, keeping the sample on ice.

Note: Use 15 s if using one lumbar spinal cord or 30 s if using pooled samples or a whole spinal cord.

5.3 Using a serological pipette, layer 12.5 mL of density sucrose buffer underneath the low sucrose buffer homogenate, taking care not to create a bubble that disrupts the density layers.

5.4 Centrifuge the tubes at 3200 x g for 20 min at 4 °C.

5.5 Once the centrifugation is complete, immediately decant the supernatant in a flicking motion.

Note: A residual volume (less than 400 µL) of sucrose buffer can be discarded if desired to produce a lower volume and cleaner final sample, but this residual volume does contain nuclei and can be preserved to maximize nuclei yield.

5.6 Using 100 µL - 1 mL of resuspension solution, resuspend the nuclei remaining on the wall. Avoid the myelin 'frown' that remains with the detergent-based preparation.

5.7 Filter the nuclei through a 30-35 µm pore-size strainer and collect in a pre-chilled tube.

5.8 Determine the nuclei yield using a hemocytometer to count nuclei under a 10X objective.

Note: Trypan blue can be added to visualize nuclei, which should appear blue. Note the amount of cellular debris.

5.9 Proceed to either Step 6 or 7.

6. **Massively Parallel snRNA-Sequencing: Academic Platform⁷**

6.1 Perform the massively parallel snRNA sequencing (e.g., Drop-Seq) method as previously described⁷ with the following modifications:⁴

6.1.1 Adjust nuclei to a final concentration of 225 nuclei per µL.

6.1.2 Prepare barcoded beads at a concentration of 250 beads per µL.

6.1.3 Prepare the lysis buffer with 0.7% sarkosyl.

6.1.4 Adjust the flow rates to 35 µL per min for beads, 35 µL per min for nuclei, and 200 µL per min for oil.

7. **Massively parallel snRNA-sequencing: Commercial Platform²⁶**

7.1 Perform massively parallel snRNA-sequencing using the commercial platform (e.g., Chromium Single Cell Gene Expression Solution) products according to the manufacturer's instructions²⁶ with the following modification:

7.1.1 Following reverse-transcription, add an additional PCR cycle to the calculated number of cycles for cDNA amplification based on the targeted cell recovery to compensate for decreased cDNA from nuclei compared to cells.

REPRESENTATIVE RESULTS:

Here, we performed isolation of nuclei from the adult mouse lumbar spinal cord for downstream massively parallel RNA sequencing. The protocol involved three main components: tissue disruption and cellular lysis, homogenization, and sucrose density centrifugation (**Figure 1**). Within seconds, the detergent-mechanical lysis yielded a crude nuclei preparation with a large number of nuclei as well as cellular and tissue debris (**Figure 2A, Table 2**). After fifteen minutes, the hypotonic-mechanical lysis yielded a crude nuclei preparation that had less debris, but also fewer nuclei (**Figure 2B, Table 2**). Both preparations underwent homogenization (**Figure 2C and D**) and sucrose density gradient centrifugation before resuspension in PBS with 0.04% BSA (**Figure 2E and F**). On an average, a mouse lumbar spinal cord ($325.5 \text{ mg} \pm 63.9 \text{ mg}$ standard error of the mean, SEM, $N = 4$) yielded 5.1×10^5 nuclei ($\pm 6.3 \times 10^4$ SEM, $N = 3$) following the detergent-mechanical lysis and 2.0×10^5 nuclei ($\pm 5.9 \times 10^4$ SEM, $N = 3$) following the hypotonic-mechanical lysis. The number of nuclei in the lumbar spinal cord was estimated from the initial crude preparation after Dounce homogenization in the detergent-mechanical lysis protocol (2.6×10^6 nuclei $\pm 4.0 \times 10^5$ SEM, $N = 3$, **Table 2**). The final sample from the detergent-mechanical lysis protocol consists of 20% of the initial nuclei ($\pm 2\%$ SEM, $N = 3$, **Table 2**). The crude nuclei preparation from the hypotonic-mechanical lysis following trituration contains 62% of the initial nuclei ($\pm 2\%$ SEM, $N = 3$, **Table 2**). The final hypotonic-mechanical lysis sample contains only 8% of initial nuclei ($\pm 1\%$ SEM, $N = 3$, **Table 2**). We did not detect any difference in the total RNA yield or the cDNA yield for a housekeeping gene (Gapdh) between the two preparation methods. Using qPCR, the detergent method yielded 463.7 ng (± 98.9 SEM, $N = 6$) of total RNA and an average detection threshold cycle of 25.2 (± 1.3 SEM) for Gapdh cDNA by qPCR and the hypotonic method yielded 419.2 ng (± 85.3 SEM, $N = 6$) of total RNA and an average detection threshold cycle of 26.1 for Gapdh cDNA (± 0.8 SEM). The two lysis options both isolate nuclei from difficult-to-dissociate tissues and provide the high-quality material for the downstream single-nucleus RNA sequencing.

Given the size of microfluidic channels for downstream massively parallel single nucleus sequencing platforms, it is critical to input a nuclei suspension free of large particles or cellular debris to prevent clogging. Following the protocol presented here, there were no instances of clogging on the platform adapted from Macosko *et al.* 2015 ($N = 17$) and one partial clog on the commercial platform ($N = 16$).

The detergent-mechanical and hypotonic-mechanical procedures were used to isolate nuclei successfully for two massively parallel droplet encapsulation platforms and representative

results are shown in **Figure 3**. Both of these approaches enabled transcriptional profiling of thousands of nuclei, and classification of cell types in the adult mouse lumbar spinal cord (**Figure 3**).⁴ These approaches resulted in comparable genes per nucleus for each cell type (**Figure 3C and D**). The rates of recovery of input nuclei between the two platforms differ. The platform adapted from Macosko *et al.* 2015 with modifications from Sathiyamurthy *et al.* 2018 recovered an estimated 0.59% of nuclei ($\pm 0.05\%$ SEM, N = 17), while the commercial platform recovered an estimated 53.7% nuclei (N = 2).

This protocol slightly enriches for neuronal nuclei in the final preparation. In lumbar spinal cord tissue sections, we found that 27% of nuclei were positive for the neuronal marker NeuN (N = 7,368 nuclei from 2 animals), while detergent-mechanical nuclei preparation of the lumbar spinal cord resulted in 31.9% of total nuclei expressing NeuN, as determined by fluorescence-activated cell sorting (FACS, $\pm 2.0\%$ SEM, N = 13 independent nuclei preparations using pooled samples from multiple animals in each preparation, **Figure 4**). This is similar to what has been observed previously for the percent of NeuN-positive nuclei in the entire spinal cord (20% to 24% depending on age),²⁷ including the cervical and thoracic regions that have more white matter and oligodendrocytes. Of note, NeuN/Rbfox3 is not expressed in all neurons and, accordingly, these numbers are likely modest underestimates. It is possible that smaller non-neuronal cells are slightly depleted during the sucrose gradient purification. In addition, downstream filtering and analysis parameters following sequencing may alter the final cell-type distribution because neurons have more genes per nucleus (**Figure 3C and D**) and, therefore, are less likely to be removed during the filtering process.

There are several key steps in this protocol that require care. First, excessive douncing or trituration (in steps 3 or 4, respectively) can lead to an increase in cellular debris and particle formation. Although filtration and sucrose density centrifugation can separate large particles, once small particles are generated during cellular lysis, they are difficult to remove. Secondly, during homogenization, do not place the homogenizer directly onto the bottom of the Oak Ridge tube. Instead, submerge the end of the homogenizer into the low sucrose solution containing resuspended nuclei, without touching the bottom of the tube. Homogenization improves nuclear isolation by removing cellular debris and reducing clumps and multiplets (**Figure 5**). Following sucrose density centrifugation, it is critical to immediately remove the Oak Ridge tube from the centrifuge, and quickly decant the supernatant in a rapid ‘flicking’ motion. When resuspending nuclei from the wall of the Oak Ridge tube, resuspend the ‘salty’ pellet from halfway between the myelin band and the bottom of the tube. Note that the pellet may not be visible. Resuspending nuclei higher along the tube may result in myelin contamination in the nuclei preparation. The cellular lysis and sucrose density centrifugation steps are the most critical to reducing particulates that may clog microfluidic channels for downstream application.

FIGURE AND TABLE LEGENDS:

Table 1: Table of Solutions.

Table 2: Yield of nuclei at each step in the protocol. The number of nuclei in the initial crude preparation after dounce homogenization in the detergent-mechanical lysis protocol was used to estimate the number of nuclei in the lumbar spinal cord. The initial nuclei yield (2.6×10^6 nuclei $\pm 4.0 \times 10^5$ SEM, N = 3) was used to calculate the nuclei yield at each downstream step for both detergent- and hypotonic-mechanical lysis protocols. The number of nuclei isolated by the hypotonic-mechanical preparation was normalized to the estimated initial nuclei. Values in the table are mean \pm SEM, N = 3.

Figure 1: Schematic of nuclear isolation. Nuclei from the adult spinal cord can be isolated using detergent-mechanical or hypotonic-mechanical cell lysis, followed by homogenization, and sucrose density gradient centrifugation.

Figure 2: Representative brightfield and DAPI-stained nuclei at key steps in the protocol. A&B. Crude nuclei following detergent-mechanical or hypotonic-mechanical lysis. **C&D.** Nuclei following homogenization. **E&F.** Nuclei resuspended in PBS with 0.04% BSA following sucrose density centrifugation. Nuclei were fixed with 2% paraformaldehyde and subsequently stained using Trypan Blue or DAPI. Images were taken at 10X (scale bar 100 μ m) using brightfield and epi-fluorescence.

Figure 3: Representative tSNE plot of sequenced nuclei: using detergent-mechanical and hypotonic-mechanical lysis. A. Results obtained from sequencing over 17,000 nuclei from the dissected adult mouse lumbar spinal cord following detergent-mechanical lysis and according to Macosko *et al.* 2015 with modifications from Sathiyamurthy *et al.* 2018. This figure has been modified with permission from Sathiyamurthy *et al.* 2018.⁴ **B.** Results obtained from sequencing 5,000 nuclei from the ejected adult lumbar spinal cord following hypotonic-mechanical lysis and a commercial microfluidic single cell encapsulation platform.²⁶ **C&D.** Average genes per nucleus results following clustering of major cell types in the adult mouse spinal cord \pm SEM. Of note, the detergent-mechanical lysis procedure followed by the Macosko *et al.* 2015 platform was performed using dissected lumbar spinal cord, while the hypotonic-mechanical lysis followed by the commercial platform was performed using ejected lumbar spinal cord (as described in this protocol). Given that ejecting the cord removes the dura and dorsal root ganglia, the meningeal/Schwann cell cluster is absent from Figure 3B and D.

Figure 4: FACS plot of NeuN⁺ nuclei following detergent-mechanical lysis. FACS plot showing fixed nuclei stained for NeuN (average 31.9% of total nuclei \pm 2.0% SEM, N = 13), isolated using the detergent-mechanical lysis protocol. For the immediate fixation, nuclei for FACS validation, a crude nuclei preparation was obtained by dounce homogenization of spinal cords using the detergent-mechanical preparation, followed by immediate fixation with 1% PFA with a 5-min incubation period. Fixation was quenched with 250 mM glycine, and nuclei were collected. Staining with anti-NeuN antibody was performed in solution. FACS was performed on fixed, NeuN stained nuclei using a cell sorter.

Figure 5: Nuclei preparation without homogenization. Nuclei were resuspended prior to sucrose density centrifugation following **A** detergent-mechanical or **B** hypotonic-mechanical lysis,

without homogenization. * Denotes cellular debris attached to nuclei **(A)** and a multiplet of nuclei attached by cellular debris **(B)**. Nuclei resuspended in PBS with 0.04% BSA following sucrose density centrifugation. Nuclei were fixed with 2% paraformaldehyde and subsequently stained using Trypan Blue or DAPI. Images were taken at 10X (scale bar 100 μ m) using brightfield and epifluorescence.

DISCUSSION:

The ultimate goal of this protocol is to isolate nuclei containing high-quality RNA for downstream transcriptional analysis. We adapted snRNA-Seq methods in order to profile all of the cell types in the spinal cord. Initially, we found that typical cell dissociation methods were ineffective for single cell RNA sequencing, as spinal cord neurons are particularly vulnerable to cell death. Furthermore, cell dissociation methods induce expression of various activity- and stress-response genes by up to several hundred-fold.^{3,4,16} Given the drawbacks associated with single cell preparations, we and others have used nuclei as an alternative.^{16-18,24} This method can also be used on human tissue, including frozen spinal cord tissue.^{4,19,24} Here, we will describe the strengths and limitations of this approach.

Strengths of this method include the avoidance of experimentally-induced IEGs as well as the ability to use both fresh and frozen tissue.⁴ Thus, this approach can be useful for probing endogenous IEGs following a behavior or stimulus.^{1,3,4} One of the benefits of this method is that it does not require specialized devices for utilization of nuclei for massively parallel single nucleus sequencing, but can use the platform developed by Macosko *et al.* 2015, with minor adjustments of lysis buffer and flow rate, or use commercially available systems. Moreover, single nucleus sequencing is proven to be a comparable method to that of single cell sequencing for the identification of cell types, lending to the strength of this approach.^{28,29} However, there are several important limitations of this approach. Nuclei contain approximately 20-50% of cellular mRNA,²⁹ and this is reflected in a lower number of transcripts per nucleus compared to single cell sequencing.^{18,29} Including intronic reads from snRNA-Seq is one approach to increase the number of detected genes.

There are several available protocols that enable isolation of nuclei from tissue.^{2,16-18,24,30} In comparison with most other methods, the protocols presented here do not require myelin removal, ultracentrifugation, or many centrifugation steps or washes that can lead to lower final numbers of nuclei. Furthermore, this protocol takes 45 minutes (detergent-mechanical) or 1 hour (hypotonic-mechanical) to complete. Commercial protocols supported on microfluidic platforms are more than double the time, and require many more centrifugation steps, increasing the risk of losing nuclei. In contrast with nuclei isolation protocols that involve only lysis and filtering, the methods presented here include a sucrose gradient to increase the purity of the final nuclei. This step is required for adult spinal cord tissue due to the large percentage of white matter and the resulting myelin debris.

The detergent-mechanical lysis protocol can be used for the complete tissue dissociation and lysis, and the hypotonic-mechanical lysis protocol can be used to control the amount of tissue

dissociation and cellular debris allowed in the downstream application. These protocols can be used for bio-bank material, difficult to dissociate tissues and for the investigation of activity-dependent transcriptional changes through the isolation of nuclei for downstream massively parallel snRNA-Seq. In addition to massively parallel single nucleus RNA sequencing, this protocol may be used to isolate nuclei for alternative applications, including immunofluorescence and FACS and epigenetic analysis such as DNA methylation studies and ChIP-Seq (Figure 4).²³

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DISCLOSURES:

We have no conflicts of interest to disclose.

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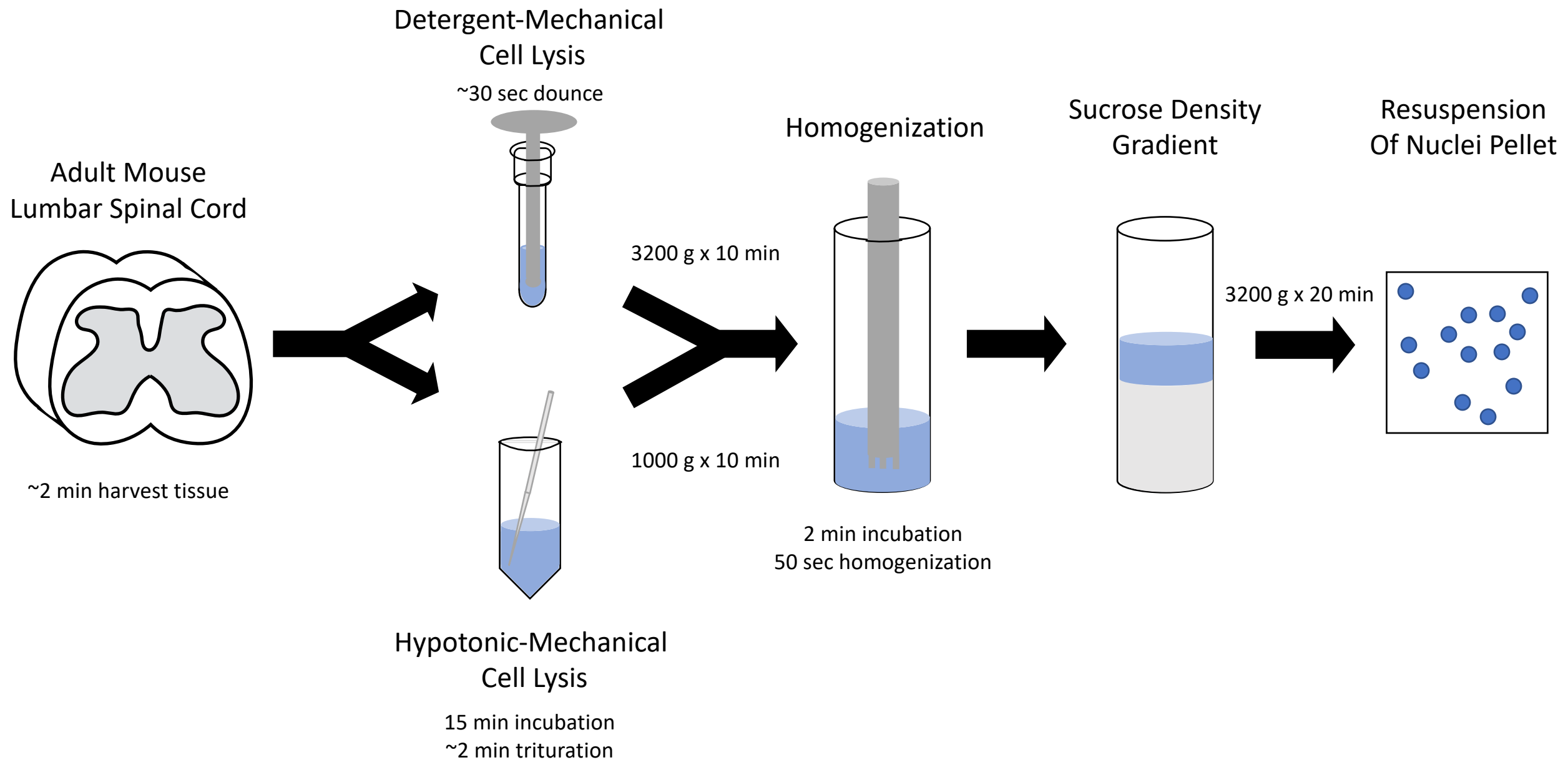
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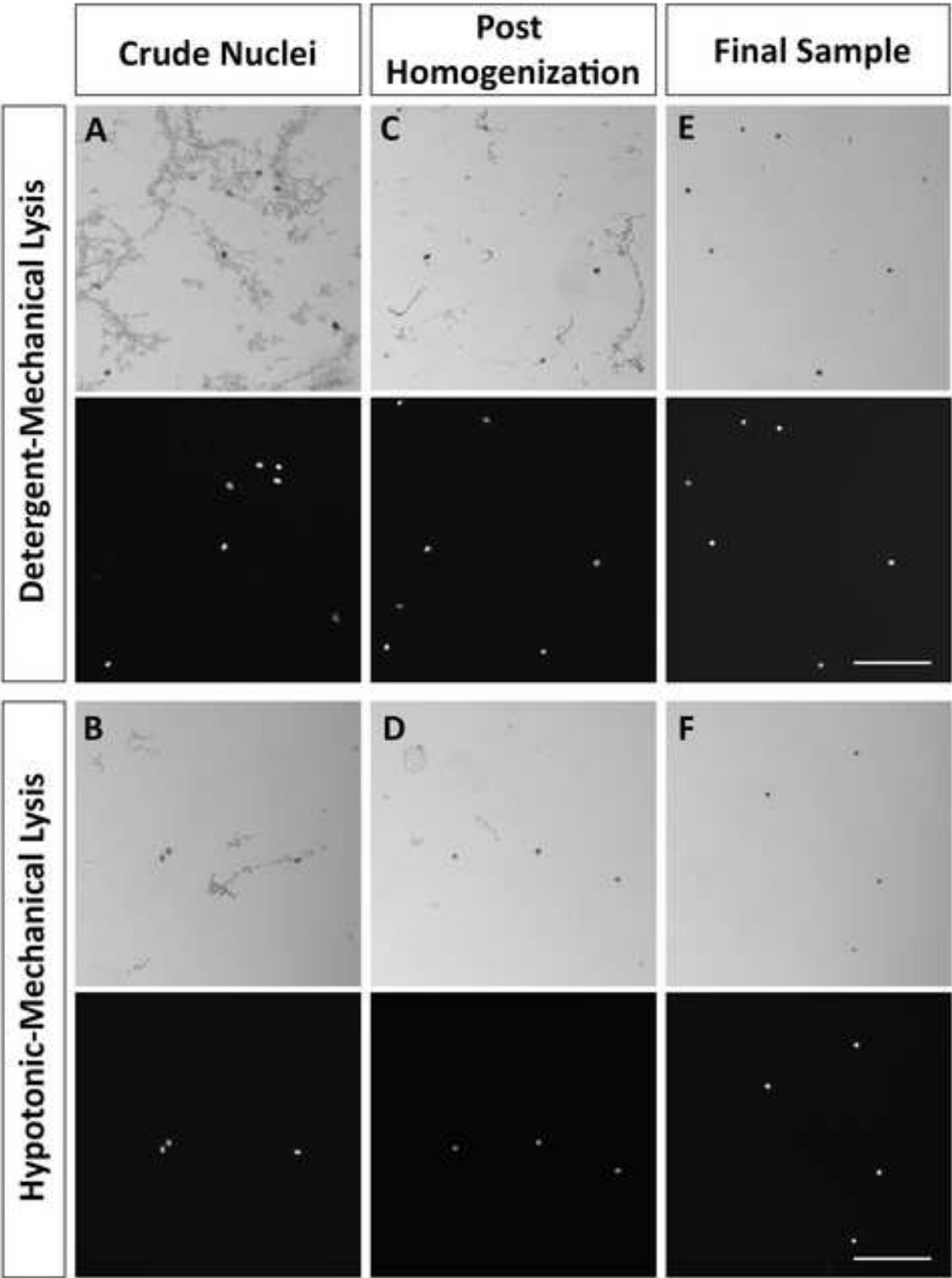
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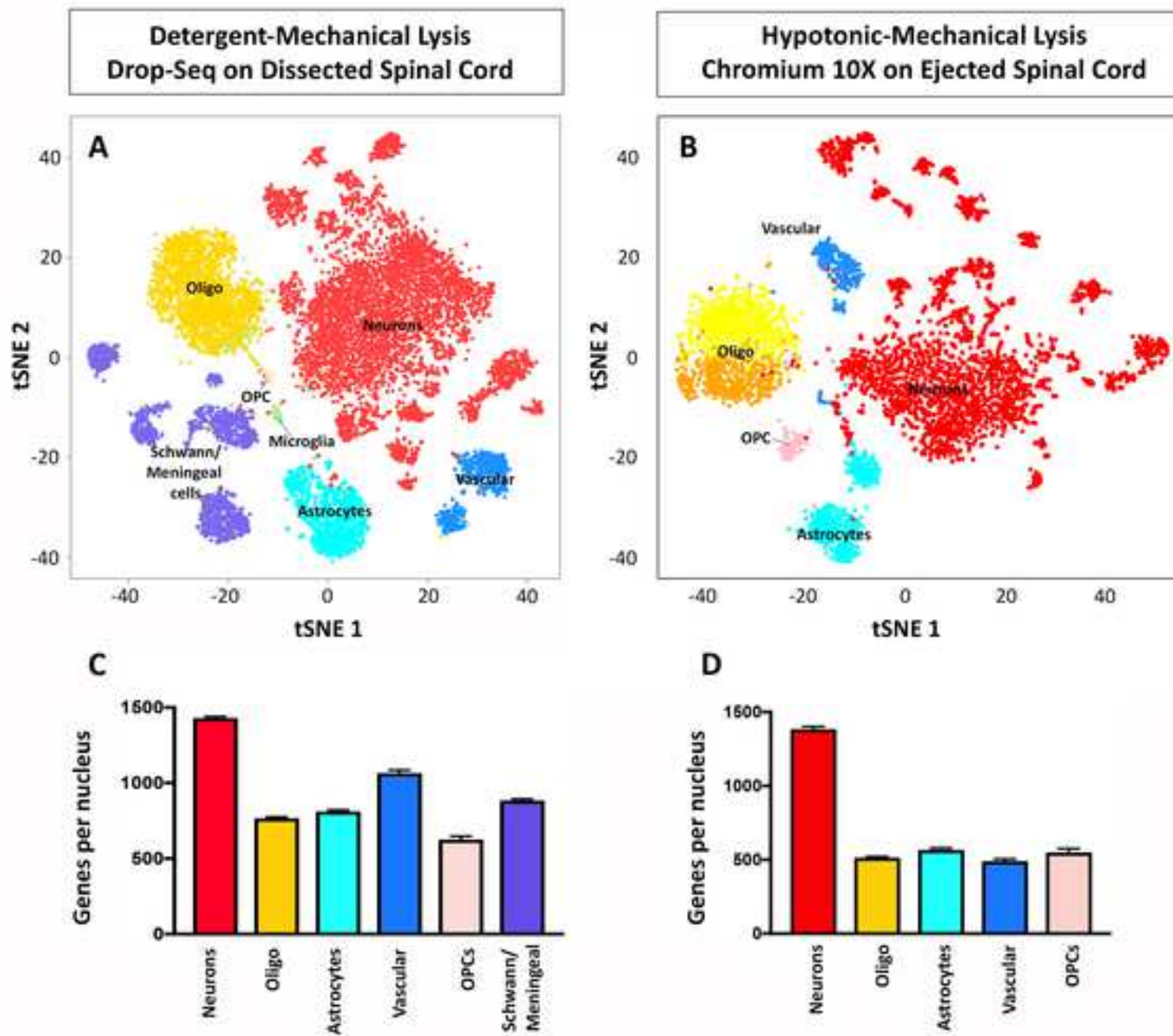
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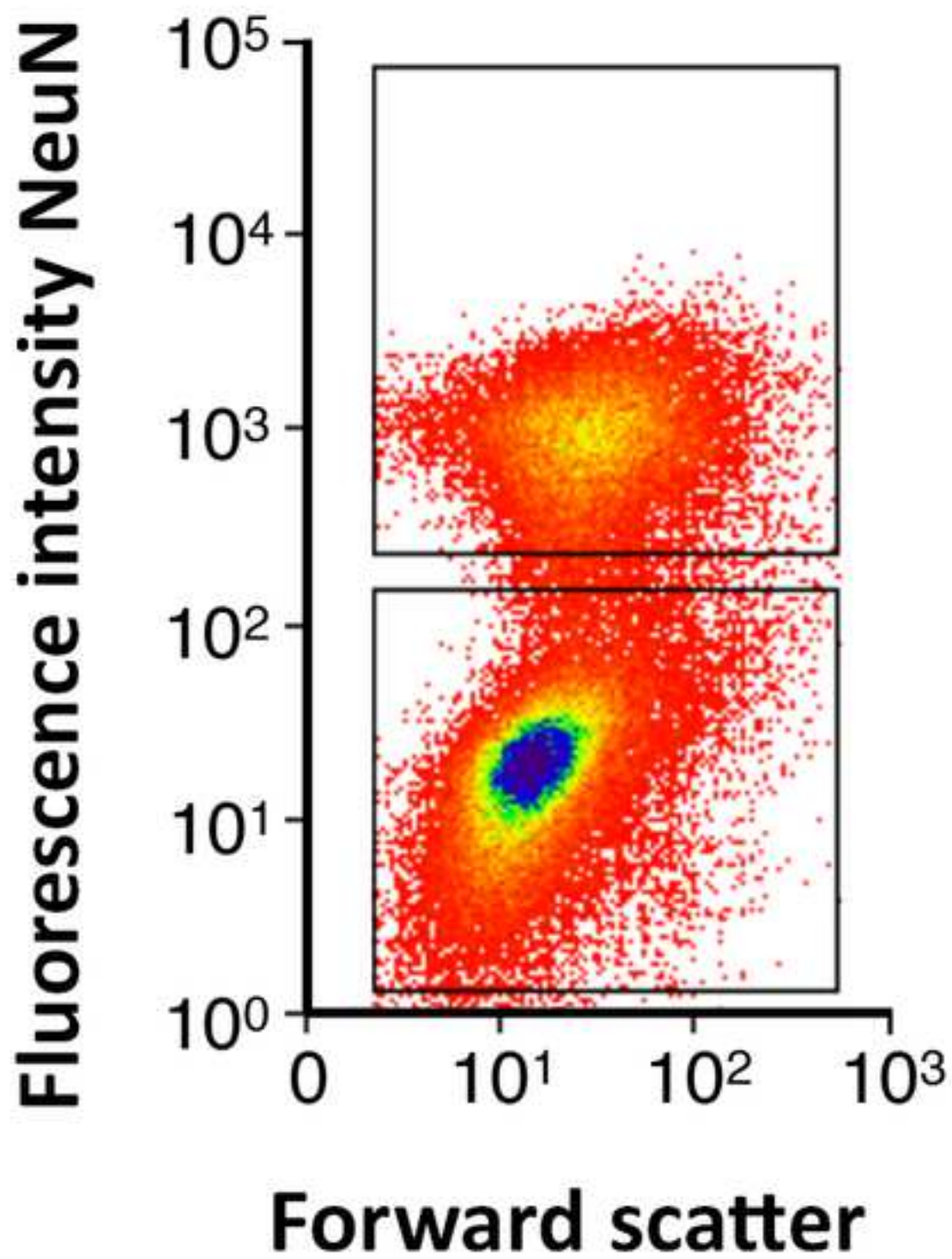
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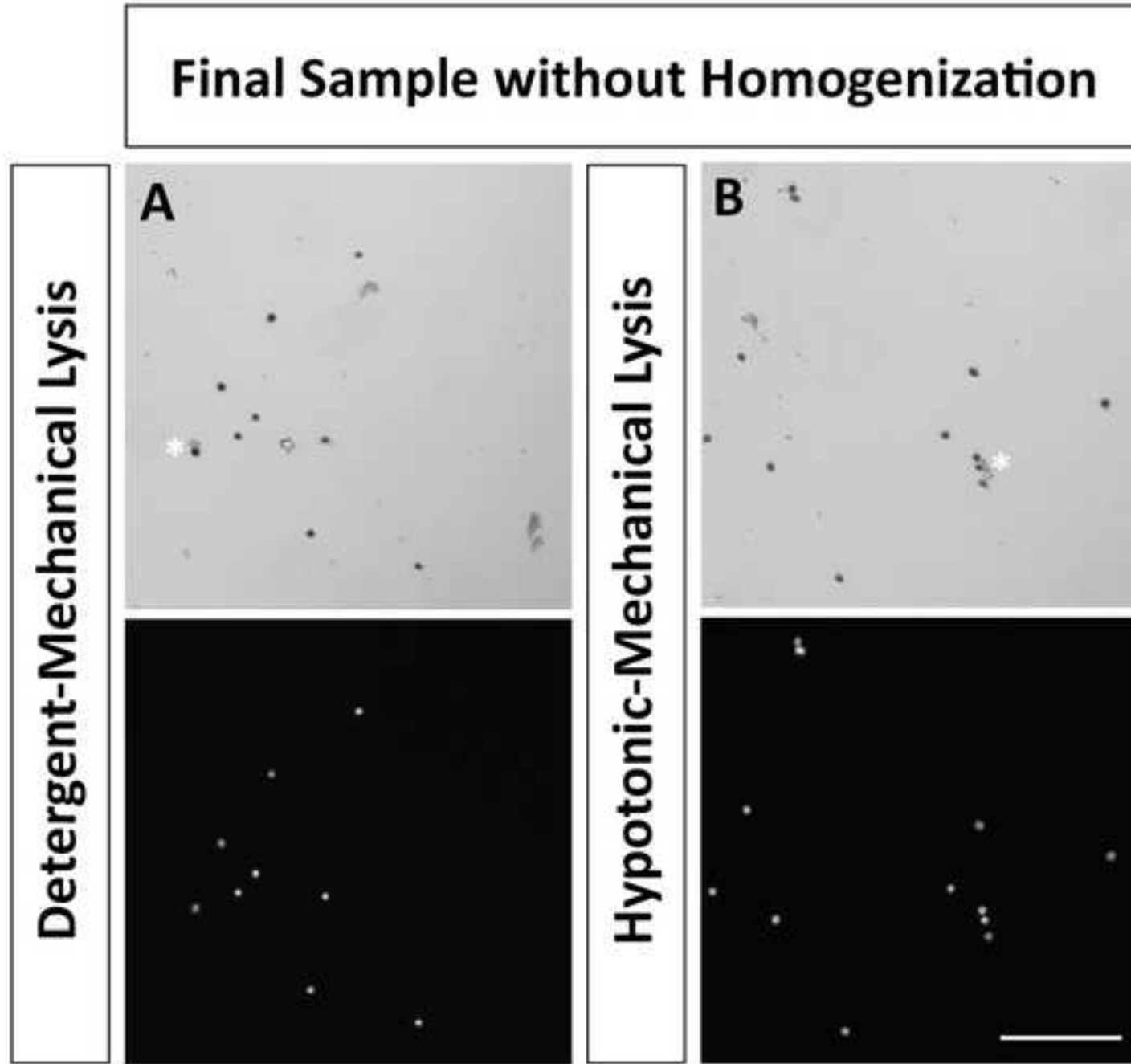
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	Crude	Homogenized	Final
Detergent-Mechanical	100 ± 15%	87 ± 9%	20 ± 2%
Hypotonic-Mechanical	62 ± 12%	35 ± 4%	8 ± 1%

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Sucrose	Invitrogen	15503-022	
1 M HEPES (pH = 8.0)	Gibco	15630-080	
CaCl ₂	Sigma Aldrich	C1016-100G	
MgAc	Sigma Aldrich	M1028-10X1ML	
0.5 M EDTA (pH = 8.0)	Corning	MT-46034CI	
Dithiothreitol (DTT)	Sigma Aldrich	10197777001	Add DTT just prior to use
Triton-X	Sigma Aldrich	T8787	
Nuclease-free water	Crystalgen	221-238-10	
1 M Tris-HCl (pH = 7.4)	Sigma Aldrich	T2194	
5 M NaCl	Sigma Aldrich	59222C	
1 M MgCl ₂	Sigma Aldrich	M1028	
Nonidet P40	Sigma Aldrich	74385	
Hibernate-A	Gibco	A12475-01	
Glutamax (100X)	Gibco	35050-061	
B27 (50X)	Gibco	17504-044	
1X PBS	Crystalgen	221-133-10	
0.04% BSA	New England Biolabs	B9000S	
0.2 U/μL RNase Inhibitor	Lucigen	30281-1	

Oak Ridge Centrifuge Tube	Thermo Scientific	3118-0050	
Disposable Cotton-Plugged Borosilicate-Glass Pasteur Pipets	Fisher Scientific	13-678-8B	
Glass Tissue Dounce (2 ml)	Kimble	885303-002	
Glass large clearance pestle	Kimble	885301-0002	
Glass small clearance pestle	Kimble	885302-002	
T 10 Basic Ultra Turrax Homogenizer	IKA	3737001	
Dispersing tool (S 10 N – 5G)	IKA	3304000	
Trypan Blue Stain (0.4%)	Thermo Fisher Scientific	T10282	
40 µm cell strainer	Falcon	352340	
MACS SmartStrainers, 30 µm	Miltenyi Biotec	130-098-458	
Conical tubes	Denville Scientific	1000799	
Sorvall Legend XTR Centrifuge	Thermo Fisher Scientific	75004505	
Fiberlite F15-6 x 100y Fixed-Angle Rotor	Thermo Fisher Scientific	75003698	
Sterological Pipettes: 5 ml, 10 ml	Denville Scientific	P7127	
Hemocytometer	Daigger Scientific	EF16034F	
Chemgenes Barcoding Beads	Chemgenes	Macosko-2011-10	

RNaseZap RNase Decontamination Solution	Invitrogen	AM9780	
Falcon Test Tube with Cell Strainer Cap (35 µm)	Corning	352235	
MoFlo Astrios Cell Sorter	Beckman Coulter	B25982	
Chromium i7 Multiplex Kit, 96 rxns	10X Genomics	120262	
Chromium Single Cell 3' Library and Gel Bead Kit v2, 4 rxns	10X Genomics	120267	
Chromium Single Cell A Chip Kit, 16 rxns	10X Genomics		
Tissue Culture Dish (60 x 15 mm)	Corning	353002	

Name of Material/ Equipment	Stock Concentration	Final Concentration	Volume / Amount
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Detergent Lysis Buffer			
Low sucrose buffer	-	-	600 µL
Triton-X	20%	0.10%	3 µL

Hypotonic Lysis buffer			
Tris-HCl (pH = 7.4)	1 M	10 mM	100 µL
NaCl	5 M	10 mM	20 µL
MgCl ₂	1 M	3 mM	30 µL
Nonidet P40	-	0.01%	1 µL
Nuclease-free water			up to 10 mL

HEB Medium			
Hibernate-A	-	-	10 mL
Glutamax	-	-	100 µL
B27	-	-	200 µL

Low sucrose buffer			
Sucrose	-	0.32 M	2.75 g
HEPES (pH = 8.0)	1 M	10 mM	250 µL
CaCl ₂	1 M	5 mM	125 µL
MgAc	1 M	3 mM	75 µL
EDTA	0.5 M	0.1 mM	5 µL
DTT	1 M	1 mM	25 µL
Nuclease-free water			up to 25 mL

Sucrose density buffer			
Sucrose	-	0.32 M	2.75 g
HEPES (pH = 8.0)	1 M	10 mM	250 µL
MgAc	1 M	3 mM	75 µL
DTT	1 M	1 mM	25 µL
Nuclease-free water			up to 25 mL

Resuspension Solution			
1X PBS	-	-	1 mL
BSA	20 mg/mL	0.4 mg/mL	20 µL
RNAse Inhibitor	40 U/µL	0.2 U/µL	5 µL



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Author(s):

Kaya J E Matson, Anupama Sathyamurthy, Kory R Johnson, Michael C Kelly, Matthew W Kelley, Ariel J Levine

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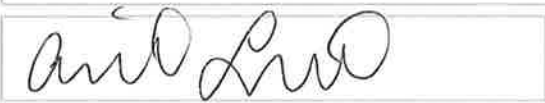
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Reviewers' comments:

We thank the reviewers very much for their thoughtful comments and believe that our responses have greatly strengthened the clarity and utility of this work. In general, we have added experimental evidence and quantification for several of the points that we previously made and have included new data using a commercially available microfluidic platform.

Reviewer #1:

Manuscript Summary:

The manuscript by Levine et al. details a protocol for nuclei isolation from mouse spinal cord that could be adapted to multiple tissue types. The protocol is well written and easy to follow. I have a few minor comments and questions listed below.

Major Concerns:

No major concerns.

Minor Concerns:

1. If cells are already lysed either by detergent mechanical disruption or hypotonic lysis to liberate nuclei, what does the additional homogenization step in low sucrose buffer do? Is it necessary? Does it result in any loss of nuclei?

We found that homogenization aids in isolation of nuclei from cellular debris and reduces the occurrence of multiplets and clumps. We now include a figure showing a representative image of nuclei from the detergent-lysis preparation or the hypotonic-lysis preparation without homogenization (Figure 5).

2. Does the density gradient centrifugation result in any damage to or loss of nuclei? How many nuclei are recovered after centrifugation - i.e. what is the input to output ratio?

We now include a table with a summary of the input nuclei (Table 1), compared to the number of nuclei at each step. This was normalized to the number of nuclei isolated in the crude detergent-mechanical lysis.

3. The authors state that nuclei isolation allows for unbiased examination of cell types, but it is unclear if they have fully examined whether their prep methods introduce any bias into the recovery of different cell types. For example, with the hypotonic lysis, given that the yield is lower, perhaps some cells are less efficiently lysed which could bias the cell types recovered?

We found that this protocol slightly enriches neuronal nuclei in the final preparation and have documented this in the results section. Unfortunately, there is no "gold standard" for the cell type composition of the spinal cord with which to compare each cell type that we observed so we focused our analysis on neurons. Previous work (for instance, Fu et al., 2013) observed that 20-24% of all spinal cord nuclei (from cervical, thoracic, and lumbar regions) expressed the NeuN neuronal marker. Similarly, in tissue sections of the lumbar spinal cord, we found that 27% of nuclei were positive for the neuronal marker NeuN, while detergent-mechanical nuclei preparation of the lumbar cord resulted in 31% of total nuclei expressing NeuN, as determined by FACS (Figure 4). It is possible that smaller non-neuronal cells are

slightly depleted during the sucrose gradient purification. In addition, downstream filtering and analysis parameters may alter the final cell-type distribution because neurons have relatively higher genes per nucleus (Figure 3C and D).

4. After the density gradient, nuclei can be pelleted and resuspended in PBS with 0.02% BSA. This is quite a low concentration of BSA given that nuclei are known to be prone to clumping. Do you find any aggregation or clumping of nuclei with this low BSA concentration?

With a concentration of 0.02% BSA in PBS, we do not see clumping. However, we now recommend using 1X PBS with 0.04% BSA. This concentration is comparable with those used successfully in DroNc-Seq and other protocols (Habib et al., 2017, Hu et al., 2018) and is the recommended resuspension buffer for cells and nuclei prior to loading the Chromium 10X Controller.

5. Have the authors tried to sort the nuclei isolated using their methods by FACS? This is another way to clean up debris and enrich for cell populations of interest and it might be useful for readers to know whether these nuclei prep methods are compatible with downstream FACS applications.

Yes, we have successfully used this method to isolate nuclei for FACS (N = 13). We thank the reviewer for this suggestion and have included a figure from FACS of the nuclei isolated using the detergent-mechanical lysis stained with NeuN (Figure 4).

Reviewer #2:

Manuscript Summary:

The authors provide a very concise summary of two protocols for nuclei isolation for single-nuclei sequencing using microfluidic platforms.

Major Concerns:

Although the authors have published with one of these protocols, it is unclear whether any of their perturbations are necessary/sufficient/superior to the published protocols for nuclei preparation. The major limitation is a lack of validity data for the final endpoint outside of their already published dataset showing that it can be done.

We offer several new points of validation including utility of particular steps, quantification and comparison of the total RNA yield, detectable quantity of mRNA for a housekeeping gene, total nuclei yield, genes per nucleus obtained following snRNA-Seq and cell-types observed following snRNA-Seq. In addition, we discuss the relative simplicity and duration of our protocol compared to previously published methods.

The authors also present two separate protocols for use downstream, but it is unclear whether either is better or worse for a specific microfluidic platform, say which is better suited for say Chromium vs Dropseq. I am unclear whether both protocols were used in microfluidic single-nuclei seq experiments or just one, so I cannot validate the efficacy of both of them.

We now include data from a hypotonic-mechanical lysis, processed on the Chromium 10X System, and have also used the detergent-mechanical lysis method successfully on Chromium

10X and the hypotonic-mechanical lysis on Drop-Seq (Figure 3). We provide a summary of the relative advantages and disadvantages of each method.

Although this could be more novel and interesting to folks not as engaged in the field, 10x now has published protocols for single-nuclei preparation of various tissues on their platform and other groups have published excellent protocols for their nuclei preparation for single-nuclei sequencing of the cord.

The protocol available for 10X takes a user 2 and a half hours to complete. Our protocol only requires 45 minutes for the detergent-mechanical lysis and 1 hour for the hypotonic-mechanical lysis. In addition to a shorter protocol that helps to preserve RNA quality, our protocol has many fewer centrifugation steps, each of which would result in a loss of total nuclei. The other detailed protocol that is available (Habib et al., 2017) does not include a density gradient and has not been validated for the 10X platform. We found that, using spinal cord tissue, a density gradient is necessary to remove medium-sized debris that could clog microfluidic channels (Figure 2). Thus, this manuscript complements existing step-by-step protocols.

This work would be much stronger if they had validated the performance metrics for say yield of nuclei per milligram of tissue, degree of clumping, integrity of the nuclear envelope, rate of Dropseq clogging, mitochondrial/cytoplasmic/spiked RNA contamination, and had some data showing the desired final readout the protocol users would care most about: single-nuclei sequencing metrics. I assume these are already data that you have from the optimization work done prior to publishing the parent manuscript, but it would be great to have the data to look at rather than just discussing anecdotal reports.

We have now included the yield of nuclei (and note the quantity of tissue) for three different stages of both protocols, the rate of microfluidic channel clogging, the genes per nucleus detected by single nuclei sequencing, and the identified cell types. In addition, we did not observe nuclei clumping (Figure 2) and found that mitochondrial contamination was 1.5-4%.

Minor Concerns:

Photographic still images would be very helpful but I assume that the video protocols will be helpful for visualizing the salty pellet and myelin frown. It would be very helpful to discuss some of the problems commonly seen and their troubleshooting, such as low yield, clumping, poor amplification, etc.

We agree that the video will provide an important supplement that enhances this protocol. In addition, we now include a paragraph at the end of the results section in which we discuss several key steps in the protocol that may need troubleshooting. This includes notes regarding douncing or trituration, homogenization and sucrose density gradient.

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