**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 validatory 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 platforms, 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.**