Point-by-point response to the reviewers' comments

We thank the editors for the helpful comments to improve our manuscript.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Our manuscript has been copy-edited by an author service.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Reuse of figures for adaptation and modification (Figure 1) is allowed by the original publisher (*Scientific Reports*). The editorial policy of the original publisher is available at the following link:
https://www.nature.com/srep/journal-policies/editorial-policies#license-agreement

3. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file.

We have uploaded the figures as .png files.

4. Please provide an email address for each author.

Email addresses of the authors are as follows:
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5. Please rephrase the Abstract to more clearly state the goal of the protocol.
6. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

According to the editor's request, we have changed the wording and added text to state the goal in the Abstract and Introduction.

7. Please define all abbreviations before use.
8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.
9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.
12. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

According to the editor's request, we have modified these points thoroughly throughout the manuscript.

10. 4.11: Please add more details to this step. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.
11. Step 8: Please add action items that direct the reader to do something.

According to the editor's request, we have added the details of those steps.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes a protocol to immunoprecipitate chromatin from specific cell types carrying flag-tagged H2B. This protocol will be useful to analyze the epigenome in specific cell types within complex mixtures of cells or tissues.

Major Concerns:

I do not have any concern with the revised version of the manuscript.

We thank the reviewer for his/her appreciative comments.

Reviewer #2:

Manuscript Summary:

Mito et al developed tandem chromatin immunoprecipitation sequencing (tChIP-Seq) that involves selective chromatin enrichment of tagged core-histones expressed/induced in a cell-type specific manner(neurons) followed by ChIP-Sequencing.

We thank the reviewer for his/her fruitful suggestions to significantly improve the manuscript.

Major Concerns:

*Line 203, the authors use as many as 8 mice brains in the optimization of this protocol. Under ideal experimental conditions getting 8 mice for the experiment is not always possible. Hence it is important for authors to test and suggest the minimum number of mice to be used to get the best signal.

In neuron tChIP-Seq, we could often use 2 ng of purified DNA by tandem immune-purification (as indicated in step 7.1) from brains of 8 mice. In our hands, 0.1 ng of DNA still provided high-quality DNA libraries for ChIP-Seq (Kadota, unpublished).

Thus, in theory, a single mouse, which corresponds to 0.25 ng tandemly immune-purified DNA, should be sufficient for library preparation. We have added this information in the manuscript. We have also suggested the ChIPmentation method as an alternative for low input in the Discussion section.

*The authors do not show any evidence that their tChIP-Seq worked by doing a q-PCR. Neither do authors show a representative genome-browser tracks of H3K4me3 at some important neural genes.

According to the reviewer's suggestion, we have added the qPCR results in Figure 2F to indicate successful enrichment of promoter regions in tChIP and genome-browser tracks along representative neuron genes from tChIP-Seq in Figure 3.

Minor Concerns:

*There are considerable number of typos and grammatical errors and hence this manuscript needs additional copy-editing.

We apologize for the inconvenience. Our manuscript has been reviewed and copy-edited by an author service.

Reviewer #3:

Manuscript Summary:

In this manuscript, Mito et al. demonstrated a variant method for ChIP-Seq, through which they can pull down nucleosomes bearing certain histone marks from a specific cell-type in given tissue, upon sequencing the related DNA to get epigenomic information. This method is novel and useful for the chromatin field of scientists.

Major Concerns:

1. The structure of this manuscript can be optimized. To me, this looks like a truncated and unbalanced version of paper, with a big head (proof of concept) and small body

(downstream sequencing, data interpretation and other know-how). Indeed, the latter is what JoVE readers care about, so that they know how to apply this method for their own research, and potentially find novel regulators. As long as there are no clear restrictions on how many figures can be in this manuscript, I would recommend to add figure 3, 4, 5…, to give more details and know-how for downstream work.

We thank the reviewer for his/her fruitful suggestions and constructive comments on our manuscript. The additional quality check data for the library preparation have been added to the manuscript; the amount of DNA isolated by anti-FLAG immuno-purification to indicate the specificity of the experiments (Figure 2C) and qPCR results to show successful enrichment of promoter regions in the tChIP library. Moreover, as mentioned above, we have also added representative genome-browser tracks (Figure 3) indicating successful neuron tChIP-Seq to facilitate interpretation of the data. We wish to add more data to the manuscript to provide detailed analyses and information based on those data. However, such data would overlap with our earlier published work (Mito *et al. Sci. Rep* 2018). Thus, here we avoided those representations but instead described the points in the Discussion section, citing our earlier work. We believe that the concrete and point-by-point procedures described in this manuscript complements our earlier work and will be appealing to the readers of *JoVE*.

2. The authors claim that they can purify chromatin in a cell-specific manner without isolation, I am wondering what makes FACS sorting less effective than this method, given the fact that single cell RNA-Seq analyses are so popular nowadays. I would encourage the authors to do more comparison of this method with known methods, to test their efficacy.

Indeed, in our earlier work (Mito *et al. Sci. Rep* 2018), we compared our tChIP-Seq method and known methods, including FACS-sorting and following RNA-Seq and TRAP (translating ribosome affinity purification)-Seq. Here we referenced that point in the Discussion section and avoided the repeated presentation of published data.

3. Although in the cartoon of figure 1, the sheered chromatin are in the state of mononucleosomes, sonication will actually yield mono-, di-, tri- nuecloesomes products. In order to get single nucleosome resolution, micrococcal nuclease (MNase) digestion can be applied (refer to JoVE2593, doi: 10.3791/2593). It will be very interesting if you can apply tChIP on mononucleosomes to see if you get better results than sonication!

We thank the reviewer for the constructive comment. We completely agree that MNase digestion, which is used for native ChIP (N-ChIP), must be an alternative option to sheer chromosomes. We have added the MNase option in the Discussion section. Although we are quite interested in such an option, we are afraid that it would be beyond the scope of this manuscript focusing on the best practices of already established tChIP-Seq. Again, we are grateful to the reviewer for sharing this excellent idea to improve our methods in the future.

Minor Concerns:

1. In Line 44, I would change the wording "epigenetic regulation" into "chromatin modifications", since epigenetics including a lot of mechanisms other than histone modification and DNA methylation, such as chromatin remodeling, X-inactivation, RNAi etc.

According to the reviewer's suggestion, we have changed the wording.

2. Through reading the core IP part, it is not clear to me what control you use, please elucidate.

We thank the reviewer for pointing out this lack of clarity. We usually use lysates from naïve mouse brains, which do not express FLAG-tagged H2B, as negative controls for the immune-purification. We have added data showing representative DNA contents obtained in the experiments (Figure 2C). We usually do not recover any detectable DNA from the negative controls.