

Dear Editor:

Thank you very much for your appreciation and reviewers' suggestions for our manuscript 'Robust Generation of Ready-to-Use Cryopreserved Motor Neurons from Human Pluripotent Stem Cells for Disease Modeling'. We are very grateful for the opportunity for improving our works. According to the recommendations of reviewers, we improve the presentation of figures, modify the article content, redo the scientific English language editing, and response to the suggestions point by point in the reply letter and highlight the changes with "Track Changes" and "Yellow Highlight Color" in the revised text. We sincerely hope this revised manuscript will be given a serious consideration for publishing in *International Journal of Molecular Sciences*.

Sincerely yours,

Chia-Yu Chang

Bioinnovation Center, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan.

No. 707, Sec. 3, Chung-Yang Rd. Hualien, Taiwan, 97002

Phone No: +886-3-856-1825 (ext. 12106)

Email Address: scata0726@hotmail.com

Reply Letter to Reviewer 1

Comments and Suggestions for Authors

Major:

Fig 1 is impossible to understand.

The quantification Figures 1q and 1r are inconsistent with the images and the text.

Reply: I'm sorry that we make the mistake on the presentation of Data set in Figure 1r. The white color bars are BiSF original method, and the black bars are CHSF-MN method, but we labeled them with opposite colors in the previous version Figure 1r. We correct the Figure 1r with the right color symbols.

In images, NF and Isl1 markers appear to be lower in the new (CHSF) method compared to the old method (including in Fig. 1r). Isn't it supposed to be just the opposite, according to the manuscript's idea? NF marker was not quantified.

Reply: In addition to the wrong symbols in Figure 1r, reader may feel like the NF expression in Figure 1g (BiSF original) is more than Figure 1o (CHSF-MN) because of there are large, aggregated cell clumps with more cells in the visual field of 1g. To avoid this possibility of misunderstanding, we change the Figure 1g with an image that have less cells and without large, aggregated cell clumps. We calculate the day 25 NF expression of BiSF ($84.57 \pm 4.92\%$) and CHSF-MN ($98.41 \pm 0.21\%$), present in Figure 1r and describe at [line 116 and line 146](#). We also calculate the NF expression of each line in Figure 2t and describe at [Line 185](#).

The Oligo2 and Isl1 are both MN related markers in the ventral-posterior spinal cord. However, Oligo2 would express at the progenitor stage (early) and the Isl1 express at pre-mature neuron stage (late). Thus, these two markers would not co-express at the same differentiation stage. They express sequentially[1-3]. At day 15 of differentiation (progenitor stage), the major expression stage of Oligo2, there are very few islet1 expression in both BiSF (Figure 1b) and CHSF-MN (Figure 1j). At day 25 of differentiation (pre-mature neuron stage), the Isl1+HB9+ cells indicate the pre-mature MN. At this stage, the Isl1 expression in CHSF-MN ($96.48 \pm 0.66\%$; Figure 1n and 1r) is much more than BiSF ($55.74 \pm 31.21\%$, about half of DAPI+ cells are lack of Isl1 and HB9 expression; Figure 1f and 1r). The data is calculated in the corrected Figure 1r and describe at [line 118, line 152 and line 154-160](#).

The Figure 1q calculate the marker expression data of day 15 and Figure 1r calculate the day 25 data. We add the information in the result and figure legend at [line 163-164 and line 175](#). Sorry for the lack of information.

In images, HB9 marker appears to be higher in the new method, but in Fig. 1r it is lower.

Reply: We are sorry for the wrong symbols in Figure 1r and correct it.

The quantifications in Fig. 1 are inconsistent with those in Fig. 2.

Reply: This is also due to the opposite color symbols in Figure 1r. Sorry for this mistake.

Fig. 1q and 1r both contain NKX2.2 marker (why?), but show opposite ratios between the two methods.

Reply: The Figure 1q is day 15 data and 1r is day 25, we add the information in the result and figure legend at [line 163-164](#) and [line 175](#). The mistake we made in the Figure 1r is also lead to confusion. The NKX2.2 is a ventral-posterior spinal cord marker that expressed in the common pool of Oligo2+ neural progenitor that can finally become interneurons (NKX2.2+ but HB9-) or MNs (HB9+ but NKX2.2-). Thus, we track the NKX2.2 expression at day 15 and day 25 to indicate the final fate of these neural progenitors. In the BiSF protocol, most of neural progenitors are NKX2.2+ at day 15 ($87.45 \pm 4.81\%$; Figure 1q), and finally about half of them become NKX2.2+ interneurons at day 25 ($52.44 \pm 4.12\%$; Figure 1r). In the CHSF-MN protocol, most NKX2.2 are inhibited at day 15 ($7.05 \pm 2.13\%$; Figure 1q) and almost undetectable at day 25 (Figure 1r), indicate the NKX2.2 inhibition may promote neural progenitors to become MNs. The role of NKX2.2 is added at [line 120-125](#) and [line 160-163](#).

Overall, it's a total mess.

Reply: We are sorry for the wrong symbols in Figure 1r.

Images in Fig. 2 have no indication at which time point they were taken. Fig. 2s is a mixture of the markers on day 15 and day 25. They should be separated to different graphs.

Reply: Thank you for your suggestions. We add the ICC timing information beside the name of each cell line in Figure 2. The Figure 2s is separated into Figure 2s (day 15) and 2t (day 25). The time information is added in the result and figure legend at [Line 186](#) and [194-195](#).

Line 163-164: "On day 25 of differentiation, $91.34 \pm 4.26\%$ – $93.54 \pm 1.34\%$ HB9 was elevated in all PSC lines (Figure 2s)." What this sentence is supposed to mean?

Reply: We correct this sentence as "[On day 25, the differentiated cells expressed \$96.92\% \pm 1.03\%\$ to \$99.23\% \pm 0.19\%\$ NF and \$91.34\% \pm 4.26\%\$ to \$93.54\% \pm 1.34\%\$ HB9 in all PSC lines](#)" at [line 185-186](#).

It looks like the scale bars should be different in the images in Fig. 2, but the legend to

Fig. 2 states they are the same.

Reply: We examine the image record of all original files that presented in Figure 2 and confirm that they are captured under 200X magnification. The cell clump size in individual image and the cell morphological difference between day 15 and day 25 may cause the visual effect and feel like the images are under different magnification.

No error bar in Supplementary Fig. S1.

Reply: sorry for the lack of error bar. We add the error bar on Figure s1 and describe the ChAT expression ratio at [line 232-234](#).

L. 401-405. There is no explanation how the cell boundaries were defined to enable cell counting. It's crucial, since all the quantifications are represented by a marker+/DAPI+ cell ratio. It's very difficult to guess individual cells in the majority of the images presented.

Reply: We calculate the DAPI+ cell nucleus that without overlapping with other ones, and can be clearly identified under 400X magnification, for quantification. We add this information at [line 434-436](#). Most of images in Figure 1 and Figure 2 are 200X magnification, to show some specific structures, such as the Rosette like neural stem cells, and for presenting an overall view of marker expression.

All the new additions to the manuscript's text were made in a hurry and written in a very bad English. Just one example: Lines 296-308. The entire manuscript, especially new additions, must undergo a professional scientific English editing.

Reply: Thank you for providing us the opportunity to improve the English presentation. This article was edited by whose native language is English before submission, but we are sorry the language quality is poor. This time we carefully examine the full article and rewrite some parts (especially the new added parts) for improving the expression, and redo the full manuscript English editing. Since there are numerous modifications while full manuscript English editing, we do not highlight all minor modified points. The major modifications are labeled with "Track Changes" and the major rewrite or new added parts are labeled with "Track Changes + **Yellow Highlight Color**" at [line 120-125, 154-164, 230-235, 259-262, 284-288, and 326-338](#). Hope our effort could improve the quality of manuscript.

References:

1. Maury, Y.; Come, J.; Piskorowski, R.A.; Salah-Mohellibi, N.; Chevaleyre, V.; Peschanski, M.; Martinat, C.; Nedelec, S. Combinatorial analysis of developmental cues efficiently converts human pluripotent stem cells into

- multiple neuronal subtypes. *Nat Biotechnol* 2015, 33, 89-96, doi:10.1038/nbt.3049.
2. Du, Z.W.; Chen, H.; Liu, H.; Lu, J.; Qian, K.; Huang, C.L.; Zhong, X.; Fan, F.; Zhang, S.C. Generation and expansion of highly pure motor neuron progenitors from human pluripotent stem cells. *Nat Commun* 2015, 6, 6626, doi:10.1038/ncomms7626.
 3. Li, X.J.; Du, Z.W.; Zarnowska, E.D.; Pankratz, M.; Hansen, L.O.; Pearce, R.A.; Zhang, S.C. Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol* 2005, 23, 215-221, doi:10.1038/nbt1063.