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Nijmegen, August 20th

Dear Jaydev,

On behalf of the authors I would like to resubmit the accompanying manuscript, entitled “**Rapid neuronal differentiation of induced pluripotent stem cells for measuring network activity on micro-electrode arrays**”, to the Journal of Visualized Experiments.

We were happy to read that the referees acknowledge the importance of reliable protocols for generating mature functional neurons. We however also realize that in the first version of our manuscript we did not support our claims with enough experimental data, as well as did not well explain the focus and context of our protocol.

Taking into consideration all the comments we have now thoroughly revised our manuscript.

For each of the editorial/reviewer’s comments, our response can be found below. We thank you in advance for reconsidering our manuscript.

Yours sincerely,

Nael Nadif Kasri, PhD

**Note:** All changes in the manuscript are highlighted in yellow. Additional changes in the author list were made and are marked by the red font color.

**Editorial comments:**

•Formatting: References – Please abbreviate all journal titles.

We thank the editor for pointing this out. All journal titles were abbreviated.  
  
•Additional detail is required:  
-Section 1.2 – How are animals euthanized and brains removed from the skull? How are embryos isolated from the dam?

This information was added to the protocol by adding extra steps to section 1.2.  
-2.1 – Where are iPSCs obtained?

An additional note was provided at the start of section 2 to indicate the source of the iPSCs that were used in our experiments.   
-Please include an additional step for downstream analyses of the differentiated cells. This does not need to be highlighted for filming, and citations can be supplied in lieu of detail.

We included an additional section in the protocol for describing downstream analyses. For the details of the analyses, we referred to other papers from our group that contain the details in their methods section.   
  
•Discussion: Please discuss the significance with respect to alternative methods and include independent citations.

We added this information to the discussion, including the relevant citations.

•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

We ignored this comment, because all figures and tables are original and not published previously.

•JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

For all references, the DOIs were included if available.   
                                               
•IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVEeditor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.                                               

**Reviewers' comments:**

**Reviewer #1:**  
*Manuscript Summary:*  
The iPSC field is in need of reproducible protocols to generate mature neurons for functional assays. The authors adjusted the protocol of Zhang et al (2013) by creating a rtTA/Ngn2-positive iPSC line before performing differentiation studies. They claimed that this increased the reproducibility and efficiency of neuronal differentiations between experiments with one iPSC line and between experiments with different iPSC lines. The authors tested and optimized conditions to perform MEA analysis, a technology which gives information about the electrophysiological properties of neuronal networks and suitable for future high throughput functional analysis of patient iPSCs.  
  
*Major Concerns:*  
-The authors should present data to support their conclusion that a stably affected rtTA/Ngn2 iPSC line resulted in more reproducible neuronal differentiation than the original protocol.

We realize that in the first version of our manuscript the emphasis was wrongly put on the comparison with the original protocol by Zhang et al. However we would like to stress that the goal of this protocol was not make a direct comparison with the original protocol. Instead we provide a protocol based on the original protocol with small adaptations that in our hands facilitated the recordings from differentiated neurons on MEAs. Since in the original protocol they did not measure neuronal network activity on MEAs, we cannot make a direct comparison. We have now rewritten, the abstract and introduction to better reflect this.

-The maturation levels should be presented, e.g. whole cell patch recordings or stainings

As suggested by the reviewer, we added results regarding the characterization of our iPSC-derived neurons. Figure 2 has been extended. We now show:

1. MAP2 and synapsin-1/2/PSD-95 stainings for independent experiments and independent IPS lines, at different times after the start of differentiation.
2. MAP2 and cortical markers stainings (SATB2, BRN2, CTIP2, FOXP2) to assess the identity of the differentiated neurons.
3. Whole-cell current/voltage-clamp recordings at different time-points after induction of the differentiation.

In addition, we expanded the results section adding more details regarding the development of the iPSC-derived neurons at the single-cell and network level.

-They should perform these experiment in more than one stably affected rtTA/Ngn2 iPSC line.

We have now included results from a second IPS line. These results can be found in **new figure 2**  
*Minor Concerns:*  
-The abstract gives the impression the authors designed a complete new protocol. The abstract should be rewritten to reflect the results properly.

We agree with the referee that this impression was raised in the original version of our abstract. The abstract was rewritten to indicate clearly what the novelty of the results and the protocol is. It is certainly not our goal to compare our protocol with the previously published protocol by Zhang et al, Instead we modified it as such that it can be used for MEA recordings.

-The protocol can be useful to explore deficits in neurological disorders in which glutamatergic neurons are involved. The authors should discuss whether the absence of inhibitory neurons affect the physiological representation of the in vivo situation.

We thank the reviewer for his kindness to point this out. The absence of inhibitory neurons is indeed a limitation of our protocol. We now included an elaborate paragraph to the discussion on the limitations of our methodology, including a few sentences on the absence of inhibitory neurons.

-The following points should be corrected:  
Page 2- 88: awkward sentence and should be rewritten. Translation from rodent to human?

We thank the reviewer for this comment. The sentence was rewritten.   
Page 3- 123: compared to rat primary neurons? 'Coupled to' or 'plated on'?

Parts of the introduction were rewritten, including this sentence. Also the term ‘coupled to’ was replaced throughout the manuscript with clearer terms.  
Page 3- 132: grammar: In particular, we added the step of generating an rtTA/Ngn2-positive iPSC line to the original protocol before the onset of differentiation

Parts of the introduction were rewritten, including this sentence.

Page 4- 135: compared to the approach used by Zhang et al. (2013), in which the iPSC line...

Parts of the introduction were rewritten, including this sentence.  
Page 5- 219: to combine, unclear. Collect in same tube?

We thank the reviewer for this comment. This sentence was changed.   
Page 6-233: replace the medium. 100%?

Indeed, all the medium is replaced. We added this missing detail to the protocol.  
Page 7-296: Add concentration of ROCK- inhibitor.

We thank the reviewer for pointing out that this detail is missing. However, this concentration cannot be provided. In the Table of Specific Materials/Reagents, we indicate that we use RevitaCell. This product of Gibco has multiple components (among others Rho-associated protein kinase inhibitors), for which the concentration is provided by the manufacturer. The E8 medium is supplemented with 1% (v/v) of RevitaCell, according to the instructions of the manufacturer. Since JoVE does not allow the use of commercial language, we could not provide more details here. Nonetheless, we think that the information in the protocol with the details provided in the Table of Specifc Materials/Reagents are sufficient for performing this step of the protocol.  
Page 8- 321: grammar: add 1 ml of the prepared E8 medium to each well.

We thank the reviewer for his kindness to correct our English. The word order was changed.  
Page 8- 342: grammar: add 1 ml of the prepared E8 medium to each well.

The word order was changed.  
Page 9- 383: 'lower' instead of 'less'

This was changed in the manuscript.   
Page 11- 432-435: unclear sentence

We thank the reviewer for pointing out the lack of clarity. We rewrote the sentences in this note; in particular, we now included the specific steps of the protocol to which the remarks refer to. This should make the remarks clearer.  
Page 11-438: concentration of ROCK- inhibitor

See the above.  
Page 11- 447-449: dissociate iPSCs by… (active instead of passive)

We changed the sentence and now use the active instead of the passive form of the verb.  
Page 12- 480: wash the culture by adding 5 ml DPBS and swish it around gently.

We thank the reviewer for his kind suggestions for improving our phrasings. We changed the sentence as suggested by the reviewer.   
Page 13- 528: the morphologically started to resemble neurons?

We changed the sentence after the suggestions by the reviewer.  
Page 14- 529: Evidence lacking for claim that neuron are connecting (forming synapses)

New stainings and whole-cell voltage clamp recordings were added to Figure 2 and to the results section to support our claim.   
Page 14- 546: coupled to MEA? You used MEAs to record the electrophysiological activity …

As written in the above, we replaced the unclear term ‘coupled to’ with clearer terms. In this particular case we replaced it with ‘cultured on’ to indicate that we plated and cultured the cells on the MEAs as described in the protocol.   
Page 14- 547: contradicting: you claim to have mature neurons at day 20 but you only see spontaneous activity at day 23?

We thank the reviewer for this comment. We rewrote the results section to include results of an earlier time point (16 days after induction of differentiation; **new figure 2M**), to include quantifications of the spontaneous and synchronous activity (**new figure 2N,O**), and to clarify our claims. We expect that these changes will make clear to the reader that neuronal networks showing spontaneous activity can be expected early in the development (e.g., 16 days after induction of differentiation). However, later in the development the spontaneous activity increases and synchronous activity can be observed that involves all electrodes of the MEA.   
Page 15- 609-612: unclear sentence

We thank the reviewer for pointing out this lack of clarity. We changed this sentence to make it clearer.   
Page 16- 620: neurons coupled to the MEA: neurons in close proximity to or on top of the electrodes of the MEA?

As written in the above, we replaced the unclear term ‘coupled to’ with clearer terms. In this particular case we replaced it with his suggestion ‘on top of’: the neurons should form network contacts directly on top of the electrodes to allow measurement of the network activity.   
  
*Additional Comments to Authors:*  
N/A

**Reviewer #2:**

*Manuscript Summary:*  
Van Gastel, Frenga, and colleagues have authored a JoVE manuscript intended to implement the Zhang method for deriving neuronal cultures from human iPSCs in the context of MEA recordings. This is a laudable goal, which if implemented well should be of considerable interest to the neuroscience community.  
Overall, however, I am less than enthusiastic about the current form of this submission. The authors describe the protocol well, although half of the paper is actually about making the supporting rat astrocytes.  
  
*Major Concerns:*  
-One of my major concern with the paper is the claim of reproducibility, while the data shown seem to come from one single experiment and from one iPSC line. The authors claim to also have used another control iPSC line and a patient line, although no data are shown and it is not specified how these iPSCs are made (source/reprogramming/characterization) or from what kind of patient. Also claims of finding a uniform population of upper layer cortical neurons is not supported by data. The figures are very minimal and of insufficient resolution to justify their claims.

We agree with the reviewers that we have not provided enough data to support our claim and that the figures in our original version were minimal. As stated in the letter to the editor the goal of the protocol was not to compare this protocol to the previously published protocol by Zhang et al, but instead to publish an adaptation to the Zhang protocol in order to facilitate recording on MEA’s. As to request of the referee and consistent with the results described by Zhang et al, our protocol generated a population of excitatory upper layer cortical neurons, confirmed by pan-neuronal (MAP2) and subtype-specific cortical markers such as BRN2and SATB2(layer II/III). We did however not observe neurons that were positive for deep layer neurons CTIP2 (layer V) or Foxp2 (layer VI). We furthermore now also included these data for a second IPS line. All data are **shown in new figure 2**

-A second major concern regards the electrophysiology data presented. The authors discuss mature intrinsic properties and later mention whole-cell recordings, yet there are no data or figures that provide quantitative data. It would be important to implement at least basic pharmacology to establish the validity of the MEA data (e.g., TTX to block APs). Overall, the physiology data is very limited and some important information is missing (e.g., the discussed whole-cell data should be provided).

We completely agree that the electrophysiological data were minimal in our original submission. As suggested by the reviewer, we added results regarding the electrophysiological characterization of the hiPSC-derived neurons. **New figure 2** now shows MAP2 and synapsin-1/2?PSD-95 staining and whole-cell voltage and current clamp recordings during development. We expanded the result section adding more details regarding the development of the hiPSC-derived neurons at the single-cell level. We show that excitatory postsynaptic currents were blocked completely by CNQX. Finally, we also added quantifications of the level of activity of neuronal networks in development.

Specific points:  
\* Title: Reproducibility is not actually shown.

The title was changed  
\* Introduction (lines 138-141): "The fast, reliable and efficient protocol presented here enables comparisons between neurons derived from different iPSC lines (e.g., different patient-derived iPSC lines) and will likely advance the study of human neurological diseases."  
\* Discussion (lines 599-602): "We have successfully generated one rtTA/Ngn2-positive iPSC line from healthy donor iPSCs and two rtTA/Ngn2-positive iPSC lines from patient iPSCs. With these lines, we have performed several experiments that indicate the reproducibility of this procedure."  
> Reliability is not actually shown. Were multiple iPSC clones per line examined? Where do these iPSC lines come from? What kind of patient(s)? Source of iPSC lines should be better described.  
> 'Several experiments' is a bit vague. It would be important to actually show the reproducibility (between iPSC lines and differentiation batches).

We agree with the reviewer that in the original submission we data presented were minimal and vague. We have now included several experiments in new Figure 2 showing reproducibility (figure 2B, M) as well as included additional IPS lines (figure 2C, F). All these data can be found in **new figure 2.**

\*Discussion (lines 614-616): "This will inevitably lead to batch-to-batch differences in astrocyte quality, which currently limits full reproducibility of this protocol."  
> This again supports my view that the reproducibility of this protocol has not been proven by the experiments provided.

We indeed have experienced that quality and the amount of astrocytes used for the culturing affects the neuronal network activity. We do not show all this information, instead now we provide clear information to control for astrocyte cell density and quality.

\*Representative Results (lines 522-526): Results are not shown and MAP2 expression does not prove upper-layer identity.

We have no wincluded data showing that most of the MAP2 positive neurons express upper layer marker but not lower layer markers (**new figure 2E,F).** These results are consistent with the data presented in the original protocol by Zhang et al.

\*Representative Results (lines 534-536): Why estimated and not quantified?

We admit that our description was unclear at this point in the manuscript and we clarified it in our revision. Our main point is that the presence of a synapsin-1/2 puncta in our picture does not necessarily mean that a functional synapse is present. Hence, we *quantified* the number of synapsin-1/2 puncta and thereby *estimated* the number of functional synapses. In addition we show that synapsin markers juxtapose PSD-95 puncta. Finally we now show quantification of whole-cell voltage clam recordings (EPSCs).   
\*Figure 2: Image resolution is very poor for all panels. In particular, the synapsin staining seems quite poor, at least at this resolution.

We thank the reviewer for pointing this out. We are not sure which format of the figure was provided to the reviewer, since we provided two figure formats (tiff and eps) with the submission. After submission, it turned out that one of the formats (tiff) had a lower resolution than expected. With our current submission, we will provide the journal with the correct figure formats, so that the resolution will be optimal.   
\*Figure 2C: Seems N=1, despite the claim to be using multiple different iPSC lines with high reproducibility.

We thank the reviewer for pointing out this lack of clarity. We clarified this in the figure legend. Indeed, the results shown in the figure are obtained with one hiPSC line. The results that were obtained with the other hiPSC lines mentioned in the text are not shown, since the results shown are representative for the other results. However we now also included data of a second IPS line in **new figure 2**   
\*Discussion (lines 582-584): Regarding the claim of the creation of a stable cell rtTA/Ngn2-positive iPSC line, please show data to characterize the stable cell line (RNA/protein expression, immuno).

All our IPSC lines are characterized through a battery of quality control tests including morphological assessment, expression of pluripotent markers measured by RT-qPCR and immunocytochemistry, ability to generate cells from the 3 germ layers when spontaneously generated, karyotyped and assessed for genomic integrity by high-density SNP arrays. We believe that it is beyond the scope of this manuscript to include all these data in the current manuscript, since the focus is on the differentiation and measurement of neural networks.

We however think that the reviewer’s request touches upon a valid point: the procedure of generating a stable cell line might affect the pluripotent properties of the hiPSC line and this may have consequences for the neuronal differentiation. However, we did not have indications that the pluripotent properties were affected. During the procedure of generating the stable cell lines, the morphology and behavior of the cells did not change for our lines. In addition and importantly, the stable lines that we generated were capable of differentiating to neurons, which was the only goal of generating the stable lines in the first place. We are therefore convinced that the protocol of generating the stable cell line is valuable for the applications that are described in the manuscript.   
  
*Minor Concerns:*  
\*Introduction (line 93): perhaps add more reference (e.g., Shi et al Nat Prot 2012) and acknowledge emerging 3D protocols

We thank the reviewer for his kind suggestions. We added the extra reference to the introduction. In our discussion, the emerging three-dimensional culturing protocols are now briefly discussed as a future perspective.

\*Protocol (section 2.1: Plate the iPSCs): Which iPSCs were used here?

We thank the reviewer for pointing out the absence of this information. The information was added as an additional note at the start of section 2.1.

\*Protocol (section 2.2.4): No titers or MOI determined?

We typically generate virus with a titer of !06. We did not calculate the MOI for each experiment, instead we performed transductions with different amounts of lentivirus in combination with positive selection, as described in the protocol. We admit that determining the titers and/or MOI would make the method more robust, but we found that it was not an important prerequisite for generating the stable lines.   
  
*Additional Comments to Authors:*  
N/A

**Reviewer #3:**  
*Manuscript Summary:*  
This manuscript describes protocol for fast production of hIPSC derived neurons and their measurement using MEA platform. As it is of major interest to study human derived neuronal networks in vitro for disease modelling and neurotoxicity for excample, this paper is of great interest for large audience.  
  
*Major Concerns:*  
The manuscripts describes optimization of previous protocol made by Zhang et al. for production of upper layer cortical neurons, with high efficiency. As original protocol of Zhang et al 2013 was modulated, authors should provide evidence that neurons they produce are indeed cortical neurons as MAP-2 staining by itself does not prove that. Thus markers of cortical neurons should be used in addition. Second, if modified protocol provides more stable production of cortical neurons, details of cortical neurons yield from different hIPSC lines should be shown. Third , authors shown result of single 6 well MEA without any details of gained signals; total spikes, burst, synchrony ect and without any data of parallel MEA's (networks derived from same or different hIPSC lines). This makes in difficult to evaluate how reproducible is production of functional networks with this protocols. Authors could also provide short trouble shooting for protocol parts which they find most challenging to accomplish.

As suggested by the reviewer, we added results regarding the characterization of our iPSC-derived neurons. Figure 2 has been extended. We now show:

1. MAP2 and synapsin-1/2/PSD-95 stainings for independent experiments and independent IPS lines, at different times after the start of differentiation.
2. MAP2 and cortical markers stainings (SATB2, BRN2, CTIP2, FOXP2) to assess the identity of the differentiated neurons.
3. Whole-cell current/voltage-clamp recordings at different time-points after induction of the differentiation.
4. In figure 2M two raster plots indicating the network activity at two different time points after the induction of differentiation. We also added the global quantification of the level of activity of neuronal networks during development.

*Minor Concerns:*  
a) 1.3.11 please provide cell density for astrocyte plating to T75 flasks

We thank the reviewer for pointing out this lack of clarity. However, the requested cell density cannot be provided because it was never determined. The astrocytes cannot be counted, as the cell suspension at this stage also contains also other glial cell populations. We added this extra information to step 1.3.11 of the protocol to make it clearer.

b) 1.4.2 please provide cell density for astrocytes in confluence

As written above, the exact cell density cannot be provided as it was never determined. Instead, we now included the percentage of confluency to make this step clearer.

c) 1.4.2.6 please provide cell density for astrocyte splitting

We thank the reviewer for pointing out this missing detail. We added this information to the protocol.

d) Is it possible to freeze astrocytes for further use?

We thank the reviewer for pointing out this missing information. Indeed, the rat astrocytes can be frozen, but we do not do this and prefer to use astrocyte cultures from fresh embryonic rat brains. This information was added to the protocol.

e) 2.1 please provide cell density for hIPSC cultures in 6 well prior harvesting

We thank the reviewer for this remark. The information has been added with an additional note in the protocol.   
f) 2.1.1 please provide final concentration or percentage of Geltrex DMEM/F12 in parenthesis, do that also for other supplements later on

The first sentence of step 2.1.1 was rewritten to include this percentage. However, for the other supplement, i.e. the ROCK inhibitor, we cannot provide the percentage/concentration because of the reasons mentioned in response to the comment of reviewer 1.   
g) 2.5 provide protocol for freezing of rtTA/Ngn2-positive hIPSC

We thank the reviewer for mentioning this lack of clarity. Unfortunately, because our protocol is already quite elaborate, we do not have additional space for including the complete protocol. However, the protocol is the same as standard freezing protocols for cells and this remark was added to the manuscript.   
h) 3 6 wells MEA have 9 embedded electrodes + 1 reference electrode (fig 1 b), please clear that up. In figure 2 C, authors are showing recordings from all 10 per each well, why?

We thank the reviewer for pointing out this lack of clarity. We added the detailed information to the protocol section of the manuscript. The analysis software that we use also plots the trace of the reference electrode. Hence, for each well of the 6 well MEA, 10 traces are shown, which includes one empty trace for the reference electrode.   
i) 3 is cell density same in both 6 well MEA's and 24 well plates with cover slips, add that information to 3.2.6 ? directional scaling to 12 or 6 wells do not necessary work by keeping the same cell density, have authors done this what they as suggesting here?

The density for the 6 well MEA is different than for the 24 well plates with cover slips. We now added a note to the protocol to explicitly mention this and to refer to figure 1, in which this difference is visualized. We have performed scaling to 12 or 6 wells by keeping the same cell density and this worked for us; therefore, we added this information to the protocol.   
j) 3.1.3 reason for using poly-ornithine instead of commonly used PEI as first coating?

We thank the reviewer for this question. The reason for this is that we also use poly-ornithine for other neuronal differentiation protocols in our lab and that we do not use PEI in this context. To be honest, we never tested PEI as a first coating, but we have to admit that it is a good suggestion for improving the protocol.   
k) Fig 1 B, MAP-2 is not marker solely for cortical neurons.

We now show co-immunostainings of MAP2 and cortical markers (SATB2, BRN2, CTIP2, FOXP2) to assess the identity of the differentiated neurons.

l) What were the measurement parameters in MEA recordings; filters, spike detection, sampling rate ect?

We added an additional section to the protocol for performing the downstream analyses. Here, we also provide references for the details of the data analysis.   
m) provide details of burst analysis

For this point, please see the above.   
n) in results, authors state that all the recording channels show synchronous events, which is not case if looking signals from orange and turquoise wells in figure 2b. more detailed data of recorded signals are needed as already stated in major comment 3.

We thank the reviewer for this comment. It is true that there is a difference in the pattern of activity shown by the different wells. However, the level of network bursting rate is not really different between the wells: the number of network burst recorded during the 5 minutes shown is 22 in well A, 18 in well E (orange) and 21 in well F (turquoise). In order to clarify the level of synchronous activity, now we show the frequency of the network burst for the experiments performed. We also added the reference for the tool used to detect synchronous events.  
o) how long the activity could be recorded from the wells?

We recorded 20 minutes of activity. We added this information in the text.  
p) discussion part: authors should make it clear that they still need rodent astrocytes in the cultures to make functional neuronal networks which can effect the results in neurotoxicology or disease modelling as rodent vs human astrocytes are not necessary similar in general and there are even subtype differences. Thus, these cultures are not purely human. Also, use of FBS there as astrocytes supporting factors can influence results as it is not the purest and well defined supplement in the field.

We thank the reviewer for these critical remarks. We think that the reviewer is absolutely right about the fact that our neuronal networks are not human neuronal networks *sensu stricto* because of the presence of the rat astrocytes. We therefore added this point to the discussion as one of the limitations of our approach and we provided our future perspectives on this. We also agree that FBS may not be the most well-defined supplement in the field. We used it because it was used in the original protocol by Zhang *et al.* (2013), but we agree with the reviewer that the protocol can be improved by finding better-defined alternatives for this serum supplement.   
  
*Additional Comments to Authors:*  
N/A