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Rapid neuronal differentiation of induced pluripotent stem cells for measuring network activity on micro-electrode arrays

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Abstract:	Neurons derived from human induced pluripotent stem cells (hiPSCs) provide a promising new tool for studying neurological disorders. In the past decade, many protocols for differentiating hiPSCs into neurons have been developed. However, these protocols are often slow with high variability, low reproducibility, and low efficiency. In addition, the neurons obtained with these protocols are often immature and lack adequate functional activity both at the single-cell and network levels unless the neurons are cultured for several months. Partially due to these limitations, the functional properties of hiPSC-derived neuronal networks are still not well characterized. Here, we adapt a recently published protocol that describes production of human neurons from hiPSCs by forced expression of the transcription factor neurogenin-212. This protocol is rapid (yielding mature neurons within three weeks) and efficient, with nearly 100% conversion efficiency of transduced cells (>95% of

	DAPI-positive cells are MAP2 positive). Furthermore, the protocol yields a homogeneous population of excitatory neurons that would allow the investigation of cell-type specific contributions to neurological disorders. We modified the original protocol by generating stably transduced hiPSC cells, giving us explicit control over the total number of neurons. These cells are then used to generate hiPSC-derived neuronal networks on micro-electrode arrays. In this way, the spontaneous electrophysiological activity of hiPSC-derived neuronal networks can be measured and characterized, while retaining inter-experimental consistency in terms of cell density. The presented protocol is broadly applicable, especially for mechanistic and pharmacological studies on human neuronal networks.
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Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

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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

TITLE:

Rapid neuronal differentiation of induced pluripotent stem cells for measuring network activity on micro-electrode arrays

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SHORT ABSTRACT:
We modify and implement a previously published protocol describing the rapid, reproducible, and efficient differentiation of human induced pluripotent stem cells (hiPSCs) into excitatory cortical neurons[12]. Specifically, our modification allows for control of neuronal cell density and use on micro-electrode arrays to measure electrophysiological properties at the network level.

LONG ABSTRACT:
Neurons derived from human induced pluripotent stem cells (hiPSCs) provide a promising new tool for studying neurological disorders. In the past decade, many protocols for differentiating hiPSCs into neurons have been developed. However, these protocols are often slow with high variability, low reproducibility, and low efficiency. In addition, the neurons obtained with these protocols are often immature and lack adequate functional activity both at the single-cell and network levels unless the neurons are cultured for several months. Partially due to these limitations, the functional properties of hiPSC-derived neuronal networks are still not well characterized. Here, we adapt a recently published protocol that describes production of human neurons from hiPSCs by forced expression of the transcription factor neurogenin-2[12]. This protocol is rapid (yielding mature neurons within three weeks) and efficient, with nearly 100% conversion efficiency of transduced cells (>95% of DAPI-positive cells are MAP2 positive). Furthermore, the protocol yields a homogeneous population of excitatory neurons that would allow the investigation of cell-type specific contributions to neurological disorders. We modified the original protocol by generating stably transduced hiPSC cells, giving us explicit control over

the total number of neurons. These cells are then used to generate hiPSC-derived neuronal networks on micro-electrode arrays. In this way, the spontaneous electrophysiological activity of hiPSC-derived neuronal networks can be measured and characterized, while retaining inter-experimental consistency in terms of cell density. The presented protocol is broadly applicable, especially for mechanistic and pharmacological studies on human neuronal networks.

INTRODUCTION:

The development of human induced pluripotent stem cell (hiPSC) differentiation protocols to generate human neurons *in vitro* has provided a powerful new tool for studying neurological disorders. Until recently, the study of these disorders was severely hampered by the lack of model systems using human neurons. Although rodents can be used to study neurological disorders, the results of such studies cannot be translated easily to humans[1]. Given these limitations, hiPSC-derived neurons are a promising alternative model that can be used to elucidate molecular mechanisms underlying neurological disorders and for *in vitro* drug screening.

In the past decade, several protocols to convert hiPSCs into neurons have been developed[2-8]. However, these protocols are still limited in many ways. First, the protocols are often time-consuming: generating neurons with adequate maturation (i.e., synapse formation) and functional activity requires months of culturing procedures, which renders large-scale studies difficult[9]. In addition, hiPSC-to-neuron conversion efficiency is low. Protocols often yield a heterogeneous population of neurons, and thus do not allow studies of specific subsets of neuronal cells. Moreover, the protocols are not reproducible, yielding different results for different iPSC lines[10,11]. Lastly, the maturation stage and functional properties of the resulting neurons are also variable [10].

To address these problems, Zhang *et al.* (2013)[12] developed a protocol that rapidly and reproducibly generates human neurons from hiPSCs by overexpressing the transcription factor neurogenin-2. As reported by the authors, differentiation occurs relatively quickly (only two to three weeks after inducing expression of neurogenin-2), the protocol is reproducible (neuronal properties are independent of the starting hiPSC line), and the hiPSC-to-neuron conversion is highly efficient (nearly 100 %). The population of neurons generated with their protocol is homogeneous (resembling upper-layer cortical neurons), allowing the investigation of cell-type specific contributions to neuronal disorders. Furthermore, their hiPSC-derived neurons exhibited mature properties (e.g., the capability to form synapses and robust functional activity) after only 20 days.

Characterizing the electrophysiological properties of hiPSC-derived neurons at the network level is an important prerequisite before hiPSC technology can be exploited for the study of human diseases. For this reason, many research groups have recently begun to investigate stem-cell-derived neurons at the network level using micro-electrode array (MEA) devices (Multichannel Systems, Reutlingen, Germany)[13-16]. The electrodes of a MEA are embedded in a substrate on which neuronal cells can be cultured. MEAs can be used to explore the electrophysiological properties of neuronal networks and the *in vitro* development of their

activity. Currently, MEAs are used only in combination with differentiation protocols that take several months to yield mature networks. Hence, combining MEAs with a rapid differentiation protocol should facilitate the use of this technology in large-scale studies of neurological disorders.

Here, we present a modification of the Zhang *et al.* (2013)[12] protocol and adapt it for use on MEAs. In particular, rather than relying on an acute lentiviral transduction, we instead created hiPSC lines stably expressing *rtTA/Ngn2* before inducing differentiation. We did this primarily to have reproducible control over the neuronal cell density, since the neuronal cell density is critical for neuronal network formation, and for good contact between the neurons and the electrodes of the MEA[17,18]. Although the Zhang *et al.* protocol is very efficient with respect to conversion of transduced hiPSCs, it is inherently variable with respect to the final yield of neurons from the number of hiPSCs plated initially (see Figure 2E in Zhang *et al*.)[12]. With a stable line, we eliminate many issues causing variability, such as lentiviral toxicity and infection efficiency. We then optimized the parameters that reliably produce hiPSC-derived neuronal networks on MEAs, obtaining network maturation (e.g., synchronous events involving a majority of the channels) by the third week. This rapid and reliable protocol should enable direct comparisons between neurons derived from different (i.e., patient-specific) hiPSC lines as well as provide robust consistency for pharmacological studies.

PROTOCOL:

All experiments on animals were carried out in accordance with the approved animal care and use guidelines of the Animal Care Committee, Radboud University Medical Centre, the Netherlands, (RU-DEC-2011-021, protocol number: 77073).

1. Glia cell isolation and culture

NOTE: The protocol presented here is based on the work of McCarthy and de Vellis[19], and a very similar detailed protocol for mouse astrocytes is available[20]. To generate primary cultures of cortical astrocytes from embryonic (E18) rat brains, a pregnant rat needs to be sacrificed, the embryos need to be harvested from the uterus, and the brains need to be isolated from the embryos. To fill a T75 flask, the cortices from 2 embryonic brains need to be combined. As an alternative, commercially-available purified and frozen astrocytes can be purchased.

1.1 Prepare the T75 culture flask

1.1.1) Dilute poly-D-lysine (PDL) in sterile, ultrapure water to a final concentration of 10 µg/mL. Add 5 mL of the diluted PDL to the T75 culture flask. Swish around gently to wet the entire growth surface. Place the flask in a humidified 37 °C incubator for 3 h.

1.1.2) Aspirate the PDL from the flask. Rinse the flask 3 times with 5 mL sterile water to remove unbound PDL. Aspirate the water completely. Leave the flask to dry in a laminar flow hood or used immediately.

1.2 Dissection of the cortices

1.2.1) Prepare 50 mL dissection medium: Lebovitz's L-15 medium with 2 % (v/v) B-27

supplement. Keep on ice.

1.2.2) Anesthetize the rat deeply with isoflurane in an induction chamber (small Plexiglas box) until respiration ceases (~5-8 min). Remove rat from the induction chamber and immediately euthanized by cervical dislocation.

1.2.3) Spray the abdomen of the rat with 70 % EtOH and wipe away the excess. Expose and remove the uterus from the dam via Caesarean section using a pair of scissors[21].

1.2.4) Cut individual embryos from their amniotic sacs with scissors, transfer to a sterile petri dish filled with cold dissection medium, and keep on ice.

1.2.5) Transfer embryos again to a new, sterile 6-cm petri dish filled with cold dissection medium. Extract brains from the embryos under a stereo microscope. To expose the brain, gently peel away the skin and skull using forceps. Gently scoop out the entire brain and transfer to a 35-mm petri dish with fresh, cold dissection medium.
NOTE: Whole brains dissected from embryos can be stored in dissection medium on ice for many hours without losing cellular viability.

1.2.6) Separate the two hemispheres of each brain by cutting through the midline with fine-tipped spring scissors or a scalpel. Carefully strip off the meninges with straight fine-tipped forceps.
NOTE: It is very important to remove the meninges completely. This prevents fibroblast contamination of the astrocyte culture. Fibroblasts are rapidly dividing cells and will eventually displace the other cells.

1.2.7) Remove the midbrain/striatum and the olfactory bulb with spring scissors or a scalpel. Also make sure to remove the hippocampus (C-shaped structure that is perimedian and caudal with respect to the cortex) with spring scissors or a scalpel. Collect the cortical hemispheres in a 15 mL centrifuge tube filled with 5 mL dissection medium. Keep on ice.

1.3 Dissociation of the cortices
1.3.1) Prepare 2 mL Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (HBSS) with 0.25 % trypsin (dissociation medium). Prepare 50 mL high-glucose Dulbecco's Modified Eagle's Medium (DMEM) with 15 % (v/v) Fetal Bovine Serum (FBS) and 1 % (v/v) penicillin/streptomycin (culture medium) and filter sterilize.

1.3.2) Let the tissue settle to the bottom of the centrifuge tube. Carefully aspirate as much of the dissection medium as possible from above the tissue. Wash the tissue with 5 mL Ca^{2+}/Mg^{2+}-free HBSS (without trypsin) and allow the tissue to settle at the bottom of the tube.

1.3.3) Carefully aspirate the HBSS. Add 2 mL dissociation medium and flick the tube gently to mix the enzyme around the tissue. Incubate in a 37 °C water bath for 5–10 min. Flick the tube a few times during incubation to agitate the tissue.

1.3.4) Immediately triturate the tissue using a 1000 µL pipette tip. Set the pipetter volume to about 800 µL. Aspirate the pieces and eject forcefully onto the side of the tube, directly above the fluid line. However, try to minimize bubbles or foaming. Repeat until the tissue is sufficiently dissociated, about 15–20 times. Add 8 mL culture medium to inactivate the trypsin. Gently mix by inverting the tube several times.

1.3.5) Pass the cell suspension through a 70 µm cell strainer placed on top of a 50 mL centrifuge tube. Rinse the 15 mL tube with culture medium and filter the medium through the cell strainer to collect the medium in the 50 mL tube with the cell suspension. Rinse the cell strainer a few times with culture medium. After rinsing, the final volume should be about 20–25 mL.

1.3.6) Pellet the cells at 200 x g for 10 min. Carefully aspirate as much medium as possible, without touching the cell pellet. Resuspend the cells in 1 mL culture medium using a 1000 µL pipette. Add 11 mL pre-warmed culture medium and mix gently (to prevent bubbles) using a 10 mL pipette.

1.3.7) Rinse the PDL-coated T75 flask once with 5 mL culture medium. Aspirate the medium and transfer the cell suspension to the flask. All cells are in the suspension are plated, and we find it generally unnecessary to count them, since astrocytes cannot be differentiated from other cells in the suspension. Place the flask into a humidified 37 °C incubator with an atmosphere of 5 % CO_2 for two days.

1.4 Expansion and maintenance of the astrocytes

1.4.1) Replace the entire medium for the first time 2 days after initial plating. Replace the entire medium afterwards every 3 days. Always pre-warm the fresh medium to 37 °C before adding to the cells.

NOTE: The astrocytes require 7–10 days to reach approximately 90 % confluency (the astrocytes appear as a densely packed tessellated monolayer, with microglia and oligodendrocytes lying on top and intermixed).

1.4.2) When the astrocytes reach approximately 90 % confluency, shake the flask to remove the contaminating glial cells:

1.4.2.1) Remove the flask from the incubator and tighten the cap (phenolic) or cover the port (filtered).To remove microglia, shake the flask on an orbital platform at 180 rpm for 1 h. Aspirate the medium. Rinse once with 5 mL pre-warmed culture medium, aspirate and replace with 12 mL culture medium.

1.4.2.2) To remove the oligodendrocytes, return the flask to the orbital platform and shake at 250 rpm, 37 °C for a minimum of 7 h, but preferably overnight.

1.4.2.3) Aspirate the medium. Rinse once with 5 mL pre-warmed culture medium, aspirate and replace with 12 mL culture medium. Return the flask to the incubator.

1.4.2.4) When 100 % confluent, split the astrocytes using standard procedures at a ratio of 1:3 to 1:2 with 0.05 % trypsin-ethylenediaminetetraacetic acid (EDTA). A T75 flask at 100 % confluency will typically yield about 4.0 x 10^6 cells in total. Under this schedule, the cultures can typically be split once per week.

NOTE: When the astrocytes reach confluency, they can be harvested and used for hiPSC differentiation as described below in protocol step 3.4. The astrocytes can be split at least once without a noticeable loss of viability. They can be maintained for up to 2 months in culture. From experience, primary embryonic day 18 rat astrocytes progressively become terminally differentiated and/or lose viability after repeated splitting. Although it is possible to freeze the astrocytes for future use, we prefer to isolate the astrocytes from fresh embryonic brains when required.

2. Generation of *rtTA/Ngn2*-positive hiPSCs

NOTE: The hiPSCs used for our experiments were generated in-house by lentiviral transduction of human fibroblasts with the reprogramming factors *cMYC, SOX2, OCT4* and *KLF4*.

NOTE: For the generation of *rtTA*/*Ngn2*-positive hiPSCs, lentiviral vectors are used to stably integrate the transgenes into the genome of the hiPSCs. The protocol for the production of the lentivirus has been published previously[22]. The details of the lentiviral packaging vectors that are used to produce the *rtTA* and *Ngn2* lentivirus particles are provided in the Table of Specific Materials/Equipment. The transfer vector used for the *rtTA* lentivirus is pLVX-EF1α-(Tet-On-Advanced)-IRES-G418(R); i.e., this vector encodes a Tet-On Advanced transactivator under control of a constitutive EF1α promoter and confers resistance to the antibiotic G418. The transfer vector used for the *Ngn2* lentivirus is pLVX-(TRE-thight)-(MOUSE)Ngn2-PGK-Puromycin(R); i.e., this vector encodes the gene for murine neurogenin-2 under control of a Tet-controlled promoter and the puromycin resistance gene under control of a constitutive PGK promoter. Hence, by using these two transfer vectors, an hiPSC line can be created for which the expression of murine neurogenin-2 can be induced by supplementing the medium with doxycyclin. For the transduction of the hiPSCs, the supernatant with the lentivirus particles is used (referred to as 'lentivirus suspension' in the remainder of the text), i.e., without concentrating the particles using ultracentrifugation.

2.1 Plate the hiPSCs (day 1)

NOTE: The volumes that are mentioned in this protocol assume that the hiPSCs are cultured in a 6 well plate and that the cells of one well are harvested. In addition, it is assumed that the cells are plated subsequently in 12 wells of a 12 well plate.

2.1.1) Prepare 10 mL cold DMEM/F12 with 1 % (v/v) basement membrane matrix (BMM) to obtain diluted BMM. Add 800 µL diluted BMM per well of a 12 well plate. Incubate for at least 1 h in a humidified 37 °C incubator with an atmosphere of 5 % CO_2. Before usage, incubate the

plate for 1 h at room temperature.

2.1.2) Warm 15 mL Essential 8 (E8) medium with 1 % (v/v) penicillin/streptomycin, 9 mL DMEM/F12 and 1 mL cell detachment solution (CDS) to room temperature. Supplement the E8 medium with Rho-associated protein kinase (ROCK) inhibitor.

2.1.3) Aspirate the spent medium of the hiPSCs and add 1 mL CDS to the hiPSCs. Incubate 3-5 min in a humidified 37 °C incubator with an atmosphere of 5 % CO_2. Check under the microscope whether the cells are detaching from one another.

2.1.4) Add 2 mL DMEM/F12 in the well, gently suspend the cells with a 1000 µL pipette and transfer the cells to a 15 mL tube. Add 7 mL DMEM/F12 to the cell suspension. Spin the cells at 200 x g for 5 min.

2.1.5) Aspirate the supernatant and add 2 mL of the prepared E8 medium. Obtain a cell suspension in which the hiPSCs are dissociated (do not form cell clumps) by putting the tip of a 1000 µL pipette against the side of the 15 mL tube and resuspending the cells gently. Check under the microscope whether the cells are dissociated.

2.1.6) Determine the number of cells (cells/mL) using a hemocytometer chamber.
NOTE: A 6 well plate well at 80-90 % confluency will typically yield 3.0-4.0 x 10^6 cells in total.

2.1.7) Aspirate the diluted BMM from the wells of the 12 well plate. Dilute the cells to obtain a cell suspension of 3.0 x 10^4 cells/mL. Plate 1 mL of the cell suspension per well of the 12 well plate. Place the 12 well plate overnight in a humidified 37 °C incubator with an atmosphere of 5 % CO_2.

2.2 Transduce the iPS cells with *rtTA* and *Ngn2* lentivirus (day 2)
2.2.1) Warm 12 mL E8 medium with 1 % (v/v) penicillin/streptomycin to room temperature. Supplement the E8 medium with ROCK inhibitor and polybrene to a final concentration of 8 µg/mL to the E8 medium.

2.2.2) Thaw the aliquots with lentivirus suspension. Add polybrene to a final concentration of 8 µg/mL to the lentivirus suspension. Aspirate the spent medium and add 1 mL of the prepared E8 medium to each well.

2.2.3) Perform the transduction with different amounts of the *rtTA*- and *Ngn2*-lentivirus suspensions. For example, transduce the hiPSCs by adding 100 µL of both the *rtTA*-lentivirus and *Ngn2*-lentivirus suspension to one well of the 12 well plate. For the other wells, use 200 µL, 300 µL, 400 µL and 500 µL lentivirus suspension instead of 100 µL. The hiPSCs of two wells of the 12 well plate should not be transduced; they will serve as controls during the selection.

NOTE: The transductions are preferably performed in duplicate, so that the transduction efficiency can be estimated more accurately after the start of the selection (see protocol step

2.2.4). The amount of lentivirus suspension that is required to efficiently transduce the majority of the hiPSCs depends on the titer of the lentivirus suspension and the hiPSC line that is used. In this study, we usually use 100-500 µL of lentivirus suspension to transduce the hiPSCs.

2.2.4) Place the 12 well plate in a humidified 37 °C incubator with an atmosphere of 5 % CO_2 for 6 h. Before the end of the 6 h incubation period, warm 12 mL E8 medium with 1 % (v/v) penicillin/streptomycin and 12 mL Dulbecco's Phosphate-Buffered Saline (DPBS) to room temperature. Supplement the E8 medium with ROCK inhibitor.

2.2.5) Aspirate the spent E8 medium. Wash each well with 1 mL DPBS. Add 1 mL of the prepared E8 medium to each well. Place the 12 well plate overnight in a humidified 37 °C incubator with an atmosphere of 5 % CO_2.

2.3 Refresh the E8 medium (day 3)

2.3.1) Warm 12 mL E8 medium with 1 % (v/v) penicillin/streptomycin to room temperature. Aspirate the spent medium from the wells of the 12 well plate and add 1 mL of the prepared E8 medium to each well. Place the 12 well plate overnight in a humidified 37 °C incubator with an atmosphere of 5 % CO_2.

2.4 Perform selection with puromycin and G418 (day 4-8)

NOTE: Depending on the cell division rate of the hiPSC line and the efficiency of the lentiviral transduction, the cells may reach 70-80 % confluency during the selection period, at which point the cultures need to be split. Because the timing of the splitting cannot be predicted in advance, it will not be mentioned in the protocol. However, instead of refreshing the E8 medium supplemented with the mentioned concentrations of puromycin and G418, one can split the hiPSC culture as a normal hiPSC culture (including plating the cells on vitronectin-coated plates). The only exception is that the E8 medium should be supplemented with the mentioned concentrations of the antibiotics to continue the selection.

2.4.1) Warm 12 mL E8 medium with 1 % (v/v) penicillin/streptomycin to room temperature. Add puromycin and G418 for selection; different amounts of the antibiotics are added during the selection period (table 1).

[Place table 1 here]

2.4.2) Estimate the efficiency of the transduction by estimating the percentage of G418- and puromycin-resistant cells. To estimate the percentage of resistant cells, estimate the percentage of dead cells (non-resistant cells) for the different conditions (the cultures transduced with the different amounts of lentivirus suspension) and for the non-transduced cells (the cells that serve as a selection control). Calculate the percentage of resistant cells as [100 % - (percentage of dead cells)].

NOTE: If the transductions with the different amounts of lentivirus suspension were performed in duplicate, the transduction efficiency can be estimated more accurately. The condition with

the non-transduced cells serves as a selection control; the percentages of dead cells for the cultures transduced with the different amounts of lentivirus suspension should be lower. The estimated percentage of resistant cells is used to choose the hiPSCs that are likely positive for both transgenes. In general, we choose the hiPSCs from the transduction condition were > 90 % of the cells survive the 5 day selection period.

2.4.3) Aspirate the spent medium of the hiPSCs and add 1 mL of the prepared E8 medium to the wells. Place the 12 well plate overnight in a humidified 37 °C incubator with an atmosphere of 5 % CO_2.

2.5 Stop the selection and start regular culturing (day 9 and later)

2.5.1) After the 5 day selection period, culture the *rtTA*/*Ngn2*-positive hiPSCs as normal hiPSCs, with the exception that the E8 medium of the cells is supplemented with G418 to a final concentration of 50 µg/mL and with puromycin to a final concentration of 0.5 µg/mL.

NOTE: The cells can now be frozen (according to standard protocols for cryopreservation of cells) to serve as a backup. This is an important step for the reproducibility of the differentiation protocol, because it allows the use of the same batch of *rtTA*/*Ngn2*-positive hiPSCs for many future differentiation experiments.

3. Differentiation of *rtTA*/*Ngn2*-positive hiPSCs to neurons on 6 well MEAs and glass coverslips

NOTE: In this protocol, the details are provided for differentiating *rtTA*/*Ngn2*-positive hiPSCs on two different substrates, i.e., 6 well MEAs (devices composed of 6 independent wells with 9 recording and 1 reference embedded microelectrodes per well) and glass coverslips in the wells of a 24 well plate. The protocols, however, can easily be adapted for larger substrates (e.g., for the wells of 12 well or 6 well plates), by scaling up the mentioned values according to the surface area.

3.1 Prepare the MEAs or glass coverslips (day 0 and day 1)

3.1.1) The day before the start of the differentiation, sterilize the 6 well MEAs at 120 °C for 1 h and subsequently expose them to ultraviolet (UV) light for 2 h. Sterilize the glass coverslips at 180 °C for 5 h.

3.1.2) Dilute the adhesion protein poly-L-ornithine in sterile ultrapure water to a final concentration 50 µg/mL. Coat the active electrode area of 6 well MEAs by placing a 100 µL drop of the diluted poly-L-ornithine in each well. Place the coverslips in the wells of the 24 well plate using sterile tweezers. Add 800 µL of the diluted poly-L-ornithine in each well. Prevent the coverslips from floating by pushing them down with the 1000 µL pipette tip.

3.1.3) Incubate the 6 well MEAs and 24 well plate overnight in a humidified 37 °C incubator with an atmosphere of 5 % CO_2. The next day, aspirate the diluted poly-L-ornithine. Wash the glass surfaces of the 6 well MEAs and the coverslips twice with sterile ultrapure water.

3.1.4) Dilute laminin in cold DMEM/F12 to a final concentration of 20 µg/mL (for the 6 well MEAs) and 10 µg/mL (for the glass coverslips). Immediately coat the active electrode area of the 6 well MEAs by placing a 100 µL drop in each well. Similarly, add 400 µL of the diluted laminin in each well of the 24 well plate to coat the coverslips. Prevent the coverslips from floating by pushing them down with the 1000 µL pipette tip.

3.1.5) Incubate the 6 well MEAs and 24 well plate in a humidified 37 °C incubator with an atmosphere of 5 % CO_2 for at least 2 h.

3.2 Plate the hiPSCs (day 1)

NOTE: The volumes that are mentioned in steps 3.2.1-3.2.4 assume that the *rtTA*/*Ngn2*-positive hiPSCs are cultured in a 6 well plate and that the cells of one well are harvested. The volumes that are required for plating the cells on the 6 well MEAs and/or the coverslips depends on the number of 6 well MEAs and/or the number of coverslips that are used in the experiment; the numbers specified in steps 3.2.6-3.2.8 allow scaling to different experiment sizes.

3.2.1) Warm DMEM/F12, CDS and E8 medium with 1 % (v/v) penicillin/streptomycin to room temperature. Add doxycycline to a final concentration of 4 µg/mL and ROCK inhibitor to the E8 medium.

3.2.2) Aspirate the spent medium of the *rtTA*/*Ngn2*-positive hiPSCs and add 1 mL CDS to the hiPSCs. Incubate 3-5 min in a humidified 37 °C incubator with an atmosphere of 5 % CO_2. Check under the microscope whether the cells are detaching from one another.

3.2.3) Add 2 mL DMEM/F12 in the well, gently suspend the cells with a 1000 µL pipette and transfer the cells to a 15 mL tube. Add 7 mL DMEM/F12 to the cell suspension. Spin the cells at 200 x g for 5 min.

3.2.4) Aspirate the supernatant and add 2 mL of the prepared E8 medium. Dissociate the hiPSCs by putting the tip of a 1000 µL pipette against the side of the 15 mL tube and resuspending the cells gently. Check under the microscope whether the cells are dissociated.

3.2.5) Determine the number of cells (cells/mL) using a hemocytometer chamber.

NOTE: A 6 well plate well at 80-90 % confluency will typically yield 3.0-4.0 x 10^6 cells in total.

3.2.6) Aspirate the diluted laminin. For the 6 well MEAs, dilute the cells to obtain a cell suspension of 7.5 x 10^5 cells/mL. Plate the cells by adding a drop of 100 µL of the cell suspension on the active electrode area in each well of the 6 well MEAs. For the coverslips, dilute the cells to obtain a cell suspension of 4.0 x 10^4 cells/mL. Plate the cells by adding 500 µL of the cell suspension to the wells of the 24 well plate.

NOTE: The final cell density on the MEAs is higher than on the coverslips (*cf*. Figure 1A and B). We found that this high cell density was required for proper recording of the network activity. In the protocol, the numbers are provided that turned out to be optimal for the assays.

3.2.7) Place the 6 well MEAs and 24 well plate in a humidified 37 °C incubator with an atmosphere of 5 % CO_2 for 2 h (MEAs) or overnight (24 well plate).

3.2.8) After 2 h, carefully add 500 µL of the prepared E8 medium to each well of the 6 well MEAs. Place the 6 well MEAs overnight in a humidified 37 °C incubator with an atmosphere of 5 % CO_2.

3.3 Change the medium (day 2)

3.3.1) The next day, prepare DMEM/F12 with 1 % (v/v) N-2 supplement, 1 % (v/v) non-essential amino acids and 1 % (v/v) penicillin/streptomycin. Add human recombinant neurotrophin-3 (NT-3) to a final concentration of 10 ng/mL, human recombinant brain-derived neurotrophic factor (BDNF) to a final concentration of 10 ng/mL, and doxycycline to a final concentration of 4 µg/mL. Warm the medium to 37 °C.

3.3.2) Add laminin to a final concentration of 0.2 µg/mL to the medium. Filter the resulting medium. Aspirate the spent medium from the wells of the 6 well MEAs and the 24 well plate and replace it with the prepared medium. Incubate the 6 well MEAs and 24 well plate overnight in a humidified 37 °C incubator with an atmosphere of 5 % CO_2.

3.4 Add rat astrocytes (day 3)

NOTE: The volumes that are mentioned in this protocol assume that the rat astrocytes are cultured in T75 culture flasks. It is critical that the rat astrocytes that are added to the cultures are of good quality. We use two criteria to check if the rat astrocytes are of good quality. First, the rat astrocyte culture should be able to grow confluent within ten days after the isolation from the rat embryonic brains. Second, after splitting the rat astrocyte culture, the rat astrocytes should be able to form a confluent, tessellated monolayer (Figure 1C). If the rat astrocyte culture does not fulfill these two criteria, we advise not to use this culture for differentiation experiments.

3.4.1) Warm 0.05 % trypsin-EDTA to room temperature. Warm the DPBS and DMEM/F12 with 1 % (v/v) penicillin/streptomycin to 37 °C.

3.4.2) Aspirate the spent medium of the rat astrocyte culture. Wash the culture by adding 5 mL DPBS and swish it around gently.

3.4.3) Aspirate the DPBS and add 5 mL 0.05 % trypsin-EDTA. Swish the trypsin-EDTA around gently. Incubate in a humidified 37 °C incubator with an atmosphere of 5 % CO_2 for 5-10 min.

3.4.4) Check under the microscope whether the cells are detached. Detach the last cells by hitting the flask a few times.

3.4.5) Add 5 mL of DMEM/F12 to the flask. Triturate the cells gently inside the flask with a 10 mL pipette. Collect the cell suspension in a 15 mL tube. Spin the tube at 200 x g for 8 min.

3.4.6) Aspirate the supernatant and resuspend the cells in 1 mL of DMEM/F12. Determine the number of cells (cells/mL) using a hemocytometer chamber.

3.4.7) Add 7.5 x 10^4 astrocytes per well of the 6 well MEAs. Add 2.0 x 10^4 astrocytes per well of the 24 well plate. Incubate the MEAs overnight in a humidified 37 °C incubator with an atmosphere of 5 % CO_2.

3.5 Change the medium (day 4)

3.5.1) Prepare neurobasal medium with 2 % (v/v) B-27 supplement, 1 % (v/v) L-alanyl-L-glutamine and 1 % (v/v) penicillin/streptomycin. Add NT-3 to a final concentration of 10 ng/mL, BDNF to a final concentration of 10 ng/mL, and doxycycline to a final concentration of 4 µg/mL. In addition, add cytosine β-D-arabinofuranoside to a concentration of 2 µM.

NOTE: Cytosine β-D-arabinofuranoside is added to the medium to inhibit astrocyte proliferation and to kill the remaining hiPSCs that are not differentiating into neurons.

3.5.2) Filter the medium and warm to 37 °C. Aspirate the spent medium from the wells of the 6 well MEAs and the 24 well plate and replace it with the prepared medium. Maintain the 6 well MEAs and the 24 well plate in a humidified 37 °C incubator with an atmosphere of 5 % CO_2.

3.6 Refresh the medium (day 6-28)

NOTE: Starting from day 6, refresh half of the medium every two days. From day 10 onwards, the medium is supplemented with FBS to support the astrocyte viability.

3.6.1) Prepare neurobasal medium with 2 % (v/v) B-27 supplement, 1 % (v/v) L-alanyl-L-glutamine and 1 % (v/v) penicillin/streptomycin. Add NT-3 to a final concentration of 10 ng/mL, BDNF to a final concentration of 10 ng/mL, and doxycycline to a final concentration of 4 µg/mL. From day 10 onwards, also supplement the medium with 2.5 % (v/v) FBS. Filter the resulting medium and warm to 37 °C.

3.6.2) Remove half of the spent medium from the wells of the 6 well MEAs and the 24 well plate using a 1000 µL pipette and replace it with the prepared medium. Maintain the 6 well MEAs and the 24 well plate in a humidified 37 °C incubator with an atmosphere of 5 % CO_2.

4. Establish the neurophysiological profile of hiPSC-derived

NOTE: Two to three weeks after the induction of differentiation, the hiPSC-derived neurons can be used for different downstream analyses. In this section, examples of some downstream analyses are given that can be performed to establish the neurophysiological profile of the hiPSC-derived neurons.

4.1) Characterize the neuronal network activity using MEAs

4.1.1) Record 20 min of electrophysiological activity of hiPSC-derived neurons cultured on MEAs. During the recording, maintain the temperature at 37°C, and prevent evaporation and pH changes of the medium by inflating a constant, slow flow of humidified gas (5 % CO_2, 20 %

O_2, 75 % N_2) onto the MEA.

4.1.2) After 1200 x amplification (MEA 1060, MCS), sample the signal at 10 kHz using the MCS data acquisition card. Analyze the data (spike and burst detection) using a custom software package[23].

4.2) Characterize the single-cell electrophysiological activity

4,2.1) Transfer the cover slips containing the hiPSC-derived neuronal cultures to a submerged fixed-stage recording chamber in an upright microscope. Record 20 min of spontaneous action potential-evoked postsynaptic currents (sEPSC) [24]. Detect the synaptic event using neuroscientific program.

4.3) Characterize the neuronal morphology and synapsin expression

4.3.1) Fix and stain the hiPSC-derived neurons for MAP2, synapsin-1/2, and PSD-95[22, 24, 25]. Quantify the number of synapsin-1/2 and PSD-95 puncta using image analysis software.

REPRESENTATIVE RESULTS:

Here we have successfully modified a protocol in which hiPSCs are differentiated directly into cortical neurons by over-expressing the transcription factor neurogenin-2[12] and we have adapted it for the use of MEAs. This approach is fast and efficient allowing us to obtain functional neurons and network activity already during the third week after the induction of differentiation.

During the course of the differentiation protocol, the cells morphologically started to resemble neurons: small processes were formed and neurons started connecting to each other (Figure 1A). We established a neurophysiological profile of the neurons derived from a healthy-control hiPSC line, by measuring their neuronal morphology and synaptic properties during development. hiPSC-derived neurons were stained for MAP2 and synapsin-1/2 at different days after the start of differentiation (Figure 2A). The derived neurons show mature neuronal morphology already three weeks after the induction of differentiation. The number of synapsin-1/2 puncta (a measure for the number of synapses) was quantified based on synapsin-1/2 immunocytochemistry stainings. The number of synapsin-1/2 puncta increased over time, suggesting that the level of neuronal connectivity is also increasing (Figure 2B). The number of synapsin-1/2 puncta 23 days after the induction of differentiation was similar in two independent IPS lines (Figure 2C). At 23 DIV most synapsin1/2 puncta were juxtaposed to PSD-95 puncta, which is indicative for functional synapses (Figure 2D).

Consistent with the results described by Zhang et al we generated a population of excitatory upper layer cortical neurons, confirmed by pan-neuronal and subtype-specific cortical markers such as BRN2 and SATB2 (layer II/III). We did not observe neurons that were positive for deep layer neurons CTIP2 (layer V) or Foxp2 (layer VI) (Figure 2E, F)

To characterize the electrophysiological activity of the hiPSC-derived neurons, we used whole-cell current and voltage clamp recordings, i.e., intrinsic properties and excitatory input onto

these neurons were measured during development. The neurons were able to generate action potentials already one week after the of differentiation and the percentage of spiking cells was increasing over time (Figure 2G,H). Furthermore, the neurons received excitatory synaptic input already one week after the induction of differentiation: both frequency and amplitude of the excitatory synaptic input increased during development (Figure 2I-K).

To better understand how single-cell activity combines to form network-level functions, it is essential to study how neurons work in concert. *In vitro* neuronal networks cultured on MEAs constitute a valuable experimental model for studying the neuronal dynamics. We recorded 20 min of electrophysiological network activity of neurons derived from a healthy-control hiPSC line cultured on 6 well MEAs (Figure 2M). Few weeks after the induction of differentiation, the neurons derived from healthy-control hiPSCs formed functionally active neuronal networks, showing spontaneous events (0.62 ± 0.05 spike/s; Figure 2N). At this stage of development (i.e., 16 days after the start of the differentiation) no synchronous events involving all the channels of the MEAs are detected (Figure 2O). The level of network activity increased during the development: during the fourth week after the induction of differentiation, the neuronal networks showed high level of spontaneous activity (2.5 ± 0.1 spike/s; Figure 2N) in all the wells of the device. The networks also exhibited synchronous network bursts (4.1 ± 0.1 burst/min, Figure 2O) with long duration (2100 ± 500 ms).

[Place Figure 1 here]

[Place Figure 2 here]

Given the results, the quality of the resulting hiPSC-derived neurons can be assessed by making a neurophysiological profile of the cells. That is, three to four weeks after the start of the differentiation, the morphology, synapsin-1/2 expression and electrophysiology of the neurons can be assessed. At that time point, the hiPSC-derived neurons are expected to show a neuronal-like morphology, to be MAP2, synapsin/PSD-95 positive when performing immunocytochemistry, and to exhibit spontaneous electrophysiological activity (both at the single-cell and network level).

FIGURE LEGENDS:

Figure 1: hiPSC differentiation into neurons. A. Three time points of hiPSCs differentiation into neurons on coverslips. **B.** Plating of hiPSCs on MEAs. **C.** Astrocytes at 100 % confluency in T75 flask (note that the cells form a tessellated monolayer). Scale bars: 150 µm.

Figure 2. hiPSC-derived neurons characterization. A. hiPSC-derived neurons were stained for MAP2 (green) and synapsin-1/2 (red) at different days after the start of differentiation. Scale bar: 10 µm. **B.** Quantification of synapsin puncta in two independent experiments. In each experiments at least 10 cells were analyzed **C.** Quantification of synapsin puncta at DIV23 in neurons derived from two independent IPS lines. **D.** hiPSC-derived neurons were stained for PSD-95 (green) and synapsin-1/2 (red) 23 days after the start of differentiation. Synapsin puncta are juxtaposed to PSD-95 puncta. E. hiPSC-derived neurons were stained for MAP2 (green) and

BRN2 (red) or SATB2 (red) 23 days after the start of differentiation. F. Percentage of MAP2 positive cells that were positive for indicated markers. G. Representative current clamp recordings showing that action action potentials can be generated as early as 7 days after the start of differentiation H. Percentage of cells at different days after the induction of differentiation that show one or more action potentials. **I.** Representative traces of excitatory postsynaptic currents (EPSCs) received by hiPSC-derived neurons at different days after differentiation. **J.** Frequency of excitatory postsynaptic currents during development. **K.** Amplitude of excitatory postsynaptic currents during development. **L**. Representative traces of EPSC recordings without (control) and with CNQX (CNQX). **M.** Neurons derived from one hiPSC line were cultured on a 6 well MEA and network activity is shown for hiPSC-derived neuronal networks 16 and 23 days after the induction of differentiation. The activity recorded from each well (sampling rate of 10 kHz) is indicated with a different color (5 min of the 20 min of recording are shown). **N.** Firing rate 16 and 23 days after the induction of differentiation. **O.** Bursting rate 16 and 23 days after the induction of differentiation.

Table 1: Concentrations of antibiotics during the selection period. Concentrations of the puromycin and G418 during the 5 days of the selection period.

DISCUSSION:

Here we have implemented an efficient hiPSC-differentiation protocol published by Zhang *et al.* (2013)[12] for measuring the network activity of hiPSC-derived neuronal networks on MEAs. We adapted the original protocol by creating an *rtTA*/*Ngn2*-positive hiPSC line before inducing neuronal differentiation. This additional step allows us to control the neuronal cell density on the MEA. Control over the neuronal density was an important pre-requisite for adapting the protocol to MEAs and for ensuring consistency. To measure the activity of neuronal networks using MEAs, the neurons need to form dense networks directly on top of the MEA electrodes[17,18]. This necessarily requires tight control over the plating density of the neurons. The *rtTA*/*Ngn2*-positive hiPSC line allows for control of neuron density because this tactic does not rely on acute lentiviral transductions of hiPSCs prior to differentiation; the *rtTA*/*Ngn2*-positive hiPSC line therefore nearly eliminates any variation in the final yield due to, for example, lentiviral toxicity and variable infection efficiency.

Another critical step of the experimental procedure is the number of the rat astrocytes that are co-cultured with the differentiating hiPSCs. Astrocytes actively contribute to the refinement of developing neural circuits by controlling synapse formation, maintenance, and elimination, all of which are important processes for neuronal functioning. The protocol presented in this paper is highly astrocyte-dependent: to fully mature and form functional synapses, the neurons require support from the astrocytes. We experienced that the number of astrocytes should be roughly equal to the number of hiPSC-derived neurons to support the maturation of the neurons and the formation of neuronal networks exhibiting spontaneous activity. Since our astrocyte protocol yields primary cell cultures with a limited life span, the isolation of rat astrocytes has to be performed regularly.

Our adaptation of the protocol published by Zhang *et al.* (2013)[12] for use with MEA technology

will likely significantly improve our ability to study the network activity of hiPSC-derived networks. Previously, protocols used for studying hiPSC-derived neuronal networks with MEAs relied on time-consuming differentiation procedures[13-16]. The protocol from Zhang *et al.* (2013) provides a rapid alternative, and our modification removes a source of variability, which makes it now more feasible to use hiPSC-derived neurons in combination with MEA technology, especially in high-throughput or pharmacological studies. In addition, because the method published by Zhang *et al.* (2013)[12] yields a homogeneous population of upper-layer cortical neurons, our adapted protocol makes possible focused studies into the network activity of this particular neuronal subset.

Nonetheless, this approach has also several limitations. First, the homogeneity of the cultures can also be considered a disadvantage, because the cultures are less likely to resemble *in vivo* networks, where different classes of neurons (i.e., inhibitory and excitatory neurons) constitute a heterogeneous network. To further enhance the use of the hiPSC-derived neurons with MEA technology, it will be important to develop rapid (transgene-based) differentiation protocols for other neuronal cell populations. If protocols become available, the in vitro networks would mimic in vivo networks more closely. Second, at present rat astrocytes must be added to the hiPSC-derived neurons for growth support, and therefore the resulting neuronal network is not a human neuronal network *sensu stricto.* Reliable protocols for differentiating hiPSCs into astrocytes may in the future solve this problem[26]. Third, two-dimensional neuronal networks, as described here, are a limited model for studying complex three-dimensional *in vivo* neuronal networks. Fortunately, protocols describing three-dimensional cultures of rat primary neurons in combination with MEA technology are already available[27,28]. Prospectively, the combination of rapid differentiation protocols for obtaining hiPSC-derived neurons and astrocytes with three-dimensional culture techniques and MEA technology should provide novel insight into the biological mechanisms underlying neurological disorders.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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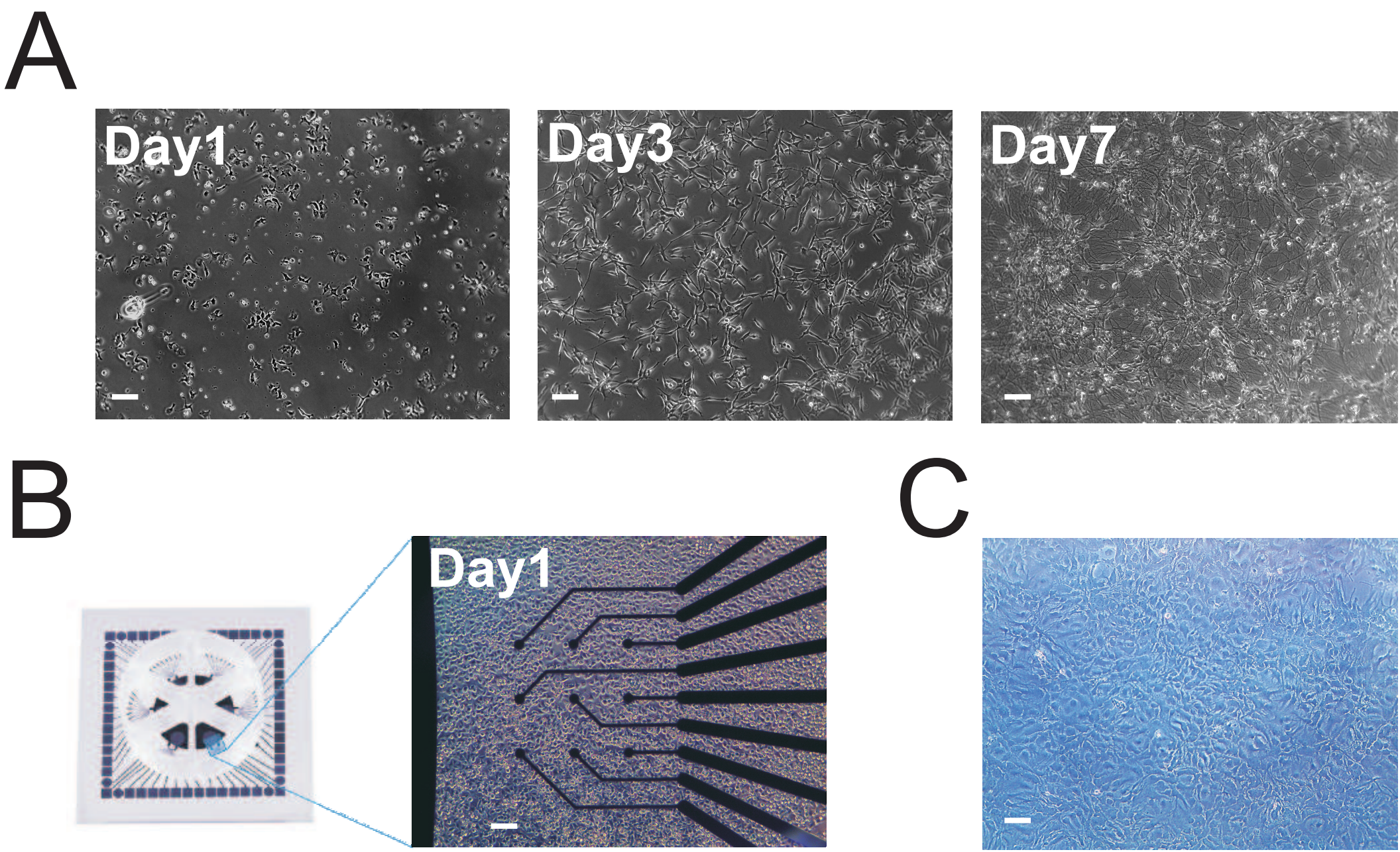

Figure 2

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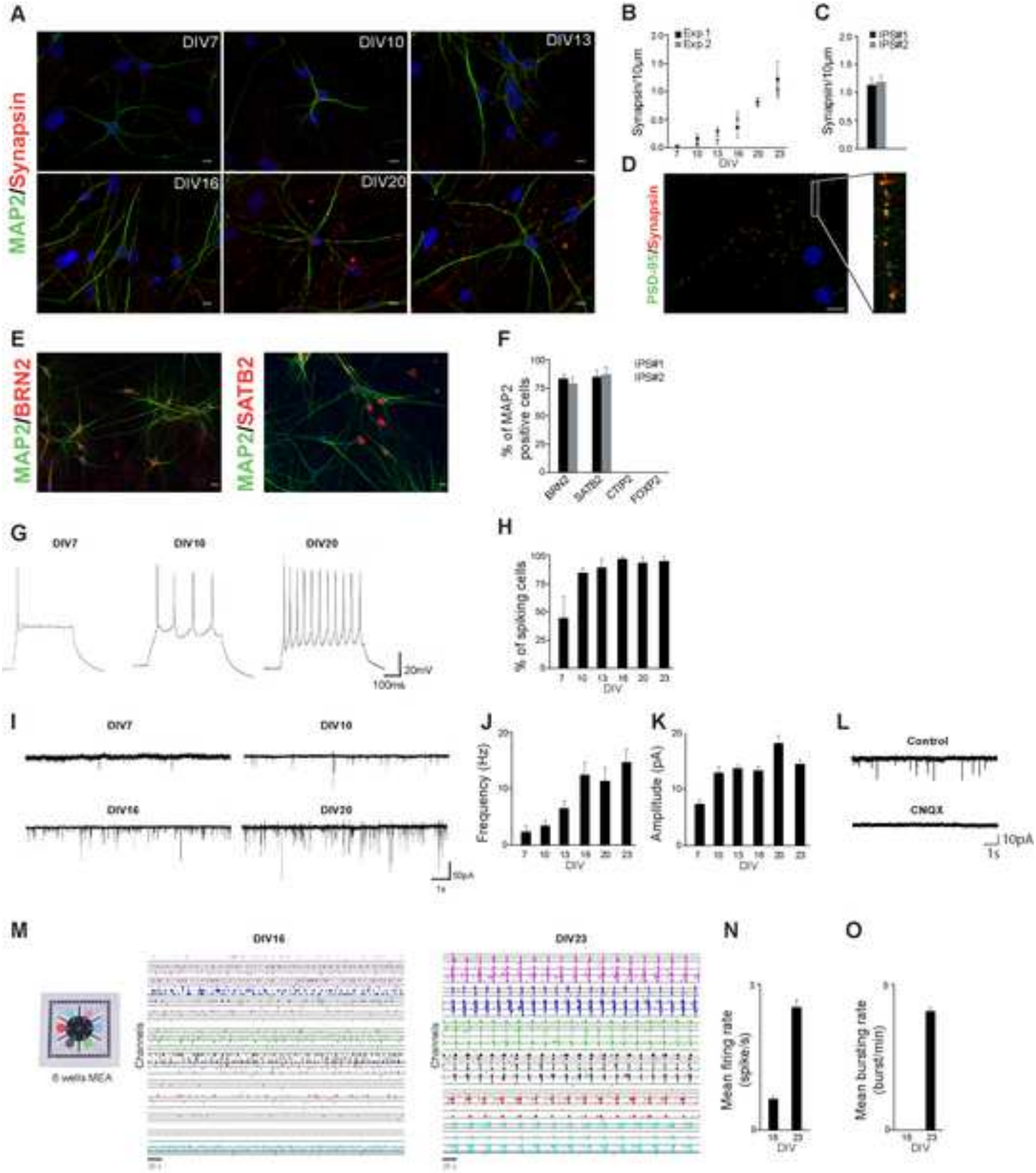

Table1

	Final concentration of G418	Final concentration of puromycin
Day 4	250 µg/ml	2 µg/ml
Day 5	250 µg/ml	2 µg/ml
Day 6	250 µg/ml	1 µg/ml
Day 7	250 µg/ml	1 µg/ml
Day 8	250 µg/ml	1 µg/ml

Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/Equipment	Company	Catalog Number	Comments/Description
Lebovitz's L-15 medium	Gibco	11415-064	
B-27 supplement	Gibco	0080085SA	
Poly-D-lysine	Sigma-Aldrich	P6407	
Ca2+/Mg2+-free HBSS	Gibco	14175-095	
0.05 % Trypsin-EDTA	Gibco	25300-054	
2.5 % Trypsin	Gibco	15090-046	
High-glucose DMEM	Gibco	11965-092	
FBS	Sigma-Aldrich	F2442-500ML	
Penicillin/Streptomycin	Sigma-Aldrich	P4333	
70 µm cell strainer	BD Falcon	352350	
DPBS	Gibco	14190-094	
psPAX2 lentiviral packaging vector	Addgene	Plasmid #12260	
pMD2.G lentiviral packaging vector	Addgene	Plasmid #12259	
Basement membrane matrix	Gibco	A1413201	
DMEM/F12	Gibco	11320-074	
Cell detachment solution	Sigma-Aldrich	A6964	
E8 medium	Gibco	A1517001	
ROCK inhibitor	Gibco	A2644501	Alternatively, ROCK inhibitors like thiazovivin can be used.
Polybrene	Sigma-Aldrich	H9268-5G	
G418	Sigma-Aldrich	G8168-10ML	
Puromycin	Sigma-Aldrich	P9620-10ML	
Vitronectin	Gibco	A14700	
6-well MEAs	Multi Channel Systems	60-6wellMEA200/30iR-Ti-tcr	
Glass coverslips	VWR	631-0899	
Poly-L-ornithine	Sigma-Aldrich	P3655-10MG	
Laminin	Sigma-Aldrich	L2020-1MG	
Doxycyclin	Sigma-Aldrich	D9891-5G	
N-2 supplement	Gibco	17502-048	
Non-essential amino acids	Sigma-Aldrich	M7145	
NT-3, human recombinant	Promokine	C66425	
BDNF, human recombinant	Promokine	C66212	
Trypsin-EDTA	Gibco	25300-054	
L-alanyl-L-glutamine	Gibco	35050-038	
Neurobasal medium	Gibco	21103-049	
Cytosine β-D-arabinofuranoside	Sigma-Aldrich	C1768-100MG	
Straight fine-tipped forceps	Fine Science Tools	11251	
Fine-tipped spring scissors	Fine Science Tools	91500-09	

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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.

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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:

a) MAP2 and synapsin-1/2/PSD-95 stainings for independent experiments and independent IPS lines, at different times after the start of differentiation.
b) MAP2 and cortical markers stainings (SATB2, BRN2, CTIP2, FOXP2) to assess the identity of the differentiated neurons.
c) 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 BRN2 and 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 !0^6. 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:

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