Ncardia
Stem cell experts

CNS.4U®
in Maestro microelectrode array (MEA) assays

User Guide

Version 1.1 / February 2019

Contents

1. General Information

This protocol covers thawing, seeding, and culturing of CNS.4U® neural cells to record the neuronal activity with microelectrode array (MEA) technology (Maestro System, Axion BioSystems, Atlanta USA). The protocol is designed for cryopreserved CNS.4U®, and 48-well and 96-well MEA plates. Please read the entire protocol before you start your experiment.

CNS.4U® neural cells are produced through a well-defined *in vitro* differentiation process from human induced pluripotent stem cells (iPSC). The iPSC line is generated by introducing defined transcription factors, described by Yamanaka[1] in a human skin fibroblast, using a non-viral system[2].

CNS.4U® neural cells comprise two key cell types of the human central nervous system (CNS): neurons (glutamatergic, GABAergic and dopaminergic) and astrocytes, and express the relevant markers (Beta III tubulin as a pan-neuronal marker, as well as tyrosine hydroxylase, vGlut and vGAT as markers for dopaminergic, glutamatergic and GABAergic neurons, GFAP as a marker for astrocytes, respectively). Based on immunofluorescence data, the composition of the neuronal cultures are 80-90% neurons and 10-20% astrocytes. Of the neurons, 90% are glutamatergic and GABAergic and 10% are dopaminergic neurons.

2. Safety Information

- CNS.4U® neural cells are intended for *in vitro* research use only. The cells are not intended for diagnostics, therapeutic or clinical use and are not approved for human *in vivo* applications.
- CNS.4U® neural cells are genetically modified human cells and therefore genetically modified organisms (GMO). They should be handled according to local directives (Biosafety level 1, US-CDC, or S1, GenTSV, Germany).
- CNS.4U® neural cells can be inactivated by autoclaving at 121°C for 20 minutes.
- CNS.4U® neural cells should be cultured in a sterile environment.

It is highly recommended that gloves and lab coats are worn when handling all reagents as some reagents contain chemicals that may be harmful. Please consult the certificate of analysis (CoA) and material safety data sheets (MSDS) for additional safety instructions where applicable.

3. Material

3.1 Cells and media provided by Ncardia

CNS.4U® are available cryopreserved with 2 million cells / vial and are offered as a bundle including cells, medium, and medium supplement (order no. Ax-B-HZ02-2M). The bundle contains ingredients for 100 ml of culture medium and allows cultivation of the cells for two weeks when cultivated in 48- or 96-well format. Bundle ingredients can also be ordered separately (see Table 1 for order numbers).

The medium for CNS.4U®, i.e., CNS.4U® Culture Medium, is prepared by adding CNS.4U® Supplement to Neuro.4U Basal Medium A (see 4.2). Neuro.4U Basal Medium A is used for thawing of the cells, while CNS.4U® Culture Medium is used for seeding and culturing of CNS.4U®.

Supplement C contains the antibiotic Ciprobay (2 mg/ml) and may be added to the culture medium where the use of antibiotics is desired. Note that the use is optional; importantly, cell growth and function is not compromised by long-term culture in the presence of Ciprobay.

Material	Container	Content	Storage	Shelf life
Cryopreserved CNS.4U® (Ax-C-HZ02-2M)	Cryo vial (x 1)	2 million cells	Liquid nitrogen	Max. 1.5 years from issue date on CoA
Neuro.4U Basal Medium A (Ax-M-NBM250)	Bottle (x 1)	250 ml	LIquid +4 °C	See expiry date on bottle label
CNS.4U® Supplement (Ax-M-DCS-DA)	Cryo vial (x 2)	1.25 ml each	Frozen -80 °C	See expiry date on cryo vial label
Axio-Supplement (Ax-M-CB-5)	Cryo vial (x 1)	250 µl	Liquid RT, dark	See expiry date on cryo vial label

Table 1: Overview of suitable CNS.4U® products for MEA microelectrode assays

3.2 Storage conditions

- **Cryopreserved cells:** Upon receipt of cryopreserved CNS.4U®, transfer the vials directly to the vapor phase of liquid nitrogen for further storage. Do not expose the vials to room temperature and do not store cells at -80 °C, as recrystallization will harm the cells.
- **Medium:** Store Neuro.4U Basal Medium A at +4°C. Avoid excessive exposure to light. For storage life see expiry date on bottle.
- **CNS.4U Supplement:** Store at -20°C upon arrival.
- **Supplement C:** Store at room temperature upon arrival. Avoid excessive exposure to light.

3.3 Required consumables

Consumables	Vendor	Cat. No.
Sterile 50 ml polypropylene tubes	Various	-
Maestro multi-well MEA (48-well)	Axion BioSystems	M768-KAP-48
50 % polyethyleneimine solution (PEI)	Sigma-Aldrich	P3143
Boric acid	Sigma-Aldrich	B7660
Sodium tetraborate	Sigma-Aldrich	221732
Trypan blue solution 0.4 %	Sigma-Aldrich	T8154

Table 2: Overview of required consumables

3.4 Required equipments

Consumables	Vendor
Maestro multi-well MEA System	Axion BioSystems
Axion Integrated Studio (AxIS)	Axion BioSystems
Laminar flow hood	Various
Cell culture incubator (37°C, 95 % humidity, 5 % CO_2)	Various
Neubauer hemocytometer	Various
Centrifuge (swinging bucket rotor)	Various
Inverse microscope	Various
Liquid nitrogen storage	Various
Microscope	Various
37°C water bath	Various

Table 3: Overview of required equipment

4. Preparations

4.1 Coating

It is recommended to seed CNS.4U® on PEI-coated MEA plates.

Preparing 0.1 % PEI solution

1. Prepare 100 ml of borate buffer by dissolving 310 mg boric acid and 475 mg of sodium tetraborate in 100 ml distilled water.
2. Adjust the pH to 8.4 using HCl.
3. Mix the borate buffer overnight.
4. Prepare a 0.1 % PEI solution in borate buffer using a 50 % PEI solution.
5. Filter the solution through a 0.22 µm filter.

Coating of MEA plates

1. Add 200 µl 0.1 % PEI solution to each well to coat the entire bottom of the wells.
2. Incubate the MEA plate for 1 hour at room temperature.
3. Aspirate the PEI solution from the wells and rinse the culture surface with sterile deionized water 4 times.
4. Air-dry the MEA plate in a laminar flow hood overnight.

4.2 Medium

1. If use of antibiotics is desired, add 250 µl Axio-Supplement to 250 ml Neuro.4U Basal Medium (final concentration of antibiotic: 2 µg / ml).
2. Thaw on ice a vial containing CNS.4U® Supplement.
3. Transfer 50 ml Neuro.4U Basal Medium A to a 50 ml polypropylene tube.

4. Homogenize the CNS.4U® Supplement by pipetting; add 1.25 ml to the Neuro.4U Basal Medium in the 50 ml tube.
5. Mix by pipetting. CNS.4U® Culture Medium will now be ready to use and should be used within 7 days. Store at 4°C.

Note
If less than 50 ml Culture Medium is required, aliquot the CNS.4U® Supplement after thawing and refreeze in adequate portions. Do not repeatedly thaw and freeze this supplement!

5. Cell Culture

5.1 Thawing

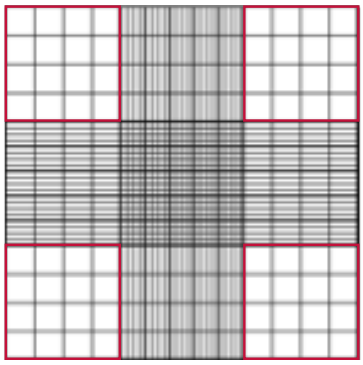
The following applies for thawing of one vial of CNS.4U®. Note that 2 vials are needed in order to fill a Maestro 96-well plate. When 2 vials are required, they may be thawed in parallel and pooled (see 5.1.3) by increasing the volume (1 ml x number of vials) of Neuro.4U Basal Medium A in the 50 ml polypropylene tube before centrifugation.

Note: Neuro.4U Basal Medium A (without supplements) should be used for thawing of cells.

1. Coat an MEA plate with PEI (see 4.1.).
2. Warm Neuro.4U Basal Medium A to room temperature (ca. 15 minutes).
3. Add 1 ml Neuro.4U Basal Medium A to a 50 ml polypropylene tube.
4. Quickly transfer cryopreserved CNS.4U® from the vapor phase of liquid nitrogen or from a transport dewar with liquid nitrogen directly to a 37 °C water bath.
5. Thaw the vial until the frozen cell suspension detaches from the bottom of the vial and only a small ice clump is visible (2 minutes).
6. Gently resuspend the cells by gently shaking the vial. Avoid repeated pipetting of the thawed neurons.
7. Transfer the cell suspension to the 50 ml tube using a 1000 µl pipette. The cells should be added dropwise to the previously-prepared Neuro.4U Basal Medium.
8. Gently mix the cell suspension by gently shaking the falcon tube.
9. Transfer a 20 µl portion of the cell suspension to a vial for counting.
10. Immediately add an additional 8 ml Neuro.4U Basal Medium A to the cell suspension in the 50 ml tube and gently mix the cell suspension; the total volume will now be 10 ml.
11. Pellet CNS.4U® by centrifugation at 260 x g for 8 min at room temperature.
12. During centrifugation, count cells (see 5.2).

5.2 Cell counting and viability determination

1. Add 20 µl trypan blue solution to 20 µl cell suspension withdrawn for counting.
2. Apply 10 µl of the 1:1 mixture to a Neubauer hemocytometer and count viable (clear), dead (blue), and total cells.
3. Count the number of cells in each of the four outer squares highlighted in red of figure 1. Calculate the mean number of cells per red square.
4. Calculate the number of cells corrected by chamber factor (1×10^4), dilution factor (2), and total volume of cell solution (e.g., 1 ml).



Calculation example
E.g.: Mean number of viable cells per square = 100
100 x 10,000 x 2 x 1 = 2,000,000
2 million living cells in the cell suspension

Fig.1 Neubauer hemocytometer

5. Calculate the viability as follows:
   100 x Number of viable cells / (number of dead cells + viable cells) = % viability

5.3 Seeding

The following applies to seeding on a Maestro multi-well MEA.

1. Aspirate the supernatant and gently resuspend the neurons in CNS.4U® Culture Medium to a density of 8,000 cells / µl. Gently mix the cell suspension by gently shaking the vial.
2. Plate cells on the pre-coated MEA plates by adding a droplet of 5 µl (40,000 cells / well) of the cell suspension in the very center, directly on the electrode field of each well.
3. Place the MEA plate in an incubator at 37°C for 1 hour to allow the neurons to settle.
4. Carefully add culture medium to each well of the MEA plate (see table 4).
5. Culture the cells in an incubator at 37°C, 5 % CO_2 and 95 % humidity.
6. Very carefully add 400 µl CNS.4U® Culture Medium to each well.
7. After 48 hours, replace the entire medium in the wells with freshly prepared CNS.4U® Culture Medium.

Note

Due to the typically small volume of medium needed for diluting the cells it is important to consider the cell pellet volume (30 µl) and the remaining small portion of medium when diluting the cells.

MEA format	Cell number	Cell volume	Media volume (µl)
48-well	40,000	Droplet (5 µl / well)	400

Table 4: Cell number and media volume

5.4 Maintenance

We recommend changing the Culture Medium three times per week during subsequent culture. For this, use CNS.4U® Culture Medium at room temperature. Carefully aspirate the medium from the edge of the well with an aspiration pump and a 2 ml aspiration pipette with a 10 µl pipette tip. Do not touch the cell layer. Slowly add the fresh media with an 8-channel pipette to each well. Careful handling is necessary in order to avoid damage to the cell layer.

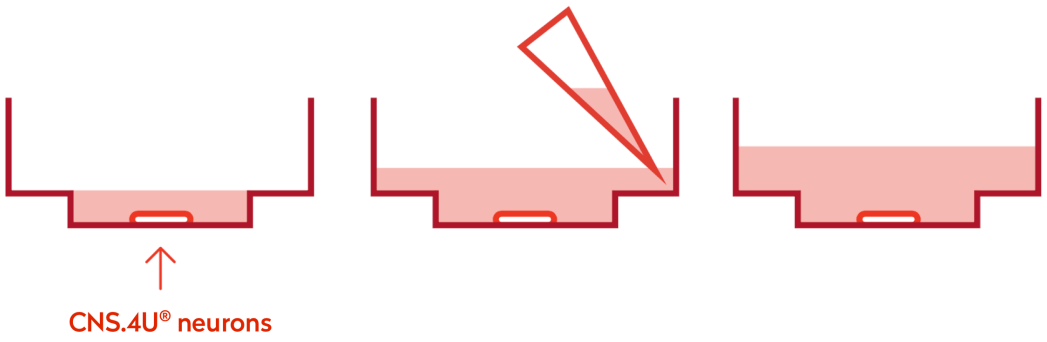


Fig.2 Illustration of Culture Medium aspiration technique

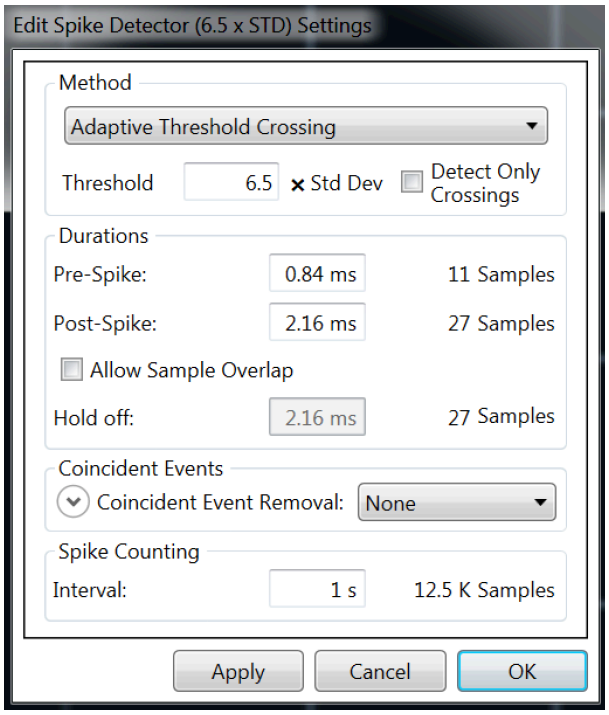
Cells are very sensitive to 100 % medium changes, while MEA measurements with compounds require a defined medium volume across all wells. Therefore, as a compromise between both requirements, the following maintenance / medium changes are recommended:

- 48 hours after seeding, change 100 % of medium (full medium replacement).
- On a weekly basis, change medium three times: once fully (e.g., Mondays) and twice by replacing 50 % of the medium only (e.g. Wednesday and Friday).

6. MEA Recordings

1. Switch on the Middleman of the Maestro MEA system.
2. Start the AxIS software and switch on the temperature control, set to 37°C.
3. Wait until the device temperature has reached 37°C.
4. Before placing a MEA plate into the Maestro device, dry the bottom of the MEA plate with a smooth tissue. It is important that the plate is dry when in contact with the measuring electrodes. Humidity on the electrodes will interfere with the measurement and result in a noisy background measurement.
5. Choose your settings for recording, as described in detail in the Maestro Handling Guide.
6. Before starting a recording, let the neuronal cells acclimatize for 5 min.
7. For spike and burst detection of CNS.4U®, we recommend the following settings:

Settings for **spike** detection



Settings for **burst / network burst** detection

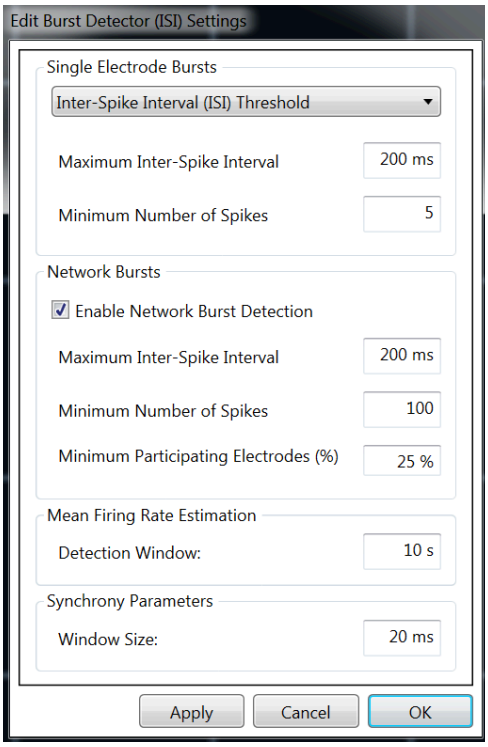


Fig.3 Settings for detection

7. References

1. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S. (2007) Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell. 2007 Nov 30;131(5):861-72.
2. Ncardia AG Patent WO2012098260 A1, A non-viral system for generation of induced pluripotent stem (iPS) cells.

8. Related Documents

1. Ncardia CNS.4U® User Manual

9. Ncardia Limited Label Use License

A. Ncardia Intellectual Property Rights

This product is covered by patent families including, but not limited to, EP1348019; EP1002080; EP1745144; EP1644485; JP4904153; JP4159358; JP3956154; JP4814875; DE10136702 and other

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C. Other patents

Ncardia products which were derived from iPS cells are covered by patents in patent family EP1970446 and US8048999 licensed from iPS Academia (Kyoto University).

Cor.At®, Cor.4U®, Peri.4U®, CNS.4U® and Mel.Cor® are registered trademarks of Ncardia AG, Cologne, Germany.

For information on the patents, patent applications, and licenses associated with the product contact the Ncardia Business Development Department at: patent@ncardia.com.

10. Appendix

10.1 CNS.4U® morphology

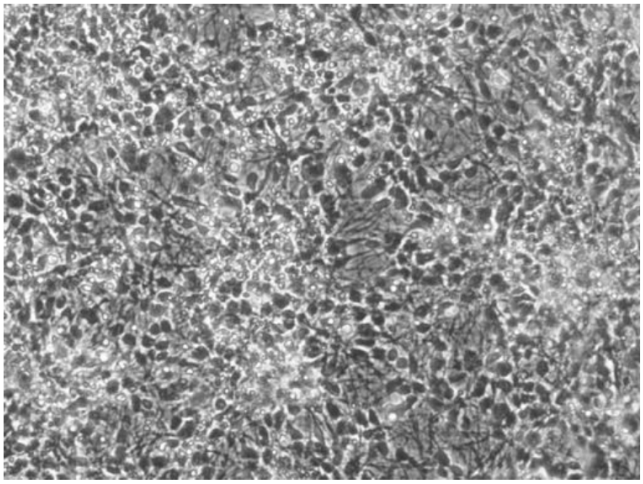

Fig.4 CNS.4U® morphology 6 days after seeding on an MEA plate. CNS.4U® were seeded in a 5 µl droplet on aa PEI-coated plate. Magnification 200x.

10.2 Example of CNS.4U® activity in a MEA well

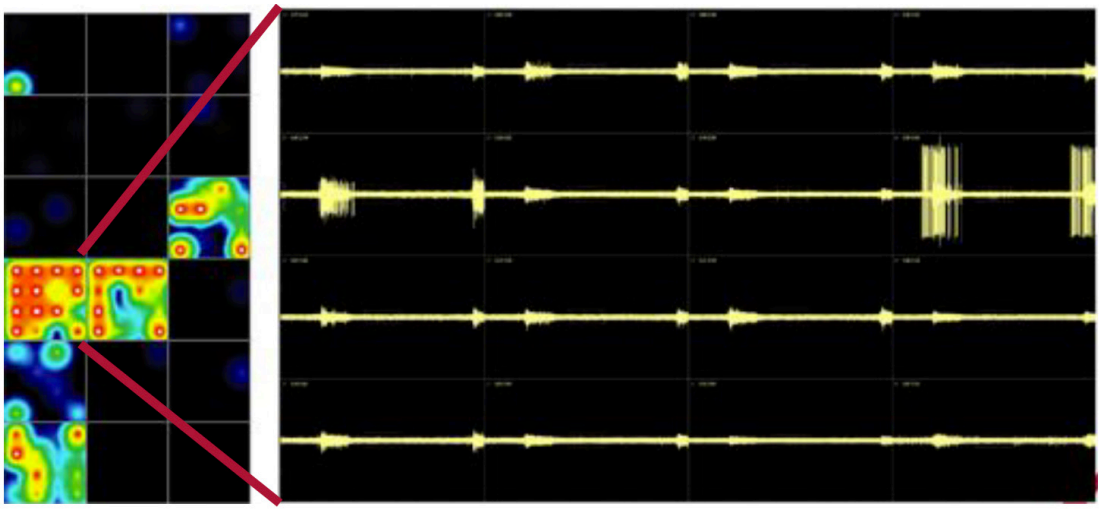

Fig.5 CNS.4U® form extensive neuronal networks and exhibit long-term synchronous network activity assessed by MEA (right), here measurement on day 34 after plating. CNS.4U® exhibit burst-like spontaneous activity, synchronized over 16 electrodes of a 48-well MEA measurement as illustrated in an activity heat map (left). Shown is a time lap of 10 sec.

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