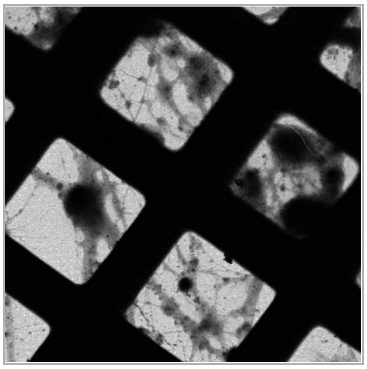

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Neural differentiation on EM grids - iNeurons sample preparation for cryo-ET and CLEM

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ABSTRACT

This is a protocol for differentiating AAVS1-TRE3G-NGN2 iPSCs and hESCs to iNeurons directly on EM grids for cryo-ET and cryo-CLEM. The protocol comprises the following steps:

- EM grids coating with Matrigel or Poly-Ornithine and Laminin;
- differentiation and seeding of iNeurons on EM grids;
- lentiviral transduction for transient expression of fluorescently-labelled proteins;
- plunge freezing.

MATERIALS

CELL LINES:

- H9 ES cells + AAVS-TRE3G-NGN2 (Ordureau et al., 2020);
- iPSCs KOLF2.0 + AAVS-TRE3G-NGN2.

CELL CULTURE MEDIA RECIPES:

Please refer to the protocols listed in the references.

EM GRIDS:

- Quantifoil Au grids, carbon or SiO_2 film, R 1/4, R2/4, R2/2, R2/1.

DISHES WITH FOUR INNER RINGS:

- Cell culture dish, 35/10 mm, four inner rings (Greiner bio-one);

GRID COATING REAGENTS:

- Matrigel (Corning);
- Poly-L-ornithine solution, 0.01%,
- Laminin from Engelbreth-Holm_swarm murin.

PLUNGE-FREEZING:

- Dynabeads MyOne, 1 µm;
- Whatman blotting paper.

INSTRUMENTS:

- Plasma-cleaner (Harrick);
- Vitrobot Mark IV (Thermo-Fisher);
- EM GP2 Plunger (Leica).

SAFETY WARNINGS

For all chemicals, please refer to Safety Data Sheets (SDS) for health and environmental hazards. Liquid nitrogen (LN2) and other cryogens, like ethane-propane, can cause severe damage to the skin and eyes. Always wear personal protective equipment when handling these cryogens. When working with lentiviruses and lentivirus-infected cells follow Biosafety Level 2 protocols and safety rules.

Maintenance of iPSCs and hESC cell lines

1 For maintenance of both induced pluripotent stem cells (iPSCs) and human embryonic stem cells (hESCs) as well as for a complete list of media and reagents, we refer to this protocol:

Protocol

NAME

Human pluripotent stem cell culture

CREATED BY

Felix Kraus

PREVIEW

Starting the neural differentiation (Day 0-Day6)

2 We based our neural differentiation protocol, for both iPSCs and hESCs AAVS1-TRE3G-NGN2 cells, on:

Protocol

NAME

Neural differentiation of AAVS1-TRE3G-NGN2 pluripotent stem cells

CREATED BY

Felix Kraus

PREVIEW

We also refer to that protocol for a complete list of media and reagents.

- Follow the neural differentiation protocol until cell splitting on Day 6;
- One well of a six well plate is enough to seed iNeurons on 10-20 **EM grids** at Day 6, depending on the desired cell density.

EM grid preparation for neuronal culture seeding (Day 5) 1d

3 **Choosing grid type:**

- Use gold (Au) grids only for mammalian cell culture; copper (Cu) is cytotoxic;
- iNeurons grow well on films with 1 µm and 2 µm holes. All following grid geometries worked in our hands: R 1/4, R2/4, R2/2, R2/1. Films with 2 µm holes may be preferred for direct TEM data acquisition on the processes.
- Both carbon and silicon dioxide (SiO_2) films work. SiO_2 is preferred when preparing samples for FIB-milling. In our hands, iNeurons did not grow on UltraAUfoil gold film grids.

Start preparing EM grids one day before seeding the iNeurons.

4 **Plasma cleaning**

Carefully place EM grids on a glass slide and plasma clean the grids for 30 seconds.

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- Prepare 3.5 mm **dishes with four inner rings** (see materials). This kind of dish allow to use smaller quantity of medium and cells for seeding.
- Use tweezers to transfer each grid inside of one inner ring of the culture dish.

6 **UV-treating**

Treat the grids with UV-light for 20 min.

7 **Coating grids**

We suggest two methods, coating grids with **matrigel** or **poly-Ornithin + Laminin (PO+L).** Both methods worked well for hESC-derived iNeurons, while iPSC-derived iNeurons seemed to grow better on POL+L coated grids.

Note

Sometimes EM grids are hard to handle during coating and washing steps, they may float to or move around the dish, which may cause breaking of the film or bending of the grid. If you experience similar problems, here are few tricks:

- use a 10 µl drop of buffer to "glue" the grid to the bottom of the dish when first placing it in the culture dish;
- never let the grids dry completely whenever removing medium during washing steps; instead, leave a thin film of liquid and increase the number of washing steps.

7.1 **Method 1: Coating grids with Matrigel** 1d

- thaw the Matrigel in ice and dissolve it in cold DMEM/F12 medium;
- dispense 100 µl on top of each grid to form a a small dome;
- incubate overnight at 37 °C;
- the grids are ready for use the next day.

7.2 **Method 2: Coating grids with PO+L**

- dispense 100 µl of poly-ornithine solution (0.01%) on top of every grid;
- incubate at RT for 4 hours in a sterile container;
- remove the PO solution and add 100 µl of Laminin (dissolved in PBS at a final concentration of 0.2 µg/ml);
- incubate overnight at RT;
- the next day wash 3 times with PBS and store with 100 µl ND2 or DMEM/F12 on top till cell seeding;

Seeding iNeurons on the grids (Day 6)

1d

8 *iPSC and hESC-derived iNeurons can be successfully seeded on grid on any day between 5 and 7.*

Detach the cells and count:

- wash the cells with PBS;
- add Accutase (1 ml for one well) and incubate for 5 minutes at 37 °C;
- add 5 ml of ND2 medium;
- use a 1 ml pipette to pipette the cells up and down to obtain single cells;
- OPTIONAL: pass the cells through a cell strainer;
- transfer to 15 ml tube and count the cells.

8.1 **Dilute to desired density and seed:** 2h

- Prepare cell dilution in ND2 medium: seed 1500 cells in a volume of 150 µl on top of each grid (*See note: How many cells should I seed on the grid?*);
- Leave the cells in the incubator for 1 or 2 hours while they attach on the grid.

Note

How many cells should I seed?

- In general, iNeurons grow better and differently on the dish compared to EM grids. On grids, the denser they are, the better they will grow by creating a thick network of processes;
- Aim for lower cell densities (1500 cells in 150 µl, 1 X 10^4 cell/ml) if you plan to image thin areas in the processes directly by TEM. If you plan instead to do FIB-milling you can seed at higher density (up to 1 X 10^5 cells/ml) to get more somata or thick processes. Pay attention, too high concentration, resulting in thicker sample, will not allow vitrification by plunge-freezing.
- Test a range of different cell densities during the first experiment.

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- After the cells attached, carefully add 2 ml of ND2 medium.

Note

Always add medium by gently pipetting it on the wall of the dish, to avoid to stress the cells and move the grids around the dish.

Medium exchange and Doxycycline withdrawal (Day 7-15)

10

- Exchange 50% of the medium every other day, as explained in the neural differentiation protocol;

- Withdraw Doxycycline on Day 10 for hESC-derived iNeurons, or around Day 15 for iPSC-derived iNeurons.

Lentiviral transduction for fluorescence marker expression ...

1h

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In order to express fluorescently-labelled proteins for CLEM experiments, we use transient expression via lentiviral transduction. Before transducing iNeurons on EM grids, determine the correct quantity of virus to add by titrating it on control cell culture dishes.

On the day of viral transduction:

- Exchange half of the medium as usual;
- If co-transducing with different viruses, pre-mix the different viruses in 200 µl of medium;
- Dilute the virus in 200 µl of culture medium;
- Apply the virus solution dropwise on top of the dish;
- Incubate the cells and do not exchange medium the following day.

Two days after viral transduction:

- Start exchanging half of the medium with fresh ND2.

Safety information

When working with lentiviruses and lentivirus-infected cells follow Biosafety Level 2 protocols and safety rules.

Note

In order to minimize background cryo fluorescence, you may want to switch to phenol-red free Neurobasal medium for ND2 preparation at this point of the sample preparation. This is especially important if you are planning to use blue fluorophores (excitation in the UV range).

Plunge-freezing of iNeurons on EM grids (Day 20-24)

1h

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- If possible, store cells in an incubator close to the plunge-freezing set up during plunging day;
- Be very careful when moving and transporting grids and dishes, shake the dishes as little as you can and minimize their time outside of the incubator. iNeurons are very fragile and have a tendency to detach from the grid when shaken.

We propose two methods for two commonly used plunge-freezing instruments, **Vitrobot Mark IV** and **Leica EM GP2**.

Safety information

Liquid nitrogen (LN2) and other cryogens, like ethane-propane, can cause severe damage to the skin and eyes. Always wear personal protective equipment when handling these cryogens

Note

- Here below we share settings that worked well for two plunge-freezing machines in our lab. However, it is extremely important that you test and find the optimal settings for your own plunge-freezing system;
- Any setting that already worked for mammalian adherent cell cultures on EM grids should work well for iNeurons.

12.1
- When preparing sample for correlative FIB-milling, apply 4 µl of medium containing 1 µm autofluorescent beads (Dynabeads, diluted 1:20) before blotting. If not, just apply 4 µl of culture medium.
- Plunge in ethane-propane or ethane.

12.2 **Method 1: Plunging with Vitrobot Mark IV**
- apply 4 µl;

Settings:
Blot force: 8; Blot time: 10; Chamber humidity: 70%.
Blot from the back: one teflon sheet + blotting paper, teflon only on front pad.

12.3 **Method 2: Plunging with Leica EM GP2**
- apply 4 µl;

Settings:
Blot time: 7.0 sec; Horizontal blot position: 43.2 mm /43.0 mm; Vertical blot position: 2.7 mm; Chamber humidity: 95%.

12.4 Store your grids in LN2. The iNeurons samples are ready to be directly imaged by Cryo-ET or imaged by cryo-FLM and subsequently FIB-milled.