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Protocol for neurophysiological imaging in human iPSC-derived neurons

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Protocol status: In development
The protocol works but we are still developing and optimizing this protocol.

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Abstract

The protocol describes how to perform neurophysiological imaging experiments on different types of human iPSC-derived neurons.

Materials

Solutions:

HEPES buffer consists of: (in mM) 140 NaCl, 5 KCl, 10 HEPES, 2 $CaCl_2$, 2 $MgCl_2$, and 10 d-glucose, adjusted to pH = 7.4 using NaOH (**Li et al. 2022**).

Electrodes:

- Focal electrodes were constructed by soldering a 50 µm tungsten wire to a gold connector inserted into a glass pipette that was pulled using a Sutter instruments pipette puller. The tip of the glass pipetted was sealed by melting the glass in the open flame of a lighter. This electrode was connected to a Grass stimulator and (30V) pulses were given at 1-20 Hz. A silver electrode was used as a reference electrode. (**Li et al. 2019**)

- For field stimulation: two Pt wire electrodes (0.5 mm diameter) were flatted and cut to from two planar side which were spaced 1 mm apart in a plexiglass rod. This rod was positioned using a Narishige 3D manipulator such that the 1 mm space covered the field of view that was under investigation. The electrode was gently put on the coverslip and current (5-10 mA) stimuli were given to depolarize the neuronal networks.

iPSC culture and maintenance

1 The iPSC cell lines (WTC11 and KOLF2.1J) are cultured in Essential 8 medium on Geltrex-coated plates. The cultures are passaged every 3 days with 0.5mM EDTA.

iPSC-derived neurons generation

2 Cells were differentiated from human iPSCs (WTC11, KOLF2.1 cell lines) according to published protocols, with some modifications to allow for easier live-cell imaging.

2.1 To generate midbrain dopaminergic-like neurons, we followed the **Kriks et al. (2011)** protocol, with the following modifications:

- Simplified basal medium:
Days 1-20: Neurobasal-A + 1% GlutaMAX + 2% B27 Supplement + 1% N2 Supplement + 1% PenStrep
Days 20+: Neurobasal-A + 1% GlutaMAX + 2% B27 Supplement + 1% PenStrep

- Additional 1:2 replating step at Day 8 of differentiation to avoid cell detachment due to overgrowth.

- For the replating step at Day 20, the cells are replated under low-density conditions (30 $\times 10^3$ - 100 $\times 10^3$ cells per cm^2) as opposed to the recommended high-density conditions (300 $\times 10^3$ - 400 $\times 10^3$ cells per cm^2)

- If there are non-neuronal cells present in the culture after Day 20, a treatment with 2 µM Ara-C for 24-48h is carried out.

2.2 To generate enteric nervous system-like neurons, we followed the **Barber et al. (2019)** protocol, with the following modifications:

- Additional replating step at Day 20-22. We plate 60 $\times 10^3$ - 100 $\times 10^3$ cells per cm^2.

- If there are non-neuronal cells present in the culture after Day 20, a treatment with 2 µM Ara-C for 24-48h is carried out.

Live-cell imaging

3 Using calcium as a proxy for neuronal activity, we assess calcium dynamics during basal conditions, as well as during stimulations.

3.1 For midbrain dopaminergic-like neurons: 1h 15m

- The cells are loaded with the calcium dye Fluo-4-AM at a concentration of [M] 50-100 nanomolar (nM) for 00:10:00 at 37 °C.
- The cells are washed 3x with HEPES and left to incubate at 37 °C for 00:05:00.
- The coverslips are mounted into an imaging holder and the cells covered with HEPES. As an easy alternative, glass bottom culture dishes can be used.
- The cells can be imaged for up to 01:00:00.

3.2 For ENS-like neurons: 1h 10m

- The cells are loaded with the calcium dye Fluo-4-AM at a concentration of [M] 1 micromolar (µM) for 00:10:00 at Room temperature, on a shaker (<50 rpm).
- The cells are washed 1x with HEPES and mounted into an imaging holder. The cells are covered with HEPES. As an easy alternative, glass bottom dishes can be used.
- The cells can be imaged for up to 01:00:00.

4 Using mitochondrial dyes such as MitoTracker (MitoTracker Green/Red/Red CMXRos), we investigate neuritic mitochondrial transport in these cells (**Van Steenbergen et al. 2022**). 1h 15m

- The cells are loaded in [M] 5-7.5 nanomolar (nM) (for dopaminergic-like neurons) or [M] 20 nanomolar (nM) (for ENS-like neurons) MitoTracker for 00:10:00 at 37 °C.
- The cells are washed 3x with HEPES and left to incubate at 37 °C for 00:05:00.
- The coverslips are mounted onto an imaging holder and covered with HEPES. As an easy alternative, glass bottom culture dishes can be used.
- The cells can be imaged for up to 01:00:00.

Stimulation

5 Depending on the experiment, we use a combination of different stimuli to assess neuronal physiology.

5.1 Electrical stimulation

- Electrodes (custom field electrodes and focal electrodes; see Materials for details)

- Stimulator: Master8 (A.M.P.I) driving the A385 Stimulus Isolator (World Precision Instruments)
- Stimulation protocols:
  - Field electrode
    - 3 - 100 pulses at 20 Hz - 100 Hz
    - Current: 5 - 10 mA
  - Focal electrode
    - Train duration: 2×1000 ms
    - Rate: 2×10 pps
    - Delay: 1×1ms
    - Duration: 3×0.1 ms
    - Volts: 3×10 volts

5.2 Local perfusion (for ENS-like cells, as in **Boesmans et al. 2019**)

- High-K^+ ([M] 75 millimolar (mM))
- Substance P ([M] 1 micromolar (µM))
- DMPP ([M] 10 micromolar (µM))

Microscopy

6 A variety of microscopes can be used. We use wide-field systems (Zeiss Axiovert, Nikon coupled to Till Vision image acquisition hard/software) and confocal systems (Zeiss LSM 780-NLO, Zeiss LSM 880 AiryScan). The image acquisition speed should be at least 1-2 Hz in order to capture the relevant physiological events.

Laser powers and exposure times (generally ~ 2 mWatt for widefield, 20-80ms exposure per 500 ms) should be adjusted to avoid bleaching and to preserve neuronal health.

Protocol references

Barber at al. 2019
Boesmans et al. 2019
Kriks et al. 2011
Li et al. 2019
Li et al. 2022
Van Steenbergen et al. 2022