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We use this protocol and it's working

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Primary neuron culture for live imaging of axonal cargoes

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ABSTRACT

This protocol describes the preparation and culture of mouse primary cortical neurons for live-imaging experiments. Cortices were dissected from mouse embryos at day 15.5. Cortical neurons were isolated by digestion with 0.25% Trypsin and trituration with a serological pipette. Neurons were plated on glass-bottom imaging dishes in Attachment Media. After 5 hours in culture, Attachment Media was replaced with Maintenance Media, and AraC was added on the next day to prevent glia cell proliferation. Neurons were transfected 16-24 hours before imaging using Lipofectamine 2000.

ATTACHMENTS

547-1143.pdf

MATERIALS

Materials:

- Dissecting microscope

Equipment	
Dumont #5 - Mirror Finish Forceps	NAME
Forceps	TYPE
Durmont	BRAND

11251-23 SKU

https://www.finescience.com/en-US/Products/Forceps-Hemostats/Dumont-Forceps/Dumont-5-Mirror-Finish-Forceps/11251-23 LINK

- Micro spring scissors (Fine Science Tools)

Equipment

Countess 3 FL Automated Cell Counter NAME

Automated Cell Counter TYPE

Thermofisher scientific BRAND

AMQAF2000 SKU

https://www.thermofisher.com/th/en/home/life-science/cell-analysis/cell-analysis-instruments/automated-cell-counters/models/countess-3-fl.html LINK

Equipment

35 mm Dish | No. 1.5 Coverslip | 20 mm Glass Diameter | Uncoated NAME

Coverslip TYPE

Mattek BRAND

P35G-1.5-20-C SKU

https://www.mattek.com/store/p35g-1-5-20-c-case/ LINK

- 15 mL conical tubes
- 10 cm cell culture dish

Reagents:

- Poly-L-lysine hydrobromide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #p1274**
- 10X HBSS **Thermo Fisher Scientific Catalog #14185-052**
- HEPES Buffer **Thermo Fisher Scientific Catalog #15630-080**
- Trypsin (2.5%), no phenol red **Thermo Fisher Catalog #15090046**
- MEM **Thermo Fisher Catalog #11095072**
- Horse serum **Thermo Fisher Scientific Catalog #16050122**
- 45% D-( )-Glucose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G8769**
- Sodium Pyruvate (100 mM) **Thermo Fisher Scientific Catalog #11360070**
- Trypan Blue Stain (0.4%) for use with the Countess™ Automated Cell Counter **Thermo Fisher Catalog #T10282**
- Neurobasal™ Medium **Thermo Fisher Catalog #21103049**
- B-27™ Supplement (50X), serum free **Gibco - Thermo Fisher Catalog #17504044**
- GlutaMAX™ Supplement **Thermo Fisher Catalog #35050061**
- Penicillin-Streptomycin (10,000 U/mL) **Thermo Fisher Scientific Catalog #15140122**
- Cytosine β-D-arabinofuranoside hydrochloride (AraC) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C6645**
- Lipofectamine™ 2000 Transfection Reagent **Thermo Fisher Scientific Catalog #11668019**
- Hibernate E Low Fluorescence Imaging Medium (BrainBits, Cat# HELF)

SAFETY WARNINGS

Take necessary precautions with sharp objects during dissection. Follow institutional recommendations for disposal of animal tissue and biohazardous materials.

Day before dissection

1 Coat glass-bottom imaging dishes with PLL.

1.1 Hydrate 100 mg PLL (Sigma) in 50 mL 0.1 Molarity (M) borate buffer, pH 8.5.

1.2 Store PLL stock solution (2 mg/mL) in 1 mL aliquots at -80 °C.

1.3 On the day before neuron dissection, dilute PLL in ddH_2O 1:20 to a final concentration of 100 µg/ml.

1.4 Add 1 mL PLL to each glass-bottom imaging dish (MatTek) and incubate Overnight at 37 °C.

1.5 Only coat the glass center with PLL.

Note

For easy handling, we find it helpful to place imaging dishes in 10 cm or 15 cm cell culture dishes.

2 Prepare HBSS, attachment media and maintenance media.

2.1 For 500 mL 1x HBSS, combine

	A	B
	10 x HBSS	50 mL
	1 M HEPES	5 mL
	ddH2O	up to 500 mL
	Filter-sterilize	

2.2 Store 1x HBSS at 4 °C and use within one month.

2.3 For 50 mL attachment media, combine

	A	B
	Heat-inactivated horse serum	5 mL
	100 mM Sodium pyruvate	500 µL
	45% Glucose	660 µL
	MEM	up to 50 mL

2.4 For 50 mL maintenance media, combine

	A	B
	GlutaMAX	500 µL
	Penicillin/Streptomycin	500 µL
	45% Glucose	660 µL
	B-27	1 mL
	Neurobasal	Up to 50 mL

2.5 Store attachment media and maintenance media at 4 °C.

Note

Maintenance Media should be used within 7 days. Attachment media can be kept at 4 °C for 3-4 weeks.

Dissection of cortical neurons

3 In the morning of the day of dissection, wash PLL-coated imaging dishes twice with sterile ddH_2O.

3.1 Add 2 mL attachment media per imaging dish and leave dishes at 37 °C in cell culture incubator.

3.2 Warm required amount of attachment media and 1x HBSS (4.5 mL for one dissection) in 37 °C water bath.

3.3 Aliquot maintenance media into 10 cm cell culture dish to equilibrate in 37 °C / 5% CO_2 cell culture incubator.

3.4 Let 2.5% trypsin aliquots thaw at Room temperature.

4 Sacrifice pregnant mouse, dissect embryos, and place embryonic brains in HBSS On ice.

4.1 Using a dissecting microscope, remove meninges from brain hemispheres with fine forceps.

4.2 Isolate cortices using fine forceps and small spring scissors.

4.3 Transfer dissected cortices into a 15 mL conical tube filled with 5 mL HBSS and keep On ice until all cortices are collected.

Note

Use clean and sterile equipment for all dissection steps to prevent bacterial contamination of neuron cultures.

Note

We find that using ice-cold HBSS helps preventing the tissue from getting sticky during the dissection. If HBSS gets too warm during the dissection, replace with fresh cold HBSS.

5

Note

Perform all following steps under a sterile tissue culture hood.

Once all cortices are collected, remove HBSS from 15 mL conical tube and add 4.5 mL warm (37 °C) HBSS and 0.5 mL 2.5% trypsin.

5.1 After adding trypsin, invert the tube to mix.

5.2 Then incubate for 00:10:00 in a 37 °C water bath. 10m

6 Remove HBSS-trypsin solution with a 5 mL serological pipette.

6.1 Wash thrice with 7 mL attachment media.

6.2 Add attachment media, then wait until cortex tissue has settled at the bottom of the conical and remove attachment media with a serological pipette to repeat the washing step.

Note

We do not recommend using a vacuum aspirator for removing HBSS and attachment media, instead use a 10 mL serological pipette.

7 Add 5 mL attachment media after the last washing step.

7.1 Triturate cortices by pipetting up and down forcefully with a 5 mL serological pipette 10 – 15 times.

Note

Trituration is complete when no tissue clumps are visible and attachment media turns turbid.

7.2 Let media with triturated tissue settle for 00:01:00 - 00:02:00 . 3m

7.3 Transfer top 4.5 mL to a new tube to remove any remaining cell clumps.

8 Mix 10 µL cell suspension with 10 µL 0.5% trypan blue in an Eppendorf tube.

8.1 Count cells using a hemocytometer or an automated cell counter.

9 Dilute cortical neurons to 1,000,000 cells/mL.

9.1 For transfection and live-imaging, plate 200,000 cells per live-imaging dish.

9.2 Place imaging dishes in 37 °C cell culture incubator.

Note

Take up cells in a 200 µL pipette, then plate drops of cells in different areas to distribute neurons evenly across the live-imaging dish.

10 After 03:00:00 - 04:00:00 use an aspirator to remove all attachment media. 7h

10.1 Replace with 2 mL pre-equilibrated maintenance media per imaging dish.

Note

Cells should be attached to the glass-bottom dish at this point. Maintenance media must always be pre-equilibrated to 5% CO_2 in 37 °C incubator before adding to cells.

Neuronal cell culture

11 On the day following the dissection, dilute AraC to [M] 10 micromolar (µM) in maintenance media and bring to 37 °C.

11.1 Add 200 µL maintenance media + AraC to each imaging dish for a final AraC concentration of [M] 1 micromolar (µM).

12 Every 3-4 days, remove 600 µL maintenance media from each dish and replace with 750 µL fresh, pre-equilibrated maintenance media.

Note

Cultured neurons are sensitive. Try to keep time outside the cell culture incubator to a minimum. If possible, use a separate incubator for primary neurons and keep openings to a minimum.

Transfection

16h 55m

13 Transfect primary neurons on DIV6-7, ~ 16:00:00 before live-imaging. 16h

14 Replace conditioned media with fresh, pre-equilibrated maintenance media (2 mL per imaging dish).

14.1 Save old media = conditioned media in a 10 cm cell culture dish at 37 °C in the cell culture incubator.

15 For each imaging dish, prepare two tubes with transfection reagents.

15.1 In tube 1, add plasmid DNA to 200 µL Neurobasal medium.

15.2 In tube 2, add Lipofectamine 2000 to 100 µL Neurobasal medium.

> **Note**
>
> It is important to use non-supplemented Neurobasal and not maintenance medium to set up the transfection reaction.

> **Note**
>
> The amount of Lipofectamine 2000 and plasmid DNA depends on the specific construct(s) used and may require optimization. We find that for transfection with one plasmid, 0.4 µg DNA and 1 µL Lipofectamine 2000 works well in most cases.

16 Combine contents of tube 1 + 2 and mix by gently pipetting up and down 4-5 times.

16.1 Incubate mix at Room temperature for 00:10:00. 10m

17 Add Lipofectamine-DNA mix to imaging dishes dropwise.

17.1 Incubate for 00:45:00 at 37 °C in cell culture incubator. 45m

18 Remove all transfection media and replace with conditioned media collected earlier.

18.1 Return cells to incubator and image on the next day.