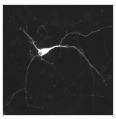

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Working

Primary Ventral Midbrain Culture

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ABSTRACT

Primary ventral midbrain cultures provide a reproducible medium to explore neuronal dynamics that are experimentally difficult to perform *in vivo*. The ventral midbrain contains two important dopaminergic-rich regions: substantia nigra pars compacta (SNc) and ventral tegmental area (VTA). These regions have been shown to be critical in regulation of goal-directed behavior and reward prediction error. Furthermore, dysfunction of the SNc and VTA has been identified as targets in Parkinson's disease and addiction, respectively. Therefore, primary ventral midbrain culture provides a model to explore the relative impact of dopaminergic modulation from molecular to neural network scales.

GUIDELINES

Ventral midbrain neuronal cultures are notoriously sensitive. Ensure that all tissue and cells are handeled as gently as possible. Additionally, general aeseptic technique is critical to preventing contamination.

MATERIALS

NAME	CATALOG #	VENDOR
200 mM L-Glutamine	G7513	Sigma
45% D-( )-Glucose	G8769	Sigma
Sylgard 184	View	Dow Corning
Sodium bicarbonate	S5761	Sigma Aldrich
Sodium chloride	S7653	Sigma Aldrich
Penicillin-Streptomycin	15140122	Gibco - Thermo Fisher
Potassium Chloride	P9541	Sigma Aldrich
MEM with no L-glutamine	11090-081	Gibco, ThermoFisher
BenchMark Fetal Bovine Serum	100-106	Gemini Bio-Products
Insulin from bovine pancreas	I5500	Millipore Sigma
Neurobasal Plus Medium	A3582901	Gibco, ThermoFisher
B-27™ Plus Supplement (50X)	A3582801	Gibco, ThermoFisher
Kynurenic acid	K3375	Sigma – Aldrich
L-Cysteine	C7352	Sigma – Aldrich
Calcium chloride dihydrate	223506	
GDNF from mouse	SRP-3200	Sigma – Aldrich
Magnesium sulfate	M7506	Sigma – Aldrich
Dextrose	D9434	Sigma – Aldrich
Ethylenediaminetetraacetic acid	E9884	Sigma – Aldrich

NAME	CATALOG #	VENDOR
Papain	LS003126	Worthington Biochemical Corporation
Hydrochloric acid solution	H9892	
Gey's Balanced Salt Solution	G9779	Sigma – Aldrich
COVER SLIPS ROUND GERMAN GLASS #1 THICKNESS	CLS-1760-012	
Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane	L2020	Sigma – Aldrich
Poly-D-lysine hydrobromide	P0899	
Trypan Blue Solution 0.4% (w/v) in PBS pH 7.5 ± 0.5	25-900-CI	Corning

BEFORE STARTING

This protocol requires multiple combinations of solutions and materials. Therefore, many of them need to be prepared before beginning the surgical protocol. The following solutions and materials should be prepared in advance:

Poly-D-Lysine Solutions (Store at -20°C)
- Stock: Resuspend to 5 mg/ml using sterile water.
- Working: Dilute to 0.1 mg/ml using sterile water.

GDNF (Store at -20°C)
- Resuspend to 0.625 µg/ml using sterile water.
- Prepare 40 µl aliquots.

Kynurenic acid (Store at -20°C)
- Resuspend to 0.5 M using 1N NaOH.

DISCO (5X) - Sterile Filter (Store at 4°C)
- 580 mM NaCl
- 27 mM KCl
- 130 mM $NaHCO_3$
- 10 mM NaH_2PO_4
- 5 mM $MgSO_4$
- 2.5 mM EDTA
- 125 mM Dextrose

Cysteine Water - Sterile Filter (Store at 4°C)
- 7mg L-cysteine
- 855 µl 100mM $CaCl_2$
- 44.1 ml ddH_2O

Glia Media - Sterile Filter (Store at -20°C)
- 65 ml MEM with no L-glutamine
- 50 ml Fetal Bovine Serum
- 100 µl Insulin
- 3.8 ml 45% glucose
- 1.23 ml 200 mM l-glutamine
- 10 ml penicillin-streptomycin

Sylgard Platform (Store at room temperature)
- Mix 10 parts elastomer base with 1 part curing agent.
- Pour into 12-well plate approximately 1/3 full.
- Cure overnight at 60ºC.

- Wrap individually with aluminum foil and autoclave.

Trituration pipettes
Using 1 ml pipette tips, seal the end with a flame and puncture through both sides with needle. Autoclave and store at room temperature.
- Large: 18G
- Medium: 21G
- Small: 25G

Washing Coverslips

1
1. Boil cloverslips in 1M HCL for 5 minutes, allow to cool. **00:05:00**
2. Wash 3X with ddH_2O.
3. Wash for 30 min in 100% ethanol. **00:30:00**
4. Wash 3X with ddH_2O.
5. Cover flask with aluminum foil and autoclave.

Coating Coverslips

2
1. Distribute 2 coverlips into each 35 x 10 mm petri dish with flamed forceps.
2. **37 °C** Dispense approximately 175 µl of 0.1 mg/ml Poly-D-Lysine on each coverslip and incubate for 1 hour. **01:00:00**
3. Wash 3X with ddH_2O.
4. Allow to air dry at least 30 minutes. **30:00:00**
5. Dilute laminin in MEM (5 µg/ml final concentration) and coat coverslips with 100 µl of solution.
6. Cover petri dishes to prevent evaporation of the solution.

Dissociation Media Preparation

3 Prepared fresh day of dissection.

Mix the following solutions in order, otherwise papain will not dissolve
1. 15.5 ml Cysteine water
2. 400 units papain
3. 4 ml DISCO
4. 20 µl kynurenic acid

Adjust pH to 7.4 with 5N HCl and sterile filter into Nunc vial

Pre-surgical preparation

4
1. Clean 10 cm glass petri dish with 70% ethanol and allow to dry
2. Using VetBond, glue a single Sylgard stage in the middle of the dish and allow to cure
3. Warm water bath to 37ºC.
4. Thaw Glia Media in water bath.
5. Transfer Dissociation media to water bath and oxygenate with 95% O_2 5% CO_2. Flow rate approximately 1 bubble per second.

Dissection

5 All orientation instructions assumes user is right-handed.
1. Place 10 cm petri dishes on ice and fill with Gey's Balanced Salt Solution (GBSS).
2. Remove post-natal day 0 pups from mother in container with bedding and place on heating pad (set to low) until ready to use.
3. Prepare a trough in ice and line with aluminum foil.
4. Place pups on ice.
5. Once movement has slowed and pups stop moving, rapidly decapitate and transfer to 10 cm petri dish.
6. Cut skin to expose skull and remove the skull by cutting around the base with 3 mm Vanna Scissors.

7. Using a spatula, gently push the brain from the skull from the front into GBSS.
8. Place brain in center of stage, ventral surface upwards and anterior pointing left.
9. Using 5A style forceps angled downward, pierce the brain through the rostral hypothalamus and pin to stage.
10. Make first cut caudal to the midbrain flexture.
11. Make second cut rostral to midbrain flexture, including caudal hypothalamus.
12. Take resulting slice and orient ventral surface to the right.
13. Hold slice in place by inserting forceps into slice at the acqueduct.
14. Remove the hypothalamus by cutting the ventral portion.
15. Isolate ventral midbrain by cutting approximately halfway between previous cut and acqueduct.
16. Remove left and right meninges.
17. Dissect ventral midbrain into 1 mm^3 sections and transfer to dissociation media using sterile transfer pipette.
18. Incubate in dissociation media for 20 minutes. **00:20:00**

Neuronal Media

6 Neuronal Media (Prepare fresh)

- 25 ml Neurobasal Plus
- 0.5 ml B-27 Plus
- 250 µl L-glutamine

For day one of plating add:

- 40 ul GDNF (1 ng/ml)
- 20 ul kynurenic acid

Warm to 37°C.

Plating

7
1. Following incubation, transfer tissue to biosafety cabinet.
2. Add 5 ml of Glia Media to a 15 ml tube and transfer tissue, ensuring to transfer as little dissociation media as possible.
3. Wash tissue 2-3x with 10 ml Glia Media, allowing tissue to settle between solution removal.
4. After final wash, resuspend tissue in 5 ml Glia Media.
5. Starting with large bore pipette, triturate tissue until it no longer decreases in size (10-20X) before switching to medium then small bore pipettes. Final appearance will be opalescent, indicating cells are in suspension. Pipette slowly to minimize the entrance of air bubbles and significant shear force.
6. Centrifuge cell suspension for 5 minutes at 1550 RPM at room temperature.
7. Remove supernatant and resuspend in 1 ml of Glia Media and pass through a 70 µm cell strainer.
8. Aspirate 10 µl of cell suspension (ensure it does not settle) and mix thoroughly with 10 µl Trypan Blue Solution and count number of live cells / µl.
9. Adjust cell concentration to 80,000 cells per 100 µl with Glia Media.
10. Pipette 100 µl of suspension directly into the laminin coating solution on each coverslip, slowly and in a clockwise motion to ensure homogenous plating.
11. Allow cells to settle for 2 hours in incubator set to 37°C with 5% CO_2. **02:00:00**
12. Gently flood dishes with 2 ml of Neuronal Media supplemented with GDNF and kynurenic acid.
13. Transduce neurons with AAV5-hSyn-GCaMP6f (number of genome copies per cell = 30,000).

Maintenance

8 Every 4 days following plating, remove 1 ml of old media and replace with 1 ml of fresh Neuronal Media. Media can be changed more often, if necessary.

DIV 9-12 is the ideal window for general neuronal health and activity.