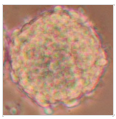

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Neurosphere Protocol - A rapid and detailed method for the isolation of spheres *in-vitro*

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ABSTRACT

The neurosphere assay is a powerful *in-vitro* tool that has been used to study neural stem cell biology. Here we demonstrate a rapid and detailed isolation approach that produces large number of neurospheres from the dorsal lateral ventricle of late embryonic mice without the need for specialized equipment. This approach can be used to generate neurospheres for genetic studies, pharmacological manipulation, immunocytochemistry, self-renewal and differentiation assays.

GUIDELINES

This protocol is designed to generate neurospheres from a single embryo. Multiply all values as needed to generate neurospheres from additional embryos.

This protocol has four general sections:
1.Set-up prior to tissue dissection
2.Tissue Dissection
3.Primary Neurosphere
4.Secondary Neurosphere

After 5-7 days, primary neurospheres should be split to maintain the health of the culture.

Please Note: Breeding and euthanasia of all animal work should be performed in accordance with an institutionally approved animal care and use protocol.

MATERIALS TEXT

Name of Material/ Equipment	Type	Company	Catalog Number	Comments	
Industrial Razor Blades	Surgical tool	VWR	55411-050		
Forceps	Surgical tool	Fine Science Tools	11251-20		
Small Scissors	Surgical tool	Fine Science Tools	14060-09		
Hanks' Balanced Salt Solution	Reagent	ThermoFisher Scientific	14185-052	Adjust to pH 7.1 after dilution to 1X	
0.25% Trypsin/EDTA (1x)	Reagent	ThermoFisher Scientific	25200-056		
MgSO4	Reagent	JT Baker	2500-01		
Dnase I	Reagent	Roche	10104159001		
BSA	Reagent	Sigma	A3912		
10% FBS	Reagent	ThermoFisher Scientific	26400044		
Penstrep	Reagent	ThermoFisher Scientific	15140-148		
Soybean Trypsin Inhibitor	Reagent	Sigma-Aldrich	T6522		
B27 Supplement	Reagent	ThermoFisher Scientific	17504-044		

EGF Recombinant Human Epidermal Growth Factor	Reagent	ThermoFisher Scientific	PHG0311		
18-gauge Needle	Dissociation tool	Becton Dickinson	305196		
21-gauge Needle	Dissociation tool	Becton Dickinson	305190		
23-gauge Needle	Dissociation tool	Becton Dickinson	305194		
Syringes	Dissociation tool	Becton Dickinson	309657		
15 ml Centrifuge Tube	Culture ware	Corning	430791		
10 cm Petri Dish	Culture ware	ThermoFisher Scientific	875713		
48 Well Plates	Culture ware	Corning	3548		
DMEM/F12	Media	ThermoFisher Scientific	11320-033		
DMEM/F12 Serum containing media	Solution			DMEM/F12 media with 10% FBS, 1X Penstrep	
Hank's-low	Solution			1x Hank's buffer with 1.2 mM MgSO4, 40 mg/ml DNaseI, 3 mg/ml BSA, and filter sterilized	
Hank's-high	Solution			1x Hank's buffer with 1.2 mM MgSO4, 40 mg/ml DNaseI, 4% BSA, and filter sterilized	
Neurosphere Media	Solution			DMEM/F12 media with 1x B27 and 10ng/ml EGF	
Trypsin Inhibitor solution	Solution			DMEM/F12 with 1 mg/ml soybean inhibitor	

BEFORE STARTING

Follow steps 1.1-1.8. Pre-make all solutions before protocol. A detail list of critical solutions can be found in the materials table.

Before you begin dissections

1 **1. Set-up prior to embryo dissection**

2 1.1) Establish breeding pairs of mice to obtain embryonic day 17 (E17) embryos. Day 0 is defined as the day a vaginal plug is detected.

3 1.2) Prepare sterile surgical tools (scissors for decapitation, #5 forceps, razor blades).

4 1.3) Add 20 mls of Hank's buffer to each of two 10 cm petri plates and place on ice. Add 5 mls Hank's buffer to a 15 ml tube and also place on ice. Reserve another 50 mls of room temperature Hank's buffer.

5 1.4) Prewarm 10 mls of Hank's-low BSA at 37 °C

6 1.5) Prewarm 5 mls of Hank's-high BSA at 37 °C.

7 1.6) Prewarm 10 mls of DMEM/F12 with serum at 37 °C.

8 1.7) Prewarm 5-15 mls of neurosphere media at 37 °C. Amount is based on number of desired wells.

9 1.8) Prewarm 2 mls of 0.25% trypsin/EDTA at 37 °C.

Tissue Dissection

10 **2. Tissue dissection**

11 2.1) Spray the abdomen with 70% ethanol, and make an incision to expose the uterus. Remove the uterus and transfer it to an empty petri plate.

12 2.2) Remove embryos from the uterus, spray desired number with 70% ethanol, and decapitate one or more embryos. Rinse each decapitated head in one petri plate containing Hank's buffer, and then transfer to the second petri plate containing Hank's buffer on ice.

13 2.3) Use forceps to remove the skin and skull. Remove the brain and place in an empty petri dish.

14 2.4) Separate the two hemispheres with a razor blade, and place one half of a brain on its lateral surface.

15 2.5) Using a stereomicroscope, identify the location of the lateral ventricle on the medial surface (the dorsal region of the lateral ventricle contains the dLGE). The ventricle is visible as a T-shaped structure that is slightly darker than the rest of the brain. Using a razor blade or a scalpel, sequentially trim away the brain surrounding the ventricle on all four sides.

16 2.6) Transfer the dissected tissue into the 15 ml tube on ice.

17 2.7) If neurospheres are to be isolated from additional embryos (e.g. because of low yield), keep tube on ice until all dissections are complete.

Primary Neurosphere culture

18 **3. Primary neurosphere culture**

19 3.1) Spin sample at 300 RCF in a clinical centrifuge for 3 min. to pellet tissue.

20 3.2) Aspirate off the supernatant and add 2 mls of pre-warmed trypsin/EDTA. Incubate at 37 °C for 15 min. with intermittent swirling.

21 3.3) Spin tube at 300 RCF for 2 min.

22 3.4) Add 10 mls of room temperature Hank's to trypsin/tissue mixture and incubate at 37 °C for 5 min. with intermittent swirling. Spin culture at 300 RCF for 3 min. and remove the supernatant.

23 3.5) Repeat wash step 3.4 two additional times.

24 3.6) Aspirate the supernatant and add 4 mls of Hank's-low BSA.

25 3.7) Triturate the tissue gently and slowly approximately 10 times with an 18-gauge needle until tissue chunks appear relatively uniform in size. Avoid creating bubbles or foam.

26 3.8) Triturate the crude cell suspension gently and slowly approximately 7-10 times with a 21-gauge needle until tissue chunks appear relatively uniform in size.

27 3.9) Triturate the suspension approximately 4-5 times with a 23-gauge needle until suspension appears uniform.

28 3.10) Add 3 mls of Hank's-high BSA solution to a 15 ml tube. Slowly add the cell suspension to the bottom of the tube underneath the Hank's-high BSA solution using a 23-gauge needle.

29 3.11) Centrifuge at 300 RCF for 5 min.

30 3.12) Aspirate supernatant and resuspend cells with 3 mls of prewarmed Hank's-low BSA.

31 3.13) Centrifuge at 300 RCF for 5 min.

32 3.14) Aspirate supernatant, and resuspend cells in 5 mls of prewarmed DMEM/F12 with serum.

33 3.15) Incubate tubes for 2-4 hours at 37 °C to reduce bacterial contamination.

34 3.16) Centrifuge at 300 RCF for 5 min.

35 3.17) Resuspend cells in 1 ml of prewarmed neurosphere media.

36 3.18) Count cells with a hemocytometer. Plate 10,000 cells in a volume of 250 ul in each well of a 48-well plate. Plate at least 8 wells to ensure adequate numbers of neurospheres.

37 3.19) Incubate at 37 °C in a humidified incubator with 5% CO2.

38 3.20) Neurospheres should form within 3-4 days. At day 3, add an additional 100 ul of neurosphere media to each well.

Secondary Neurosphere culture

39 **4. Secondary neurosphere culture**

40 After 5-7 days, primary neurospheres should be split to maintain the health of the culture.

41 4.1) Prewarm 1 ml of Trypsin/EDTA at 37 °C.

42 4.2) Prewarm 10 mls of DMEM/F12 (without serum or antibiotics) at 37 °C.

43 4.3) Prewarm 2-15 mls of neurosphere media at 37 °C. Amount is based on number of desired wells.

44 4.4) Prewarm 1 ml of trypsin inhibitor at 37 °C.

45 4.5) Collect all neurospheres from at least 8 wells and transfer to a 15 ml tube.

46 4.6) Spin down neurospheres at 300 RCF for 5 min.

47 4.7) Remove supernatant and add prewarmed trypsin. Incubate at 37 °C for approximately 5-10 min., depending on the size of neurospheres.

48 4.8) Add 1 ml of trypsin inhibitor solution and swirl cells for 15 secs to inactivate trypsin.

49 4.9) Spin down culture at 300 RCF for 5 min.

50 4.10) Resuspend cells in 4 mls of DMEM/F12.

51 4.11) Triturate the crude cell suspension gently and slowly approximately 7-10 times with a 21-gauge needle until tissue chunks appear relatively uniform in size.

52 4.12) Triturate the suspension approximately 4-5 times with a 23-gauge needle until suspension appears uniform.

53 4.13) Spin down dissociated cells at 300 RCF for 5 min.

54 4.14) Resuspend cells in 1 ml of DMEM/F12.

55 4.15) Count cells using a hemocytometer, and plate 10,000 cells in a volume of 250 µl neurosphere media in each well of a 48-well plate. Plate at least 8 wells to ensure adequate numbers of neurospheres.

56 4.16) Incubate at 37 °C in a humidified incubator with 5% CO2.