Video Article

Organotypic Slice Cultures for Studies of Postnatal Neurogenesis

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Abstract

Here we describe a technique for studying hippocampal postnatal neurogenesis in the rodent brain using the organotypic slice culture technique. This method maintains the characteristic topographical morphology of the hippocampus while allowing direct application of pharmacological agents to the developing hippocampal dentate gyrus. Additionally, slice cultures can be maintained for up to 4 weeks and thus, allow one to study the maturation process of newborn granule neurons. Slice cultures allow for efficient pharmacological manipulation of hippocampal slices while excluding complex variables such as uncertainties related to the deep anatomic location of the hippocampus as well as the blood brain barrier. For these reasons, we sought to optimize organotypic slice cultures specifically for postnatal neurogenesis research.

Video Link

The video component of this article can be found at https://www.jove.com/video/52353/

Introduction

Adult neurogenesis in the mammalian hippocampus represents a remarkable example of the brain's innate capacity for adaptability and plasticity. Dentate granule cells (DGCs) are generated from a renewable pool of neural progenitor cells in the hippocampal dentate gyrus, which is one of the two presently well-characterized neurogenic regions in the mammalian brain, and is thought to be particularly important for learning and memory. The hippocampus is part of the limbic system and has a deep location within the mammalian brain, which makes it a difficult target for precise pharmacological manipulation. Additionally, aberrant neurogenesis has been implicated in conditions, such as epilepsy, schizophrenia, and Alzheimer's disease, which has prompted interest in understanding the influence of various pharmacological agents during the maturation and survival of newborn neurons. The distinction between postnatal and adult neurogenesis is blurred and previous studies have shown that many features of *in vivo* neuronal development in the early postnatal period and adulthood are similar[25]. Here we emphasize postnatal neurogenesis and suggest possible applications to adult neurogenesis.

Organotypic slice cultures provide an efficient *in vitro* method for studying various physiological properties of the mammalian hippocampus. The value of slice cultures prepared from rodent brains can be summarized in three main qualities: 1) the protocol is straightforward and requires readily available materials; 2) slice cultures allow for pharmacological studies that eliminate complex variables such as the deep anatomic location of the hippocampus and circumvents the blood brain barrier[1]; and 3) the well characterized structure of the hippocampus and tri-synaptic circuit is preserved[2]. Previous investigators have used the organotypic hippocampal culture to study synaptic development and physiology[3,4], gliogenesis[5-7], ischemic brain damage[8,9], neuroprotection and neurorepair[10-12] as well as epilepsy[13-15].The slice cultures could also provide a useful model system allowing for the monitoring of cell development in conjunction with labeling of cells with green fluorescent protein (GFP) or other vital markers.

Slice cultures have also been previously employed to study postnatal hippocampal neurogenesis[16-19], but one important factor in the majority of these studies is the well-characterized degeneration that results from explanting tissue from adult animals after approximately 2 weeks *in vitro*[20,21]. For this reason, slice cultures are typically prepared from early post-natal (P5-P10) mice or rat pups, which utilizes the improved viability of early postnatal brain tissue for culturing[22]. While previous studies have shown that the early postnatal and adult hippocampus differ with regards to synaptic physiology and the expression of specific neuronal subtypes[23,24], there is substantial conservation of the choreographed developmental program that newborn dentate granule cells proceed through during maturation[25]. Additionally, recent studies have suggested that the physiological characteristics of newborn DGCs in culture are very similar to immature neurons in the acute hippocampal slice preparation[16].

Protocol

NOTE: All animal procedures conformed to the animal health and welfare guidelines of the Department of Comparative Medicine at the University of Toronto.

1. Preparation of Hippocampal Slices

1. Sterilize the following instruments using the dry autoclave at 125 °C: Scalpel handle (#3) (2), Standard pattern forceps, large (1), Small dissector scissor (angled to side) (1), Micro spoon (spoon and flat spatula ends) (1), Micro-spatulas (rounded and rounded tapered ends) (2), Fine paintbrush (1), Fire polished Pasteur pipette (2), Gauze squares, 2 x 2 inches (5).
2. When sterile, put the instruments into a sterile container and keep covered until use. Immediately before dissection, immerse instruments in a 70% ethanol solution.
3. Prepare a 6-well culture plate with culture insert before beginning the dissection procedure by adding 1 ml of culture medium/well and storing the plate in the incubator at 35 °C and 5% CO_2.

2. Arrange Dissection Tools in Sterile Laminar Flow hood

1. Spray the laminar flow hood with 70% ethanol and remove sterilized dissection instruments from alcohol. Allow the instruments to dry while resting on a sterile Petri dish to avoid contact between the alcohol and the dissected brain tissue.
2. Deposit 5-7 ml of sterilized ice-cold dissecting solution in 2 large sterile Petri dishes. One dish will chill and clean the head (dirty), the other one for cooling and rinsing the scooped out brain (clean). Place a sterilized filter paper in one of the Petri dish lids for dissecting out the brain.
3. Place a small, sterilized filter paper in one of the small Petri dish lid for dissecting out the hippocampus. Deposit 3-5 ml of sterilized ice-cold dissecting solution in 2 small, sterile Petri dishes. One dish will hold the scooped out hippocampus, one will hold sections during separation of hippocampal sections under dissection microscope.
4. Prepare the tissue chopper by taping a piece of sterilized filter paper to the cutting stage and mounting a sterile razor blade. Wet the filter paper with sterilized dissecting solution.
5. Spray a clean bio-bag with alcohol and place it in laminar flow clean bench.

3. Hippocampal Dissection

1. Spray the P7 Sprague Dawley rat pup with 70% ethanol outside of the laminar flow clean bench and quickly decapitate the animal using large sterile surgical scissors inside the laminar flow bench. Let the head drop into ice-cold dissecting solution in one of the Petri dishes.
2. In the Petri dish, rinse off the blood and quickly transfer the head to sterilized filter paper, ventral side down.
3. Using the scalpel, cut along the dorsal surface in the sagittal plane to expose the underlying skull. Cut through the skin, but not the underlying bone, which is soft and easily penetrable in rats of this age. Set aside this “dirty” scalpel and do not use on brain tissue.
4. Using the small dissector scissors (angled to side) and forceps, cut open the skull along sagittal suture of the skull to bregma, the anatomical point on the skull where the coronal suture is intersected perpendicularly by the sagittal suture. Use forceps to pull skull flaps up and away from the midline of the skull.
5. Place the micro spoon on the underside of the brain, beneath the brain stem, to gently lift the brain out of the skull. Lift the brain to expose the optic nerves and olfactory bulb on basal surface of brain. Cut these structures with small scissors to fully detach the brain from skull. Remove and transfer the intact brain to the other large Petri dish containing ice-cold dissecting solution.
6. Using the micro spoon, transfer the brain to a small Petri-dish lid containing sterile filter paper. With a sterile Pasteur pipette, rinse the brain with a few drops of dissecting solution to keep tissue moist.
7. Using a “clean” scalpel blade cut the two hemispheres apart. Transfer the left hemisphere back to large Petri dish with micro spoon and place hemisphere pia side down in ice-cold dissecting solution for subsequent use.
8. View the medial face of the right hemisphere and identify the edge fornix, a prominent band of white matter along the medial edge of the hippocampus. Using a sterile scalpel, make a sagittal cut through the fornix, but take care because only 0.5 cm of the scalpel tip will be sufficient to cut the fornix.
9. Using 2 micro-spatulas, remove the first hippocampus from right hemisphere by placing the right-hand-spatula on the brain stem and lifting the overlying cortex with the left-hand spatula. Gently lift the cortex to reveal the lateral ventricle and medial surface of the hippocampus. A white curved line, the fimbria, should now be visible.
10. Align the curvature of spatula with the curvature of the fimbria and gently press the spatula under the fimbria. Slide the spatula left and ride along rostral-caudal axis and then lift spatula in dorsal direction to remove hippocampus.
11. Transfer the hippocampus to a 2nd small Petri dish with ice-cold dissecting solution. Repeat the same procedure on the left hemisphere to remove the left hippocampus.
12. Using a micro spatula, carefully transfer the hippocampi to the tissue chopper stage. Arrange them adjacent and parallel to each other and perpendicular to the axis of the chopper blade. Use a paintbrush to position the tissue and add a few drops of the dissecting solution on top of the hippocampi.
13. Cut the tissue in 400 µm slices without pausing to remove individual slices (usually they will not adhere to the blade). After the whole hippocampus has been cut, use the paintbrush to gently transfer the sections to a 2nd small Petri dish with dissection solution.
14. Under a dissecting microscope, carefully separate the floating slices using amicro-spatula and paintbrush.
15. Remove the pre-prepared culture plate with culture insert from the incubator and place in a laminar flow hood.
16. Using a fire-polished Pasteur pipette, draw 4-5 slices into the pipette and transfer slices to the apical surface of culture insert membrane. Next, adjust the positioning with a paintbrush and leave space between individual sections and the border of the culture insert.
17. Using a sterile Pasteur pipette, remove the excess dissecting solution from apical surface of membrane.

NOTE: While removing solution avoid drawing tissue sections into the pipette. Alternatively, use a regular pipette (P200 or P1000) with sterilized pipette tips to slowly remove solution.

18. Place the culture plate with serum-containing culture medium and the hippocampal slices back into the incubator at 35 °C and 5% CO_2.
NOTE: If experiment calls for generating cultures from multiple animals, thoroughly clean the laminar flow clean bench between dissections. Return instruments to alcohol solution and replace all Petri-dishes, filter papers and sterile razor blades prior to second dissection.

4. Feeding and Maintaining Organotypic Slices

1. Feed cultures in a sterile laminar flow clean bench.
2. Perform the first feeding of the cultured sections 2 days post-dissection. Aspirate old culture medium using sterilized glass pipette.
3. Use sterile 5 ml serological pipette to add 1 ml of fresh, sterile, serum-containing medium to the wells.
4. Gently replace the culture insert and take care to remove any air bubbles that may have formed underneath membrane surface.
5. After the first feeding, change medium every other day.

5. Incubating Tissue Slices with Thymidine Analogues to Label Newborn Neurons

1. In order to study the maturation and integration of dentate granule cells in the hippocampus, incubate the organotypic slices with a thymidine analogue, such as Bromodeoxyuridine (BrdU) or Chlorodeoxyuridine (CldU). Here, we use CldU because of its higher solubility in saline.
2. After 3DIV, add 1 µl of 10 mg/ml CldU stock solution in saline to 1 ml of culture medium for a final concentration of 10 µg/ml. If BrdU is used, correct the concentration for differences in molecular weight. Add medium with CldU to culture wells and incubate tissue with CldU-containing medium for 2 hr at 35 °C.
3. Following 2 hr of incubation at 35 °C, remove the CldU-containing medium and replace with regular feeding medium to resume normal feeding schedule (outlined above).

6. Tissue Fixation and Storage

1. Establish a timeline for applying treatments and fixing tissue samples before starting culture experiments **(Figure 2B)**.
2. At a predetermined day post-dissection, prepare the following in a laboratory fume hood: a 10-50 ml beaker containing 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS); an empty beaker for discarded culture medium; small forceps; and a 1,000 ml pipette with disposable tips.
3. Remove the culture plate(s) from incubation chamber and transfer to a fume hood. Tilt the individual well plate inserts with forceps. Next, use a pipette to remove culture medium and transfer to a disposal beaker. Tilt the culture plate at an angle to help ensure that all culture medium is removed.
4. Finish removing medium for one culture plate at a time. When medium has been removed, add 1 ml of 4% PFA to each culture well and seal well plate with parafilm. Repeat for as many culture plates as necessary and transfer plates to refrigerator at 4 °C for 24 hr.
5. After 24 hr, prepare the following in a fume hood: 10-50 ml beaker containing 0.1% Sodium Azide in PBS; an empty beaker for discarded PFA (follow safety precautions when discarding toxic substances); small forceps; and 1,000 ml pipette with disposable tips.
6. Follow the procedure outlined in 6.4 for adding PFA, but add 1 ml of PBS with sodium azide instead. Once completely transferred, seal well plates for future use by wrapping edges in parafilm and storing in refrigerator at 4 °C.

7. Sectioning Tissue for Immunohistochemistry

1. Perform tissue sectioning using a vibratome. The following series of steps help maximize the yield of usable tissue sections from organotypic cultures.
NOTE: Immediately following hippocampal dissection and plating of slices, the tissue has a thickness of approximately 400 µm. However, after 2-3 weeks in the incubation chamber, tissue slices will begin to flatten, resulting in a final section thickness of 150-300 µm.
2. Prepare the following items to section cultured tissue: a #11 scalpel blade and handle, a glass Petri dish containing ice cold PBS, a micro-dissection forceps, and a clean vibratome cutting stage to mount tissue.
3. Use a scalpel to carefully cut along the perimeter of the circular insert membrane so that it can be detached from the plastic insert housing. Leave ample space between the cultured slice and the scalpel.
4. Transfer the detached insert membrane to a Petri dish containing PBS. After rinsing in PBS, use forceps to transfer the membrane to the vibratome mounting stage.
5. Next, use the scalpel to eliminate excess membrane surrounding cultured slices and cut away excess material to create clean edges. This step will ensure the membrane is flat and can easily adhere to the cutting surface.
6. Place 1-2 drops of adhesive on the vibratome cutting stage and spread in even layer using a 22 G needle. Spread adhesive into a rectangular shape with the long edge parallel to the cutting blade of the vibratome. Perform this step quickly, to prevent the adhesive from drying.
7. Use forceps to transfer the trimmed membrane containing hippocampal slices to the cutting stage and gently position the membrane on glue and ensure that there are no air bubbles.
8. As the superglue dries, transfer the vibratome stage with the glued membrane back to the PBS containing Petri dish. Prepare the vibratome blade and a 48 well plate containing sodium azide to store tissue sections.
9. Use the vibratome to generate 30 µm sections of the organotypic slice tissue and transfer to a 48 well plate containing sodium azide for storage and subsequent immunohistochemical staining.
10. Refer to pre-existing protocols for immunohistochemical staining[26,29].

Representative Results

Determining if organotypic cultures would be suitable for adult neurogenesis research required that they satisfy two main criteria: 1) that slices maintain characteristic morphological features of hippocampal slices after 10-21 days *in vitro* (DIV), and 2) that newborn DGCs can be quantified using standard immunohistochemical techniques commonly employed in adult neurogenesis research. Regarding the first criterion, **Figure 1A** and **1B** highlight the preserved hippocampal morphology. Characteristic features such as the dentate gyrus (DG), CA1, and CA3 regions are easily identifiable.

Regarding the second criterion, **Figure 1C** (upper panel) provides a representative sample of newborn DGCs co-expressing the endogenous neuronal marker, Doublecortin (DCX) in green and the exogenous thymidine analogue, 5-Chloro-2′-deoxyuridine (CldU) in red. These neurons are located in the sub-granular zone of the hippocampal DG. In order to ensure correct birth dating of neurons, we identify CldU+ nuclei that co-express DCX. Confocal microscopy is needed at this stage to successfully identify double-labeled neurons because candidate cells must co-express the marker of interest throughout the Z-axis of the cell. Sample data obtained with such double-labeling yield approximately 17% of CldU+ cells that expressed DCX and 35% of CldU+ cells that expressed CaBP at 12 days after CldU application. The DCX value is very similar whereas the CaBP value is considerably lower than comparable figure obtained *in vivo*[26]. Standard tissue culture conditions may be responsible for a relatively low percentage of the CaBP+ cells.

Figure 2A presents the dissection steps that proceed from left to right (1-8): starting with decapitation of animal (1), removal of brain (2), transfer of brain to ice-cold dissection solution (3), dissection of hippocampus from left and right hemispheres (4), storage of dissected hippocampi in ice-cold dissection solution (5), transfer of both hippocampi to Stoelting tissue chopper and sectioning at 400 µm (6), separation of individual slices under dissecting microscope (7), and plating tissue on cell culture inserts (8). Proceeding in this manner helps maintain a sterile environment throughout the culture process.

Lastly, since the time-course of development is an important feature of hippocampal neurogenesis, we chose to incubate the cultured slices with CldU for exactly 2 hr after 3 DIV to label dividing neural stem cells. The narrow time window for CldU administration was chosen to improve the likelihood that labeled neurons constituted a homogeneous population of cells at approximately the same maturational stage (**Figure 2**). With regards to CldU labeling, one critical feature of neurogenesis for hippocampal function is that at a given time there is a heterogeneous population of dentate granule cells at various maturational stages[27,28].

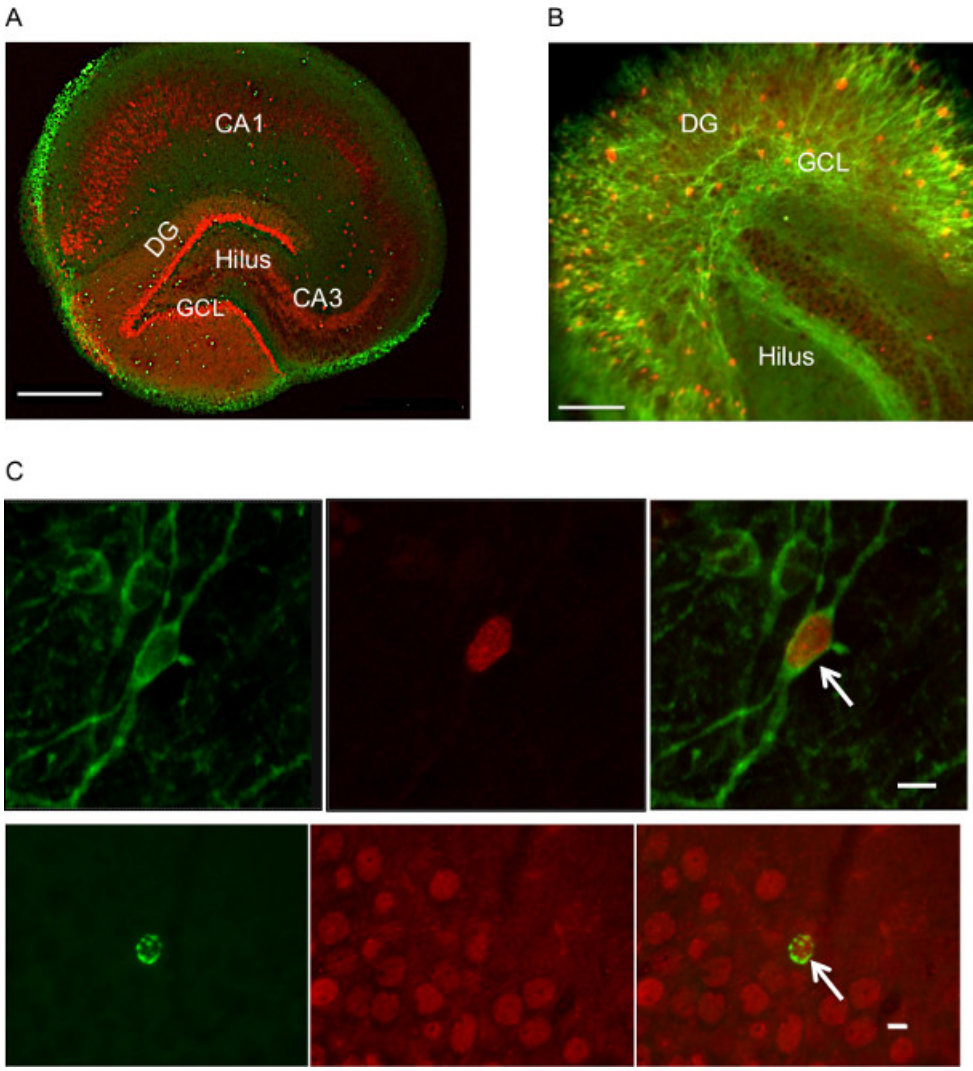

Figure 1. Sample fluorescence microscope photographs highlighting preserved hippocampal morphology. (A) Organotypic slice from 12 days post dissection immunolabeled for CldU (green) and CaBP (red), 20x air composite image (Scale bar= 500 µm). **(B)** Sample micrograph of slice from 21 days post dissection immunolabeled for CldU (red) and DCX (green) (opposite color-scheme of **Figure 1A**), 20x air (Scale bar= 100 µm). **(C)** Upper panel. Representative confocal microscope photograph of cells co-expressing ClXdU and endogenous immature neuronal marker, DCX. DGCs co-expressing DCX (green) and CldU (red) are counted as newborn neurons. Arrow indicates a double-labeled cell at early stage of development, 40X oil-immersion (Scale bar = 10 µm). Comparable cells have been observed 10 days post-labeling *in vivo*[26]. Lower panel. Representative fluorescent images of cells co-expressing CldU (green) and CaBP (red). Arrow indicates a double-labeled cell, 40X fluorescent micrograph (Scale bar = 10 µm). DG-dentate gyrus. GCL-granule cell layer. Please click here to view a larger version of this figure.

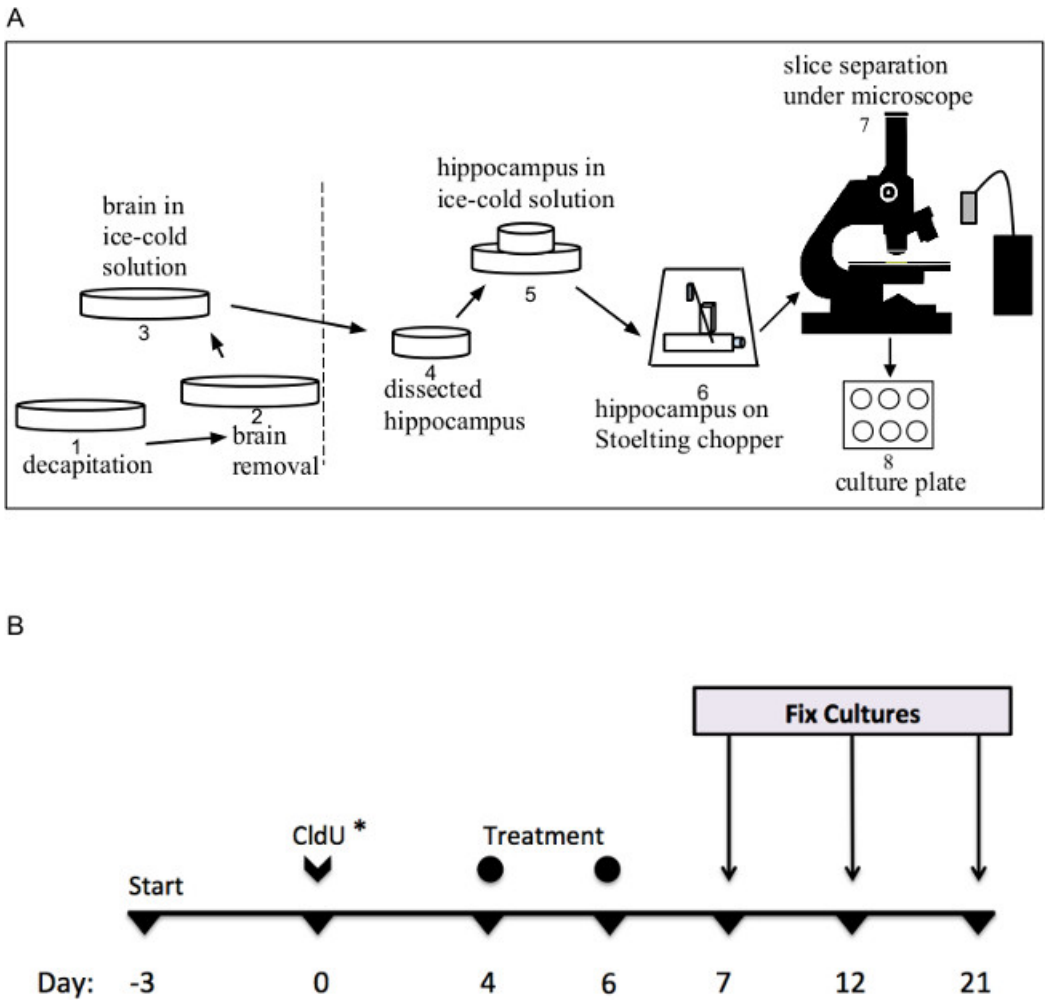

Figure 2. (A) Illustration of the sequential steps for hippocampal dissection in laminar flow clean bench. Dotted line indicates "unsterile" (left) and "sterile" (right) zones of the dissection area. **(B)** Timeline for organotypic slice cultures prepared from P7 rat pups (start). Notations indicate application of thymidine analogue, CldU*, and "treatment," which can include various pharmacological agents suited to the experimental question. Cultures are fixed with paraformaldehyde at desired dwell times from CldU application (Fix Cultures). Please click here to view a larger version of this figure.

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
5-chloro-2'-deoxyuridine (CldU)	MP Biomedicals	105478	Hazardous, Carcinogenic
Cell culture inserts, 30 mm diameter, 0.4 µm pore size	Thermo scientific	140660	Nuclon delta coating on these inserts provides better tissue adhesion and improves slice quality.
Conical Centrifuge tubes (sterile)	Fisher Scientific	14-432-22	
Dissector scissors (angled to side)	Fine Science Tools	14082-09	
Minimum essential medium (MEM)	Gibco	11095; liquid	Store at 4 °C
Eclipse Ni-U fluorescent microscope	Nikon		
Glue for tissue	Krazy Glue	KG585	Use minimum amount of glue to achieve adhesion as any tissue exposed to glue will be unusable for IHC.
Hank's Balanced Salt Solution (HBSS) (500 ml)	Gibco	14025-092	Store at 4 °C
Horse Serum Heat Inactivated (500 ml)	Gibco	16050-122	Make 50 ml aliquots and store at -20 °C
Kimwipes	Kimberly-Clarke	TW 31KYPBX	
Modified glass pipettes (bottom of Pasteur pipette removed and edge smoothed with Bunsen flame)			
Petri Dish (100 mm x 15 mm) and (60 mm x 15 mm)	Fisher Brand	FB0875712 and FB0875713A	
Scalpel blades #11	Fine Science Tools	10011-00	
Scalpel handle #3	Fine Science Tools	10003-12	
Serological Pipettes	Sorfa Medical Plastic Co.	P8050	
Standard Pattern forceps	Fine Science Tools	11000-12	
Sterile vacuum filter	Thermo-Scientific	565-0020	
Surgical Scissors	Fine Science Tools	14054-13	
Syringe driven filter unit	Millipore-Millex	SLGP033RS	
Tissue chopper with moveable stage	Stoelting	51425	
Fine tip paintbrush			

Table 1. Supplies and Reagents

Solution	Ingredients and Instructions
Dissection solution	a) 500 ml of Hank's Balanced Salt Solution (HBSS) (Gibco-14025-092).
	b) Add 2.2 g D-glucose.
	c) Add 0.5 g Sucrose.
	d) Add 1.787 g HEPES.
	e) Mix for 30 min with magnetic stir plate.
	f) Use pH meter to ensure solution has a final pH= 7.4.
	g) Use osmometer to ensure final osmolality= 320-330 mOsm.
	h) Sterilize solution in sterile laminar flow hood using vacuum filtration through 0.2 µm filter.
Serum-containing culture medium: 100 ml Minimum Essential Medium (MEM) (Gibco 11095), 50 ml Horse serum (Gibco 16050-122), 50 ml HBSS.	a) Add the following to 50 ml HBSS in beaker and dissolve in 37°C water bath. Mix with magnetic stirrer.
	b) 1.3 g D-glucose.
	c) 36 mg $MgSO_4$.
	d) 17.6 mg Ascorbic acid.
	e) 5 µl of 2M $CaCl_2$ stock solution.
	f) Add 50 µl Antibiotic-Antimycotic (100x stock, sterile; Gibco 15140-062).
	g) 1 µg/ml Insulin.
	h) Sterilize above solution by filtration through a 0.2 µm filter.
	i) Mix filtered solution with 100 ml MEM and 50 ml Horse serum in laminar flow hood.
	j) Make 50 ml aliquots in sterile conical centrifuge tubes (Fisher Scientific-14-432-22) and store at 4°C.
4% Paraformaldehyde fixative solution.	a) Prepare phosphate buffered saline (PBS) by adding the following to 300 ml of distilled H_2O and mixing on magnetic stir plate.
	b) Add 2.7 g sodium phosphate monobasic (NaH_2PO_4).
	c) Add 11.5 g sodium phosphate dibasic ($NaHPO_4$).
	d) Add 9.0 g sodium chloride (NaCl).
	e) Heat approx. 700 ml of distilled H_2O to 55 °C and turn off heat.
	f) Add 40 g paraformaldehyde (PFA) and stir into 700 ml of water using magnetic stir plate.
	g) Combine the PBS (a,b,c,d) and PFA (e,f) solutions, adjust the pH to 7.4 and top up to final volume of 1,000 ml.
0.1% Sodium Azide Solution	a) Add 1g of powdered sodium azide (NaN_3) to 1 L of PBS solution.
	b) Mix using magnetic stir plate and store at 4°C.

Table 2. Solutions and Recipes

Discussion

Following CldU (or BrdU) administration, the timeline of application of pharmacological agents can be chosen to target newborn DGCs during particular developmental windows. For example, a hypothetical agent can be applied during the second week post-CldU injection, which is proposed to coincide with the age of immature neurons that are at a developmental stage where GABA is depolarizing. Future studies using this protocol could adapt the pharmacological agent and the window of exposure to “tailor” the approach to the specific experimental question of interest.

An important criterion for determining that slice cultures are a valid model for postnatal neurogenesis research is the ability to stain and quantify newborn neurons in the hippocampus. The two main findings in support of this hypothesis were that microscopic analysis revealed immunohistochemical reactivity for CldU and endogenous protein markers in the same neurons. When used in combination with endogenous neuronal markers, thymidine analogues such as BrdU and CldU are powerful tools for neurogenesis research.

The application of thymidine analogues, such as BrdU, via intraperitoneal injections is commonly utilized in neurogenesis research to label neurons undergoing S-phase of mitosis[29]. Similar approaches can be employed in organotypic cultures with certain modifications. For example,

previous studies administered BrdU (0.5 µM for 3 days) to slice cultures after ~14 DIV[18]. Reviewing the data presented in that paper reveals that some of the metrics used for quantifying neurogenesis do not employ the standard techniques used in the neurogenesis field, *i.e.*, stereological quantification[30]. For example, when reporting the co-expression of BrdU and Neuronal-nuclei (NeuN) positive cells, they indicate a total number of cells "per culture" instead of providing information regarding tissue area or volume.

Subsequent studies improved on this method by sectioning the cultured slices to individual 10 µm sections, which improved visualization and immunohistochemistry protocols by allowing antibodies to more readily permeate the tissue samples[31]. Bunk *et al.*[28] reported double labeling with BrdU (10 µM for 3 days) as the number of co-labeled cells per 10 µm section, but did not provide information about the comparative area or specific hippocampal region studied *i.e.,* CA1, CA3 or DG. Additionally, analysis of the confocal and fluorescence microscopy images does not convincingly show that hippocampal morphology was successfully maintained.

Importantly, both studies used a BrdU exposure period of 3 days, which has associated drawbacks. BrdU labeling has greatly aided neurogenesis studies by allowing investigators to track newly divided cells in various brain regions. However, BrdU toxicity has also been well characterized. Its use has been shown to cause morphological and behavioral abnormalities[32,33] and negative effects on cell cycle, differentiation, migration and survival of neural stem cells[34-36]. The prolonged administration of BrdU in the previously mentioned studies may have introduced confounding variables that altered hippocampal physiology and while some side effects from BrdU administration may be unavoidable, our experimental protocol was designed to limit some of these complications by incubating the tissue with thymidine analogues for 2 hr. Additionally, we chose to use CldU instead of BrdU because it showed better solubility than BrdU when preparing the incubating solution. Although the 3 day protocol may be useful for certain experimental designs *e.g.,* maximizing the labeling of proliferating cells, this 2 hr protocol has an advantage of pulse-labeling of a relatively small population of cells which can be studied at desired survival times (see **Figure 2B**).

By comparing the level of neuronal production following two different methods of BrdU application, Namba *et al.* made an important contribution to labeling techniques in organotypic slice cultures[37]. The authors compared intraperitoneal (I.P.) injection of BrdU (50 mg/kg) in postnatal day 5 (P5) rats with *in vitro* cultures that received culture medium containing 1 µM BrdU for 30 min immediately following explantation of tissue. They report no statistically significant difference between in-vivo and early *in vitro* BrdU injection in cultured tissue. The authors did not present clear images outlining the hippocampal structure but they report BrdU immunoreactivity as percentages of total cells in the granule cell layer. While they employ stereological counting, providing a measure by area or volume would be valuable. In general, the cited studies present organotypic cultures as a thorough detailing of postnatal hippocampal slice cultures, with applications for the study of neurogenesis and pharmacological perturbations. Using this technique, hippocampal slices can be maintained for up to 21 days *in vitro* (DIV) and drugs can be added to the medium at any point in the culture period to study the effect on neurogenesis.

Our aim was to label a discrete, relatively homogenous population of DGCs by providing a brief 'pulse' application of CldU for 2 hr. One commonly employed strategy for studying neurogenesis involves identification of a neuron's maturational stage via immunohistochemical staining for various endogenous markers with a thymidine analogue. Confocal and fluorescence microscopy confirmed the presence of nuclei that incorporated the thymidine analogue CldU and were therefore actively undergoing mitosis during the culture period. **Figure 2** provides evidence that immunohistochemical protocols commonly used for *in-vivo* tissue analysis can be adapted for slice cultures.

Specifically, a commonly used method in neurogenesis research is to perform immunohistochemical staining for the microtubule associated protein, DCX, which is predominantly expressed in immature neurons from day 3-21 and the mature neuronal marker, Calbindin (CaBP), which is fully expressed following 28 days post-mitosis. The phenotype of CldU+ cells was determined using these endogenous markers[26].

Improved methods for maintaining slice cultures for longer periods may have the additional benefit of allowing more CldU+ neurons to reach the mature, CaBP+ stage. At present, one limitation of the organotypic culture approach is that the tissue is continuously changing during the culture period. For example, immediately following hippocampal dissection and plating of slices, the tissue has a width of approximately 400 µm. However, after 2-3 weeks in the incubation chamber, tissue slices will begin to thin, which results in a final width between 250-350 µm. This limits the amount of tissue that can be used for immunohistochemistry and should be considered when planning how many animals to use for a project. Additional experiments will help characterize the functional changes in hippocampal physiology that occur *in vitro*.

The protocol for sectioning and staining hippocampal slices was developed to analyze cellular and morphological changes taking place during the culturing period. Slice cultures provide an opportunity to test the effect of various pharmacological agents as hippocampal DGCs pass through distinct developmental stages during maturation and represent a valuable tool for future adult neurogenesis studies.

Disclosures

The authors declare that they have no competing financial interests.

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