METHOD ARTICLE

REVISED Quantitative approach to numbers and sizes: Generation of primary neurospheres from the dorsal lateral ganglionic eminence of late embryonic mice [version 2; peer review: 2 approved]

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Abstract

Background: The neurosphere assay is a powerful *in vitro* tool to investigate neural stem cells in the dorsal lateral ventricle (dLGE). In the dLGE, metrics of sizes and numbers of neurospheres generated using this assay has not been completely characterized. The objective of this protocol is to provide a stepwise method from a single isolation that predicts the average number of neurospheres generated and to estimate an approximation of its sizes after several days *in vitro*. The advantage of this protocol is that no expensive and specialized equipment is needed for tissue isolation. Estimates about the numbers and sizes of neurospheres will provide investigators with quantitative data to advise on how much starting dLGE tissue is required to generate the appropriate number of spheres for the implementation of downstream applications, including immunocytochemistry, self-renewal and differentiation assays.

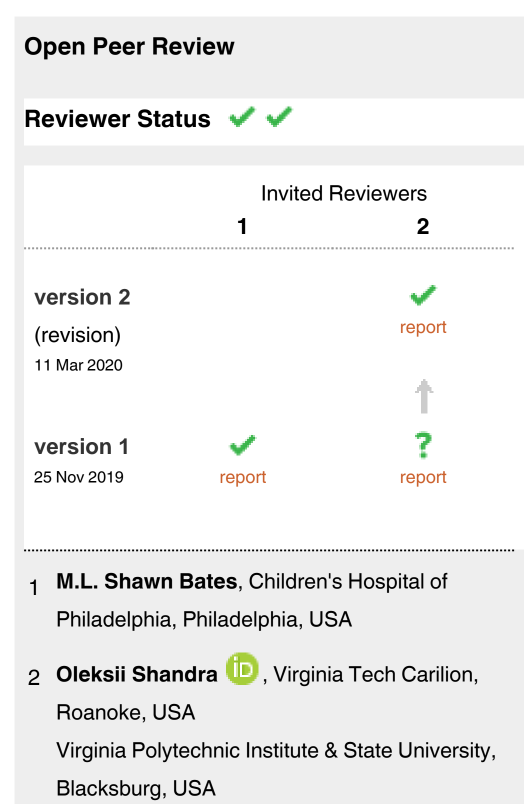
Methods: Our method is based on a simple dissection technique, where tissue surrounding the dorsal lateral ventricle from a single mouse embryo is trimmed away to enrich for neural stem cell and progenitor populations. Following this dissection, tissue is mechanically dissociated by trituration. Cells are then cultured in media containing epidermal growth factor and other supplements to generate healthy primary neurospheres.

Results: Using this approach, we found reproducible number of primary neurospheres after 7 days *in vitro* (DIV). Furthermore, we observed that this method yields an average range of neurospheres sizes greater than 50 µm, but less than 100 µm after 7 DIV. Lastly, using an anti-GFAP antibody, we show that these neurospheres can be stained, confirming their use in future immunocytochemistry studies.

Conclusions: Future use of this protocol provides metrics on the generation of primary neurospheres that will be useful for further advances in the area of stem cell biology.

Keywords

Embryonic, mechanical dissociation, neurosphere, neural stem cell, progenitor cell



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Author roles: Blackwood C: Conceptualization, Investigation, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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REVISED **Amendments from Version 1**

We would like to thank the peer reviewers for their comments. The comments improved the manuscript. The revised version of this manuscript contains changes to the abstract, data & acquisition, figures, and discussion sections. The abstract was modified to clearly state the goals of the protocol. The data and acquisition section was changed to add additional details about the camera and working distance used to generate data. An additional picture was added to the Figure 4 to show that immunocytochemistry can be performed on both small and large neurospheres. The discussion was updated to include alternative approaches such as a cell strainer to achieve single cell suspension.

Any further responses from the reviewers can be found at the end of the article

Introduction

Neural stem cells are a tissue-specific subtype of self-renewing and multipotent cells that will produce several mature cell types. The neurosphere assay is an important tool that has been extensively employed to study neural stem cell biology[1]. Since its introduction some 25 years ago[2], neurospheres have been used to study neurogenesis[3], genes that regulate self-renewal[4,5], and molecular mechanisms that control neuronal and glial differentiation[3,6–9]. Although there are many neurosphere protocols, the expected number and size of neurospheres generated after a week *in vitro* is not entirely characterized.

We developed a simple dissection technique that helps to maximize the number of neurospheres that can be produced in culture. Furthermore, we characterize the expected sizes of neurospheres after a week *in vitro*. With some other techniques, a brain slicer or other means are used to obtain thick slices of brain tissue from late embryonic stages[10,11]. The area surrounding the ventricle is then microdissected from a given slice of tissue to enrich for neural stem/progenitor cells. This approach, while effective, can be painstaking and may require expensive specialized equipment. Additionally, many protocols do not provide metrics on expected numbers and sizes of neurospheres generated. Thus, it is unclear whether researchers can generate sufficient numbers of neurospheres in a particular range of sizes. In contrast, our approach requires no specialized equipment. The lateral ventricle is visualized with a stereomicroscope, and the surrounding tissue is simply trimmed away using a razor blade or scalpel. This method requires only half of a single brain, and generates reproducible numbers of neurospheres in a few days. Furthermore, using our method neurospheres small appears as early as 3 days. Another advantage of this protocol is that it can generate neurospheres with average sizes of 50 μm - 100 μm after 5–7 days *in vitro*.

Methods

Mice

The animals were housed in the AAALAC-accredited East Campus Research Facility and Transgenic Mouse Core Facility in the Veterinary College of Cornell University. All animal procedures were performed in accordance with the guidelines outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, eighth Edition. Animals were approved by Cornell University's Animal Care and Use Committee (IACUC; #01-75). Mice were maintained on a mixed 129Sv/C57BL/6 background and housed on a reverse light-dark cycle. Food and water were continuously available. Male and female mice mated overnight. The following morning females were separated and checked for vaginal plug. Pregnant mice were euthanized using CO_2 asphyxiation followed by cervical dislocation consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and the Cornell IACUC. Embryonic day 17.5 (E17.5; E0.5 was defined as the first detection of the vaginal plug) male and female embryos were dissected. A total number of n=5 mice were used for primary neurospheres experiments.

Collection of tissue

The mouse brain was sliced down the center into two hemispheres. Using one hemisphere, placed sagittal orientation, dissect out the dorsal lateral ventricle (see Figure 1). The dissections were guided using stereotaxic coordinates (A/P 1 mm, M/L 1 mm, D/V 2.3 mm) from Paxinos and Franklin (2007) atlas source.

Immunofluorescence

Neurospheres were fixed with 4% paraformaldehyde, and blocked in goat serum containing 0.5% Triton. Immunofluorescence analysis of protein expression was performed using rabbit anti-Glial Fibrillary Acidic Protein (GFAP) Antibody (Millipore; RRID:AB_2109645; ab5804; 1:100). Secondary antibodies used were biotinylated goat anti-rabbit (Abcam; RRID:AB_2661852; ab64256; 1:1000) and streptavidin alexa fluor 488 conjugate (ThermoFisher Scientific; RRID:AB_2315383; S11223; 1:500).

Data acquisition and statistics

Images were taken with a Canon EOS Rebel XS camera. (Canon USA; Melville, NY). The optimum magnification is approximately 5x with 3888 x 2592 dimensions. Camera was connected to the trinocular port of the stereomicroscope (Carl Zeiss Stemi 305; White Plains, NY) using Mount Adaptor EF-EOS (6098B007AA; Canon; Melville, NY). The working distance was defined as the amount of room required between the top of the neurosphere and the bottom of the objective lens in order

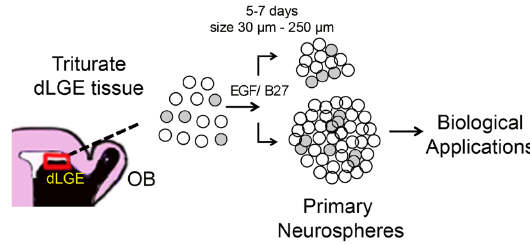

Figure 1. Schemetic illustration. Tissue from the dLGE is dissected and dissociated. Single cells are grown in media containing EGF and B27. After 7 days *in vitro* neurospheres are on average between 50 μm to 100 μm in size. Neurospheres generated can be used for various downstream applications.

for the image to be in focus. The steromicroscope was used at a working distance of ~110 mm. Due to the variation in neurosphere size, 110 mm should be adjusted to focus on the desire region of the neurosphere to provide optimal focus. The field of view represents a length of 783 μm and a width of 522 μm. Data is derived from single random pictures of each well. Per animal, 3–4 wells were analyzed. A total of 5 individual animals were analyzed. Size measurements and neurosphere counts were analyzed in Adobe Photoshop (version 10.0.1) CS3 Extended (Adobe; San Jose, CA) using the measurement function (ImageJ is an open-access alternative that can be used to perform this function). Statistical analyses were carried out as previously described[12,13]. Briefly, data were analyzed using PRISM 8 (version 8.3.0) (GraphPad Software; San Diego, CA) by performing one-way ANOVA. If the main effect was significant ($p < 0.05$), Bonferroni's multiple comparison post hoc test were used to compare the different replicates. The null hypothesis was rejected at $p < 0.05$. Data is made available on figshare open access platform (Metrics of Primary Neurospheres). Error bars represent standard error of the mean (±SEM).

Neurosphere assay protocol

This protocol is designed to generate neurospheres from a single embryo. Multiply all values as needed to generate neurospheres from additional embryos. See Table 1 and Table 2 for premade solutions and materials needed.

1. Set-up prior to tissue dissection

NOTE: *Breeding and euthanasia of all animals should be performed in accordance with an institutionally approved animal care and use protocol. Sterilize all surgical instruments packed in aluminum foil in an autoclave at 121°C (15 psi) for 30 mins. This includes a scissors, forceps, and razor blades. Before starting all premade solutions should be warmed to 37°C.*

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.

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

Table 1. Equipment, reagents, and catalog information.

Name of material/equipment	Type	Company	Catalog Number
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 (Adjust to pH 7.1 after dilution to 1X)	Reagent	ThermoFisher Scientific	14185-052
0.25% Trypsin/EDTA (1x)	Reagent	ThermoFisher Scientific	25200-056
$MgSO_4$	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
100 mm Petri Dish	Culture ware	ThermoFisher Scientific	150466
35 mm Petri Dish	Culture ware	ThermoFisher Scientific	150460
48 Well Plate	Culture ware	Corning	3548
EOS Revel Camera model # 1894C002	Imaging	Canon	3548
Digital Incubator, model #311D	Incubator	The lab Depot	15311-D

Table 2. Premade solutions for neurosphere assay.

Solutions	Ingredients
DMEM/F12 Serum containing media	DMEM/F12 media with 10% FBS, 1X Penstrep
Hank's-low	1x Hank's buffer with 1.2 mM MgSO4, 40 mg/ml DNaseI, 3 mg/ml BSA, and filter sterilized
Hank's-high	1x Hank's buffer with 1.2 mM MgSO4, 40 mg/ml DNaseI, 4% BSA, and filter sterilized
Neurosphere Media	DMEM/F12 media with 1x B27 and 10ng/ml EGF
Trypsin Inhibitor solution	DMEM/F12 with 1 mg/ml soybean inhibitor

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.

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

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

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

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

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

2. Tissue dissection

NOTE: *Make freshly prepared 70% ethanol spray prepared. Have a petri dish (100 mm) prepared with ice-cold Hank's buffer kept on ice, which will be used to collect embryos after dissection. Afterwards, additional petri dishes will be needed to place in each of the dissected brains (35 mm).*

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.

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 ice-cold Hank's buffer, and then transfer to the second petri plate containing Hank's buffer on ice.

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

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

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.

2.6) Transfer the dissected tissue into the 15 ml tube of Hank's buffer on ice.

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.

3. Primary neurosphere culture

NOTE: Before starting warmed to 37°C the following solutions: trypsin/EDTA serum media, Hank's-low, and Hank's-high. In this section you will need the 18-gauge, 21-gauge, 23-gauge needle will be needed for trituration steps. Trituration should be performed gently and slowly to avoid killing cells. Hemocytometer will be needed to count cells.

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

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.

3.3) Spin tube at 300 RCF for 2 min.

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.

3.5) Repeat wash step 3.4 two additional times.

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

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.

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.

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

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.

3.11) Centrifuge at 300 RCF for 5 min.

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

3.13) Centrifuge at 300 RCF for 5 min.

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

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

3.16) Centrifuge at 300 RCF for 5 min.

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

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

3.19) Incubate at 37°C in a humidified incubator with 5% CO_2.

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

Secondary neurospheres

The extended version of our protocol can be used to obtain secondary neurospheres (https://dx.doi.org/10.17504/protocols.io.823hygn).

Results

This approach generates consistent number of primary neurospheres

Figure 1 shows a general overview of the neurospheres assay. A picture representation of primary neurospheres grown for 7 days *in vitro* is given in Figure 2A. The statistical analysis using a one-way ANOVA revealed no significant difference between the average numbers of neurosphere per field of view ($F_{(4,13)} = 0.666$; $p = 0.6268$; N=5) (Figure 2B).

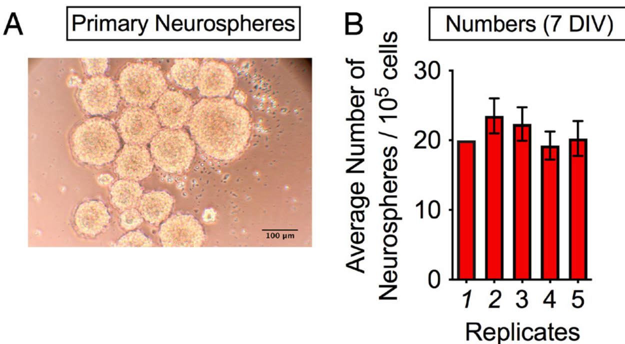

Figure 2. Primary neurospheres generated from the dorsal lateral ventricle. (**A**) Average size of primary neurospheres per field of view after 7 days *in vitro*. (**B**) Average number of neurospheres per field of view after 7 days *in vitro* (N=5). Scale bar = 100 µm.

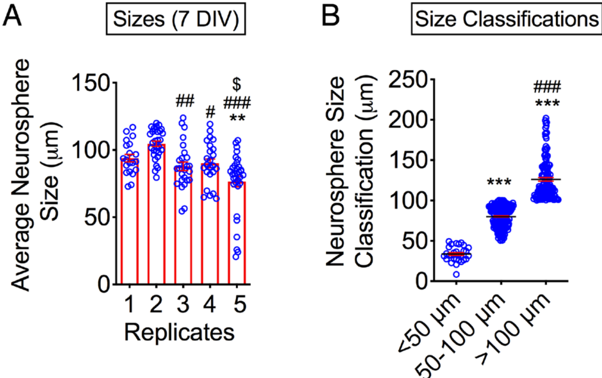

Figure 3. Size classification of primary neurospheres from the dorsal lateral ventricle. (**A**) Average size of primary neurospheres per field of view after 7 days *in vitro* (N=5). (**B**) The comparison of the numbers of neurosphere that are less than 50 µm, between 50-100 µm, and greater than 100 µm. Key to statistics **, *** = p <0.01, 0.001, respectively, in comparison to NS less than 50 µm or replicate 1. #, ##, ###, = p < 0.05, 0.01, 0.001, respectively, in comparison to NS between 50-100 µm or replicate 2. $ = p <0.05 in comparison to replicate 3 (minimum of 5 independent samples; N=29, N=214, N=136, respectively to NS<50 µm, NS between 50 µm -100µm, NS>100 µm).

This protocol generates different sizes of neurospheres

Figure 3 shows in the variation in sizes of neurospheres grown for 7 days *in vitro* (Figure 3A). The statistical analysis using one-way ANOVA revealed a significant difference in the sizes of neurospheres between the replicates ($F_{(4,129)} = 11.666$; $p < 0.0001$) (Figure 3A). Using similar analyses, we found significant differences between the size classification of neurospheres that were less than 50 µm, between 50–100 µm, and greater than 100 µm ($F_{(2,379)} = 424$; $p < 0.0001$) (Figure 3B). Post hoc analysis using Bonferroni's multiple comparison revealed a significant difference between the primary neurospheres that were greater than 100 µm compared to neurospheres that were less than 50 µm ($p < 0.0001$) or between 50–100 µm ($p < 0.0001$) (Figure 3B). Similarly, we found a substantial difference between primary neurospheres that were greater than 100 µm compared to neurospheres that were between 50–100 µm ($p < 0.0001$) (Figure 3B). Numbers and sizes of neurospheres, alongside the raw images used to produce these values, are available as *Underlying data*[14].

Primary neurospheres at 7 days *in vitro* can be used for immunocytochemistry

Neurospheres can be used for a variety of purposes, including immunocytochemistry. Figure 4 is a picture of a small (Figure 4A–B; arrowhead) and a larger (Figure 4C; arrowhead) primary neurosphere immunostained using an anti-GFAP antibody and counterstained with DAPI (Figure B; arrowhead). Additional neurosphere staining using other antibodies can be found in previous published studies[3] from our lab.

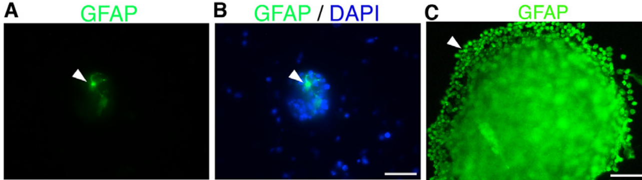

Figure 4. Immunocytochemistry of small and large primary neurospheres. Visual representation of immunocytochemistry staining of a small neurosphere using (**A**) anti-GFAP antibody (green) and (**B**) counterstained with DAPI (blue) after 7 days *in vitro* (7 DIV) Scale bar = 100 μm. (**C**) Anti-GFAP staining of a large neurosphere 7 DIV. Scale bar = 50 μm Arrowheads represent positive GFAP signal.

Discussion

There are several key steps that are important to maximize the yield and health of neurospheres. The most important step is to incubate the final triturated culture in the prewarmed DMEM/F12 with serum for 2–4 hours. This incubation time is necessary in order for the antibiotics in the media to inhibit growth of bacteria. A sign of bacteria contamination is reduced visibility of the media. Another marker of an unhealthy culture is a large number of differentiated neurons surrounding neurospheres. Indicators of differentiation are the large presence of axons and dendrites in your cultures. This can be caused by depletion of growth factors. If this is the case, it is recommend that you increase the concentration of EGF. Another cause of differentiation is too many cells in your prep. This leads to over crowdedness. It is recommend to split the culture to a lower density or decrease the number of neurons that are plated per 48 well.

Another essential step is to perform the trituration as gently as possible. Over-trituration, or trituration with great force, will result in increased cell death. After trituration, if a uniform suspension has not been achieved an alternative method used in previous protocols are strainers[15].

Additional modifications to the approach may be required. We have provided general guidelines for the number of cells to be plated per well and the expected number of neurospheres per field of view. If too many cells are plated per well, differentiation may occur. If so, either reduce the number of cells plated per well or increase the concentration of EGF to 20 ng/ml. If desired, trypsin inhibitor can be substituted for the FBS during the generation of primary neurospheres to inactivate proteolytic activity.

Our approach utilizes a mechanical trituration to dissociate cells. By sequentially processing the cells through successively higher gauge needles, we obtain very few clumps of cells. This eliminates the need for a cell strainer, which can reduce yield, as well as the time needed to perform enzymatic dissociation. Although cell death can be increased with mechanical dissociation, in our hands, this does not seem to affect neurosphere yield or health.

One advantage of our approach is the ease with which tissue surrounding the lateral ventricle can be isolated from the rest of the brain. Although this dissection is relatively crude, it is easier and faster than other approaches, which may require a brain slicer and/or microdissection[10,11]. This method eliminates the need for specialized equipment while generating high numbers of neurospheres from a single brain. This method has been shown in other studies to generate neurospheres greater than 300 μm in size when grown for 12 days *in vitro*[3].

Data availability

Underlying data

Figshare: Metrics of Primary Neurospheres.xlsx. https://doi.org/10.6084/m9.figshare.10280288.v1[14].

This project contains the following underlying data:

- Spreadsheet containing numbers and sizes per field of view.xlsx (details on numbers and sizes of neurospheres produced from each mouse).
- All raw, unprocessed microscope images used to produce results.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgements

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Open Peer Review

Current Peer Review Status:

Version 2

Reviewer Report 25 March 2020

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All the comments made to the previous version have been addressed.

Please, consider correcting a few minor typos:
Data Acquisition and Statistics:
Typo:
1. “steromicroscope” should be “stereomicroscope”.
2. “focus on the desire region” should be “focus on the desired desired region”.
3. “Data is” should be “data are”.

Section 3. Primary neurosphere culture:

Repetition in the “NOTE”: “will need”, “will be needed”.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunohistochemistry, glial cells, astrogliosis, epilepsy, traumatic brain injury, two photon microscopy, neuronal and astrocytic calcium signaling

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 24 December 2019

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This method article written by Dr. Blackwood is aimed to provide a simple and reproducible protocol generating neurospheres from lateral ganglionic eminence in late embryonic mice and demonstrate its applicability for further immunocytochemical assays. Several aspects and protocol steps, however, require clarification:

1. This protocol includes multiple sections with crucial steps. One suggestion that could make the protocol even greater for educational purposes is providing a brief paragraph to sections 1, 2 and 3 (prior to individual steps) that will explain what is important in this section and list all the necessary reagents and tools that will be used. This will help anyone who is using this protocol for the first time to prepare all required items for this protocol section easier rather than finding the specific reagents and volume from each individual step.

2. Table 1 details equipment, reagents and catalogue numbers; however, the digital camera used for acquiring the images is not listed. The model of stereo stereomicroscope (step 2.5) should be added to table 1, as well as the optimum magnification required or used in this protocol. The type and model of humidified incubator in step 3.19 would also be helpful for reproducibility.

3. Section 3 called “primary neurosphere culture” is the main section explaining the method of neurosphere generation. In other publications, e.g. Raponi et al. (2007)[1], a filtration using a 40um-mesh-nylon cell strainer to dissociate cells after triturating. It may be valuable to add a note after trituration step that at this point similar step could be done if the purpose is to obtain uniform suspension from tissue.

4. The “Data acquisition and statistics” section requires more details. Specifically, the additional valuable information could be addressing the distance from the culture plate, whether this is an important factor for further size measurements and if so what was the distance between the camera and the well. What was the zoom factor used here?

5. The section “Primary neurospheres at 7 days *in vitro* can be used for immunocytochemistry” is aimed to demonstrate applicability of this protocol. The GFAP/DAPI is used as an example. While it is stated in the “Introduction” that neurospheres have been used to study molecular mechanisms controlling differentiation of both neural and glial cells, it is not very clear why GFAP was used for this example. Maybe author could clarify or explain why more specific markers were not used here instead of GFAP. From the Fig. 4 it looks as only 1 cell within the neurosphere was GFAP+, hence I believe using a more specific antibody could produce more representative result. Several references provided to this review detail markers that could also be used.

6. Lastly, I believe another interesting question that could be potentially valuable here is the step explaining the reader, which factors or characteristics of the neurosphere help to determine that the neurosphere is mature.

In summary, after addressing these comments, I believe this method article is a good protocol that can be used as a reference and educational tool for both researchers that are familiar with similar techniques and those who approach it for the first time.

References

1. Raponi E, Agenes F, Delphin C, Assard N, et al.: S100B expression defines a state in which GFAP-expressing cells lose their neural stem cell potential and acquire a more mature developmental stage.*Glia*. 2007; **55** (2): 165-77 PubMed Abstract | Publisher Full Text

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunohistochemistry, glial cells, astrogliosis, epilepsy, traumatic brain injury, two photon microscopy, neuronal and astrocytic calcium signaling

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 08 Mar 2020

Christopher Blackwood, Cornell University, Ithaca, USA

Comment 2.1: This protocol includes multiple sections with crucial steps. One suggestion that could make the protocol even greater for educational purposes is providing a brief paragraph to sections 1, 2 and 3 (prior to individual steps) that will explain what is important in this section and list all the necessary reagents and tools that will be used. This will help anyone who is using this protocol for the first time to prepare all required items for this protocol section easier rather than finding the specific reagents and volume from each individual step.

Response 2.1: Thank you for your comments. We have revised accordingly and provided a brief paragraph to section 1, 2, and 3 prior to the individual steps.

1. Section 1:**Set-up prior to tissue dissection**

NOTE: *Breeding and euthanasia of all animals should be performed in accordance with an institutionally approved animal care and use protocol. Sterilize all surgical instruments packed in aluminum foil in an autoclave at 121°C (15 psi) for 30mins. This includes a scissors, forceps, and razor blades. Before starting all premade solutions should be warmed to 37°C.*

1. Section 2: **Tissue dissection**

NOTE: *Make freshly prepared 70% ethanol spray prepared. Have a petri dish (100 mm) prepared with ice-cold Hank's buffer kept on ice that will be used for embryos after dissection. Afterwards, additional petri dishes will be needed to place in each dissected brain (35 mm)*

Section 3:**Primary neurosphere culture**

NOTE: Before starting warmed to 37°C the following solutions: trypsin/EDTA serum media, Hank's-low, and Hank's-high. In this section you will need the 18-gauge, 21-gauge, 23-gauge needle will be needed for trituration steps. Trituration should be performed gently and slowly to avoid killing cells. Hemocytometer will be needed to count cells.

Comment 2.2 Table 1 details equipment, reagents and catalogue numbers; however, the digital camera used for acquiring the images is not listed. The model of stereo stereomicroscope (step 2.5) should be added to table 1, as well as the optimum magnification required or used in this protocol. The type and model of humidified incubator in step 3.19 would also be helpful for reproducibility.

Response 2.2. We have revised the Table 1 to add the Camera and Humidifier. We also included information for the optimum magnification to Data acquisition and statistics section.

"Images were taken with a Canon EOS Rebel XS camera. (Canon USA; Melville, NY). The optimum magnification is approximately 5x with 3888 x 2592 dimensions. Camera was connected to the trinocular port of the stereomicroscope (Carl Zeiss Stemi 305; White Plains, NY) using Mount Adaptor EF-EOS (6098B007AA; Canon; Melville, NY).

Comment 2.3 Section 3 called "primary neurosphere culture" is the main section explaining the method of neurosphere generation. In other publications, e.g. Raponi et al. (2007)[1], a filtration using a 40um-mesh-nylon cell strainer to dissociate cells after triturating. It may be valuable to add a note after trituration step that at this point similar step could be done if the purpose is to obtain uniform suspension from tissue.

Response 2.3. Thank you have revised the "primary neurosphere culture" section accordingly. "After trituration, if a uniform suspension has not been achieved an alternative method used in previous protocols are strainers [14]."

Comment 2.4 The "Data acquisition and statistics" section requires more details. Specifically, the additional valuable information could be addressing the distance from the culture plate, whether this is an important factor for further size measurements and if so what was the distance between the camera and the well.

Response 2.4: We have revised the Data acquisition and statistics" section according to your

recommendation. The scale bar should be used to advise the size of the neurosphere. Additionally, we have included information about the working distance to the "Data acquisition and statistics" section.

"The working distance was defined as the amount of room required between the top of the neurosphere and the bottom of the objective lens in order for the image to be in focus. The stereomicroscope was used at a working distance of ~110 mm. Due to the variation in neurosphere size, 110 mm should be adjusted to focus on the desire region of the neurosphere to provide optimal focus."

Comment 2.5: The section "Primary neurospheres at 7 days *in vitro* can be used for immunocytochemistry" is aimed to demonstrate applicability of this protocol. The GFAP/DAPI is used as an example. While it is stated in the "Introduction" that neurospheres have been used to study molecular mechanisms controlling differentiation of both neural and glial cells, it is not very clear why GFAP was used for this example. Maybe author could clarify or explain why more specific markers were not used here instead of GFAP. From the Fig. 4 it looks as only 1 cell within the neurosphere was GFAP+, hence I believe using a more specific antibody could produce more representative result. Several references provided to this review detail markers that could also be used.

Response 2.5: To provide more evidence that this protocol can be used to stain neurospheres, we have included an additional picture to the panel (Figure 4C). Larger neurospheres will contain more GFAP-expressing neurons that can be labeled. Furthermore, we have reference our previous published report that demonstrated other immunocytochemistry using other antibodies.

Comment 2.6: Lastly, I believe another interesting question that could be potentially valuable here is the step explaining the reader, which factors or characteristics of the neurosphere help to determine that the neurosphere is mature.

Response 2.6: Thank you for the recommendation. We have revised the manuscript to expand this point in our discussion.
"A sign of bacteria contamination is reduced visibility of the media. Another marker of an unhealthy culture is a large number of differentiated neurons surrounding neurospheres. Indicators of differentiation are the large presence of axons and dendrites in your cultures.This can be caused by depletion of growth factors. If this is the case, we recommend that you increase the concentration of EGF. Another cause of differentiation is too many cells in your prep. This leads to over crowdedness. We recommend splitting the culture to a lower density and decreasing the number of neurons that are plated per 48 well."

Competing Interests: No competing interests.

Reviewer Report 19 December 2019

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M.L. Shawn Bates

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This manuscript describes a protocol that can be used to generate primary neurospheres from the tissue surrounding the lateral ventricle in mice in the late embryonic period. The method is a relatively simple technique that will provide investigators with viable neurospheres that can be used in further studies.

The protocol described here is important, and will likely be useful for many researchers who utilize neural stem cells. However, I have some constructive criticism and points that may require clarification, see below:

1. How long does it usually take to generate neurospheres? Is the fact that this protocol takes 7 days the rationale for developing it?

2. Is there a benefit to generating different sizes of neurospheres?

3. Why only use anti-GFAP (in essence, my question is why only visualize glia)?

4. Under the “Data acquisition and statistics subsection”:
    - The field of view represent**s** a length of...
    - Bonferroni's multiple comparis**o**n post hoc test…
    - A total of 5 individual animal**s** were analyzed…

5. The significance of the protocol is somewhat understated. I would add language to the Discussion to highlight the novelty of this approach. I would also include more suggestions for potential ways that others can use the protocol.

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: My area of expertise is behavioral neuroscience, including in vivo electrophysiology, and various learning and conditioning methods

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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