Video Article

Isolation of Distinct Cell Populations from the Developing Cerebellum by Microdissection

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Abstract

Microdissection is a novel technique that can isolate specific regions of a tissue and eliminate contamination from cellular sources in adjacent areas. This method was first utilized in the study of Nestin-expressing progenitors (NEPs), a newly identified population of cells in the cerebellar external germinal layer (EGL). Using microdissection in combination with fluorescent-activated cell sorting (FACS), a pure population of NEPs was collected separately from conventional granule neuron precursors in the EGL and from other contaminating Nestin-expressing cells in the cerebellum. Without microdissection, functional analyses of NEPs would not have been possible with the current methods available, such as Percoll gradient centrifugation and laser capture microdissection. This technique can also be applied for use with various tissues that contain either recognizable regions or fluorescently-labeled cells. Most importantly, a major advantage of this microdissection technique is that isolated cells are living and can be cultured for further experimentation, which is currently not possible with other described methods.

Video Link

The video component of this article can be found at https://www.jove.com/video/52034/

Introduction

The cerebellum is comprised of multiple cell layers, each containing distinct cell types. During development, the EGL contains proliferating granule neuron precursors (GNPs) while the molecular layer and Purkinje layer contain Bergmann glia and Purkinje neurons, respectively. Deep within the cerebellum lies the white matter, which contains neural stem cells (NSCs) and oligodendrocytes[1].

Sonic hedgehog (Shh) plays a critical role in regulating cerebellar development and in particular, it promotes the proliferation of GNPs via binding to its receptor Patched (Ptc), a negative regulator of Shh signaling[2-4]. Aberrant activation of Shh signaling generates medulloblastoma (MB), the most common malignant brain tumor in children[5,6].

Ptc mutant MB transgenic mouse models are powerful tools for the study of MB and develop tumors resembling human MB[7,8]. Using these mice, it was discovered that GNPs are the cell of origin for hedgehog-type MB[7]. Furthermore, in addition to GNPs, we have recently identified a unique population of neuronal progenitors within the EGL of the developing cerebellum that can also give rise to MB. These cells express high levels of the type VI intermediate filament protein, Nestin, and are termed NEPs[9]. NEPs are located within the deep part of the EGL and exist transiently only during neonatal cerebellar development. They are committed to the granule cell lineage as GNPs but are distinct as they are quiescent and do not express Math1, a well-established marker for conventional GNPs[10]. In addition, NEPs give rise to MB more efficiently than GNPs after activation of the Shh signaling pathway[9], which makes them a novel origin for MB tumorigenesis.

We previously isolated NEPs from the cerebellar EGL at postnatal day 4 (P4) by the microdissection technique described here[9]. Microdissection via direct microscopic visualization of the tissue allows for specific dissection of the cerebellar EGL. This is necessary as Nestin is also expressed by NSCs in the cerebellar white matter and by Bergmann glia in the molecular layer[1,7,11,12] and it was crucial that these cells not be included, as they would confound analysis. Cells isolated from microdissected tissue can be used immediately for molecular analysis or they can be cultured for further applications.

To aid in specifically microdissecting the EGL and to further purify NEPs, *Math1-GFP* (green fluorescent protein) mice were crossed with *Nestin-CFP* (cyan fluorescent protein) mice. The transcription factor Math1 is specifically expressed by GNPs and GFP expression is clearly visible in the EGL[9,13]. *Nestin-CFP* mice express a nuclear form of CFP and can be easily visualized in the molecular layer[9,14]. Together, GFP and CFP expression create boundaries for microdissection of the EGL (see **Figure 1**). CFP-positive NEPs were then isolated by FACS, following enzymatic dissociation of the dissected EGLs.

Currently, the most well utilized method to isolate cells from the EGL is Percoll gradient centrifugation of whole cerebellar tissue[15,16]. This method, however, is unable to completely exclude Nestin+ cell populations from the molecular layer and white matter and therefore cannot be used for the study of NEPs. Laser capture microdissection, which uses the transfer of laser energy to remove cells of interest, is another method

used to isolate specific cells within a heterogeneous tissue[17,18] but captured cells can only be used for recovery of DNA, RNA and protein and are not able to be cultured.

This protocol provides a way to specifically isolate an alive, pure population of NEPs from the EGL. This technique can also be applied to different tissue types that have either recognizable anatomical architecture or fluorescently labeled cells/regions. Therefore, the major advantage of incorporating this novel microdissection technique into cell isolation protocols is that cells can be isolated from specific tissue regions to eliminate contaminating neighboring tissue and the cells can be collected immediately for analysis or cultured for other applications.

Protocol

Use of animals in this protocol has been performed in accordance with procedures approved by the Fox Chase Cancer Center Animal Care and Use Committee.

1. Preparation of Instruments, Solutions, and Coverslips

1. Clean two sets of #5 fine forceps, one set of #7 fine curved forceps, one large surgical scissor, one microdissecting scissor, one spatula and one perforated spoon. Place the instruments in a self-sealing sterilization pouch and autoclave. Keep instruments in pouch until ready for use.
2. Prepare a 3% low melting temperature agarose solution.
    1. Add 1.5 g agarose into 50 ml of sterile Dulbecco's Phosphate Buffered Saline (DPBS) then place in microwave and heat until bubbles appear. Swirl flask and reheat until all agarose has dissolved. Mix solution with a stir bar prior to heating if clumps appear.
    2. Store solution in a 37 °C water bath until needed.
    3. For long-term storage, store the agarose solution for future use at room temperature or at 4 °C for months. Reheat in microwave to liquefy. For repeated heating of the agarose solution, weigh the flask before heating to maintain its initial weight with warm sterile distilled water.
3. Prepare the following solutions for papain-based cell dissociation as previously described[2]:
    1. A papain solution with papain (100 U/ml), cysteine (0.2 mg/ml) and DNase (250 U/ml) in sterile phenol red-containing DPBS.
    2. An ovomucoid solution with bovine serum albumin (BSA; 8 mg/ml), soybean trypsin inhibitor (8 mg/ml)) and DNase (250 U/ml) in sterile phenol red-containing DPBS.
    Filter sterilize both solutions and adjust pH to return original color of phenol red-containing DPBS.
4. Prepare cell culture media (NB/B27): supplement basal media with 1 mM sodium pyruvate, 2 mM L-glutamine, 1% penicillin-streptomycin (P/S) and B27 supplement. Filter sterilize and protect from light. Equilibrate media at 37 °C and 5% CO_2 before use.
5. Prepare FACS buffer: 5% fetal bovine serum (FBS) in DPBS. Add 2.5 ml FBS to 47.5 ml DPBS.
6. Prepare poly-D-lysine (PDL)-coated coverslips.
    1. Autoclave new glass coverslips.
    2. Coat the coverslips with PDL (100 µg/ml in sterile distilled water) and incubate for 2 hr at 37 °C or room temperature overnight.
    3. Wash coverslips with sterile distilled water. Wash once with NB/B27 media. Let coverslips dry completely in tissue culture hood prior to use.

2. Dissection and Preparation of Tissue Slices

1. On the day of dissection, wipe down the dissection area with 70% ethanol and cover with an absorbent pad.
2. Remove dissection tools from the sterilization pouch. Add ice cold, sterile DPBS/1% P/S to a small Petri dish and place on ice.
3. Decapitate P4 *Math1-GFP*/*Nestin-CFP* mouse pups with large surgical scissors then hold the head down with fine curved forceps. Remove the skin and gently peel away the skull with #5 fine forceps then scoop out the brain from the skull using a spatula and transfer it to the Petri dish containing DPBS/1% P/S.
    1. Using #5 fine forceps, carefully separate the cerebellum from the rest of the brain. Be sure to dissect one pup at a time as quickly as possible.
    2. Collect a minimum of 8 cerebella in order to obtain enough NEPs for molecular and functional analyses.
4. Fill an embedding mold measuring 2 x 2 x 2 cm with 3% low melting temperature agarose solution. Ensure that the temperature of agarose is no more than 37 °C.
5. Remove excess liquid from cerebellum by dabbing gently on a laboratory tissue. Place cerebellum into mold in a vertical position. Place the mold on ice to allow it to solidify quickly. Use ~4 cerebella per block.
6. Obtain living tissue sections with a vibrating blade vibratome. Trim cerebella-containing agarose block with a razor blade. Glue the agarose block to the vibratome plate with cerebella oriented in a vertical position.
7. Fill the tray of the vibratome with ice-cold sterile DPBS/1% P/S and place ice underneath.
    1. Cut 600 µm sections through the whole length of cerebellum. Collect sections from the ice tray using a perforated spoon. Place sections in a Petri dish filled with DPBS/1% P/S on ice.

3. Microdissection and Cell Dissociation

1. Place the Petri dish containing tissue slices under a fluorescent dissecting microscope.

1. Carefully separate the EGL from the rest of the cerebellar section using fine forceps by dissecting in between the CFP+ molecular layer and the GFP+ EGL (refer to dotted line in **Figure 1**). Be careful to not include any part of the molecular layer, which will result in contamination of Nestin-expressing Bergmann glia.
2. Complete this step as quickly as possible and keep the tissue on ice to avoid cell death.
3. Remove the agarose surrounding the tissue before proceeding to the next step.

2. Digest the microdissected EGL tissue using a papain-based protocol as previously described[2] to obtain a single cell suspension.
   1. Re-suspend cells in FACS buffer for collection of CFP-positive cells via FACS.

4. Cell Sorting and Plating

1. Sort cells for CFP fluorescence using a sterile, high speed flow cytometer containing an appropriate CFP filter and collect cells in FACS buffer on ice. Obtain approximately 100,000 NEPs from each P4 cerebellum.
2. Centrifuge cells at 300 x g for 5 min. Use cells immediately for molecular analysis or culture for further experimentation.
3. To culture cells, re-suspend the cells in pre-warmed NB/B27 media and plate on PDL-coated coverslips as previously described[2].

Representative Results

As shown in **Figure 1A**, cerebellar slices were prepared from P4 *Math1-GFP*/*Nestin-CFP* mice, in which the cerebellar EGL was dominated by GFP-expressing GNPs and the molecular layer was enriched by CFP-positive glial cells. To isolate NEPs that are located in the deep part of the EGL, slices were microdissected between the EGL and the molecular layer (along dashed line; **Figure 1A**). Dissected EGLs (**Figure 1B**) were then collected for enzymatic dissociation followed by FACS.

As expected, a majority (more than 85%) of cells in the dissected EGL were conventional GNPs, which were positive for GFP (**Figure 2A**). NEPs (CFP+) account for only around 5% of the EGL cell population. Almost none of the cells were double-positive for GFP and CFP based on the FACS analysis. CFP+ cells purified by FACS, were cultured *in vitro* to examine the differentiation potential. As shown in **Figure 2B**, NEPs exclusively gave rise to β-tubulin+ neurons after 4 days in culture, suggesting NEPs are lineage-restricted neuronal progenitors.

By this microdissection protocol, NEPs and GNPs were also purified from the EGL of *Ptc* deficient cerebellum. Upon intracranial transplantation, NEPs develop MBs more rapidly compared with GNPs, indicating that NEPs are particularly susceptible to oncogenic transformation[9].

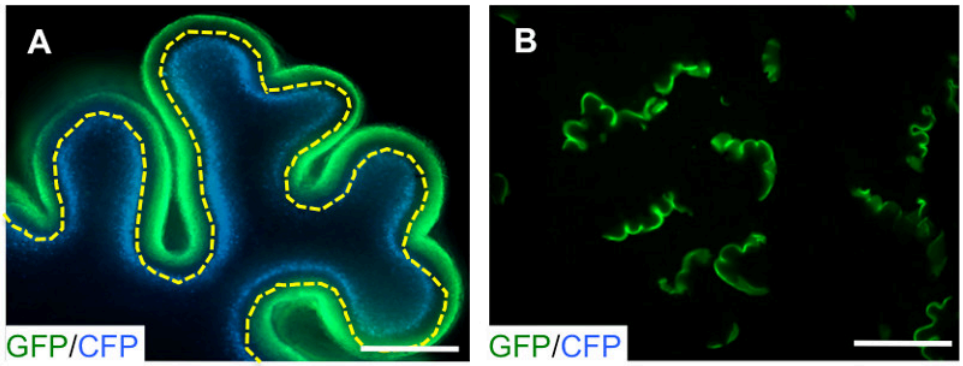

Figure 1. Identification of the region for EGL microdissection. Fluorescent images from P4 *Math1-GFP/Nestin-CFP* animals. **(A)** GFP expression is located in the EGL, while the majority of CFP expression is located in the molecular layer. Microdissection was done along the yellow dotted line. **(B)** EGLs were collected for tissue dissociation. This figure has been modified from Li *et al*[9].

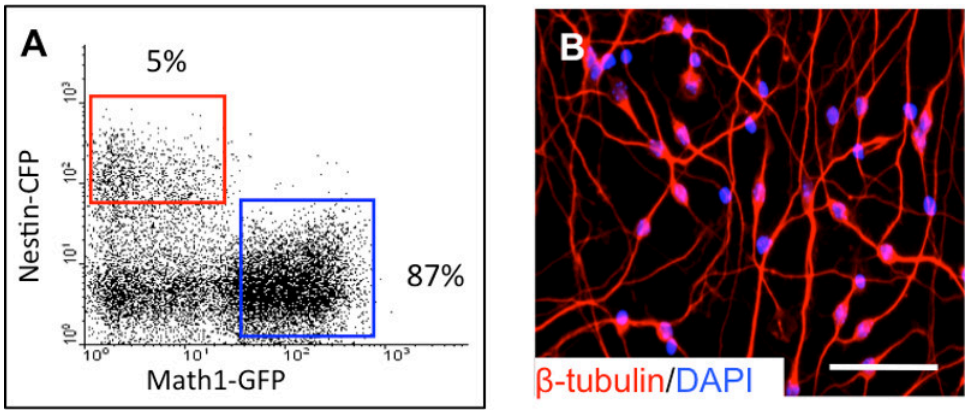

Figure 2. NEPs represent a small population of cells in the EGL and are lineage-restricted neuronal progenitors. (A) Flow cytometry analysis of CFP+ cells isolated from microdissected EGL. **(B)** NEPs were cultured under differentiation conditions for 4 days and stained for β-tubulin (red) and counterstained with DAPI (blue). This figure has been modified from Li *et al.*[9]

Discussion

The microdissection method described here is the first procedure able to isolate a pure population of living cells for use in molecular and functional analyses. As demonstrated by Li *et al.*[9], NEPs obtained by this method can be cultured and incorporated into various experiments and because of their high purity, can also be used for microarray analysis.

It is very important to take time to become proficient with this microdissection technique. NEPs are located in the deep region of the EGL in very close proximity to the molecular layer, in which Nestin-expressing Bergmann glia could contaminate the NEP cell isolation and compromise purity. Microdissection should therefore be done conservatively to make sure contaminating cells in adjacent tissue are not included. The major advantage of the microdissection technique described here is the ability to obtain living cells that can be cultured for further experimentation. Because of this, it is crucial that all steps during this procedure be done quickly and that tissue be kept on ice as much as possible. Also, all instruments and reagents must be sterile. Following these steps will reduce cell death and cell culture contamination. In addition, mounting multiple cerebella per agarose block will aid in reducing the procedure time.

This technique is not limited to isolation of NEPs and can be used to collect cells from other areas of the cerebellum, such as glial cells from the molecular layer and neural stem cells from the white matter. Beside the cerebellum, microdissection can be used with other tissues that contain recognizable regions, such as the hippocampus, for example. The use of fluorescent transgenes, as utilized here, can aid in creating borders or identify regions for dissection, which can be especially helpful in tissues that lack easily identifiable areas and would otherwise not be able to be microdissected. This technique could therefore aid in cell purifications in a wide range of research areas and applications.

Disclosures

The authors have nothing to disclose.

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