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PROTOCOL

Isolating astrocytes and neurons sequentially from postnatal murine brains with a magnetic cell separation technique

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Abstract Understanding the mechanism of developmental brain injury is crucial for the progress of discovering neuroprotective strategies and interventions. However, the pathophysiology is complex which involves interactions and crosstalk of diverse neural cell types. Isolating viable and pure populations of these brain cells is a valuable tool to study the particular cell properties and understand the physiologic and pathophysiologic mechanisms. Here we present a magnetic cell sorting approach to separate astrocytes and neurons sequentially from the same neonatal (postnatal day 9 or 10) CD-1 mouse brain samples. The procedure which involves positive selection of astrocytes by the ACSA-2 antibody followed by a negative depletion of non-neuronal cells from the flow through yields relatively enriched neuronal cells. The sorted fractions are highly pure and viable and can be used for further applications and analyses.

Keywords: astrocytes, CNS, MACS, magnetic cell sorting, neurons

BACKGROUND

Understanding the physiologic and pathologic mechanisms underlying developmental brain injury is challenging. Many types of cells contribute to the processes including neurons, astrocytes, microglia and oligodendrocytes. There is crosstalk and paracrine signaling in neural cells during the injury process that still remains to be investigated. Particularly astrocytes and neurons show a wide range of interactions important not only for synaptogenesis, modulation of neurotransmission [1], energy storage [2] and detoxification [3], but also for intrinsic injury repair [4]. Revealing this endogenous neuroprotective potential might lead to the development of new therapeutic strategies to attenuate or prevent irreversible neuronal injury to the developing brain. Isolation of diverse neural cell types from acutely injured brains therefore, permits the detailed study of mechanisms related to these interactions.

During the last three decades, magnetic cell sorting (MACS) and fluorescence-activated cell sorting (FACS) have been established as state-of-the-art cell isolation technologies [5]. The advantages of MACS are the high separation capacity [6], the speed of processing, excellent purity and minimal stress to the cells[5]. It is based on high gradient magnetic cell separation with super-paramagnetic microparticles, a concept first described by Molday and Molday in 1984 [7]. Miltenyi and colleagues subsequently optimized this method and published and patented it in 1990 [6]. Cells are immunologically labeled with super-paramagnetic nanoparticles and applied to separation columns containing ferromagnetic steel wool. By placing these columns in a magnetic field, the labeled cells are attracted to the magnet, retained in the columns, and may thus be separated from the unlabeled cells. Elution of the labeled cells is possible after removing the column from the magnetic field [5].

MACS technology has been increasingly used in neuroscience research. In this protocol, instead of isolating neurons and astrocytes separately from different brains, we describe a novel application of magnetic cell sorting approach to purify them sequentially from the same mouse brain sample. For astrocytes, we used the ACSA-2 antibody, which specifically recognizes an epitope that is comparably expressed as the astrocyte-specific L-glutamate/L-aspartate transporter GLAST (EAAT1, Slc1a3) [8,9]we describe a new monoclonal antibody, ACSA-1, which was generated by immunization of GLAST1 knockout mice. The antibody specifically detects an extracellular epitope of the astrocyte-specific L-glutamate/L-aspartate transporter GLAST (EAAT1, Slc1a3. The use of ACSA-2 microbeads with magnetic separation columns allows the isolation of pure astrocytes by positive selection from brain single-cell suspension. Pure neurons are acquired by 'depletion' of non-neuronal cells from the flow through after removal of astrocytes [10]. This method allows direct comparison and accurate analysis of cell type-specific physiological function and response to injuries.

MATERIALS

Animals

Postnatal day 9 or 10 (P9 or P10) old CD-1 mice were used (Harlan Labs, Livermore, CA, USA). Animal research was approved by the

University of California San Francisco Institutional Animal Care and Use Committee and was performed with the highest standards of care under the U.S. National Institutes of Health guidelines. The pups were euthanized and perfused for collection of brain tissue. 40 µl of Euthasol for each mouse was used to achieve unresponsiveness to paw pinch. 10 ml ice cold HBSS with Ca^{2+} and Mg^{2+} at a perfusion pump speed of 5 ml/min was used for transcardial perfusion of the brain before tissue removal.

Equipment

- ✓ GentleMACS Dissociator (Miltenyi Biotec, San Diego, CA, USA, # 130-093-235)
- ✓ OctoMACS Separator (Miltenyi Biotec, # 130-042-109) (suitable magnet for MS column)
- ✓ QuadroMACS Separator (Miltenyi Biotec, # 130-090-976) (suitable magnet for LS Column)
- ✓ 2 fire polished glass Pasteur pipettes (ca. 0.7 mm and 1 mm diameter)
- ✓ Centrifuge (suitable for 15 and 50 ml tubes)
- ✓ 15 ml centrifuge tubes
- ✓ Pre-Separation Filters (70 µm) (Miltenyi Biotec, # 130-095-823)
- ✓ LS Columns (Miltenyi Biotec, # 130-042-401)
- ✓ MS Columns (Miltenyi Biotec, # 130-042-201)
- ✓ C Tubes (Miltenyi Biotec, # 130-093-237)
- ✓ Hemocytometer for cell counting

Reagents

- ✓ Neural Tissue Dissociation Kit Postnatal Neurons (Miltenyi Biotec, # 130-094-802)
- ✓ Myelin Removal Beads II, human, mouse, rat (Miltenyi Biotec, # 130-096-733)
- ✓ Anti-ACSA-2 MicroBead Kit (Miltenyi Biotec, # 130-097-678)
- ✓ Neuron Isolation Kit (Miltenyi Biotec, # 130-098-752)
- ✓ D-PBS with Ca^{2+} and Mg^{2+} (Invitrogen, Grand Island, NY, USA, # 14040-133)
- ✓ PBS (Cell Culture Facility (CCF), University of California San Francisco)
- ✓ HBSS with Ca^{2+} and Mg^{2+} (CCF)
- ✓ Bovine Serum Albumin (BSA) (Merck Millipore, Billerica, MA, USA, # 126609)
- ✓ Euthasol (Virbac AH, Fort Worth, TX, USA, # 200-071)
- ✓ BD BioCoat 96-well plate (Becton Dickinson, Franklin Lake, NJ, USA, # 356461)
- ✓ Paraformaldehyde (Fisher Scientific, Pittsburg, PA, USA, # 04042-500)
- ✓ Neurobasal Medium (Life Technologies, Carlsbad, CA, USA, # 12348-017)
- ✓ B-27 Supplement (Life Technologies, # 17504-044)
- ✓ GlutaMax Supplement (Life Technologies, 35050-061)
- ✓ NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA, # 78833)
- ✓ BCA Protein Assay Reagent (Pierce, # 23228)
- ✓ LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, # L-3224).
- ✓ Anti-Glial Fibrillary Acidic Protein Antibody, mouse monoclonal (Merck Millipore, 1:100, # MAB3402)
- ✓ Anti-Glial Fibrillary Acidic Protein, rabbit polyclonal (Dako, Carpinteria, CA, 1:500, # Z0334)
- ✓ Doublecortin, mouse monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA, 1:100, # sc-271390)
- ✓ Anti Iba-1, rabbit polyclonal (Wako Chemicals, Richmond, VA, 1:500, # 019-90741)
- ✓ Anti-MAP2, mouse monoclonal (abcam, Cambridge, MA, 1:500, # ab11267)

PROCEDURE

The following procedure is based on a Magnetic Cell Sorting Technique with reagents and instruments purchased from Miltenyi Biotech. This method follows mainly the manufacturer's protocol, we focused here on steps with novel and significant modifications and integration of separate procedures of astrocyte and neuron isolation for sequential purification from the same brain samples.

In order to improve cell viability and increase cell yield, pre-cooled reagents should be used to avoid antibody internalization by the cells [11]. Even the tubes and separation columns can be pre-chilled at 4°C.

In this protocol, 4 cortical hemispheres were combined to achieve a cell yield that allows extracting enough protein for western blot analysis.

Tissue Dissociation

1. Collect the brains of the transcardially perfused mice in HBSS with Ca^{2+} and Mg^{2+} and place the samples on ice.
2. Dissect the brains and collect the cortices in D-PBS with Ca^{2+} and Mg^{2+} (DPBS w) and place the samples on ice.
3. Perform Neural Tissue Dissociation according to the manufacturer´s protocol, by using the C Tubes and the GentleMACS Dissociator.
4. Caution: Tissue pieces can stick to the wall of the C Tube and not be dissociated properly.
5. Combining the automated dissociation with manual trituration with the fire polished pipettes can increase the yield. The last addition of enzyme mix 2 should therefore be followed by a short centrifugation to collect the cells at the bottom of the tube (30 s at 300 × g).

6. Use the bigger Pasteur pipette (ca. 1 mm diameter) after rinsing with DPBS w + 0.5% BSA and slowly pipette the cell solution 10 - 15 times up and down. No air bubbles should be formed. Repeat with the smaller pipette (ca. 0.7 mm diameter).
7. Tip: The sample should turn into a milky solution without observable tissue pieces.
8. Prepare a 15 ml tube with a 70 µm Pre-Separation Filter by rinsing with 1 ml of DPBS w + 0.5% BSA before the cell suspension is applied onto the strainer. Use 10 ml of DPBS w + 0.5% BSA to rinse the C Tube, recollect the buffer and apply it onto the filter.
9. Spin the solution for 15 min at 300 × g at room temperature.
10. Remove the supernatant carefully and proceed to the Myelin Removal step.

Myelin Removal

11. Resuspend the cell pellet in 360 µl of PBS supplemented with 0.5% BSA and add 40 µl of Myelin Removal Beads and incubate for 15 min at 2-8°C. Agitate the tube every 5 min to assure homogenous binding of the antibodies. Then wash the solution by adding 2000 µl of PBS + 0.5% BSA.
12. Spin the cells for 10 min at 300×g at room temperature.
13. Prepare the LS Column with a 70 µm Pre-Separation Filter by rinsing with 3 ml of PBS + 0.5% BSA.
14. Resuspend the cell pellet in 1000 µl of PBS + 0.5% BSA and apply it onto the cell column.
15. Wash the column with 2 × 1 ml of PBS + 0.5% BSA and collect the flow through which contains the unlabeled cells.
16. Perform a cell count using a hemocytometer to keep track of your yield.

Astrocyte Separation

17. Spin the cells for 10 min at 300 × g at room temperature and aspirate the supernatant carefully.
18. Resuspend the pellet in 160 µl of PBS + 0.5 % BSA and add 20 µl FcR Blocking Reagent. After 10 min incubation at 2-8°C, add 20 µl Anti-ACSA-2 MicroBeads and incubate for another 15 min. Manual agitation of the tubes by gentle flipping can help to equally label the cells.
19. Add 4 ml of PBS + 0.5% BSA to the cells, spin them down for 10 min at 300 × g at room temperature and aspirate the supernatant.
20. Resuspend the pellet in 1 ml of PBS + 0.5% BSA and pipette the cell suspension onto a LS Column rinsed with 3 ml of the resuspension buffer.
21. Wash the column with 3 × 3 ml of PBS + 0.5% BSA. Collect the flow through since it contains the unlabeled cells for further isolation of neurons.
22. Remove the LS Column that contains the retained astrocytes from the magnetic field and place it on a collection tube. Immediately pipette 6 ml of pre-equilibrated and pre-warmed cell medium onto the reservoir of the column (Alternatively, appropriate buffers can be used to elute the cells depending on the downstream applications).
23. Press the plunger onto the columns and elute the ACSA-2 labeled cells.

    NOTE: A high flow rate helps to elute the cells from the column.
24. Perform a cell count using a hemocytometer.

Neuron Separation

25. Centrifuge the flow through from step 19 which contains the unlabeled cells at 300×g for 8 min. Remove the supernatant and resuspend the cell pellet in 80 µl of DPBS w + 0.5% BSA.
26. Add 20 µl of Non-Neuronal Cells Biotin-Antibody Cocktail and incubate for 5 min at 2-8°C.
27. Wash the cells with 1 ml of DPBS w + 0.5% BSA and spin them down at 300 × g for 8 min.
28. After aspiration of the supernatant add 80 µl of DPBS w + 0.5% BSA to the cell pellet and incubate with 20 µl of Anti-Biotin MicroBeads for 10 min at 2-8°C.
29. Prepare a MS Column by rinsing with 500 µl of DPBS w + 0.5% BSA.
30. Fill the sample volume up to 500 µl with DPBS w and apply it onto the MS Column placed in the magnetic field.

31. Wash the column with 2 × 500 µl of DPBS w + 0.5% BSA. Collect the flow through as it contains the non-labeled neuronal cells.
32. Spin the collected solution down at 300 × g for 10 min and resuspend the cells in pre-equilibrated and pre-warmed culture medium or an appropriate buffer depending on further applications.
33. Perform a cell count using a hemocytometer.

ANTICIPATED RESULTS

Cell and Protein Yield

The cell yield was determined by cell counts immediately after isolation of the respective fractions. Around 1 × 10^6 ACSA-2-positive astrocytes, and around 0.45 × 10^6 neurons were obtained per P9-P10 CD-1 mouse brain (n=2) (**Table 1**). Cells were lysed and nuclear and cytoplasmic fractions were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents. The protein concentration was measured with BCA Protein Assay. From 3-4 cortical hemispheres, we could get 91.69 µg cytoplasmic protein, and 15.49 µg nuclear protein for astrocytes. The respective yield for the neuronal fraction was 85.07 µg cytoplasmic and 13.99 µg nuclear protein (**Table 2**).

Cell Viability

Cell viability was visualized using a fluorescence-based Live/Dead Cell Viability assay. Dead and live cells were distinguished by propidium iodide and calcein-AM, respectively, which were incubated with the cells immediately after isolation. Approximately 97.3% ACSA-2 positive astrocytes are viable (2.7% cell death) and 73% neurons are viable (27% cell death, see troubleshooting) (**Fig. 1**).

Cell Purity

For immunocytochemistry, cells were plated into a BD BioCoat 96-well plate. Cells were kept at 37°C in a humidified incubator with 5% CO_2 in Neurobasal Medium with 2% B-27 and 0.5 mM GlutaMax supplement. After 2 days cultured *in vitro*, cells were fixed with 4% Paraformaldehyde in PBS at room temperature and stained with specific cell markers for astrocytes (GFAP), neurons (MAP2 and Doublecortin (Dcx)) and microglia (Iba1) (**Fig. 2-4**). The percentage of GFAP-positive cells was 92% from astrocyte preparation (n=3), and MAP2-positive cells accounted for 85.79% from neuron preparation (n=3).

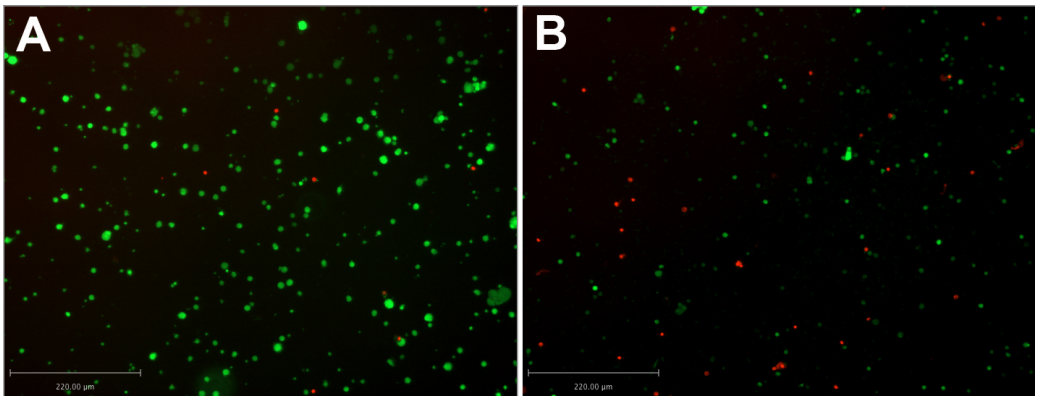

Figure 1. Live/Dead Cell Viability assay of freshly isolated cells. Dead cells were stained with EthD-1 (red), live cells were stained with Calcein-AM (green). **A.** ACSA-2 positive astrocytes, 2.7% cell death. **B.** Neuronal cells, 27% cell death.

Table 1. Cell count after subsequent separation steps using a starting material of 4 cortical hemispheres (two representative cell counts).

Type of cells	Cell count	
	n1	n2
After myelin removal	5.69 × 10^6	6.66 × 10^6
Astrocytes	2.28 × 10^6	2.28 × 10^6
Neurons	0.98 × 10^6	0.88 × 10^6
Non-neuronal cells	1.99 × 10^6	2.20 × 10^6

Table 2. Protein yield of isolated cells in µg using 3-4 cortical hemispheres (n=4).

	Nuclear fraction	Cytosolic fraction
Astrocytes	15.49	91.69
Neurons	13.99	85.07

TROUBLESHOOTING

In the following section the most common issues and some solutions are listed. Additional help with troubleshooting can be found in the company's detailed data sheets.

Cell Yield

Make sure the tissue is completely dissociated before the labeling and separation of the cells is started. The automated programs of the MACS Tissue Dissociator can be repeated as needed. All tissue should be in the range of the rotor of the C Tube to ensure a proper dissociation. During incubation of the solution at 37°C, constant slow agitation can help to equally dissociate the tissue. A rotator can be placed in a 37°C incubator for this purpose.

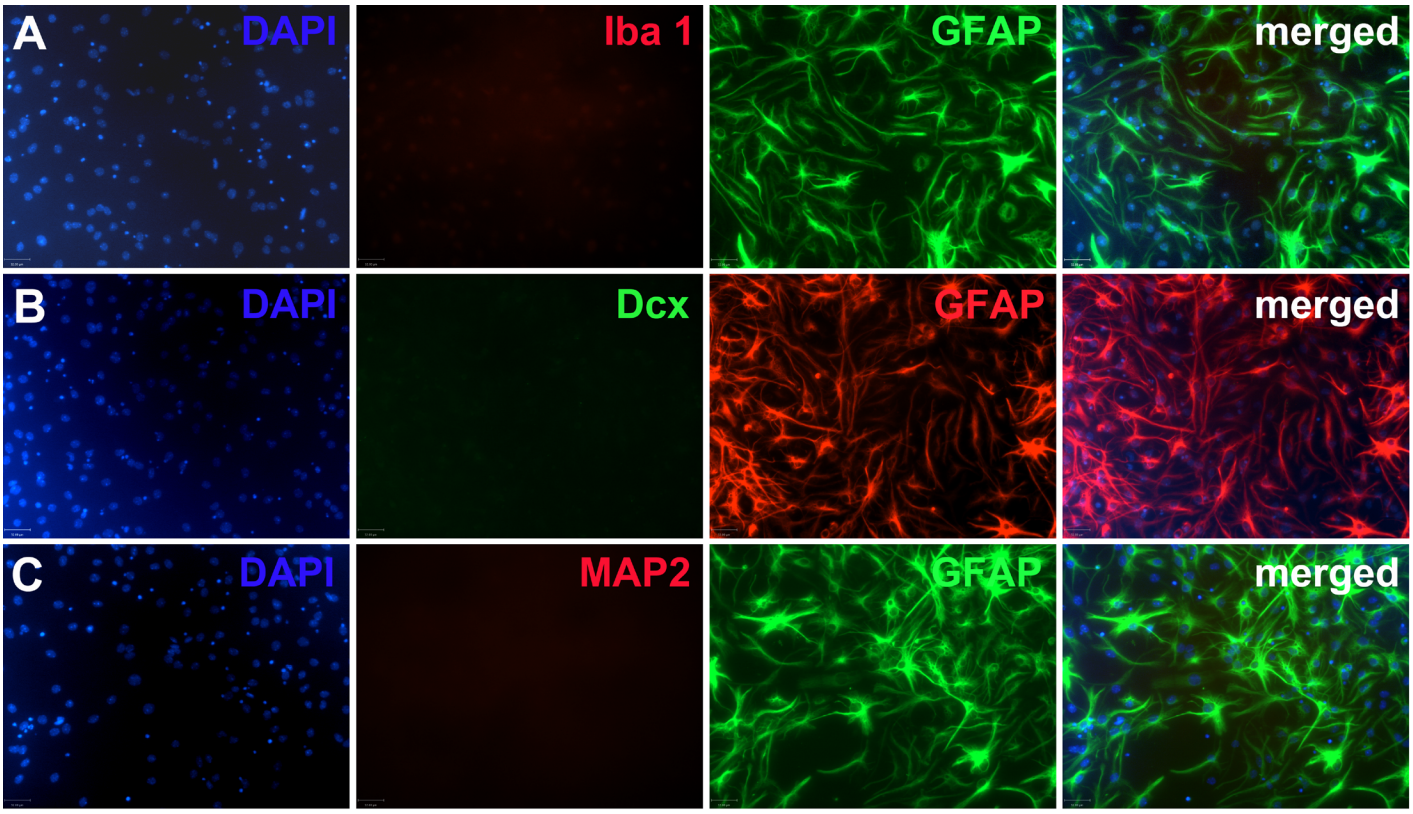

Figure 2. Purity of astrocytes after magnetic isolation and 2 days cultured *in vitro*. Cells were stained with DAPI (blue), Iba1 (red in A), MAP2 (red in C), GFAP (green in A, C; red in B), Dcx (green in B). The last panels are the merged images.

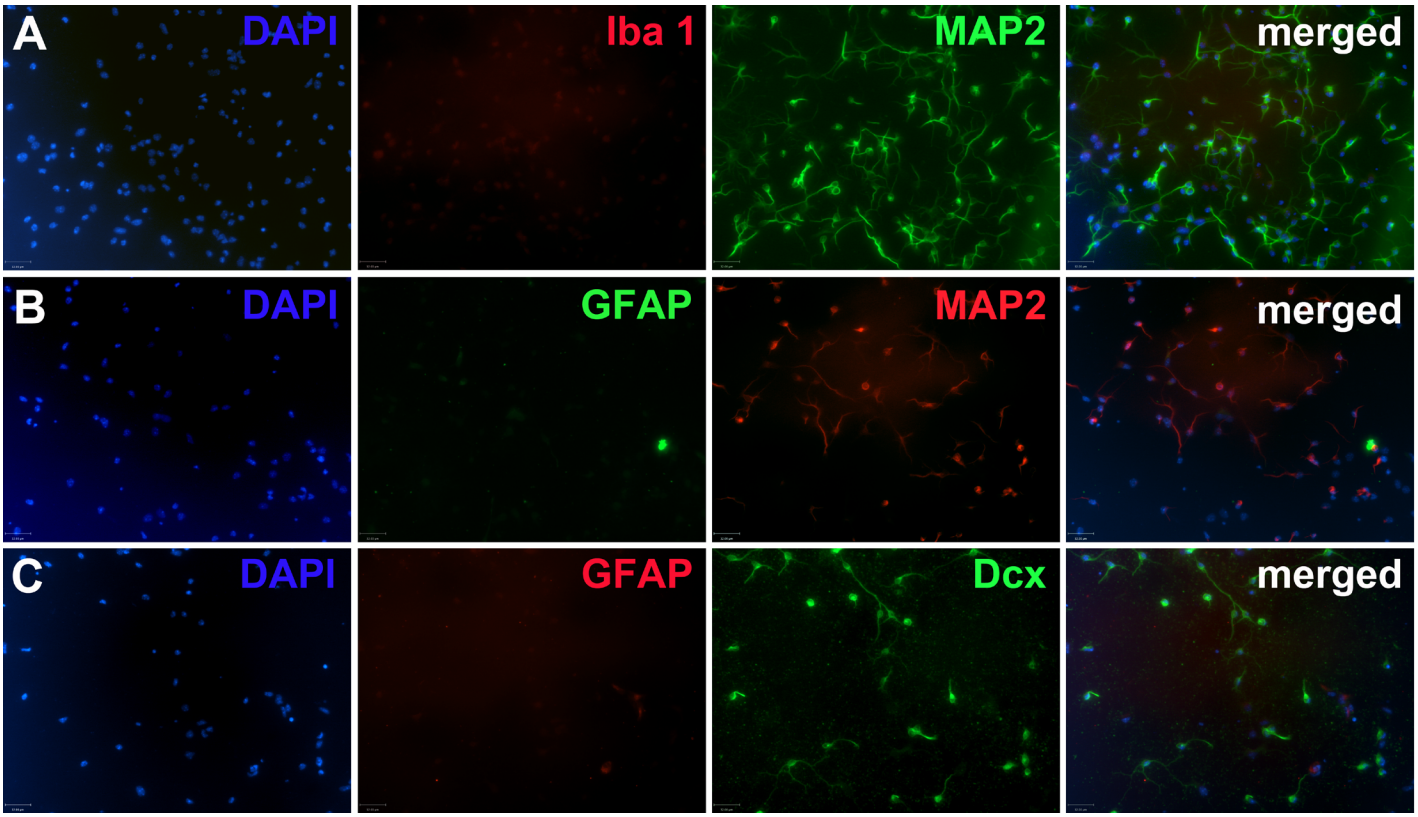

Figure 3. Purity of neurons after magnetic isolation and 2 days cultured *in vitro*. Cells were stained with DAPI (blue), Iba1 (red in A), MAP2 (green in A; red in B), GFAP (green in B; red in C), Dcx (green in C). The last panels are the merged images.

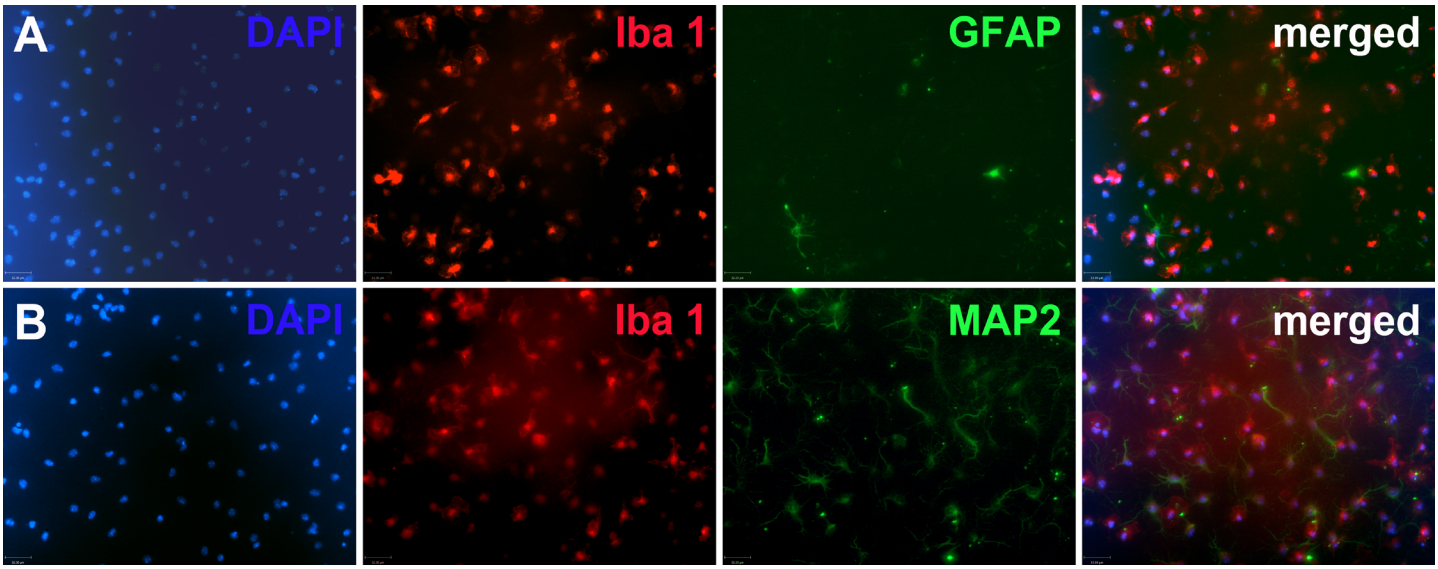

Figure 4. Non-neuronal cells after magnetic isolation and 2 days cultured *in vitro*. Cells were stained with DAPI (blue), Iba1 (red), MAP2 (green in B), GFAP (green in A). The last panels are merged images.

Cell Viability

To increase the viability of the cells, fast processing is crucial. After the dissociation steps, using pre-cooled solutions and a cooled centrifuge can help to protect the cells.

Many dead cells in the neuronal fraction: The sorting of neurons is based on negative depletion of the non-neuronal cells. Dead cells that are not labeled and removed with the Non-Neuronal Cells Biotin-Antibody Cocktail could be left in the flow through with the neuronal fraction. Due to non-specific staining, dead cells can be labeled and retained by the microbeads as well. To increase this effect of non-specific removal of dead cells the antibody concentration can be increased. The use of LD Columns (Miltenyi Biotec, # 130-042-901) can additionally help to sort out the dead cells, since it creates a higher magnetic gradient and thereby retains labeled cells more effectively. However a higher loss of neurons to the retained fraction due to non-specific staining has been observed which may result in lower yield.

Purity

Using a different type of separation column can help to improve the purity. Expected number of labeled cells and capacity of the column should be taken into consideration. The design of the LD Column is optimized for negative depletion of cells and could improve the purity of the neuron isolation. However, cell yield could be suffered by higher cell loss.

CONCLUSIONS

While isolation of ACSA-2-positive astrocytes has been tested for mice younger than eight days (< P8) [9], and isolation of "untouched" neurons was only tested from P1 mice [10], our protocol shows that astrocytes and neurons could be successfully isolated from P9 - P10 CD-1 mice. Our magnetic cell sorting technique allows simultaneous preparation of fresh, pure and viable astrocytes and neurons from the same injured neonatal mouse brain, rather than from different brain samples, and thereby provides the opportunity to investigate the properties and interplay of viable cells directly ex vivo. These freshly isolated cells could be used immediately for downstream applications, such as FACS or fluorescence microscopy for further characterizing subpopulations and activation states of cells; real-time PCR or western blotting for evaluation of induced gene transcription and translation patterns in individual cell types. These applications represent more closely the behavior of the cells *in vivo* at the time of isolation than the *in vitro* injury paradigm on primary cells. It can help us understand the role of one or more particular cell types in physiology and shed light into highly complex cellular based injury responses in a disease setting.

LIMITATIONS

1. Although the purity of astrocytes (92%) and neurons (85.79%) is high with our procedure, we do see contamination of other cell types (DAPI-positive cells without staining of GFAP or MAP2/Dcx). These could be either other subpopulations of astrocytes that do not express GFAP or other neuron subtypes that do not express MAP2/Dcx, or other cells types resulting from non-specific labeling. FACS analysis using more cell specific markers would help identify these cells.
2. There are some MAP2-positive cells in the populations of "Non-neurons", which suggests that the efficiency of neuron-sorting is not high enough, although the yield of neurons is good enough for subsequent downstream analysis (**Fig. 4**). This could be improved by repeating the steps of non-neuronal cell depletion to recover all neurons remained in the non-neuronal fractions.
3. Although ACSA-2 is a specific marker for astrocytes, it co-expresses with other common astrocyte markers such as GLAST (EAAT-1), GFAP and S100β [9]. The question remains whether ACSA-2 labels all astrocytes in our brain samples or whether it represents a subpopulation of astrocytes. The heterogeneity of astrocytes is poorly understood; therefore, further work is needed for a comprehensive classification of astrocyte subtypes and neuron subtypes in our preparations.

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