Isolation of sex specific PND 1/2 rat cortical primary astrocytes

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We use this protocol and it's working

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ABSTRACT

Many xenobiotic metabolizing enzymes, such as P450s, SULTs, UGTs, and sulfatases, are expressed in the brain, including astrocytes, the most abundant cell type in the brain. To obtain a purified culture of primary astrocytes, a method was developed and confirmed through flow cytometry. Primary glial cells are isolated separately from male and female postnatal day 1/2 (PND1/2) rat pups to isolate developing astrocytes. Sex is first determined by the anogenital distance and then confirmed through PCR followed by gel electrophoresis. Once isolated, the medium is changed every 3-4 days with additional incubation at room temperature for 10 min on a plate shaker before aspirating media to release microglia. When flasks reach confluency, cells are passaged and reseeded with 2 x 10^5 cells per flask. Once the cells reached passage 3, or appear to be confluent with only astrocytes, flow cytometry is performed utilizing Anti-Iba1 and GFAP primary antibodies.

MATERIALS

Chemical List:
S-MEM (minimal essential medium with Earle's salts, modified for suspension cultures; ThermoFisher, 11380-037)
Pen/strep (Live Technologies; 100 U/mL penicillin, 100 µg/L streptomycin final; ThermoFisher, 10378-016)
3 U/mL dispase (ThermoFisher, 17105-041)
MEM (minimal essential medium with Earle's salts; ThermoFisher, 11095080)
Heat-inactivated horse serum (ThermoFisher, 26050-088)
15000 U DNase I from bovine pancreas (type IV; Sigma 69182)
Sigmacote (Sigma, SL2-100ML)
Trypan blue (ThermoFisher, 15250-061)
Extract-N-Amp Tissue PCR kit (Millipore Sigma, XNAT2)
DNase- and RNase-free water (ThermoFisher, 10977-015)
Agarose (ThermoFisher, 18300-012)
10x TAE buffer(ThermoFisher, 15558-042)

EtBr (ThermoFisher, 15585-011)
DNA ladder for gel electrophoresis (ThermoFisher, 10416-014)
Gel electrophoresis loading buffer (ThermoFisher, 10816015)
Anti-Iba1 antibody (EPR16588) (ab178846) for flow cytometry (Abcam, (EPR16588) (ab178846))
Anti-GFAP (Cell Signaling 3670) (Cell Signaling Technology, 3670T)
Donkey Serum (Millipore Sigma, D9663-10ML)
Goat serum (ThermoFisher, 50197Z)
DPBS (ThermoFisher, 14190144)
1x Trypsin EDTA (0.25%) (ThermoFisher, 25200056)
Paraformaldehyde (diluted to 2%) (ThermoFisher, J19943.K2)
0.05% Triton-X in PBS (ThermoFisher, HFH10)
Goat anti-rabbit 488 (Life Tech) (diluted 1:500) (ThermoFisher, A-11008)
Donkey anti-mouse 647 (Life Tech) (diluted 1:200) (ThermoFisher, A-31571)
Autoclaved water

Accessories:
Filter flask (150 mL, 0.2 µm pore size, cellulose acetate) (Corning, 431154)
Filter flask (1 L, 0.2 µm pore size, cellulose acetate) (Corning, 431098)
0.2 µm syringe filter (cellulose acetate) (Millipore Sigma, WHA10462200)
Pasteur pipets (Fisher Scientific,22-183632)
Cotton-tipped swabs (1 per Pasteur pipet)(Fisher Scientific, 22-363-157)
Fine-angled micro-dissecting scissors (Fisher Scientific, 08-953-1B)
Forceps for dissection(Fisher Scientific, 08-953F)
Scissors for dissection (Fisher Scientific, 13-804-18)
Sterile gauze pad (1 per excised brain) (Fisher Scientific, 19-090-729)
50 mL conical polypropylene tube (1 per brain section or 1 per 10 brains from PND1/2) (Fisher Scientific, 14-432-22)
15 mL conical polystyrene centrifuge tubes (5 per 50 mL beaker) (Fisher Scientific, 14-959-53A)
10 mL glass serological pipet (Fisher Scientific, NC9868325)
T75 cell culture flasks (Fisher Scientific 50-809-260)
1.5 mL centrifuge tube (1 per pup) (Fisher Scientific, 05-408-129)
12x75 mm falcon tubes (Corning, 352052)
50 mL beaker with stir bar autoclaved (1 per 50 mL conical polypropylene tube)
Ice bucket

Instruments:
Stir plate (FisherSci 11520495H)
37°C, 95% air/5% CO2, 95% relative humidity cell culture incubator (Hera cell 150, 51026334)
Inverted phase-contrast microscope (Olympus CKX41)
Eppendorf centrifuge 5810 R (5811 08785)
Swinging bucket rotor (Eppendorf A-4-62 Swing Bucket Rotor; Fisher Scientific 05-

414-138)
Hot water bath set to 37ºC (Fisher Scientific, Isotemp Model 2320)
Autoclave (Easter Services, Inc. 1620E)
Portable power pipettor (Eppendorf easypet 4430000018)
Gel electrophoresis power supply, instrument, and combs (ThermoFisher PS0300; ThermoFisher A2-BP)
Hot water bath set to 95ºC (Fisher Scientific, Isotemp 210, 15-462-10)
Biosafety cabinet (The Baker Company, SG403)
BD Accuri™ C6 Flow Cytometer (BD Biosciences, 660519)
BD Accuri C6 Software
Bunsen burner
Microwave

Before you begin

1 Prepare **complete S-MEM**

1.1 Add 5 mL pen/strep solution to the 500 mL S-MEM.

1.2 Store complete S-MEM medium for up to 2 months at 4ºC

2 Prepare **dissociation medium** and warm to 37ºC - MUST MAKE FRESH EACH ISOLATION DAY. Prepare 10 mL of dissociation medium per sex per litter of PND1/2 pups (20 mL total).

2.1 Add **187.5 U dispase for cortex isolation** per 125 mL complete S-MEM in an autoclaved container.

2.2 Sterilize with a 150 mL sterile filter flask (0.2 µm pore size, cellulose acetate)

2.3 Prewarm in 37ºC hot water bath for 1 hour before use.

3 Prepare **astrocyte growth medium.**

3.1 Add 900 mL MEM, 10 mL pen/strep, and 100 mL heat-inactivated horse serum to a 1 L sterile filter flask.

3.2 Sterilize with a 1 L sterile filter flask (0.2 µm pore size, cellulose acetate) by pulling vacuum through the apparatus.

3.3 Store the astrocyte growth medium for up to 1 month at 4ºC.

4 Prepare **DNase I solution, 8000 U/mL.**

4.1 Add 15,000 U DNase I from bovine pancreas (type IV) to 1.875 mL autoclaved water.

4.2 Mix gently, do NOT vortex. DNase I from pancreas is vulnerable to inactivation by physical damage and should not be vortexed.

4.3 Sterilize with a 0.2 µm syringe filter (cellulose acetate).

4.4 Store in 100 µL aliquots at -20ºC.

5 Fire polish and Sigmacote treatment of pipets for cell isolation.

5.1 **Fire polish the tip of a 9-in Pasteur pipet by placing the small end in an open flame from a Bunsen burner for a few seconds.** This will slightly melt the glass and will produce a smooth tip to the pipet, which helps prevent damage to the cells. However, if the opening becomes too small, increased shear forces during titration may lead to increased cell damage.

5.2 Plug the large end of the fire-polished Pasteur pipets with cotton removed from a cotton-tipped swab or, alternatively, small pieces of cotton balls.

5.3 Treat fire-polished Pasteur pipets and 10 mL glass, cotton-plugged serological pipets with Sigmacote by drawing the viscous Sigmacote solution up into the pipet with a portable power pipettor. Do not let the solution touch the cotton plug. Allow Sigmacote to drain back into the bottle, and allow the pipets to drain and dry completely in a beaker lined with paper towels.

5.4 Place Sigmacoted pipets in a metal container with lid and autoclave.

6 Autoclave fine-angled micro-dissecting scissors, large scissors, and forceps in a beaker wrapped with aluminum foil.

Brain and Tail Dissection

7 Sex the pups based on anogenital distance (females will have a much shorter distance than males, compare pups to one another to be able to sex pups efficiently).

8 For pups at PND1/2, use between 8 and 15 pups and **decapitate using sterile large scissors.**

9 Place the head on a sterile gauze pad. Each head will be placed on a separate gauze pad.

10 Take a tail snip of approximately 0.5-1 cm (too much tissue may not be digested with the DNA extraction kit in the steps to follow) and place the tail in a 1.5 mL centrifuge tube, label the tube according to the sex of the pup, and then place it on ice.

11 Remove brain from head.

11.1 Secure the head by holding down the snout.

11.2 With fine-angled micro-dissecting scissors, cut the skin along the midline from the base of the skull to the eyes.

11.3 Use 0.8 mm forceps to separate skin and expose the skull as necessary.

11.4 Cut skull along midline with micro-dissecting scissors.

11.5 Cut away the skull flaps for easier removal of the brain, remove the brain with spatula, and place the brain on a sterile gauze pad.

Brain Tissue Dissection for Cortex

12 Split the brain in half down the midline to separate each hemisphere from one another. Place each hemisphere with the inside facing up to examine the hippocampus on the sterile gauze pad.

13 Gently remove darker hippocampal crescents with forceps (dissecting microscope can be used here to assist in locating the different brain regions).

14 Remove remaining meninges with forceps (look for any veins and flip the brain section over a few times to use the sterile gauze pad to help remove the meninges).

15 Place sections in a sterile 50 mL conical polypropylene tube containing 10 mL complete S-MEM on ice labeled with the proper sex

16 Repeat steps, placing all cortical sections in a single 50 mL tube on ice per sex (all pups from the same litter with the same sex can be placed in the same 50 mL tube).

Confirm sex of pups

17 Add 100 µL extraction solution from Extract-N-Amp Tissue PCR kit to each centrifuge tube containing a small tail snip for a pup (collected in step 10).

18 Add 25 µL tissue preparation solution from Extract-N-Amp Tissue PCR kit

19 Incubate at room temperature for 10 min

20 Incubate in 95ºC hot water bath for 3 min

21 Remove from hot water bath and add 100 µL neutralization solution B from Extract-N-Amp Tissue PCR kit

22 Vortex

23 Remove tail snip and discard

24 Store tissue extract at 4ºC or use for PCR immediately

25 PCR amplification of *Sry* gene

25.1 Prepare master mix with the following per tissue extract: **10 µL** extract-N-amp PCR reaction

mix, **4 µL** RNase- and DNase-free water, **1 µL** of 25 µM forward primer (5'- TAC AGC CTG AGG ACA TAT TA -3'), **1 µL** of 25 µL reverse primer (5'- GCA CTT TAA CCC TTC GAT GA -3'). An autosomal gene, *Actb* that encodes for beta-actin was used as control with the following set of primers: Forward: 5'- AGC CAT GTA CGT AGC CAT CC -3'; Reverse: 5'- TGT GGT GGT GAA GCT GTA GC -3'.

25.2 To PCR tubes labeled for each tail snip extract, add 16 µL of master mix

25.3 Add 4 µL tissue extract to each designated PCR tube containing the master mix

26 Run PCR program

26.1 94ºC for 5 min

26.2 35 cycles of: 94ºC for 30 seconds, 50ºC for 30 seconds, and 72ºC for 1 min

26.3 72ºC for 7 min

27 Prepare 1% agarose gel

27.1 Prepare 1x TAE buffer by diluting 10x TAE buffer with DI water (1:10).

27.2 Add 1 g agarose to 100 mL 1x TAE buffer

27.3 Microwave in 1 min intervals, swirling the flask each min until clear

27.4 Let agarose cool to ~50ºC (warm to touch and able to grab)

27.5 Add 2 µL EtBR or use another nucleic acid stain, such as GelRed per manufacturer's instructions such that final stain concentration is 1x.

27.6 Pour gel into gel tray with well comb(s) in place

27.7 Let cool at room temperature for 1 hour (until solidified)

27.8 When solidified, remove the combs carefully without damaging the gel.

27.9 Orient the gel in the gel electrophoresis apparatus such that the combs are placed towards the cathode. The DNA will be run from cathode to anode.

28 Fill the gel electrophoresis box with 1xTAE buffer until the gel is fully covered

29 To each PCR-amplified DNA sample (20 µL), add 4 µL of loading dye (6x concentration).

30 Carefully load 10 µL of the ladder followed by samples to each well

31 Run gel at 100 V for 1 hour

32 Turn the power off and disconnect the electrodes

33 Visualize under UV light to confirm sex of pups. The samples for males should show a band for *Sry* gene, amplified product of 317 bp while all samples should show a band for *Actb* gene, amplified product of 220 bp.

Dissociate cells extraction

34 Carefully remove as much complete S-MEM as possible with a sterile, cotton-plugged, 9' Pasteur pipet, taking care to retain all pooled brain sections.

35 Add 7 mL **prewarmed dissociation medium** to a 50 mL sterile beaker with a stir bar and carefully pour sections into the beaker.

36 Gently triturate the sections 7-8 times using a Sigmacote-treated 10 mL serological pipet.

37 Stir for 10 min at low speed on a stir plate inside the biosafety cabinet.

38 While this is stirring, prepare 5 of the 15 mL centrifuge tubes for each extraction by adding 5 mL room temperature astrocyte growth medium to each tube.

39 Additionally, thaw DNase I solution and place on ice

40 After 10 min of gentle stirring, remove the beaker from the stir plate and place at ~45º angle for 3 min to allow the non-dissociated tissue to collect at the bottom of the beaker (e.g. using a 1.5 mL centrifuge tube holder).

41 Carefully aspirate the dissociated cells in medium with a Sigmacote-treated 10 mL glass serological pipet (take care not to remove the undissociated tissue pieces).

42 Place this suspension into a 15 mL centrifuge tube that contains astrocyte growth medium

43 Invert the 15 mL centrifuge tube 2-3 times to mix and then allow the tube to sit undisturbed during the continuing extractions.

44 Add 5 mL prewarmed dissociation medium to the 50 mL beaker containing undissociated tissue pieces.

45 Add 100 µL of 8000 U/mL DNase I solution

46 Stir for 10 min at low speed on a stir plate inside the biosafety cabinet.

47 After 10 min of gentle stirring, remove the beaker from the stir plate and place at ~45º angle for 3 min to allow the non-dissociated tissue to collect at the bottom of the beaker (using a 1.5 mL centrifuge tube holder works nicely here).

48 Carefully aspirate the dissociated cells in medium with a Sigmacote-treated 10 mL glass serological pipette (take care not to remove the undissociated tissue pieces).

49 Place this suspension into a 15 mL centrifuge tube that contains astrocyte growth medium.

50 Invert the 15 mL centrifuge tube 2-3 times to mix and then allow the tube to sit undisturbed during the continuing extractions.

51 Add 5 mL prewarmed dissociation medium to the 50 mL beaker containing undissociated tissue pieces. Do not add any more DNase I solution for the remaining extractions.

52 Once you have dissociated a total of 3 times, remove undissociated tissue from the 15 mL tubes by inserting a sterile, fire-polished, cotton-plugged, Sigmacote-treated 9" Pasteur pipette to the

bottom of the tube and carefully aspirate the undissociated tissue.

53 Place this tissue back into the 50 mL beaker and perform the final two extractions, adding another 5 mL prewarmed dissociation medium to the 50 mL beaker each time.

Pellet Cells

54 Pool dissociated cells and medium from all 15 mL centrifuge tubes into a singular 50 mL conical tube.

55 Centrifuge for 10 min at 1000 rcf at 4ºC, in a swinging bucket rotor to pellet the suspended cells.

56 Carefully aspirate medium from the cell pellet.

57 Resuspend in 20 mL **astrocyte growth medium** by gently pipetting with a Sigmacote-treated 10 mL glass serological pipette.

58 Allow cells to sit for 5 min and then discard any sedimented tissue from the bottom of the tube by using a fire-polished Sigmacoted 9" Pasteur pipette.

Assess cell number and viability

59 Gently mix 100 µL cell suspension with 100 µL of 0.08% trypan blue staining solution

60 Allow cells to take up the trypan blue for 3 min

61 Determine the total cell number and cell viability with a hemacytometer and an inverted phase-contrast microscope (intact cells are able to exclude trypan blue while dead or damaged cells retain the dye).

Plate and grow cells

62 Plate 5+ T75 cell culture flasks with $7.5x10^6$ cells per flask in 9 mL astrocyte growth medium.

63 Place plated cells in a 37°C, 95% air/5% CO2, 95% relative humidity incubator.

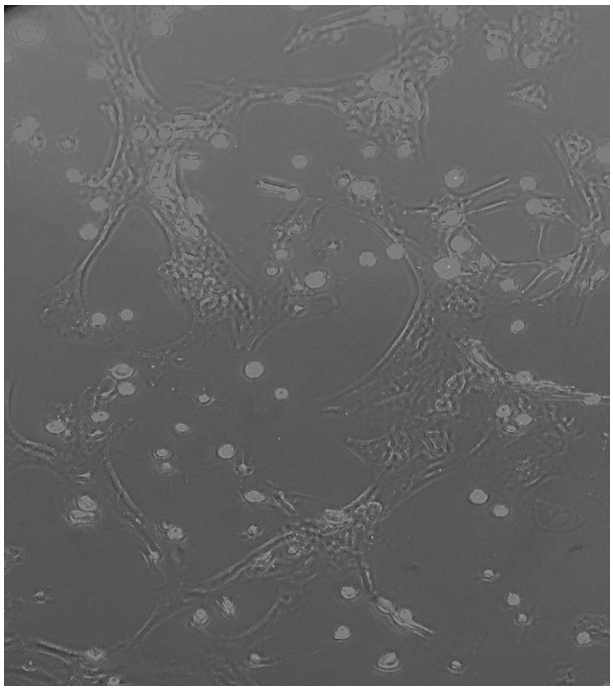
64 After 18-24 hours, remove the old growth medium with a sterile 5" Pasteur pipette attached to a vacuum source.

65 Add 9 mL of fresh astrocyte growth medium to each flask.

66 Change medium twice per week (every 3-4 days) as described below.

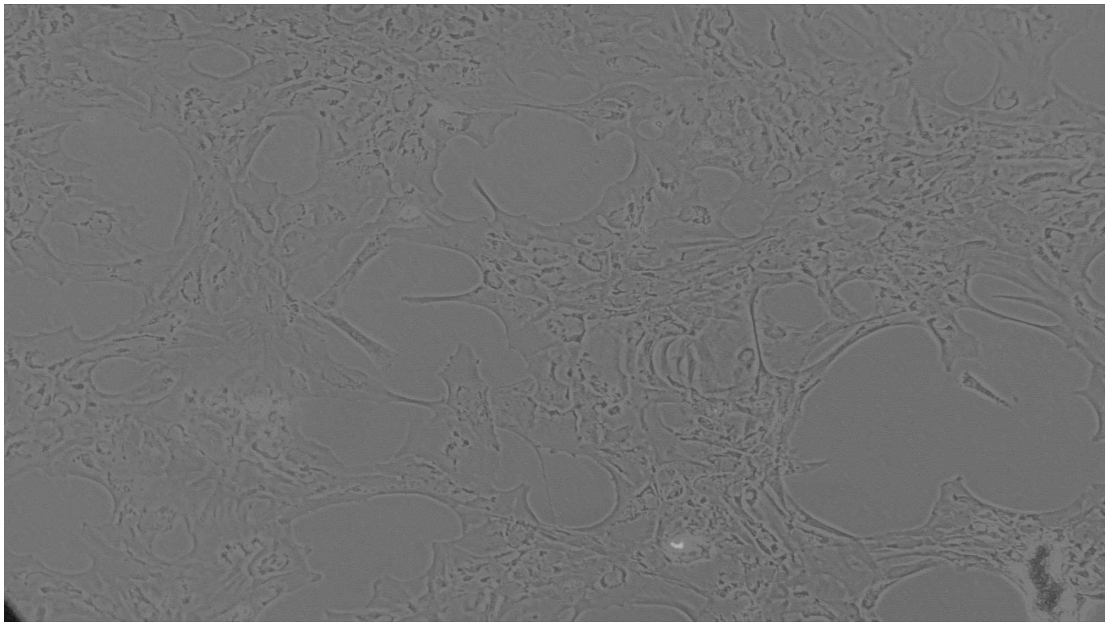
66.1 For primary astrocytes that are passage 0-2 (P0-P2), check cells under the microscope, shake flasks for 10 minutes at a medium-low speed on a plate shaker, check cells under the microscope again to make sure astrocytes are still adhering to the flask while the microglia have popped off, aspirate media, add 5 mL DPBS per T75 flask, rinse plate well by moving

DPBS back and forth across flask bottom, aspirate, add 9 mL growth media per T75 flask, and then place back in the 37°C, 95% air/5% CO2, 95% relative humidity incubator.



Glial cells. All round cells are microglia that will "pop" off the flask with shaking.

66.2 For primary astrocytes that are either confluent astrocytes, or are passage 3+ (P3+), check cells under the microscope, aspirate, add 9 mL growth media per T75 flask, check cells once again under the microscope, and then place back in the 37°C, 95% air/5% CO2, 95% relative humidity incubator.



Pure astrocyte culture.

Flow Cytometry to confirm purity of astrocytes

67 Harvest and wash cells for flow cytometry

67.1 Aspirate media from T75 flask

67.2 Rinse plate with 10 mL DPBS

67.3 Aspirate DPBS

67.4 Add 2 mL 37ºC 1x trypsin EDTA (0.25%)

67.5 Incubate for 5 min in a 37°C, 95% air/5% CO2, 95% relative humidity incubator

67.6 Tap sides of flask to allow cells to detach

67.7 Add 10 mL **FACS media** (DPBS + 2% heat-inactivated horse serum)

67.8 Centrifuge at 4000 rcf at 4ºC, in a swinging bucket rotor for 10 minutes to pellet cells

67.9 Aspirate media

67.10 Resuspend in approximately 1 mL of fresh FACS media

67.11 Adjust cell number to a concentration of 1-5x10^6 cells/mL

67.12 Transfer 200 uL cell suspension to polystyrene round-bottom 12x75 mm falcon tubes

67.13 Centrifuge at 4000 rcf at 4ºC, in a swinging bucket rotor for 10 minutes to pellet cells

67.14 Add 200 uL/well paraformaldehyde (2%)

67.15 Incubate at room temperature for 15 min on shaker

67.16 Centrifuge at 4000 rcf for 5 min at 4ºC

67.17 Aspirate media using a fire-polished, cotton-plugged Pasteur pipette

67.18 Permeabilize (remove more cellular membrane lipids to allow large molecules like antibodies to get inside the cell easily) with 200 uL 0.05% Triton-X (in PBS)

67.19 Incubate at room temperature for 5 min on shaker

67.20 Centrifuge at 4000 rcf at 4ºC, in a swinging bucket rotor for 5 minutes to pellet cells

67.21 Aspirate media using a fire-polished, cotton-plugged Pasteur pipette

68 Blocking the non-specific binding of the staining antibody reagents

68.1 Prepare block solution: 1% donkey serum, 1% goat serum, 1x PBS; 490 µL PBS + 5 µL donkey serum + 5 µL goat serum

68.2 Add 100 µL blocking solution to each washed tube

68.3 Block for 20 min on ice

68.4 Centrifuge at 4000 rcf at 4ºC, in a swinging bucket rotor for 5 minutes to pellet cells

68.5 Aspirate blocking solution using fire-polished, cotton-plugged Pasteur pipet

69 Addition of primary antibody

Add 0.1-10 µg/mL (100 uL) of each primary labeled antibody (we use primary antibody diluted

69.1 1:200 in blocking solution).

69.2 Incubate primary antibody for at least 30 min at room temperature

69.3 Centrifuge at 4000 rcf at 4ºC, in a swinging bucket rotor for 5 minutes to pellet cells

69.4 Aspirate primary antibody solution using fire-polished, cotton-plugged Pasteur pipette

70 Addition of secondary antibody

70.1 Dilute secondary antibodies in blocking solution according to dilution according to dilution factor listed in materials.

70.2 Resuspend cells in secondary antibodies (100 uL each)

70.3 Incubate for 1 hour at room temperature in the dark (cover with aluminum foil and turn lights off

71 Wash 3x

71.1 Centrifuge at 4000 rcf at 4ºC, in a swinging bucket rotor for 5 minutes to pellet cells

71.2 Aspirate antibodies using fire polished, cotton-plugged Pasteur pipette

71.3 Resuspend in 200 µL of ice-cold FACS media

72 Analyze on flow cytometer as soon as possible. Flow cytometry data analysis will involve identifying a population of cells through subsequent steps called gating and subjecting that population to various parameters through subsequent gating steps.

72.1 First, make forward scatter (FSC-A) and side scatter (SSC-A) density plots for the cell events using the BD Accuri C6 Software. The first gate will be applied to remove debris (dense accumulation of events on the bottom left corner). Thus, the gate will select all events measured by the flow cytometer except the debris, these events should all therefore be cells.

72.2 In the subsequent density plot of FSC-A x Width, the single cells are gated and selected, removing any clumps of cells from analysis.

72.3 Followed by this, the selected cells are analyzed for each of the cell-specific markers. First, microglia-specific marker Iba-1 was assessed by selecting an appropriate channel on the flow cytometer that is suited for the goat anti-rabbit 488 antibody detection. The Iba-1 positive cells are expected to be seen in the top left quadrant of the FSC-A x Iba-1 density plot.

72.4 Similarly, in the FSC-A x GFAP plot, GFAP-positive cells (astrocytes) are expected in the top left quadrant.

72.5 By combining the gates from steps 72.3 and 72.4, both cell populations can be viewed on the same plot. Gates are applied as shown to determine astrocyte purity.