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# Concomitant isolation of primary astrocytes and microglia for protozoa parasite infection

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October 30th, 2019

To:
**Jaydev Upponi, Ph.D.**
Science Editor, Journal of Visualized Experiments (JoVE)
&
**Alisha DSouza, Ph.D.**
Senior Review Editor, Journal of Visualized Experiments (JoVE)

Dear Dr. Upponi and Dr. DSouza,

We have revised our manuscript according to the comments of the Reviewers. A list of point-by-point replies is included.

We believe we have a significantly improved manuscript with all the points raised by reviewers addressed during the round of revision. We hope you find this re-revised version suitable for publication in the *Journal of Visualized Experiments* (JoVE)

Yours sincerely,

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**TITLE:**

# Concomitant Isolation of Primary Astrocytes and Microglia for Protozoa Parasite Infection

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**KEYWORDS:**

Glia cells, astrocytes, microglia, protozoa infection, *T. cruzi*, *T. gondii*

**SUMMARY:**

The overall goal of this protocol is to instruct how to extract, maintain, and dissociate murine astrocyte and microglia cells from the central nervous system, followed by infection with protozoa parasites.

**ABSTRACT:**

Astrocytes and microglia are the most abundant glial cells. They are responsible for physiological support and homeostasis maintenance in the central nervous system (CNS). The increasing evidences of their involvement in the control of infectious diseases justify the emerging interest in the improvement of methodologies to isolate primary astrocytes and microglia in order to evaluate their responses to infections that affect the CNS. Considering the impact of *Trypanosoma cruzi* (*T. cruzi*) and *Toxoplasma gondii* (*T. gondii*) infection in the CNS, here we provide a method to extract, maintain, dissociate and infect murine astrocytes and microglia cells with protozoa parasites. Extracted cells from newborn cortices are maintained in vitro for 14 days with periodic differential media replacement. Astrocytes and microglia are obtained from the same extraction protocol by mechanical dissociation. After phenotyping by flow cytometry, cells are infected with protozoa parasites. The infection rate is determined by fluorescence

microscopy at different time points, thus enabling the evaluation of differential ability of glial cells to control protozoan invasion and replication. These techniques represent simple, cheap and efficient methods to study the responses of astrocytes and microglia to infections, opening the field for further neuroimmunology analysis.

**INTRODUCTION:**

The CNS is mainly composed of neurons and glial cells[1–3]. Microglia and astrocytes are the most abundant glia cells in the CNS. Microglia, the resident macrophage, is the immunocompetent and the phagocytic glia cell in the CNS[3,4], while astrocytes are responsible for maintaining homeostasis and exert supportive functions[5].

Despite glial cells being classically known to be responsible for the support and protection of neurons[6,7], emerging functions of these cells have been described in the recent literature, including their responses to infections[8–11]. Thus, there is a push to develop methods to isolate these glial cells to understand their functions individually.

There are some alternative models to study glial cells rather than primary cultures, like immortalized cell lineages and in vivo models. However, immortalized cells are more likely to undergo genetic drifting and morphological changes, while in vivo studies impose limited manipulation conditions. Conversely, primary cultures are easy to handle, better resemble in vivo cells and also allow us to control experimental factors[12,13]. Here, we describe guidelines on how to extract, maintain and dissociate murine astrocytes and microglia primary cells in the same protocol. Furthermore, we also provide examples on how to work with protozoa infection in these cultures.

CNS cells extracted from neonatal mice (up to 3-day-old) were cultured for 14 days on differential media that allows the preferential growth of astrocytes and microglia cells. Since microglia rest above the attached astrocytes, cell populations were mechanically dissociated in an orbital incubator. Next, we collected all the supernatant containing microglia and added trypsin to detach astrocytes. Isolated glial cells were phenotypically evaluated by flow cytometry and plated according to the desired experiment.

We also provided examples on how to infect these isolated microglia and astrocytes with protozoa parasites. *T. gondii* is a highly neurotropic protozoan responsible for toxoplasmosis[14], while *T. cruz*i is responsible for Chagas disease which can leads to development of neurological disorders in the CNS[15,16]. Furthermore, it has also been reported that infection with *T. gondii*[17,18] or *T. cruzi*[19–21] were the presumable cause of death in immunocompromised patients. Therefore, the elucidation of immunologic role of glial cells from the CNS in controlling protozoa infections is of great importance.

**PROTOCOL:**

All experimental procedures involving mice were carried out in accordance to the Brazilian National Law (11.794/2008) and approved by the Institutional Animal Care and Use Committees (IACUC) of the Federal University of São Paulo (UNIFESP).

## 1. Glial cells extraction, maintenance and dissociation

NOTE: The number of mice used for the glial cells extraction depends on the quantity of cells required to perform the desired experiments. In this protocol, a total of 2.7 x $10^7$ astrocytes and 4 x $10^6$ microglia were obtained from six neonatal C57BL/6 mice. All procedures were performed under sterile condition in a class II biosafety cabinet.

### 1.1. Day 1

1.1.1. Prepare pure Hank's balanced salt solution (HBSS) and HBSS + 10% of heat-inactivated fetal bovine serum (FBS) (see **Table of Materials**).

1.1.2. Prepare supplemented Dulbecco's Modified Eagle Medium (DMEM)/F12 (10% FBS + 0.08 mM Penicillin + 0.09 mM Streptomycin + 12.5 mM HEPES + 30 mM sodium bicarbonate, pH 7.2) and filter it with a 0.22 µm filter (see **Table of Materials**).

NOTE: All culture media should be stored at 4 °C, but it is necessary to prewarm them at 37 °C for 20 min before starting the experiment.

1.1.3. Sterilize all surgical instruments (scissors, spatula and tweezers) in an autoclave and use 70% ethanol during the procedure.

1.1.4. Put newborns (up to 3-day-old) mice in a sealed chamber containing cotton soaked with isoflurane for 5 min for profound anesthesia.

1.1.5. Spray the mouse pup with 70% ethanol and decapitate the animal with scissors.

1.1.6. Make sagittal cut (posterior to anterior) with scissors along the cranium to open it and expose the brain. Separate the skull from the brain using tweezers. Remove the brain using a micro spatula to maintain the brain integrity.

1.1.7. Place the brain in a dry Petri dish (6 cm diameter). Remove the olfactory bulb and cerebellum using a micro spatula. Move the cortex to another Petri dish (6 cm diameter) containing HBSS + 10% FBS (2 mL/Petri dish).

1.1.8. Cut the brain tissue into small pieces with sterile scissors and using a p1000 micropipette transfer each brain tissue with HBSS + 10% FBS to different 15 mL conical tubes. Make sure that the final volume is 2 mL/tube. If not, complete with HBSS + 10% FBS.

NOTE: The protocol can be paused here. If so, conical tubes with CNS tissue must be placed on ice up to 1 h.

1.1.9. Wash the cut brain tissue with 3 mL of HBSS + 10% FBS (per tube) and after decantation.

Carefully remove the supernatant. Repeat this step 3 times.

NOTE: This step aims to remove the debris and to recover the extracted tissue.

1.1.10. Wash the cut brain tissue with 3 mL of pure HBSS (per tube) and after decantation, carefully remove the supernatant. Repeat this step 3 times.

NOTE: This step aims to remove the remaining serum from the previous washes, as cells will be trypsinized in the next step.

1.1.11. Add 3 mL of trypsin per tube and place in a water bath at 37 °C for 30 min, gently shaking the tubes every 5 min. Avoid bubbles by inverting or abruptly mixing the tubes.

NOTE: This step aims to digest the collected tissue.

1.1.12. To inactivate the trypsin, wash the tissue with 3 mL per tube of HBSS + 10% FBS and, after decantation of the tissue, carefully remove the supernatant. Repeat this step 3 times.

1.1.13. Wash the tissue with 3 mL per tube of pure HBSS and carefully remove the supernatant. Repeat this step 2 times.

1.1.14. Add 7 mL per tube of pure HBSS and homogenize the tissue through successive passages in pipettes: first with the 10 mL serological pipette, followed by the 5 mL and finally with the p1000 micropipette.

1.1.15. After homogenization, centrifuge tubes at 450 x *g* for 5 min at 4 °C.

1.1.16. Discard the supernatant and resuspend the pellet with 4 mL of HBSS + 10% FBS per tube.

1.1.17. Transfer cells to a pretreated T-75 flask for optimal cell culture adhesion (see **Table of Materials**).

NOTE: Process tissue from each animal per flask.

1.1.18. Place the flask in the incubator at 37 °C and 5% $CO_2$ for 30 min for adherence.

1.1.19. Add 10 mL of supplemented DMEM/F12 per flask and incubate at 37 °C and 5% $CO_2$.

NOTE: The final volume is 14 mL per flask.

**1.2. Day 3**

1.2.1. Remove 7 mL of culture medium from the T-75 flask and add 7 mL of fresh supplemented DMEM/F12.

NOTE: The flasks will contain a lot of cellular debris and a cloudy appearance.

**1.3. Day 5**

1.3.1. Remove all the medium from the T-75 flask and add 14 mL of fresh supplemented DMEM/F12.

NOTE: Medium is replaced from the flasks to remove debris and non-adherent cells.

1.3.2. After each 48 h, remove 6 mL of the supernatant and add 7 mL of fresh supplemented DMEM/F12 medium until day 14 of culture.

**1.4. Day 14**

1.4.1. After removing 6 mL of the supernatant and adding 7 mL of fresh supplemented DMEM/F12 medium, close the T-75 flasks tightly.

1.4.2. Place them in the floor orbital shaker at 200 rpm and 37 °C overnight to mechanically dissociate microglia from astrocytes.

**1.5. Day 15**

1.5.1. Take the flasks from the shaker and vigorously wash them with their own medium in order to optimize cell dissociation and harvest the maximum number of microglia. Then, collect the supernatant (containing microglia) and transfer to a 50 mL conical tube.

1.5.2. Next, add 4 mL of trypsin into each flask and incubate for 5 min at 37 °C in order to detach the astrocytes.

1.5.3. Add 5 mL per flask of supplemented DMEM/F12 to inactivate the trypsin. Wash the flasks with their own medium and transfer contents to a 50 mL conical tube.

1.5.4. Centrifuge all tubes containing dissociated microglia and astrocytes at 450 x *g* for 5 min at 4 °C.

1.5.5. Discard the supernatant. Resuspend the microglia pellet in 1 mL and the astrocytes in 10 mL of supplemented DMEM/F12.

1.5.6. Proceed to cell counting in a Neubauer chamber or an automatic cell counter. Plate the cells with supplemented DMEM/F12 at the desired density in an appropriate flat bottom cell culture plate (pretreated for optimal cell attachment; see **Table of Materials**). Incubate cells at 37 °C and 5% $CO_2$ for 24 h to allow them to attach.

NOTE: Reserve 1.5 x $10^6$ of each cell population to confirm their purity by flow cytometry.

1.5.7. Perform a flow cytometric analysis to verify the purity of cell populations.

NOTE: We consider microglia $CD11b^+/CD45^+/GFAP^-$ and astrocytes $CD11b^-/CD45^-/GFAP^+$. Iba1 and TMEM119 staining might be considered in order to fully characterize microglia[22].

1.5.8. Add a FMO (Fluorescence Minus One)[23] and an unstained sample for each cell population (astrocytes and microglia) as controls of flow cytometry assay.

1.5.9. For each cell population, reserve 5 x $10^5$ cells for each stained and unstained samples. For FMO, reserve two other tubes of microglia containing 2.5 x $10^5$ cells each and also reserve another tube of 5 x $10^5$ astrocytes.

NOTE: Since microglia numbers can be a limiting factor, it is possible to use fewer cells for unstained and FMO controls.

1.5.10. Centrifuge all microtubes at 450 x *g* for 5 min at 4 °C. Discard the supernatant and resuspend the pellet with 500 µL/microtube of FACS buffer (phosphate-buffered saline (PBS) + 0.5% bovine serum albumin (BSA) + 2 mM EDTA, pH 7.2).

1.5.11. Centrifuge all microtubes at 450 x *g* at 4 °C for 5 min. Discard the supernatant and resuspend the pellet with 100 µL/microtube of anti-CD16/CD32 (clone 93) (1:50) diluted in FACS buffer. Incubate for 10 min at room temperature.

1.5.12. Add 500 µL/microtube of FACS buffer into all microtubes and centrifuge at 450 x *g* at 4 °C for 5 min. Discard the supernatant. Resuspend astrocytes and microglia staining tubes and also astrocyte FMO tube with 50 µL/microtube of surface markers mix: anti-CD45 (PE, clone 30-F11) and anti-CD11b (eFluor 450, clone M1/70) (both diluted at 1: 100 in FACS buffer).

1.5.13. For unstained cells, resuspend with the same volume of FACS buffer. For microglia FMO, incubate each microglia tube with anti-CD45 or anti-CD11b. Incubate all samples at 4 °C for 20 min in the dark.

1.5.14. Add 500 µL of FACS buffer per microtube. Centrifuge at 450 x *g* at 4 °C for 5 min. Discard the supernatant and resuspend the pellet with 100 µL/microtube of fixation buffer (see **Table of Materials**) for 20 min at room temperature in the dark.

1.5.15. Add 500 µL/microtube of permeabilization buffer 1x (see **Table of Materials**) and incubate at 4 °C for 5 min in the dark.

1.5.16. Centrifuge all the microtubes at 450 x *g* at 4 °C for 5 min. Discard the supernatant. Resuspend astrocytes and microglia staining tubes and also microglia FMO tubes with 50 µL/microtube of intracellular antibody anti-GFAP (APC, clone GA5) in a 1:100 dilution in 1x permeabilization buffer. For unstained cells and astrocyte FMO, resuspend the cells with the

same volume of FACS buffer. Incubate all samples at 4 °C for 30 min in the dark.

1.5.17. Wash all tubes by adding 500 µL/microtube of 1x permeabilization buffer. Centrifuge all the microtubes at 450 x *g*, for 5 min at 4 °C. Resuspend each microtube in 200 µL of FACS buffer. Acquire 1 x $10^5$ events/sample on the flow cytometer.

1.5.18. For analysis, cells must be gated first on CD11b x CD45 and then GFAP histogram.

NOTE: Unstained and FMO controls are useful to determine gates.

**2. Infection and evaluation of infection rates**

**2.1. Infection of glial cells with *T. gondii***

2.1.1. Plate 3 x $10^4$ microglia or astrocytes with supplemented DMEM/F12 at 37 °C and 5% $CO_2$ for 24 h in a pretreated 96-well flat plate (see **Table of Materials**).

2.1.2. Infect each cell population with tachyzoites from *T. gondii* RH strain expressing yellow fluorescent protein (YFP) at a multiplicity of infection (MOI) of 1:1 (parasite:cell) diluted in 200 µL/well of supplemented RPMI (3% FBS + 0.16 mM Penicillin + 0.18 mM Streptomycin + 12.5 mM HEPES + 30 mM sodium bicarbonate, pH 7.2) and filter it with a 0.22 µm filter (see **Table of Materials**). Incubate at 37 °C and 5% $CO_2$ for 48 h.

2.1.3. Then, discard the supernatant and fix the cells by adding 100 µL/well of 1% paraformaldehyde (PFA) diluted in PBS at 4 °C for 24 h.

2.1.4. Replace PFA witrh100 µL/well of PBS at pH 7.2.

2.1.5. Carefully remove the supernatant and stain cells' nuclei by pipetting 50 µL/well of DAPI (5 mg/mL) diluted in PBS pH 7.2 (1:1000) for 1 min at room temperature in the dark.

2.1.6. Replace DAPI staining solution with 100 µL/well of PBS (pH 7.2) and analyze it by fluorescence microscopy.

**2.2. Infection of glial cells with *T. cruzi***

2.2.1. Plate 3 x $10^4$ microglia or astrocytes with supplemented DMEM/F12 at 37 °C and 5% $CO_2$ for 24 h in a pretreated 96-well flat plate (see **Table of Materials**).

NOTE: For each time point of infection, use a different plate.

2.2.2. Infect the glial cells with trypomastigotes from *T. cruzi* Y strain at a MOI of 5:1 (parasites:cell) diluted in 200 µL/well of supplemented RPMI and incubate at 37 °C and 5% $CO_2$ for 2 h.

NOTE: This step is important for the cell invasion by the parasite.

2.2.3. Remove all of the supernatant and wash the wells by adding 200 µL/well of supplemented RPMI to remove all the extracellular parasites. Remove supernatant and add 200 µL/well of fresh supplemented RPMI.

NOTE: This step intends to remove all parasites that did not invade the adherent cells.

2.2.4. Incubate infected cells for 2 h, 48 h and 96 h to evaluate *T. cruzi* replication in glial cells[11].

2.2.5. After each time point, remove the supernatant and fix the cells with 100 µL/well of methanol for 15 min at room temperature and then replace methanol with 100 µL/well of PBS (pH 7.2).

2.2.6. After fixation, prepare the cells for the immunofluorescence assay as follows.

2.2.6.1. To avoid autofluorescence, treat the cells with 100 µL/well of 50 mM $NH_4Cl$ diluted in PBS (pH 8.0) for 15 min. Wash the cells by adding 100 µL/well of PBS and immediately remove it (repeat this step 3 times).

2.2.6.2. Next, permeabilize the cells by adding 100 µL/well of 0.5% Triton diluted in PBS for 15 min. Wash cells by adding 100 µL/well of PBS and immediately remove it (repeat this step 3 times).

2.2.6.3. Then, incubate the cell culture with 100 µL/well of blocking solution (5% non-fat milk + 2% BSA diluted in PBS [pH 7.2]) for 1 h at room temperature. Wash cells by adding 100 µL/well of PBS and immediately remove it (repeat this step 3 times).

2.2.6.4. In order to stain amastigotes, incubate with 30 µL/well of non-commercial monoclonal antibody (mAb) 2C2 anti-Ssp-4 protein (1:200) diluted in blocking solution for 1 h at room temperature.

2.2.6.5. Wash cells by adding 100 µL/well of PBS and immediately remove it (repeat this step 3 times). Incubate the plate with 30 µL/well of secondary anti-mouse antibody (1:500) diluted in PBS for 1 h at room temperature.

NOTE: The non-commercial mAb 2C2 anti-Ssp-4 protein was a kind gift from Prof. Dr. Renato A. Mortara from Department of Microbiology, Immunology and Parasitology of Federal University of São Paulo.

2.2.6.6. Carefully remove the supernatant and stain nuclei by adding 50 µL/well of DAPI (5 mg/mL) diluted in PBS pH 7.2 (1:1000) for 1 min at room temperature in the dark.

2.2.6.7. Replace DAPI staining solution with 100 µL/well of PBS pH 7.2 and analyze it by fluorescence microscopy.

**REPRESENTATIVE RESULTS:**

On the 14$^{th}$ day, glial cells culture (**Figure 1A**) underwent mechanical dissociation. Isolated cell populations were analyzed by flow cytometry according to CD11b, CD45 and GFAP markers. We could observe a purity of 89.5% for the astrocyte population and 96.6% for the microglia population (**Figure 1B**). After isolation, cells were plated in a 96-well flat plate and after 24 h they were ready to be infected by *T. cruzi* or *T. gondii* according to the respective infection protocols. Here we provided a time-course infection of *T. cruzi* as an example of infection rate evaluation in these glial cells.

Astrocytes and microglia (3 x $10^4$ cells/well) were infected with *T. cruzi* at MOI = 5:1 (parasites:cell) for 2–96 h (**Figure 2**). Infection rate was evaluated by immunofluorescence by counting the number of cells and the number of parasites stained with a DNA intercalator (DAPI). Briefly, we could observe that *T. cruzi* is able to invade microglia and astrocytes at similar rates. Moreover, *T. cruzi* replicates in both cell types, reaching the highest infection rate at 96 h post infection (p.i.). Interestingly, at 96 h p.i. the infection rate is more pronounced in astrocytes than in microglia, suggesting that microglia cells are able to control the parasite replication more efficiently than astrocytes, as we previously described[11].

It is important to note that the use of genetically modified parasites expressing fluorescent reporters or parasites labeled with specific fluorescent antibodies could improve the immunofluorescence microscopy, since they are better distinguished from cell nucleus (**Figure 3**). Moreover, the infection rate of fluorescent parasites can be determined by other techniques such as flow cytometry. Here we provided examples of *T. gondii* RH strain constitutively expressing YFP (**Figure 3A**) and *T. cruzi* Y strain stained with non-commercial mAb 2C2 anti-Ssp-4 protein (**Figure 3B**).

Altogether, this protocol describes how to extract, maintain, dissociate and infect murine microglia and astrocytes primary cells, which could be a powerful tool to study, for example, immune responses of glial cells to protozoa infection as briefly elucidated here.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Primary astrocytes and microglia cells.** (**A**) Images of C57BL/6 murine glial cells on the 14$^{th}$ day of culture were obtained by inverted microscope (400x). The red arrow indicates the layer of astrocytes and the black arrow indicates the microglia. (**B**) After mechanical dissociation, astrocytes and microglia were stained (5 x $10^5$ cells) with fluorescent anti-CD11b, anti-CD45 and anti-GFAP and evaluated by flow cytometry (1 x $10^5$ cells acquisition). Astrocytes are considered as CD11b$^-$/CD45$^-$ and GFAP$^+$ cells, whereas microglia are CD11b$^+$/CD45$^+$ and GFAP$^-$ cells.

**Figure 2: Time-course of *T. cruzi* infection in glial cells.** Astrocytes and microglia from C57BL/6 mice were infected with *T. cruzi* Y (MOI = 5:1). After 2 h, 48 h and 96 h, chambers were fixed with

methanol and stained with cell nucleus marker DAPI (blue) and anti-GFAP (red). Images were obtained by inverted fluorescence microscope (400x). The number of amastigotes inside the cells and the frequency of infected cells were evaluated by the fluorescence microscope software (see **Table of Materials**).

**Figure 3: Astrocytes and microglia infection with fluorescent protozoan parasites.** (**A**) 3 x $10^4$ astrocytes and microglia from C57BL/6 mice were infected (MOI = 1:1) with *T. gondii* RH YFP (green) for 48 h, chambers were fixed with 1% PFA and stained with DAPI (blue). (**B**) 3 x $10^4$ astrocytes and microglia from C57BL/6 mice were infected (MOI = 5:1) with *T. cruzi* Y for 96 h, and chambers were fixed with pure methanol and stained with DAPI (green) and mAb 2C2 anti-Ssp-4 (orange). Images were acquired using an inverted fluorescence microscope.

**DISCUSSION:**

The importance of studying isolated glial cells functions in distinct biological contexts has been expanding in the last two decades. Understanding the CNS beyond neurons is still a growing field in cell biology, especially under infections or inflammatory conditions[8,9,24]. Glial cells are crucial not only for neurons physical support (as it was previously known), but also in many other physiological situations such as neuron energy supply, neurometabolism, immune surveillance, synaptic pruning, shaping and modulation of the tissue, among others[3,25–29].

Since astrocytes and microglia can be differentially modulated during infection or sterile inflammation, it is of great importance to understand the individual and relative role of these glial cells. Although microglia are known to represent the mononuclear phagocyte system (MPS) in CNS, astrocytes have also been described as a pivotal player in the CNS immune responses[8]. In order to compare the role of each glial subset in the context of infection and/or inflammation, it is mandatory to obtain them from the same extraction and conditions. There are few reports about the effector responses of microglia and astrocytes to control infections, especially in regard to protozoan parasites. In this sense, we recently demonstrated the requirement of NLRP3 inflammasome to the control of *T. cruzi* replication in microglia but not in astrocytes by a mechanism involving nitric oxide (NO) secretion[11].

This protocol describes a method that is focused on simultaneously extracting the two most abundant glial cells populations, astrocytes and microglia, from postnatal (up to 3-day-old) mice. Newborn mice older than 3-day-old can be used, but we experienced that the yield of both cells decreases slightly over time (data not shown). Our method differs from previously described protocols for astrocytes or microglia isolation because we focused on obtaining and isolating both cell types in the same extraction, under the same conditions, thus optimizing the usage of experimental animals and improving the study of the relative role for those cells in immunological contexts. Moreover, our protocol also optimized the yield of both cell types. The yield is usually 3–5 x $10^6$ astrocytes and 3–5 x $10^5$ microglia per flask. Compare this to other protocols that result in less cells using more animals per T-75 flask (some protocols use 2–3 animals per flask)[30,31].

Another advantage of this protocol is the time required to obtain isolated astrocytes and microglia. Within 14 days it is possible to obtain mature microglia. In fact, it is important to

highlight that, as Flode and Combs demonstrated, microglia older than 14 days present diminished ability to secrete cytokines; it is very important to consider this in neuroimmunological contexts[30]. Despite the fact that the mature makers for astrocyte are not completely understood, GFAP is largely used to characterize responsive and active astrocytes. Some protocols achieve levels of 90% GFAP positive cells only after 21 or 28 days of culture. For that reason, we proposed a method that provided, cells that are immunoresponsive and mostly mature within 7–14 days. Although thepurity can be lower than other methods for both astrocyte[31] and microglia[30], the main focus was to obtain microglia and astrocytes in greater quantities in a concomitant extraction. We understand that cell populations could be further sorted or isolated with column separation to improve their purities. For this, additional cell markers might be included, such as Iba1 or TMEM119 for microglia; Aquaporin-4 or S100B for astrocytes, CC1 or O4 for oligodendrocytes and NeuN for neurons[22,31–33]. However, an important cell loss at the end of the protocol should be considered.

Thus, we provided a modified method to concomitantly obtain microglia and astrocytes in an efficient and cheap protocol. Moreover, this protocol provide the advantages of a great yield allowing the comparative studies of glial subsets in neuroimmunological contexts, as illustrated here during *T. cruzi* and *T. gondii* infection.

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1 Primary astrocytes and microglia cells R1 Click here to access/download;Figure;Fig1 Primary astrocytes and microglia cells R1.pdf

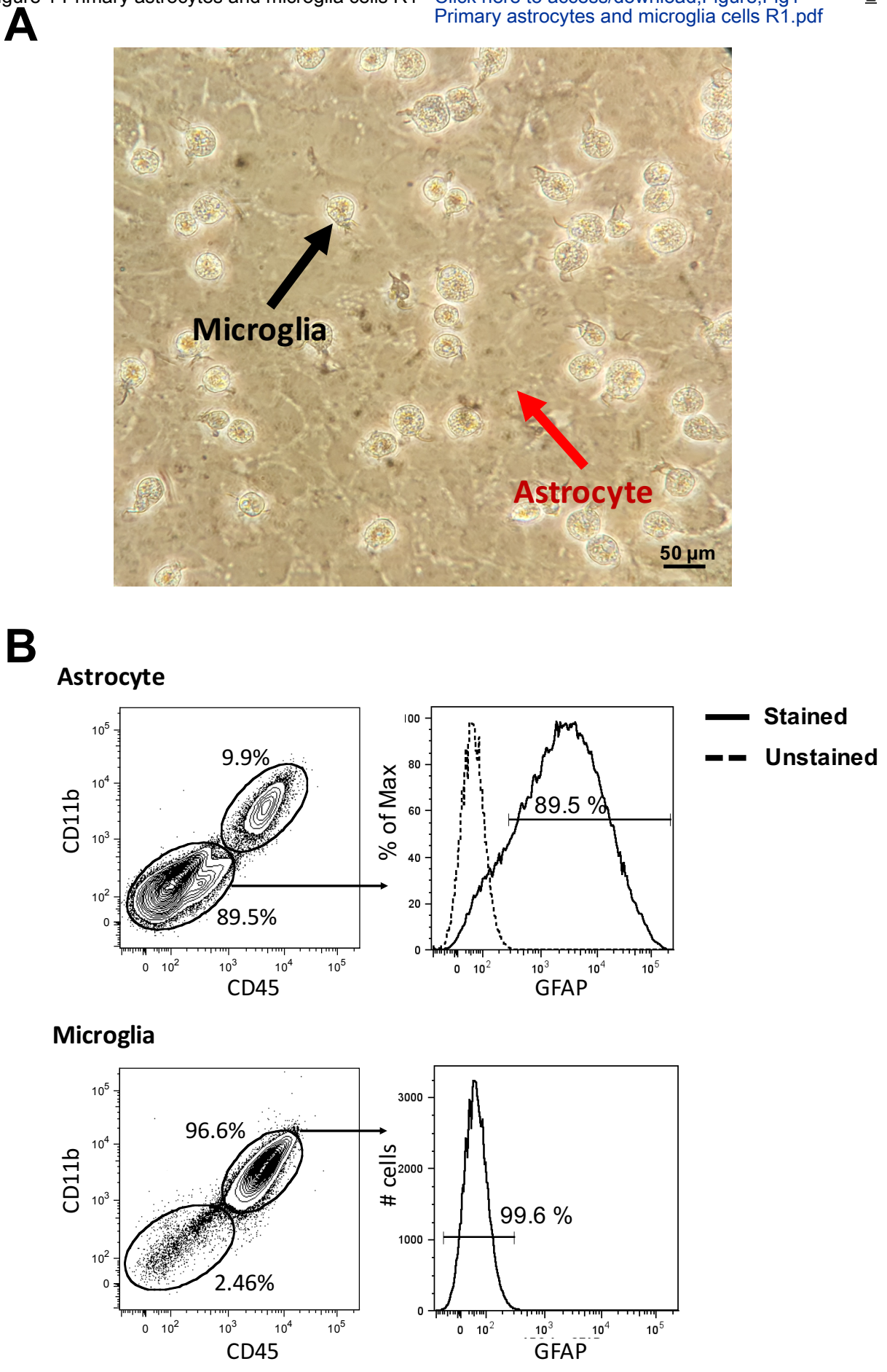

Figure 2 Time-course of T cruzi infection in glial cells R1



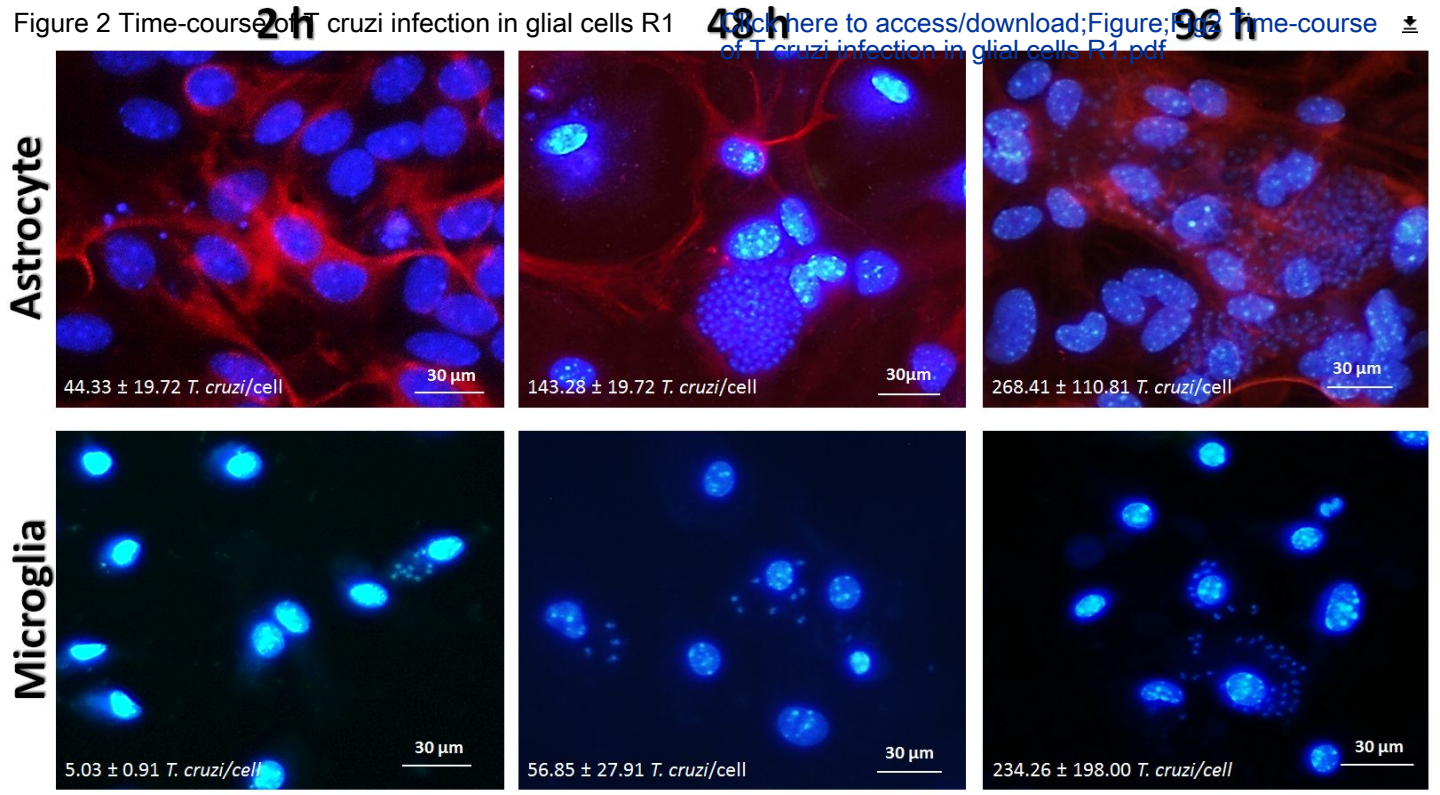

Figure 3 Astrocytes and microglia infection with fluorescent protozoan parasites

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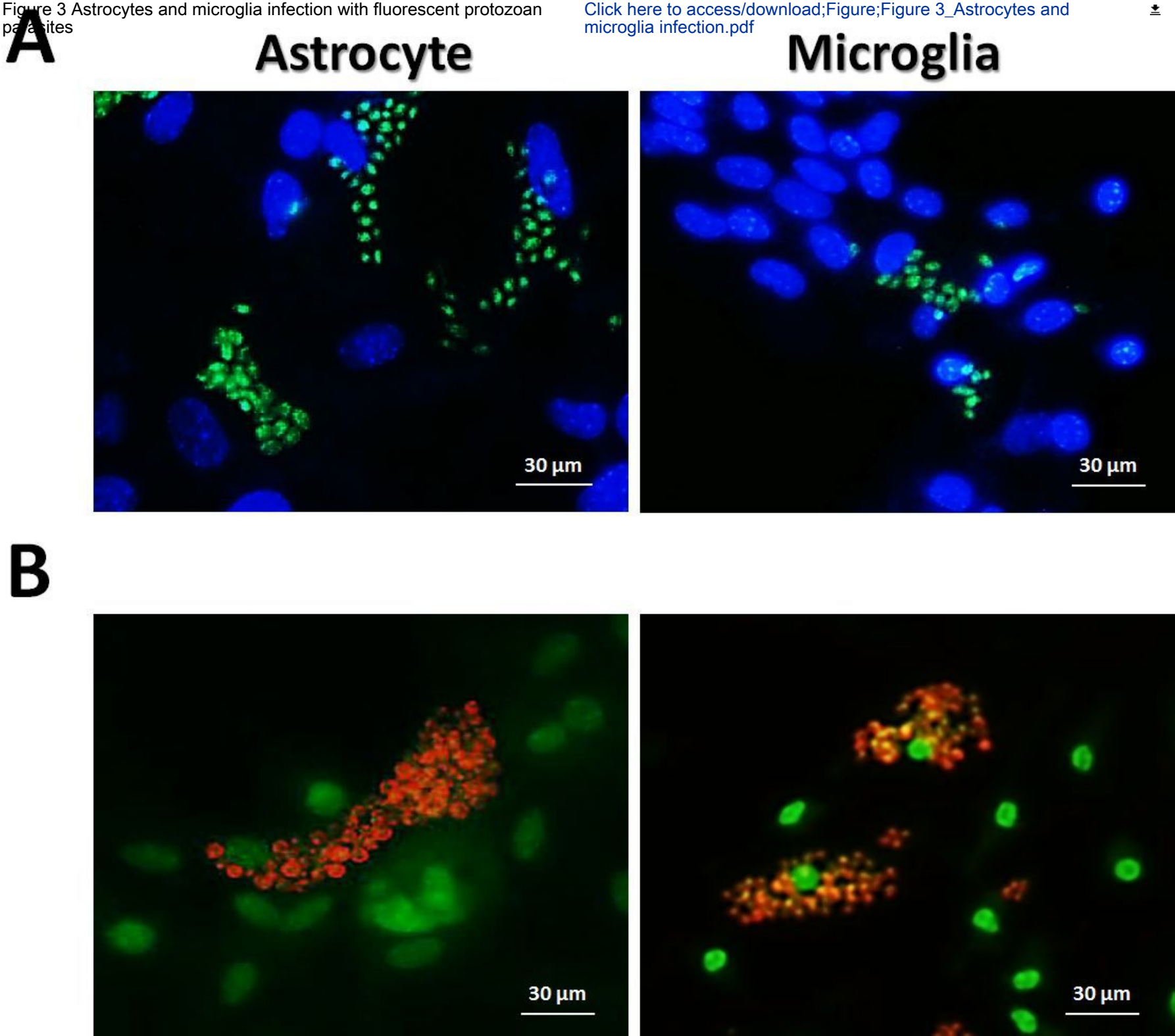

Table of Materials

Click here to access/download;Table of Materials;Table of materials.xlsx

| Name of Material/Equipment | Company | Catalog Number |
|---|---|---|
| 70% Ethanol | Dinâmica Química Contemporânea | Cat: 2231 |
| 75 $cm^2$ Flask | Corning | Cat: 430720U |
| 96 well cell culture plate | Greiner Cellstar | Cat: 655090 |
| Ammonium Chloride ($NH_4Cl$) | Dinâmica Química Contemporânea | Cat: C10337.01.AH |
| Anti-GFAP antibody | Abcam | Cat.: ab49874 |
| Bottle Top Filter 0.22 mm CA | Corning | Cat: 430513 |
| Bovine Serum Albumin (BSA) | Sigma Aldrich | Cat: A7906 |
| CD11b (FITC) | BD Pharmigen | Cat.: 553310 |
| CD45 (PE) | Invitrogen | Cat.: 12-0451-83 |
| Centrifuge | Eppendorf | Cat: 5810R |
| Centrifuge | Eppendorf | 5415R |
| Class II biosafety cabinet | Pachane | Cat: 200 |
| $CO_2$ Incubator | ThermoScientific | Model: 3110 |
| Conical tubes 15 mL | Corning | Cat: 430766 |
| Conical tubes 50 mL | Corning | Cat: 352070 |
| Countess automated cell counter | Invitrogen | Cat: C10281 |
| DAPI | Invitrogen | Cat.: D1306 |
| Digital Microscope Camera | Nikon | Cat: DS-RI1 |
| Dulbecco's Modified Eagle Medium (DMEM) | Gibco | Cat: 12800-058 |
| Ethylenediaminetetraacetic acid (EDTA) | Sigma Aldrich | Cat: E9884 |
| F12 Nutrient Mixture | Gibco | Cat: 21700-026 |
| FACS Canto II | BD Biosciences | Unavaiable |
| Fetal Bovine Serum (FBS) | LGC Biotechnology | Cat: 10-bio500-1 |
| Flow Jo (software) | Flow Jo | Version: Flow Jo_9.9.4 |
| Fluorescence intenselight | Nikon | Cat: C-HGFI |
| GFAP (APC) | Invitrogen | Cat.: 50-9892-82 |
| Goat - anti-mouse IgG (FITC) | Kirkeegood&Perry Lab (KPL) | Cat.: 172-1806 |
| HBSS - Hank's Balanced Salt Solution | Gibco | Cat: 14175079 |
| HEPES | Sigma Aldrich | Cat: H4034 |
| IC Fixation Buffer | Invitrogen | Cat: 00-8222-49 |
| Inverted microscope | Nikon | Model: ECLIPSE TS100 |

| | | |
|---|---|---|
| Isoflurane | Cristália | Cat: 21.2665 |
| Methanol | Synth | Cat: 01A1085.01.BJ |
| Micro spatula | ABC stainless | Unavaiable |
| Microtube 1.5 mL | Axygen | Cat: MCT-150-C |
| Monoclonal antibody (mAb) 2C2 anti-Ssp-4 | Non commercial | Non commercial |
| Multichannel Pipette (p200) | Corning | Cat: 751630124 |
| NIS Elements Software | Nikon | Version 4.0 |
| Non-fat milk | Nestlé | Cat: 9442405 |
| Orbital Shaker Incubator | ThermoScientific | Model: 481 Cat: 11 |
| Paraformaldehyde (PFA) | Sigma Aldrich | Cat: P6148 |
| PBS | Non commercial | Non commercial |
| Penicillin G | Sigma Aldrich | Cat: P-7794 |
| Permeabilization Buffer (10X) | Invitrogen | Cat: 00-8333-56 |
| Petri dish 60x15 mm (Disposable, sterile) | Prolab | Cat: 0303-8 |
| pH meter | Kasvi | K39-1014B |
| RPMI 1640 Medium | Gibco | Cat: 31800-014 |
| Scissors | ABC stainless | Cat: LO9-W4 |
| Serological pipette 10 mL | Corning | Cat: 4101 |
| Serological pipette 5 mL | Corning | Cat: 4051 |
| Single Channel Pipette (p1000) | Gilson Pipetman | Cat: F123602 |
| Single Channel Pipette (p200) | Gilson Pipetman | Cat: F123601 |
| Sodium bicarbonate | Sigma Aldrich | Cat: S6297 |
| Streptomycin sulfate salt | Sigma Aldrich | Cat: S9137 |
| Triton X-100 | Sigma Aldrich | Cat: T9284 |
| Trypsin | Gibco | Cat: 27250-018 |
| Tweezers | ABC stainless | Cat: L28-P4-172 |
| Water Bath | Novatecnica | Model: 09020095 |

| Comments/Description |
| --- |
| Sterilize |
| Plastic material |
| Cell culture |
| Remove autofluorescence |
| Immunofluorescence antidoby |
| Culture medium filter |
| FACS Buffer preparation |
| Flow cytometry antibody |
| Flow cytometry antibody |
| Centrifugation |
| Centrifugation |
| Biosafety cabinet for sterile procedures |
| Primary cells maintenance |
| Plastic material |
| Plastic material |
| Cell counter |
| Immunofluorescence antidoby |
| Capture images on microscope |
| Cell culture medium |
| FACS Buffer preparation |
| Cell culture medium |
| Flow cytometer |
| Cell culture medium supplement |
| Data analysis |
| Fluorescence source |
| Flow cytometry antibody |
| Immunofluorescence antidoby |
| Cell culture medium |
| Cell culture medium supplement |
| Cell fixation for Flow Citometry |
| Microscope |

Inhaled anesthetic
Fixation for Immunofluorescence
Surgical material
Plastic material
Immunofluorescence antidoby
Pipette reagents
Acquire and analyse images
Blocking solution for immunofluorescence
Dissociate microglia from astrocytes
Fixation for Immunofluorescence
Neutral Buffer
Cell culture medium supplement
Cell permeabilization for Flow Citometry
Plastic material
Calibrate pH solution
Cell culture medium
Surgical material
Plastic material
Plastic material
Pipette reagents
Pipette reagents
Cell culture medium supplement
Cell culture medium supplement
Permeabilization for immunofluorescence
Digestive enzyme
Surgical material
Digeste tissue at 37 ºC with trypsin

Thank you for your time and effort in communicating our manuscript. We have revised our manuscript according to your comments and the reviewers' suggestions. A list of point-by-point responses is included.

We hope that these additional descriptions of the protocol and revisions bring our manuscript to the standards of the journal and answer the reviewers' questions satisfactorily. We would like to thank the reviewers for their constructive comments and suggestions.

## Editorial Comments:

• The manuscript will benefit from thorough language revision as there are a number of grammatical errors throughout. Please thoroughly review the manuscript and edit any errors.

We want to thank the editor for the constructive comments regarding our manuscript, which were used to substantially improve its accuracy and quality. As suggested, we revised the manuscript and corrected grammatical mistakes. Please find below a point-by-point reply to the suggestions and comments that addresses the questions raised.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Examples:
1) 1: Mention mouse strain used.
2) 1.6: Please mention all details. How is the meninges handled? What care instructions do you recommend? How are the cortices handled?
3) 1.7: Should the medium be chilled?

As pointed by editor, a several steps were included in the description of methods and table of materials (all alterations in the manuscript are marked in red). As the examples cited:
1) Line 118 includes mouse strain
2) Lines 139-145 describe in more details how the cortices are handled. In fact, we do not specifically remove meninges and other cells that are present in the CNS (such as neurons and oligodendrocytes). But since we use a differential media that favors the outgrowth of astrocytes and microglia cells, we believe that the frequency of these other cells is despicable. Furthermore, we discuss about other cell contaminant and how to deal or overcome this issue in the discussion section (lines 394-443).
3) Culture medium should be prewarmed before use it, as mentioned in lines 128-129.

In addition, we also decided to change the name of the article to a more authentic one according to its content. We decided to a new clearer and coherent title: **"Concomitant isolation of primary astrocytes and microglia for protozoa parasite infection"**

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. Please add a one-line space after each protocol step.

As requested, the format of sections headlines and steps were straighten out accordingly with JoVE's instructions.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

We highlighted in yellow all steps requested by the editor.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

As requested, the discussion was revised to cover the questions raised by the editor (lines 394-443).

• **Figures:** Add a space before "h" in fig 2.

We thank the referee for point out this mistake that was properly corrected.

• **Figure/Table Legends:** Define the format of the numbers on fig 2.

We thank the referee for point out this mistake that was properly corrected.

• **References:** Please spell out journal names.

We included the journals full names at the References section.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Nikon Eclipse TS100 / NIS, NIS Elements (Nikon),
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We have replaced all commercial sounding names, such as Nikon TS100 and BD FACS Canto for the generic terms microscope and flow cytometer. They are explicit only in table of materials, as requested.

• Please define all abbreviations at first use (e.g, SFB)

All abbreviations mentions were properly corrected.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All figures and tables submitted here are original and unpublished material. They were handled and elaborated specifically for the JoVE's publication.

**Comments from Peer-Reviewers:**

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:
The protocol and its content is sufficient. I believe it would be a nice reference for other researchers.

We thank the reviewer for taking time to evaluate our work and the overall positive comments.

**Reviewer #2:**

In this manuscript the authors describe the protocols to obtain astrocytes and microglia from newborn mice. Purified cells, in a good percentage of each population, were infected with *T. cruzi* or *T. gondii*. Although, the manuscript seems interesting, the authors fail to describe in detail some methodological aspects, which is fundamental in JOVE.

- For instance, in this same journal Schildge, et al., in 2013 described the purification and culture of primary mouse astrocytes. Similarly, Floden and Combs 2007, described a method to culture neonatal microglia for 14 days. There are also manuscripts

describing infection of primary mouse astrocytes with *T. cruzi* and *T. gondii*, but not literature on infection in primary mouse microglia.

We appreciate the point raised by the reviewer 2 that we used to significantly improve our manuscript. The introduction (lines 75-108) and discussion (lines 394-443) about the advantages of our protocol was now updated in a new version

- Protocol
  We can assume that authors used the infective forms of both parasites, but tachyzoites and trypomastigotes are no mentioned in the methods.

  As pointed by reviewer, the information about tachyzoites and trypomastigotes were included and can be found in lines 283 and 305, respectively.

- Staining protocols and quantities of reagents were not described

  As pointed by reviewer, the detailed description about immunofluorescence can be found at the section 2.2.5.

- Staining with the monoclonal antibody for *T. gondii* was not described. Is the fluorochrome PE?

  As mentioned in section 2.1.2, we used genetically modified *T. gondii* that constitutively expresses the yellow fluorescent protein (YFP).

- A counterstain was used in some fluorescence assays, its no in the methods.

  A counterstain used was DAPI staining. As requested, the information was included in lines 294-295 and 335-336.

- Results
  Figure 1A does not have a scale bar. It is difficult to determine it is 400x magnification. There are two arrows indicating specific cells, but it is very difficult to distinguish the morphology. If the flow cytometry density plots showed high percentages of specific cells, what other kind of cells are in figure 1B.

  We thank the referee for point out this mistake that was properly corrected.
  A discussion about contaminant cells in primary glial cell cultures can be found in the discussion section (lines 394-443).

- Figure 1B. Astrocytes (CD11b and CD45 negatives) need negative staining control in the histogram.

  We thank the referee for bringing our attention to this missing information that was properly included.

- Cells in figure 1B (CD11b and CD45 positives) are markers of microglia. Microglia tend to be CD45 intermediate. To rule it out blood contamination, microglia needs further characterization, for instance staining for TMEM119.

  We would like to thank the referee for the suggestion. Although we do not have the anti-TMEM119 to characterize microglia at this moment, the information was included in lines 230-231 and 436.

- In figure 2 and 3 the authors claim differences in infection rate, but they need more data (numbers and replicas).

  We would like to thank the reviewer for the comment. In fact, we did not discuss it deeply, since here we just want to provide a protocol on how to infect glial cells by protozoa parasites. For further explanation about differential infection rate in these cells please see our recent published article (Pacheco *et al* – DOI https://doi.org/10.1002/JLB.4AB1118-416RR).

- Discussion
  There is not discussion of their own data and the results are repeated in this section.

  As requested, the discussion section was properly modified. (lines 394-443).

- References
  References 7, 15 and 23 maybe are not appropriate for the statements.
  There are 11 reviews out 27 references.
  Check names order in reference 27

  We are really thankful for the careful examination of our manuscript in the light of the literature. As suggested by the reviewer, these references were replaced for better suitable ones.

**Reviewer #3:**

Manuscript Summary:
In the paper "Astrocytes and microglia infection with protozoa parasites" Amaral et. al. have written a protocol about astrocyte and microglia isolation, culturing and subsequent protozoa infection. They provide a step by step guide on how to perform the experiments. Furthermore, they explain why and how certain steps are important for the experimental procedure. They also provide FACS-graphs and immunofluorescent pictures to validate their result and conclusions. Even though they have written a detailed protocol there are lots of things that needs to be clarified and described in more detail.

We thank the reviewer for taking the time to evaluate our work and the overall positive comments. We have addressed all questions raised (please see the point-by-point below).

- Major Concerns:
  Some of the critical steps are not written in detail which makes it hard to follow. A protocol needs to be simple and straightforward so that people who never done this before should be able to follow. For example, how is the cortex isolate (from rest of the brain), how and when were the tissue flasks and plates coated with poly-L-lysine?

  We appreciate the point raised by the reviewer 3. The protocol was reviewed and we made a clearer step-by-step. Besides, we also believe that during the video recording, it will be more straightforward. Cortex isolation information can be found in lines 134-151. The treated plates for tissue culture that allow optimal cell attachment were obtained from Greiner CellStar, as described in the table of materials.

- *Line 132, The isolation of the cortex needs to be described in more detail, consider adding photos of step 6 and 7 Day 1.

  We are really thankful for the careful examination of our manuscript. We included more details about the cortex isolation (lines 134-151). Since these steps will be recorded in the video, we did not add photos.

- *Line 157, When were the plates coated with poly-L-lysine, what concentration was used, from which company? This is very unclear and needs to be described.

  We apologize for the misunderstanding. The treated plates for tissue culture were obtained from Greiner CellStar, as described in the table of materials.

- *Line 186, The authors says that the cells should be washed but they do not mention with what solution the cells were washed.

  We appreciated this important question raised by the reviewer. In fact, the cells were washed with supplemented DMEM/F12 culture medium, as described in lines 208-211).

- *Line 198-199, The FACS protocol needs to be expanded and explained in detail (incubation times, concentrations of antibodies and so on).

  As requested, we added a detailed FACS protocol for astrocyte and microglia analysis. Please find this information in lines 228-275.

- Minor Concerns:
  *Line 85, The authors mention that primary cells are more suitable to study glial cell function (lines 88-92) but reference number 8 refers to a paper based on virus infection in a cell line, instead changing the reference to paper that investigate the role of primary astrocytes/glial cells in viral infection would be more appropriate.

  We thank the referee for point out this mistake that was properly corrected.

- *Line 123, What is "Hanks"? Write either HBSS or Hank's balanced salt solution if that is what the authors are referring to (was antibiotics used in dissection medium?).

  We thank the referee for point out this mistake that was properly corrected for HBSS. The corrected information can also be found in the table of materials. Furthermore, no antibiotics were used in the HBSS medium.

- *Line 124, Cells were cultured in DMEM/F12 without antibiotics?

  We thank the referee for point out this misinformation. We added more details about the supplemented DMEM/F12 in lines 125-127.

- *Line 127 - 128, It is hard to follow what was done

  We thank the referee for bringing attention to this point of the protocol. A new sentence is provided in lines 134-135.

- *Line 134-135, Brain tissue was put into a dry 15 mL tube?

As described in lines 147-149,  2 mL of HBSS medium was added to cortex tissue and transferred to a 15 mL conical tube. We also added an extra sentence to make sure that the final volume is 2 mL (lines 147-149).

- *Line 204 and 213, Were cells plated on uncoated plates? If not how and when were the plates coated?

  We thank the referee for bring attention for this absent information. The treated plates for tissue culture were obtained from Greiner CellStar, as described in the table of materials.

  *Line 210 and 223, Describe the protocol of the immunofluorescent staining

  We thank the referee for bring attention for this absence information. The methods section was completed with step-by-step immunofluorescent protocol. Please find this information in lines 319-339.

- *Line 219, wash with what?

  We washed the cells with 200 µL per well with supplemented RPMI. This information was added in lines 309-311.

- *Have the figures in correct order, Figure 2 appears before figure 1

  We apologize for the inconvenience, but we had some troubles to upload on the platform. This will be fixed in this new upload phase.

- *Line 242, what are the "last" and "first" cells? Needs to be clarified. Rather write astrocytes and microglia.

  We thank the referee for point out this mistake that was properly corrected

- *Line 250-251, indicate affiliation of Dr. Renato A. Mortara

  We thank the referee for point out this mistake that was properly corrected (lines 331-333 and 446-447).

- *Line 269, what kind of microscope?

  We used an inverted fluorescence microcospe (line 383), more information about the microscope can be accessed on table of materials.

- *Double check the Material/equipment list, poly-L-lysine is missing, some material might be omitted from this table.

  We thank the referee for point the missing information. The table of materials were updated and completed as required.

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| Author(s): | **Marcelo Pires Amaral, Aline de Oliveira Lima Pacheco, Ingrid Sancho de Farias, Luiza Zainotti Miguel Fahur Bottino, Karina Ramalho Bortoluci** |

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