# Journal of Visualized Experiments

## Obtaining Human Microglia from Adult Human Brain Tissue

--Manuscript Draft--

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| **Article Type:** | Invited Methods Collection - Author Produced Video |
| **Manuscript Number:** | JoVE61438R1 |
| **Full Title:** | Obtaining Human Microglia from Adult Human Brain Tissue |
| **Section/Category:** | JoVE Neuroscience |
| **Keywords:** | Human microglia,<br>microglia isolation,<br>cell culture,<br>tissue culture,<br>glial cells,<br>neurosurgery,<br>autoimmunity,<br>TBI,<br>MS,<br>glioblastoma |
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**TITLE:**

# Obtaining Human Microglia from Adult Human Brain Tissue

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**KEYWORDS:**

Human microglia, microglia isolation, cell culture, tissue culture, glial cells, neurosurgery, autoimmunity, TBI, MS, glioblastoma

**SUMMARY:**

This protocol is an efficient, cost effective and robust method of isolating primary microglia from live, adult, human brain tissue. Isolated primary human microglia can serve as a tool for studying cellular processes in homeostasis and disease.

**ABSTRACT:**

Microglia are resident innate immune cells of the central nervous system (CNS). Microglia play a critical role during development, in maintaining homeostasis, and during infection or injury. Several independent research groups have highlighted the central role that microglia play in autoimmune diseases, autoinflammatory syndromes and cancers. The activation of microglia in some neurological diseases may directly participate in pathogenic processes. Primary microglia are a powerful tool to understand the immune responses in the brain, cell-cell interactions and dysregulated microglia phenotypes in disease. Primary microglia mimic in vivo microglial properties better than immortalized microglial cell lines. Human adult microglia exhibit distinct properties as compared to human fetal and rodent microglia. This protocol provides an efficient method for isolation of primary microglia from adult human brain. Studying these microglia can provide critical insights into cell-cell interactions between microglia and other resident cellular populations in the CNS including, oligodendrocytes, neurons and astrocytes. Additionally, microglia from different human brains may be cultured for characterization of unique immune responses for personalized medicine and a myriad of therapeutic applications.

## INTRODUCTION:

The central nervous system (CNS) is constructed of a complex network of neurons and glial cells[1]. Among the glial cells, microglia function as the innate immune cells of the CNS[2,3]. Microglia are responsible for maintaining homeostasis in the healthy CNS[4]. Microglia also play an important role in neurodevelopment, by pruning synapses[2]. Microglia are central to the pathophysiology of several neurological diseases including but not restricted to; Alzheimer's disease[5], Parkinson's disease[6], stroke[7], multiple sclerosis[8], traumatic brain injury[9], neuropathic pain[10], spinal cord injury[11] and brain tumors such as gliomas[12].

Studies related to CNS homeostasis and diseases utilize rodent microglia due to a dearth of cost efficient and time efficient human primary microglia isolation protocols[13]. Rodent microglia resemble primary human microglia in expression of genes such as Iba-1, PU.1, DAP12 and M-CSF receptor and have been effective in understanding normal as well as diseased brain[13]. Interestingly, the expression of several immune related genes such as TLR4, MHC II, Siglec-11 and Siglec-33 varies between human and rodent microglia[13]. The expression of several genes also varies in temporal expression and in neurodegenerative diseases in both species[14,15]. These significant differences make human microglia an essential model to study microglia function in homeostasis and disease. Primary human microglia can also be an effective tool for preclinical screening of potential drug candidates[16]. The above mentioned reasons underline the growing need for cost effective protocols for isolation of primary human microglia.

We have developed a protocol for isolation of primary human microglia from adult human brain tissue collected as a result of surgical window created for tumor resections or other surgical resections. The method here is considerably different from existing methods. We were able to isolate and culture microglia after a transit time of about 75 minutes from the tissue collection site to starting the isolation protocol in the laboratory. We have used the supernatant of L929 fibroblast cells to promote the growth of isolated microglia. This method specifically focuses on the culture and development of only primary microglia. The resulting culture prepared is 80% microglia. While other protocols provide a enriched culture of microglia by density gradient centrifugation, flow cytometry and magnetic beads, the protocol is a rapid, simple, robust and cost effective way to culture primary human microglia[17-20]. The ability to utilize surgically removed live adult brain tissue instead of fixed brain tissues from cadavers proves an added advantage of this method in contrast to existing procedures[18,21].

## PROTOCOL:

All tissues were acquired after ethical clearance from the institute ethics committees of Indian Institute of Technology Jodhpur and All India Institute of Medical Sciences (AIIMS) Jodhpur.

### 1. Tissue acquisition and processing (Day 0)

1.1. Collect the tissue in a 50 mL tube containing 10 mL of ice cold artificial cerebrospinal fluid (aCSF) (2 mM $CaCl_2 \cdot 2H_2O$, 10 mM glucose, 3 mM KCl, 26 mM $NaHCO_3$, 2.5 mM $NaH_2PO_4$, 1 mM $MgCl_2 \cdot 6H_2O$, 202 mM sucrose)[20]. Ensure that the tube is kept on ice if the tissue needs to

be transferred to a different location.

NOTE: Prepare aCSF in autoclaved distilled water. Filter it with 0.22 µm syringe filter in the laminar hood. This can be stored for 1 month at 4 °C.

1.2. Wipe the collection tube carefully with 70% alcohol and transfer to an aseptic laminar air flow chamber.

1.3. Discard aCSF carefully and weigh tissue in an aseptic condition. Tissue weight is essential to calculate the volume of trypsin-EDTA needed for subsequent steps.

1.4. Keep the tissue in fresh warm aCSF at 37 °C for 5 minutes. This step is critical to avoid cell death.

1.5. Discard aCSF and wash tissue once with 1x PBS (phosphate buffered saline) at 37 °C. Ensure all blood is washed away with repeated PBS washes (as needed).

1.6. Incubate the tissue in warm PBS, at 37 °C, for 5 min.

1.7. Discard PBS carefully and transfer tissue to a sterilized Petri dish. PBS may be removed with a pipette. This will prevent any loss of tissue.

1.8. Dice the tissue into small (at least 1 $mm^3$) pieces using a sterile scalpel. Finely diced tissue provides higher tissue surface area for tissue dissociation by trypsin-EDTA ensuring higher yield.

1.9. Transfer the diced tissue to a 50 mL tube containing 10 mL/g tissue of 0.25% trypsin-EDTA and mix by pipetting through a 10 mL serological pipette. Add 2 mL of trypsin/EDTA to a Petri dish and wash the plate thoroughly with the help of pipette. Add this trypsin back to the falcon tube. This minimizes loss of tissue and cells while dicing.

1.10. Incubate the tube on a shaker for 30 minutes at 37 °C at 250 rpm. This step increases the dissociation of cells from tissue.

1.11. At the end of the incubation, add 10 mL of neutralizing medium (50% DMEM/50% F12 with glutamine, 1% penicillin-streptomycin, 10% FBS) to neutralize trypsin. Mix with a 10 mL serological pipette. The amount of neutralizing media added should be equal to the amount of trypsin used.

1.12. Centrifuge the tube at 2,000 x g at 4 °C for 10 minutes.

1.13. Discard the supernatant and re-suspend the pellet in 1 mL of culture medium (50% DMEM/50% F12 with glutamine, 1% penicillin-streptomycin, 20% L929 supernatant, 10% FBS).

NOTE: L929 cells are culture in DMEM (DMEM with glutamine, 1% penicillin-streptomycin, 10% FBS). ATCC recommended culture method should be followed for cell culture. Supernatant must be collected from the culture flask which is at least 75% confluent. It can be collected in bulk and stored at -80 °C to prevent degradation of growth factors. It is recommended to add L929 supernatant separately in flasks instead of adding to the stock culture medium.

1.14. Plate the cells in a T-25 flask, suited for adherent cells, and add 4 mL of additional culture medium. Incubate the flask at 37 °C with 5% $CO_2$. Carefully shake the flask to homogenously disperse the tissue. Avoid bringing the media to the neck of the flask, while shaking, as this may increase the chances of contamination.

**2. Cell culture (Day 2)**

2.1. Collect the media from the T-25 culture flask prepared on day 0 in three 1.5 mL centrifuge tubes by collecting equal volume of media in each tube. Wash the flask once with 1 x PBS. Shake the flask gently to remove any remnant tissue fragments left. Avoid harsh shaking of the flask as any remnant fragments will not adversely affect the culture. Add 5 mL fresh culture media to the flask.

2.2. Centrifuge the collected media at 1,466 x g (4000 rpm) at 4 °C for 4 minutes.

2.3. Discard the supernatant from each tube and add 1 mL of culture medium to one of the tubes. Mix thoroughly with pipette. Serially add the mixed media with cells to other tubes. Mix thoroughly with pipette and pool the cells in one tube.

2.4. Plate the cells in a separate T-25 flask, suited for adherent cells. Add 4 mL of culture medium and incubate the flask at 37 °C with 5% $CO_2$.

**3. Cell culture (Day 4)**

3.1. Discard the media from both flasks and add fresh 5 mL of culture media to the flask.

3.2. Incubate the flask at 37 °C with 5% $CO_2$ for 2 days.

**4. Cell Culture (Day 6)**

4.1. Cells will be ready for further experiments.

**REPRESENTATIVE RESULTS:**

By using the above-mentioned protocol (**Figure 1**), we were able to isolate primary human microglia from live surgically resected brain tissues. Cultured cells were stained with *Ricinus communis agglutinin-1* (RCA-1) lectin for microglia (green) and with Glial fibrillary acidic protein (GFAP) for astrocytes (red) (**Figure 2**) as previously described[22-26]. 4′,6-diamidino-2-phenylindole (DAPI) was used to stain nuclei (blue). On the sixth day from the starting of the experiment the

cells were ready for further experiments. Stained cells were counted blind for microglia and astrocytes present in the culture. About 80% of the primary culture were microglia (**Figure 2**).

**FIGURE LEGENDS:**

**Figure 1: Schematic of primary microglia isolation from adult brain**. Surgically removed tissue was collected in ice cold 10 mL of aCSF in a 50 mL tube and transferred to the laboratory. The tissue was washed with aCSF and PBS respectively and finely diced, dissociated with the help of trypsin-EDTA and plated in a T-25 flask. On the second day the media was collected and centrifuged. Pellet was mixed in fresh media and plated in a T-25 flask. Fresh media was added to the first flask. Media was changed in both the flasks on alternate days. Cells were ready for further experiments on day 6.

**Figure 2: Immunocytochemistry of isolated primary human microglia.** (**A**) Isolated cells were plated in a two well chamber slide and were stained with GFAP for astrocytes (Green-first panel) or RCA for microglia (Green-second panel). Nuclei were stained blue with DAPI. The control for RCA and secondary antibody control for GFAP is shown in inset. (**B**) Isolated cells were plated in a two well chamber slide and were stained with RCA for microglia (green) and GFAP for astrocytes (red). The second row shows the control for RCA and secondary antibody control for GFAP. Nuclei were stained blue with DAPI. (**C**) Cells were counted by blinded control. Quantification is representative of counting by one blinded control. About 80% of the cells were microglia.

**DISCUSSION:**

Microglia ensure homeostasis in the normal brain and play central roles in the pathophysiology of various neurological diseases[4]. Microglia are central to neurodevelopment and formation of synapses[2]. Microglial studies have proven pivotal in understanding the development and progression of diverse neurological diseases[4]. Rodent microglia are the prevalent model of choice for primary microglial studies, even though, rodent microglia are different from primary human microglia in key aspects[13]. Development of cost effective, high-yielding, protocols for isolation of primary human microglia may help bridge this gap. We have developed a protocol for isolation of primary human microglia from live, surgically resected, adult, human brain tissue. We were able to achieve microglial purity of about 80% as checked on the 7$^{th}$ day.

One of the most critical steps of the protocol was the transportation of acquired tissue to the laboratory for processing. As the transit time was about 75 minutes, it was probable that we may not be able to isolate any cells. We managed this by using a 50 mL tube with only 10 mL of aCSF. aCSF provided the required nutrients and the remaining space in the tube helped aerate the aCSF and tissue. There is the possibility that there was considerable death of neurons and other cells during the transit period. While this helps with the isolation of microglia, this protocol may not be efficient for isolation of other neurological cells. We were able to isolate microglial cells from 268 mg of dissected tissue. We were also able to achieve significant purity of microglia by also avoiding the coating of flask by poly-D-lysine. While this may have resulted in some loss of microglia, this also avoided other glial population from adhering to the flask. Additionally, this avoided an extra step of shaking the flask and collecting microglia. It was

possible that some of the cells might have not adhered in the flask prepared on day 0. We collected non adherent cells from the initial culture and plated it again in another flask on day 2, which also yielded microglia cells. It should be noted that finely dicing the tissue is important as it will increase the surface the area of the tissue. This will allow trypsin to access most of the tissue and dissociate more cells.

To promote the growth of microglia in the culture, we have conditioned the culture medium with the supernatant of L929 cells[27,28]. This provides a rich source of Granulocyte-macrophage colony stimulating factor (GM-CSF) as a supplement, which enhances macrophage proliferation[27,29]. This helped reduce the cost for additional expensive growth supplements that are a mainstay of several microglial primary isolation protocols. Adding L929 supernatant is crucial for the efficient isolation and growth of microglia in the protocol. However for the labs without L929 cell culture, this becomes a limiting step considering the overall cost of the protocol as additional growth supplements will be needed. We were able to get a microglial population of about 80% in the culture conditions. This is less than some published protocol but this can be overcome by having an additional round of isolation through specific protocols like using magnetic beads for specific microglial markers. At 80% culture purity, the protocol is efficient for many experiments like immunocytochemistry. However, for experiments like protein purification, protein identification and western blotting, additional purification of the culture may be needed. Even with high purity of the primary cultures, there is always a possibility that other cells present in the culture might increase with longer culture duration. We have successfully cultured isolated microglia for 9 days by passaging them once. While the culture conditions in the protocol favors the isolation and growth of microglia, the presence of other cells should be considered when maintaining the culture for longer duration.

This protocol for isolating primary microglia is effective, robust and cost efficient. Such protocols for isolation of primary human microglia from adult brain tissue will enable timely research on immune functions, cell physiology and disease responses in the adult brain. Additionally, patient derived primary microglia may aid in developing personalized, future therapeutics.

**ACKNOWLEDGMENTS:**

SJ's laboratory was established with institutional grants from IITJ and is funded by grants from the Department of Biotechnology (BT/PR12831/MED/30/1489/2015) and Ministry of Electronics and Information Technology Government of India (No.4(16)/2019-ITEA). The human brain tissue sections were obtained from the All India Institute of Medical Sciences (AIIMS) Jodhpur after institutional ethics committee clearance. We thank Mayank Rathor, B.Tech Student member of Design and Arts Society IIT Jodhpur, for videography support.

**DISCLOSURES:**

Authors have nothing to disclose.

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Figure 1



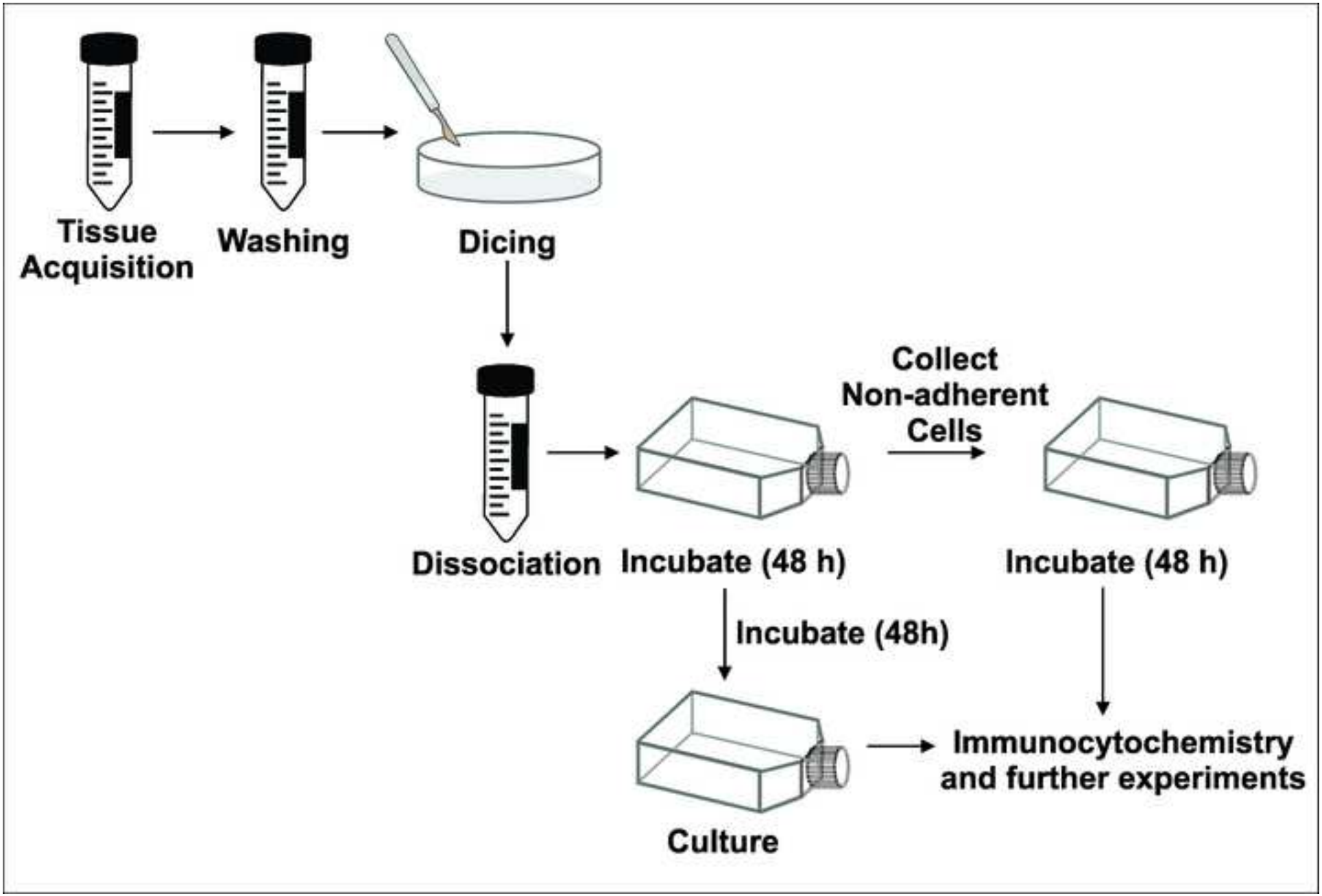

Figure 2 a

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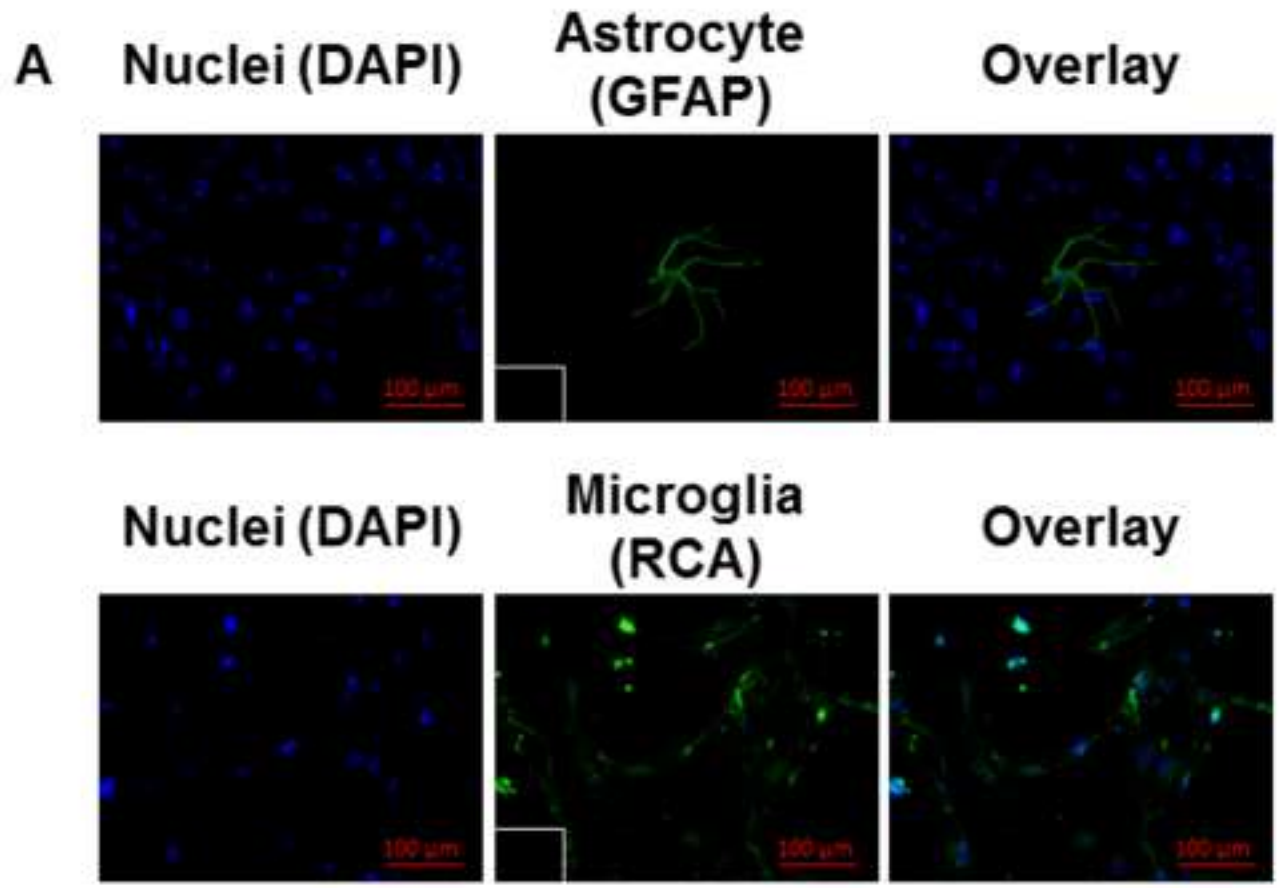

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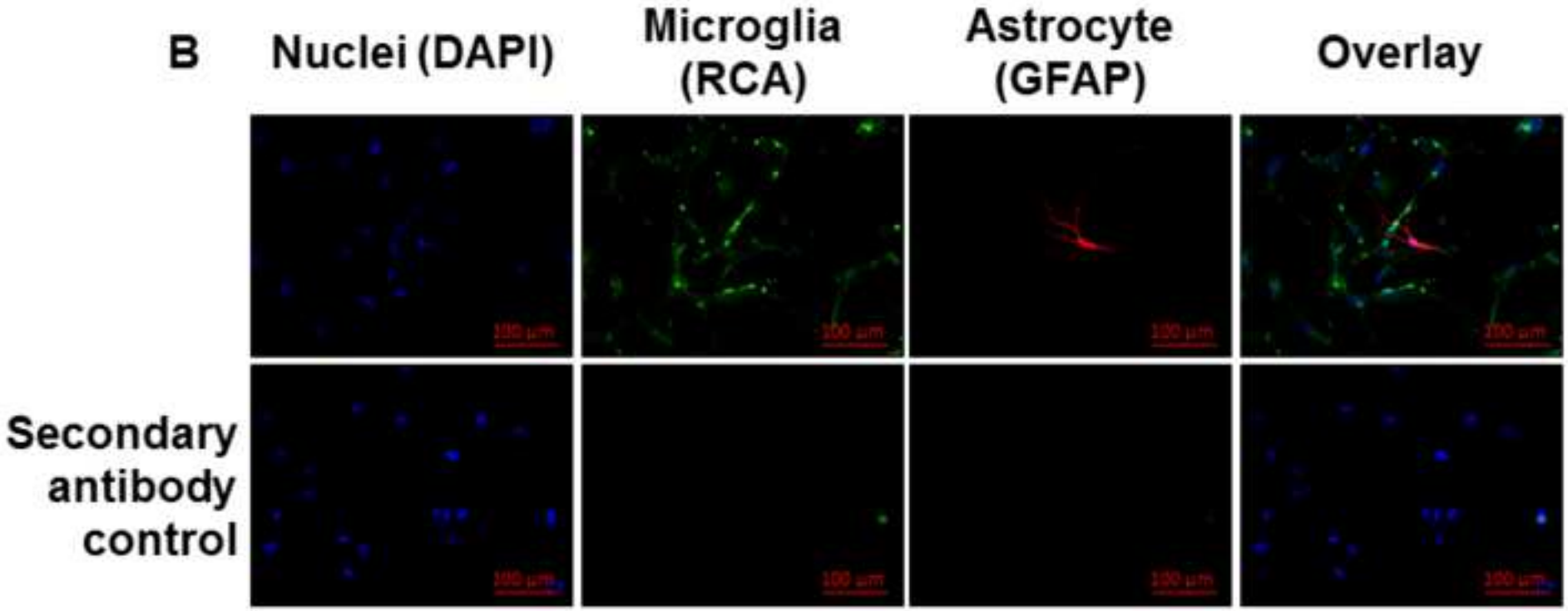

Figure 2 c

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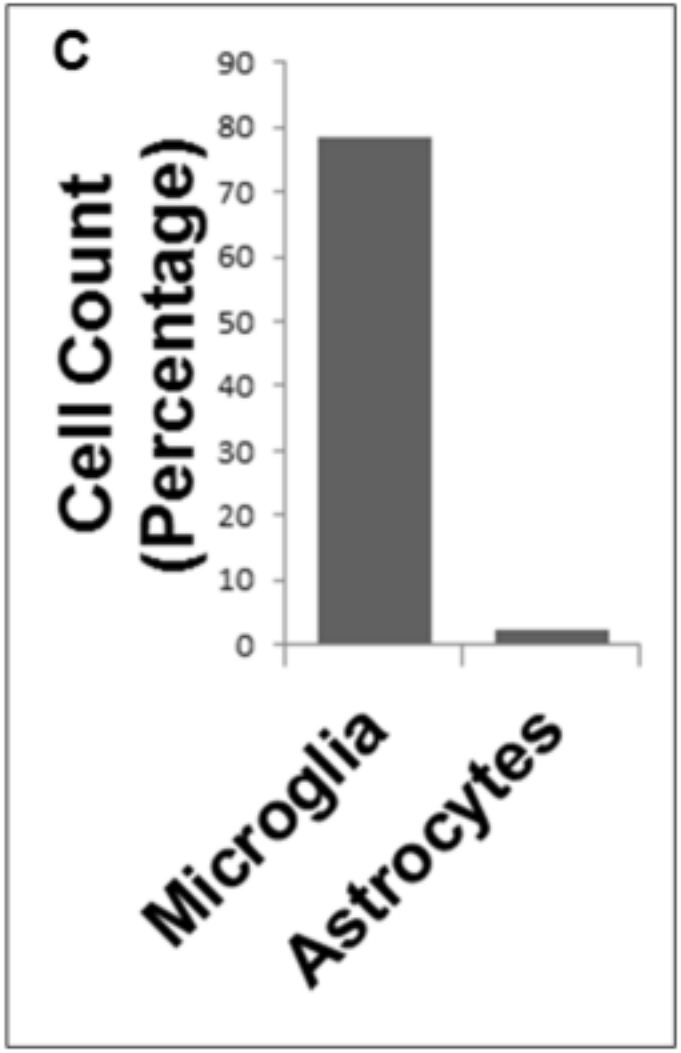

| Name of Material/Equipment | Company |
|---|---|
| Antibiotic-Antimycotic solution | Himedia |
| Calcium chloride | Sigma |
| Centrifuge (4 °C) | Sigma |
| Centrifuge tubes | Abdos |
| $CO_2$ incubator | New Brunswik |
| D-Glucose | Himedia |
| DMEM medium with glutamine | Himedia |
| Fetal bovine serum | Himedia |
| Flacon tube (50 ml) | Thermo Fsiher Scientific |
| Fluorescein Ricinus communis agglutinin-1 | Vector |
| Fluorescent microscope | Leica |
| Fluoroshield with DAPI | Sigma |
| GFAP antibody | GA5 |
| Incubator shaker | New Brunswik Scientific |
| L929 cell line | ATCC |
| Laminar air flow | Thermo Fsiher Scientific |
| Magnesium chloride | Himedia |
| Monosodium phosphate | Merck |
| Nutrient Mixture F-12 Ham Medium | Himedia |
| Petri dish | Duran Group |
| Phosphate buffered saline | Himedia |
| Potassium chloride | Himedia |
| Serological pipette | Labware |
| Sodium bicarbonate | Himedia |
| Sucrose | Himedia |
| Syringe filter (0.2µ, 25 mm diameter) | Axiva |
| T-25 tissue culture flasks suitable for adherent cell culture. | Himedia |
| Trypsin-EDTA (0.25%) | Gibco |

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MB043
LW-SP1010
MB045
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25200-056

We thank the Editor and reviewers for their careful reading of our manuscript. We have now edited the manuscript as per the reviewers' suggestions. All edits have been highlighted yellow for the reviewers' convenience.

## Editor's comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We thank the editor for the suggestion. We have read the manuscript carefully and corrected all spelling and grammatical mistakes.

2. Please submit each figure as a vector image file to ensure high resolution throughout production:(.psd, ai, .eps.,. svg).

We thank the editor for the suggestion. We are submitting all image files as ".psd".

3. Figure 1: Please include a space between all numbers and units: 48 h instead of 48h

We thank the editor for careful reading of the manuscript. We have included spaces between all numbers and units in the figure as suggested.

4. Figure 2: Please revise the scale bar to say micrometer (lower case m) instead of micromolar (capital M). Please include a space between all numbers and units: 100 um, etc.

We apologize for this error. We have now revised the scale bars to say micrometer (um). We have also included space between numbers and units as suggested.

5. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

We thank the editor for the suggestion. We have reviewed the table of essentials. We have also sorted the Materials Table alphabetically by the name of the material.

6. Please revise the title to be more concise. Obtaining Human Microglia from Adult Human Brain Tissue.

We thank the editor for the suggestion. We have edited the title as suggested.

7. Please provide an email for each author.

We have included the email ids of all authors.

8. Please discuss limitations of the technique in the Discussion.

We thank the editor for the suggestion. We have now discussed the limitations of the technique in the discussion.

## Changes to be made by the Author(s) regarding the video

1. Please try to minimize audio gaps to ensure that all steps of the protocol have narration. Avoid long lapses without any narration or audio.

We thank the editor for this suggestion. We have tried to minimize audio gaps in the protocol narration and have edited the video.

2. On-Screen Text and Graphics:

Please ensure that background colors in all text title cards and figure drawings fully extend to the edges of the screen. Meaning, there should be no black bars on the sides in the chapter title cards such as at 01:06, or in the figures at 09:38. Please make the backgrounds white to match the cards.

We thank the editor for this suggestion. We have now ensured that background colors in all text title cards and figure drawings fully extend to the edges of the screen.

3. Narration Audio:

Narration should be in mono channel audio, meaning the same volume should be heard in the right and left channels. The following sections have narration in stereo, and should be converted to mono.

- 01:46-02:09
- 03:07-06:27
- 06:46-08:41
- 09:00-10:22

Please make sure the audio level peaks are between -12 and -6 dB.

We thank the editor for this suggestion. We have converted the audio to mono channel as suggested.

4. Editing:
Jump Cuts
In the video there are some edit points that feature shots with very similar content transitioned over a single frame, causing the content to “jump” on screen. This style of edit

is not appropriate for JoVE videos since it causes confusion and draws attention away from the content. When needing to join very similar shots in succession, use a cross dissolve or fade so the transition between shots is smooth. Here are some points noticed in the video that should be converted to dissolves:

- 01:20
- 01:42
- 01:47
- 02:44
- 03:40
- 03:42
- 04:18
- 04:44
- 05:00
- 05:39
- 06:26
- 07:11
- 07:29
- 07:46

We thank the editor for this suggestion. We have avoided jump cuts and have added dissolve to the transition slides as suggested.

## Pacing Considerations

- 02:58 Consider shortening this shot of the washing of the sample.
- 07:46-08:43 Consider shortening and cutting down this section a bit. Some of the actions can be assumed and some are repeated. Edit it down a bit and move the narration to correspond what we're seeing on screen.

We thank the editor for this suggestion. We have shortened the protocol on suggested time points. We have also shortened the video at some other time points as recommended.

## Editing Glitches

- 09:51 The image behind the dip to white doesn't change before the white starts fading out, so there is a flash of the previous figure here. Move the edit point so that it lines up with center of the fade to white

We thank the editor for this suggestion. We have corrected the suggested editing glitch in the video.

## Reviewer #1

The protocol of primary cultures of microglia from adult human tissue is very useful. The explanatory video is exhaustive and complete, but the writing of the protocol deserves to be more detailed.

1. 1.1 Which part of the brain? how many mg of tissue it takes to have a number of cells useful for an experiment? a scheme describing mg of tissue and approximately number of cells may be appropriate. Is aCSF sterile solution?

We thank the reviewer for this suggestion. The tissue was collected from different sections of the brain. This is one of the advantages of the protocol that it can be applied to tissue collected from any part of the brain. However, as the population of microglia varies in different parts of brain the output of isolation may vary. In the discussion, we have mentioned the least amount of tissue from which we were able to successfully isolate microglia. We have not used tissue less than 268 mg, but, efficiently following the protocol may give results from less amount of tissue. Yes, aCSF is a sterile solution. aCSF was prepared in autoclaved distilled water and filtered with a 0.22 µm syringe filter in a laminar hood to ensure sterility.

2. 1.3 in aseptic condition?

We thank the reviewer for this suggestion. We have mentioned "in aseptic condition" at the following step in the protocol.

3. 1.4 Warm PBS?

We thank the reviewer for the suggestion. At step 1.4 the tissue was kept in aCSF but not PBS as mentioned in the protocol. We have now included keeping the tissue in warm aCSF.

4. 1.8 in discussion : how important is it to cut dice tissue?

We thank the reviewer for the valuable suggestion. We have discussed the step in the discussion section emphasizing the importance of this step.

5. 1.9 0.25% of trypsin -EDTA is in PBS?

We thank the reviewer for careful reading of the manuscript. The 0.25% trypsin we are using is purchased from Gibco (Catalog no. 25200-056). We are using trypsin directly from this stock. We are not diluting trypsin in PBS or any other solution.

6. 1.11 is the quantity of neutralizing medium (10 ml) fixed or proportionate to the added trypsin-EDTA?

We thank the reviewer for careful reading of the manuscript. The volume of trypsin used is proportionate. We have now mentioned this in the protocol.

7. 1.14 I don't think it is necessary to specify "suited for adherent cells", unless we need plastic coated, in this case what kind of coating?

We thank the reviewer for the valuable suggestion. We have specifically used "suited for adherent cells" as we are not using any coating to coat our flask as sued in other protocols. However different manufacturers sell flasks specifically mentioning the suitability (or not) of the flask for adherent cell culture. That is why we have specifically used "suited for adherent cells" so that the flasks which are not suitable for adherent cell culture may not be used by anyone following the protocol. There is no separate coating needed in our protocol but the use flask which are not suited for adherent cell might vary the results.

8. 2. it is not clear why this step is necessary. Please discuss.

We apologize for the lack of clarity. We have now discussed the step in the discussion.

9. 2.1 how many pipes? how many ml per tube?

We thank the reviewer for the valuable suggestion. We have now added the details in the protocol.

10. 2.2 1466xg? exactly 1466xg are needed, 1500xg can't go well? Many centrifuges do not allow this type of setting; it would become an important limitation of the protocol.

We thank the reviewer for the valuable suggestion. We performed centrifugation at 4000 rpm. But the Jove author guidelines recommend mentioning speed in the terms of Xg. For our centrifuge 4000rpm is 1466 Xg. That is why we have mentioned the speed as 1466 Xg.

## Reviewer #2:

### Manuscript Summary:

In this manuscript Agrawal et al. propose and demonstrate a method for isolating pure cultures of human brain microglia derived from neurosurgical tissue. The manuscript is easy to follow and the method is well demonstrated visually. Superficially the method appears to work as described, however, their final characterization of microglial purity and phenotype is inadequate and should be further validated. Ensuring the proper classification of the derived cell population is essential to all downstream applications and the overall utility of this method and must be confirmed should this manuscript be suitable for acceptance.

Major Concerns:

1. The authors utilize RCA 1 to confirm microglial purity in their culture and demonstrate low contamination with astrocytes via GFAP staining. However, RCA 1 is a lectin, not a specific antibody towards microglial antigens. It can also label endothelial populations which following extended passage can also proliferate and contaminate a culture. Further, it is sometimes used to distinguish tumour vs. non tumour tissue and the authors obtained some samples from surgical resections of brain tumour patients, where the degree of tumour contamination is unclear.

The authors should utilize various microglial-specific antibodies to ensure the RCA 1 labelled cells are truly microglia. Suggestions for microglia include PU.1, CSF1R, TREM2, and/or DAP12 as these remain on microglia with extended culturing, unlike certain microglial-specific markers (e.g., TMEM119, P2YR12) which demonstrate downregulation. Additionally, utilising other various cell type-specific antibodies (OPCs, oligodendrocytes, endothelia, neurons etc) would be beneficial to appreciate the undescribed contaminating populations as this can alter interpretations of derived functions.

We thank the reviewer for the valuable suggestion. We would like to mention that the culture condition of primary endothelial cells and primary microglia cells are very different. We also culture primary endothelial cells in our lab. The coating of the flask with gelatin is one of the major determinants of the successful endothelial cell isolation and culture. The media is different and supplementation of the media with low serum growth supplement is also very critical for survival and proliferation of endothelial cells. It is highly unlikely that in our culture conditions endothelial cells will survive. Cells other than microglia are present as the purity of the culture is 80% and an additional round of purification might be needed for certain experiments. This is one of the limitations of the protocol which we have now discussed in detail in the discussion. We would also like to mention that RCA1 is routinely used for staining microglia in vivo. Some references are mentioned here:

1) Leslie Freeman, Haitao Guo, Clément David, June Brickey, **Sushmita Jha*** and Jenny Ting*, NLR members NLRC4 and NLRP3 mediate sterile inflammasome activation in microglia and astrocytes, **The Journal of Experimental Medicine**, DOI: 10.1084/jem.20150237 | Published April 12, 2017, ***co-corresponding author**

2) **Sushmita Jha** et.al, The inflammasome sensor, NLRP3, regulates CNS inflammation and demyelination via caspase-1 and IL-18. ***The Journal of Neuroscience***,2010,30(47):15811–15820).

3) Plant, S. R. et al. Lymphotoxin beta receptor (Lt betaR): dual roles in demyelination and remyelination and successful therapeutic intervention using Lt betaR-Ig protein. The Journal

of neuroscience : the official journal of the Society for Neuroscience. 27 (28), 7429-7437, (2007)

4) Arnett, H. A. et al. The Protective Role of Nitric Oxide in a Neurotoxicant- Induced Demyelinating Model. The Journal of Immunology. 168 (1), 427, (2002).

5) Arnett, H. A. et al. TNFα promotes proliferation of oligodendrocyte progenitors and remyelination. Nature Neuroscience. 4 (11), 1116-1122, (2001).

2. The authors obtain microglial cultures with ~80% purity in their final method (if the RCA 1 labelling holds true as microglial-specific). Whilst this may be suitable for various applications, including immunocytochemistry, where various functions can be attributed to microglia via the use of co-staining, this could complicate methods where the bulk cell homogenate is used, for example, protein extractions/western blotting or cytokine secretion analysis from cell media. The authors should discuss such caveats of their system.

We thank the reviewer for careful reading of the manuscript. We have now discussed this and other limitations of the protocol in the discussion.

3. The authors state "To further assure the quality of the culture we checked the response of isolated microglia for pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). The expression of cytoplasmic markers was similar to the reported results in the literature where the isolation has been done through different protocol."

There is no such data in the manuscript, or no reference to a published paper using this method to produce these results. As such, this should be removed entirely as this is not supported by the available data. Such an inclusion would be incredibly welcomed and would significantly strengthen the appropriateness of this method in downstream applications. As it stands, it is unclear 1) whether RCA 1+ cells are truly microglia, and 2) if so, whether these microglia truly function like microglia in vivo. Obviously these results are imperative to this being a useful protocol for others to follow.

We agree with the reviewers' comments and concerns. We have now removed this section as suggested.

Minor Concerns:

1. More details are required for the L929 supplement as this appears to be a critical source of growth factors for microglial survival. Most obviously, it is not clear what media these are grown in, and therefore what the base media for this supplement is. The authors should briefly discuss the culture of these cells so that other groups can easily reproduce this

protocol. It would also be interesting if the authors could briefly discuss what growth factors this supplement contains. The available growth factors, e.g., TGFb, M-CSF, or IL-34 can significantly alter microglial phenotypes.

We thank the reviewer for careful reading of the manuscript. We have mentioned the basal medium for L929 cell culture. We have provided the reference which can be followed to prepare and collect L929 supernatant. We have also mentioned the use of L929 supernatant as the primary source of GM-CSF and included the reference.

2. The authors state that cells are ready for use on the sixth day of culture. However, it is unclear which days the purity quantification comes from. It would be very useful to know how long the authors can maintain this purity. Certain experiments will likely require cells to remain in culture for several days post-plating, but if these cultures are overrun by other proliferating cells this could prove problematic. The authors should demonstrate this, or at least comment on their experience with this.

We thank the reviewer for the valuable suggestion. We have now mentioned the day, which is $7^{th}$, on which we checked the purity of the culture in the discussion section. We have cultures the cells till 9 days and performed Immunocytochemistry experiments with the cells, after passaging them once, on day 9 with satisfactory results. We are also using L929 media supernatant to enhance the growth of microglia cells. However it is possible that the number of other cells might increase with time. This is one of the limitations of the protocol and we have now discussed this in discussion.

3. The panel in Figure 1 suggesting to collect supernatant into a new flask may confuse readers. The supernatant typically refers to the remaining liquid after solids (hereby containing cells) is centrifuged out. What is being taken here is unattached cells which can then have the chance to attach in a new flask. I would suggest changing this to "non-adherent cells", or a similar synonym, rather than supernatant as this implies only soluble factors.

We thank the reviewer for careful reading of the manuscript. We have edited the figure and mentioned non-adherent cells in place of supernatant.