A refined rat primary neonatal microglial culture method that reduces time, cost and animal use.

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Background: Primary microglial cultures have been used extensively to facilitate the development of therapeutic strategies for a variety of CNS disorders including neurodegeneration and neuropathic pain. However, existing techniques for culturing these cells are slow and costly.

New Method: Here, we report a refined protocol based on our previously published methods described by Clark *et al.*, which reduces in the time, reagents and the number of animals used for each culture whilst yielding high number and excellent quality microglial cells.

Results: Our refined protocol offers an isolation of > 96% microglia from a mixed glial culture after only four days of incubation. It results in a high yield of microglia, in excess of one million cells per cortex with predominantly resting morphology and a low level of cell activation.

Comparison with Existing Method(s): Compared to conventional procedures our refined protocol requires only one third of the time to prepare high quality microglial cultures, cuts the cost more than four-fold, and significantly reduces the number of animals used per culture.

Conclusion: Our consistent, reliable, and time/cost effective microglial culture protocol is crucial for efficient *in vitro* screening of potential therapeutics. By dramatically reducing the culture time from 2 weeks to just 4 days and increasing the laboratory research output it has implications for the Reduction, Refinement and Replacement policies endorsed by many government funding agencies and animal research regulatory bodies.

Subject terms: cell culture

Keywords: cell culture, microglia, tissue culture dish, neonatal rats, cortex, *in vitro*

Introduction

Microglia are a population of CNS-resident macrophage-like cells [1]. In their non-activated resting state microglia have a ramified morphology, which is not seen in other macrophage populations [2, 3]. An important function of microglia is their ability to generate innate and adaptive immune responses [4]. Resting microglia can be activated by a variety of CNS pathologies, such as infection, injury, or neurodegenerative disease. *In vitro*, these cells may also be activated directly by lipopolysaccharide (LPS) or pro-inflammatory cytokines [5]. Upon activation, microglia undergo morphological transformation, proliferation, migration, upregulation of several bioactive molecules, such as reactive oxygen species, nitrogen intermediates and cytokines [6]. Due to their integral mechanistic roles in the CNS [7], namely immune regulation, microglia are of great interest in the research areas of neurodevelopment, neuroinflammation, neurodegeneration, and neuropathic pain [8-10]. Therefore, i*n vitro* microglial preparations have been used extensively in preclinical research for the development of novel therapeutic strategies for nervous system disorders and traumas.

Several protocols for culturing primary microglial cells from rodent CNS tissue have been published [11-19]. However, the majority of existing protocols vary with regard to the origin of the tissue used (e.g. mouse or rat), the reagents used, the postnatal stages at which the tissue is harvested, the types of CNS tissues used (e.g. cortex, whole brain or the spinal cord), and the isolation techniques of microglia from mixed glial cultures, which consists of microglia and astrocytes. Some existing protocols use proliferation stimulating factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) to increase the proliferation of microglia following the initial harvest and hence allow isolation of microglia at several time points [20, 21]. This is a good approach for increasing the total yield of microglia, if subculture of the cells is necessary. However, this should be applied to situations in which the addition of supplementing reagents does not interfere with the experimental aim or collection of

microglia at different stages *in vitro* is desirable. Even with the use of supplementing reagents the typical time required to culture microglia by published conventional protocols is 10 to 14 days, sometimes even up to 3 weeks, and it requires a mixed glial culture seeded into tissue culture flasks [11, 21-24]. Existing conventional protocols also require in general a large amount of animal tissue (e.g. 3 rodent cortices or 1-2 whole brains per flask [11, 23, 25] and a large volume of culture media (e.g. 13 to 15 ml, changed every 3 days) to maintain the cultures. This can be problematic because regular medium changes increase the risk of bacterial contamination and also the risk of activation of microglia due to mechanical disturbance and to temperature fluctuation during these routine culture procedures.

We have developed a refined protocol based on our previously published protocol reported by Clark *et al.*[25] and Staniland *et al.* [26], which used a unique combination of digestive reagents that allow effective dissociation of the rat brain tissue. However, apart from the reagent combinations our previous protocol is similar to other existing conventional protocols in terms of plastic ware and incubation duration. Although these protocols are well established, a common difficulty is maintaining high quality non-stimulated, resting microglia in cultures prior to the desired experimental treatment. In the international scientific networking platform Research Gate, there are thousands of views and reads on topics related to issues with culturing primary microglial cells. There is not apparent consensus in the scientific community with regard to the most efficient method for microglial cell culture to achieve consistent cell yields and consistent microglial resting phenotype. Published protocols vary dramatically for microglial isolation; some propose only a minute of vigorous shaking of the flask, while others propose shaking the flask on an orbital rotator for 3h to 24h. Some protocols also describe overnight trypsinization as the most effective method of cell separation but this discrepancy between methods could have a huge impact on the final quantity and quality of the cells. Therefore, there is a need for a refined culture protocol that overcomes the difficulty of obtaining high quality resting cells and improves consistency.

Furthermore, existing conventional protocols require a large amount of resources per culture, including culture media, reagents, and even animals, which is not ideal considering ongoing global cuts to science budgets. Here we have established a simplified and refined protocol that results in consistently reproducible high quality resting microglial culture with significant reductions in the time, reagents and the number of animals used. Thus, in comparison to existing conventional microglial protocols, the procedures described here significantly reduce the following: 1) the incubation time prior to microglia harvest (Supplementary Table S5); 2) the volume of culture media and related reagents used (Supplementary Table S4) per culture; 3) the number of neonatal rodents required per culture (Supplementary Table S4); and 4) the mechanical disturbance to cells upon plating. Meanwhile, the consistency of the cultures is ensured because the tissues are taken from a discrete brain area (cortex). Furthermore, the reduction in the number of animals used per culture subsequently reduces the time needed for handling and dissection of the tissues. Therefore, the total time required from initial plating to high quality cultures of microglia is substantially shorter than existing conventional protocols.

Methods

A list of the reagents and equipment used in our refined protocol and their corresponding catalogue numbers are provided in Supplementary Table S2 and Supplementary Table S3.

Animals and tissue collection

All procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedure) Act of 1986 and were approved by the College of Life Sciences and Medicine Ethics Review Board at

the University of Aberdeen. Wistar female and male rat breeders were purchased (Charles River, UK) and maintained in the breeding facility with free access to food and water. Approximately 25 days following mating rat neonates at postnatal day 3-6 (DIV3-6) were culled humanely by neck dislocation.

Dissection of neonatal rat cortex

Procedures for dissection of rodent cortex have been described only briefly in previous publications [13, 27, 28]. Since this dissection procedure is critical to the success of our protocol and to the good quality of these cultures we include a detailed step-by-step guide. Our experience has found the following gross and fine dissection technique to be the most efficient method for isolation of the rat cortex used for the microglial cultures.

Once collected from the animal facility, the live rat neonates between P3-6 were culled by cervical dislocation and dissected (Figure 1a-j) one at a time to prevent necrosis of the tissue. Following cervical dislocation each pup was wiped with 70% ethanol and decapitated with large sterile scissors (Figure 1b). The head was then pinned through the nose cavity on a sterile polystyrene surface using a dissecting pin or syringe needle (Figure 1c). After removing the skin from the skull using fine scissors (Figure 1d), an incision was made along the sagittal suture starting from the foramen magnum all the way to the frontal bone using fine scissors (Figure 1e). To prevent damage to the brain the blade of the fine scissors was guided through the midline of the skull by applying gentle pressure on the skull bone. Using sharp, fine Dumont forceps the skull covering the cortex was crushed in a lateral direction starting from the midline of the parietal bone all the way to the frontal bone (Figure 1f). This step was performed quickly and confidently to remove the entire skull around the cortex and small bone fragments were not allowed to perforate the brain.

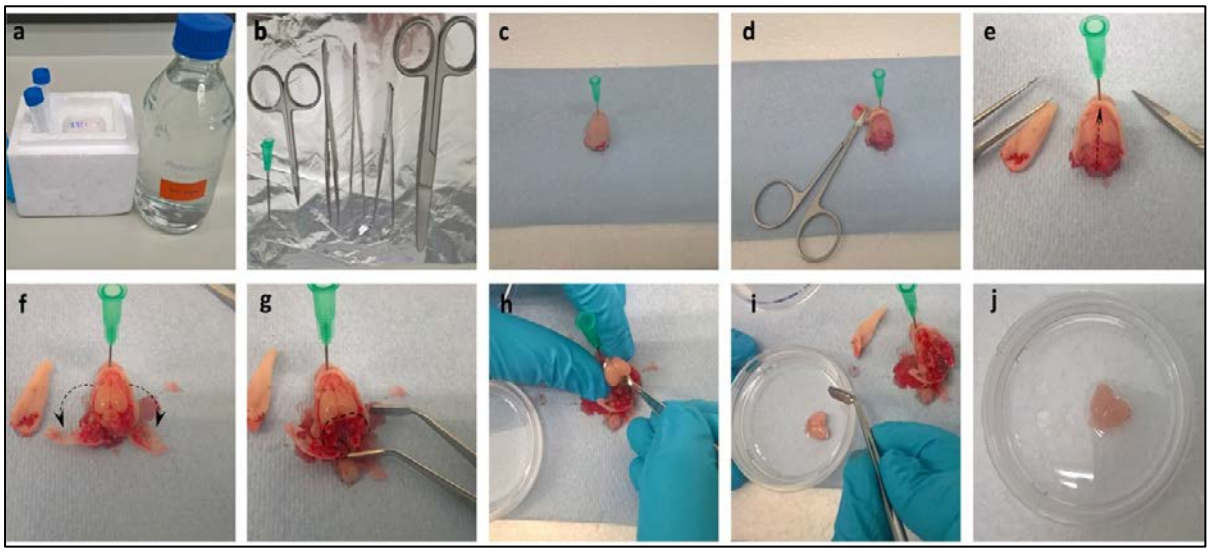
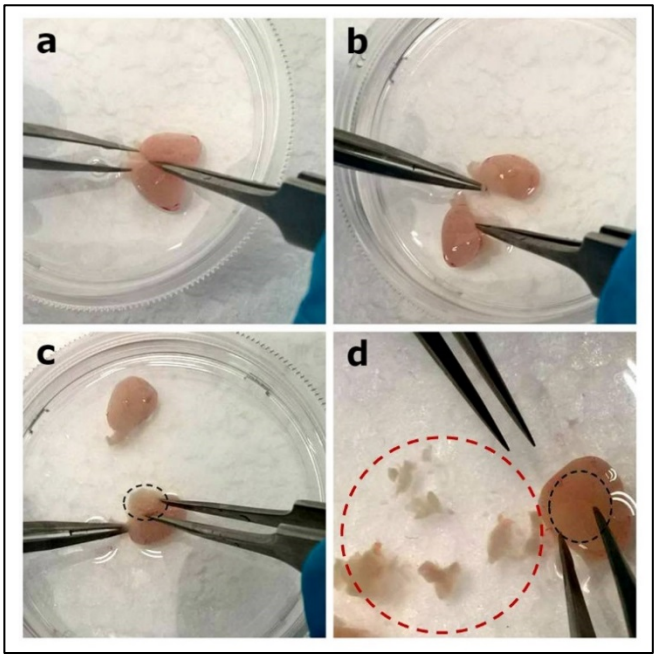


Figure 1. Step-by-step gross dissection of neonatal rat cortex. (a) Centrifuge tube(s) and 60 mm dissection dishes must be placed on ice well in advance prior to dissection. 70% ethanol should be readily available to sterilise the work area, the instruments and the surface of the neonatal head. (b) The following instruments are essential for the dissection of the cortex: 16 mm needles (Fisher 12329179, UK, small scissors (FST14090-09, Germany), 2 x straight Dumont forceps (FST11252-20, Germany), cover glass forceps (FST 11073-10, Germany), and large scissors (FST 14007-14, Germany). They should be cleaned and sterilized with ethanol immediately prior to use. (c) The head of the rat pup is pinned through the nose cavity onto a sterile polystyrene surface using a dissecting pin or a syringe needle. (d) The skin covering the skull is removed using fine scissors and forceps. (e) An incision is

made along the sagittal suture starting from the foramen magnum all the way to the frontal bone using fine scissors. (f) The skull covering the cortex is crushed in a lateral direction starting from the midline of the parietal bone all the way to the frontal bone. (g) The hindbrain is removed using cover glass forceps, leaving only the two hemispheres and the olfactory bulbs. (h) The cortex is lifted from the skull base using the cover glass forceps. (i-j) The tissue is quickly transferred in the 60 mm dish containing ice cold HBSS.

Once the entire cortex was exposed cover glass forceps were used to remove the hindbrain immediately, leaving only the two hemispheres and the olfactory bulbs (Figure 1g). The tissue was collected quickly in the 60mm Petri dishes containing Hank's Balanced Salt Solution (HBSS) 1x without $CaCl_2$ and $MgCl_2$ (Invitrogen 14175053, UK) on ice by gently holding the skull with the non-dominant hand and using the cover glass forceps with the dominant hand to lift the brain from the skull base. The procedure was repeated for all brains and the tissue kept on ice until further dissection (Figure 1j).

Under a dissecting microscope, closed sharp Dumont forceps were passed along the inter-hemispheric fissure and the two cortices were separated (Figure 2a-b). By holding the olfactory bulb with the forceps (to prevent tissue damage to the cortices), each cortex was flipped on its lateral surface (Figure 2c). It is important to remove the meninges as one unit without contaminating the clean cortex. To do this whilst maintaining the integrity of the cortex (Figure 2c-d) remove the inner contents of the brain first (hippocampus, hypothalamus, thalamus), leaving removal of the meninges until last.



There is a distinct border that can be seen between the cortex surface and the surface of the midbrain and the hippocampus (Figure 2c). With several snips of the Dumont forceps, the midbrain, hypothalamus, and hippocampus were separated from the two hemispheres of the cerebral cortex and left aside (Figure 2d). The cortex was flipped with its lateral surface facing up and a second set of Dumont forceps was used to remove the sheet of meninges covering the cortex.

Figure 2. Fine dissection of cortices prior to the removal of meninges. (a-b) Separation of the two cortical hemispheres using closed Dumont forceps. The best way of handling the cortex is to hold the olfactory bulb while moving it around so the rest of the tissue is not damaged. (c) Once flipped on its lateral side hold the cortical hemisphere at the base of the olfactory bulb. There is a distinct border (appears as an indentation in the tissue) that can be seen between the cortex surface and the surface of the midbrain and the hippocampus (black circle). The tissues in the middle of the hemisphere could be removed by a few snips of the forceps, leaving clean cortex underneath. (d) The midbrain and the hippocampus have been removed from the middle area of the cortical hemisphere (black circle) and are seen here as waste floating in the HBSS (red dotted circle).

Fibroblasts originating from the meninges proliferate rapidly in the first couple of days following seeding [23]. Therefore, special care must be taken to completely remove the cortex meninges, otherwise the cultures are likely to become contaminated by fibroblasts. Cortices or parts of cortices that are not cleaned of all meninges therefore should not be used further, i.e. not dissociated chemically (see digestion solution in reagent set up below) or seeded in the tissue culture dishes.

The most efficient approach for complete removal of the meninges following the steps in Figure 2c-d, is by flipping the cortex, so the side covered with meninges is facing the observer. Under a dissecting microscope with a good light source the meningeal layer is identified easily due to the presence of blood vessels within it. The first step in the process is to remove the olfactory bulbs with a few snips of the forceps, which exposes the border between the cortex tissue and the meninges. The removal of the bulbs creates an edge of the meningeal layer that can be grasped with forceps in the dominant hand and peeled away from the cortex tissue, whilst simultaneously securing the cortex in place at the anterior part of the hemisphere with the forceps in the non-dominant hand. Once the beginning of the meningeal layer is caught, it is pulled/peeled gently toward the posterior of the hemisphere, while the anterior part of the hemisphere is stabilised with the forceps in the non-dominant hand at all times. This stabilisation should move in a posterior direction as the peeling progresses to permit sufficient pressure to be applied near the peeling site constantly. Once the whole sheet of meninges is completely peeled the clean cortex is gently transferred with forceps to the 7ml bijou tube with fresh, ice cold HBSS prior to. Each tube can hold tissue from up to three whole rat brains (6 cortices).

Timing of the culture

The duration of the entire culture procedure depends on the number of pups to be used. Adequate time should be allowed to collect the pups from the animal facility, adding 1 minute for dislocation of the neck per pup. The gross dissection of the brains should not take more than 1 minute per pup. Once the brains are taken out of the skull they should be transferred into a dish containing ice cold HBSS and kept on ice until further processing. If the tissue requires to be transported between facilities, this should require no more than 15 minutes and fine dissection of the cortex should commence as soon as possible after the pups have been sacrificed. The fine dissection of the cortex must be performed as quickly as possible and the tissue should be stored on ice until the digestion step to preserve cell viability [21, 22]. For best results, no more than a minute per cortex should be allowed for the removal of the meninges.

Reagents setup

Digestion solution was prepared by mixing 50 ml Earle's Balanced Salt Solution (EBBS) 1x with $CaCl_2$ and $MgCl_2$ (Invitrogen 24010, UK), 150 mg bovine serum albumin (BSA) fraction V (Sigma A-4503, UK), 7 mg trypsin from bovine pancreas (Sigma T-9935, UK), 8.5 mg DNase type 1 (Sigma DN25, UK), 500 µl Penicillin/Streptomycin (Invitrogen 10378-016, UK). The mixed solution should be filter sterilised using 0.2 µm syringe filters (Triple red FPE204025, UK) or any analogue.

The culture medium was prepared by mixing Dulbecco's Modified Eagle's Medium (DMEM) high glucose, GlutaMAX™ Supplement, HEPES (Invitrogen 32430-027, UK), 15% Foetal bovine serum (FBS) heat inactivated (Invitrogen10500-064, UK) and 1% Penicillin/Streptomycin (Invitrogen 10378-016, UK).

For the dissection of the cortices HBSS solution was prepared by mixing Hank's Balanced Salt Solution (HBSS) 1x without $CaCl_2$ and $MgCl_2$ (Invitrogen 14175053, UK) with 1% Penicillin/Streptomycin. For every one to three brains dissected, one 60 mm dish (to dissect the tissue in) and one 7 ml bijou tube (to store the dissected tissue) filled with 5 ml of HBSS were prepared in the laminar flow hood, capped and

place on ice for at least 30 minutes before use. All solutions were pre-warmed to 37ºC prior to use for cell culture. In addition, all instruments that were used for the dissection of the tissue or for insertion of coverslips in the wells were sterilized prior to use with either by autoclaving or with 70% ethanol.

The 60 mm 21.2 cm² tissue culture dishes (BIOFIL Triple red TCD010060, UK) mm were coated for 1 to 2 hours prior to seeding of the cells in the laminar hood with 5 ml Poly-D-lysine coating solution (the solution was added to the dishes, the lid closed, and the dishes were left aside for the required time, while other culture related procedures were carried out). A 1mg/ml stock solution was prepared by adding sterile distilled water to the powdered Poly-d-Lysine (70 to 150 kDa; Sigma P6407, UK). The stock solution was then stored at -20°C and a 0.01 mg/ml working solution was prepared immediately prior to use by adding 50 µl of the stock solution to 5 ml of double distilled H_2O. Once coated the solution was removed from the dishes and they were left for several minutes to air dry. The surface of the dishes was washed twice with sterile distilled water and again left to dry until use (usually this is performed while the cell suspension is incubated in digestive solution or centrifuged to form a pellet).

Cell suspension preparation

The cleared from meninges cortex tissue was left to settle in the HBSS on the bottom of the 7 ml tube (maximum of three cortex/ 2 hemispheres each in a tube) after which the supernatant was discarded by pipetting out the liquid carefully with a P1000 pipette. 3 ml of pre-warmed digestion solution was then added to the bijou tube and the cortices were transferred to a 15 ml centrifuge tube with a serological 5 ml pipette. An additional 3 ml of digestion solution (total 6 ml digestion solution for cortices from 3 whole rat brains – in other words 2ml digestion solution for cortex tissue from 1 brain) was added to the tissue and the suspension was gently triturated 15-20 times using the serological 5 ml pipette. After mechanically breaking down the tissue the lid of the tubes was closed and the tubes were placed in the incubator (37°C, 5% CO_2) for 7 minutes. After an additional gentle swirl the tubes were again placed in the incubator for further 7 minutes. To stop the digestion reaction an equal volume (6 ml) of pre-warmed FBS was added to each tube and the mixture was triturated gently 10 times using a fresh serological 5 ml pipette. It is important to note that the suspension should be cloudy at this stage and once triturated sufficiently it should be free of large fragments of tissue. Take care to not over triturate the cell suspension, which may reduce yield of viable cells.

The tubes were then centrifuged for 10 minutes at 200xg at 4ºC and care was taken to set the centrifuge break to maximum to avoid the cell suspension forming a spiral column as the centrifuge slows in speed. The supernatant was removed and 10 ml pre-warmed DMEM supplemented medium was added to the cell pellet. The suspension was triturated well until cloudy and filtered through a 70 µm cell strainer into a 50 ml centrifuge tube. The strainer was washed through with another 5 ml of culture medium into the same tube resulting in a total volume of 15 ml cell suspension that was equally separated into three 60 mm tissue culture dishes pre-coated with Poly-d-lysine. The dishes were then incubated in a humidified environment at 37ºC, 5% CO_2, 95% air atmosphere for 4 days without changing the culture medium.

Microglial plating

The procedures related to the isolation of microglial cells took approximately 45 minutes (followed by initial incubation of 2 hours and a total incubation time of 24 hours). Four days after the initial seeding and incubation, the dishes were examined under a light (phase contrast) microscope. The presence of two layers of cells was confirmed. One layer of astrocytes was firmly attached to the surface of the dish and a layer of microglia were visible as large spherical phase bright cells (Figure 3d) on top of the astrocyte layer.

The dishes were then swirled gently for 1 minute by hand in a clockwise and an anticlockwise direction. Alternating the directions every 30 second helps the cells to detach easier. The swirling motion is performed with care, while supporting the lid with hand and not letting the base of the dish to lift from the surface of the laminar hood. The movement of the dish around its cylindrical axis should be fast enough to make the medium inside swirl and cause microglial cell detachment from the base astrocytes layer, but slow enough to not allow medium to spill out of the dish or to touch the inside surface of the lid. It must be remembered that although the culture dish is closed, it is not a sealed vessel, so great care must be taken to ensure that the medium has no contact with the outside environment, which would lead to contamination. The medium was then carefully collected using a serological pipette and placed in a 15 ml centrifuge tube. Another 5 ml of fresh DMEM supplemented medium was added to the dish and another gentle swirl for 1 min was performed as described above.

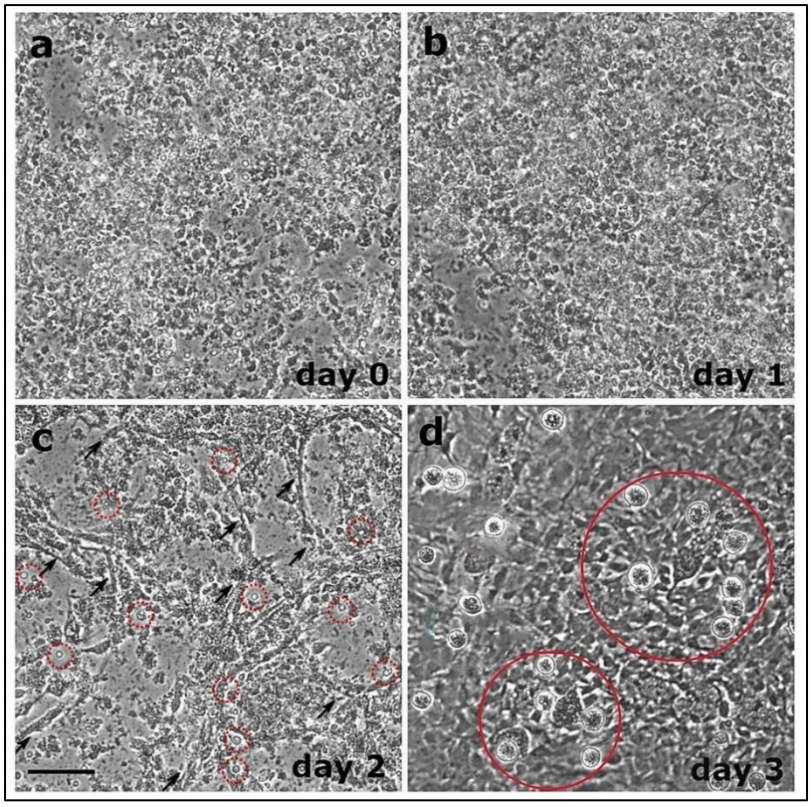


Figure 3. Mixed glial cultures at different stages prior to microglial harvest. (a) At DIV0 a mixed astrocyte and microglial layer is formed at the bottom of the dish. All cells appear small and can hardly be distinguished at this stage. (b) At DIV1 astrocytes cover the bottom of the dish, while microglia start to proliferate on top, visible as a brighter layer of cells. (c) At DIV2 the astrocyte layer becomes denser as astrocytes also proliferate (black arrows) and their processes form connections. Microglia, which appear phase bright here start increasing in size and number (red dotted circles). Some microglia start to attach loosely to the astrocyte layer. After roughly 24 h the empty spaces on the culture dish base would be covered by a dense astrocyte layer. (d) At DIV3 microglia have increased in size significantly and start forming larger, round, phase bright colonies (red circles) on top of the very dense, phase dark astrocyte layer. Once microglia form dense colonies they are ready to be harvested (DIV4). DIV=days in vitro. Scale bar 100 μm.

The medium collected from the dish was then added to the 15 ml centrifuge tube and the dish was checked under the microscope for any residual microglial cells. In most cases, two repetitions of the swirling step are sufficient for all microglia to be detached. However, if any residual microglial cells are noticed in the dish, the steps could be repeated once more. More repetition of the steps beyond 3 times, could increase the risk of astrocyte detachment from the basal layer and contamination of the culture. At the end of the procedure 5 ml fresh DMEM supplemented medium was added and the dish was returned to the incubator for future astrocyte harvesting. The tubes were centrifuged for 10 minutes at 200xg, 4ºC and the supernatant was discarded by gently withdrawing it with a 5 ml serological

pipette. 1 ml of fresh pre-warmed DMEM culture medium was added to the cell pellet. Following trituration with 5 ml serological pipette the cells were counted using an automated cell counter or a haemocytometer and diluted with DMEM culture medium to the desired cell density as required for the *in vitro* experiment (60 000 to 100 000 cells/ml for our experiments).

Next, 18 mm uncoated sterile glass coverslips (best sterilised by autoclave to preserve adhesion properties) were placed into 12-well cell culture plates. 150 µl of the mixed cell suspension was plated onto each coverslip, ensuring that the liquid surface tension is conserved on the coverslip surface and does not flow onto the underlying plate surface. Such overspill would encourage the cell suspension to run under the coverslip by capillary action. The surface tension on the glass should be sufficient to retain a volume of cell suspension up to 200 µl.

The cells were then incubated for 2 hours at 37ºC, 5% CO_2, 95% air atmosphere and then examined with a light microscope to confirm that they had attached to the surface of the coverslip and had started extending cell processes. After the cells had settled sufficiently 300 µl fresh pre-warmed DMEM medium was added to each well and the dishes were left in the incubator overnight.

Results

This protocol was developed to culture microglia for testing compounds with the potential to modulate microglial activation. Microglia obtained from this protocol were of a higher quality, with a significantly higher percentage of cells expressing a resting morphology compared to cultures produced by existing conventional methods. For the purpose of our experiments we stimulated our cultured microglia to an activated state using LPS [29-31]. Microglia cultured following our protocol showed activation after 4 hours of LPS stimulation (10 µg/ml). We analysed microglial proliferation and activation using immunocytochemical staining (method in Supplementary Methods 1) with antibodies to Iba-1 (microglia/macrophage-specific calcium-binding protein; a pan marker for microglia) and iNOS (pro-inflammatory microglial marker). In addition, RT-qPCR was used to measure gene expression profiles of pro-inflammatory mediators in microglia including TNF-alpha, IL-6and iNOS (details in Supplementary Methods 2, Supplementary Table S1).

Microglia seeded into a 60 mm dish proliferated faster than those in a T75 flask

Comparisons were made of cell proliferation and the confluence rate of astrocyte and microglial layers for mixed cell suspensions seeded into T75 flasks (typical for most existing conventional protocols) and into 60 mm dishes (our refined protocol). In the 60 mm poly-d-lysine coated dishes, microglia appeared to proliferate three times faster than those grown in T75 flasks, reaching ~90% confluence at DIV4 in comparison to DIV10-14 for T75 flasks. The peak of proliferation in the dish happened at DIV2 and DIV3, as confirmed by Iba-1/IdU double labelling (Supplementary Figure S1; Supplementary Figure S2). More than 70% of all Iba-1 immunoreactive cells were also labelled with IdU during DIV2 and DIV3, which indicated high rates of proliferation. The proliferation rate then significantly decreased at DIV4, as about 20% of the total Iba-1 immunoreactive cells expressed IdU.

In addition, the basal layer of astrocytes formed within only one to two days when cultured in 60 mm dishes (Figure 3b-c) compared to four to six days when cultured in T75 flasks. Furthermore, these mixed cultures in 60 mm dishes did not require medium changes until the time of harvesting, whereas the T75 flask cultures required medium changes every three days. The density of microglia in T75 flasks at the time of harvesting appeared to be much lower than that in 60 mm dishes (Figure 4) despite three cortices being used for the flask culture but only one cortex was used per 60 mm dish.

The effective surface area for cell attachment in a T75 flask is 75 cm^2 and for our 60 mm dishes it is 21.2 cm^2. Therefore, there was a higher cell number at harvest in a 60 mm dish despite the similarity in the initial plating density, i.e. one cortex per 25 cm^2 in the T75 flask and one cortex per 21.2 cm^2 in the 60 mm dish. This was confirmed by cell yield counts following harvest, i.e. yields at DIV14 ranging from 960 000 to 1 600 000 cells from 3 cortices seeded in the T75 flask vs. yields at DIV4 ranging from 750 000 to 1 200 000 from 1 cortex seeded in the 60 mm dish.

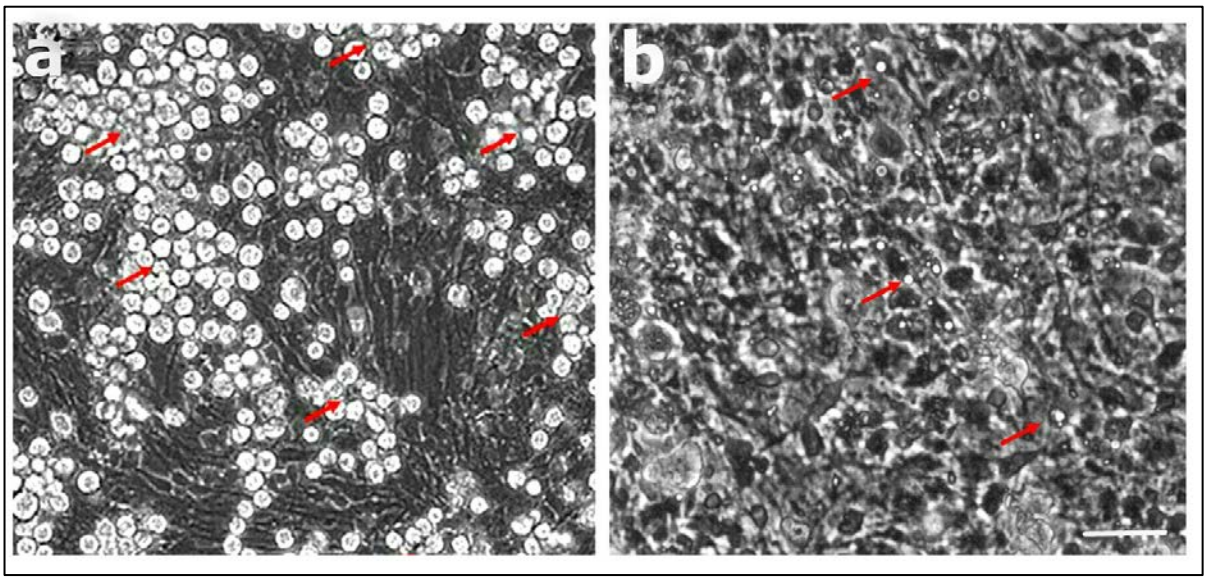


Figure 4. Cultured microglial morphology at harvest using our refined protocol compared to the conventional protocol. (a) Using our refined protocol microglia at DIV4 appear as bright spherical cell colonies (red arrows) loosely attached to the underlying astrocyte layer. (b) Using the conventional protocol microglia at DIV10 still appear small and sparse compared to those at DIV4 prepared using our protocol. Microglia do not form colonies. Scale bar 100µm.

The purity of the microglial culture exceeded 96%

Quantitative analysis of double labelling with Iba-1 antibody and Hoechst nuclear dye showed that our refined protocol resulted in microglial cultures that were ≥96% pure (Figure 5, Supplementary Figure S3). Immunostaining with the astrocyte marker GFAP across biological replicates showed occasional negligible astrocyte contamination in the subcultures following microglial harvest, which equalled to less than 1% (Supplementary Figure S3d).

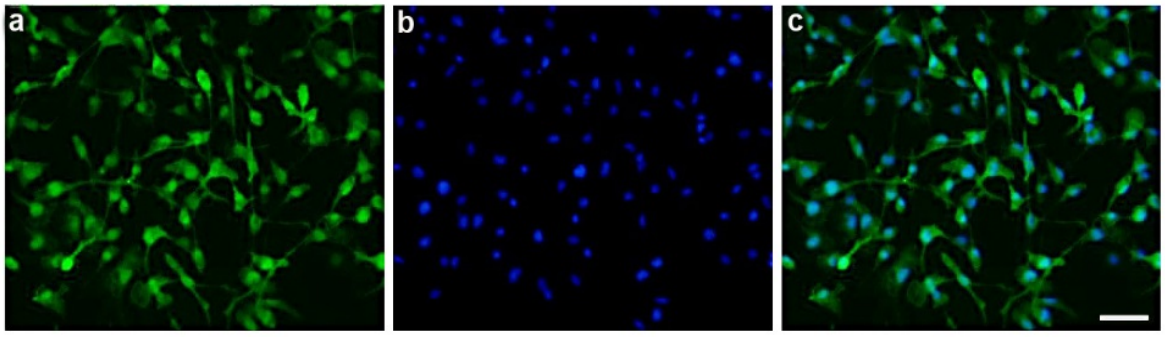


Figure 5. Representative images showing microglial purity using our refined protocol. (a) Immunofluorescent staining of microglia with Iba-1, a pan marker for microglia (green). (b) Nuclear staining with a Hoechst dye (blue). (c) An overlay of (a) and (b), demonstrating over ≥ 96 % Iba-1 positive and Hoechst positive cells. Scale bar 100 µm.

Microglia grown in 60 mm dishes exhibited resting bipolar morphology 24 hours after plating

One hour after plating microglial cells settled down onto the uncoated coverslips and started to extend processes. By 24 hours the cells were firmly attached to the coverslips and showed a typical bipolar resting morphology (Figure 6). The cells were used for experiments between 24 hours and 72 hours after plating and were activated effectively by LPS. Cells treated with 10 µg/ml LPS (positive control) developed an activated/amoeboid morphology following 4 hours of exposure (Figure 6). The concentration and the application time of the activating agent used, whether LPS, IFN-gamma, or combination of both, will need to be adjusted to suit the experimental requirements of labs adopting this method.

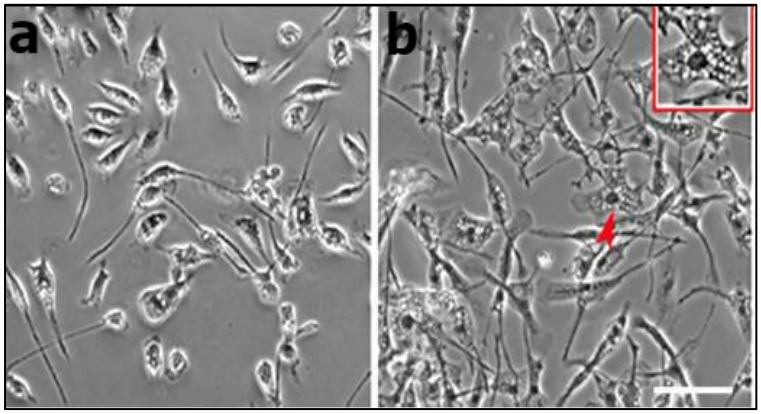


Figure 6. Representative images of resting and activated microglial morphologies. (a) At a resting state (control), e.g. within 24 hours after plating, most microglia appear to have a bipolar morphology with long and slender processes. (b) In comparison, when exposed to LPS (10 µg/ml) for 4 hours in culture, most microglia appear to be activated; having hypertrophic cell bodies with many vacuoles in the cytoplasm (magnified in the red box insert), with their processes thickened. Scale bar 100 µm.

Microglia obtained by our protocol did not express iNOS and release nitrite

Following fixation with 4% paraformaldehyde (PFA) microglia were stained with primary antibodies for Iba-1 and iNOS to identify activated microglia. This was done to confirm the resting state of the cells for comparison with microglia stimulated with LPS (10 µg/ml). In untreated microglia, no iNOS expression was observed (Figure 7c). However, a significant increase in iNOS expression was observed in LPS-treated microglia (Figure 7d). Furthermore, untreated microglia did not show any increase in expression of the pro-inflammatory genes iNOS, TNF-α, and IL-6, as seen in microglial cells treated with LPS (Supplementary Figure S4).

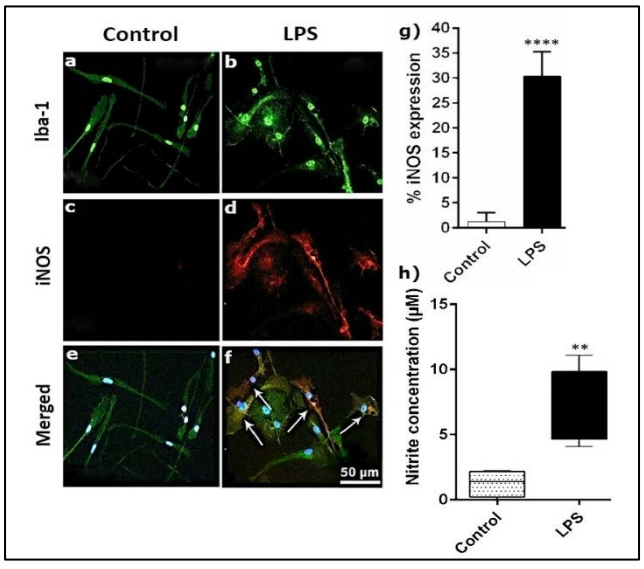


Figure 7. Comparison of pro-inflammatory status between resting and activated microglia using our refined protocol. Iba-1 staining for microglia (green) is shown in (a) control treatment with serum free medium and (b) LPS (10 µg/ml) treated for 4 hours. iNOS staining (red) is shown in (c) control treatment and (d) LPS-treated for 4 hours where increased iNOS expression was observed. (e) and (f) are merged images of (a/c) and (b/d) respectively. (g) Quantitative image analysis showing a significant difference in iNOS immunoreactive cell numbers between control microglia and LPS-treated microglia. (h) Greiss assay demonstrated a significant increase of nitrate concentration in

the supernatant collected from LPS-treated microglial cultures when compared to that of the control. SEM=standard error means. N=3 (biological replicates)/n=9 (technical replicates) per treatment. Student *t*-test; **p<0.01, ****p<0.0001.

Prior to fixation, the culture media in which the cells had been maintained for 4 hours were collected. The levels of nitrite in the media from untreated and the LPS-treated microglial cultures were then measured by the Greiss assay (Figure 7h). The nitrite release from the resting microglial cells was significantly lower than the nitrite concentration released in the media from LPS-treated microglia. The microglial cells obtained by our protocol are suitable for drug treatments, including different activating agents and therapeutic compounds, thus making them a useful *in vitro* model to screen potential therapeutics.

Discussion

The majority of published protocols for microglial culture are similar in regard to initial tissue dissociation techniques and incubation period. Most established methods still use T75cm^2 poly-d-lysine coated flasks for initial seeding of the cells, long incubation times (between 10-14 days) and two main methods for isolation of the microglial cells - vigorous shaking of the flask or enzymatic separation of the two cell layers (trypsinization). These techniques are found in many publications from the past 5 to 10 years [22, 23, 32, 33] but there are differences between protocols in terms of the duration and the more specific execution of the harvesting methods; duration of the vigorous separation of the two cell layers varies between 1 and 24 hours performed manually or on an orbital shaker, with or without the presence of a digestive enzyme. Hence, these protocols are not always easily reproducible and do not always result in consistent primary microglial cultures.

Here, we have demonstrated a refined protocol that provides a reproducible and straight forward method of culturing high quality primary microglial cells from rat neonatal cortex tissue. One of the most significant advantages of our protocol is that it allows the preparation of resting microglial cultures in significantly less time (4 days) compared to existing conventional protocols (2 to 3 weeks) (Figure 8a-b; Supplementary Table S5). Thus, our refined protocol saves significant resources, which is increasingly important for preclinical medical research as science budget cuts continue globally. The refined protocol reduces the cost of supplies and reagents by 75% compared to existing conventional protocols (Figure 8a-b; Supplementary Table S4). Importantly, our refined protocol reduces the number of animals required per protocol. The microglial cells obtained by our protocol are suitable for drug treatments, including various activating agents and therapeutic compounds making them a useful *in vitro* model to screen potential therapeutics.

Here we have shown, that there is a faster and more effective method for culturing resting primary microglial cells than the existing conventional methods prevalent during the past 10 years. We cultured microglial cells in only four days in a 60mm tissue culture dish, without changing medium during the incubation period. Our data showed that microglial cells proliferate at very high rates during DIV2 and DIV3 in culture and that the proliferation decreased significantly at DIV4, when we harvested the cells, as they had reached confluency.

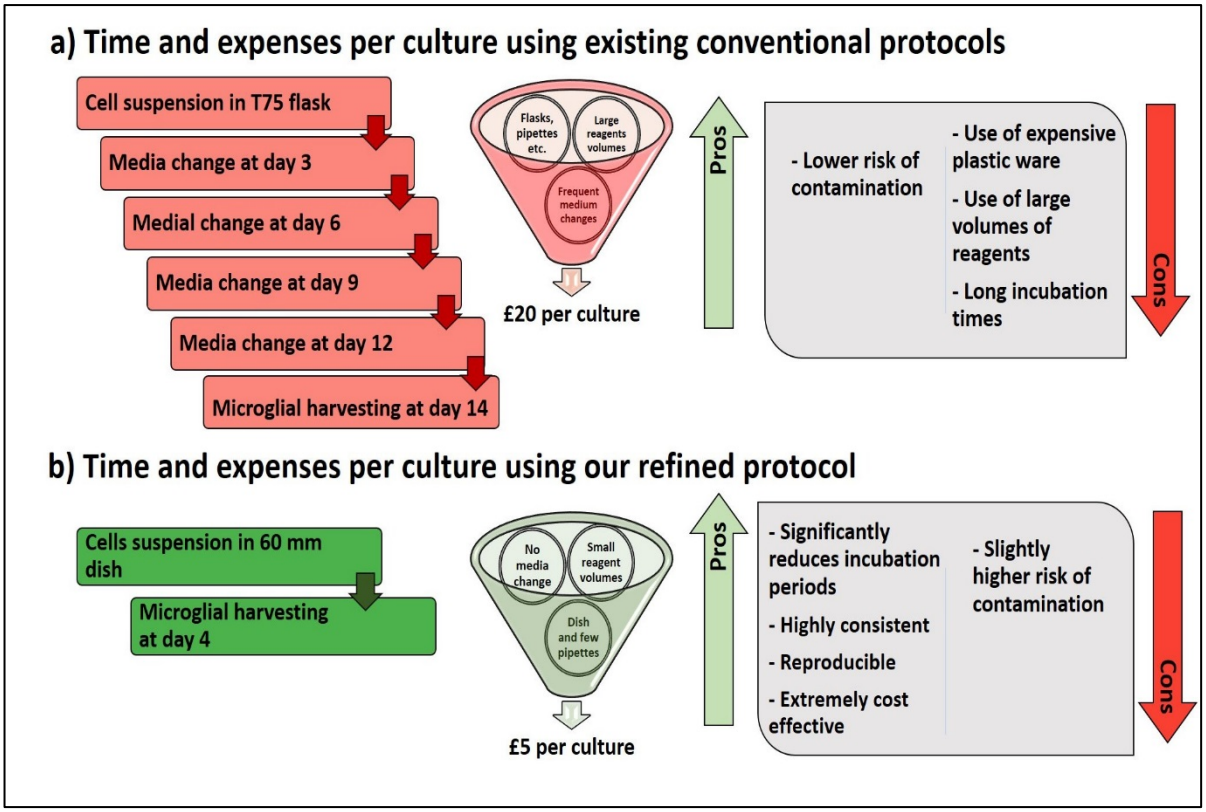


Figure 8. Schematic diagram representing the advantages of our refined protocol compared to existing conventional protocols containing a list of the pros and cons of our method. In comparison to the conventional microglial protocols (a), the most important advantages of our refined protocol (b) include a significant decrease in the incubation culture time prior to microglia harvest, considerable reduction in the usage of culture media and related reagents per culture, and significant reduction in the number of neonatal rodents required per culture.

By using our refined culture protocol, tissue from one cortex seeded in a 60 mm tissue culture dish regularly yielded more than 1 000 000 microglial cells harvested at DIV4. By using our refined protocol cell grouping is evident at DIV3 and a dramatic increase in cell size and cell number at DIV4, when the cells appear as large shiny spheres distributed across the area of the dish. In comparison, these changes were not observed when the established protocol was used and the density of the microglial cell layer was much lower than that in the tissue culture dish used in our refined protocol. The higher final cell yield per cortex and large microglial cells size obtained by our refined protocol using the 60 mm dish may be attributable to following four factors.

Firstly, seeding of the cells in this particular type of plastic ware potentially may have led to increased proliferation of microglia and hence reaching of confluency by DIV4. Secondly, the microglial cells from our refined protocol were maintained in the same culture medium for the whole period of incubation. Multiple medium changes involved during the incubation period of the existing conventional protocols may remove important soluble factors released from the microglial cells and/or astrocytes, such as TGF-β2, CSF-1/IL-34, and cholesterol, which have been shown to promote microglial growth and cell survival [34]. Fewer medium changes may also have prevented the loss/washing away of the microglial cells in the upper cell layer.

Thirdly, microglia grown by our refined protocol were maintained in culture only for four days. Changes of microglial phenotype, as a result of cell ageing in culture (from DIV2 to DIV16) have been reported previously by Caldeira *et al.* [35]. In addition to morphological changes, these authors report decreased phagocytic ability of the microglial cells together with decreased migration ability as a consequence of prolonged incubation. Furthermore, the same publication reports a decrease of the reactive abilities of microglial cells that have stayed in culture for 16 days; namely, decreased ability of the cells to respond to chemotactic signals in the medium. These evidence suggest that the cell-to-cell communication between the microglial cells grown in the dish for four days is increased, which may have promoted cell growth and proliferation.

Lastly, the incubation time for microglia required by existing conventional protocols is between 10 to 14 days, and in some protocols up to 3 weeks. By that time microglial cells still present in the flask would have firmly attached to the underlying astrocyte layer and vigorous mechanical shaking would be required to harvest the microglial cells. Even this harsh separation method does not always result in successful harvest of 100% of microglia from the flask. In comparison, microglia in the 60 mm dish were observed to loosely attached to the underlying basal astrocyte layer, which allowed easier removal of the microglial population by just gentle swirling. Easy separation of the microglial cells in our refined protocol improves the quality of the cultures dramatically by minimising mechanical stress to the cells.

It is important to note that the cells that were harvested using our refined microglial culture protocol were resting microglial cells with the usual bipolar non-activated shape. More than 96% of the cells produced by the refined method were positive for the pan microglial marker – Iba-1 and only less than 1% of the total harvested cells were positive for GFAP – an astrocyte marker. The microglial cells obtained using our refine protocol were also negative for iNOS – a pro-inflammatory marker for microglial activation, and they did not release nitrite in the culture medium, suggesting their resting phenotype.

There are a few key points in our refined protocol that ensure a high-quality microglial culture is achieved. The age of the rat pups that could be used in this protocol varies between postnatal days P3 to P6. However, in our experience the best results are obtained with tissues collected from P5 pups. The fine dissection step of the cortex is also essential for good quality and reproducible cultures, hence detailed information about the best approach for the procedure is given in the methods section.

Another important step of our refined protocol is the air-drying process of the poly-d-lysine coating. The procedure is to ensure even surface charge of the dishes, as cells may adhere less well to areas that are not dried fully. A major improvement of the protocol is the fact that the culture does not require changes of the media in the dishes until isolating and plating microglia onto the coverslips. However, if the medium appears to be turning yellow (which happens very rarely) as the cells grow and use nutrients from it (indicating change in pH) an extra 1-2 ml of fresh, pre-warmed DMEM supplemented medium should be added to the dish to sustain the cells until they are ready for harvest. There is enough space in the dish to accommodate the addition of that volume of medium, so no replacement of media is necessary.

We found that the brand of plastic culture dishes was vital to success. Our refined protocol works best with 60 mm 21.2 cm^2 tissue culture dishes (BIOFIL TCD010060; distributed by Triple Red, UK). Our work indicates that the protocol is not compatible with Greiner CELLSTAR® 60 mm cell culture dishes (628160CI), which may be attributed to the different surface charge of this type of dishes. We did not test other brands here but such testing may prove instructive in the future.

It is advisable to keep the dishes in the incubator until they are ready to harvest or until they are required experimentally. In a practical sense this means limiting the number of times dishes are removed from the incubator for observation or other manipulation. Unlike T75 flasks, which have a screw cap, the 60 mm culture dishes are not sealed. Therefore, each time the dishes are removed from the incubator there is increased risk of airborne infection. Additionally, this protocol has been designed to generate non-activated microglial cultures for experimental use. The changes of temperature-associated removal of the dishes from the incubator may possibly increase the risk of microglial phenotype change [36, 37].

The refined protocol that we have established is easy to follow and can be replicated easily by users with varying levels of experience. Given adequate training in aseptic technique and observation of the critical steps described in the methods, this protocol has been used successfully in our laboratory by 17 students ranging from undergraduate to PhD levels (4 summer undergraduate summer students, 5 BSc, 4 MSc, and 4 PhD students) without difficulty in obtaining high quality microglial cultures. It is therefore unlikely that any major difficulties will arise, providing the procedures are followed closely and precisely. To analyse the morphology and activation of cultured microglia appropriately users would also need basic training in light microscopy, immunocytochemical staining methods and fluorescence microscopy.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions statement

M.G. wrote the main manuscript text and prepared the final version of all figures. M.G., A.P., and M.D. performed the experiments and analysed the data presented in this manuscript. M.M. provided training with the original protocol. A.M.R. is the second PhD supervisor of M.G. and participated in review of the manuscript and helped with the response letter. W.H. is the principal PhD supervisor of M.G. and planned the experiments and participated in the writing and review of the manuscript as well as helping with the response letter. All authors reviewed the manuscript.

Competing financial interests

The author(s) declare no competing financial interests.

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Supplementary Materials

A refined rat primary neonatal microglial culture method that reduces time, cost and animal use.

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Running title: **A refined rat primary neonatal microglial culture method**

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Supplementary materials and methods

Method 1: Immunocytochemical Staining

Cells were fixed using 4% paraformaldehyde and then double stained with the following antibodies: Primary: rabbit anti-Iba-1(Wako, Japan 019-19741) 1:1000, mouse anti-iNOS (BD Biosciences, 610329) 1:100; Secondary: 488 goat anti-rabbit AlexaFluor (Molecular Probes Inc., Invitrogen, USA) and 568 goat anti-mouse AlexaFluor (Molecular Probes Inc., Invitrogen, USA), respectively. All samples were counterstained with a nuclear dye using Hoechst 33342 solution (Sigma, UK). Samples were then mounted on microscopic slides using glycerol solution. Images were taken using a Nikon Ti-E Eclipse Inverted Microscope (Nikon, Japan) with Nikon DS-Fi1c camera (Nikon, Japan) at 10x and 20 x objective magnifications, and were analysed using cell counting software including ImageJ and Photoshop.

To assess proliferation of the microglial cells at different stages in culture double-labelling with rabbit Iba-1 (Wako, Japan 019-19741) 1:1000 and mouse monoclonal IgG1 against IdU (Abcam, ab8955) 1:200 was performed. Briefly at DIV2, DIV3, and DIV4 IdU (5-iodo-2'-deoxyurine, Sigma, I7125) at working concentration 2μg/ml (stock concentration 2mg/ml) was added to the tissue culture medium and cells were incubated for 45 minutes. The cells were then briefly washed with cold PBS and fixed with 4% Paraformaldehyde (PFA) for 10 minutes. Following 3 washes with PBS, the cells were permeabelised with MeOH at -20ºC for 5 minutes. Following 3 washes with PBS the DNA was melted with 2M HCl at 37ºC for 30 minutes. The reaction was neutralised with 0.1M sodium borate buffer at pH 8.5. The cells were washed with PBS and usual protocol for immunostaining with the above-mentioned antibodies was followed. Images were taken using 10x objective magnification and analysed to assess the number of Iba-1 immunoreactive cells that were also labelled with IdU.

Method 2: RT-qPCR

RNA was isolated from cultured microglia cells using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The concentration of the eluted RNA was quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific, UK), which was calibrated using RNase-free water as a blank before measuring the samples.

cDNA was synthesised using superscript II reverse transcriptase (Invitrogen, USA) following the manufacturer's protocol to synthesise 0.5 μg cDNA. The cDNA was then diluted 10 fold and run using SYBR master mix (Applied Biosystems, USA) and primers on an AB7900 quantitative real-time PCR machine (Applied Biosystems, USA).

Method 3: Phosphate Buffer Saline (PBS) 0.01 M

For making 4 litres Phosphate Buffer Saline (PBS) 0.01 M mix the following reagents and still on a magnetic stirrer:

- 35.04 g NaCl
- 0.80 g KCl
- 200 ml 0.2M phosphate buffer
- 3.8 l distilled H2O

Table S1: Primers selection used for qRT-PCR

Primer sets used for quantitative real-time PCR (qRT-PCR) analysis		
Gene	**Forward Sequence**	**Reverse Sequence**
YWHAZ	5'-GCTACTTGGCTGAGGTTGCT-3'	5'-TGCTGTGACTGGTCCACAAT-3'
Il-6	5'-TCCTACCCCAACTTCCAATGCTC-3'	5'-TTGGATGGTCTTGGTCCTTAGCC-3'
TNF-α	5'-ATCCGAGATGTGGAACTGGCA-3'	5'-CCGGACTCCGTGATGTCTAAGT-3'
iNOS	5'-CACCTTGGAGTTCACCCAGT-3'	5'-ACCACTCGTACTTGGGATGC-3'

Table S2: Reagents used for the preparation of the culture

Products	**Company and Catalogue number**
Dulbecco's Modified Eagle's Media(DMEM) high glucose, GlutaMAX™ Supplement, HEPES	**Invitrogen** 32430-027, UK
Earle's Balanced Salt Solution(EBBS) 1x with $CaCl_2$ and $MgCl_2$	**Invitrogen** 24010, UK
Hank's Balanced Salt Solution(HBSS) 1x without $CaCl_2$ and $MgCl_2$	**Invitrogen** 14175053, UK
Phosphate Buffer Saline(PBS) 0.01 M, pH 7.4 - (prepared in house)	Supplementary Method 3
Penicillin/Streptomycin	**Invitrogen** 10378-016, UK
Bovine serum albumin (BSA) fraction V	**Sigma** A-4503, UK
DNAse type 1	**Sigma** DN25, UK
Trypsin from bovine pancreas	**Sigma** T-9935, UK
Fetal bovine serum (FBS) heat inactivated	**Invitrogen** 10500-064, UK
Poly-d-Lysine (70 – 150 kDa	**Sigma** P6407, UK
Trypsin-EDTA 0.25%	**Invitrogen** 25200072, UK

Table S3: Equipment used during the preparation of the culture

Product	**Company and Catalogue number**
Z™ Series Coulter Cell Counter	**Beckman Coulter**, USA
Large scissors	**FST** 14007-14, Germany
Small scissors	**FST**14090-09, Germany
2 x straight Dumont forceps	**FST**11252-20, Germany
Cover glass forceps	**FST** 11073-10, Germany
16 mm needles	**Fisher** 12329179, UK
7 ml bijou tubes	**Thermo Scientific** 129A, UK
15 ml centrifuge tubes	**Triple red** CFT011150, UK
60 mm 21.2 cm^2 tissue culture dishes	**Triple red** TCD010060, UK
50 ml centrifuge tubes	**Triple red** CFT011500, UK
12-well plates	**VWR**734-2324, UK
24-well plates	**VWR** 734-1605, UK
18 mm circular coverslip	**VWR** 631-1580, UK
70 µm cell strainers	**Falcon** 352350, UK

0.2 µm syringe filters	**Triple red** FPE204025, UK
25 ml syringes	**Fisher Scientific** 12951031, UK
Pipette motorized controller	**Triple Red**, UK
5 ml serological pipette	**Costar** 4487, UK

Supplementary Results

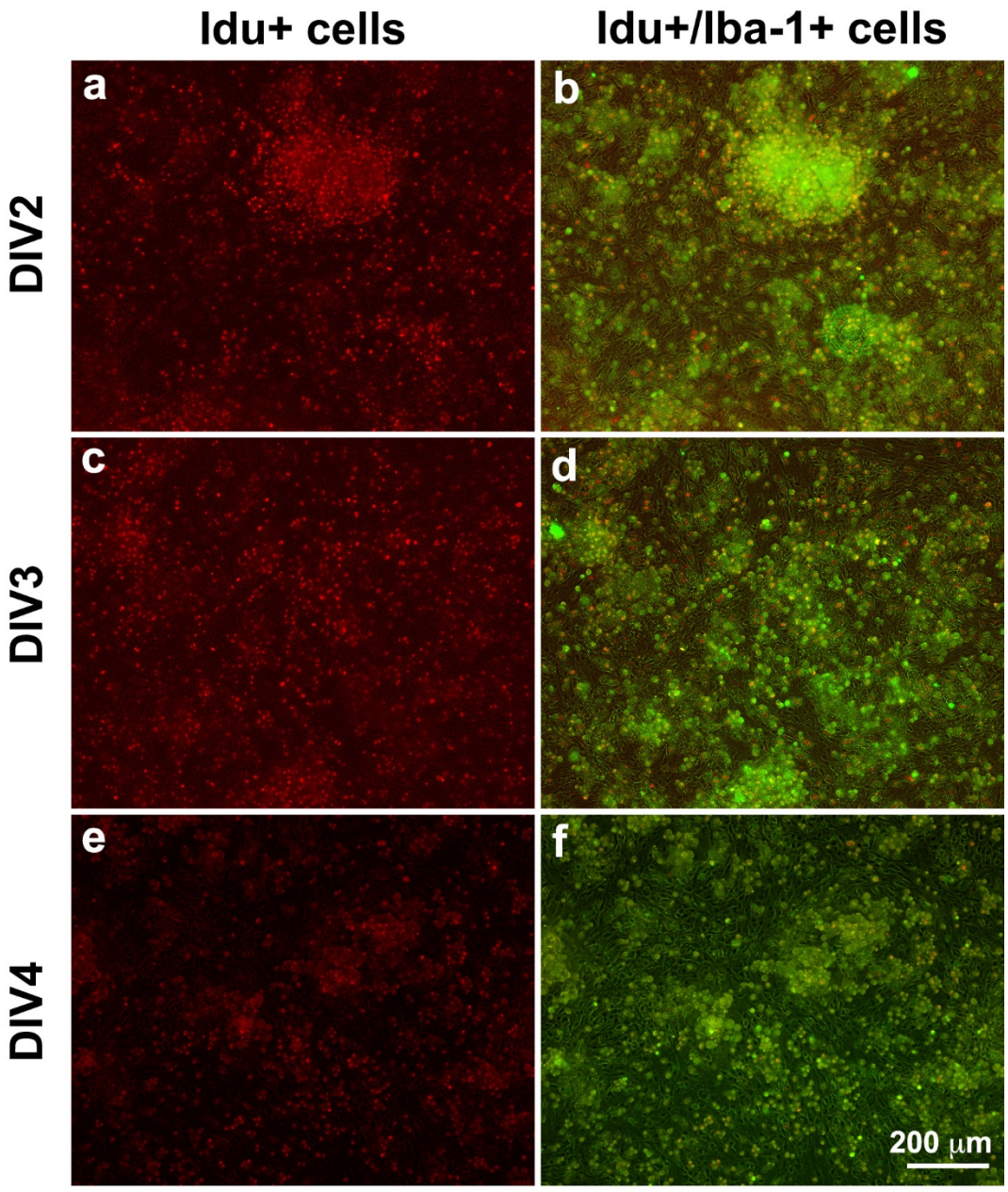

Figure S1. Immunostaining for Iba-1 (a pan microglial marker) and IdU (a cell proliferation marker) in microglial cultures from DIV2 to DIV4. (a-b) Microglial cells from DIV2 in culture stained with Iba-1 were highly reactive for IdU antibody, showing a high proliferation of the cells. (c-d) The majority of microglial cells from DIV3 in culture stained with Iba-1 were also reactive for the IdU antibody, showing that the high proliferation of the cells continued at DIV3. (e-f) The proliferation of the microglial cells in culture from DIV4

were decreased significantly compared to the two previous days, as seen by the reduced expression of IdU by the Iba-1 reactive microglia. N=3 biological replicates per group/day.

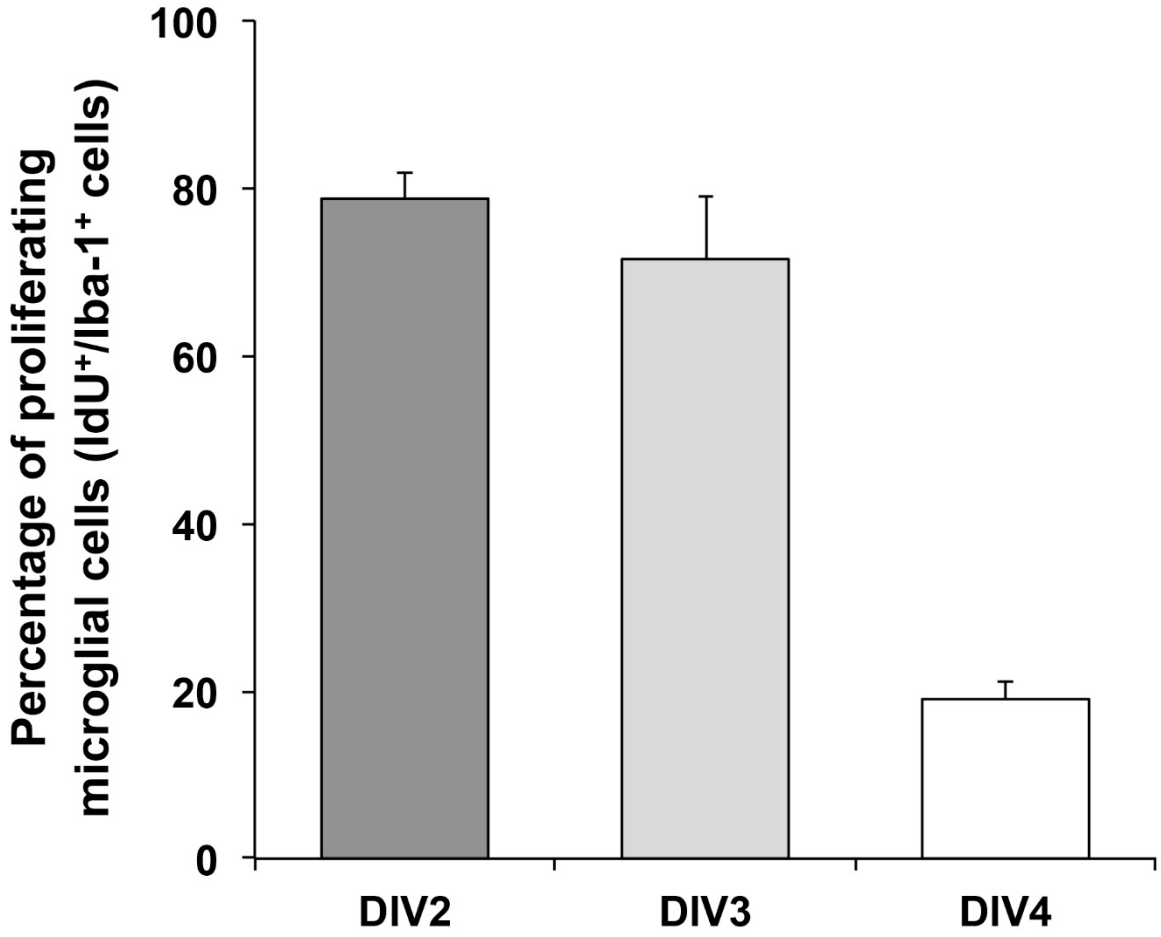

Figure S2. Quantification of the IdU immune-reactivity in Iba-1 labelled microglial cells. High microglial cell proliferation was observed during DIV2 and DIV3. The analysis showed that more than 70% of Iba-1+ microglial cells also expressed the IdU marker in both days and no significant difference was observed between the two days. However, the percentage of Iba-1+ microglial cells expressing Idu was decreased significantly at DIV4 to 20%, comparing to those at DIV2 and DIV3 ($p<0.05$). Error bars are SEM; N=3 biological replicates per group/day.

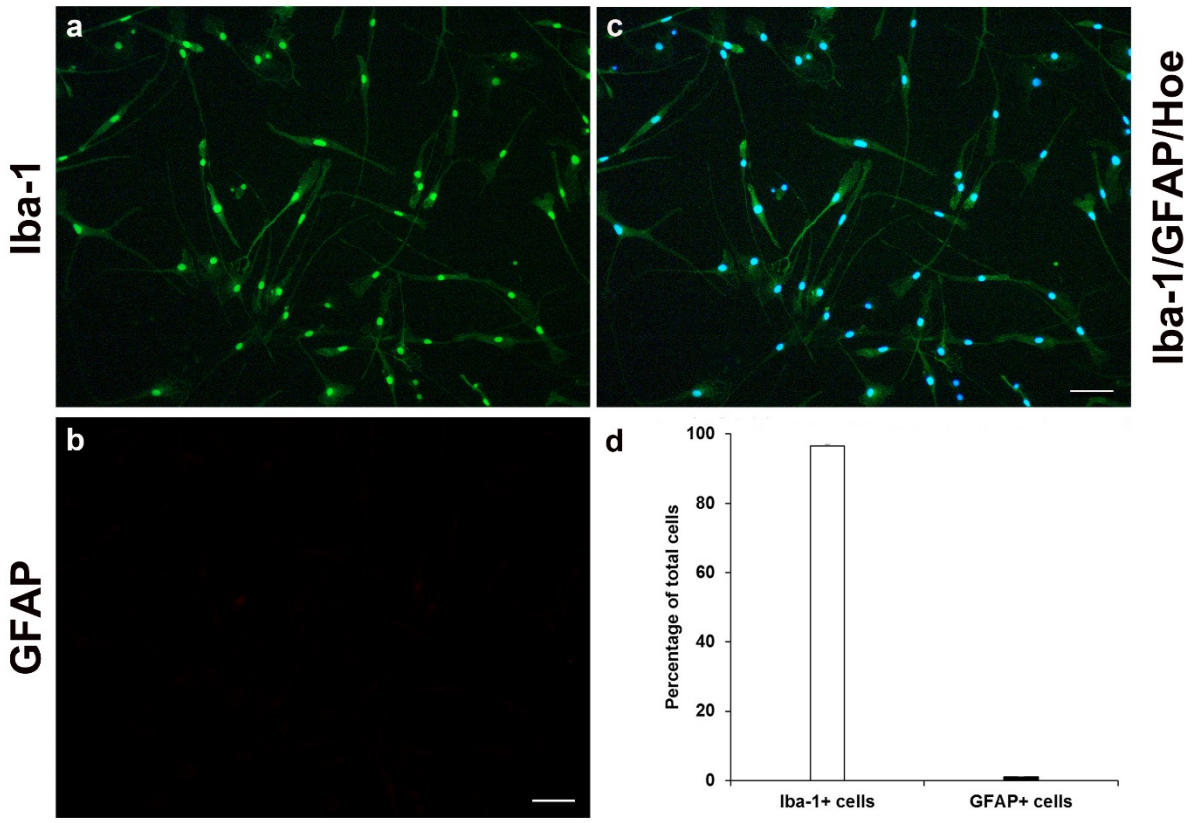

Figure S3. Quantification of triple immunocytochemistry with Iba-1 (a pan marker for microglia), GFAP (a marker for astrocytes) and Hoechst 33342 (a nuclear dye) of microglial cell at DIV4. (a) Resting bipolar microglial cells obtained from our refined protocol at DIV4 stained for Iba-1. (b) A representative image of GFAP staining in the microglial culture obtained from our refined protocol at DIV4. (c) Merged images of Iba-1 (a), GFAP (b), and Hoechst (not shown) staining. All Hoechst labelled nuclei match the reactive Iba-1 microglial cells and there is no presence of astrocytes following plating of the microglial cells, as shown by the lack of GFAP staining. (d) Quantification of the immunocytochemistry analysis showing that the microglial cultures produced by our refined protocol exceed 96% purity and the presence of astrocytes in the culture is less than 1%. Only highly fluorescent Iba-1 reactive cells we counted towards the quantification of the culture purity. In the rare occasions, when there were cells that express Iba-1 weakly, these cells were not counted as microglia even though they did not express other cell markers. Therefore, although the astrocyte contamination in the culture equals less than 1%, the culture purity is represented as >96% instead of 99%, to account for these weakly stained cells. N=3 biological replicates, n=9 technical replicates. Error bars are SEM. Scale bar -100μm.

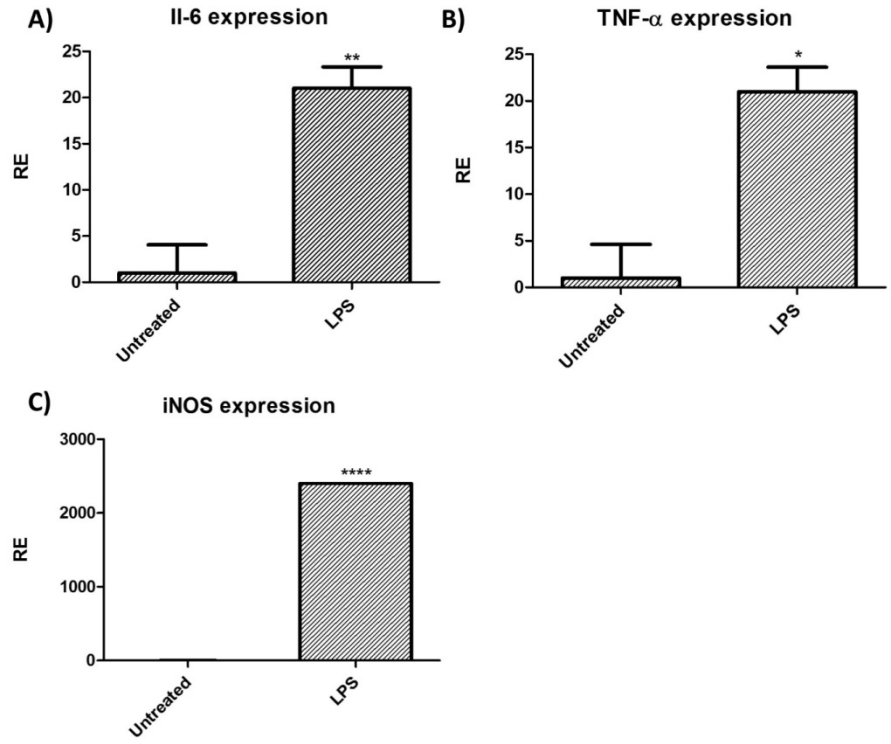

Figure S4. Comparison of pro-inflammatory gene expressions of IL-6, TNF-α, and iNOS between resting and activated microglia using our refined protocol. Quantitative qRT-PCR analysis of untreated and LPS treated (10 µg/ml, 4 hours) microglial samples showing the relative expression of the following genes: (A) IL-6 (Untreated n=4, LPS n=5). (B) TNF-α (Untreated n=3, LPS n=5). (C) iNOS (Untreated n=6, LPS n=7). Microglia cells not treated with LPS showed none or low expression of the pro- inflammatory mediators IL-6, iNOS, and TNF-α. In comparison, the LPS treated samples showed significantly higher expression of each gene. Error bars are SEM. Statistical analysis was an unpaired Student's t-test. n.s. = not significant ($p>0.05$), *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$, ****$p\leq0.0001$. 'n' represents technical replicates.

Table S4: Comparison of costs between conventional and refined microglia protocols.

Materials and Reagents (minimum requirements for microglia primary culture)	Conventional Protocol	Price (GBP) per flask	Refined Protocol	Price (GBP) per dish
Tissue required	3 x cortices	9.00	1 x cortices	3.00
HBSS for dissection	15 ml for 3 cortices	0.45	5 ml for 1 cortex	0.20
Culture plasticware	1 x T75 flask	2.50	1 x 60 mm dishes	0.60
Poly-D-Lysine coating	T75 flask (minimum 200 µl)	2.60	60 mm dish (50 µl)	0.65
Serological pipettes for medium change	6	3.00	0	0.00
DMEM medium (for initial plating)	15 ml	0.45	5 ml	0.15
DMEM medium (for medium change)	3 x 15 ml (45 ml)	1.35	0 ml	0.00
		Total: £19.60		**Total: £4.60**

Table S5: Comparison of culture times between conventional and refined microglia protocols.

Time	Conventional Protocol	Refined Protocol
Dissection time	6-10 minutes	2-3 minutes
Incubation time prior to harvest	10 – 14 days	4 days
Medium changes	30 minutes (10 minutes per medium change x3)	0 (initial plating only)
	Total culture time: 14 days	**Total culture time: 4 days**