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

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## HIGHLIGHTS

- A refined microglia culture protocol that significantly reduces incubation period.
- Reproducible techniques resulting in consistent high quality microglia cells.
- Harvesting methods minimising the mechanical disturbance of the cells upon plating.
- Significant cut in the costs for culture related reagents and culture plastic ware.
- Procedures which are successfully reproduced by students from all education levels.

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## ABSTRACT

*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.

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## 1. Introduction

Microglia are a population of CNS-resident macrophage-like cells (Tremblay et al., 2011). In their non-activated resting state microglia have a ramified morphology, which is not seen in other macrophage populations (Kettenmann et al., 2011; Saijo and Glass, 2011). An important function of microglia is their ability to generate innate and adaptive immune responses (Yang et al., 2010). 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 (Dheen et al., 2007).

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Upon activation, microglia undergo morphological transformation, proliferation, migration, upregulation of several bioactive molecules, such as reactive oxygen species, nitrogen intermediates and cytokines (Imai and Kohsaka, 2002). Due to their integral mechanistic roles in the CNS (Rivest, 2009), namely immune regulation, microglia are of great interest in the research areas of neurodevelopment, neuroinflammation, neurodegeneration, and neuropathic pain (Ginhoux et al., 2010; Olson and Miller, 2004; Tsuda et al., 2003). Therefore, *in 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 (Tamashiro et al., 2012; Chen and Gaitri Sadadcharam, 2013; Ni and Aschner, 2010; Gingras et al., 2007; Calvo et al., 2011; Lastres-Becker et al., 2014; Godbout et al., 2004; Bonora et al., 2014; Saura et al., 2003). 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 (Esen and Kielian, 2007; Yu et al., 2017). 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 10–14 days, sometimes even up to 3 weeks, and it requires a mixed glial culture seeded into tissue culture flasks (Tamashiro et al., 2012; Yu et al., 2017; Lin et al., 2017; Stockley et al., 2017; Urrutia et al., 2013). 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 (Tamashiro et al., 2012; Stockley et al., 2017; Clark et al., 2010) and a large volume of culture media (*e.g.* 13–15 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. (2010) and Staniland et al. (2010), 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 3 h to 24 h. 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.

## 2. 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.

### 2.1. 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.

### 2.2. Dissection of neonatal rat cortex

Procedures for dissection of rodent cortex have been described only briefly in previous publications (Ni and Aschner, 2010; Albuquerque et al., 2009; Chen et al., 2007). 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 rat neonates between P3-6 were culled by cervical dislocation and dissected (Fig. 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 (Fig. 1b). The head was then pinned through the nose cavity on a sterile polystyrene surface using a dissecting pin or syringe needle (Fig. 1c). After removing the skin from the skull using fine scissors (Fig. 1d), an incision was made along the sagittal suture starting from the foramen magnum all the way to the frontal bone using fine scissors (Fig. 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 (Fig. 1f). This step

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