Purification of retinal ganglion cells

Purification of retinal ganglion cells from postnatal rats by magnetic cell sorting

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Introduction

Retinal ganglion cells (RGC) are the projection neurons of the retina, which transmit light-evoked signals via the optic nerve to the visual centers of the brain. Degeneration of RGCs causes loss of vision and represents a hallmark of several ophthalmic diseases including glaucoma and inflammation of the optic nerve in multiple sclerosis. Therefore, RGCs are subject to intense preclinical R&D efforts aiming to develop neuroprotective treatments. RGCs were one of the first CNS neurons that could be purified to up to 99.5%¹ and cultured under defined conditions.² Cultures of purified RGCs have allowed the study of the influence of non-neuronal (glial) cells upon CNS neurons.³,⁴ In the original ‘immunopanning’ protocol, retinae are first dissociated in a multi-step process requiring papain and ovomucoid/trypsin inhibitor. RGCs are then purified by depletion of unwanted cell types using anti-rat macrophage serum and by enrichment using hybridoma supernatant specific for CD90.1/Thy1.1. However, the protocol is very time-consuming (approximately 6 hours per preparation) and requires considerable technical skill. Here we compare the original method with new and simplified protocols for tissue dissociation and isolation based on magnetic cell sorting (MACS® Technology).

Materials and methods

Immunopanning protocol

RGCs were purified from postnatal day 5–7 (P5–7) rats by the original ‘immunopanning’ protocol (Barres et al., 1988; see http://pfrieger.gmxhome.de/work/intro.html for a detailed description). Briefly, two culture dishes for depletion were coated with goat anti-rabbit IgM, while a dish for selection was coated first with goat anti-rabbit IgM antibody and then with CD90.1/Thy1.1 hybridoma supernatant. Retinae were incubated in D-PBS, papain and DNase for 30 min and agitated every 10 min. The cells were then incubated with ovomucoid solution, anti-macrophage serum, and more DNase before trituration by four rounds of 10 passages through three 1-mL pipette tips of increasingly small diameter. The suspension was then washed twice by centrifugation for 13 min, resuspended in D-PBS/BSA and filtered through a nylon mesh. Cells were then allowed to adhere to the first depletion plate for approximately 36 min with gentle shaking every 12 min. The non-adherent cells were collected after this time and put through an identical round of depletion before being applied to a selection plate and incubated for a further 45–60 min with gentle shaking every 15 min. Finally, after washing up to 10 times, cells adhering to the selection plate were trypsinized and centrifuged for 13 min before being plated for cell culture.

MACS Technology–based protocol

Alternatively, RGCs were isolated by a novel method that employs new kits for tissue dissociation and magnetic cell sorting. The retinae were first dissociated using the Neural Tissue Dissociation Kit – Postnatal Neurons (Miltenyi Biotec) according to the manufacturer’s instructions. Briefly, washed retinae were incubated with Enzyme Mix 1, containing a low concentration of papain, in the MACSmix™ Tube Rotator for 15 min. After adding Enzyme Mix 2, retinae were then dissociated by 10 passages through a serological 5-mL pipette (Sarstedt) and incubated for a further 10 min in the MACSmix Tube Rotator. More Enzyme Mix 2 was added and tissue pieces were further dissociated by 35 passages through a fire-polished Pasteur pipette. The cell suspension was then passed through a 40-µm filter and washed by centrifugation. Prior to magnetic cell sorting, the single-cell suspension was incubated with CD32/FcγII Receptor (BD Bioscience) for 2 min to block undesired antibody binding to Fc receptors. RGCs were then isolated using the Retinal Ganglion Cell Isolation Kit (Miltenyi Biotec) according to manufacturer’s instructions. Briefly, RGCs were
incubated for 5 min with CD90.1/Thy1.1 MicroBeads and then for an additional 10 min with a biotinylated depletion antibody. After washing, cells were resuspended in 750 µl of D-PBS/BSA and mixed with Anti-Biotin-MACSiBead™ Particles (Miltenyi Biotec) for 15 min. Unwanted cells labeled by the biotinylated antibody–MACSiBead complex were then depleted using a MACSImag™ Separator. Non-depleted RGCs labeled with CD90.1 MicroBeads were subsequently enriched using two MS Columns and an OctoMACS™ Separator. Cells were then eluted from the second MS Column and plated for routine culture. RGCs isolated by either protocol were subsequently cultured in serum-free MACS Neuro Medium (Miltenyi Biotec) supplemented as described previously.

Results and discussion

Our goal was to compare the efficiency of a new method to purify RGCs with the established immunopanning technique. The new approach consisted of a new dissociation protocol, the Neural Tissue Dissociation Kit – Postnatal Neurons, and of the Retinal Ganglion Cell Isolation Kit for magnetic cell sorting. For comparison, both protocols were performed in parallel in order to minimize site- and preparation-dependent differences. First, the total number of cells was determined in single-cell suspensions by flow cytometry. In three independent preparations, the previously published procedure delivered a two-fold higher total number of retinal cells than the Neural Tissue Dissociation Kit (means ± SD; 19 ± 8 × 10^6 cells vs. 9 ± 6 × 10^6 cells) but a lower percentage of target cells (1.16% vs 1.41%, data not shown). This difference may result from the operator-dependent dissection of retinae. This indicates that the low papain activity used by the Neural Tissue Dissociation Kit – Postnatal Neurons is sufficient to dissociate RGCs and that the use of trypsin inhibitor/ovomucoid is not necessary. We next compared the percentage of RGCs after antibody-based cell selection and found that both methods achieved a high enrichment of RGCs up to 97% purity (fig. 1). Overall, the RGC Isolation Kit delivered less variable purities and a higher total number of RGCs per 10 rats (12 ± 8×10^4 cells) compared to immunopanning (5.3 ± 2.8×10^4 cells).

A subpopulation of CD90.1^+CD48^+ endothelial cells was efficiently removed (fig. 2). These differences may be due to several advantages of magnetic cell sorting. MACS Technology uses titrated antibodies and beads for both cell depletion and enrichment, whereas immunopanning involves undefined antibody concentrations in serum and hybridoma supernatant. Moreover, the ratio of monoclonal antibody to bead is constant, while adhesion of antibody to the panning plate can vary. Another advantage is that MACS Technology–based purification does not require trypsin for the detachment of target cells from immobilized antibodies.
which can damage cells and thereby diminish the yield of RGCs. Finally, the classical immunopanning protocol takes about 6 hours (including preparation of panning dishes and dissection of retinae), whereas the Neural Tissue Dissociation Kit – Postnatal Neurons and Retinal Ganglion Cell Isolation Kit take only 3 hours.

A prime use of purified RGCs is the study of their development and function under defined culture conditions. We compared the morphology of RGCs prepared by different protocols after a few days in vitro in order to determine whether RGCs prepared by MACS Technology were suitable for primary culture. RGCs prepared by immunopanning or MACS Technology survived and showed extensive neurite outgrowth in vitro (fig. 3).

Taken together, our results demonstrate that the new protocols for dissociation and purification of RGCs are valid and have several advantages over immunopanning. The two kits provide a shorter, simpler protocol and, most crucially, produce highly purified neuron populations in a more reproducible manner.

References

<table>
<thead>
<tr>
<th>MACS® Product</th>
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<tr>
<td>Neural Tissue Dissociation Kit – Postnatal Neurons</td>
<td>130-094-802</td>
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<tr>
<td>Retinal Ganglion Cell Isolation Kit</td>
<td>coming soon</td>
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<td>CD90.1 MicroBeads, mouse and rat</td>
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<td>MACS Neuro Medium</td>
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Figure 3  Retinal ganglion cells retain their morphology following cell separation and culture in vitro. Phase-contrast micrographs of retinal ganglion cells purified from postnatal rats by either immunopanning or MACS Cell Separation and cultured for 1 or 7 days under defined, serum-free conditions. Bar = 40 μm.