

Comparative analysis of three purification protocols for retinal ganglion cells from rat

Fengjuan Gao,¹ Tingting Li,¹ Jianyan Hu,¹ Xujiao Zhou,² Jihong Wu,² Qiang Wu¹

¹Department of Ophthalmology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China; ²Eye & ENT Hospital, Fudan University, Shanghai, China

Purpose: To make comparative analyses of the common three purification protocols for retinal ganglion cells (RGCs), providing a solid practical basis for selecting the method for purifying RGCs for use in subsequent experiments.

Methods: Rat RGCs were isolated and purified using three methods, including two-step immunopanning (TIP) separation, two-step immunopanning-magnetic (TIPM) separation, and flow cytometric (FC) separation. Immunocytochemical staining, quantitative real-time PCR, flow cytometry, electrophysiology, and Cell Counting Kit-8 (CCK-8) analyses were performed to compare the purity, yield, and viability of the RGCs.

Results: The RGC yields from the TIP, TIPM, and FC methods were $24.60 \pm 15.98 \times 10^4$, $5.28 \pm 4.42 \times 10^4$, and $5.4 \pm 2.7 \times 10^3$ per retina, respectively. We easily controlled the relative purity of the RGCs with the FC method and even reached 100% of the maximum expected purity. However, the RGC purity was only $80.97 \pm 5.45\%$ and $95.41 \pm 3.23\%$ using the TIP and TIPM methods, respectively. The contaminant cells were mainly large, star-shaped, glial fibrillary acidic protein (GFAP)-positive astrocytes and small, round, syntaxin 1-positive amacrine cells with multiple short neurites. The RGCs purified with FC could not be cultured successively in our study; however, the TIP-RGCs survived more than 20 days with good viability, while the TIPM-RGCs survived less than 9 days.

Conclusions: The three protocols for purifying the RGCs each had its own pros and cons. The RGCs isolated by the TIP method exhibited the highest viability and yield but had low purity. The purity of the RGCs isolated with the FC method could reach approximately 100% but had a low yield and cell viability. The TIPM method was reliable and produced RGCs with considerable purity, yield, and viability. This study provides a solid practical basis for selecting the method for purifying RGCs for use in subsequent experiments.

Retinal ganglion cells (RGCs) are the sole output neurons that aid in extending axons throughout the optic nerve to receive, process, and relay light-evoked signals to the brain via the optic nerve [1]. RGCs are one of the most important retinal cells. Their anatomic or functional impairment is associated with or a consequence of many ophthalmic disorders, such as diabetic retinopathy or glaucomatous optic neuropathy [2-4], central retinal artery or vein occlusion, etc. [5], and may eventually result in optic neuropathy and vision loss [6]. Unfortunately, why and how the disease-associated RGCs degenerate are largely unknown [5]. Therefore, it is of vital importance to obtain an in-depth understanding of the mechanisms of RGC death to identify new therapeutic strategies for protecting RGCs.

An in vitro analysis of RGCs will be a crucial and almost indispensable tool for the study of retinal visual physiology and pathophysiology associated with various retinopathies and neuropathies, which cannot easily be realized in animal models. For instance, RGCs can be studied in isolation and

observed over time, ruling out the effects of other types of cells in the retina. The RGC receptors and signaling pathways can be precisely and quantitatively perturbed using specific chemical factors or pharmacological agents or by introducing genes of interest, and the consequences for cell biology could be evaluated using molecular biology, electrophysiological, or imaging techniques. Using these techniques in situ within an animal model would be technically challenging. Based on their high research value and urgent need, several types of culture models, including mixed retinal cells [7], purified RGCs [8], transformed RGC cell lines [9,10], retinal explant cells [11,12], embryonic stem (ES) cells, and induced pluripotent stem (iPS) cell cultures [13-15] have been established. However, most studies have limitations. For example, the immortalized RGC-5 cell line has been widely used to study the neurobiology of RGCs. However, Krishnamoorthy et al. demonstrated that the purported rat ganglion cell line RGC-5 is in fact of mouse origin and contaminated with 661W cells; therefore, any findings using RGC-5 cells as an in vitro model for RGCs must be carefully interpreted [16], thus largely limiting their usefulness [7]. RGC explant cultures are a mixed culture of different retinal cell types, and studies have shown that RGCs constitute only 5% of the total retinal cells in the mixed culture, thus limiting the application of RGCs

Correspondence to: Qiang Wu, Department of Ophthalmology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, 600 Yishan Road, Shanghai 200233, China. Phone: t86 18930174421; FAX: t86 021 64701361; email: Qiang.wu@shsmu.edu.cn

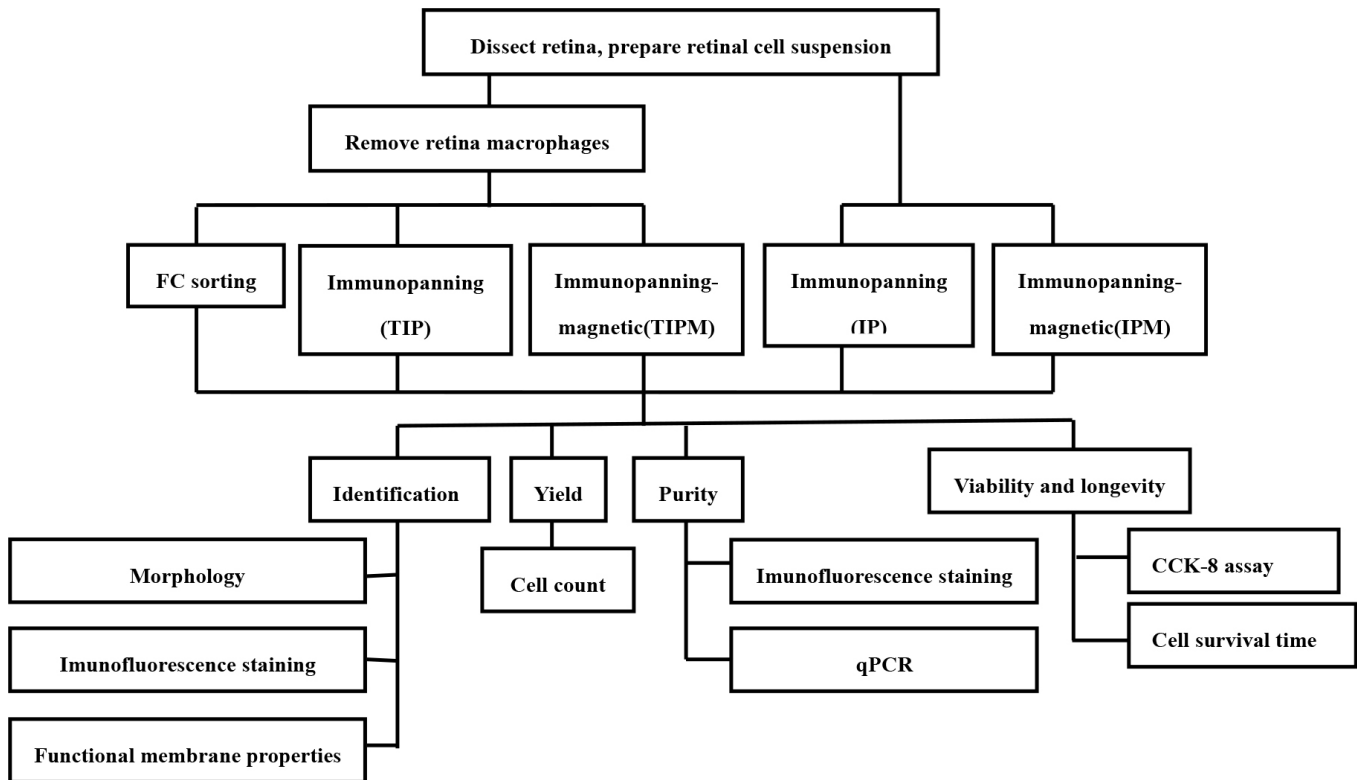


Figure 1. The protocol steps for isolating and analyzing RGCs are outlined and illustrated in this schematic procedure workflow.

in the study of RGC function [17]. IPS cells can directly differentiate into RGCs but require highly sophisticated techniques, and the cells often exhibit a low differentiation rate. Therefore, there is a mounting need to establish an effective system for isolating primary RGCs.

RGCs comprise the innermost layer of the retina and represent less than 1% of the total population of various types of retinal neurons and non-neuronal cells [8], making the purification of the cells more difficult. There have been many methods for the isolation, purification, and culture of RGCs from retinas [18-24]. As each method has its pros and cons, identifying the optimal method for purifying RGCs for specific applications can be difficult. Until recently, no systematic study has analyzed the strengths and weaknesses of every approach. In this work, we used the three most common methods for purifying RGCs from newborn rat retinas and compared the efficiency of each method, providing a practical basis for selecting the method for purifying RGCs for use in subsequent experiments.

METHODS

Experiments were approved by the Shanghai Jiao Tong University Institutional Animal Care and Use Committee (IACUC) and adhered to the ARVO Statement for Use of Animals. Here we describe the three most common methods for purifying the RGCs from newborn rat retinas and compared the efficiency of each method with different experimental methods. An overview of the method is provided in the flowchart (Figure 1).

Preparation of the retinal cell suspensions: The retinal tissues were separated from the enucleated eyeballs of newborn Sprague-Dawley rats on postnatal days 1 to 4 and incubated in precooled calcium-free and magnesium-free Earle's Balanced Salt Solution (EBSS; Gibco, Grand Island, NY) and Hank's Balanced Salt Solution (Life Technologies, Grand Island, NY) containing 5 mg/ml of papain, 0.24 mg/ml of L-cysteine, and 10 U/ml of DNase I for 30 min. Then, an ovomucoid solution containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO), 0.1% ovomucoid (Sigma-Aldrich), and 1% DNase I (4 mg/ml, Sigma-Aldrich) in minimum essential media (MEM, Gibco) was subsequently used to fully quench any residual papain activity. After centrifugation at 200 ×g for 10 min, the cells were resuspended in MEM containing 0.5 mg/ml of BSA, and

then the cell suspension was filtered through the mesh filter (pore size 40 μm , BD Falcon, Franklin Lakes, NJ) to yield a single cell suspension. The procedures were conducted at room temperature in a laminar flow hood.

RGC purification:

Preparation of panning dishes and cell culture dishes/plates—The antibody-coated 10-cm Petri dishes (one dish per every eight rats) were prepared for negative or positive selection by adding 15 μl of a rabbit anti-rat macrophage/Thy-1 antibody and 7 ml of 50 mM Tris-HCl (pH 9.5) per dish. The plates were swirled until the surfaces were evenly coated with the antibody-Tris solution. The panning plates were incubated overnight at 4 °C. Immediately before use, the plates were rinsed three times with Dulbecco's PBS (1X; 0.9 mM CaCl_2 , 0.49 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 137.9 mM NaCl, 2.67 mM KCl, 8.06 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.47 mM KH_2PO_4 , pH 7.4; D-PBS, Gibco). Then 1 \times Poly-D-lysine stock (PDL, Sigma-Aldrich) was added to the cell culture plates (50 μl for the 96-well plates, 100 μl for the 24-well plates, and 500 μl for the six-well plates), and the plates were incubated overnight at room temperature. The plates were rinsed three to four times with sterile H_2O and aspirated to dryness. Mouse laminin (1 mg/ml) was diluted to a final concentration of 50 $\mu\text{g}/\text{ml}$ by adding 10 μl of the laminin stock to 5 ml of Neurobasal medium (Gibco, Grand Island, NY). The diluted laminin solution was mixed well, added to the dried cell culture plates, and incubated in a 37 °C incubator for 2 h. The plates were rinsed with D-PBS three times before use. To isolate RGCs with immunopanning (IP), we adapted a protocol from Cold Spring Harbor Protocols [25].

Removing the retinal macrophages: In the retina, at least two types of cells, RGCs and macrophages, express Thy-1; most are macrophages [8]. Thus, it was necessary to deplete the macrophages and other Thy 1-positive contaminants that are not RGCs and then select the Thy 1-positive RGCs. Therefore, the retinal cell suspensions were first incubated in flasks coated with an anti-rat macrophage monoclonal antibody (OX-41; 1:50) for 45–60 min to exclude the macrophages; the plate was agitated every 15 min to ensure good cell adhesion. The non-adherent cells were collected. This procedure allowed us to rapidly isolate a population of pure RGCs with excellent viability.

IP for RGCs: The RGCs were isolated as previously described [8,25]. The retinal cell suspension excluding macrophages (or not) was incubated in a 100-mm Petri dish coated with a mouse anti-rat Thy-1 antibody (1:50 dilution; Abcam, Cambridge, MA) for 1 h; the plate was shaken every 15 min during this time period. The supernatant was discarded, and

the plate was washed six times with D-PBS. The adherent cells were collected as RGCs. (Note: we abbreviated the direct immunopanning in a plate coated with an anti-rat Thy-1 antibody as IP and the immunopanning in two plates coated with anti-rat macrophage and Thy-1 antibodies as TIP.)

Immunopanning-magnetic (IPM) for RGCs: The retinal cell suspension excluding macrophages was centrifuged at 300 g for 10 min at 4 °C. After the supernatant had been completely aspirated, the cell pellet was resuspended in a concentration of 10^7 cells per 90 μl buffer. Subsequently, 10 μl of CD90.1 MicroBeads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany) per 10^7 total cells was added, mixed well, and incubated at 4 °C for 15 min. Then, the cells were carefully washed twice with magnetic cell sorter (MACS) separation buffer. After being resuspended, the cell suspension was applied onto the MS column (Miltenyi Biotec GmbH) in the magnetic fields of a MiniMACS (Miltenyi Biotec GmbH). When the column reservoir was empty, the column was washed with 1.5 ml of MACS separation buffer and then removed from the magnetic field. To increase the purity of the RGCs, the eluted fraction was enriched over a second MS column. The magnetic separation procedure was repeated as described with a new column. Upon completion, the column was removed from the separator, and the retained cells were eluted as a magnetically labeled RGC fraction. (Note: we abbreviated the direct magnetic separation as IPM; if the retina macrophages had been removed before the magnetic separation, we referred to it as TIPM.)

Flow cytometry (FC) method for RGCs: The non-adherent cell suspension in flasks coated with an anti-rat macrophage monoclonal antibody was centrifuged at 300 \times g for 10 min at 4 °C. After the supernatant had been completely aspirated, up to 10^6 cells were resuspended in 45 μl of buffer and incubated with 5 μl of phycoerythrin (PE)-conjugated anti-rat CD90.1 or PE-conjugated IgG1 isotype control antibodies (eBioscience, San Diego, CA) for 15 min at 4 °C. The cells were then carefully washed twice. After being resuspended, the cell suspension was sorted with a flow cytometer (FACS Caliber; Becton Dickinson, San Jose, CA). The cells were counted automatically, and cells that were not incubated with the PE-conjugated anti-rat CD90.1 antibody served as the blank (Figure 2A). To improve the RGCs' yield, the sort gates were set as R10 and R21 (Figure 2); the RGCs in R21 were high purity (HP-RGCs), while the cells in R10 were low-purity RGCs (LP-RGCs).

Cell culture: The purified RGCs were seeded at the desired density on the PDL- and laminin-coated coverslips in 24-well or six-well plates in prewarmed RGC growth medium and maintained in a 37 °C cell culture incubator with a

humidified atmosphere containing 5% CO₂ and 95% air. The RGC growth medium was improved from the formulation described in the Cold Spring Harbor Protocols [25], based on our repeated experiments, and contained Neurobasal medium, BSA (0.1 mg/ml; Sigma), transferrin (0.1 mg/ml; Sigma), progesterone (60 ng/ml; Sigma), putrescine (16 µg/ml; Sigma), selenium (40 ng/ml; Sigma), 3,5,3-triiodothyronine T3 (40 ng/ml; Sigma), thyroxine T4 (40 ng/ml; Sigma), B27 (20 µl/ml; Invitrogen), sodium pyruvate (1 mM; Gibco), glutamine (2 mM; Gibco), N-acetyl-L-cysteine (NAC, 5 µg/ml; Sigma), insulin (5 µg/ml; Sigma), forskolin (5 µM; Sigma), brain-derived neurotrophic factor (BDNF, 50 ng/ml; PeproTech, Rocky Hill, NJ), ciliary neurotrophic factor (CNTF, 10 ng/ml; PeproTech), basic fibroblast growth factor (bFGF, 10 ng/ml; PeproTech), and penicillin-streptomycin (100 U/ml; Gibco). Half of the medium was replenished every 3 days. However, although a high level of purity was obtained

with FACS, the level of cell survival was low. The causes of this low survival are unclear.

Cell Counting Kit-8 assay for RGC viability: The RGCs were seeded in 96-well plates at a density of 3×10⁴ cells/100 µl and were cultured for 1, 3, 5, 7, 9, 11, 14, 17, and 20 days. Then, 10 µl of Cell Counting Kit-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well of the plate. After a 4-h incubation at 37 °C, the plates were analyzed with a Tecan Genios (Synergy H1, BIOtAK) at 450 nm. All values are reported as the mean ± standard error of the mean (SEM) of at least three wells and at least three separate experiments.

RGC purity and identification:

Immunocytochemical staining—Two days after seeding, the RGCs isolated using the TIP and TIPM methods were fixed with 4% paraformaldehyde for 30 min, treated with 0.1% Triton X-100 (Sigma) for 20 min, and then blocked

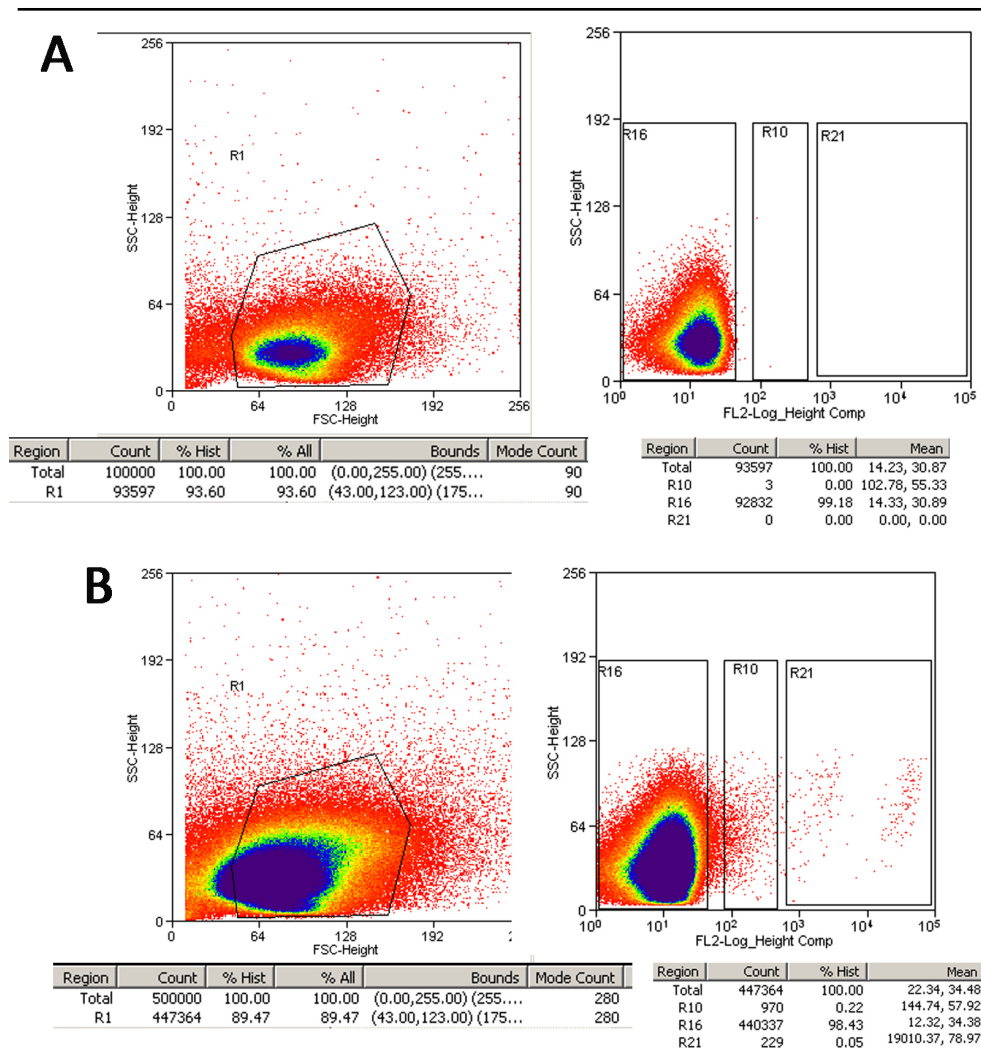


Figure 2. Design of FC cell sorting gates. **A:** Blank control. Staining with control antibodies conjugated with phycoerythrin (PE). **B:** Retinal ganglion cell (RGC) sorting strategy. RGCs were identified by staining with PE-conjugated mAb directed against Thy-1 (R10 and R21). R10 is regarded as a low-purity group and R21 as a high-purity group. R16 consists mainly of non-RGCs. Y axes = side scatter (SSC), X axes = forward scatter (FSC), fluorescence intensity on the FL2 channel.

with 3% BSA (Sigma-Aldrich) for 1 h. The cells were incubated with anti-rat γ -synuclein (1:400 dilution; Abcam), anti-rat Thy-1 (1:800 dilution; Abcam), anti-rat microtubule associated protein-2 (MAP2, 1:800 dilution; Abcam), anti-rat neuron-specific class III beta tubulin (TUJ-1, 1:400 dilution; Abcam), anti-rat Brn3b (1:400 dilution; Abcam), anti-rat Islet1 (1:400 dilution; Abcam), astrocyte-specific anti-rat glial fibrillary acidic protein (GFAP; 1:100 dilution; Abcam), and amacrine-specific anti-rat syntaxin 1 antibodies (1:50 dilution; Abcam) overnight at 4 °C. They were then exposed to the appropriate fluorescent secondary antibodies (1:400 dilution, Life Technologies) for 1 h at room temperature. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies). The cells were imaged with a confocal microscope (Leica SP8).

Quantitative real-time PCR analysis: The RNA extraction and cDNA synthesis were performed as previously described [26]. The gene-specific primers for β -actin, Thy-1, TUJ1, Brn3b, syntaxin 1, and GFAP were verified before use (Table 1 for primer sequences). Quantitative real-time PCR (Applied Biosystems, 7500 Fast) was performed in duplicate with 10 ng of cDNA and 10 pmol of each primer. The thermal conditions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The specificity of the detected signals was confirmed with a dissociation curve, which consisted of a single peak. Using the SYBR Green data, the relative RNA expression was normalized to β -actin. All samples were run in triplicate in each experiment. The data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Flow cytometry analysis: The RGCs' purity was evaluated using a flow cytometer (FACSCaliber; Becton Dickinson). As most of the Thy-1 antigens on the surface of the RGCs

were bound to the corresponding antibody during the separation and purification process, we adopted another RGC-specific marker, Brn3a, as a surrogate marker for the RGCs. The steps are summarized as follows: The RGCs that had been separated and purified using the three methods were separately fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Then, the cells were centrifuged and resuspended in PBS containing 0.4% Triton X-100 (Sigma), and 3% BSA was added to block non-specific binding of the antibodies. Each sample was subsequently centrifuged and incubated with a rabbit anti-rat Brn3a antibody (1:50, Abcam) for 60 min at 4 °C. After washing with PBS, the cells were exposed to fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG; 1:400, Life Technologies) in 1.0 ml of PBS for 1 h at RT. After washing twice with PBS, the cells were excited with a 488-nm laser and collected in the FITC (515–545 nm) channels. Cell Quest Acquisition and Analysis software (Becton Dickinson) was used to quantify the intensities of the fluorescent signals and to construct dot density plots.

Electrophysiology of purified RGCs: Electrophysiological recordings were performed on the primary cultured RGCs that had been purified with the TIP and TIPM methods on the second to fourth day. The bath solution contained the following components: 125 mM NaCl, 3 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4 , and 15 mM glucose, pH 7.4. The pipette solution for the current-clamp recordings consisted of the following components: 120 mM K-gluconate, 10 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM $CaCl_2 \cdot 2H_2O$, 4 mM ATP-Mg, 0.3 mM GTP-Na, and 10 mM phosphocreatine. The pH was adjusted to 7.2 with KOH, and the osmolarity was adjusted to 280–290 mOsm/l. The pipette resistance was typically 3–7 M Ω after it was filled with the internal solution. The spontaneous action potentials were recorded in the whole-cell current-clamp configuration using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) with a Digidata 1440A data acquisition board. The data were digitized at 10 kHz with a 1 kHz low-pass filter. The data were analyzed using Clampfit 10.02 (Axon Instruments, Foster City, CA). The membrane potentials were maintained at approximately –55 mV.

Statistical analysis: The TIP, TIPM, and FC methods were separately repeated 18, 15, and five times, respectively, and approximately 50–60 Sprague-Dawley rats were used for each experiment. The RGC yield, purity, and quantitative RT-PCR data were expressed as the mean \pm standard error of the mean (SEM) and compared with the Kruskal–Wallis one-way ANOVA (ANOVA) using the PASW Statistics 18 for

TABLE 1. LIST OF THE PRIMER SEQUENCES FOR PCR STUDIES.

Target	Sequence (5'-3')
β -actin	F: TGACGTGGACATCCGCAAAG R: CTGGAAGGTGGACAGCGAGG
Thy1.1	F: GGCAGTGAAGAGGCAGGATA R: AGGCACAGACACAGTCCAAC
TUJ1	F: TGCTGGCCATTCAGAGTAAGA R: TGCTGGCCATTCAGAGTAAGA
Brn3b	F: GGCTGGAGGAAGCAGAGAAA R: TTGGCTGGATGGCGAAGTAG
syntaxin 1	F: AGGCACGCAGGAAGAAGAT R: CAGGGAGACCCATCCAGAA
GFAP	F: ACCGCATCACCATTCTGTGA R: GCATCTCCACCGTCTTTACC

Windows, version 18.0.0 (SPSS, Chicago, IL). A *p* value of less than 0.05 was considered statistically significant.

RESULTS

RGC yield: Approximately 4.24 ± 0.62 million cells were collected per retina. First, we tried to leave the macrophages in the suspension and directly incubated the retinal cells with the Thy 1 antibodies. The results (Figure 3A and Table 2) show that $136.29 \pm 50.20 \times 10^4$ and $64.40 \pm 29.98 \times 10^4$ RGCs were achieved per retina using the IP and IPM methods, respectively, which accounted for $33.04 \pm 9.23\%$ and $14.26 \pm 5.90\%$ of the total retinal cells. These values were far higher than the actual numbers [8]. However, when the macrophages were removed first, the RGC yields were $24.60 \pm 15.98 \times 10^4$ ($5.91 \pm 3.38\%$) and $5.28 \pm 4.42 \times 10^4$ ($1.31 \pm 0.94\%$) using the TIP and TIPM methods, respectively. This yield is within (TIP) or below (TIPM) the statistical error of the 110,000 ganglion cells per adult rat retina that was previously determined [27-29]. Flow cytometry is a versatile technique for cell sorting. It is quantitative, highly reproducible, and relatively easy to perform. As the density of Thy-1 on the surface varies greatly in specific subsets of RGCs, two regions, R10 and R21, would appear when flow cytometry is used to measure the Thy-1 protein, as shown in Figure 2B. We regarded the cells in the R21 region as high-purity RGCs (HP-RGCs) and the cells in the R10 region as low-purity RGCs (LP-RGCs). In our experiment, we obtained only approximately 2.7×10^3 RGCs per retina in the R21 region, while 5.4×10^3 RGCs were obtained in R21+R10, comprising only 0.05–0.35% of the total retinal cells.

RGC viability and longevity: The data from the Cell Counting Kit-8 (CCK-8) assay showed (Figure 3B) that there was no significant difference in RGC5 viability between the TIP and TIPM methods after the cells were cultured for 24 h. Then, the viability of the cells that were purified using both methods increased during the first 3 days and with prolonged culture times. The TIP-RGC viability was always greater than that of the TIPM-RGCs ($p < 0.05$). However, the viability of the TIPM-RGCs began to decrease, and most of the cells had died by the 9th day (the absorption value was 0.09). Interestingly, the viability of the TIP-RGCs reached the highest value at the same time point (the absorption value was 1.2) and began to slowly decrease until the 20th day (the absorption value was 0.48). Therefore, we can see that the TIP-RGCs could survive for more than 20 days with higher viability, while the TIPM-RGCs survived for less than 9 days.

RGC identification: The RGCs were identified by their morphology, immunofluorescence staining and whole-cell patch-clamp recordings. Neurons typically have dendrites

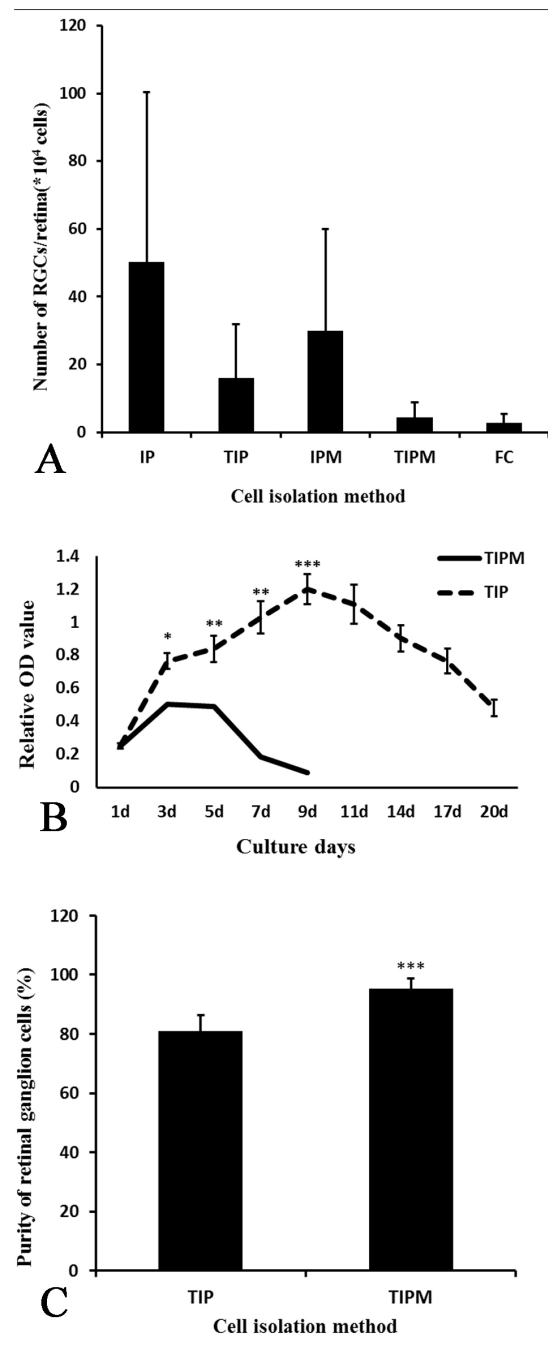


Figure 3. The yield, purity, and viability of RGCs from different methods. **A:** The yield of retinal ganglion cells (RGCs) from IP is the highest, while that from flow cytometry (FC) is the lowest. **B:** The viability of two-step immunopanning–retinal ganglion cells (TIP-RGCs) was increased at the first 9 days with culture time and was much higher than that of the two-step immunopanning–magnetic–retinal ganglion cells (TIPM-RGCs). Although the activity of the TIP-RGCs began to decline slowly, they still survived for 20 days, which was far longer than the TIPM-RGCs. **C:** The purity of the RGCs isolated from TIP is around 81% and much lower than that from the TIPM method (about 95%).

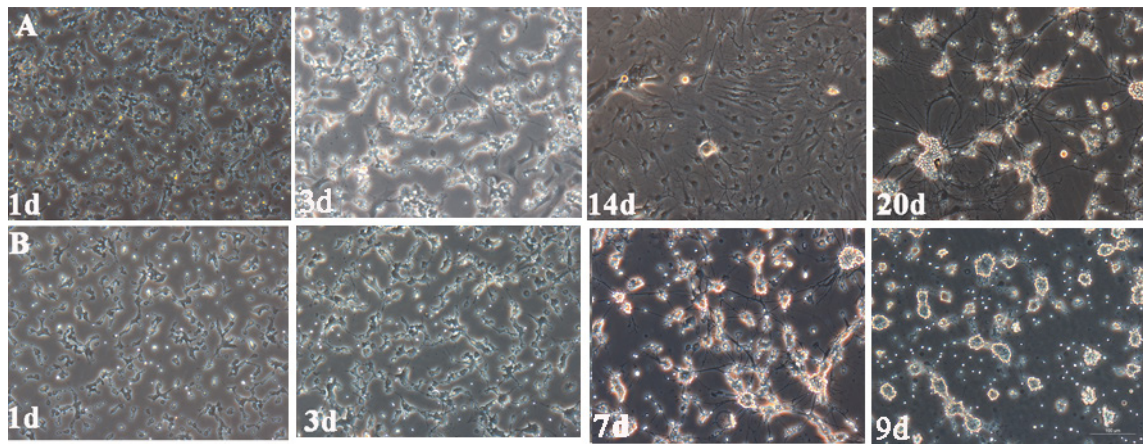


Figure 4. Morphological changes in RGCs. **A:** Two-step immunopanning–retinal ganglion cells (TIP-RGCs). **B:** Two-step immunopanning–magnetic–retinal ganglion cells (TIPM-RGCs). Scale bar = 10 μ m.

and axons that appear at opposing poles of the cell. We screened the survival and morphology of RGC isolated by the two immunopanning methods (TIP, TIPM) every day as shown Figure 4. Under standard culture conditions, the RGCs tended to form cell clusters with small to medium round cell bodies, and some extended fine neurites after a 24-h culture, but there were no significant differences between the two groups. However, from the third day, the RGCs purified using the two methods showed obvious differences. The majority of the TIP-RGCs exhibited uniform, round cell bodies and their neurites gradually extended to connect each other. At day 14, most cells developed a complex dendritic network with numerous neurites and branches. By replenishing half of the medium with fresh medium every 3 to 4 days, the TIP-RGCs were maintained for up to one month in culture, which was the longest time point in our examination (Figure 4A). However, the TIPM-RGCs neurites were obviously shorter than those from the TIP-RGCs beginning at day 3, and its cell body began shrinking. Although its neurites were still

extended, they ultimately survived for no more than 9 days (Figure 4B).

In retinas, many types of cells, such as rod and cone photoreceptors, horizontal, bipolar and amacrine cells, have neurites and round cell bodies, similar to the RGCs [30]. Therefore, we could not identify the RGCs by morphology alone. Several cell-specific markers have been suggested to be expressed in RGCs, such as Thy1 [31], Brn3a/b [32, 33], β III tubulin [31], MAP2 [34], Islet-1 [35,36], and γ -synuclein [37]. As none of these are specifically expressed in all RGC subgroups, we used six common RGC-specific immunocytochemical markers to provide a more accurate identification of the RGCs and further analyzed their expression in RGCs. γ -Synuclein and MAP2 (Figure 5A-D) were expressed in the soma and neurites in almost all of the RGCs, and their expression perfectly coincided with each other. TUJ1 and Thy1 were expressed in the cytoplasm of the soma and neurites in some of the RGCs, particularly those with long axons, while Brn3b and Islet-1 were predominantly localized in the nuclei in all

TABLE 2. COMPARATIVE ANALYSIS OF THREE PURIFICATION PROTOCOLS FOR RGCs.

Various	TIP	TIPM	FC
Yield(*10 ⁴)	24.60±15.98	5.28±4.42	0.54±0.27
RGCs/retina cell (%)	5.91±3.38	1.31±0.94	0.05–0.35
Purity (%)	80.97±5.45	95.41±3.23	Controlled, up to 100
viability	+++	++	-
Longevity (days)	>20	≤9	-
operation time (hour)	3–4	2–3	3–4
stability	+	++	+++
cost	+	++	+++

+ :low, ++: medium, +++:maximum -:we did not get.

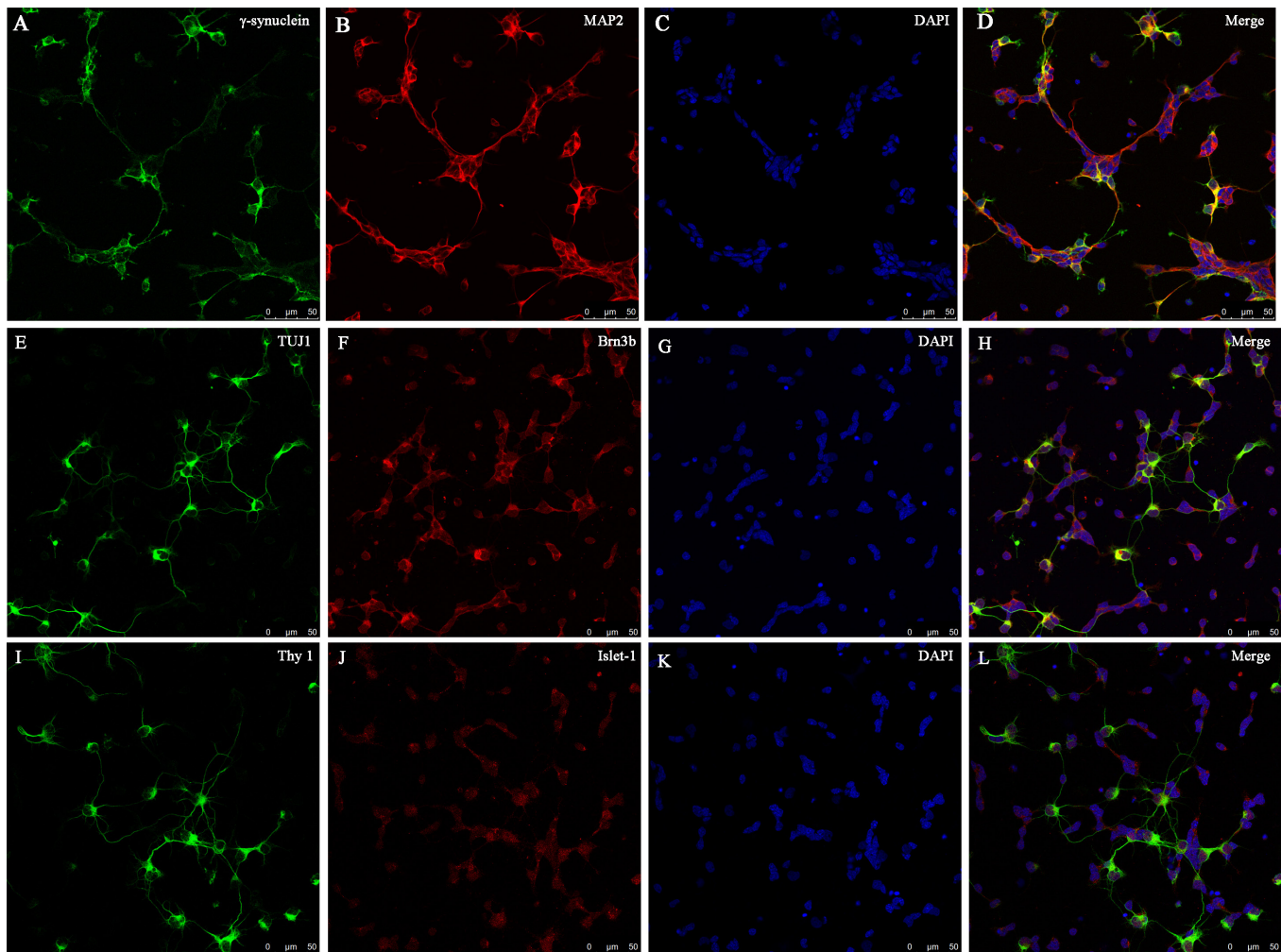


Figure 5. RGC identification. Immunofluorescence of retinal ganglion cells (RGCs) at day 2 of culture shows costaining of γ -synuclein (green) and MAP2 (red; A–D), TUJ1 (green), and Brn3b (red; E–H), Thy-1 (green), and Islet 1 (red; I–L). 4',6-diamidino-2-phenylindole (DAPI) nuclear staining is shown in C, G, and K, and the merged images are shown in D, H and L. Scale bar = 50 μ m.

of the RGCs, and little expression was observed in the cytoplasm (Figure 5E–L).

To further investigate whether the primary cultured RGCs have functional membrane properties, we performed whole-cell patch-clamp recordings of the RGCs on day 2 to 4 after they were cultured. For the majority of the analyzed primary cultured RGCs (TIP-RGC: 86.7%; TIPM-RGC: 63.1%, n=15), action potentials could be spontaneously elicited in current-clamp mode (Figure 6A). These action potentials could be blocked by tetrodotoxin (TTX), a specific inhibitor of Na⁺ ion channels (not shown). For the other mixed cells, such as glial cells, action potentials could not be spontaneously produced in current-clamp mode (Figure 6B). Action potentials could not be elicited by depolarizing the membrane from -90 mV to +50 mV at 10 mV intervals (not shown).

RGC purity: The purity of the RGCs isolated with the two methods was first determined with immunofluorescence staining (Figure 7). Nearly all of the TIPM-RGCs were positive for Thy-1 and MAP2, and only three cells in the field were negative (Figure 7D). The number of negative TIP-RGCs was much higher, as approximately seven cells in equal visual fields were not labeled (Figure 7H). To further analyze the source of these negative cells, we stained the cells for the expression of GFAP (the marker for glia cells) and syntaxin-1 (the marker for amacrine cells). As shown in Figure 8, large, star-shaped, GFAP-positive astrocytes (Figure 8B) and small, round, syntaxin 1-positive amacrine cells with multiple short neurites (Figure 8A) were rarely observed in the entire field.

The quantitative RT-PCR data for Thy-1, syntaxin 1 and GFAP were calculated as a relative RNA ratio to the housekeeping gene, β -actin (Figure 9A). The GFAP levels in

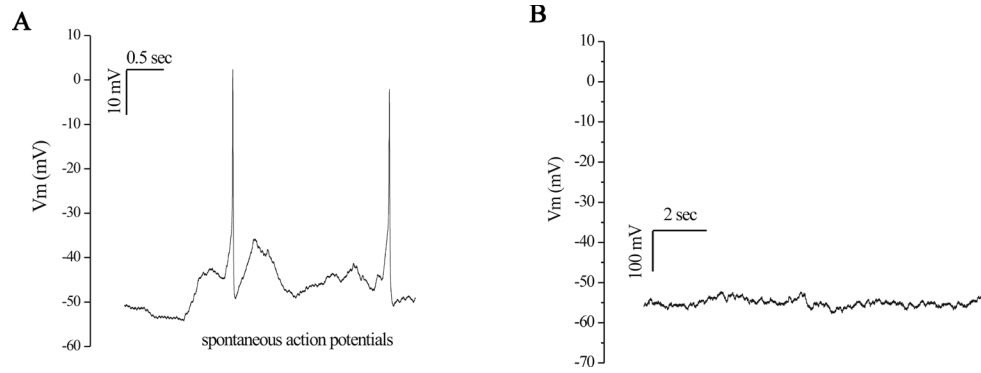


Figure 6. Representative traces of membrane potential in current-clamp mode. Spontaneous action potentials recorded from primary cultured RGCs purified by TIP and TIPM method at the second to the fourth day. No current injection was applied. Membrane potential was current-clamped at around -55 mV (A). In some cells action potentials were not produced spontaneously, indicating these were not RGCs (B).

the TIP- and TIPM-RGCs were obviously higher than in the FC-RGCs ($p < 0.01$), whereas the syntaxin 1 levels in the TIP-RGCs were significantly higher than in the other two groups ($p < 0.05$), while there were no significant differences in the TIPM- and FC-RGCs. This is consistent with the immunofluorescence staining results. As we had divided the FC-RGCs into HP-RGCs and LP-RGCs, an additional, more detailed quantitation of the quantitative RT-PCR data was performed

to examine whether our assumption was valid (Figure 9B). The expression of the Thy-1, TUJ 1, Brn3b, syntaxin 1, and GFAP mRNAs was analyzed in the HP and LP groups, and to our surprise, there were no significant differences in the mRNA expression levels, except for GFAP.

We next used flow cytometry to further compare purity, and RGCs isolated using the TIP and TIPM methods were immunostained with an anti-Brn3a antibody and analyzed

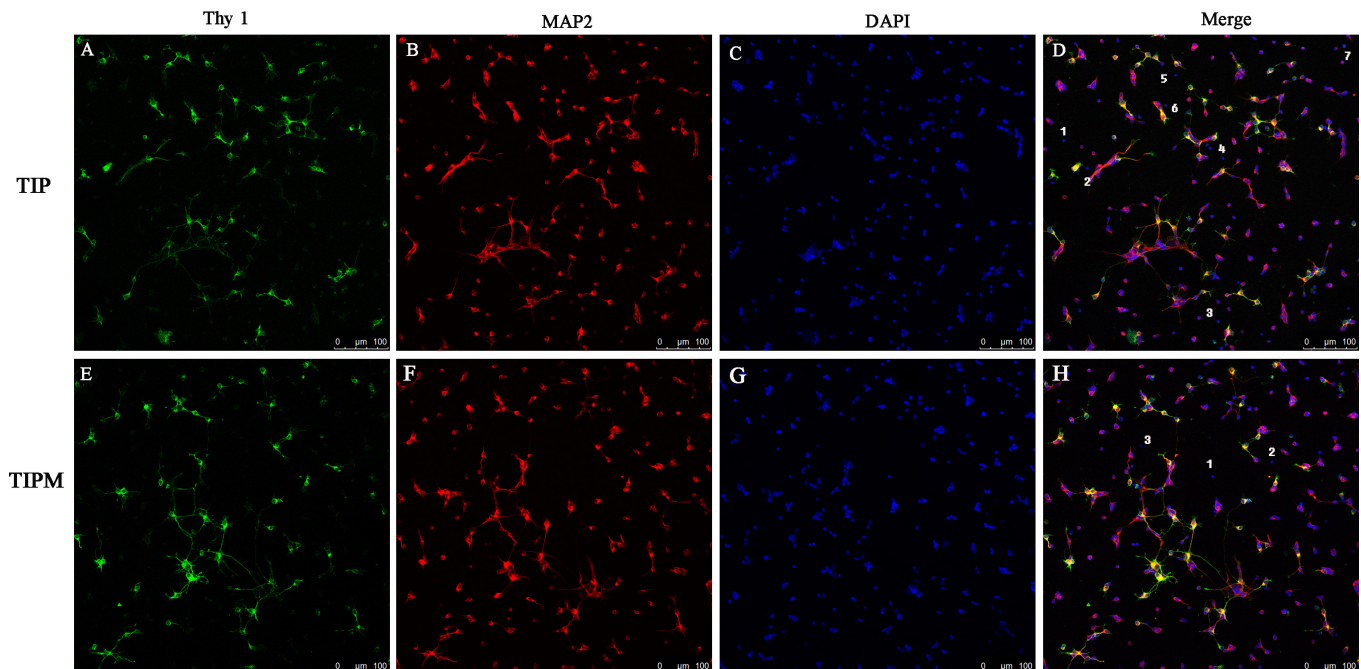


Figure 7. RGC purity. Confocal double immunofluorescence of retinal ganglion cell (RGCs) from the two-step immunopanning (TIP) and two-step immunopanning-magnetic (TIPM) methods shows the costaining of Thy-1 (A and E) and MAP2 (B and F). 4',6-diamidino-2-phenylindole (DAPI) nuclear staining is shown in C and G, and in the merged images in (D) and (H). Cells negative for Thy-1 and MAP2 are regarded as non-RGCs and are marked with Arabic numbers (D and H). Visual counting of immunostained cells seeded at the same density demonstrates that seven cells in a 200X visual field were not labeled by Thy-1 or MAP2 in the TIP groups while only three cells were negative in the TIPM group. Scale bars = 100 μ m.

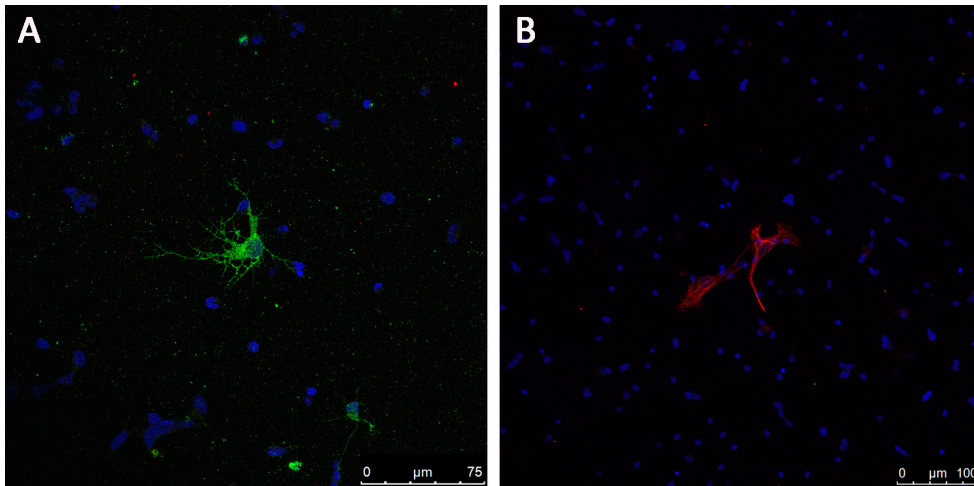


Figure 8. Rare labeling of astrocytes and amacrine cells. Syntaxin 1-labeled amacrine cells (A, green) and glial fibrillary acidic protein (GFAP)-labeled astrocytes (B, red) cells were rarely detected in the fields of cells. Nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bars = 100 μ m.

with FACS. The results (Figure 10) showed that $80.97 \pm 5.45\%$ of the cells from the TIP method and $95.41 \pm 3.23\%$ of the cells from the TIPM method were Brn3a-positive, indicating that the TIPM method obtained higher-purity RGCs (Figure 3C).

DISCUSSION

Cultured RGCs will be an important and almost indispensable tool for the study of retinal visual physiology and pathophysiology. Until recently, numerous approaches, including TIP methods [25], magnetic separation methods [19,23], retrograde labeling with fluorescent dyes combined with a pull-off technique [24,38], and flow cytometric methods [20]

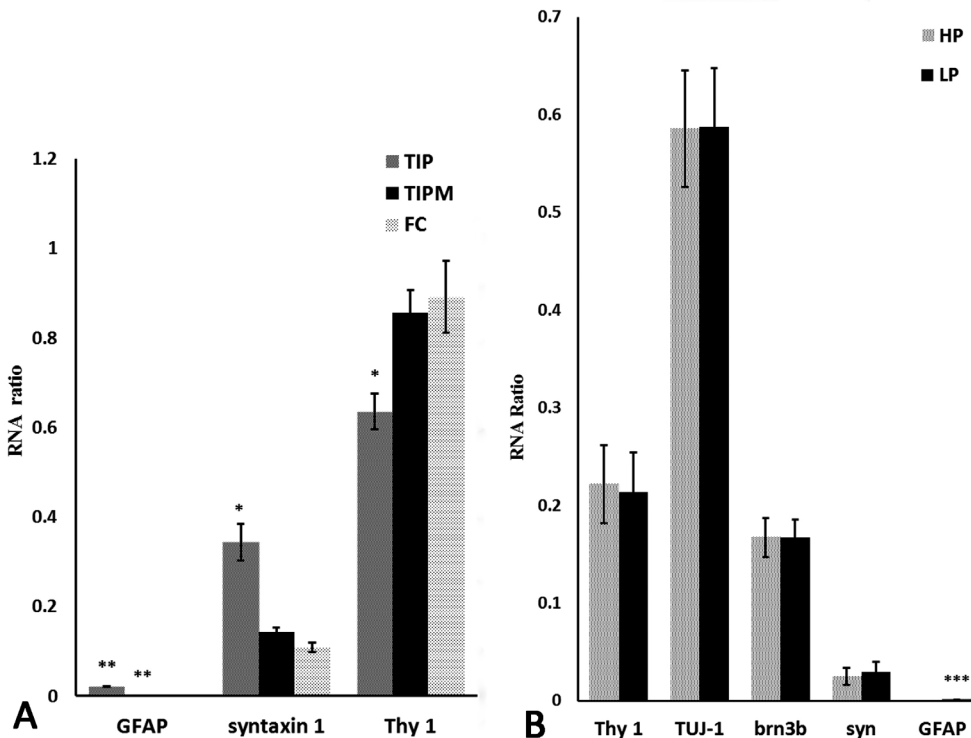


Figure 9. Quantitative expression of cell markers in isolated RGCs. A: Quantitative real-time PCR for glial fibrillary acidic protein (GFAP), syntaxin 1, and Thy-1 of retinal ganglion cells (RGCs) isolated with the two-step immunopanning (TIP), two-step immunopanning-magnetic (TIPM), and flow cytometry (FC) methods. The Thy-1 levels in FC were the highest ($p < 0.05$), and the syntaxin 1 levels were the lowest ($p < 0.05$), while GFAP were zero. This indicates that the purity of the RGCs from FC is the highest, followed by TIPM, and the RGC purity of TIP is the lowest of the three methods. B: Relative mRNA levels of Thy-1, TUJ-1, Brn3b, syntaxin 1 (syn), and GFAP from RGCs isolated with the FC method (high purity and low purity). There were no significant differences in the mRNA

expression levels, except for GFAP. Data expressed as relative expression to the housekeeping gene β -actin. All relative RNA ratios expressed as the mean \pm standard error of the mean (SEM; $n = 9$).

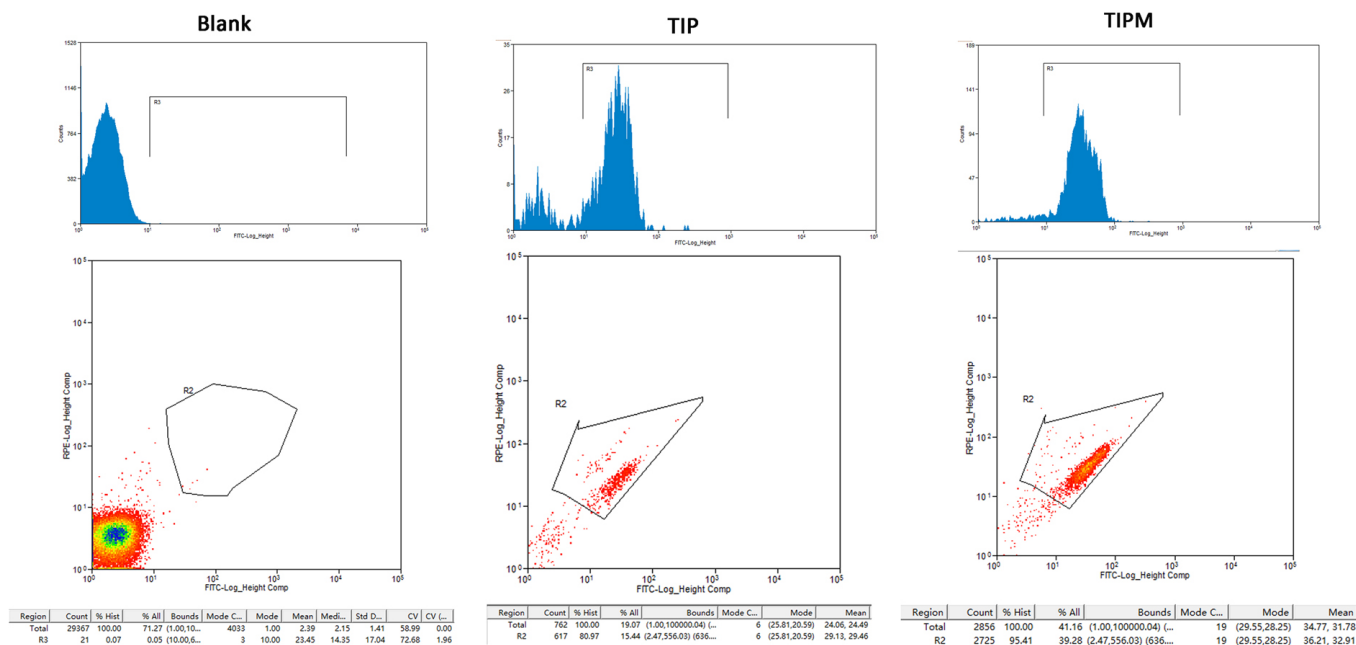


Figure 10. Flow cytometry analysis of the purity of RGCs sorted with the TIP and TIPM methods. The retinal ganglion cells (RGCs) were identified by staining with fluorescein isothiocyanate (FITC)-conjugated mAb against Thy-1 (Ex: 490 nm; Em: 520 nm).

have been established to isolate and purify RGCs in attempts to study retinal pathophysiology. Labeling RGCs with retrogradely transported fluorescent marker that had been injected into a fiber tract or target nucleus isolated RGCs from post-natal retinas with nearly 100% purity; however, the yield was sacrificed for purity, and the experiment is time-consuming and requires expensive instrumentation [38]. The TIP, TIPM, and FC methods are currently the most commonly used methods, but they vary greatly in experimental principles, procedures, and RGC purity, yield, and viability. The basis and key to a successful study is selecting the most effective and appropriate method for purifying RGCs. However, there has been no specific comparison with which to benchmark these methods and determine which are the most appropriate.

The TIP method has been widely used to purify RGCs. It has been reported that the purity of RGCs isolated with TIP ranges from 50% to 99.5% [8,21,30], but we achieved approximately 81% in our study, even though we had repeated the whole experiments dozens of times. To find the main reasons for this discrepancy, we coated the panning dishes only with 50 mM Tris-HCl as negative control but found that many cells were still attached to the plate, suggesting that most of the contaminating cells had adsorbed on the plate, even though we used a low absorption plate (Nunc, Thermo, Denmark). Therefore, we insist that purity higher than 90% is difficult to achieve, and it is difficult to maintain a constant yield. Furthermore, the binding affinity between the RGCs

and the anti- Thy-1 antibody is not strong, and plate swinging, cell clusters, and undigested retinal tissue may, in part, cause the variable yield. However, although the purity of the RGCs from the TIP method was not as high as that from the TIPM method, their viability and longevity were much higher, which are essential and particularly important in some studies. This method is particularly ideal for experiments in which the presence of a small number of contaminating cells could be ignored without any obvious impact on the experimental results but that require a large quantity of highly viable RGCs for a long period.

The TIPM method introduced by Samin Hong [19] is faster and less complicated than the TIP method, as summarized in Table 2. The TIPM method not only has the advantage of a more stable yield but also has a much higher purity compared with the TIP method, even though the TIP-RGCs have a short cell survival time and lower viability. However, if the experimental cycle is short and/or highly pure RGCs with specific activity are required, TIPM-RGCs are the best source. Nevertheless, TIPM requires highly skilled hands, and the cost was slightly higher than that of the TIP method.

Flow cytometry has been used for cell sorting for many years. Samin Hong et al. compared the purity of the RGCs from the TIP and TIPM methods in 2012 [20], but they did not analyze FC, which is an ideal and efficient approach for cell sorting. Using FC, several million cells can be screened within a short time, and subpopulations and single cells can

be isolated from within mixed-cell populations, even when the cells are present at frequencies as low as 10^{-6} within the population [39]. In our experiment, $5.4 \pm 2.7 \times 10^3$ RGCs were obtained per retina, and this reduced yield is due to the increased vulnerability of the cells to the intricate process and the long-term mechanical separation, which may also reduce the activities of RGCs. Although this number is not sufficient for protein analyses or additional biochemical approaches that require a great quantity of cells, their purity can almost reach 100%, and we can easily control the relative purity of RGCs. Therefore, FC is a suitable choice for studies that require extremely pure RGCs. If a large number of RGCs with 100% purity are required, the method could be scaled up by increasing the number of retinas and extending the sorting time. However, the reduced viability of cells collected by this method will limit their use to experiments of short duration only.

Collectively, this is the first report to compare the three most common methods for purifying RGCs from newborn rat retinas by analyzing the purity, yield, and viability. Our study found that the RGCs isolated with the TIP method possess the highest viability and yield but low purity; the TIPM method was the most reliable, as the RGCs produced using this method exhibited considerable purity, yield, and viability. The purity of the RGCs from the FC method can reach approximately 100%, but their yield was low with the same number of retinas. Although factors such as increasing the amount of starting tissue and reducing the isolation and sorting times may improve yield and viability by the FC method, an area that may have greater impact on cell viability of RGCs isolated by all methods is modification of the culture conditions of these cells to permit greater survival.

In summary, our study demonstrates that TIP, TIPM, and FC can isolate RGCs from newborn rats, and each approach has its own pros and cons. The research provided a solid practical basis or at least a reference for selecting the method for purifying RGCs for subsequent experiments.

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