Simultaneous Mice Sub-Retinal Cell Enrichment Using FACS Sorting Strategy

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Abstract. The retinal tissue consists of many cell types with distinct characteristics and functions. Many eye diseases are initiated by a dysregulated retinal cell subtype within the tissue. A feasible method is needed for single-cell transcriptomic studies. It is possible to enrich retinal cell subtypes by combining specific epitopes expressed in rod cells, cone cells, Müller cells, and other cell types. This study aims to optimize sorting strategies to simultaneously enrich murine rod, cone, and Müller cells using FACS. Methods: Standard retinal cell dissociation protocols using proteases, then cell viability assay using trypan blue staining and MTT. In the optimal condition, dissociated cells were stained with several central differentiation (CD) markers, including CD73, CD133, CD147, CD29, SSEA-1, and CD44, before sorting by FACS. Results: This study shows that the sorting strategy achieves rods, cones, and Müller cells simultaneously. These results suggest that FACS can be used for complex experimental setups for downstream applications such as RNA-seq. Conclusion: The strategy successfully sorted viable murine rods, cones, and Müller cells.

Keywords: FACS, Transcriptomic Biomarker, Inherited Retinal Dystrophy, Cluster Differentiation, Mouse Retinal Cells.

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1 Introduction

Inherited retinal dystrophy (IRD) is a rare genetic eye disease that causes irreversible blindness in children and teenagers. The mutation causes the rapid, premature death of retinal cells. These cell deaths are temporally and spatially diverse. For example, photoreceptor cell death in Stargardt disease typically begins in the macula, whereas retinitis pigmentosa usually starts in the peripheral retina [1]. In animal model evidence, specific mutations affect certain subtypes of cell death and then trigger other subtypes [2–4]. This hardens the study of the IRD etiology. However, it is possible to be evaluated at the single-cell resolution.

Finding a transcriptomic biomarker using the bulk RNA sequencing approach is less precise. The biomarker in certain types of retinal cells will become diluted and fade. In single-cell resolution, biomarkers are preserved by accumulating within each cell type. Generally accepted Single-cell RNA sequencing (scRNAseq) was from 10x Genomics and BD Rhapsody.

Droplet-based scRNA-seq from 10x Genomics, combined with Illumina short-read sequencing, provides high-resolution transcriptomic data at high cost per sample. However, it is not designed to identify spatial biomarkers. To address this, 10x Genomics offers Visium, a technology that enables spatial visualization of gene expression. This molecular profiling approach classifies tissue from FFPE or cryosections based on total mRNA content. However, less single-cell precise than droplet-based.

After numerous scRNAseq investigations of the retina samples, many important cells along with their biomarkers were identified. Fluorescence-activated cell sorting (FACS) may be a promising alternative for studying cell-specific transcriptomic biomarkers [5]. This approach provides a cost-effective option for larger sample sizes and is more focused on specific cells. Flow cytometry technology utilizes specific epitopes or cluster differentiation (CD) markers in each retinal cell subtype [6,7]. This research aims to investigate an improved sorting strategy for mouse retina tissue samples in FACS applications.

2 Methods

2.1 Animal

The experiment was reviewed and approved by the Ethical Commission (No.: KE/FK/0482/EC/2024). The C57BL mice were obtained from a local supplier and were maintained in an animal facility at the Faculty of Medicine, Gadjah Mada University. Mice were maintained in accordance with standard animal welfare. Four adult mice were terminated by neck dislocation, and the eyes were enuculated immediately. Single cells underwent FACS.

2.2 Retinal Tissue Dissociation

Retinal tissue dissociation was performed by protease digestion as described previously [8]. All surgical procedures were performed in a cold environment. The four left and

right eyes were pooled in cold D-PBS. The retina was detached from the sclera and lens using Vannas scissors under a dissection microscope at the Laboratory of Pharmacology and Therapy, Faculty of Medicine, Universitas Gadjah Mada. Retinal tissues were incubated in 250 µL of D-PBS containing 40 µL Liberase (Sigma-Aldrich). The incubation was performed at 8 °C for 40 minutes. Fully dissociated tissue was achieved by gently pipetting the suspension 10 times – 15 times with a P1000 pipette. Trituration was stopped once the retina appeared fully dissociated. Subsequently, 700 µL of ice-cold washing buffer was carefully layered beneath the cell suspension. The suspension was then centrifuged at 200 × g for 5 minutes at 4 °C using a swinging-bucket rotor. After discarding the supernatant, the pellet was resuspended in 500 µL of D-PBS supplemented with 0.04 % BSA and DNase I (100 µg · mL^{-1}, E1010; Zymo Research). The cell suspension was passed through a 40 µm filter to remove debris. Cell viability was determined by mixing 10 µL of the cell suspension with 10 µL of trypan blue (T8154-20ML; Sigma-Aldrich), followed by staining with trypan blue for further viability assessment.

2.3 Cell Sorting

Retinal cells were stained using anti-CD73, CD133, CD147, CD29, SSEA-1, and CD44 as per the manufacturer's recommendation (supplemental file). Aliquot 100 µl of the cell suspension into a 5 mL round-bottom tube.

Dilute the conjugated antibody 100 times, then add it to each determined group. Mix well and incubate at 4 °C for 30 minutes in the dark. Add 2 mL of Staining Buffer, centrifuge at 300 g – 400 g for 5 minutes, and discard the supernatant. Resuspend the cells in 2 mL of Staining Buffer, centrifuge at 300 g – 400 g for 5 minutes and discard the supernatant. Load the tube into the BD FACSAria III instrument at LRT, Faculty of Medicine, Universitas Gadjah Mada (UGM), which is configured with 2 lasers and 7 detection channels, enabling multi-color flow cytometry analysis. Overall, the instrument is in good operational condition. Testing was performed using a 100 µm nozzle, which functioned properly and produced a well-defined stream. Daily performance evaluation using the CS&T module resulted in a PASSED status, with a warning on the APC channel where the Bright Bead% rCV was 6.35 %, slightly above the recommended specification of 6 %. Air pressure was maintained at 85 psi, providing stable fluidics for both sorting and analytical processes. Store the sorted cells in cold PBS.

2.4 RNA Isolation

Whole RNA isolation of sorted cells using the commercial kits FavorPrep Total RNA Extraction HE Mini Kit (Favorgen Biotech Corp.) to allow a higher RNA yield by passing the liquid through the filter twice. Whole RNA quality was assessed using Nanodrop.

3 Results

Cell reading using FACS indicated that a combination of CD markers bound to the mice's retinal cells. The protease type selection for FACS is also correct. The FACS analysis shows Liberase yields more cells than other proteases.

A diagram was drawn to visualize the optimized sorting strategy (Fig. 1). First, cells were sorted from debris based on FSC-A and SSC-A, followed by singlets sorted by FSC-A and SSC-H. Then, CD29 and SSEA-1 exclusion was used to only include non-differentiated cells.

Only include cells that have CD73 and CD133 for rod immature, whereas CD133 and CD147 for cone. Only include area Y from Fig. 2, and CD73 for rod mature. For Müller, only include cells that are negative for SSEA-1, then positive for CD29 and CD44.

After sorting is finished, the plot can be generated to visualize the number and type of cells (Fig. 1).

RNA isolation from sorted cells needs further improvement for further analysis. Like downstream analysis, qPCR, NGS, and long-read sequencing. The number of cells was not sufficient to achieve the minimum RNA yield in the current attempt.

Population	#Events	%Parent	%Total
All Events	1,092,225	####	100.0
Cells	10,013	0.9	0.9
Singlet	9,170	91.6	0.8
Induk	2,080	22.7	0.2
Rod Mature	283	13.6	0.0
Cone Mature	1	0.0	0.0
Rod Immature	216	10.4	0.0
P1	1,510	16.5	0.1
Muller	1,432	94.8	0.1

Fig. 1. The FACS plot according to each cell's identity.

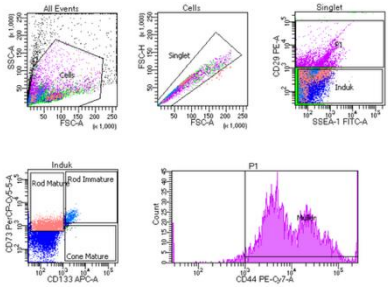

Fig. 2. The sorting strategy for specific cell enrichment

4 Discussion

The rapid cell death in IRD patients remains hard to correct [9]. Whole-exome sequencing has high resolving power for determining causative mutations, comparable to panel

sequencing [1,10]. Need transcriptomic biomarker discovery to prevent the onset of IRD.

This study optimizes a flow cytometry-based strategy to isolate specific retinal cell subpopulations from mouse tissues using a panel of CD markers.

The five cell types successfully isolated from the bulk of retinal cells in this research are the prospective start. This achievement indicates that the combination of cell surface epitope labeling is able to effectively distinguish retinal cell subpopulations. The scenario enables the involvement of more cells, with complex experimental details (i.e., gradual dying level or even death), with a large sample. Moreover, the setup is relatively affordable compared to single-cell transcriptomic methods for the same sample size.

The limitation of FACS sorting requires more laborious effort for cell dissociation and instrument setup. The preservation of the cell's metabolic state requires further evaluation. This technique also requires a homogeneous cell suspension free of particles that interfere with flow or clog the narrow channels of the instrument. The detection limit of sensitive instruments often requires a pre-enrichment step, making it challenging to quantify very rare cell populations accurately. Sorting efficiency also decreases drastically if the target cells are less than 1 % of the total population, which can result in significant cell loss. The binding of specific antibodies also has the potential to activate intracellular signals and trigger functional changes in cells. This requires careful selection of phenotypes for sorting. In addition, FACS requires specialized high-cost equipment, as well as a complex calibration and optimization process [11, 12]. Cell death begins in the rod cells, followed by the cone cells. Inherited retinal dystrophy (IRD) is a rare genetic eye disease that causes irreversible blindness in children and teenagers. The mutation causes rapid, premature retinal cell death. These cell deaths are temporally and spatially diverse. For example, photoreceptor cell death in Stargardt begins in the macula, while retinitis pigmentosa begins in the peripheral retina [1]. In animal model evidence, specific mutations affect certain subtypes of cell death and then trigger other subtypes [2–4]. This hardens the study of the IRD etiology. However, it is possible to be evaluated at the single-cell resolution.

5 Conclusion

This study has several limitations. First, although both the right and left retinas from each animal were analyzed, the use of only one mouse per protease group limits the statistical power and generalizability of the findings. Second, processing small tissues such as mouse retinas requires technically demanding and repetitive procedures, which may increase variability and affect cell yield and viability. Third, the expression of markers such as CD133 and CD44 is not entirely restricted to a single retinal cell population, potentially leading to overlap between subpopulations during cell sorting. Further optimization of the tissue dissociation and sorting protocols is therefore required to better preserve cell viability, metabolic status, and marker specificity. In addition, transcriptomic comparisons between FACS-sorted and non-sorted samples are warranted to validate sorting specificity and to evaluate potential transcriptional changes induced by the cell isolation and sorting process.

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