Computational Biology-Assisted and Experimentally Validated of Various Protease Cleavage Activities on Surface Proteins Using the Murine Retinal Tissue

Ayudha Bahana Ilham Perdamaian[1-2] Titi Marsifah[3], Nina Sakina Lessy[3], Dewi Kartikawati Paramita[4-5-6] Bjorn Voss[7], Muhammad Bayu Sasongko[2], Supanji Supanji[2*]

[1]Doctorate Program of Medicine and Health Science, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia;
[2]Department of Ophthalmology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia;
[3]Master Program of Biomedical Science, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia;
[4]Department of Histology and Molecular Biology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia;
[5]Study Center for Biotechnology, Universitas Gadjah Mada, Yogyakarta, Indonesia;
[6]Integrated Research Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia;
[7]RNA-Biology and Bioinformatics, Institute of Biomedical Genetics, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany.
supanji@ugm.ac.id

Abstract. Efficient dissociation of retinal cells requires careful selection of proteases to preserve surface epitopes critical for downstream analyses. This study aimed to identify the most suitable protease for murine retina dissociation without compromising key surface markers. Retinas were harvested from normal C57BL/6 mice and treated with various proteases, including papain, trypsin, Liberase (consisting of collagenase and thermolysin), elastase, and proteinase K. In silico analyses using Expasy Peptide Cutter, Rapid Peptide Generator, and HADDOCK docking. This in silico analysis was followed by experimental validation through retinal tissue dissociation and assessment of cell viability. Results showed that trypsin produced the highest cell yield, followed by Liberase, while papain and trypsin minimally cleaved surface epitopes. Immunofluorescence confirmed that Liberase preserved the SSEA-1, CD73, and CD147 epitopes best, followed by papain. These findings underscore the significance of protease selection for retinal cell dissociation and provide a framework for optimizing procedures to preserve functional surface proteins in downstream applications.

Keywords: FACS, Machine Learning, In Silico, Cell Dissociation, Specific Protease.

V. D. S. Handayani et al. (eds.), *Proceedings of the 6th International Conference on Bioinformatics, Biotechnology, and Biomedical Engineering (BIOMIC 2025)*, Advances in Health Sciences Research 94,
https://doi.org/10.2991/978-94-6239-762-0_15

1 Introduction

Tissue dissociation is a routine procedure used in various analyses, including flow cytometry and Single-cell RNA sequencing (scRNA-seq). To prepare single-cell suspensions from tissue samples, various proteases with different specificities are used. Many studies also compare lists of proteases for specific tissues, such as brain tissue [3], fish retina [1,2], or use a cold-activated protease for tissue dissociation to reduce artificial stress in single-cell RNA sequencing studies [4,5].

The retina is considered a good model to study protease cleavage activity on surface proteins, a complex tissue consisting of many cell types connected by various types of cell junctions [6]. Well-documented cell-cell junctions include zonula adherens at RPE and N-cadherin and retinal-cadherin [7,8]. Retinal cells are delicate and thus require smooth cell dissociation. Several studies utilized various types of proteases for cell dissociation, such as papain [9], proteinase K [10], Liberase [11], trypsin [12], and collagenase [13].

Each retinal cell subtype has distinct specific epitopes; for example, rod and cone cells express CD73 and CD133 [14-17], while Müller cells possess CD44 and CD29 [18]. These epitopes are beneficial for cell sorting and must be preserved. An optimization study should be conducted to evaluate several proteases to maintain the integrity of these epitopes. By using available virtual structures of both the epitope and the protease, potential cleavage sites can be identified. The findings will be verified using an Immunofluorescent (IF) microscope. Flow cytometry technology relies on epitope cluster differentiation (CD), so the epitope(s) must remain intact. Excessive or improperly selected proteases could annihilate the recognized site of an epitope, rendering it undetectable by FACS. An example of excessive protease activity is the red blood cell surface antigen eraser that utilizes papain [19,20].

Several studies have shown that the type of protease used in tissue dissociation can differentially affect the integrity of cell surface epitopes. Previous research has shown that dissociating human lung tissue with dispase decreases the expression of the immune markers CD4, CD8, CD69, and CD103 and alters the composition of epithelial and immune cell populations. This research also demonstrated that collagenase produces a distinct profile, with an increase in AT1/AT2 cells and a decrease in endothelial cells [21]. Other studies have reported that dispase treatment significantly impairs the detection of surface molecules important for identifying immune cell subsets. This effect can persist for more than 24 hours and impact T cell proliferation [22]. Furthermore, epitope sensitivity to proteases varies. Specific epitopes can be resistant to trypsin but susceptible to other enzymes, thus affecting the accuracy of antibody detection [23]. In flow cytometry-based proteomic analysis, mild trypsin treatment does allow most surface proteins to remain detectable. However, approximately 7.9 % of epitopes exhibit signal loss, potentially leading to false-negative results, particularly for proteins with multiple potential cleavage sites (>30). Both enzymatic and mechanical dissociation have been shown to produce significant variations in the presence of surface antigens and can introduce experimental bias [24].

Despite their importance, there have been no systematic studies addressing the effects of proteases such as papain, trypsin, collagenase, elastase, and liberase on the integrity

of retinal surface markers. Therefore, optimization studies are needed to identify proteases that maximize live cell yield without damaging epitopes essential for cell identification and function. Potential cleavage sites can be identified using virtual structures of available epitopes and proteases.

This study aims to investigate the most appropriate protease for the dissociation of mouse retinal tissue. An in silico approach will be employed to predict the cleavage susceptibility of retinal surface proteins, followed by experimental validation through retinal tissue dissociation, assessment of cell viability, and CD marker detection.

2 Methods

2.1 Animal Model

Six C57BL mice were provided by a local supplier and then placed in an animal facility at the Faculty of Medicine, Universitas Gadjah Mada. The mice were maintained according to standard procedures. Five adult mice were randomly assigned to five experimental groups, each corresponding to a specific protease for activity assessment. The control group underwent mechanical dissociation. Before retinal enucleation, the mice were euthanized by cervical dislocation. In each mouse, both retinas (right and left) were collected and pooled to be used as a tissue sample per treatment group. Ethics for this study involving animal models (No KE/FK/0482/EC/2024) was issued by the Ethical Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (FMPHN UGM).

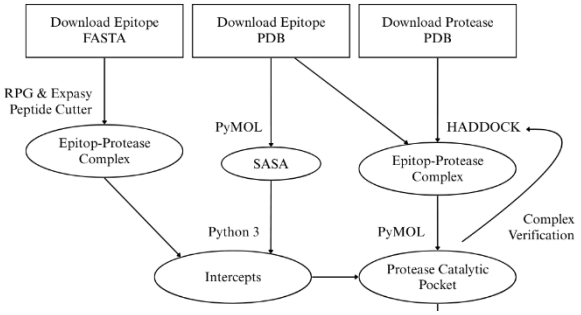

Fig. 1. Schematic diagram of the computational study of a selected protease. Figure created by the author based on references [25-29].

2.2 Computational Protease-Substrate Binding Analysis

Screening of potentially cleave sites of CD73 (PDB ID: 6TVG_1; uniport: Q61503), CD44 (PDB ID: 4mre; P15379), CD147 (PDB ID: 3qqn; P18572), CD133 (PDB ID: 6DUM; O54990), CD29 (PDB ID: 7NWL_2; P09055) was done using Rapid peptides generator (RPG) [29] and Expasy peptide cutter [27]. Each epitope's Solvent-accessible

surface area (SASA) data were retrieved using PyMOL (The PyMOL Molecular Graphics System, Version 3.0, Schrödinger, LLC). The data of intercepted, cleaved, and exposed sites were stored (Fig. 1).

The protease evaluated, including papain (9PAP), Liberase® consisting of [collagenase I (966C), collagenase II, and thermolysin (8TLN)], elastase I (1ESB), proteinase K (2PRK), and trypsin (2PTN) catalytic triad, were acquired from the Merops database and various publications. The pdb_selaltloc function at the PDB-tool website was used to fix residue or atom duplicates before uploading to HADDOCK3 installed on the cloud [28]. An "expert" assessment allowed a simulation without prior knowledge.

2.3 Retina Collection and Retinal Tissue Dissociation

Following euthanasia, ocular enucleation was performed under cold conditions to preserve tissue integrity. Retinal isolation was conducted under a dissection microscope using Vannas scissors. Retinas from both eyes of each mouse were pooled into a petri dish containing cold Dulbecco's Phosphate-Buffered Saline (D-PBS) for subsequent analysis.

Retinas were incubated with different protease solutions; each was prepared in a final volume of 250 µL using D-PBS. Concentrations for each protease were based on laboratory optimization. The conditions included 20 U · mL^{-1} Papain (Worthington), 9 µL of Elastase (E1250-10MG; Sigma-Aldrich), 100 µg · mL^{-1} Trypsin (Trypsin/Lys-C Mix V5071; Promega), 20 µL of Liberase™ TL Research Grade (100 µg/mL; 5401020001; Sigma-Aldrich), and 5.2 µL of Proteinase K (D3001-2-5; Zymo Research) diluted in 244.8 µL D-PBS. Liberase™ is a purified enzyme blend containing collagenase and thermolysin. Retinal samples were incubated at 8 °C for 40 minutes with each respective enzyme. For each sample, mechanical trituration was carried out by pipetting the suspension 10 to 15 times using a P1000 pipette. Trituration was halted once the retinal tissue appeared fully dissociated, after which 700 µl of ice-cold washing solution was carefully added beneath the cell suspension. Each dissociated retinal suspension was centrifuged at 200 × g for 5 minutes at 4 °C using a swing-bucket rotor, followed by removal of the supernatant; the cell pellet was resuspended in 500 µl of DPBS supplemented with 0.04 % BSA and DNase I (100 µg · ml^{-1}, E1010; Zymo Research), then filtered through a 40 µm cell strainer. Cell viability was assessed by adding 10 µL of the sample to 10 µL of trypan blue (T8154-20ML; Sigma-Aldrich).

2.4 Cell Sorting

As many as 100 µL of each dissociated retinal cell was transferred into individual 5 mL round-bottom tubes. The cells were labeled with antibodies against CD73, CD133, CD147, CD29, SSEA-1, and CD44, following the manufacturer's instructions (see supplementary file). The prepared samples were examined using the BD FACSAria III flow cytometer, located in the Integrated Research Laboratory at FMPHN UGM. Sorted cells were collected in cold PBS and subsequently subjected to intracellular biomarker staining.

3 Results

3.1 Computational Study of Protease-Substrate Binding

The result of the first stage of the computational analysis can be seen in Fig. 2. Papain and trypsin are predicted to cause the least harm to the epitopes. On the other hand, proteinase K and elastase 1 can cleave the epitopes at many cleavage sites. Liberase as protease, bends of collagenase and thermolysin, in this research was represented by thermolysin. Based on the evaluated epitopes, the most prone epitope was CD133, while CD147 was the least prone epitope.

Liberase at 40 µL achieved the highest cell viability (97 %), followed by Trypsin (88 %) and Mechanical dissociation (84 %). In contrast, Papain at 40 µL showed the lowest viability (28 %). Lower concentrations of Liberase, Papain, and Elastase produced moderate viability levels, while Proteinase K and Elastase showed around 78 % and 53 % – 78 % viability (Fig. 3).

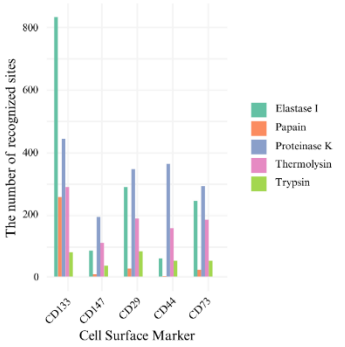

Fig. 2. The number of cleavage sites per protease of each CD marker.

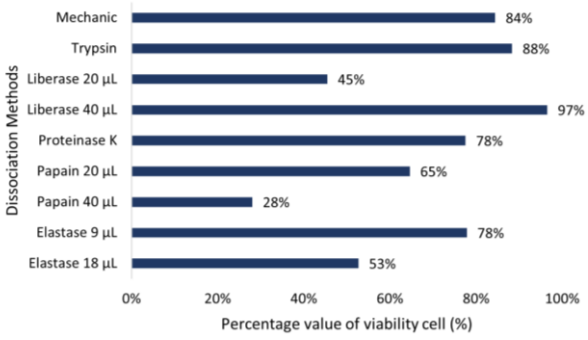

Fig. 3. Cell viability percentage after different dissociation methods.

The cell viability obtained from various dissociation methods is shown in Fig. 4, which presents the results of the Trypan Blue test on retinal cells using six different treatments, including the mechanical method. Overall, these results indicate that the

type and concentration of enzyme greatly influence the efficiency of retinal dissociation and the number of cells that can be obtained.

Among the dissociation methods tested, Papain yielded the highest cell viability (87 %) and consistently high marker expression (CD44: 97 %, CD73: 86 %). Trypsin and mechanical dissociation produced moderate viability (32 % and 48 %, respectively) with relatively high CD44 and CD73 expression. In contrast, Liberase resulted in complete cell death (0 % viability) and minimal marker expression.

It should be noted that Proteinase K and Elastase samples have not yet been processed for FACS analysis.

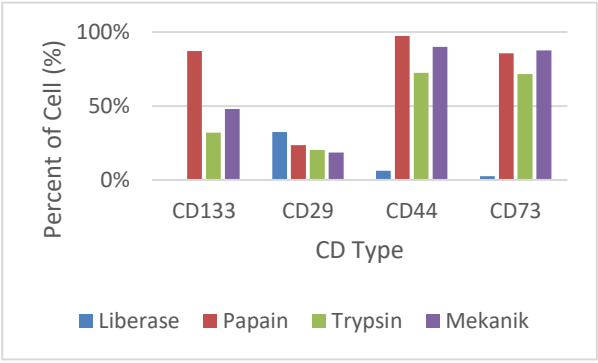

Fig. 4. Percentage of cells expressing different CD markers after various dissociation methods.

3.2 Retinal Cell Yield After Enzymatic Dissociation

The total number of dissociated retinal cells was determined manually using a Neubauer chamber. The results showed that the viability of dissociated retinal cells varied between 28 % – 97 %, with the lowest viability observed in cells dissociated using Papain 40 µL, and the highest viability (97 %) obtained from cells dissociated using Liberase 40 µL, which was comparable to that achieved through mechanical dissociation. Based on cell counts (Table 1), Trypsin produced the highest number of cells, reaching 1.6×10^7, indicating a very high efficiency in dissociating retinal tissue. Liberase was used at a volume of 40 µL, which resulted in 8.3×10^6 cells. In comparison, a lower volume of 20 µL yielded only 2.2×10^6 cells, indicating a positive correlation between enzyme volume and the number of cells obtained. Elastase at 9 µL yielded 3.7×10^6 cells, which was higher than the 1.4×10^6 cells obtained at 18 µL, indicating that the effectiveness of this enzyme does not always increase with a larger volume. Proteinase K yielded 1.6×10^6 cells, while Papain at 40 µL and 20 µL resulted in substantially lower yields of 3.3×10^5 and 1.0×10^6 cells, respectively.

Table 1. Number of Retinal Cells Obtained from Two Retinas Based on Dissociation Methods.

Methods	Total of Retina Cells
Enzymatic Dissociation	
Elastase 18 µL	$1.4x10^6$
Elastase 9 µL	$3.7x10^6$
Papain 40 µL	$3.3x10^5$
Papain 20 µL	$1.0x10^6$
Proteinase K	$1.6x10^6$
Liberase 40 µL	$8.3x10^6$
Liberase 20 µL	$2.2x10^6$
Trypsin	$1.6x10^7$
Mechanical Dissociation	
Mechanic	7.2×10^6

3.3 Dissociation Computational Study of Protease-Substrate Binding

Liberase at 40 µL achieved the highest cell viability (97 %), followed by Trypsin (88 %) and Mechanical dissociation (84 %). In contrast, Papain at 40 µL showed the lowest viability (28%). Lower concentrations of Liberase, Papain, and Elastase produced moderate viability levels, while Proteinase K and Elastase showed around 78 % and 53 % – 78 % viability (Fig. 3).

3.4 Retinal Cell Yield After Enzymatic Dissociation

The cell viability obtained from various dissociation methods is shown in Fig. 4, which presents the results of the Trypan Blue test on retinal cells using six different treatments, including the mechanical method. Overall, these results indicate that the type and concentration of enzyme greatly influence the efficiency of retinal dissociation and the number of cells that can be obtained.

Among the dissociation methods tested, Papain yielded the highest cell viability (87 %) and consistently high marker expression (CD44: 97 %, CD73: 86 %). Trypsin and mechanical dissociation produced moderate viability (32 % and 48 %, respectively) with relatively high CD44 and CD73 expression. In contrast, Liberase resulted in complete cell death (0 % viability) and minimal marker expression (Fig. 5).

4 Discussion

Our study demonstrates that the combination of CD markers with immunofluorescence validation provides reliable evidence that sufficient cell numbers and intact surface proteins can be obtained after protease treatment. Among the enzymes tested, trypsin and papain offered the most favorable balance between dissociation efficiency and preservation of cell surface markers. The sorted rod, cone, and Müller cells yielded RNA of high integrity, suitable for both short-read and long-read RNA sequencing. These findings suggest that protease selection is a critical parameter in retinal cell dissociation protocols, not only for ensuring effective FACS-based isolation but also for maintaining the biological relevance of downstream transcriptomic analyses.

Aligning the computational predictions with in vitro experimental results is crucial for validating the model. A close match between predictions and experimental outcomes indicates robust computational performance. CD147 exhibited a clear linear relationship, whereas CD133 showed no such trend. CD73, in contrast, demonstrated moderate linearity. Thermolysin on Liberase, which is also comparable to papain specificity (Table 1), aligns with in vitro validation.

The results of computational studies indicate that each protease has a different tendency to recognize and cleave epitopes. Initial evaluation based on solvent-accessible surface area (SASA) values showed that papain and trypsin were predicted to have the lowest potential to damage epitopes. In contrast, proteinase K and elastase showed a tendency to cleave epitopes at many sites. This indicates a more aggressive proteolytic activity. Among the epitopes evaluated, CD133 was the most susceptible to degradation, while CD147 was the most resistant.

These results are in line with in vitro experimental data, where the number of retinal cells successfully isolated from the two retinas was highly dependent on the type and concentration of protease used. Among the tested enzymes, trypsin emerged as the most efficient protease, as reflected by both in silico and in vitro findings. Computational analysis predicted minimal cleavage sites on key epitopes such as CD73 and CD133, indicating low potential for surface marker degradation.

This prediction is consistent with the results of trypsin dissociation, which yielded the highest number of viable retinal cells (1.6×10^7 cells from two retinas) while maintaining the integrity of CD markers. These results are in agreement with previous studies, which have shown that trypsin digestion of primary cortical neurons from neonatal rats yields higher neuron numbers, larger cell bodies, longer axons, and fewer impurities compared to papain. Neurons dissociated with trypsin showed slightly higher transfection efficiency and better morphology after several days in culture 26.

In contrast, although computationally, papain enzyme appears to be less aggressive, the number of viable cells is much lower (3.3×10^5–1.0×10^6 cells). Factors that may influence this include suboptimal enzymatic conditions or incubation times in vitro [31,32]. Papain remains relevant despite its relatively low yield, as it exerts a gentle action with minimal epitope disruption. This is supported by AO/PI staining results showing that papain-digested suspensions display low agglomeration rates and reduced cellular adhesions compared to mechanical grinding or Liberase + DNase I digestion [33]. Flow cytometric analysis further confirmed that papain, similar to trypsin, facili-

tates single-cell dispersal, but without the risk of impairing antibody binding often associated with trypsin digestion. Moreover, papain has been shown in CNS dissociation studies to achieve cell viabilities above 80 % and reliable recovery of mononuclear cells [34]. From a biochemical perspective, papain is highly stable across a broad range of pH and temperature conditions [35]. Thus, although yield may initially be lower than trypsin or Liberase, papain's gentle enzymatic activity and tunable conditions (e.g., incubation time, enzyme concentration) underscore its reliability and potential for refinement in preparing fragile tissues for flow cytometry or transcriptomic analyses.

In silico, proteinase K and elastase tend to recognize multiple cleavage sites. Both enzymes produce a moderate number of cells (1.4×10^6 cells $- 3.7 \times 10^6$ cells). In other words, both enzymes cause degradation that may be excessive or non-selective.

Liberase, a mixture of collagenase and thermolysin, consistently produced the highest number of viable cells. From these results, Liberase can be selected as an effective option for retinal tissue dissociation. The addition of thermolysin can increase dissociation efficiency. However, its activity must be carefully controlled, as higher concentrations or prolonged incubation have been shown to degrade proteolysis-sensitive epitopes such as B220, CD4, and CD62L [36]. Collagenases cleave the rigid triple helix structure of collagen, which is resistant to common proteases, thereby allowing efficient release of cells from the extracellular matrix [37,38]. However, only thermolysin could be evaluated through in silico analysis, as structural data for collagenase were not available for modelling. This represents a limitation, since the role of collagenase in epitope preservation cannot yet be computationally validated, even though its enzymatic properties are well established in the literature. Taken together, the in vitro evidence strongly supports Liberase as a robust dissociation enzyme, while the combination of computational and experimental approaches underscores the importance of selecting the appropriate protease. For fluorescence-activated cell sorting (FACS), either Liberase or papain can be recommended, particularly when working with fragile retinal tissues.

The researchers hypothesized that the use of a broad-spectrum protease, such as elastase, would damage most surface proteins (epitopes), including CD markers. This condition would affect the staining results. However, immunofluorescence (IF) results showed that the intensity of the light signal produced was relatively uniform for all proteases tested. This indicates that epitope damage by proteases may not be as severe as previously thought. Furthermore, the detection method used may have limitations in distinguishing these differences. It is worth noting that confocal microscopy is qualitative and has limitations in detecting and distinguishing multiple fluorochrome labels simultaneously. In this context, the instrument cannot effectively read eight fluorochrome labels due to the limited number of excitation and emission wavelengths that can be captured simultaneously. Therefore, not all CD marker expression can be fully elucidated using this approach.

Given the cellular heterogeneity of the retina, uniform binding across all CD markers cannot be assumed. To minimize bias, our screening approach was designed to cover the entire slide area, ensuring comprehensive spatial representation. Further evidence from higher-resolution experiments is necessary to strengthen these findings. The detection capabilities of the IF microscope constrain the integrity of the epitope. The light

intensities of the fluorochromes across different protease-treated groups are indistinguishable. However, a more refined and quantitative assessment can be achieved using FACS.

This study used only murine retinas without disease induction. Further research in degenerative models such as RP could help determine the influence of disease-induced changes in the extracellular matrix or cell fragility on protease performance. Furthermore, functional assays beyond antibody detection, such as signaling or electrophysiological assays, are also needed to ensure that cells remain biologically intact.

5 Conclusion

Computational and experimental integration suggests that trypsin and Liberase are optimal for retinal dissociation. Papain can also be used with appropriate optimization to increase cell viability. Protease selection should consider dissociation efficiency and functional epitope integrity to achieve accurate cell sorting and transcriptomic profiling.

Acknowledgments. The authors want to acknowledge Gadjah Mada University for providing the RTA research grant (5286/UN1.P1/PT.01.03/2024). The authors would like to thank the Bonvin lab, the Computational Structural Biology group at Utrecht University, Heidelberglaan 8, 3584 CS Utrecht, The Netherlands, for providing the "expert" access in Haddock. The authors would like to thank the Biomic Committee for assisting with the English editing.

Disclosure of Interests. The authors have no competing interests to declare that are relevant to the content of this article.

References

1. Allan, K., Dicicco, R., Ramos, M., Asosingh, K., & Yuan, A. (2020). Preparing a Single Cell Suspension from Zebra fi sh Retinal Tissue for Flow Cytometric Cell Sorting of Müller Glia. International Society for Advancement of Cytometry, 638–646. https://doi.org/10.1002/cyto.a.23936
2. Wiesmann, U. N. (1999). Segregating Cells - Proteases in Tissue Culture (Issue 031).
3. Volovitz, I., Shapira, N., Ezer, H., Gafni, A., Lustgarten, M., Alter, T., Horin, I. Ben, Barzilai, O., Shahar, T., Kanner, A., Fried, I., Veshchev, I., Grossman, R., & Ram, Z. (2016). A non - aggressive, highly efficient, enzymatic method for dissociation of human brain - tumors and brain - tissues to viable single - cells. BMC Neuroscience, 1–10. https://doi.org/10.1186/s12868-016-0262-y
4. Adam, M., Potter, A. S., & Potter, S. S. (2017). Psychrophilic proteases dramatically reduce single-cell RNA-seq artifacts: a molecular atlas of kidney development. Development, 1, 3625–3632. https://doi.org/10.1242/dev.151142
5. Denisenko, E., Guo, B. B., Jones, M., Hou, R., Kock, L. De, Lassmann, T., Poppe, D., Clément, O., Simmons, R. K., Lister, R., & Forrest, A. R. R. (2020). Systematic assessment of tissue dissociation and storage biases in single- cell and single-nucleus RNA-seq workflows. Genome Biology, 21, 1–25.

6. Kong, Y., Naggert, ürgen K., & Nishina, P. M. (2018). The Impact of Adherens and Tight Junctions on Physiological Function and Pathological Changes in the Retina. Advances in Experimental Medicine and Biology. https://doi.org/10.1007/978-3-319-75402-4_66
7. Adil, M. S., Narayanan, S. P., & Somanath, P. R. (2021). Cell-cell junctions: structure and regulation in physiology and pathology. Tissue Barriers, 9(1). https://doi.org/10.1080/21688370.2020.1848212
8. Naylor, A., Hopkins, A., Hudson, N., & Campbell, M. (2020). Tight junctions of the outer blood retina barrier. International Journal of Molecular Sciences, 21(1). https://doi.org/10.3390/ijms21010211
9. Benjamin R. Fadl, Seth A. Brodie, Michael Malasky, Joseph F. Boland, Michael C. Kelly, Matthew W. Kelley, Erich Boger, Robert Fariss, Anand Swaroop, & Laura Campello. (2020). An optimized protocol for retina single-cell RNA sequencing. Molecular Vision, 26(July), 705–717.
10. Chen, Y., Dong, Y., Yan, J., Wang, L., Yu, S., Jiao, K., & Paquet-Durand, F. (2022). Single-Cell Transcriptomic Profiling in Inherited Retinal Degeneration Reveals Distinct Metabolic Pathways in Rod and Cone Photoreceptors. International Journal of Molecular Sciences, 23(20). https://doi.org/10.3390/ijms232012170
11. Voigt, V., Andoniou, C. E., Schuster, I. S., Oszmiana, A., Ong, M. L., Fleming, P., Forrester, J. V, & Degli-esposti, M. A. (2018). Cytomegalovirus establishes a latent reservoir and triggers long-lasting inflammation in the eye. PLOS Pathogens, 14(5), 1–23. https://doi.org/https://doi.org/10.1371/journal.ppat.1007040
12. Samardzija, M., Corna, A., Mohamed, R. G., Jarboui, A., Armento, A., Roger, J. E., Petridou, E., Haq, W., Eberhart, F. P., De, P., Günther, V., Christian, Z., Patricia, G., & Marius, B. (2021). HDAC inhibition ameliorates cone survival in retinitis pigmentosa mice. Cell Death & Differentiation, 28, 1317–1332. https://doi.org/10.1038/s41418-020-00653-3
13. Chavkin, N. W., Walsh, K., & Hirschi, K. K. (2022). Isolation of Highly Purified and Viable Retinal Endothelial Cells. Journal of Vascular Research, 58(1), 49–57. https://doi.org/10.1159/000510533
14. Feodorova, Y., Koch, M., Bultman, S., Michalakis, S., & Solovei, I. (2015). Quick and reliable method for retina dissociation and separation of rod photoreceptor perikarya from adult mice. MethodsX, 2, 39–46. https://doi.org/10.1016/j.mex.2015.01.002
15. Koso, H., Minami, C., Tabata, Y., Inoue, M., Sasaki, E., Satoh, S., & Watanabe, S. (2009). CD73, a novel cell surface antigen that characterizes retinal photoreceptor precursor cells. Investigative Ophthalmology and Visual Science, 50(11), 5411–5418. https://doi.org/10.1167/iovs.08-3246
16. Lakowski, J., Welby, E., Budinger, D., Di Marco, F., Di Foggia, V., Bainbridge, J. W. B., Wallace, K., Gamm, D. M., Ali, R. R., & Sowden, J. C. (2018). Isolation of Human Photoreceptor Precursors via a Cell Surface Marker Panel from Stem Cell-Derived Retinal Organoids and Fetal Retinae. Stem Cells, 36(5), 709–722. https://doi.org/10.1002/stem.2775
17. Welby, E., Lakowski, J., Di Foggia, V., Budinger, D., Gonzalez-Cordero, A., Lun, A. T. L., Epstein, M., Patel, A., Cuevas, E., Kruczek, K., Naeem, A., Minneci, F., Hubank, M., Jones, D. T., Marioni, J. C., Ali, R. R., & Sowden, J. C. (2017). Isolation and Comparative Transcriptome Analysis of Human Fetal and iPSC-Derived Cone Photoreceptor Cells. Stem Cell Reports, 9(6), 1898–1915. https://doi.org/10.1016/j.stemcr.2017.10.018
18. Shinoe, T., Kuribayashi, H., Saya, H., Seiki, M., Aburatani, H., & Watanabe, S. (2010). Identification of CD44 as a cell surface marker for Müller glia precursor cells. Journal of Neurochemistry, 115(6), 1633–1642. https://doi.org/https://doi.org/10.1111/j.1471-4159.2010.07072.x

19. Hyono, A., Mazda, T., Okazaki, H., Tadokoro, K., & Ohshima, H. (2008). Analysis of enzyme-treated red blood cell surface and haemagglutination using a theory of soft particle electrophoresis. Vox Sanguinis, 95(2), 131–136. https://doi.org/10.1111/j.1423-0410.2008.01070.x
20. Kwan, D. H., Constantinescu, I., Chapanian, R., Higgins, M. A., Kötzler, M. P., Samain, E., Boraston, A. B., Kizhakkedathu, J. N., & Withers, S. G. (2015). Toward efficient enzymes for the generation of universal blood through structure-guided directed evolution. Journal of the American Chemical Society, 137(17), 5695–5705. https://doi.org/10.1021/ja5116088
21. Bondonese, A., Craig, A., Fan, L., Valenzi, E., Bain, W., Lafyatis, R., Sembrat, J., Chen, K., & Snyder, M. E. (2023). Impact of enzymatic digestion on single cell suspension yield from peripheral human lung tissue. Cytometry, 103(10), 777–785. https://doi.org/10.1002/cyto.a.24777
22. Autengruber, A., Gereke, M., Hansen, G., Hennig, C., & Bruder, D. (2012). Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function. European Journal of Microbiology and Immunology, 2(2), 112–120. https://doi.org/10.1556/eujmi.2.2012.2.3
23. Fritschle, K., Mielke, M., Seelbach, O. J., Mühlthaler, U., Živanić, M., Bozoglu, T., Dötsch, S., Warmuth, L., Busch, D. H., Skerra, A., Kupatt, C., Weber, W. A., Randall, R. E., Steiger, K., & Morath, V. (2025). The V5-Epitope Tag for Cell Engineering and Its Use in Immunohistochemistry and Quantitative Flow Cytometry. Biology, 14(7), 1–20. https://doi.org/10.3390/biology14070890
24. Nowak-Terpiłowska, A., Śledziński, P., & Zeyland, J. (2021). Impact of cell harvesting methods on detection of cell surface proteins and apoptotic markers. Brazilian Journal of Medical and Biological Research, 54(2), 1–7. https://doi.org/10.1590/1414-431X202010197
25. Abraham, M. J., Murtola, T., Schulz, R., Páll, S., Smith, J. C., Hess, B., & Lindah, E. (2015). Gromacs: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. SoftwareX, 1–2, 19–25. https://doi.org/10.1016/j.softx.2015.06.001
26. De Vries, S. J., Van Dijk, M., & Bonvin, A. M. J. J. (2010). The HADDOCK web server for data-driven biomolecular docking. Nature Protocols, 5(5), 883–897. https://doi.org/10.1038/nprot.2010.32
27. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2005). Protein Identification and Analysis Tools on the ExPASy Server. In (eds) The Proteomics Protocols Handbook. Springer Protocols Handbooks. Humana Press. https://doi.org/https://doi.org/10.1385/1-59259-890-0:571
28. Honorato, R. V., Koukos, P. I., Jiménez-García, B., Tsaregorodtsev, A., Verlato, M., Giachetti, A., Rosato, A., & Bonvin, A. M. J. J. (2021). Structural Biology in the Clouds: The WeNMR-EOSC Ecosystem. Frontiers in Molecular Biosciences, 8(July), 1–7. https://doi.org/10.3389/fmolb.2021.729513
29. Maillet, N. (2020). Rapid Peptides Generator: fast and efficient in silico protein digestion. NAR Genomics and Bioinformatics, 2(1), 1–10. https://doi.org/10.1093/nargab/lqz004
30. Hu, C. Y., Du, R. L., Xiao, Q. X., & Geng, M. J. (2022). Differences between cultured cortical neurons by trypsin and papain digestion. Ibrain, 8(1), 93–99. https://doi.org/10.1002/ibra.12028
31. Heyman, E., Devriendt, B., De Vlieghere, E., Goethals, K., Van Poucke, M., Peelman, L., & De Schauwer, C. (2024). Evaluation of enzymatic protocols to optimize efficiency of bovine adipose tissue-derived mesenchymal stromal cell isolation. Npj Science of Food, 8(1), 1–10. https://doi.org/10.1038/s41538-024-00313-7

32. Shcherbakova, A., Utkina, M., Valyaeva, A., Pachuashvili, N., Bondarenko, E., Urusova, L., Popov, S., & Mokrysheva, N. (2024). Factors Affecting Cell Viability during the Enzymatic Dissociation of Human Endocrine Tumor Tissues. Biology, 13(9), 1–11. https://doi.org/10.3390/biology13090665
33. Wang, Q., Zhu, X., Zhang, X., Xia, Y., & Tian, X. (2025). Comparison of methods for the preparation of single-cell suspensions of rat retina and immunophenotyping by flow cytometry. Journal of Neuroscience Methods, 417(November 2024), 110384. https://doi.org/10.1016/j.jneumeth.2025.110384
34. Hussain, R. Z., Miller-Little, W. A., Doelger, R., Cutter, G. R., Loof, N., Cravens, P. D., & Stüve, O. (2018). Defining standard enzymatic dissociation methods for individual brains and spinal cords in EAE. Neurology: Neuroimmunology and NeuroInflammation, 5(2), 1–10. https://doi.org/10.1212/NXI.0000000000000437
35. Babalola, A. B., Ifeolu Akinwande, A., Otunba, A. A., Ebenezer Adebami, G., Babalola, O., & Nwuofo, C. (2024). Therapeutic benefits of Carica papaya: A review on its pharmacological activities and characterization of papain. Arabian Journal of Chemistry, 17(1), 105369. https://doi.org/10.1016/j.arabjc.2023.105369
36. Park, J. Y., Chung, H., Choi, Y., & Park, J. H. (2017). Phenotype and tissue residency of lymphocytes in the murine oral mucosa. Frontiers in Immunology, 8(MAR), 1–10. https://doi.org/10.3389/fimmu.2017.00250
37. Bhagwat, P. K., & Dandge, P. B. (2018). Collagen and collagenolytic proteases: A review. Biocatalysis and Agricultural Biotechnology, 15(May), 43–55. https://doi.org/10.1016/j.bcab.2018.05.005
38. Yamada, E., Dominick, K., Kulchar, R. J., Twohig, J., Khavandgar, Z., Beach, M., Pelayo, E., Baer, A., Perez, P., & Warner, B. M. (2025). Multiparameter flow cytometry enables immune profiling and IFN pathway analysis in human minor salivary glands. Frontiers in Dental Medicine, 6(June), 1–13. https://doi.org/10.3389/fdmed.2025.1590516