Research Academy for Young Scientists

Validating Candidate Cell Surface Markers for Purification of hESC-derived Photoreceptor Progenitors

Aleksander Purik
aleksander.purik@gmail.com

under the direction of
Sarah Saietz, PhD student

Karolinska Institutet,
Department of Clinical Science,
Intervention and Technology,
Division of Obstetrics and Gynecology

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Abstract

Stem cell-based photoreceptor therapies may be a possible method of combating blindness caused by retinal degenerative diseases. In this paper, flow cytometry and fluorescent microscopy were used to evaluate previously identified candidate surface markers for negative sorting. It was shown that cryopreservation of photoreceptor progenitor cells is biased towards which cell types are viable after thawing. The expression of candidate surface markers was different in pre- and post-cryo populations, suggesting that different markers have to be chosen depending on whether cells are sorted before or after cryopreservation. In addition, the expression of *NEUROD1* was lower post-cryo, suggesting that photoreceptor progenitors have low tolerance to cryopreservation.

Acknowledgements

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Contents

List of Abbreviations

AMD Age-related Macular Degeneration.

CM Candidate Marker.

FACS Fluorescense-activated Cell Sorting.

hESC Human Embryonic Stem Cells.

IF Immunofluorescence.

MACS Magnetic-activated Cell Sorting.

PhR Photoreceptor.

RPE Retinal Pigment Epithelium.

1 Introduction

Photoreceptors (PhRs) are photosensitive neurons found in the human eye. PhR cells play a central role in the visual transduction cascade, in which light is converted into biological signals that can be interpreted by the brain [1]. When PhR cells become damaged due to retinal degenerative diseases, phototransduction may be disrupted in some areas of the retina, leading to loss of vision [2]. PhRs, like other mammalian neural cells, cannot regenerate [3]; therefore, vision loss due to PhR degeneration in an adult is permanent and as yet untreatable. However, stem cell-based therapies hold the potential to regenerate retinal tissues and improve vision even in patients with a late-stage retinal degenerative disease [4].

1.1 Age-related Macular Degeneration

Age-related macular degeneration (AMD), a retinal degenerative disease, is one of the leading causes of blindness in people over 60 years old [2]. In AMD, PhRs are lost as

retinal pigment epithelial (RPE) cells degenerate. This is because RPE cells, which are directly in contact with PhR cells (see Figure 1), play an important homeostatic role, which involves nourishing the PhR layer [2]. When PhRs degenerate within the macula — the region responsible for the sharp vision in the center of the visual field — all tasks involving perception become difficult due to a large central scotoma, or blind-spot.

1.1.1 Possible Therapies

Currently, there are multiple AMD therapies in clinical trial, some of which involve cell or gene therapies [4]. The most studied stem cell therapy for AMD is RPE transplantation [5]. Injecting healthy RPE cells in a degenerating retina may rescue PhRs which would otherwise die [4]. However, in late-stage AMD, replacing RPE cells is not effective, as too many PhRs have already been lost. Therefore, PhR transplantation may have the potential to treat even late-stage AMD. Currently, PhR transplantation is understood to aid the retina in two ways: (1) Donor PhR cells can form synaptic connections with other neurons within the retina and take the place of degenerated host PhR cells, thus

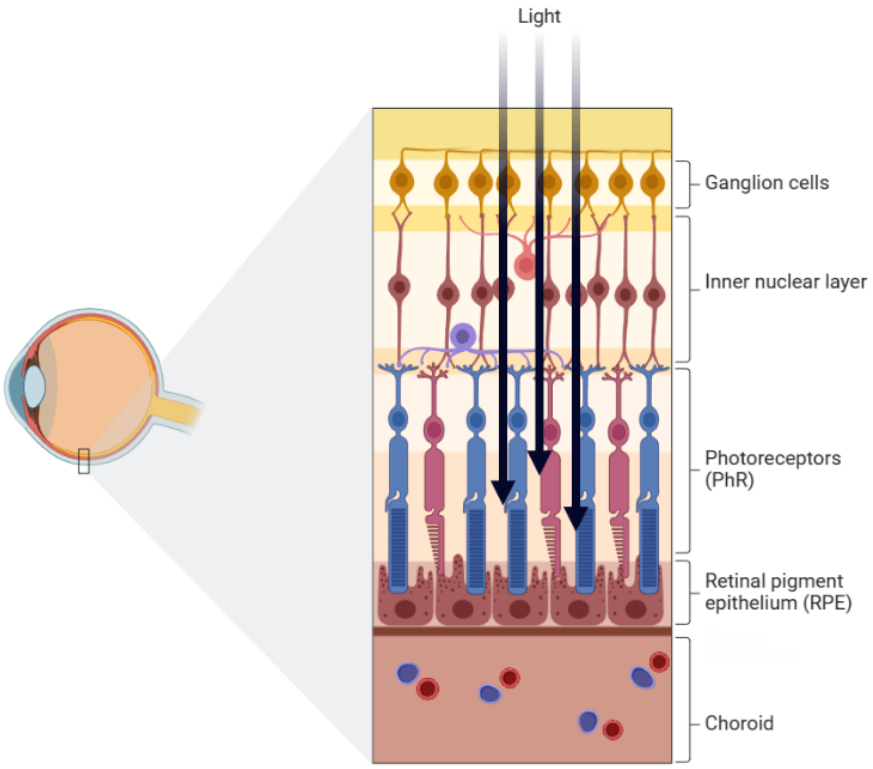

Figure 1: Layers of the retina. Created using Biorender.com.

allowing for phototransduction [4]; and (2) donor PhR cells can transfer various molecules and even whole organelles to host PhRs, which may promote survival of host PhRs [6]. PhR progenitors — cells that will become PhRs — can be transplanted instead of mature PhRs, as PhR progenitors have been shown to mature while in the host retina [7].

1.2 Stem cells

Stem cells are undifferentiated cells capable of self-renewal [8]. Human embryonic stem cells (hESCs) are obtained from the inner cell mass of a blastocyst, before the mass becomes an embryo. hESCs are pluripotent, which means that they can become any cell in the human body [8]. Stem cells can be differentiated to become specialized cells via the addition of growth factors in the medium in which they grow. Therefore, stem cells could be used to derive healthy RPE or PhR progenitor cells *in vitro* which may be transplanted into patients with AMD (Figure 2).

1.2.1 Culture

Many protocols have been established for culturing PhRs, with varying culture time and purity of final product [9]. In many cases, culturing PhRs involves generation of organ-like structures called organoids. Retinal organoids are 3D structures containing eye-related cells similar to optic vesicles in a developing embryo [10]. Organoid formation is induced by culturing cells in a suspension, also called 3D culture. However, the isolation of retinal organoids and the final desired product is a manual process that can take over 200 days [9]. If in place of suspended culture, the cells are grown using adherent, or 2D, culture, a more streamlined procedure and smaller batch-to-batch variation could be possible. In adherent culture, the cells are attached to a plate instead of forming complex 3D structures (see Figure 2). A strength of adherent culture is that it relies more on artificially added (exogenous) growth factors, rather than internal (endogenous) signalling between the cells within the organoid's 3D structure [11]. In adherent culture, most cells are exposed to the same growth factors, whereas in 3D culture, cells secrete growth factors which affect only neighboring cells [12]. Since cells are exposed to similar growth factors in 2D culture, the

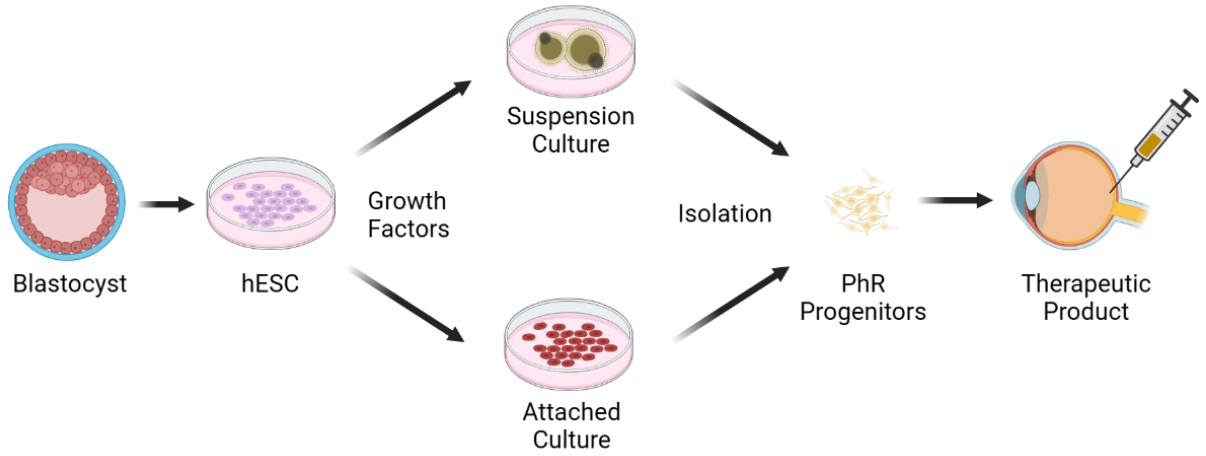

Figure 2: Stem cell-based therapy for degenerative retinopathy. Growth factors can be added to promote a PhR progenitor fate within an attached culture, or an organoid-based protocol may be used derive PhR progenitors.

result is a more homogeneous population of cells, requiring fewer cells to be sorted out before a pure final product is achieved. This paper is concerned only with 2D PhR culture and is a part of the in-house research aimed towards developing an effective and reliable protocol for PhR culture.

1.2.2 Preservation

It is desirable to maintain a stem cell line as long as possible; by using the same, genetically identical cells every time, consistent experimental results and replicability may be achieved [13]. This can be achieved by submerging cells in liquid nitrogen in a process called cryopreservation, which enables the storage of cells for a long period of time while stalling their further development is [14]. Cryopreservation can be used to store a large number of cells for potential future use while minimizing the risk of mutations and the cost of maintenance [13]. Cryopreservation is also desirable when going into clinical trial; when a candidate for transplantation is found, cells can be thawed at short notice [15].

1.3 Introduction to Tools

1.3.1 Immunostaining

By exposing cells to antibodies that target specific proteins, cells that express a particular gene can be marked. When the antibodies are conjugated to a fluorophore, cells that are marked may emit light when exited by light at a specific wavelength. In flow cytometry and fluorescent microscopy — two techniques used in this study — lasers at a specific wavelength are used to excite the fluorophores, and the intensity of emitted light can be recorded and used to draw conclusions about the cell population [16]. If the protein to be stained is intracellular, the antibody must enter the cell, which is made possible by permeabilizing the cell membrane. However, as the permeabilizing process results in cell death, intracellular staining cannot be utilized in a protocol whose goal is to derive a product suitable for transplantation. Staining a protein which is expressed on the surface of the cell, however, can be done without affecting the vitality of a cell. Surface proteins are therefore relevant when developing a protocol which is meant to deliver a therapeutic cell product.

1.3.2 Immunofluorescence

Staining may be achieved out in one of two ways: either direct or indirect immunofluorescence (IF) may be used. In direct IF, antibodies that are directly conjugated fluorophores are used. When these antibodies bind to their targets, they can be detected immediately. In indirect IF, antibodies without fluorophores — primary antibodies — are used, which bind to their targets, but cannot be detected. Secondary antibodies, which bind to primary antibodies and are conjugated to a fluorophore, must be added. The advantage of direct IF is using one directly conjugated antibody, which leads to less unspecific binding. The main advantage of indirect IF is its ability to generate a stronger signal when multiple fluorophore-conjugated secondary antibodies attach to a primary antibody [17]. Both direct and indirect IF are used for immunostaining in this paper.

1.3.3 Fluorescent Microscopy

Fluorescent microscopy can be used to image cell cultures marked with fluorophores. In contrast to cytometry, which is strictly quantitative, fluorescent microscopy can provide qualitative data about the cells and the targeted markers. In this paper, fluorescent microscopy is used to verify that the surface markers targeted are indeed expressed on the surface of the cells. In combination with bright-field microscopy, fluorescent microscopy also allows for the verification of morphology of surface-marker positive cells. For example, if a certain marker is often expressed by cells with a neuron-like morphology, it may be assumed not to be a good marker for a negative sort.

1.3.4 Cell Sorting

To filter out other retina-related, non-PhR cells which might be present in the culture, two methods are available: (1) positive sorting — cells expressing a certain gene can be selected and other unwanted cells discarded; and (2) negative sorting — cells expressing a specific gene can be discarded. Magnetic- or fluorescence-activated cell sorting (MACS and FACS, respectively) can be used to this end. FACS operates in a similar way to flow cytometry, where lasers are used to identify cells with fluorescent labels and separate them from the population. MACS works by attaching antibodies to magnetic particles, which bind to cells; these cells can then be separated out by a magnetic field [18].

Often, it is desirable to remove antibodies that have not attached after an incubation period — if free fluorescent antibodies are not removed prior to fluorescent microscopy, emissions from these antibodies will be visible in the images and make it difficult to interpret the data. To remove unattached antibodies, the cells suspension is centrifuged, which results in the formation of a solid layer at the bottom of the vial — called a pellet — and the formation of a liquid layer on top — called the supernatant. In a process called washing, the liquid layer, containing light particles such as antibodies, is removed by suction, also called aspiration.

1.3.5 Stains Used

In this paper, the proteins NEUROD1 and OTX2 are immunostained. The gene *NEUROD1* codes for NEUROD1 and is expressed by adult PhRs within the retina [19]. In flow cytometry, checking whether a surface marker is positive when genes characteristic for the cell-type are positive allows for the discovery of characteristic surface markers. *OTX2* is a gene expressed by multiple types of retinal cells, including PhR progenitors [20], and coding for the protein OTX2. The combination of NEUROD1 and OTX2 makes it easier to identify PhR progenitors, as a NEUROD1/OTX2 double-positive cell can more confidently be assumed to be a PhR progenitor.

1.4 Aim

This paper connects to research which aims to use hESC-derived PhRs cultured in a Good Manufacturing Practice compliant way for transplantation in retinopathic patients. This paper is concerned with the purification step of PhR culture; the aim of this paper is to find novel surface markers, expressed by retinal cells other than PhR progenitors, to be used in negative sorting.

In this paper, ten unique surface markers, which were identified in a previous in-house surface marker screening, are validated to find candidates for cell sorting. Due to pending patents, the surface markers are not disclosed in this paper. Instead, they will henceforth be referred to as 'candidate markers' (CM1–10, or simply CM).

2 Method

2.1 Stem Cell Culture

Human embryonic stem cells (KARO1) were differentiated towards a PhR fate using an in-house protocol. Approximately 27 million cells were cryopreserved in liquid nitrogen for several weeks prior to staining.

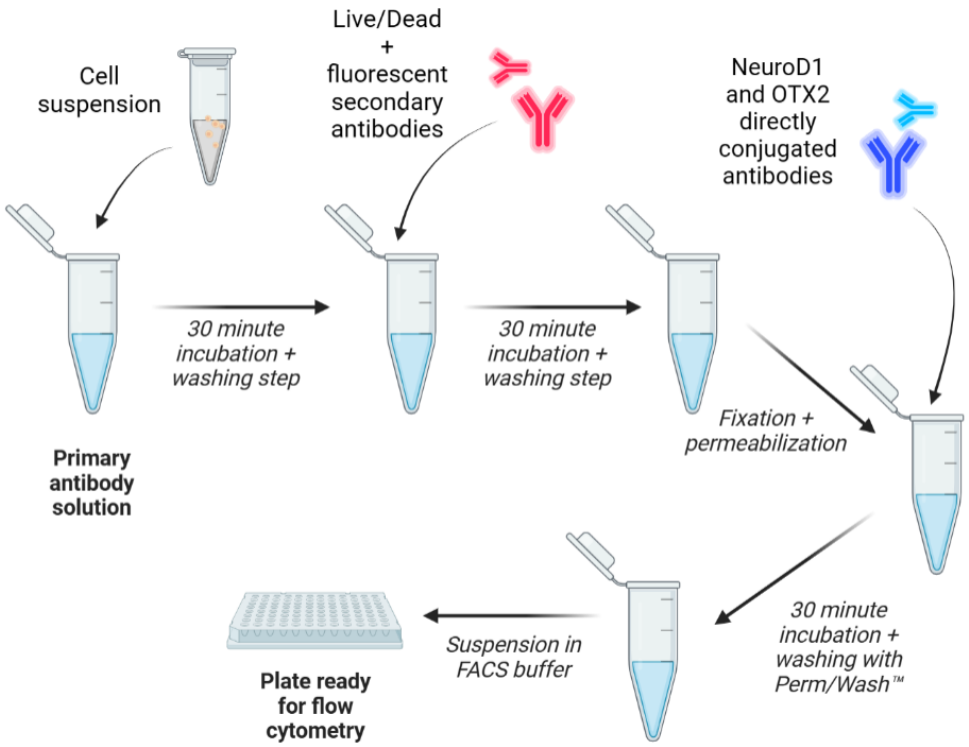

Figure 3: Schematic illustration of method used to prepare cells for flow cytometry after cells have been washed and thawed. This procedure was done for each CM in a separate vial. The final plate contained all 10 CM stains plus a stem cell control.

2.2 Flow Cytometry

In order to analyze the cell cultures, cells were stained both intra- and extracellularly. Three vials of 0.5 million KARO1 stem cells and 9 vials of 2 million differentiated cells were rapidly thawed by submersion in a 37 °C water bath. Both stem cells and the differentiated cells were placed in 15 mL tubes and PBS was added to both tubes. The solutions were centrifuged (300 RCF for 3 min) and the supernatant was aspirated. Washing with PBS was done with the purpose of removing any cytotoxic cryoprotective agents present in the solution. Both the differentiated cell pellet and the stem cell pellet were resuspended in PBS.

2.2.1 Surface Staining

20 µL of each CM primary antibody suspension were selected from BD Lyoplate™ Human Cell Surface Marker Screening Panel and placed into brown 2 mL reaction tubes. 100 µL of differentiated cell solution was added into each tube containing antibodies (see

Figure 3, first step). 100 µL of stem cell solution was placed into a tube without antibodies. The tubes were incubated for 30 minutes at 4 °C. Tubes were thereafter washed twice with 1-2 mL PBS. The secondary antibody was added, and all tubes were stained with LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (ThermoFisher). The cells were incubated for 30 minutes at 4 °C and thereafter washed twice with PBS.

2.2.2 Intracellular Staining

BD Pharmingen™ Transcription Factor Buffer Set was used for intracellular staining. The cells were fixed and permeabilized according to manufacturer's instructions. BD Pharmingen™ PE Mouse Anti-NeuroD1 and Human Otx2 Alexa Fluor® (Novum/bio-techne) antibodies with directly conjugated fluorophores were added to the tubes, which were incubated for 30 min at 4 °C. After incubation, the tubes were twice washed with BD Perm/Wash™. All vials were centrifuged at 500 RCF for 5 minutes; the supernatant was aspirated and the cells resuspended in 150 µL of FACS buffer (in-house). The suspensions were placed in a 96-well V-bottom plate and stored at 4 °C overnight. The following day, CytoFLEX S (Beckman Coulter) was used for flow cytometry. FlowJo v10 was used to analyze the cytometric data.

2.3 Fluorescent Microscopy

An 8-well chamber slide was coated with human recombinant laminin-521 (usage 1:10; BioLamina) in DPBS 1X (Hyclone/VWR) and stored overnight at 4 °C. The following day, the chamber slide was incubated for 1 hour at 37 °C to allow the laminin to polymerise. Two vials of 2 million differentiated cells were thawed in a water bath (37 °C). The cells were placed in a 15 mL vial and washed once with NutriStem® hPSC XF GF-free and then resuspended in NutriStem®. The chamber slide was removed from the incubator and the cells were plated into the four central wells (Figure 4, first step). The cells were cultured in a normoxia incubator for 6 days; the medium was changed every 48 hours.

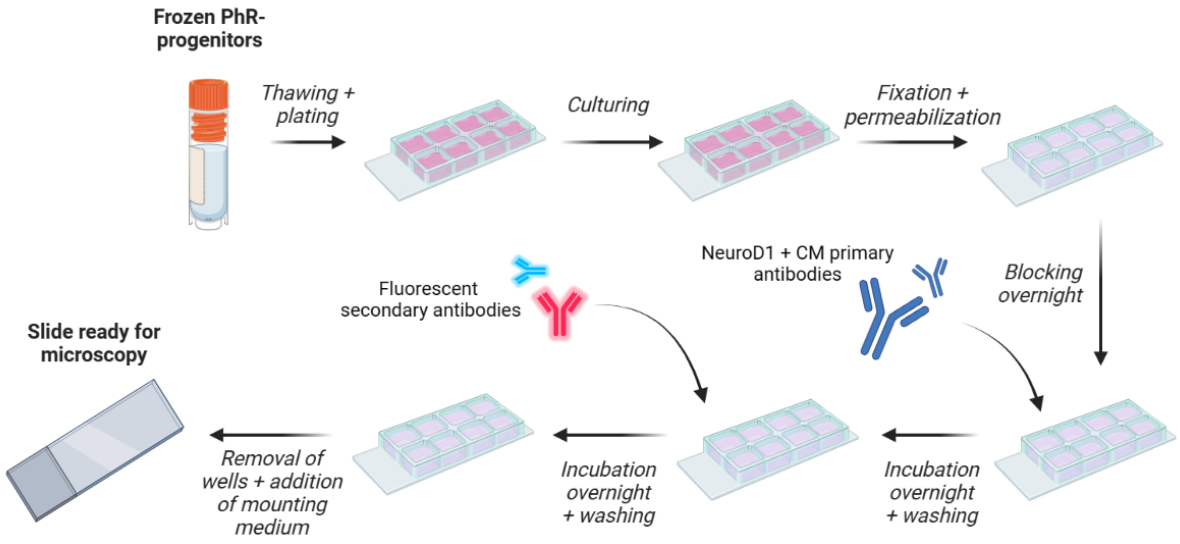

Figure 4: Schematic illustration of method used to prepare cells for fluorescent microscopy. Four central wells on the chamber slide were filled with cells; each well was stained with one CM.

2.3.1 Immunostaining

After 6 days, the cells were removed from the incubator and 4% paraformaldehyde (ThermoFisher Scientific) in PBS was added. The cells were incubated for 15 minutes at 4 °C; thereafter washed with PBS. Cells were permeabilized using 0.3% Triton™ X-100 in PBS for 10 min at 4 °C. Cells were washed and incubated in a blocking buffer (0.1% TWEEN® 20; 5% horse serum in PBS) overnight. The following day, primary antibodies, CM1, CM5, CM7, CM9; one type per well, and human/mouse NeuroD1 (Biotechne; all 4 wells) were added; the cells were incubated overnight at 4 °C. All wells were washed with blocking buffer and secondary antibodies were added. Cells were incubated overnight at 4 °C; thereafter washed twice with blocking buffer and twice in PBS. Mounting medium (with DAPI) was added after the wells were removed. The slide dried at room temperature for several hours. Fluorescent microscopy was carried out using the confocal microscope 'Tweety' at Karolinska Live Cell Imaging core facility in Flemingsberg. Images were analyzed using ImageJ.

3 Results

Cell cultures containing PhR progenitors were thawed and immunostained. Previous cytometric data on the same batch of PhR progenitor cells (technical replicate) obtained before cyropreserving the cells is also included in this section.

3.1 Flow Cytometric Data

Flow cytometric data was used to determine the amount of cells positive for each CM; a summary can be seen in Figure 5. Levels of gene expression were calculated relative to a stem cell control in a process called gating (Figure 7.2). When looking at OTX2 against NEUROD1, a distinct population of more NEUROD1 positive cells is found in pre-cryo. In post-cryo, a distinct population of OTX2 positive but NEUROD1 negative cells is found in all marker screenings (Figure 6). Post-cryo cells were on average less often positive for NEUROD1 and had more debris (Table 2; Figure 7.1).

CM1, CM8, CM9, and CM10 were the least double-positive markers for a pre-cryo population — 2.82%, 2.67%, 2.30%, 1.14%, respectively (next best is 3.96%). Double positivity is determined by calculating how many of the cells to be filtered out by negatively sorting for the CM would also be NEUROD1 positive. For post-cryo cells, CM5, CM7, and CM10 were least double-positive markers (9.27%, 11.6%, 12.3%, next best is 15.3%). Data for each CM is found in Table 1.

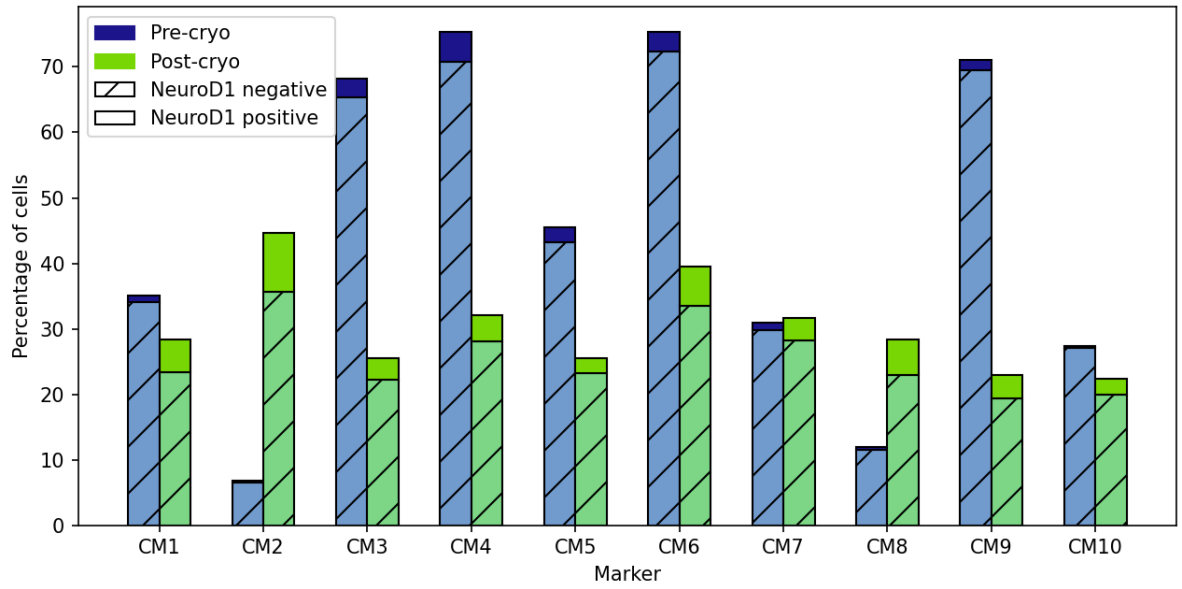

Figure 5: Comparison of candidate markers (CM) both before cryopreservation and after cryopreservation. The total height (striped + solid) corresponds to total percentage of cells expressing a specific surface marker.

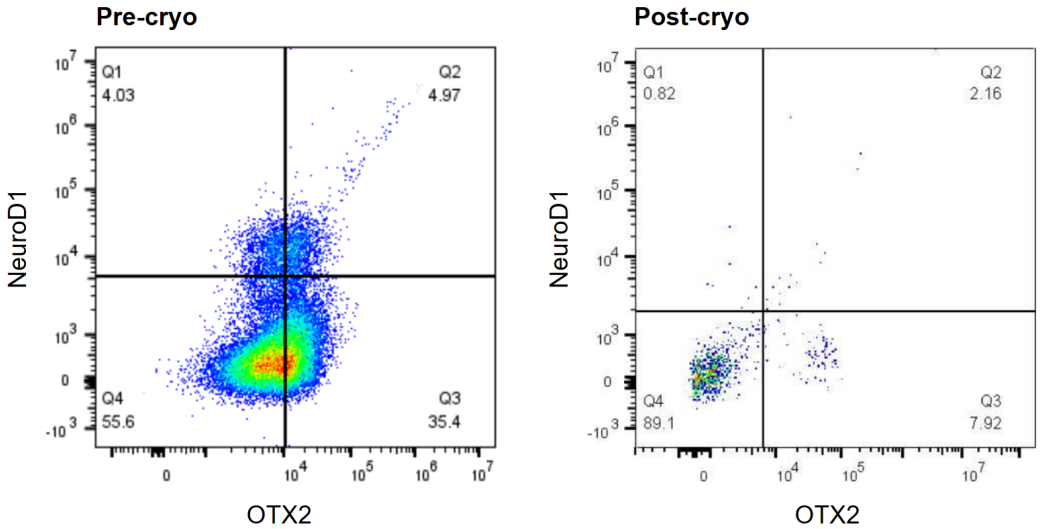

Figure 6: OTX2 and NEUROD1 pre- and post-cryo (CM5 stained). One datapoint represents one cell analyzed by the cytometer; color signifies point density. Two black bars represent thresholds for being positive/negative. Two distinct populations of cells can be identified in both plots. The location of these populations was consistent in every screening.

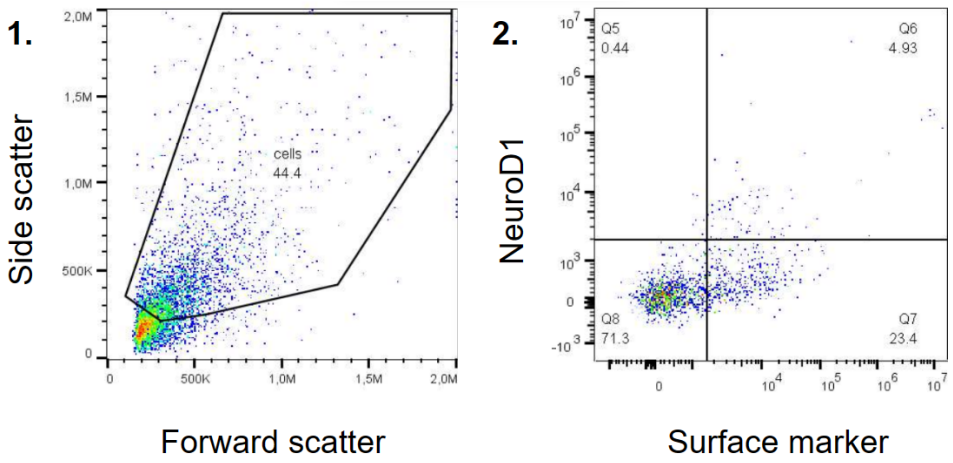

Figure 7: Flow cytometric data for CM1 post-cryo. In subfigure 1, one colored datapoint represents one particle analyzed by the cytometer. Particles can be cells, parts of cells, or debris. In subfigure 2, one datapoint is one cell. Subfigure 1 illustrates how gating is made to exclude debris based on forward scatter and side scatter. Subfigure 2 illustrates the relationship between CM1 and NEUROD1 in the population stained with CM1.

Table 1: Measure of double-positivity for various CMs. A cell is double-positive when it is both NEUROD1 positive and CM positive.

Marker	Double-positive pre-cryo (%)	Double-positive post-cryo (%)
CM1	2.82	21.1
CM2	5.70	24.9
CM3	4.24	15.3
CM4	6.45	14.0
CM5	5.30	9.27
CM6	4.07	18.1
CM7	3.96	11.6
CM8	2.67	23.8
CM9	2.30	18.0
CM10	1.14	12.3

Table 2: A comparison of total percentage of NEUROD1 positive cells and percentage of particles identified as cells in both pre- and post-cryo cultures.

	Condition	Mean %	Standard Deviation
Cells	Pre-cryo	68.5	5.57
	Post-cryo	43.2	2.89
NEUROD1+	Pre-cryo	7.03	3.00
	Post-cryo	4.81	1.95

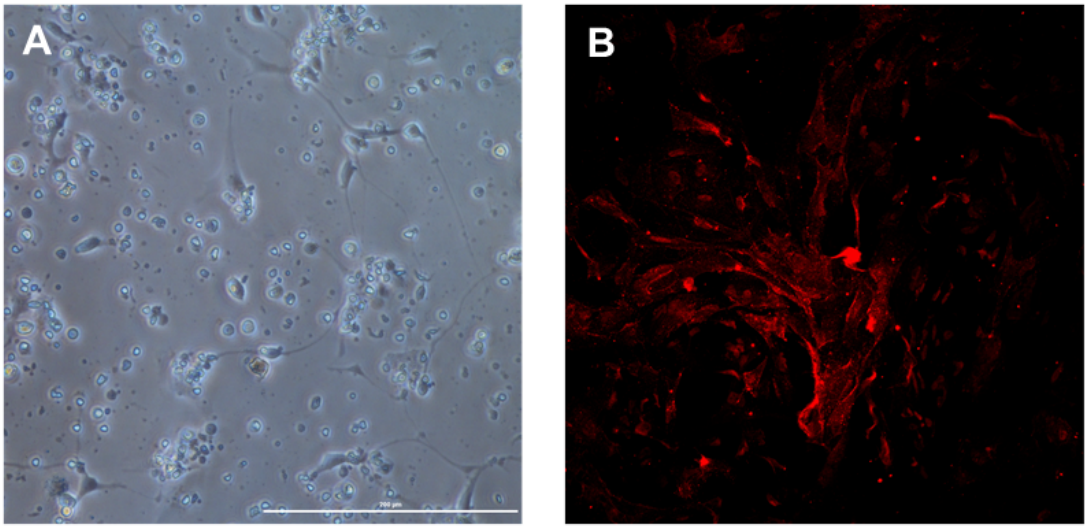

Figure 8: A) Bright-field image of attached PhR culture one day after thawing and plating. Neural projections from PhR progenitors can be seen. Scale bar = 500 µm. B) Fluorescent microscopy illustrating the affinity of CM5 for fibroblast-like cell surfaces.

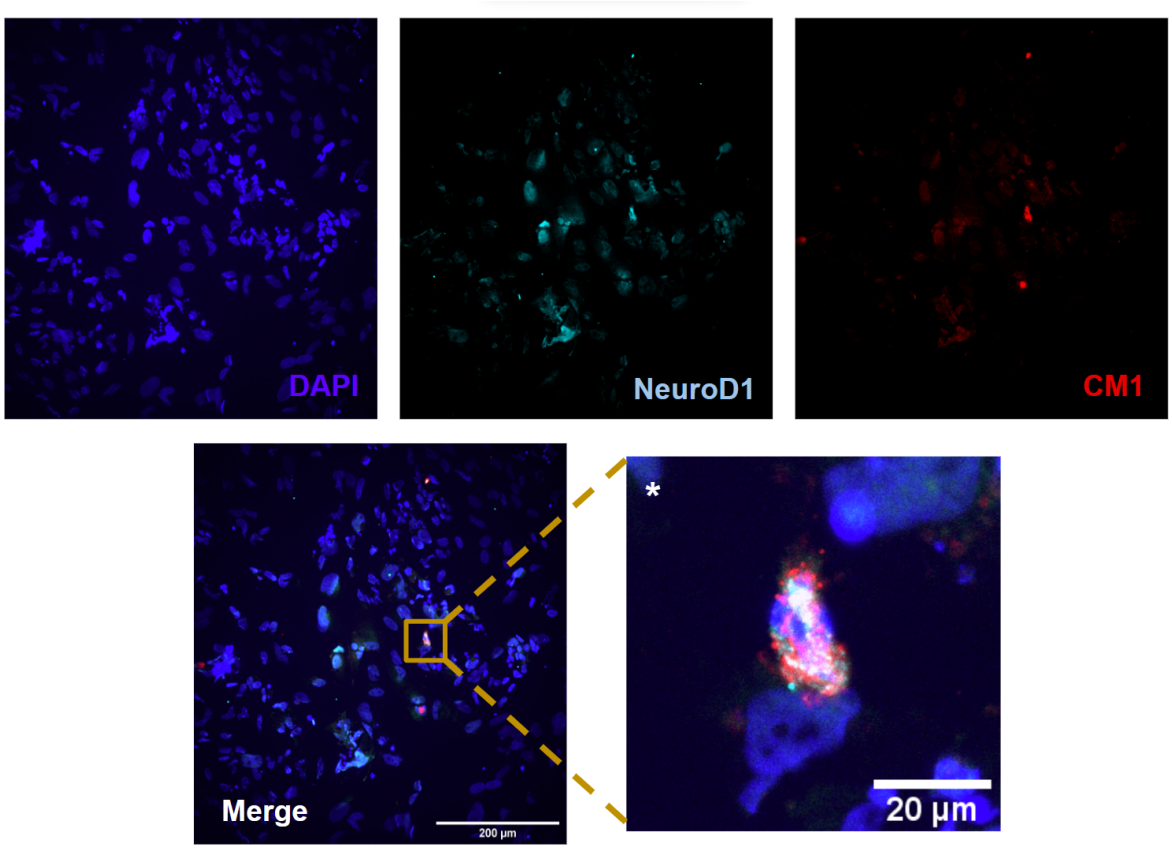

Figure 9: Fluorescent staining showing DNA (DAPI) in blue, NEUROD1 in cyan, and CM1 in red. Image marked with * is an example of a CM being expressed on the surface of a cell.

3.2 Microscopic Images

It can be seen that some neuron-like cells survived cryopreservation and attached to the plate (Figure 8A). Dead cells, characterized by their small round morphology, and other debris can also be observed. Moreover, many cells with fibroblast-like morphologies are visible. When stained with CM5, confocal microscopy reveals that CM5 has affinity for the surfaces of these non-PhR cells (Figure 8B). CM1 can be seen expressed on the surface of a cell (Figure 9).

4 Discussion

Based on the results obtained in this paper, several aspects of PhR culturing with the aim of transplantation will be discussed; namely, cryopreservation of PhR progenitors; the timepoint at which sorting occurs; and choice of surface markers to stain for.

4.1 Desirable Markers

When selecting markers for negative sorting, a number of things has to be considered: (1) whether there is a large percentage of double-positives; and (2) what total percentage of cells expresses this marker. For example, sorting out CM10 would minimize the chance of sorting out NEUROD1 positive cells (Figure 5), while CM4 would mean removing a considerable number of cells, especially when considering that mean *NEUROD1* expression was recorded to be 7.03%. For pre-cryo cells, CM9 is a promising marker which is widely expressed and negative sorting may involve removing only a small fraction of NEUROD1 positive cells. However, post-cryo, CM9 becomes less promising as a marker. This suggests that different markers may be selected depending on whether sorting is done pre- or post-cryo.

4.2 Effect of Cryopreservation

Cryopreservation may introduce a bias in the cell population, as some cell types are more likely to survive cryopreservation than others. In theory, cryopreservation could be used

as a filtering step, where cells that are desirable happen to be tolerant to cryopreservation, and therefore make up a greater percentage of the total population post-cryo. However, the data collected suggests that the percentage of PhR progenitor cells decreased in the total population, as NEUROD1 is lower in post-cryo (Table 2). Therefore, cryopreservation does not seem to be beneficial for purification of the cell culture. Figure 6 illustrates that there is a change in how many cells express *NEUROD1* and *OTX2* after cryopreservation. This suggests that there is a bias introduced by cryopreservation, which may be the cause of the difference in CM expression seen in Figure 5 between pre- and post-cryo.

In Figure 8A, it can be seen that some neuron-like cells have survived cryopreservation. However, dead cells, characterized by their small round morphology, and other debris can also be observed. After seeding the cells, many cells with fibroblast-like morphologies could also be observed. When stained with CM5, fluorescent microscopy reveals that CM5 has affinity for the surfaces of these non-PhR cells, suggesting that CM5 is a possible negative marker.

4.3 Sources of Error

A factor which might have influenced the results post-cryo is that the antibodies used were in solution for a longer time than permitted by the manufacturer. As a consequence, it is possible that the antibodies had greater unspecific binding, or that fewer viable antibodies were added to the cell solution. If fewer antibodies were used, compared to the first experiment, the data concerning the expression of specific proteins might be invalid.

In some fluorescent images, large clumps of cells that seemed to have been rolled up or dragged in a particular direction could be seen, suggesting that the coverslip might have moved after it was placed on the microscope slide. This makes it harder to interpret the images, as clumps of cells appear positive for all stains because of the amount of antibodies collected in one place. In addition, aggregates, or clumps of larger cells which existed in the culture and were not dissociated, were flattened when the coverslip was installed. These flattened aggregates could not be used to draw any conclusions about the population, and potentially concealed cells which would have been relevant to study.

4.4 Further Studies

Biological replicates — different PhR cultures made using the same in-house protocol — would allow for a better understanding of batch-to-batch difference in candidate surface marker expression. In addition, running this CM screening on multiple biological replicates would allow for statistical analysis of the results.

Since cryopreservation is desirable, further inquiry into how the viability of thawed PhR progenitor cells cryopreserved at different timepoints changes would be valuable. Moreover, no protocol has been published for cryopreservation of PhR progenitors [21]. Therefore, experimentation with different cryoprotectants and freezing techniques could be relevant. It is possible that PhR progenitors frozen in this paper were too mature, or that the cryoprotectant used was not suitable. Studying whether the viability of PhR progenitors frozen at an earlier timepoint is greater may be important in developing a protocol which allows for PhR generation with short notice. In addition, studying how the expression of CMs changes when the cells are frozen at different timepoints may help in creating a protocol which leverages both negative sorting and cryopreservation.

In practice, sorting would involve either FACS or MACS. FACS could be used to create complex gating rules, such as picking a specific desirable population of cells, which is defined by two stains. Therefore, it might be relevant to explore whether a desirable population can be isolated based on the expression of two surface markers. However, FACS is associated with high cell stress, why MACS might be preferred as a sorting mechanism. In negative MACS sorting, the desirable population is not stained, and is exposed to minimal stress.

4.5 Conclusion

Several CMs, including CM1, CM8, CM9, and CM10, are promising markers expressed on the surface of retina-related cells, which might be stained in the process of a negative sort with the goal of removing non-PhR cells from the culture. However, as post-cryopreservation viability of PhR progenitors is low, more research into how cryopreservation affects PhR progenitors and other retinal cells is needed to draw any conclusions

about the viability of CMs as negative markers post-cryo.

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