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Bachelor Thesis

Optimizing Fluorescent Immunostaining Protocols for Retinal Organoids

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Abstract

Retinal degenerative diseases (RDs) are a leading cause of visual impairment, requiring advanced models for better understanding and treatment. Retinal organoids (ROs), derived from human induced pluripotent stem cells (hPSCs), have emerged as promising tools due to their ability to mimic human retinal structures more closely than traditional animal models. This study aims to optimize immunofluorescence (IF) staining techniques for ROs to enhance the visualization of cellular structures and improve the accuracy of disease modelling and therapeutic testing. The research focused on two primary methods: IF staining of retinal organoids cryosections and of whole retinal organoids. The whole organoid staining protocols were adjusted for the three-dimensional structure of the ROs, incorporating a clearing step to allow deeper imaging. Detailed imaging using a Zeiss confocal microscope provided comprehensive insights into the effectiveness of different staining conditions. Optimal antibody concentrations were identified, with specific protocols improving the visualization of cell morphology and positions within the retinal tissue. It also highlighted the benefits of a simple immersion clearing technique, which improved imaging depth and the visibility of internal structures by decreasing light scattering. The study highlights the importance of optimized IF staining protocols for advancing retinal organoid research. Refining these techniques aims to improve the understanding of retinal diseases, enabling more precise drug testing and the development of effective therapeutic strategies. These advancements hold significant potential for enhancing the consistency and reproducibility of retinal disease models, ultimately contributing to better patient outcomes.

Contents

Chapter 1

Introduction

Retinal degenerative diseases (RDs) are the leading causes of visual impairment among millions of people [1]. Despite this, much remains to be understood about the mechanisms driving these diseases. That is why there is a critical need for more accurate human in vitro models to advance our understanding of retinal diseases. The evolution of eye-on-chip research has made it possible to grow pluripotent stem cell-derived retinal organoids (ROs), changing our understanding and treatment of retinal diseases. Human-induced pluripotent stem cells (hPSCs) are particularly promising creating complex retinal structures that closely mimic the human retina. This advancement presents significant opportunities for developmental studies and new therapeutic strategies for retinal diseases. [2] By using ROs as models, researchers can test different treatments in a human-like environment, without having the limitations of traditional animal models, such as mice. Even though the research in mouse models has proven great insights into the basic concepts of retinal diseases [3], they don't represent the human retinal anatomy due to species differences. The development of hPSC-differentiated retinal organoids holds the potential to advance drug development and provide insights into retinal diseases.

Retinal organoids are three-dimensional, miniature versions of the human eye derived from hPSCs, designed to mimic the structure and function of a human retina. These organoids contain various retinal cell types including photoreceptors and ganglion cells. They are valuable for modelling retinal diseases, drug testing, gene therapy, and regenerative medicine, offering a consistent, reproducible, and ethical alternative to animal models [4]. By enabling detailed research of conditions like RDs, retinal organoids potentially lead to more promising and accurate therapeutic strategies. However, they face challenges such as complexity in creation, potential immaturity compared to adult retinas, and variability between samples. [4]

The ability to properly stain and visualize these cells within ROs is important for obtaining essential information, including morphological structure, cell structure and cell function. Immunofluorescence (IF) staining techniques are commonly used for identifying and analyzing specific antigens and cellular structures within complex tissues. [5] Without precise and optimized staining protocols, critical details about cellular interactions and pathology could be missed, obstructing the research and development progress in this field. Furthermore, several imaging techniques are used to study retinal organoids, each providing unique insights into their structure and function. Advanced methods like Confocal microscopy offer high-resolution, three-dimensional images and deep tissue visualization with minimal photobleaching. [6] One disadvantage of using these techniques is that fluorescence microscopy is restricted in its ability to image deep within three-dimensional ROs due to light scattering. Using clearing methods, however, it is possible to image deeper into the tissue by making the tissue transparent allowing the light to penetrate deeper within the tissue. [7]

This paper aims to optimize fluorescent staining protocols for retinal organoids using advanced fluorescence microscopes. There are two methods for visualizing ROs, and both are being treated in this paper: i) IF staining of retinal organoid cryosections and ii) staining of whole retinal organoids. With the staining of RO cryosections, evaluation of different antibodies and optimal antibody concentrations are tested. Cryosectioning is a technique where tissues are rapidly frozen and then sliced into thin sections using a Cryostat. These sections are used for various types of microscopic analysis, often to preserve cellular and tissue structures in an almost original state. This process allows visualization of the various cell morphologies and positions, improving the understanding of how different retinal cells look and their spatial distribution within the tissue.

For IF of whole retinal organoids a similar protocol is used for cryosections. Alternatively, changes in concentrations and incubation times are used so it is adjusted to the three-dimensional structure of the

ROs. The primary objectives are to achieve clear visualization of various cell types and layers and to evaluate the depth of imaging achievable within a 3D retinal organoid. By optimizing these parameters, this study aims to improve the understanding of cellular structure within ROs, providing a more complete view compared to 2D cryosections. To achieve a better depth measurement, a clearing solution is added to the organoids. Different clearing methods are known such as solvent-based clearing, simple immersion, hyperhydration and hydrogel embedding.[7] In this research simple immersion clearing is tested with a clearing solution made from 60% (v/v) Glycerol and 2.5M Fructose. The advantages of this technique are its ease of use and compatibility with various organoid types and staining protocols compared to the other techniques. Simple immersion makes the tissue more transparent and matches the refractive index of the tissue with that of the surrounding medium, making it possible for the light of the microscope to go deeper because of the decrease in scattering. This process improves the visibility of internal structures without having to section the organoids.

Optimizing fluorescence immunostaining techniques for retinal organoids represents a critical advancement in the field of eye-on-chip research. By optimizing protocols for both cryosections and whole organoids, more precise visualization can be achieved of the morphology and spatial distribution of retinal cells. This enhancement is important for understanding retinal structures and strengthens the potential of retinal organoids as models for studying retinal diseases and developing therapeutic strategies.

Chapter 2

Materials and Methods

2.1 Materials

For antibodies and fluorescent stains used in the protocols, see Table 2.1. For the fixation of the cryosections, Acetone is used. A hydrophobic PAP-pen is used (Merck KGaA, Darmstadt, German) for the cryosection protocol and imaging of the ROs. The blocking buffer is made of 1% BSA and 0.025% (v/v) Triton X-100 in PBS. For the antibody buffer, 1% BSA in PBS is used and for washing the cryosections, normal PBS is used. For the whole RO staining, before the cells are fixated dPBS is used for washing them. The fixation is done with 1:10 PFA/PBS. Also, an organic washing buffer is used for washing the ROs and for diluting the antibodies, this is made of 2% BSA and 1:10 Normal Donkey Serum in PBS with 0.1 % (v/v) Triton X-100. The clearing solution is made of 60% (v/v) Glycerol and 2.5M Fructose with dH2O. The ROs are stored in PBS. Slides and ROs were imaged using the EVOS FL2 Auto (Thermo Scientific, Bothell, USA) and the Zeiss LSM-880(Carl Zeiss AG, Oberkochen, Germany).

Table 2.1 The antibodies and fluorescent stain used in the IF protocols for staining RO cryosections.

Product	**Designation**	**Source or reference**	**Purpose**
Antibody	Mouse anti-Rhodopsin	NBP2-59690, NovusBio	Staining for rod photoreceptors
Antibody	Goat anti-Arrestin-3	NBP1-37003, NovusBio	Staining for cone photoreceptors (bulk in outer segments)
Antibody	Mouse anti-SNCG	H00006623-M01A, Abnova	Staining for Retinal Ganglion Cells
Antibody	Donkey anti-Goat Alexa Fluor™ 546	A11056, ThermoFisher	Secondary antibody
Antibody	Donkey anti-Mouse Alexa Fluor™ 488	A21202, ThermoFisher Scientific	Secondary antibody
Fluorescent stain	DAPI	D1306, ThermoFisher Scientific	Minor groove of A-T rich regions of DNA.
Fluorescent stain	Lectin PNA Alexa Fluor™ 568	L32458, ThermoFisher Scientific	Staining outer segments in cone and in rod

2.2 Immunostaining Retinal Cryosections

This experiment is done on 347-day-old retinal organoid cryosections with a thickness of 7 μm. Here five slides were prepared, each with different antibody concentrations as seen in table 2.2. Four slides contained three tissue sections each, and are used for the fluorescent immunostaining. One slide serves as a negative control, which involves only the addition of secondary antibodies. This ensures that any observed staining is specific to the target antigen, minimizing the possibility of non-specific binding or background signal. This control is essential for validating the reliability and accuracy of the staining results. For the secondary antibodies, the same antibodies and concentrations are used on all slides, Alexa Fluor 488 (1:400 dilution) and Alexa Fluor 546 (1:400 dilution). Similarly for the fluorescent markers PNA-Lectin (1:100 dilution) and DAPI (1:400 dilution). The final protocol that is used is shown in Appendix A.1.

Table 2.2 A list of the primary antibodies used in the RO cryosection experiments. The slides, antibody positions, antibodies and their concentrations are presented. Slide 5 shows the negative control where exclusively secondary antibodies are added.

Position \ Slide	A		B		C	
	Antibodies	Concetration	Antibodies	Concetration	Antibodies	Concetration
1	SNCG	1:500	SNCG	1:500	SNCG	1:500
	Arrestin-3	1:500	Arrestin-3	1:500	Arrestin-3	1:500
2	Rhodopsin	1:500	Rhodopsin	1:500	Rhodopsin	1:500
	Arrestin-3	1:500	Arrestin-3	1:500	Arrestin-3	1:500
3	Rhodopsin	1:500	Rhodopsin	1:500	Rhodopsin	1:500
	Arrestin-3	1:100	Arrestin-3	1:250	Arrestin-3	1:500
4	SNCG	1:500	SNCG	1:500	SNCG	1:500
	Arrestin-3	1:100	Arrestin-3	1:250	Arrestin-3	1:500
5	Neg. control		Neg. control		-	

2.2.1 Immunostaining Procedure

After bringing the cryosection slides to room temperature, they are submerged in acetone for 10 minutes. For safety and health reasons, this process is conducted in a fume hood, where the slides are left to evaporate for at least 20 minutes. Acetone causes rapid dehydration of the tissue, leading to protein denaturation. This process fixes the tissue structures and stabilizes the molecular components.

A blocking buffer made of 1% BSA in PBS with 0.025% Triton X-100 is used to prevent non-specific binding of antibodies and permeabilizes cell membranes allowing the antibodies and blocking buffer to access intracellular targets. First, a hydrophobic barrier is drawn around the tissue sections with a PAP pen to hold the solutions within the selected area. The benefit of using this compared to a sequenza system is that a PAP pen allows testing multiple solutions and concentrations on a single slide, when having multiple tissue sections on the same slide, reducing the number of slides needed. The slides are then blocked for 1.5 hours at room temperature by carefully pipetting the blocking buffer within the hydrophobic barrier. Ensure the solution does not overflow the PAP ink. To prevent evaporation, place wet tissues beneath the slides in the slide box during the blocking step. It is crucial to prevent the tissue slices from drying out, as this can cause shrinkage, distortion, and artefacts.

To remove the blocking buffer, gently tap the fluid of the slides on a piece of paper and carefully move them backwards through PBS. The slides are then placed in a Hellendahl cuvette with PBS and washed three times for 5 minutes by carefully emptying and refilling the cuvette with PBS. The primary antibodies are diluted in an antibody buffer containing 1% BSA in PBS. After removing the blocking buffer by gently tapping the slides on a tissue, the primary antibody solution is applied using the same technique, ensuring it does not evaporate, and incubated overnight at 4°C. The solution is again removed by gently tapping the antibody dilution on a paper and then is washed three times for 5 minutes using the same technique as washing off the blocking buffer.

After diluting the secondary antibodies, DAPI, and PNA-Lectin in the antibody buffer, the slides are incubated for 1 hour at room temperature. To minimize photobleaching, work in the dark and cover the slides with aluminium foil in bright spaces. Wet tissues are again placed in the slide box to prevent evaporation. The slides are washed four times for 5 minutes using the Hellendahl cuvette technique, ensuring all dilutions are removed to avoid unwanted fluorescence signals.

2.2.2 Preparation for Imaging

For imaging preparation, the following steps are conducted in the fume hood. First, excess PBS is drained by gently tapping the side of the slide on a tissue. Any remaining solution is carefully dried with a paper tissue, being careful not to touch the tissue slices. The slides are then mounted by applying a streak of mounting solution along the side of the glass slide and positioning the coverslip at a 45-degree angle. Then it is slowly lowered until it makes full contact with the mounting solution, which spreads across the coverslip. Avoid large bubbles; if present, they can be removed with forceps, making sure not to break the glass coverslip. The mounting solution needs to dry in the fume hood for at least 2 hours, working in

the dark and covering the slides with aluminium foil to prevent photobleaching. The slides are stored at 4°C in the dark. Slides were imaged using EVOS FL2 Auto and Zeiss LSM-880.

2.3 Retinal Organoid Fluorescence Immunostaining

The whole organoid IF staining protocol involves two experiments conducted on retinal organoids (ROs) of different ages: the first experiment on 252-day-old ROs (RO 1-3) and the second on 157-day-old ROs (RO 4-9). Following the initial experiment, adjustments are made to optimize antibody concentration. Additionally, the PNA-Lectin concentration is modified in the second experiment and a clearing step is added(RO 7-9). The primary antibody concentrations with whole ROs differ from the cryosections. This is because the cryosections require lower antibody concentrations due to their thinness and easy penetration, while whole ROs need higher concentrations to ensure antibodies reach all cells within the thicker structure. Again, the same secondary antibody and concentrations are used: Alexa Fluor 488 and 546 (1:400). In the table 2.3 below the used primary antibody and fluorescent marker concentrations are shown. The final protocol is seen in Appendix A.2.

Table 2.3 Distribution of the selected primary antibodies, fluorescent markers, and their concentrations for the whole RO experiments.

RO	1st Antibodies	Concentration	Fluorescent markers	Concentration
1	Arrestin-3	1:50	PNA-Lectin	1:100
	Rhodopsin	1:100	DAPI	1:400
2	Arrestin-3	1:100	PNA-Lectin	1:100
	Rhodopsin	1:100	DAPI	1:400
3	Arrestin-3	1:100	PNA-Lectin	1:100
	Rhodopsin	1:200	DAPI	1:400
4 & 7	Arrestin-3	1:50	PNA-Lectin	1:100
	Rhodopsin	1:50	DAPI	1:1000
5 & 8	Arrestin-3	1:50	PNA-Lectin	1:200
	Rhodopsin	1:100	DAPI	1:1000
6 & 9	Arrestin-3	1:100	PNA-Lectin	1:100
	Rhodopsin	1:50	DAPI	1:1000

2.3.1 Sample Fixation

The chosen retinal organoids are transferred to a new low-adherence well plate prior to fixation. The medium is carefully aspirated to ensure the organoids are not accidentally removed. The organoids are then washed twice for 5 minutes with dPBS, ensuring it is not PBS because dPBS is free of calcium and magnesium ions, helping to prevent cell clumping and detachment. For fixation, a 1:10 formaldehyde (PFA) in PBS solution is added to the wells and incubated for 45 minutes at room temperature. For thicker, more complex organoids, overnight incubation can be used. Following PFA/PBS fixation, the organoids are washed three times for 5 minutes with (d)PBS. During the first two washes, formaldehyde is collected in a special tube due to its toxic and carcinogenic properties.

2.3.2 Blocking and Immunolabeling

For blocking, the organoids are incubated three times for 1 hour in Organoid Washing Buffer (OWB) at room temperature made, of 1:10 Normal Donkey Serum in PBS with 0.1% Triton X-100. After aspirating the OWB, the OWB diluted primary antibodies are added and incubated overnight at 4°C with gentle orbital shaking. The primary antibodies are then aspirated, and the organoids are washed three times for 1 hour with PBS at room temperature. Afterwards, the secondary antibodies, PNA-Lectin and DAPI are added and incubated overnight at 4°C with mild orbital shaking. After aspirating the secondary antibodies, the organoids are washed again three times for 1 hour with PBS.

2.3.3 Preparation for Imaging

For imaging the whole ROs, two techniques are used: i) with a custom-made PDMS print as seen in Figure 2.1A, ii) and by squeezing the ROs underneath a coverslip glass seen in Figure 2.1B. For the first

technique, a rectangular PDMS design is 3D printed and adhered to a coverslip. The design includes six circular wells, each approximately 3 mm in diameter, capable of accommodating an organoid in PBS. Organoids are carefully transferred into the wells using a large-bore pipette tip with the end cut off, and the tip is coated with 1% BSA/PBS solution to prevent the ROs from sticking. Ensuring that the organoids settle at the bottom of the wells is crucial for proper imaging, as the objective's working distance requires the organoids to be near the coverslip glass for optimal imaging. The wells are then filled with PBS to prevent the organoids from drying out. During imaging, it may be necessary to refill the wells with PBS to prevent drying out the ROs.

For the second technique, a 1x2 cm rectangle is drawn in the centre of a slide using a PAP pen. Multiple layers of double-sided sticky tape are applied at both ends of the rectangle, typically 2 to 3 layers depending on the size of the organoid. A 1000-µm pipette tip, with a part of the end cut off and coated in 1% BSA-PBS, is used to transfer the organoid into the rectangle. A coverslip is then placed on top by first positioning it on the left side of the sticky tape and slowly lowering it to the right side, allowing the fluid to spread evenly. This is left for about one minute to ensure proper fluid distribution. Gentle pressure is then applied on both sides to secure the coverslip to the sticky tape, making the preparation ready for imaging.

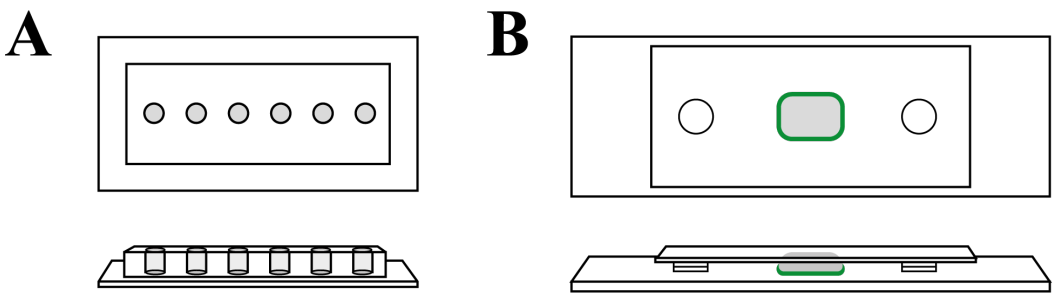

Figure 2.1 The two imaging techniques used for imaging the retinal organoids. A: The coverslip glass with a 3D printed PDMS print on it with 6 circular holes for the ROs to sit into for imaging. B: The slide where with a hydrophobic pen a rectangle is drawn between which the RO sits in PBS or clearing solution. The coverslip glass is attached on top with two layers of circular double-sided tape.

2.3.4 Clearing step

For imaging deeper into the tissue, a clearing step is included. First, PBS is removed from the wells, followed by the addition of the clearing solution made from 60% v/v fructose and 2.5M glycerol. Careful attention is given to gently resuspending the solution due to its high viscosity, minimizing the formation of bubbles. This is then incubated for a minimum of 20 minutes. As a result of this process, organoids become nearly transparent, making them hard to see. For imaging, organoids can be transferred in a 1% BSA-PBS coated 1000-m pipette tip, as described earlier. The organoids can remain in the clearing solution for up to one week at 4°C or six weeks at -20°C.

Chapter 3

Results

3.1 Immunofluorescence Experiments on Retinal Organoid Cryosections

This section presents the results of the IF experiments on the retinal organoid cryosections. Detailed images were captured using an EVOS fluorescence microscope and a confocal microscope. With this, evaluations and comparisons of the performance of the different staining conditions, the various cell morphologies and cell positions are accomplished. These findings contribute to optimizing the fluorescence immunostaining process, enhancing its accuracy and efficiency for future applications.

3.1.1 Initial Retinal Organoid Cryosection Experiment and Antibody Testing

To evaluate the expression and the location of various proteins within the retinal, cryosections from retinal organoids were stained with antibodies against SNCG, Rhodopsin, Arrestin-3, DAPI and PNA-Lectin. To test the antibodies and the different concentrations of the antibodies, an initial experiment is done on six RO cryosection slices, using the concentrations as seen in table 2.2. In Figure 3.1, the results of the first cryosection experiment are depicted. In 3.1A-B, the IF staining reveals photoreceptor rod cells stained for Rhodopsin (red) and ganglion cells stained for SNCG (red). The nuclei are visible with DAPI staining (blue), and the outer segments of the photoreceptors are stained with Peanut Agglutinin Lectin (PNA-Lectin)(yellow). The negative control is portrayed in Figure 3.3C-D. Observations show a scarcity of ganglion cells, mainly located deeper within the tissue. Also, a lack of photoreceptor cone cells stained by Arrestin-3 is seen within the outer layer, visible as small green dots. The negative control demonstrates the absence of primary antibodies leading to no specific antigen binding. This control confirms the specificity of the IF technique, ensuring that observed fluorescence signals bind only to targeted antigens.

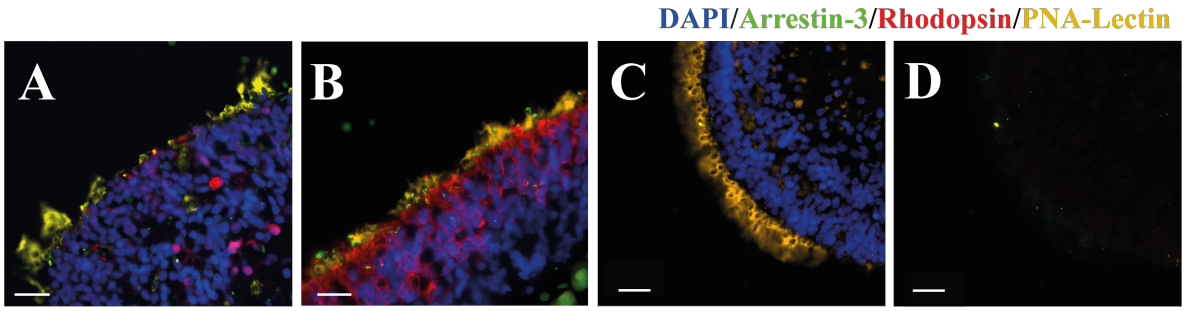

Figure 3.1 Cryosections of retinal organoids made with an EVOS microscope. The antibody concentrations used are illustrated in table 2.2, slides 1, 2 and 5. The spatial localization of the different retinal cells is seen. The outer segments are marked with PNA-Lectin (yellow), visible as flame-like structures on the cell exterior. The nuclei, stained with DAPI (blue), are present throughout the RO sections except in the outermost regions. (**A**) RO cryosections where ganglion cells are stained with antibody SNCG (red) appear as red ovals within the cryosection. (**B**) RO cryosection where rod photoreceptors are stained with antibody Rhodopsin (red) rod-shaped forms located on the outer ring of the section. (**C-D**) Negative control of the retinal cryosections where only secondary antibodies and fluorescent markers are added. (**C**) Negative control with DAPI and PNA-Lectin; (**D**) Negative control without DAPI and PNA-Lectin. Scale bars indicate a) 50 µm b) 30 µm c) 30 µm d) 30 µm.

3.1.2 Optimizing Primary Antibody Concentrations for Arrestin-3

Concluding on the findings from the initial experiment, varying concentrations of the primary antibody Arrestin-3, a marker for cone photoreceptor cells, were tested to optimize staining conditions. The different dilutions used are 1:100, 1:250 and 1:500, as seen in table 2.2. Figure 3.2 illustrates the outcomes of these different concentrations, revealing Arrestin-3 predominantly as small green dots, notably concentrated around the edge of the tissue. With the highest dilution, seen in 3.2A-C, a lot of non-specific binding is noticed, pictured as the green dots outside of the cell and deeper within the retinal cryosection. This non-specific binding decreases with lower antibody concentrations, with minimal non-specific staining at the lowest concentration with a dilution of 1:500.

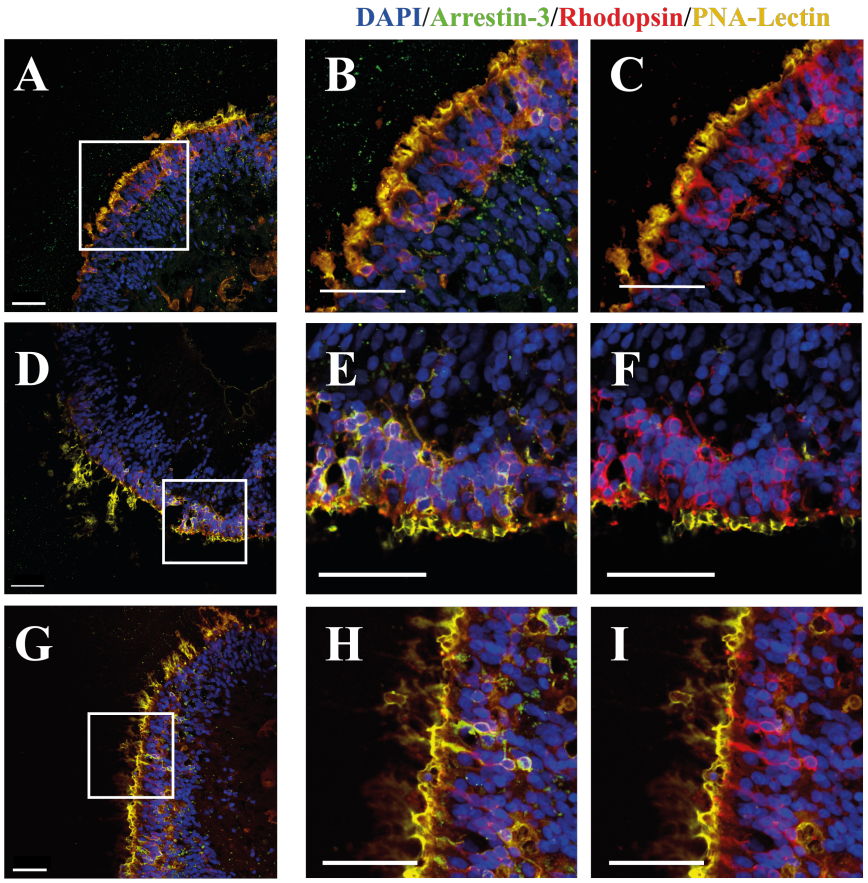

Figure 3.2 Retinal organoid cryosections with different cone photoreceptor staining Arrestin-3 (green) concentrations made with a Zeiss confocal microscope. The concentrations used are depicted in table 2.2, slide 3 & 4. The nuclei are stained with DAPI (blue), the rod photoreceptors with Rhodopsin (red) and the photoreceptor outer segments with PNA-Lectin (yellow). (**A-C**) At an Arrestin-3 dilution of 1:100, significant non-specific binding is observed as green dots both outside and deeper within the RO section; (**D-F**) At 1:250 dilution, there is reduced non-specific binding, though some remains; (**G-I**) At 1:500 dilution, there is minimal non-specific binding with expected staining along the outer regions of the photoreceptors. (**C, F, I**) RO cryosections without the Arrestin-3 staining. Here the photoreceptor rods are clearly visible as bright red, rod-shaped structures. Scale bars indicate 50 µm.

3.2 Immunofluorescence Experiments of Whole Retinal Organoids

In this section, the results of the whole retinal organoid staining are presented. Multiple images are made at various depths and stacked, using a Zeiss confocal microscope to capture the three-dimensional structure of the ROs. A total of nine ROs were tested across three experiments, varying the concentrations of DAPI, Arrestin-3, Rhodopsin, and PNA-Lectin, as detailed in Table 2.3. Six ROs were used in the first two experiments, where the antibodies were tested with varying concentrations. The remaining three

ROs used in the third experiment underwent a clearing step with a clearing solution to enable deeper imaging within their three-dimensional structure.

3.2.1 Optimizing Antibody Concentrations

To test antibodies on whole retinal organoids, six ROs were stained in two experiments with varying concentrations of PNA-Lectin for photoreceptor outer segments, DAPI for nuclei, Arrestin-3 for cone photoreceptors, and Rhodopsin for rod photoreceptors, as detailed in Table 2.3. Figure 3.3 presents representative images of these ROs. The first three ROs lacked outer segments, indicated by the absence of PNA-Lectin staining on the exterior of the ROs. This is visualized in the brightfield image (3.3A-B) and the immunofluorescence (IF) images (3.3C-G). Figure 3.3C-E shows that signal loss increases with imaging depth, resulting in fewer visible cells in the centre of the organoid. The other three ROs did have clear outer segments as seen in 3.3H-I. The outer segments stained with PNA-Lectin are visible but the signal in general is less strong compared with the other antibodies. Additionally, staining with Rhodopsin did not produce strong signals in these experiments, unlike the results seen in the cryosections in Figure 3.1. The Arrestin-3 generally has a strong signal and despite a reduced concentration of DAPI in the second experiment, the nuclei signal remains effective. The variation in photoreceptor rod staining Rhodopsin and cone staining Arrestin-3 concentrations is minimal and a higher concentration doesn't show undesirable non-specific staining.

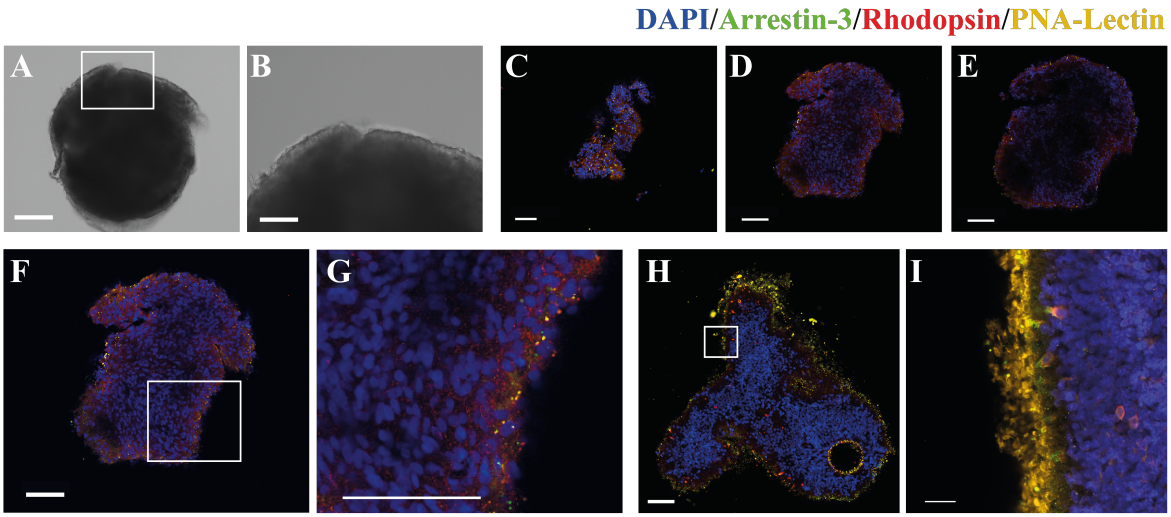

Figure 3.3 Whole retinal organoids imaged with a Zeiss confocal microscope, representative of experiments one and two using the dilutions shown in Table 2.3, ROs 1 to 6. The nuclei are stained with DAPI (blue). (**A-B**) Brightfield images of an RO with no outer segments visible. (**C-E**) Retinal organoid slices at different depths. (**F-G**) Zoomed images of the ROs show that the photoreceptor rod stain Rhodopsin (red) and the photoreceptor cone stain Arrestin-3 (green) do not stain effectively, and no outer segments stained with PNA-Lectin (yellow) are visible. (**H-I**) Images of an RO with the outer segments staining, visualized by the yellow flame-like structures. Scale bars indicate a) 100 µm b) 50 µm c-e) 100 f-g) 100 µm h) 70 µm i) 20 µm.

3.2.2 Enhanced Imaging Depth with Clearing Techniques

A simple immersion clearing step was tested to enhance imaging depth within the three-dimensional RO tissue by making the cells more transparent and matching the refractive index of the tissue with that of the surrounding medium, allowing light to penetrate further into the tissue. Figure 3.4A presents an organoid without the clearing step, where the outer segments are visible, but Rhodopsin is not observed deeper within the tissue. Figures 3.4B and 3.4D show retinal organoids with the clearing step applied. All antibodies are distinctly visible, highlighting the effectiveness of imaging deeper within the tissue. In contrast to 3.4A, Figure 3.4B shows that the clearing step enables clear visualization of Rhodopsin deeper within the tissue. Figures 3.4C and 3.4D compare the depth measurements between non-cleared and cleared samples, displaying that the clearing step allows for significantly deeper imaging.

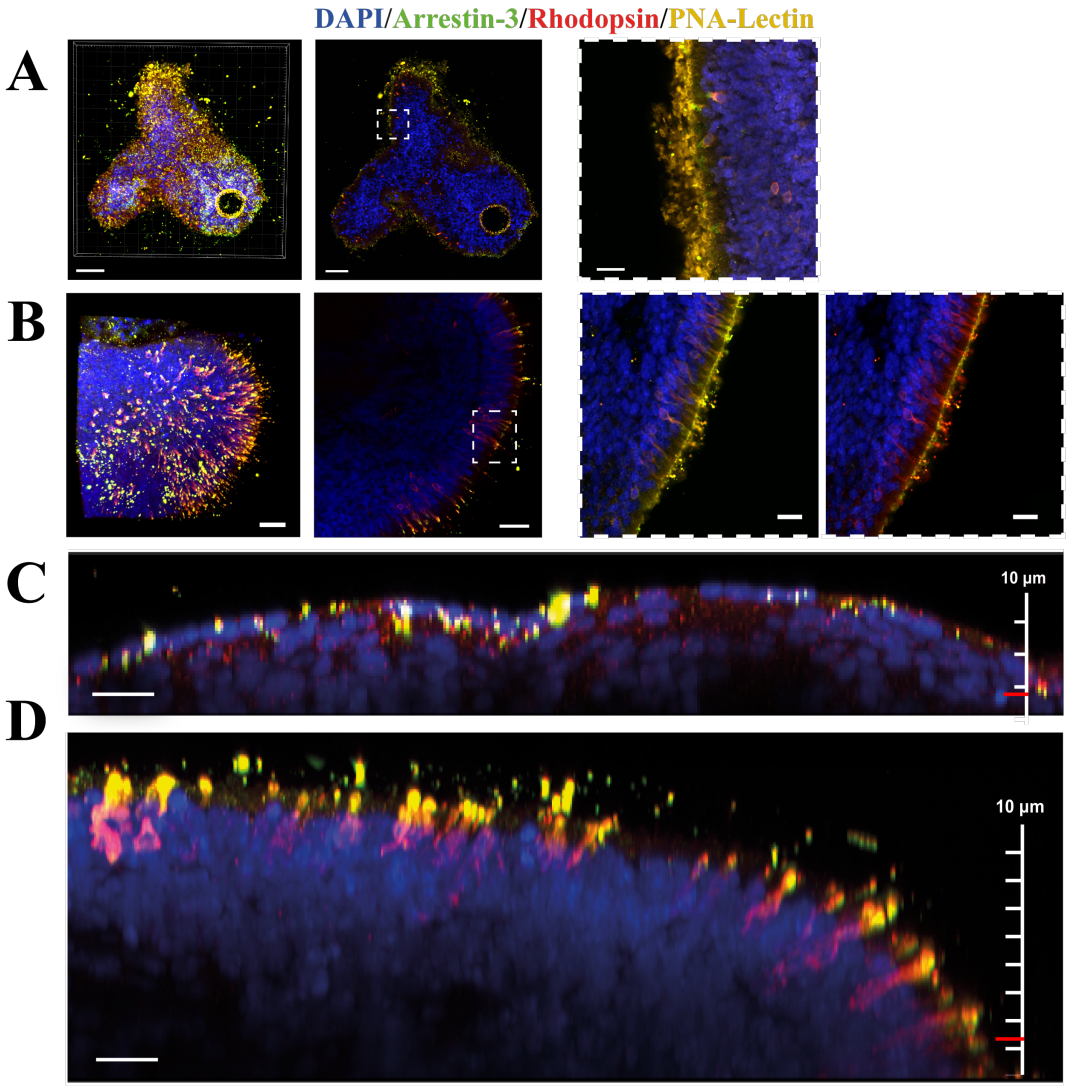

Figure 3.4 Results of the third experiment showing optimized antibody staining combined with the clearing step. The staining used includes DAPI for nuclei (blue), Arrestin-3 for photoreceptor cones (green), Rhodopsin for photoreceptor rods (red), and PNA-Lectin for the outer segments of photoreceptors (yellow). (**A**) Top view, slice, and zoomed image of Retinal Organoid 5 without clearing. (**B**) Top view, slice, zoomed image with all antibodies and zoomed image without Arrestin-3 respectively, of Retinal Organoid 9 with clearing. (**C**) Section of Retinal Organoid 5 without clearing, showcasing a thickness of approximately 32 µm, indicated by the red line. (**D**) Section of Retinal Organoid 9 with clearing, showcasing a thickness of approximately 77 µm, indicated by the red line. Scale bars indicate a) 100 µm, 70 µm, 20µm b) 50 µm, 40 µm, 20 µm, 20 µm c) 20 µm d) 20 µm.

Chapter 4

Discussion

This research aimed to optimize immunofluorescence (IF) staining protocols for both retinal organoid cryosections and whole retinal organoids to enhance the visualization of specific cellular components. The focus was on optimizing antibody concentrations to minimize non-specific binding and improve staining quality. Additionally, employing a clearing technique was explored to improve staining effectiveness and imaging depth. Insights from the experiments highlight the importance of protocol optimization and error minimization for achieving consistent and reliable IF staining results.

4.1 Optimizing Immunofluorescence Staining Protocols for Retinal Organoid Cryosections

The first notable observation in this study was the absence of ganglion cells in the retinal cryosections, a higher number of ganglion cells would be expected.[8] However, the observed morphology and spatial arrangement of the remaining cells were consistent with regular RO structure. The likely explanation for the reduced number of ganglion cells is the age of the ROs used in the experiments. At 347 days old, these organoids are well past the 125-day mark, after which ganglion cell loss is commonly reported. This age-related decline declares the observed shortage in ganglion cells.[8] The photoreceptor rod stain, Rhodopsin, was tested resulting in strong and clear staining. The rod-shaped red structures located on the outer ring of the section were distinctly visible, confirming that a 1:500 dilution is effective for staining photoreceptor rods in retinal organoid cryosections.

The results from the second retinal cryosection experiments showed overall improved visibility of Arrestin-3 staining compared to the initial experiment, yielding more representative outcomes[9] as seen in Figure 3.2. Nevertheless, it also shows that decreasing the concentration of Arrestin-3 led to reduced non-specific binding, which is visible as the green dots outside the tissue, without significantly affecting the cone-specific, expected binding. This finding suggests that the higher concentration used in the second experiment likely didn't contribute to the improved staining quality. Comparing the two experiments, it is clear that proper cone staining was present in both, but in lower quantities in the first experiment even when the same concentration was used. This is seen by comparing figure 2.2 with figure 3.2C, where the Arrestin-3 concentration that is used is the same. This indicates that the initial suboptimal staining was not due to an insufficient concentration of the antibody. The difference in staining quality could potentially be attributed to human errors. This concludes that the optimal photoreceptor cone stain Arrestin-3, has an optimal dilution of 1:500 for RO cryosections. Overall, these findings highlight the importance of optimizing antibody concentrations and minimizing procedural errors to achieve consistent and reliable IF staining results for retinal organoids.

Furthermore, it was observed that after a few days of the imaging preparation, air bubbles formed under the coverslip glass, complicating the imaging of the tissues. This issue likely occurred from the use of a PAP pen, which creates a hydrophobic barrier that can hinder the even distribution of the mounting solution across the slide. Therefore, air trapped by this barrier may gradually seep under the coverslip. To prevent this problem, using a larger volume of mounting solution or employing a sequenza system instead of a PAP pen could be helpful. However, a limitation of using a sequenza system is that it does not allow for testing different antibody concentrations on the same slide.

Lastly, the negative control confirmed the specificity of the IF staining, as expected, by showing no staining in the absence of primary antibodies. This result validates the effectiveness and reliability of the IF staining protocol.

This study has successfully optimized immunofluorescence staining protocols for retinal cryosections,

emphasizing the importance of optimizing antibody concentrations to minimize non-specific binding and enhance overall staining quality. The experiments revealed that reducing the concentration of Arrestin-3 improved specificity by minimizing non-specific binding while still binding to cone cells. The absence of ganglion cells in the cryosections was attributed to the advanced age of the retinal organoids used. The challenges with air bubbles under the coverslip glass after several days of imaging preparation highlighted the need for careful handling techniques and alternative methods to prevent this issue. Validation through negative controls confirmed the specificity of the IF staining protocol. Optimal concentrations were identified: 1:100 for PNA-Lectin, 1:500 for Rhodopsin, 1:500 for Arrestin-3, and 1:1000 for DAPI, as seen in the protocol in attachment A.

4.2 Optimizing Immunofluorescence Staining Protocols for Whole Retinal Organoids

Optimizing antibody concentrations is crucial for achieving precise and reliable immunostaining results in both whole retinal organoids and cryosections. In the cryosection experiment, varying concentrations of Arrestin-3 revealed unwanted, significant non-specific binding at higher concentrations. However, optimization efforts for whole retinal organoids revealed that higher concentrations of primary antibodies barely lead to non-specific binding. The most optimal concentrations tested were the dilutions of 1:100 for Arrestin-3, 1:50 for Rhodopsin, and 1:100 for PNA-Lectin. This conclusion takes into account the accuracy and strength of the signal, ensuring it is clear from unwanted non-specific staining. Nonetheless, it is important to keep in mind that using higher concentrations may not always be optimal due to potential antibody waste and increased costs. Furthermore, a lower concentration of DAPI (1:1000 dilution) still provided a strong signal, indicating effective staining.

Although there were generally good results from the photoreceptor outer segment staining with PNA-Lectin, the staining was suboptimal in a few experiments. Here hardly any outer segments were visible, even though their presence was confirmed with a bright field microscope. ROs may have a lower number or smaller size of outer segments, which can result in less noticeable or sparse staining.[10] Another result was that not all cells showed outer segment formation, as indicated by a negative PNA-Lectin signal. This highlights the significance of initially confirming outer segment presence and quantity through bright-field microscopy to avoid unnecessary use of outer segment staining antibodies. It is also important to consider the age of the ROs, as outer segments typically begin to appear after approximately 125 days.[8] Increasing the gain and/or laser power on the confocal microscope to improve the signal, revealed slight PNA-Lectin staining of the nucleus. This indicates potential non-specific binding of PNA-Lectin, however, there is limited literature confirming this particular observation.

Another issue is that the photoreceptor rod stain Rhodopsin was less effective in whole organoids compared to cryosections. This is likely due to the depth of the tissue. This was noticeable as Rhodopsin staining was primarily visible around the outer segments and not deeper within the tissue, as seen in cryosections in Figure 3.2. While higher concentrations of the Rhodopsin antibody resulted in slight improvements, the signal remained less strong and specific as in cryosections. One of the reasons for this difference may be due to the new batch of Rhodopsin antibody used in the following experiments (RO 4-9), highlighting the need for consistency and effectiveness in antibody batches to ensure reliable results. Another reason could be that the tissue density and composition in whole organoids differ from cryosections, restricting antibody penetration. Whole organoids have a denser 3D structure, creating physical barriers for the antibodies. In contrast, cryosections are thinner and offer a more accessible surface for antibody binding, resulting in more effective staining. To achieve more valid staining in whole retinal organoids, additional permeabilization, like a different clearing step, and longer incubation times steps may be beneficial. The use of the simple immersion clearing step improved the Rhodopsin signal, allowing deeper imaging of photoreceptor rods. This technique proves to deliver a clearer and more detailed view of the ROs structure and distribution. This improvement is important for accurately studying the morphology and functionality of photoreceptor cells in retinal organoids.

The main goal of adding the simple immersion clearing step was to enable deeper imaging by making the tissue more transparent by matching the refractive index of the tissue. The results from this research

confirmed the effectiveness of this approach. The microscope lights could penetrate deeper within the tissue, and as an additional benefit, the clearing step also seemed to enhance the effectiveness of Rhodopsin staining of the photoreceptor rods as seen in Figure 3.4. This improved both the depth and clarity of imaging, allowing for a more detailed and comprehensive analysis of the retinal organoid cells. Future research can explore alternative clearing methods like solvent-based clearing to enhance imaging depth and clarity even further. [7]

Lastly, an issue was that during imaging some organoids(RO 1-3) dried out slightly due to the use of PBS that evaporated during imaging with a confocal microscope. This can occur because the heat generated by the microscope's light source increases the temperature of the sample environment making it evaporate the PBS. For longer imaging sessions it is recommended to put the organoids in a solution, for example, a clearing solution or mounting media, that doesn't evaporate at those temperatures. The ROs were not exposed without PBS for an extended period, but it nevertheless may have impacted their morphology. Although this change was not visible in the images, it is an important factor to consider for future studies.

In conclusion, this study focused on optimizing immunofluorescence staining protocols for retinal organoids to improve the visualization of specific cellular components. Varying antibody concentrations and employing a clearing technique were key strategies to enhance staining effectiveness and imaging depth. The most optimal dilutions were 1:100 for Arrestin-3, 1:50 for Rhodopsin, 1:100 for PNA-Lectin and 1:1000 for DAPI. Challenges such as suboptimal outer segment staining and evaporation of PBS during imaging were noted, highlighting the need for careful experimental considerations and alternative solutions like clearing solutions or mounting media. Clearing techniques improved Rhodopsin staining and imaging depth, providing clearer insights into photoreceptor morphology. In summary, these findings highlight the importance of optimizing staining protocols to achieve precise imaging outcomes in retinal organoid research.

4.2.1 Imaging Preparation

For the first technique (Figure 2.1A), although the plan seemed promising, it proved less effective in practice. The small size of the holes was intended to prevent the ROs from moving during imaging. However, this made it difficult to insert the ROs into the mount. Contrary to expectations, the ROs did not easily sink to the bottom, which is necessary for imaging as the working distance of the objective requires the organoids to be at the bottom near the coverslip glass. Despite this, the technique is quicker and more convenient, with the added benefit that the ROs can be reused after imaging by pipetting them back into the well plate with PBS. A potential solution is to enlarge the holes slightly, ensuring the organoids sink directly to the bottom and don't stick to the sides. This would also address the issue of PBS drying out during imaging due to the limited volume in the small holes, although it compromises the purpose of having small holes to make sure the ROs don't move during imaging. Another disadvantage is the difficulty in properly placing the ROs into the holes when using a viscous clearing solution. One last disadvantage is that it is mounted on a coverslip glass instead of a normal glass slide. This could be problematic if there is no confocal mount that accommodates only a glass coverslip.

While the second technique (Figure 2.1B) is easier to use and more practical it is more time-consuming to prepare and presents challenges in retrieving the ROs. After the slide and coverslip are secured with tape it is challenging to recover the organoids, as the coverslip tends to break easily when removed from the double-sided tape. However, using the viscous clearing solution allows the slides to be stored in the slide, so there is no need for retrieving the RO. In contrast, PBS evaporates over time, making the slide useless. Despite this, the technique remains superior due to its relatively straightforward preparation and ease of use, particularly when working with the viscous clearing solution, which is more manageable with this method.

In conclusion, the second technique is preferred for its effectiveness, practicality, and ease of use. The custom PDMS print on the coverslip could be effective if the holes were made larger, though this might risk the RO moving during imaging.

4.2.2 Future Research

Future research in retinal organoid (RO) studies holds promising directions for advancing our understanding and treatment of for example retinal degenerative diseases (RDs). Using two-photon microscopy could significantly enhance the imaging depth and resolution, allowing for better visualization of deeper structures within the ROs.[11] Two-photon microscopy uses two photons of lower energy that simultaneously excite a fluorescent molecule, which then emits a single photon of higher energy. This technique allows for deeper tissue imaging with less photobleaching compared to traditional fluorescence microscopy. The use of infrared light enables better tissue penetration and minimizes scattering. This technique, combined with optimized staining protocols, could provide more detailed insights into the cellular and molecular dynamics of retinal tissues.

Additionally, exploring co-culture and tri-culture systems within retina-on-chip (RoC) platforms will enable researchers to study the complex interactions between different cell types, retinal pigment epithelium and vascular components, in a controlled environment.[9] Such systems can closely mimic the in vivo retinal environment, offering more accurate models for disease studies and drug testing. The development of disease-specific genetic models using human induced pluripotent stem cells (hPSCs) will further enhance our ability to study RDs.[12] By creating ROs from patients with specific genetic mutations, researchers can investigate the disease process of these conditions in a human-like environment, leading to more targeted and effective therapeutic strategies.[2]

Incorporating these advanced techniques and models into retinal research will provide a deeper understanding of RDs and support innovative treatments. These efforts will contribute to the development of personalized medicine approaches for retinal diseases, improving the quality of life for millions affected by visual impairment.

4.3 Conclusion

In conclusion, this study successfully optimized immunofluorescence staining protocols for retinal organoid cryosections and whole retinal organoids, emphasising the critical role of antibody concentration in enhancing staining quality. The findings highlight the importance of accurate protocol optimization and error reduction to achieve consistent and reliable results in IF staining. In conclusion, the optimized antibody concentrations were successfully identified, ensuring minimal non-specific binding and enhanced staining quality. The optimized antibody concentrations are displayed in Table 4.1. The clearing technique proved beneficial, delivering the expected results by enabling deeper imaging. Moving forward, integrating advanced imaging techniques and exploring co-culture models will further advance the understanding and treatment strategies for retinal degenerative diseases, promising more accurate therapeutic strategies.

Table 4.1 Optimized dilutions of the primary antibodies: Arrestin-3 and Rhodopsin; and fluorescent markers: PNA-Lectin and DAPI.

Optimized dilutions	**Arrestin-3**	**Rhodopsin**	**PNA-Lectin**	**DAPI**
RO Cryosection	1:500	1:500	1:100	1:1000
Retinal Organoids	1:100	1:50	1:100	1:1000

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Appendix A

Protocols

A.1 Immunohistochemistry of Cryosections

Immunohistochemistry of Cryosections Category Stainings
Author Meike Blaauw
Labels: immunohistochemistry cryosections

Chemicals:
* Acetone
* PBS (+++)
* Triton X-100
* BSA
* Primary antibodies:
o Rhodopsin (1:500)
o Arrastin-3 (1:500)
o SNCG (1:500)
* Secondary antibodies:
o Alexa Fluor 488 (1:400)
o Alexa Fluor 546 (1:400)
* DAPI (1:1000)
* PNA-Lectin (1:100)
* Mounting Medium

Solutions:
* Blocking buffer:
o 1% BSA
o 0.025% v/v Triton X-100
o PBS
* Antibody buffer:
o 1% BSA
o PBS

Equipment:
* Glass slide dish with holder (Hellendahl cuvette)
* PAP pen (hydrophobic marker)
* Glass coverslips
* Slide box
* Tissues
* Aluminium foil
* Forceps
* Sequenza System (opt.)

Sample fixation:
1. Take the slides out of the freezer and let them come to RT
2. Submerge in acetone at RT for 10 minutes NOTE: Work in the fume hood!
3. Take the slides out of the acetone and let the acetone evaporate in the fume hood for at least 20 minutes
4. Wash 2x with PBS a. Use a Hellendahl cuvette for the washing steps.

5. Check under microscope where your samples are, if the sections are hard to see, mark them with a permanent marker (soluble with EtOH!) on the other side of the slide (not on the sections!). Ideally, circle the sections so you can image them later and find the samples easier with the microscope.

Immunostaining procedure:
1. Circle the samples with a PAP pen otherwise mount them on the Sequenza system. NOTE: work in the fume hood for that!
2. Block for 1.5h at RT with blocking buffer. Sequenza: 100µL per slide. Adjust the volume to the area so that it does not easily spill over but also does not dry out too quickly. To avoid evaporation, put slides in a slide box with wetted (water or PBS) tissues under the slides (not too wet, so no water flows on or under the slides)
3. Dilute the primary antibodies in antibody buffer, remove blocking buffer from glass slides, and add antibody-solution. Incubate overnight at 4ºC.
4. Wash 3x5 min with PBS a. Tap off fluid from glass slides on paper b. Move glass slide backward through PBS c. Place glass slide in Hellendahl cuvette PBS
5. Dilute the secondary antibodies in antibody buffer and incubate for 1h at RT. Close the slice box, re-wet the tissues if needed. a. NOTE: work in the dark from now on to avoid fluorophore quenching.
6. Wash 3x5 minutes with PBS.
7. Dilute DAPI (if wanted) in PBS and incubate the slides 20 minutes with DAPI at RT
8. Wash 3x with PBS and leave in PBS
9. In the fume hood, prepare forceps, glass cover slips and mounting solution
10. Prior to mounting, let excess PBS drain onto a tissue or gently shake the slide.
11. Mount the slides by adding a streak of mounting solution on the side of a glass coverslip. Set the slide on the coverslip at a 45º angle and let the side of the glass slide (with the sample) come into contact with the mounting solution. Slowly lower the slide down on the cover slip and let the mounting solution spread. Avoid big bubbles, strike them out with the forceps or your finger, be mindful not to break the glass or slide the glass over the sample.
12. Image or store at 4ºC in the dark until use. Let come to RT prior to imaging.

Attachments This procedure was originally created by Lena Sophie Koch

A.2 Organoid/Spheroid Immunostaining

Organoid/Spheroid Immunostaining Category Stainings
Author Meike Blaauw
Labels: immunohistochemistry 3D organoid Spheroid

Chemicals:
* PBS
* PFA (Paraformaldehyde)
o make a 4% in PBS dilution
* BSA (or Normal Donkey Serum)
* Triton X-100
* Clearing solution
* Primary antibodies
* Secondary antibodies
* if needed structural marker (actin)
* DAPI 1:1000
* PNA Lectin

Solutions:
* Organoid Wash Buffer (OWB):
o 2% BSA (2 gr/100 mL)
o 0.1% v/v Triton X-100
o OPTIONAL: Normal Donkey Serum 1:10
Store at 4ºC.
* Fructose-Glycerol clearing solution

o 60% (v/v) Glycerol
o 2.5 M Fructose
o dH2O

Equipment:
* PAP pen (hydrophobic marker)
* Glass slides
* Glass coverslips
* Tissues
* Dubble sided tape

Clearing solution:
1. Combine 29,72 gram Fructose with 7 ml dH2O in a small beaker or 100 ml glass bottle
2. Stirr until dissolved (this takes time), put on heater plate (up to 65°C, do not let the sugar burn or caramelize!) to speed up the process
3. Let it cool to room temperature
4. Add 33 ml of glycerol and stirr to combine o Refractive index = 1.4688 at room temperature (RT: 19-23 °C)
o Stored at 4ºC fridge in Histology lab.
o Let come to RT before handling, otherwise it is too viscous!!

Fixation (LAF-hood):
1. Carefully aspirate as much medium from the organoids as possible
2. Wash 2x 5 min with dPBS
3. Add 4% PFA/PBS (1:10) ~300/400 µL
4. Incubate 30 - 45 min at RT with PFA depending on the thickness of the tissue (up to overnight at 4ºC for highly complex organoids)
5. Wash 3x 5 min with (d)PBS a. Don't aspirate the first two times, but put the PFA solution in a separate tube b. Organoids can be stored in PBS for up to 7 days (ideally continue asap)

Blocking and Immunolabeling:
Organoids can be stained in original container (plate or chip) but can be transferred to a suspension 24 wp if more organoids/spheroids are to be stained at the same time. EBs grown in V/U well plates are recommended to be transferred to low adherence (coat with anti-adherence solution if needed) 24/48/96 wp.
1. Incubate in OWB at RT for 1 hour.
2. Aspirate OWB and repeat step 1.
3. Aspirate OWB and add primary antibodies diluted in OWB. Incubate overnight at 4ºC with gently rocking/shaking movements. a. If not all solution can be aspirated. Aspirate 50% of blocking OWB and add 50% 2x concentrated primary antibodies in OWB
4. Aspirate primary antibody and wash 3x 1 hour with PBS at RT with mild orbital shaking.
5. Aspirate PBS and add secondary antibodies in OWB and incubate overnight at 4ºC on shaker.
6. Aspirate secondary antibody solution and wash 3x 1 hour in PBS at RT with mild orbital shaking.
7. Add DAPI (1:1000) (and PNA-Lectin 1:100) in OWB and incubate for 60 minutes.
8. Wash 3x 20 min in PBS at RT with mild orbital shaking.
9. For imaging, clearing the organoids is recommended.

Clearing step:
1. Remove as much of the PBS as possible.
2. Add fructose-glycerol clearing solution (minimum of 50 µl at RT) using either a large-bore 200µl tip or 200µl tip with ends cut off and resuspend gently. Careful not to produce bubbles a. The clearing solution is extremely viscous. Pipet slowly and make sure it is at RT!
3. Incubate at RT for 20 min (at least). If needed swirl the organoid around with a 10 ul pipet tip or needle to really resuspend organoid in CS. b. Clearing causes shrinkage, but structures remain intact.

Organoids will become almost translucent and very hard to see! c. Organoids can be imaged in CS. No need to refresh d. Organoids can be stored at 4 °C (for at least 1 week) or -20 °C (for at least 6 months).

Imgaging preparation:
Organoids can be imaged in the plate if quick check is wanted, but can also be transferred to a coverslip:
1. Draw a 1 × 2-cm rectangle in the middle of a slide, using a PAP pen.
2. Place a 1-cm-long piece of sticky tape at both sides of the rectangle. Use 1, 2 or 3 layers, depending on the organoid size.
3. Use a large-bore pipet tip or cut off the end of a 200-µl tip and use it to place the organoids in the middle of the rectangle. Use 20 µl per layer of tape.
4. Place a coverslip on top. Place the left side of the coverslip on the left stack of sticky tape and then slowly lower the right side of the coverslip until it touches the right stack of sticky tape; then let go of the coverslip.
5. Wait for 1 min to allow the fluid to spread out.
6. Gently apply pressure to both sides of the coverslip to firmly attach it to the double-sided sticky tape.
7. The slide is now ready for imaging.

Attachments This procedure was originally created by Lena Sophie Koch