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Identifying Ciliary Proteins in Mammalian Retinas using a Gentle Extraction Method

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Abstract:

The retina, a complex structure in the internal layer of the eye that converts light signals to neural impulses to the brain. The structure consists of a junction between photoreceptors called the inner segment (IS)/outer segment (OS) line. This line has highly modified cilia that develop the primary cilia of the retina and connect the IS to the OS. The primary cilia structure is important because defects in ciliary proteins are associated with diseases such as retinitis pigmentosa (RP), a hereditary retinal degeneration. Currently, we lack an understanding of protein complexes involved in forming and maintaining primary cilia of the retina. We lack optimized protocols to obtain samples with primary cilia from retinal photoreceptors to use in high-throughput proteomic studies (Wheway et al., 2014). Here, bovine eyes were used to conduct the research on ciliary proteins in the retina due to their availability and size which allows a high yield of proteins harvested per retina. The eyes were dissected in a darkroom to avoid light stimulation, a Mammalian ringer solution was used to gently release the retina from the back of the eye and optic nerve (Panfoli et al., 2022). A lysis buffer was used with a vortex to break the retina cells. The lysed cells were centrifuged leaving a supernatant. Once the proteins were retrieved, detergent compatible protein assay was used followed by mass spectrometry to identify proteins present. Researching the retina allows an in depth look at the structure and function of a highly modified and specialized primary cilia. Future plans to extend this work include a co-fractionation mass spectrometry pipeline to identify protein complexes in retinal photoreceptors; this will allow us to map the molecular network of disease relevant cells.

Introduction:

The most important organ of our senses are the eyes. They are responsible for 80% of all impressions by means of our sight (Why Good Vision is so Important, 2021). Our eyes are our

windows into the world however, according to O'Neal & Luther "1 in 4,000 Americans suffer from retinitis pigmentosa (RP) and there are no cures" (O'Neal & Luther, 2022). RP is a hereditary disease that is caused by defects in the primary cilia. Within the human body there are two types of cilia, motile and non-motile. Motile cilia are projections on the outside of cells that help the cell move or help move debris through the body by using a whip-like motion. The structure of motile cilia comprises of dynein ATPase motor microtubules that cause the cilia to move. Non-motile cilia, on the other hand, lack this motor and have a different structure altogether (Ishikawa, 2017). Non-motile cilia, also known as primary cilia, consist of a unique structure of an axoneme and a basal body. Primary cilia are concentrated in the retina. The retina is a thin piece of tissue lining the back of the eye. It is responsible for conducting visuals from the eye to the brain. With any damage to the retina most vision would be lost. Primary cilia are an important part of the retina. These cilia are part of the outer and inner segments of the retina (Figure 1). The outer segment role is to absorb light and transduce the signal into a nerve signal. The inner segment responsibility is to regulate these nerve signals and keep the outer segment attached to the retina. (Baker & Kerov, 2013). With damage to the outer/inner segment the information that your eyes intake would never reach your brain and therefore, you would not be able to understand what you were looking at.

My research is to study the primary cilia and the proteins that are responsible for keeping these cilia viable. To understand the proteins involved we needed to construct a gentle extraction method that can keep the cilia intact to attempt to keep our sample as densely rich with primary cilia and proteins. Currently, there is a lack of understanding of protein complexes involved in forming and maintaining primary cilia of the retina as well as protocols to obtain samples with primary cilia from retinal photoreceptors to use in high-throughput proteomic studies. Our goal is

to determine which protein complexes in primary cilia are present and to characterize them in disease-relevant tissues by creating a new gentle extraction protocol.

Methods:

The bovine eyes were obtained from Innovative Research's website. The eyes were US Origin Bovine eye dark adapted retina (*Innovative Grade US Origin Bovine Eye Dark Adapted Retina*, 2023).

Thirty bovine eyes were delivered to the lab and the dissection began 40 minutes after delivery, however, the sooner the eyes are dissected after delivery the better. We turned off the overhead lights and closed blinds to all windows in the room to reduce light exposure to the retinas. The lens is cut off, and the vitreous humor is discarded to expose the back of the eye and the retina. Mammalian Ringer solution is added to a basin with the half-cut backside of the eye. The eyes were covered with foil and gently shaken with an orbital shaker for 20 minutes at 90 speed with no sign of retinas detaching. We increased the speed to 125 for 30 minutes. The basin was taped down to avoid spillage. After incubation, we used tweezers to gently release the thin retinal tissue from the back of the eye. (Panfoli et al., 2022).

Once the retina is released, the optic nerve is cut, and 15 retinas are placed in a 50 mL centrifuge grade tube and the other 15 retinas are placed in another 50mL centrifuge grade tube. To the tubes, 500uL of Buffer A (10 mM Pipes, pH 7.0, 5 mM $MgCl_2$, and 1× proteinase inhibitor mixture) with 50% (w/v) sucrose is added. Both tubes were vortexed to dislodge the cilia. The tubes were centrifuged for 20 minutes at 13,000 g, 4C.

Once the 20 minutes were finished, we gently removed the tubes to not disturbed the pellet at the bottom of the tubes. The supernatant was a dark red color and was removed in thirds

and put in a new test tube. We diluted the sample with a 1:1 ratio of Buffer A without sucrose. We gently pipetted up and down to mix the sample.

A sucrose cushion is gently added to the bottom of the tube with a blunt edge needle. There should be a clear phase separation. Before placing the two samples in the centrifuge again, weigh the tubes and carefully add Buffer A to even out the weights. Our tubes weighed approximately 400 uL each. We centrifuged both tubes again for 20 minutes at 13,000 g, 4C.

Carefully remove tubes from the centrifuge to not disrupt the phase separation. The cilia are in the interphase separation zone and are recovered using a micropipette. We added the two interphases into one tube (about 7mL total). Dilute sample into a 1:1 ratio with Buffer A (no sucrose) and centrifuge for 1 hour, 13,000g, 4C. After centrifugation, there is a pellet at the bottom of the tube which is incubated in Buffer B (10 mM Pipes, pH 7.0, 5 mM MgCl2, 1% Triton X-100, 1 mM DTT, and 1× proteinase inhibitor mixture). The sample is centrifuged again, and the supernatant is filtered and saved (Lui et at., 2007). The supernatant contains proteins that are found in primary cilia.

The sample was injected into a machine called High-performance Liquid Chromatography (HPLC). HPLC separates the components of the sample down its column and divides them into fractions from large to small (Wan et al., 2015). Once the HPLC identifies the sample constituent and collects the sample data, we estimated the size of proteins and compared it to previous protein standards. The full protocol is found in *Appendix A*.

Results:

The goal of this research was to find a gentle extraction method and identify protein complexes within primary cilia. Our research started with dissection then led to HPLC and Mass Spec runs (Figure 2). Our research was run twice, once in January 2023 and once in May 2023.

During our first run, we did not run a successful HPCL sample, however, we sent two replicate samples to Mass-Spec and received a list of ~1,200 proteins within both samples. To analyze this data, we compared our data with the Syscilia database. Syscilia's database records all cilia within the human proteasome. After merging our data with the syscilia database we found 23 genes that our bovine sample had in common with humans.

With the data from the two replicates and the syscilia database we created a bar graph to indicate which genes were similar and which genes were the most abundant (Figure 4). The most common gene DPYSL2 with an abundance of ~80 psm is associated with neuronal development and axon growth. Within the 23 genes found, three of them were directly linked to human ciliopathies. Genes CNGB1 and STX3 are associated with retinitis pigmentosa which is a disease that affects how the retina responds to light. Both genes had an abundance of roughly 13 psm. CNGB1 is a coactivator for transcription, cell adhesion, and neurogenesis. Gene STX3 is important for docking synaptic vesicles and for membrane fusion. The other gene linked to human ciliopathies is LZTFL1. This gene corresponds with Bardet-Biedl Syndrome (BBS). BBS is a ciliopathy that affects rod and cone development in the retina, renal formation, mental development, and obesity (Elawad et al., 2022). LZTFL1 has an abundance of ~5 psm.
Using the Go Enrichment database, we looked at the top 40 most abundant genes in our Mass Spec data and found that they were enriched with non-motile cilium, cilium structure, and photoreceptor annotations. As a result, we know that our 40 most abundant genes are associated with the structure of primary cilia or photoreceptors in the retina.

Using the bovine data identified by mass-spec, I looked into a paper that conducted a similar experiment with mouse eyes authored by Liu and colleagues. Looking at their raw data I formulated a graph of their mouse retina protein data to our bovine retina protein data and

created a scatter plot (Figure 3). The scatter plot places the abundance of bovine proteins from our raw Mass-Spec data on the y axis and the abundance of Liu and colleagues' paper on the x-axis. I made the graph coordinated as logs because the raw plot was congested within the 1-20 psm regions. The Spearman correlation was 0.394 showing that our data is correlated with Liu's however, it is not as correlated as I predicted. This could be due to the use of different species, differences in sample preparation, or the use of different mass spec machines which hinders the chances of having perfect correlation. When investigating the highest abundant proteins in both datasets I found that there are eight proteins that are similar in abundance with both Lui et al. data and our bovine data. The protein with the highest abundance of both species is a spectrum beta chain known as Fodrin which plays a role in the central nervous system and cell secretion. This confirms that our gentle extraction method produced proteins found in both bovine and mouse specimens.

During our first run we had issues with our HPLC plot. Our data was not consistent with our control group. However, during our second run, our data formed distinct peaks that correlated with proteins. For our HPLC run we tested two replicate samples split into 51 fractions that we combined into nine larger fractions. We found the highest peak to be between 41-45 fractions or within the fifth fraction out of nine with a normalized value of over 1. The second highest peak in our HPLC data was within the second fraction where we observed a normalized value of 0.4. The rest of the nine larger fractions showed normalized values around 0.25.

With this information, we decided to perform a protein assay to test how much protein actually obtained in the nine fractions. We used BIORAD DC Assay which is a detergent compatible protein assay that is colorimetric to protein concentration. It is meant to mimic the

Lowry assay with a faster incubation period. Figure 6 indicates the concentration of protein in each of the 9 fractions. Fraction three contained the highest protein concentration of around 14.35ng. Our HPLC data did not correlate with this data as our highest peak of protein absorbance was in fraction 3 but our highest HPLC peak was in fraction 5. We calculated our total protein concentration and concluded that we did not have enough protein to accurately run a Mass-Spec session. We found that we had between 4.12ng and 14.35 ng of protein concentration in our second run. We needed each fraction in protein assay to be 5ug therefore, we decided to not send our second experimental run through a mass-spec analysis.

Discussion:

In this study we wanted to find a gentle extraction method to detach primary cilia from the retina due to the gap in the proteomics field of understanding protein complexes involved in forming and maintaining primary cilia of the retina and because we lack optimized protocols to obtain samples with primary cilia from retinal photoreceptors to use in high-throughput proteomic studies. To shrink this gap, we created a new protocol based on Panfoli and colleagues' protocol.

We used buffers, centrifugation, mass-spec, and HPLC to obtain a high yield of proteins and identify them. During run 2 we took a small sample of raw bovine retina to view the primary cilia's original form under a microscope, however, we could not find the structure. This could be because we did not use enough retina to be able to view the primary cilia or that the retina we used did not have the number of primary cilia we expected it to have. This could also be the reason why we did not have enough protein concentration in run 2 to use the mass-spec machine. This lack of proteins was not what we were expecting because in run 2 we tripled the number of bovine eyes from 10 used in the first run to 30 in the second run, yet, we still had extremely low

protein concentration. In run 1 we pooled all fractions together to be able to obtain 5 microliters of protein concentration to run the mass-spec machine and identify protein within the sample (Figure 4). Based on the results, we found 23 bovine proteins associated with human proteins however, since we pooled all purified fractions together, we did not have data on individual protein abundance in each fraction. In run 2, due to the lack of protein concentration in the 9 pooled fractions, we focused on getting an accurate HPLC sample. We pooled fractions 12-63 into 9 fractions. Fraction 1 pooled fractions 12-18, fraction 2 pooled 19-25, 26-33 fraction were pooled for fraction 3, fraction 4 included 34-40, fraction 5 was 41-45, fraction 6 had 46-50, fraction 7 included 51-55, fraction 8 pooled 56-59, and fraction 9 included 60-63. We observed that pooled fraction 5 containing fractions 41-45 had the tallest peak in both runs. This indicates that there were high protein levels there (Figure 5).

We compared our data to previous work from Liu and colleagues who studied mouse retinas. We took the ~1200 proteins we identified in our mass-spec data and Liu's data to create a scatter plot based on abundance. The plot exhibited many proteins in both data sets with low abundance being similar. However, the most abundant protein in both mouse and bovine is a spectrum beta chain also known as Fodrin. Fodrin plays a role in the development of the central nervous system and cell secretion. Fodrin being the most abundant in both species is understandable because of the large role retinas have in the central nervous system. The most abundant protein in the mouse sample is a cytoplasmic dynein heavy chain 1 which is an ATPase and a motor protein for vesicles and organelles along microtubules. One of primary cilia's main functions in the retina is to convert light to images and to this many vesicles are used. Having an abundance of this protein in the mouse sample is consistent with the cilia's function. The most abundant bovine protein is a pyruvate kinase which regulates cell metabolism (Israelsen &

Vander Heiden, 2015). Comparing bovine proteins to mouse proteins, the protein with the highest abundance in bovine and lowest abundance in the mouse sample is a brain acid soluble protein I that functions as an interference of oncogenic capacity of MYC. MYC is a disease protein coder (Hartl et al., 2020). On the other hand, the highest protein abundance in mice and lowest in the bovine sample is a rootletin which is a major component of ciliary rootlets that aids in ciliogenesis. It is unclear as to why the bovine sample had such a low abundance of an important ciliary protein. It may be due to how we prepared our sample for the mass-spec run. The lack of rootlin proteins could also be due to the way we harvested the retina. The rootlin is connected to the inner segment of the primary cilia. When we dissected the eye to obtain the retina, we may have left the inner segment still attached to the back of the eye. Another probable cause could be our detergents are too strong and denatured this specific protein. We identified more unique proteins with 1,653 total proteins greater than two peptide spectral matches than the Liu data set with only 1,059 total proteins greater than two spectral matches (Liu et al., 2007).

Interestingly, the three proteins found in Figure 4, CNGB1, STX3, and LZTFL1, are correlated with human ciliopathy diseases are not associated with the bovine proteins that are in relation with Liu and colleagues mouse data.

In future experiments, we will discuss why our protein concentration has been so low regardless of the number of eyes used. We will look into the company that supplied the eyes and perhaps try to reach out to a local farm to obtain fresh cow eyes as suggested in the Panfoli et al. paper. The fresh eyes may decrease the deformation of primary cilia and proteins as well. We may also need to increase our cow eye sample from 30 to 50-100 eyes. However, this increase causes an issue of needing to quickly and efficiently dissection the eyes to avoid degradation of

proteins. Another change we could make in the methods is to decrease the use of buffers to obtain a more concentrated sample. This may increase the protein concentration as well.

In sum, this research is important to understand the proteomics of primary cilia, to procure a gentle extraction method to understand what constitutes these cilia, and to be able to research diseases associated with proteins within the cilia. With this information, we hope to aid disease research in areas such as retinitis pigmentosa and bardet-biedl syndrome that deal with primary cilia structure and proteins.

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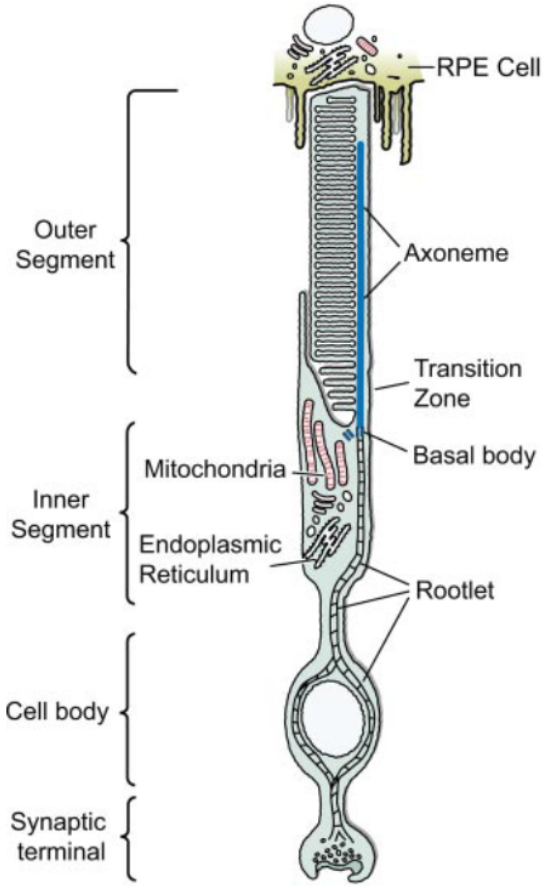


Figure 1: **A diagram of the structure of a primary cilium from Lui et al.**

The outer segment (OS) is where the primary cilia is located. The OS initiates visual signal transductions. The G-protein coupled receptor Rhodopsin is arrayed in disks in the outer segment of the rod cells. Light activates rhodopsin thereby hyperpolarizing the rod cell and sending a signal to downstream on-center bipolar cells. The inner segment (IS) contains the mitochondria and is where metabolism and regulation of membrane potential take place. The basal body is the specific name for the centrosome in the primary cilia. The axoneme consists of nine doublet microtubules. It lacks the central pair of microtubules, radial spokes, and out/inner dynein that are seen in motile cilia. These differences in the structure make the primary cilia non-motile.

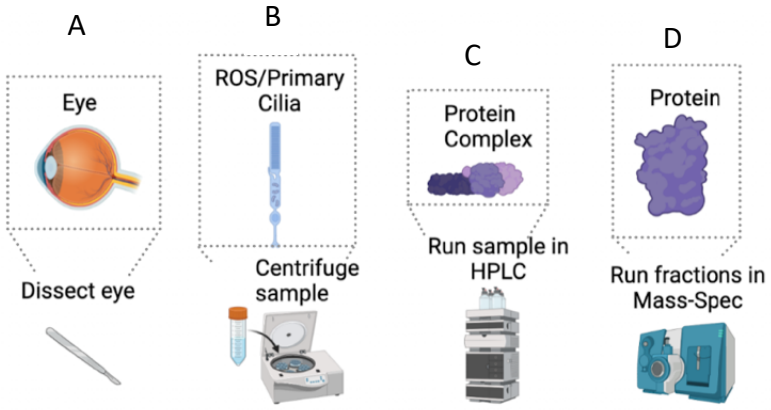


Figure 2: **Infographic of Methods**

A schematic of the methods used to conduct this experiment. The bovine eye was dissected using tools such as scalpels and forceps. *B* the ROS/Primary cilia are separated from the retinal tissue using centrifugation techniques. *C* the purified samples are ran in the HPLC to fractionate the sample to identify the protein interactions. *D* the proteins in the centrifuged samples are identified using a mass-spec machine.

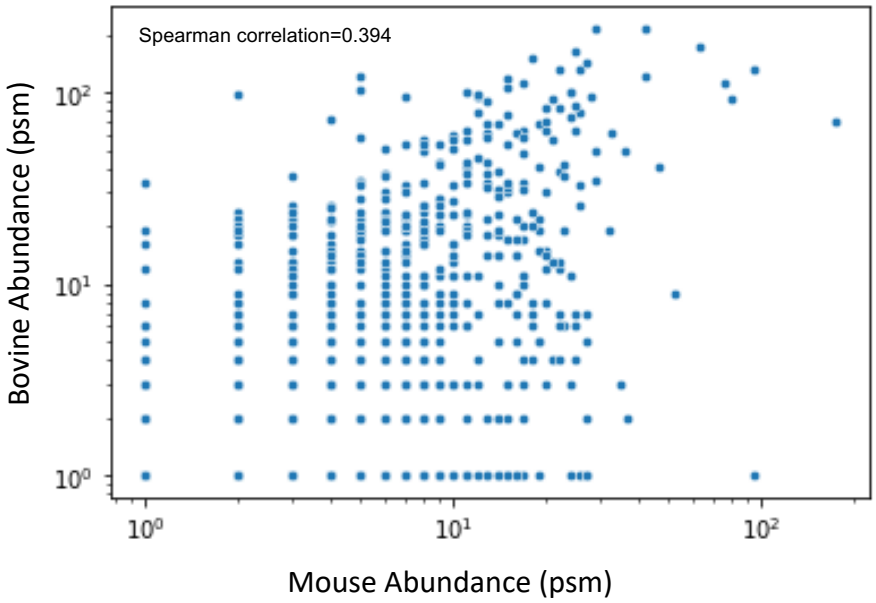
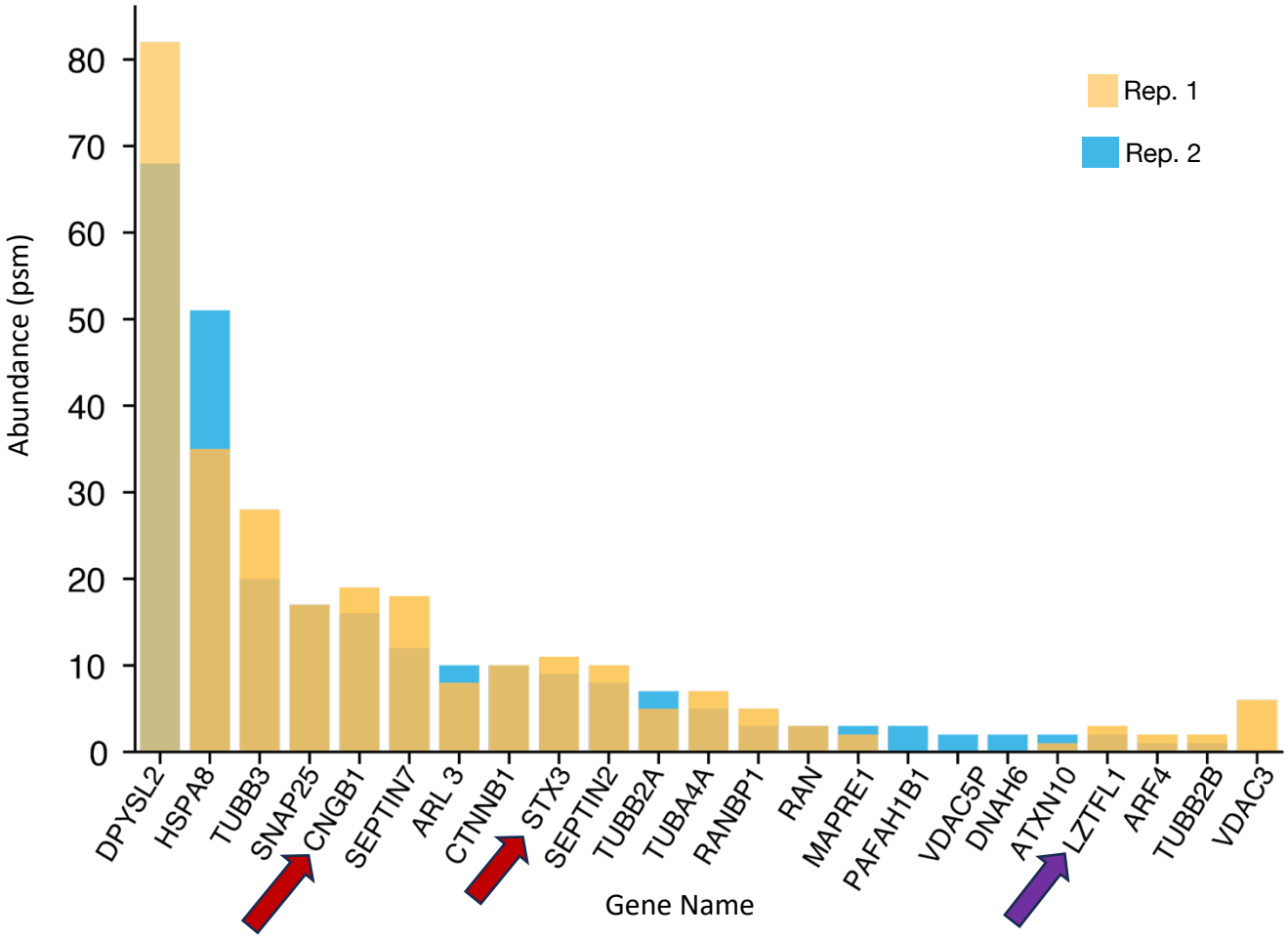


Figure 3: **Scatter plot of bovine data vs. Lui et. al mouse data**

A depiction of our bovine protein abundance data compared to a study by Liu et al. who used a similar purification process with mouse retinas. Both samples contained similar proteins at a low abundance and few proteins correspond with a high abundance in each sample.



Associated with
Retinitis Pigmentosa

Associated with
Bardet-Biedl Syndrome

Figure 4: **Mass-Spectrometry Data**

From our MS analysis we identified ~1200 bovine proteins; using GO enrichment analysis the 40 most abundant were enriched with non-motile cilium, cilium structure, and photoreceptors annotations. We cross referenced the proteins in our sample with the Syscilia database gold standard and found 23 proteins associated with cilia in our sample. The graph shows the abundance of 23 ciliary proteins within replicate samples. The genes CNGB1 and STX3 are associated with the hereditary disease called Retinitis pigmentosa that causes the retina to degenerate. The gene LZTFL1 is associated with Bardet-Biedl Syndrome with affects how cilia are structured. This disease causes blindness, obesity, and low mental capacity.

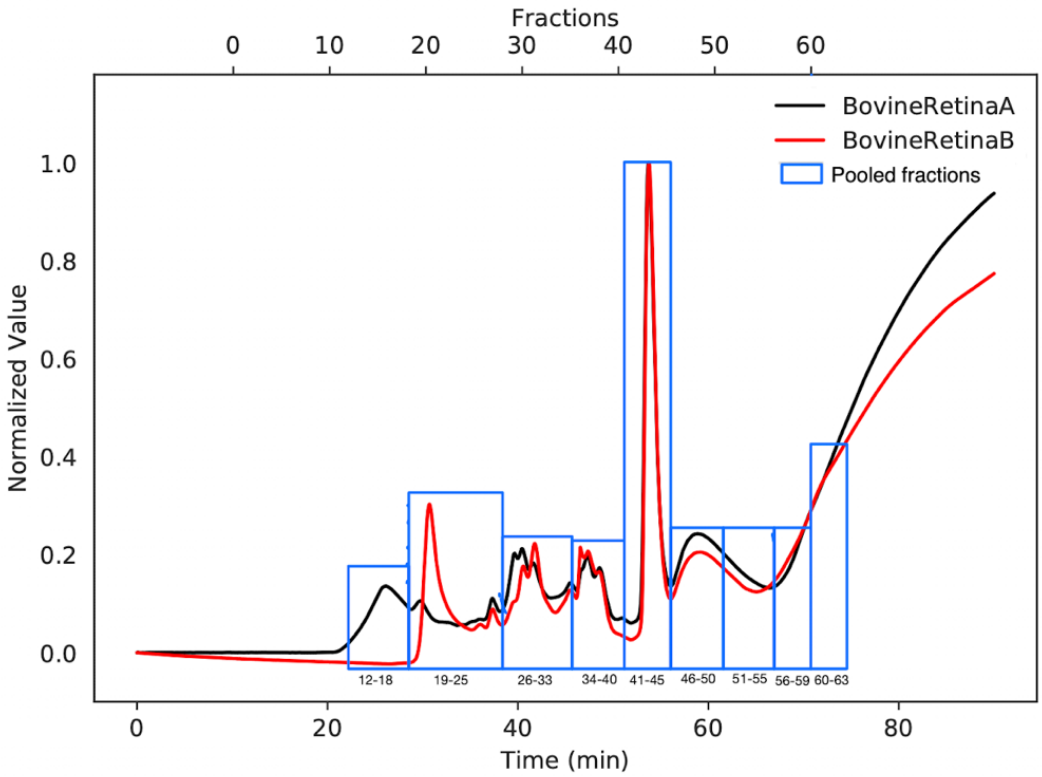


Figure 5: **HPLC Data**

In the second run of the experiment, we used HPLC to identify the fractions with the highest protein concentration. Two replicated samples were in the HPLC to determine if there were differences in the sample. The sample contained 12-63 wells that were pooled into nine fractions as seen above. The fraction with the highest peak and protein concertation was number five with wells 41-45 pooled together.

Fraction Well	ng protein remaining in well
G1	9.23
G2	9.23
G3	14.35
G4	9.23
G5	9.23
G6	4.12
G7	9.23
G8	4.12
G9	4.12

Figure 6: **Protein Assay Data**

In the second run of the experiment protein assay was used to indicate that pooled fraction with the highest protein concentration. Based off of this data fraction three comprised of wells 26-33 contained the highest protein concentration. However, this fraction did not depict the highest peak in Figure 5. The protein concentration in each fraction should have been within the range of ~5ug. This sample contain only 4-14 ng of protein. This run was not analyzed by mass spec due to the lack of protein concentration in each well.

Appendix A:

This procedure will be performed in 3 parts on the same day. Part 1 is retina dissection/extraction. Part 2 is ciliary complex purification. Part 3 is the HPLC protocol, this is detailed in a separate document. Preparation of buffers/reagents, tools, and workspace should be done the day before.

*Eye specimen will arrive at approximately 10AM

Buffers

Mammalian Ringer (MR) 1L

Mix the following into 500mL MQ water, add solution to 1000mL graduated cylinder and bring to volume. pH to 6.9

- 157 mL 1M NaCl
- .5 mL 1M KCl
- 3.5 mL 200mM Na_2HPO_4
- 4 mL 200mM NaH_2PO_4
- .05 mL 1M $MgCl_2$
- .05mL 1M $CaCl_2$
- 2mM glucose (0.36g)

Aliquot enough MR solution for experiment.

- add protease inhibitor (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail) 1 tablet per 10mL buffer

Filter sterilize solution.

Buffer A 1L

- 10mM Pipes
- 5mM $MgCl_2$
- Protease inhibitor
- pH 7

Buffer B 1L

- 10 mM Pipes,
- pH 7.0,
- 5 mm $MgCl_2$,
- 1% NP-40,
- 1× protease inhibitor mixture

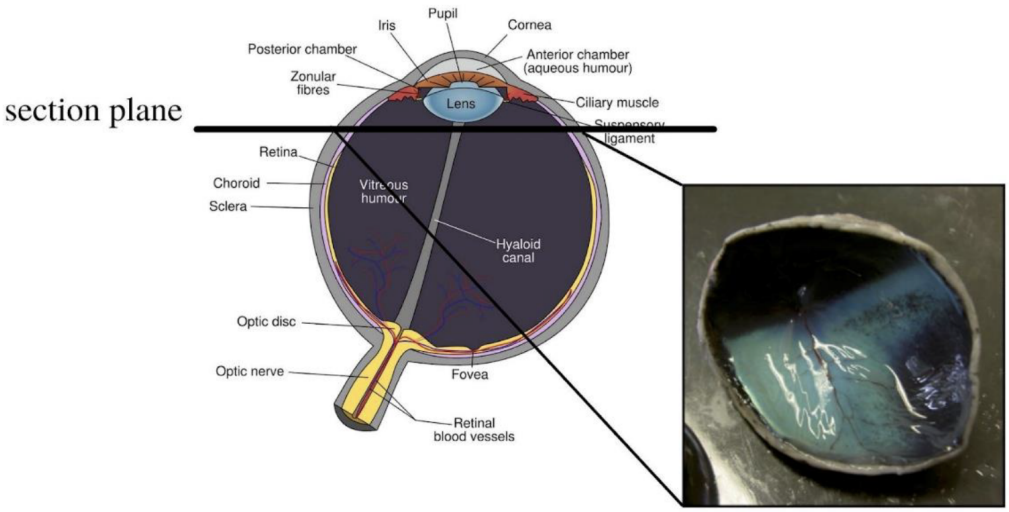
Part 1 Separating the Eye Semi-cup from the lens and vitreous humor

based on**:** *Panfoli, I. et al. (2022). Maximizing the Rod Outer Segment Yield in Retinas Extracted from Cattle Eyes. Bioprotocol 12(14): e4474. DOI: 10.21769/BioProtoc.4474.*

***All steps for Part 1 must be carried out at room temperature in a dark room.**

Be sure to don proper PPE before beginning, lab coat, gloves, cut resistant gloves, and eye protection. Cut resistant gloves go UNDER nitrile gloves.

1. Starting with whole bovine eyes, remove excess tissue with dissecting scissors. A lot of tissue and muscle will be present.
    a. This step should be done ASAP upon receipt of bovine eye specimen
2. Be sure to work slowly, so as not to squirt vitreous humor (eye goop) outside the dissection tray. Puncture the eye just below the lens with a scalpel. Then use scissors to cut a larger hole in the eye below the lens. Used forceps to hold the eye and scissors to cut. The retina is a blueish white opalescent layer. This will leave you with the lens and the "semi-cup" containing the retina. refer to the image below taken from the bioprotocol
    a. This step should be done in a nalgene tub or dissection tray lined with aluminum foil for easier cleanup



3. Place the eye semi-cups in a secondary container for retina detachment with MR
4. Fill the secondary container from step 3 with enough MR to completely cover the eye semi cups.
5. Incubate the eyes w/ MR for 10-15 min, cover tray/tub with aluminum foil. Gently shake the container every couple of minutes, or place on an orbital shaker at low speed if available. You will know when it's done as the retina can be seen freely floating inside the eye "semi-cup", it will only be attached by the optic nerve.
6. Once the retina is detached (it will just be hanging on by the optic nerve) quickly invert the eye "semi-cup" , dispose of the MR and use scissors to cut the optic nerve, fully releasing the retina.

   a. Invert the eye "semi-cup" over a waster container to catch the unwanted MR solution
   b. Once inverted, move the specimen over the 50mL conical tube for collection and cut the optic nerve
7. All retinas should be placed in 50mL centrifuge tubes. Split the number of retinas between two tubes for centrifuge balancing

Part 2 Isolation of Cilia

based on**:** *Liu et al. 2007* https://pubmed.ncbi.nlm.nih.gov/17494944/

Precool the Sorvall centrifuge. Put a sticker on the centrifuge that you need for this machine. Turn on machine: login 4109, pass 1234. If it doesn't work, login and pass are on a fridge in our lab. Put an empty rotor in a machine, tighten the screws and select it via the 'Rotor' button. You need 4°C, so select the temperature and spin the empty rotor for 15mins, at low rpm (1.5-2k).

1. Starting with (2) 50mL conical tubes containing the retinas, add 500uL of Buffer A (w/ 50% (w/v) sucrose) per retina.
2. Vigorously vortex the tubes from step 1, to dislodge cilia.
3. Centrifuge the tubes from step 2 for 20 minutes, 13,000 g, 4C
   a. Use the Sorvall centrifuge, the rotors are in the cold room
4. Gently remove the tubes from the centrifuge so as not to disturb the pellet
   a. Place the 50 mL tube in a single tube rack
   b. There will appear to be multiple layers in the supernatant, but treat all layers the same.
5. Remove the supernatant in thirds starting from the top, and put in a new tube
6. Dilute the collected sample from step 5 1:1 in Buffer A (without sucrose)
   a. Mix by gently pipetting up and down
7. In each tube from step 6 add an equal volume of 50% sucrose solution(50%w/v sucrose in buffer A).
   a. This is done carefully with a long blunt edge needle and syringe. The syringe tip is placed near the bottom of the tube and the plunger is depressed to slowly fill the bottom with the sucrose cushion. There should be clear phase separation (a line at the interface of the sample and sucrose cushion)
   b. Weigh to ensure centrifuge will be balanced. Add extra buffer A on top (very carefully and slowly) to bring to correct mass
8. Centrifuge Sample from Step 7 for 20 minutes, 13,000 g, 4C.
9. Carefully remove the tubes from the centrifuge so as not to disturb the phase separation. Our cilia will be at the interphase zone (around the phase separation line mentioned above)
10. Use a micropipette to remove the interphase zone resulting from step 9, and place in new tube. Use smallest tubes possible, see note in step 12 for reason.

11. Dilute the collected fraction from step 10 in buffer A(dilute 1:1) and spin down 1h, 13,000 g, 4°C.
    a. Mix by gently pipetting up and down
    b. Create a balance tube by weight with DI water if needed.
    c. The protein should sediment out.
12. Incubate pellet in Buffer B, 1h, 4°C (in ice). vortex or pipette up and down with a cut-off pipette tip
    a. Small tubes should be used in step 10. In our first experiment, our sample ended up too dilute, a smaller tube would make it easier to cover the pellet with a lesser volume of buffer B
    b. looking to hit 5-10mg/ml of protein for the HPLC run
13. Transfer mixture to multiple lo bind microcentrifuge tube(s) perform clarifying ultracentrifugation (~100 x g ,45')
14. Aliquot supernatant(s) into new lobind tube
15. Centrifuge 4C 10 min at 13,000 g
16. Aliquot supernatant(s) into new lobind tube
17. Use the DC assay to determine protein concentration.
18. Mix 400uL supernatant and 100uL PBS in a new lobind tube
19. Add 500uL supernatant+PBS to prerinsed centrifuge filter (filters should be prewashed with 500uL LCMS optima water 2 min 12,000g)
20. Spin sample 4C 2 min 12,000g
21. Filter may become clogged and multiple filtrations may be necessary to filter all 500uL
22. Repeat steps 16-19 with treatment for HPLC run