

Identifying Ciliary Proteins in Mammalian Retinas using a Gentle Extraction Method

Adeline S. Fredrick¹, Erin R. Claussen¹, Samantha N. Fischer¹, Samson Balasanyants¹, Akshaya Rajaraman¹, Andi C. Rosner¹, Kevin Drew^{1§}

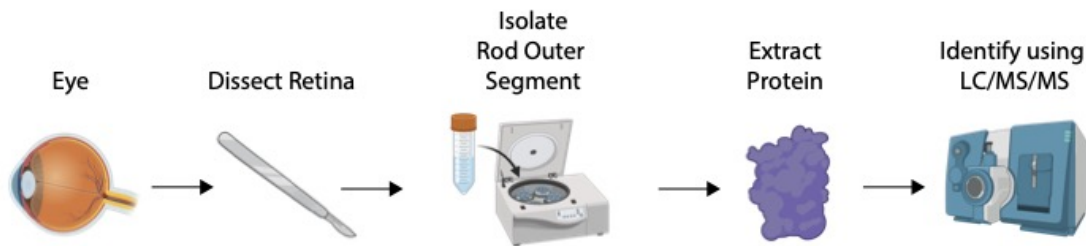
¹Biological Sciences, University of Illinois at Chicago, Chicago, Illinois, United States

§To whom correspondence should be addressed: ksdrew@uic.edu

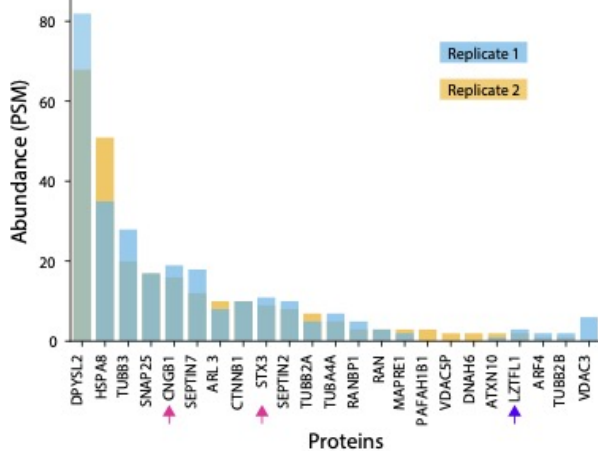
Abstract

Mutations in retinal primary cilia are responsible for human blindness but the mechanisms are not fully understood (Whewy et al., 2014). Characterizing the proteome of an organelle such as cilia, is a fruitful way to understand its function but methods often require considerable sample quantities. Here we develop a method to isolate the primary cilia of photoreceptor cells from bovine retinas. Through LC/MS/MS proteomics analysis we identify proteins enriched for cilia function including ciliopathy disease. This study shows our method can be used to isolate retinal primary cilia to obtain sufficient quantities of native protein samples.

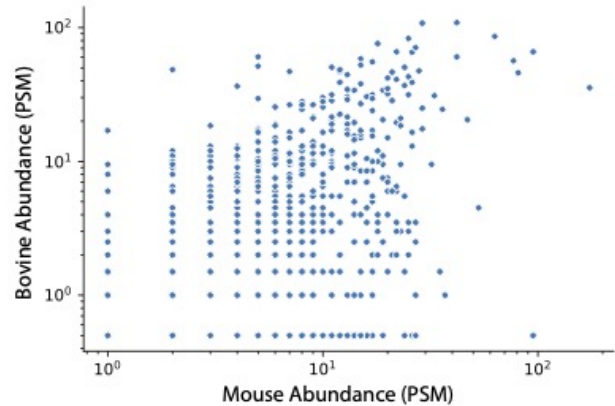
A



B



C



D

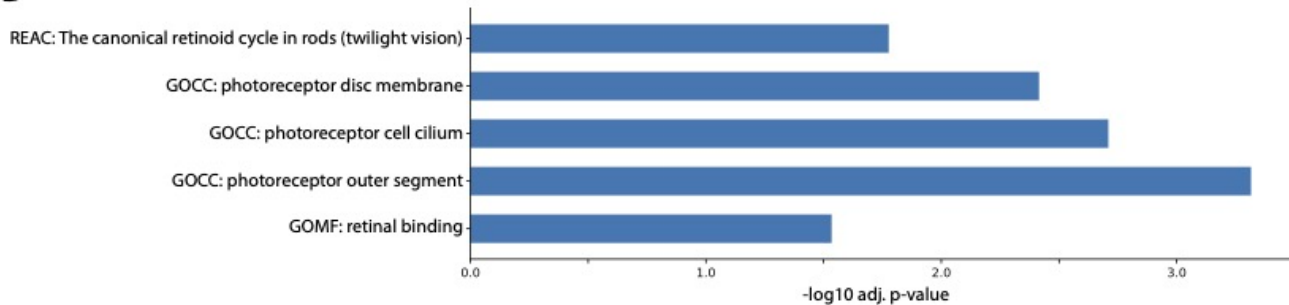


Figure 1. Isolation and proteomic analysis of primary cilia from bovine retinas:

(A) A schematic of the methods used to conduct this experiment. The bovine eye was dissected using scalpels and forceps. Next, the rod outer segment of the primary cilia was separated from the retinal tissue using centrifugation. The proteins were extracted and then identified using mass spectrometry (LC/MS/MS). (B) From our MS analysis we identified ~1200 bovine proteins; 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. Pink arrow=RP associated proteins and purple arrow=Bardet-Biedl Syndrome associated protein. (C) Comparison of our MS bovine protein abundance data compared to proteins identified in mouse retinas (Liu et al., 2007). (D) Annotation enrichment analysis of the 40 most abundant proteins in our sample and select significant annotations. Proteins were analyzed by filtering the top 40 proteins by total PSMs across both replicates. (REAC=Reactome, GOCC=Gene Ontology Cellular Component, GOMF=Gene Ontology Molecular Function, PSM = peptide spectral match) (Images in Figure 1A were created with BioRender).

Description

Retinitis pigmentosa (RP) is a rare eye disease that results in vision loss for 1 in 4000 in the United States and has no cure (O'Neal & Luther, 2024). A common cause of RP is disruptions in the primary cilia of the retina, the tissue responsible for conducting visuals from the eye to the brain through neural connections. To better understand the molecular causes of RP, studies have focused on identifying proteins in retinal primary cilia (Liu et al., 2007) but have been limited in detailing the full protein interaction network. High throughput proteomics experiments that characterize protein complexes and protein interactions often require a large amount of biological material (Drew et al., 2020; Mallam et al., 2019). Large mammals such as bovine (*Bos taurus*) are an attractive model to fill this need. In particular, the bovine eye is comparable in size to the human eye and ~10x larger than the mouse eye (Kralik et al., 2023). Moreover, bovine eyes are diurnal (same as humans) and highly visual as opposed to mice being nocturnal, making bovine retinas a better model for human vision (Kralik et al., 2023). The goal of this research is to develop a gentle extraction method to isolate native complexes from primary cilia of bovine retinas. Here we describe our method to obtain retinal primary cilia sample and demonstrate the identification of proteins associated with retinal primary cilia.

Figure 1A describes our experimental workflow. We first acquired and dissected 10 bovine eyes to extract retinal tissue. We then isolated the modified primary cilia of the retina known as rod outer segments through centrifugation. We measured the final protein concentration of our sample to be 0.5mg/ml and resulted in an estimated ~1 mg total protein. Finally, we prepared the sample for bottom-up proteomics for protein identification using LC/MS/MS. Two replicate samples were sent for proteomic analysis where ~1,200 proteins were identified (see ExtendedData Table 1). To analyze these data, we compared our identified proteins with the Syscilia database, a collection of human ciliary proteins (Vasquez et al., 2021). Comparing our data with the Syscilia database, 23 proteins were found that our bovine sample had in common with human ciliary associated proteins (Figure 1B). Of these 23 proteins, three of them were directly linked to human ciliopathies. Specifically, CNGB1 and STX3 are associated with RP which affects how the retina responds to light. CNGB1 is a coactivator for transcription, cell adhesion, and neurogenesis. STX3 is important for docking synaptic vesicles and for membrane fusion. We also identify LZTFL1 which is linked to Bardet-Biedl Syndrome (BBS), a ciliopathy that affects rod and cone development in the retina, renal formation, mental development, and obesity (Marion et al., 2012). To demonstrate our identification of the ciliary proteome in an orthogonal analysis, we used GO enrichment to identify enriched functional annotations within our identified proteins. Specifically, we searched for annotations that were enriched in the 40 most abundant proteins and found terms associated with the photoreceptor outer segment (GO:0001750, $p_{adj} 4.836 \times 10^{-4}$), photoreceptor cell cilium (GO:0097733, $p_{adj} 1.952 \times 10^{-3}$) and other related terms (Figure 1D). Collectively, this is evidence that the proteins identified through our retinal cilia extraction method from bovine retinas are associated with human ciliopathy diseases and generally, retinal ciliary proteins.

We finally compared the proteins identified from our bovine retinal sample to a sample obtained in a similar manner from mouse retinas (Liu et al., 2007) (Figure 1C). The Spearman correlation coefficient between the two datasets was 0.394 showing that our data is partially correlated with the mouse retina data. We do however see overlap among the most abundant proteins including Pkm, Map1b, Sptbn1(Fodrin), and Atp1a3, which are known to localize and function in neurons. In total, we identify 1625 proteins (PSM > 1) compared to 1058 identified proteins by Liu et al. (PSM > 1). Taken together, this suggests that the high abundant proteins identified by our gentle extraction method are in agreement with previous studies and our study extends the number of identified proteins in the retinal primary cilium overall. 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.

Methods

We followed the protocols outlined by Panfoli and colleagues ([Panfoli et al., 2022](#)) for the dissection of retinas from 10 bovine eyes. In a dark room, we cut around the lens of the eyes with a scalpel and removed the vitreous humor. Once the eyes were hollowed, we incubated them in a mammalian ringer solution for 15 minutes on an orbital shaker at low speed. Using scissors, we severed the optical nerve from the back of the retina. We used forceps to remove retinas and placed them in a 50ml conical tube. Once retinas were isolated, we followed the protocols from Liu and colleagues ([Liu et al., 2007](#)) to isolate rod outer segments. We applied the sample to a 50% sucrose cushion in Buffer A and centrifuged for 20 minutes at 13,000 g. We recovered the interphase, diluted in 1:1 buffer A, and centrifuged for 20 minutes at 13,000g. We then incubated the pellet in buffer B on ice for 1 hour, occasionally pipetting up and down. To determine protein concentration in the sample, we used the detergent-compatible BioRad DC protein assay kit. The sample was then processed twice as two separate technical replicates for bottom-up proteomics. To prepare samples for MS analysis, samples were diluted to a final concentration of 5% TFE by adding 880 μ l of digestion buffer. Trypsin was added to a final concentration of 1:25-1:100 enzyme:protein (2 μ g trypsin for 1-2 mg/ml). Samples were desalted using C18 spin tips. Peptides were analyzed using a ThermoScientific Acclaim PepMap 100 C18 HPLC Column (75-minute gradient) followed by nano electrospray-ionization and tandem mass spectrometry on a Thermo Q Exactive HF. Data-dependent acquisition was activated, with parent ion (MS1) scans collected at high resolution. Ions with charge 1 were selected for collision-induced dissociation fragmentation spectrum acquisition (MS2) (HCD, collisionEnergy=28.0). MS was acquired in the UIC Proteomics Core Facility. MSBlender was used for mass spectra lookup for protein identification ([Kwon et al., 2011](#)) using Uniprot bovine unreviewed proteome (UP000009136_9913). For annotation enrichment analysis we used G Profiler ([Kolberg et al., 2023](#)). Additional description of method details can be found in Extended Data Text: Identifying Ciliary Proteins. In addition, we performed label-free quantification (LFQ) using Fragpipe (version 21.1) built-in workflow “LFQ-MBR” with default settings, using the same database as before. The mzML files were converted from raw data using Proteowizard (version 3.0.20287) ([Kessner et al., 2008](#)) MSConvert. MSFragger (version 4.0) was performed using default closed search parameters with mass calibration and parameter optimization ([Kong et al., 2017](#)). Peptide-spectral match validation was performed using Percolator ([Käll et al., 2007](#)). Protein inference and FDR filtering were performed with ProteinProphet ([Nesvizhskii et al., 2003](#)) from the Philosopher (v. 5.1.0) toolkit ([da Veiga Leprevost et al., 2020](#)). These results can be found in ExtendedData Table 2.

Data Availability:

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE ([Perez-Riverol et al., 2021](#)) partner repository with the dataset identifier PXD051952.

Reagents

Mammalian Ringer Solution (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, 0.5 mL 1M KCl, 3.5 mL 200mM Na ₂ HPO ₄ , 4 mL 200mM NaH ₂ PO ₄ , 0.05 mL 1M MgCl ₂ , 0.05mL 1M CaCl ₂ , 2mM glucose (0.36g), cOmpete, EDTA-free Protease Inhibitor Cocktail, Roche
Buffer A (1L)	10mM Pipes, 5mM MgCl ₂ , cOmpete, EDTA-free Protease Inhibitor Cocktail, Roche, pH 7
Buffer B (1L)	10 mM Pipes, pH 7.0, 5 mm MgCl ₂ , Triton X-100, 1 mm DTT 1%, EDTA-free Protease Inhibitor Cocktail, Roche
Digestion Buffer	50mM Tris-HCl 2mM CaCl ₂ pH 8
Bovine Eyes	Sierra For Medical Science, Whittier, CA

Extended Data

Description: ExtendedData Table 1 Bovine Retina Mass Spec. Resource Type: Dataset. File: [ExtendedDataTable1 Bovine retina mass spec 20240525.xlsx](#). DOI: [10.22002/wj77d-mpj78](#)

Description: ExtendedData Table 2 Bovine Retina LFQ Mass Spec. Resource Type: Dataset. File: [ExtendedDataTable2 Bovine retina LFQ mass spec.xlsx](#). DOI: [10.22002/5nnjr-9fy77](#)

Description: Identifying Ciliary Proteins. Resource Type: Text. File: [Identifying Ciliary Proteins FredrickPDF.pdf](#). DOI: [10.22002/qjwch-e0t24](#)

References

da Veiga Leprevost F, Haynes SE, Avtonomov DM, Chang HY, Shanmugam AK, Mellacheruvu D, Kong AT, Nesvizhskii AI. 2020. Philosopher: a versatile toolkit for shotgun proteomics data analysis. *Nature Methods* 17: 869-870. PubMed ID: [32669682](#)

Drew K, Lee C, Cox RM, Dang V, Devitt CC, McWhite CD, et al., Wallingford JB. 2020. A systematic, label-free method for identifying RNA-associated proteins in vivo provides insights into vertebrate ciliary beating machinery. *Dev Biol* 467(1-2): 108-117. PubMed ID: [32898505](#)

Käll L, Canterbury JD, Weston J, Noble WS, MacCoss MJ. 2007. Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nature Methods* 4: 923-925. DOI: [10.1038/nmeth1113](#)

Kessner D, Chambers M, Burke R, Agus D, Mallick P. 2008. ProteoWizard: open source software for rapid proteomics tools development. *Bioinformatics* 24(21): 2534-6. PubMed ID: [18606607](#)

Kolberg L, Raudvere U, Kuzmin I, Adler P, Vilo J, Peterson H. 2023. g:Profiler—interoperable web service for functional enrichment analysis and gene identifier mapping (2023 update). *Nucleic Acids Research* 51: W207-W212. DOI: [10.1093/nar/gkad347](#)

Kong AT, Leprevost FV, Avtonomov DM, Mellacheruvu D, Nesvizhskii AI. 2017. MSFragger: ultrafast and comprehensive peptide identification in mass spectrometry-based proteomics. *Nature Methods* 14: 513-520. DOI: [10.1038/nmeth.4256](#)

Kralik J, van Wyk M, Leonardon B, Schilardi G, Schneider S, Kleinlogel S. 2023. The Bovine Ex Vivo Retina: A Versatile Model for Retinal Neuroscience. *Investigative Ophthalmology & Visual Science* 64: 29. DOI: [10.1167/iovs.64.11.29](#)

Kwon T, Choi H, Vogel C, Nesvizhskii AI, Marcotte EM. 2011. MSblender: A probabilistic approach for integrating peptide identifications from multiple database search engines. *J Proteome Res* 10(7): 2949-58. PubMed ID: [21488652](#)

Liu Q, Tan G, Levenkova N, Li T, Pugh EN Jr, Rux JJ, Speicher DW, Pierce EA. 2007. The proteome of the mouse photoreceptor sensory cilium complex. *Mol Cell Proteomics* 6(8): 1299-317. PubMed ID: [17494944](#)

Mallam AL, Sae-Lee W, Schaub JM, Tu F, Battenhouse A, Jang YJ, et al., Drew K. 2019. Systematic Discovery of Endogenous Human Ribonucleoprotein Complexes. *Cell Rep* 29(5): 1351-1368.e5. PubMed ID: [31665645](#)

Marion V, Stutzmann F, Gérard M, De Melo C, Schaefer E, Claussmann A, et al., Dollfus H. 2012. Exome sequencing identifies mutations in LZTFL1, a BBSome and smoothed trafficking regulator, in a family with Bardet-Biedl syndrome with situs inversus and insertional polydactyly. *J Med Genet* 49(5): 317-21. PubMed ID: [22510444](#)

Nesvizhskii AI, Keller A, Kolker E, Aebersold R. 2003. A Statistical Model for Identifying Proteins by Tandem Mass Spectrometry. *Analytical Chemistry* 75: 4646-4658. DOI: [10.1021/ac0341261](#)

O'Neal TB, Luther EE. 2024. Retinitis Pigmentosa. *StatPearls Publishing*. PubMed ID: [30137803](#)

Panfili I, Calzia D, Ravera S, Bianchini P, Diaspro A. 2022. Maximizing the Rod Outer Segment Yield in Retinas Extracted from Cattle Eyes. *Bio Protoc* 12(14). PubMed ID: [35978578](#)

Perez-Riverol Y, Bai J, Bandla C, García-Seisdedos D, Hewapathirana S, Kamatchinathan S, et al., Vizcaíno. 2021. The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Research* 50: D543-D552. DOI: [10.1093/nar/gkab1038](#)

Vasquez SSV, van Dam J, Whewey G. 2021. An updated SYSCILIA gold standard (SCGSv2) of known ciliary genes, revealing the vast progress that has been made in the cilia research field. *Mol Biol Cell* 32(22): br13. PubMed ID: [34613793](#)

Whewey G, Parry DA, Johnson CA. 2014. The role of primary cilia in the development and disease of the retina. *Organogenesis* 10(1): 69-85. PubMed ID: [24162842](#)

Funding:

This work was supported by grants from the National Institutes of Health (R00 HD092613 and L40 HD096554 to Kevin Drew) and from UIC Undergraduate Honors College (Adeline S. Fredrick). We would also like to acknowledge the UIC Mass

6/13/2024 - Open Access

Spectrometry Core Lab for processing proteomics samples.

Author Contributions: Adeline S. Fredrick: data curation, formal analysis, methodology, investigation, writing - original draft, writing - review editing, funding acquisition. Erin R. Claussen: formal analysis, methodology, data curation. Samantha N. Fischer: methodology, formal analysis, data curation. Samson Balasanyants: methodology. Akshaya Rajaraman: methodology. Andi C. Rosner: methodology. Kevin Drew: conceptualization, formal analysis, funding acquisition, project administration, supervision, writing - review editing.

Reviewed By: Anonymous

History: Received April 30, 2024 **Revision Received** May 31, 2024 **Accepted** June 12, 2024 **Published Online** June 13, 2024 **Indexed** June 27, 2024

Copyright: © 2024 by the authors. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International (CC BY 4.0) License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Citation: Fredrick, AS; Claussen, ER; Fischer, SN; Balasanyants, S; Rajaraman, A; Rosner, AC; Drew, K (2024). Identifying Ciliary Proteins in Mammalian Retinas using a Gentle Extraction Method. microPublication Biology. [10.17912/micropub.biology.001218](https://doi.org/10.17912/micropub.biology.001218)