

BIO 216 Lab: Inquiry driven research in Molecular Biology

Dr. Jennifer Larimore
jlarimore@agnesscott.edu

OFFICE LOCATION: BSC 201W

OFFICE HOUR: Tuesday & Thursday 1 PM - 1:50 PM

LAB: Thursday 2 - 5PM G15.2

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1. Required Text and Materials:

1. *Molecular Biology of the Cell*. WW Norton Press. 6th edition. Bruce Alberts. ISBN number is ISBN: 978-0-393-87094-7.

Any format is fine is fine for this class.

2. Lab Description: General Lab Direction

We are analyzing if endosomal proteins are altered in cells a mouse model of autism spectrum disorder. The mice we will be using are C57/Black 6 (the control mouse with no mutation) and the SHANK3B mutant mouse (autism mouse model). We will compare the protein levels between C57/B6 (control) and the SHANK3B mutant (ASD model).

****UNDERSTAND** science is messy and rarely follows a schedule. Because this is an inquiry based lab, the dates in the syllabus are merely a suggestion. These dates will change.

We will be using western blot (measures protein levels) analysis and immunohistochemistry (to visualize protein location) to analyze our hypothesis.

Credit and workload: BIO 216 Lab is a 1-credit course. Here is the workload:

- 3 hours in-class time + 3 hours out of class work
- read the manual/watch tutorial videos (about 60 minutes)
- read the articles for background about SHANK3B and endosomes (about 90 minutes)
- work on presenting your poster (about 30 minutes)

3. Lab GRADE:

Poster presentation in lab	20 points
SpARC presentation	40 points
Scotties with Nerves Presentation	40 points
Pre-Lab assignments	50 points
Post-lab deliverables	50 points
Peer Evaluations	30 points

The following grading scale will apply for converting numerical grades into final letter grades:

93 to 100: A, 90 to 92.9: A-, 87 to 89.9: B+, 83 to 86.9: B, 80 to 82.9: B-, 77 to 79.9: C+, 73 to 76.9: C, 70 to 72.9: C-, 67 to 69.9: D+, 63 to 66.9: D, 60 to 62.9: D-, Lower than 60: F

**lab grade is separate from lecture *Dr. Larimore may add assignments/points through the semester*

If you miss more than 2 labs, your grade will be reduced by 1 letter grade.

No late work is accepted. If you have any extenuating circumstances - health or family crisis - that would require an extension on more than 1 assignment, you will need to reach out to the Office of Accessible Education. They can confidentially receive any of your information and email ALL of your professors simply stating that due to a current crisis, you will need extended time on an assignment.

4. Academic Honesty for your work as a scientist according to our Honor Code:

- You are responsible. Violations of the honor code result in consequences ranging from failure of the assignment, failure of the course, to expulsion from the college. Speak with your professors if you need clarification
- By placing your name on ANY assignment, you are stating that you completed that assignment with academic honesty.
- do not allow another party to do your work/exam
- do not submit the same or similar work in more than one course without permission
- Cheating includes (a) doing work for another person (b) looking on another person's exam for answers (c) using exams from previous classes without permission (d) using unauthorized notes or resources or (e) helping another student (or receiving help) on work/exam that is supposed to be completed independently.

Plagiarism:

- Plagiarism can include portraying another's work or ideas as your own, buying a paper online and turning it in as if it were your own work, or not citing or *improperly citing* references on a reference page or within the text of a paper.
- If you are using a source and citing the source, the information from that source **STILL** must be reworded in your own voice.
- Do not cut and paste from the slide, your book, your neighbor, Wikipedia, or the internet.

Intellectual Fraud:

- do not falsify or create data and resources or alter a graded work without the prior consent of your professor.
- do not make up a reference for a works cited page or statistics or facts for academic work.

Results of Dishonesty:

- Academic dishonesty is reported to medical schools and graduate schools as per their request.
- Anyone caught cheating relinquishes the privilege of asking for a letter of recommendation
- academic dishonesty will receive a 0 on the assignment.
- Acts of academic dishonesty will be turned over to Honor Court.

5. Lab Components:

Pre-LABs: There are pre-lab assignments (individual assignments) due at 5:00 PM on Tuesday so that I can look them over before you come to lab. Late work is not accepted.

For each lab, you are expected to read ahead to understand what protocols we are running in lab that week. You will need to bring a personal copy of your lab manual to lab.

Because of the way the lab runs, if you do not complete the pre-lab assignment on time, you will not be allowed to attend lab.

Post-LABs: There are After lab assignments (post lab deliverables and group assignment) - they are due at 11:59 PM on the day of lab. Late work is not accepted.

PPE: Because of some of the chemicals, closed toed shoes and protective eye wear is necessary. As we are looking at protein levels, we do not want to contribute personal protein to the samples, so hair needs to be out of the way - whatever that looks like on an individual basis is fine.

Email: Instructors will make announcements regularly via email. It is your responsibility to check your Agnes Scott email account daily. When responding to a professor via email, take care that your email is professional.

Technology: You will need access to a tablet or laptop for each class period. Contact the professor or ITS if you need assistance with this. Cell phones **MUST** be in the silence mode prior to entering the classroom or lab. They should never be visible during class time.

6. Lab Policies:

Expected Classroom Etiquette and Letters of Recommendations: This is a team based, problem solving curricular approach. As such, you need to be present to work well with your team. And you need to be prepared for the discussions. Additionally, many of the topics we cover are sensitive. It is expected that you treat all your peers with the utmost respect as we discuss sensitive issues. If you ask the professor for a letter of recommendation, then it is expected that you came to the majority of classes, you came prepared to join in the discussions and your actions and language reflected respect for ALL of your peers.

Content Warning: This course will explore the human brain and behavior, which might raise issues of racism, sexism, classism, heterosexism, cissexism, ableism, and other kinds of privilege. I invite you to come see me if want more information. If you feel you will be unable to fully participate in the course requirements, set up a meeting with the course instructor to determine appropriate accommodations. This course adheres to the principles of diversity and inclusion integral to the Agnes Scott community. We respect people from all backgrounds and recognize the differences among our students, including racial and ethnic identities, religious practices, and gender expressions. We strive for our campus to be a safe space in which all students feel acknowledged and supported. At the same time, we understand that course content, critical inquiry, and classroom dialogues give us opportunities to examine topics from a variety of perspectives. Such discourse is a defining feature of a liberal arts education, and can compel debates that challenge beliefs and positions, sometimes causing discomfort, especially around issues related to personal identities. While we uphold and preserve the tenets of academic freedom, we request and invite your thoughtful and constructive feedback on ways that we can, as a community of learners, respectfully assist and challenge one another in our individual and collective academic work.

e-mail/tech: Instructors will make announcements regularly via e-mail so check your Agnes Scott email account daily. When responding to a professor over email, take care that your email is professional. Cell phones should be in the silence mode prior to entering the classroom or lab. Minimize potential distractions if you are using a laptop or tablet in class to take notes.

Deadlines: Late work is not accepted. It is your responsibility to keep up with the class material. Students are expected to attend all class periods, except in cases of documented illness or emergency. If a missed class cannot be avoided, it is **STRONGLY** recommended that you contact the instructor **IN ADVANCE**, or within 24 hours of the class period.

Course Evaluations: At the end of the semester you will receive an e-mail asking you to submit an evaluation of the course. Please give feedback! Your input is important to the college as a whole and to us as instructors.

ADA: If you have a disability that may have some impact on your work in this class and for which you may require accommodations, please the Office of Academic Advising to register for services.

Title IX: For the safety of the entire community, any incidence of or information about sexual misconduct must be reported immediately to the Title IX Coordinator.

Inclusion: This course adheres to the principles of diversity and inclusion integral to the Agnes Scott community. We respect people from all backgrounds and recognize the differences among our students, including racial and ethnic identities, religious practices, and gender expressions. We strive for our campus to be a safe space in which all students feel acknowledged and supported. At the same time, we understand that course content, critical inquiry, and classroom dialogues give us opportunities to examine topics from a variety of perspectives. Such discourse is a defining feature of a liberal arts education, and can compel debates that challenge beliefs and positions, sometimes causing discomfort, especially around issues related to personal identities. While we uphold and preserve the tenets of academic freedom, we request and invite your thoughtful and constructive feedback on ways that we can, as a community of learners, respectfully assist and challenge one another in our individual and collective academic work.

SCHEDULE:

- 1/11 Lab Expectations, Final Lab Product – the poster, Skim the manual
- 1/18 Lab #1 Background on Mice and Endosomes
- 1/25 Lab #2 Background on Assigned Protein Marker
- 2/1 Lab #3 Introduction Writing
- 2/8 Lab #4 WB sample prep
- 2/15 No Lab
- 2/22 Lab #5 WB with primary AB added Friday
- 2/23 – add secondaries *Saturday 2/24 – store in TBS (Dr. L will do this)*
- 2/29 Lab #6 WB figure created using Image J
- 3/7 Peak Week and 3/14 Spring Break
- 3/21 Lab #7 IF prep
- 3/28 Lab #8 Image slides
- 4/4 No LAB
- 4/11 Lab #9 analyze IF data/make figure/review your final poster
- 4/18 Lab #10 present your poster
- 4/25 Present your poster at SpARC
- 4/26 (Friday) Present your poster at Scotties with Nerves 4:30 PM - 6 PM

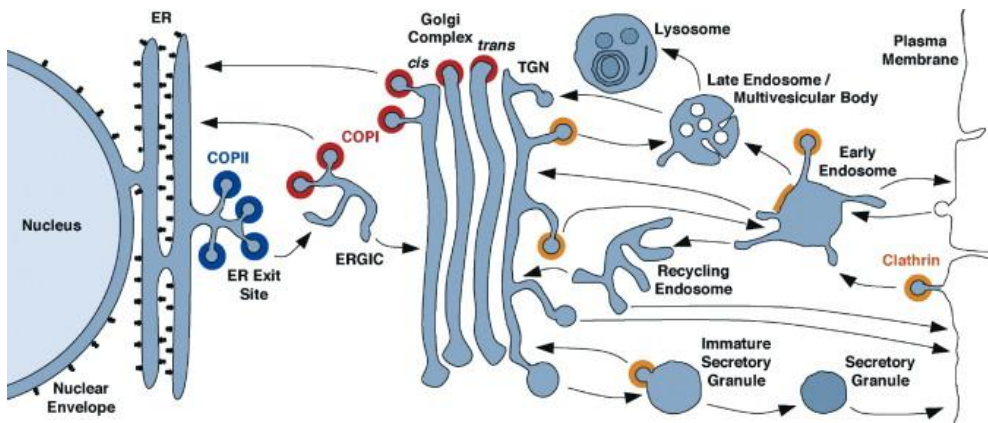
BIO 216: Molecular Biology Lab Manual

General Lab Background:

We are analyzing if endosomal proteins are altered in cells a mouse model of autism spectrum disorder. The mice we will be using are C57/Black 6 (the control mouse with no mutation) and the SHANK3B mutant mouse (autism mouse model). We will compare the protein levels between C57/B6 (control) and the SHANK3B mutant (ASD model).

Neurodevelopmental disorders, such as autism and schizophrenia, are pervasive disorders with impacts on individuals, caregivers, educational systems, and society. Autism spectrum disorders (ASDs) impact 2.2% of the U.S. population (~5.4 million individuals) and are characterized in part by impairments in social interactions, social communication, sleep, learning and memory. Aberrant connectivity among neurons underlies autism phenotypes, and that altered connectivity is a result, in part, of altered dendritic spine volume and density of patients with autism (Martinez-Cerdeno 2018). Schizophrenia impacts 1.2% of the U.S. Population (~3.2 million individuals) and is characterized in part by impairments in social interactions, sleep, learning and memory, and disorganized speech. Previous studies have reported altered dendritic arborization, spine volume and spine density in patients with schizophrenia (Glausier and Lewis, 2014). This study will examine the AGAP1 dependent regulation of the endosomal pathway in dendritic spines in neurodevelopment, which could provide insight into common molecular pathways perturbed in various neurodevelopmental disorders.

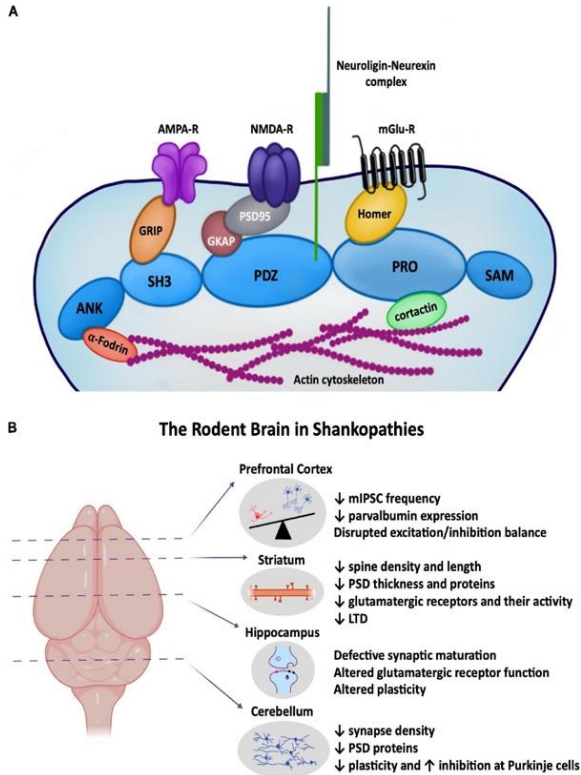
Importantly, AGAP1 is reported with copy number variation (CNV) in several GWAS studies in patients with Autism (Wassink et. al., 2005, Pacault et. al., 2019). AGAP1, adaptor proteins of the endosomal pathway as well as BLOC1 has also been implicated in developmental disorders as well (Gejman et al 2011).



Endosomal vesicle trafficking is one of the most fascinating aspects of neuronal development because of the multitude of subcellular functions that are regulated by endosomal vesicle trafficking and multitude of proteins that regulate proper vesicle formation, targeting and tethering. Vesicles that traffic through the endosomal pathway are

derived from two distinct donor membranes – the Golgi complex or the plasma membrane. Those vesicles originating from the Golgi complex can be trafficked directly to the plasma membrane, to an early endosome for further sorting, or, in the case of mis-folded proteins, vesicles traffic directly to the late endosome (Bonifacino and Glick, 2004, and Figure 1 in Background). Each of these sub-compartments within the endosomal system is regulated by specific Arfs, Arf GAPs, Arf GEFS, coat proteins, and coat associated proteins (Randazzo et.al., 2000, and Figure 2 in the background). When considering the early endosome and recycling endosome, AGAP1 localizes to both compartments,

indicating a role in vesicle coating at these sub-compartments (Arnold et.al. 2016, Nie et. al., 2002). AGAP1 also binds to AP3, a coat protein involved in the formation of vesicle coats at the early endosome (Nie et.al., 2003) and interacts with dysbindin, a coat-associated protein located at the early endosome (Arnold et.al. 2016). Of note, AGAP1, AP3, and BLOC1 have been implicated in neurodevelopment as they are genetically altered in several diseases including Autism Spectrum Disorder, Schizophrenia, and Trisomy 23 (Rudolph et. al., 2016, Arnold et. al., 2016, Wassink et. al., 2005, Talbot et. al., 2004, Talbot et. al., 2011, Chapleau et. al., 2009, Pacault et.al., 2019, Larimore et.al., 2013, Chahrour et. al., 2008, Hartwig et. al., 2017, Gejman et. al., 2011). The exact role of AGAP1-dependent endosomal trafficking in typical neurodevelopment remains unknown.



SHANK3B codes for key PSD proteins at glutamatergic synapses. Shank3B is part of the glutamate receptor protein complex that physically links ionotropic NMDA receptors to metabotropic mGlu5 receptors, a linkage which is necessary for plasticity induction (Figure 5) (Heavner et al 2021). Reduction of Shank3B protein results in spatial memory deficits and alterations in excitatory neurotransmission (Balaan et al 2019, Brown et al 2018, Chen et al 2020, Cope et al 2023, Dhamne et al 2017). As such, the SHANK3^{Btm2Gfng/+} mice make an excellent model to study how synaptic morphology is regulated in development.

The SHANK family of proteins is responsible for synapse formation and synaptic plasticity at glutamatergic synapses. SHANK3B codes for key postsynaptic density (PSD) proteins at glutamatergic synapses (Jiang and Ehlers 2013, Mullin et. al., 2013, Grabrucker et. al., 2011, Foss-Feig et. al., 2017). Shank3B is part of the glutamate receptor protein complex that physically links ionotropic NMDA receptors to metabotropic mGlu5 receptors, a linkage which is necessary for plasticity induction (Figure 4).

This linking of NMDA receptors to mGluRs is through interactions of Shank3B with scaffolding proteins PSD95-GKAP-Shank3-Homer.

Each lab group will be assigned a protein marker of an endosomal compartment or organelle. To date, this has not been examined in the SHANK3B mice. You will research your assigned protein marker in other model systems to create a hypothesis stating what you expect the protein levels will look like in the SHANK3B mice compared to control (increase, decrease or remain unchanged).

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We will be using western blot (measures protein levels) analysis and immunohistochemistry (to visualize protein location) to analyze our hypothesis.

The Poster Presentation (Final Lab Product):

Your final project for lab is a poster presentation. You will be graded individually. The rubric is on Canvas.

Sections of the poster will be due throughout the semester as a draft. This is to offer feedback prior to presenting. See Canvas for due dates.

Your lab group is responsible for printing your poster. Not the professor. Do not submit the poster until you have presented your draft to your lab group and received feedback.

To do so, you will use the Ci- Saw space in the ground level of Alston. You MUST fill out this form by 4/16 at 5PM. Failure to do so will result in loss of points.

This form will ask you to upload a PDF of your poster. and include your poster dimensions. Your poster can be no more than 40 inches tall by 55 inches wide. Please print on matte paper.

<https://docs.google.com/forms/d/e/1FAIpQLSfBytzi7wCEJdJlZ-Ab5cNpIpjslh-riOj281jTxpQjaoCQ/viewform?vc=0&c=0&w=1&flr=0>

They will email you when the print is complete.

Talk to your lab professor with any questions.

Your entire Lab group will be presenting your results. Each group will present for approximately 10 - 15 minutes. If you rush through this, people will not understand what you are saying. This is your lab grade. Take your time. Each person in the lab group is expected to do equal parts of the talking.

Symposium Expectations 101: During the poster session, your poster stays up the whole time. You will need to dress and act professionally. Phones and devices stay put away. If you are not presenting, you should be visiting other posters to gain academic experience.

Your lab group will need to save a PDF version of your poster and upload it to Canvas. All group member names should be in the filename.

For EACH section – make sure you have rehearsed your section at least 4-5 times. Your information should be understandable to someone who DID NOT conduct this research, but someone who is familiar with science. Consider practicing your information with a non-science major friend and see if they understand your presentation. Your information should be well organized, well explained. You need a good balance of text and diagrams for each section!

To make a poster, open PPT or Google slides. White background only

In preferences, set the page format as 4 feet tall by 3 feet wide. White background/black font – Times New Roman.

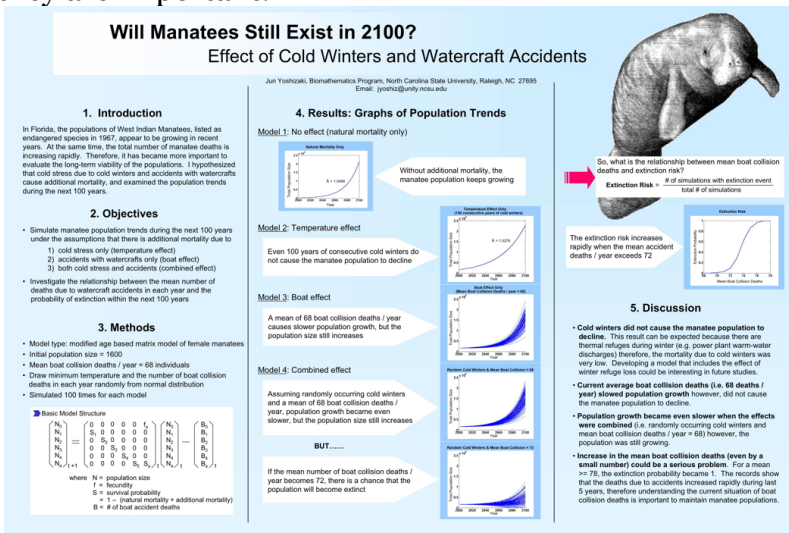
Poster Sections:

Author Line: Usually, authors write this from who contributed the most to the least, and the last author is the professor whose lab the research was conducted in. For this assignment, your group should list your group members in alphabetical order and your professor's name should be last.

Abstract and Introduction section: give background information to help our class understand your topic. Why is this experiment important? What background information will help us understand what was done? State the hypothesis. If you can give this information in a figure or a flow chart, it is way more appealing to people looking at your poster than a bunch of words!

Methods section: highlight the techniques used to gather the data. Explain what we are measuring and what we used for those measurements. You don't have to give step by step on how to do it, just what these are and why we are using them for our experiment. The underlying concept to cover is why were these techniques used and how will they address the question that is stated in the hypothesis? If you can do this with a flow chart, it looks much better than a bunch of words!

Results: the graphs, the tables, etc - should include a figure legend and a figure title. Any time you present a piece of data, always explain what the audience is looking at, what you did to arrive at the data you are seeing (what method you used), and what the data mean (your interpretation) and why they are important.



Conclusion: simple statements (bullet points work) to describe the key, take-away points from the experiment and their importance in the field of study. How did the study contribute to the hypothesis? Also, this should include future studies – what would propel this study if there was something more to be added to it.

Molecular Biology Lab #1: Background information on our (1) mouse models and (2) on the endosomal pathway.

Lab “To Dos”:

- (1) Use PubMed (search engine for research papers) to search what is known about SHANK3B mutant mouse.
- (2) You also need background information on what autism looks like in humans as well as in mouse models.
- (3) Finally, you need to understand the endosomal pathway, which was first introduced to you in BIO 110. Use this review to fully understand the endosomal pathway: <https://pubmed.ncbi.nlm.nih.gov/14744428/>

Pre -Lab Assignment (This is an individual assignment):

Answer the questions below in the space below. Then, cut and paste the questions and answers to the text box provided on Canvas for this lab. These must be submitted before lab, otherwise you will not be allowed to attend lab. This is an individual assignment

- 1. What are some of the symptoms in patients with Autism Spectrum Disorder (ASD)?**
- 2. What are some of the genes mutated in patients with ASD? What are those gene’s function?**
- 3. What is SHANK3B’s role in a neuron?**
- 4. Why are we using C57/B6 mice?**
- 5. Describe (not list) the organelles of the endosomal pathway (Chapter 12 and 13 in your textbook).**

Post-Lab Deliverables (This is a group assignment/group turn in):

- (1) Create a Google Doc with your group. Today’s Google Doc will be a list of notes you have about
 1. The 2 mice genotypes we will use
 2. ASD in humans
 3. ASD in mice
 4. The endosomal pathway

Cut and paste links to PubMed articles on your potential protein of interest. This document will help your group write the abstract, the introduction, and the hypothesis. Turn this in to Canvas.

- (2) Create the skeleton of your poster. At the end of lab, a copy of your updated poster (Title and author names) is due on Canvas as well as your google doc with notes. This is a group assignment. To make a poster, open PPT or Google slides.

In preferences, set the page format as 4 feet tall by 3 feet wide. White background/black font – Times New Roman.

Molecular Biology Lab #2: Background information on your protein marker

Lab “To Dos”:

- (1) Use PubMed to continue searching what is known about your protein marker. Look at other Autism models or in the genetic wide association studies (GWAS) in patients to better understand your specific marker. You will research your assigned protein marker in other model systems to create a hypothesis stating what you expect the protein levels will look like in the SHANK3B mice compared to control (increase, decrease or remain unchanged).

Group # : Assigned Protein Marker

Group #1: Rab11

Group #2: Dysbindin

Group #3: AP-3

Group #4: LAMP1

- (2) Write your abstract.

Pre -Lab Assignment (This is an individual assignment):

Answer the questions below in the space below. Then, cut and paste the questions and answers to the text box provided on Canvas for this lab. These must be submitted before lab, otherwise you will not be allowed to attend lab.

- 1. What is your group’s assigned protein marker?**
- 2. What compartment/region of the endosomal pathway does this marker localize to?**
- 3. What role does that endosomal compartment play? What is its purpose?**
- 4. What is the role of your protein marker? (use genecard.org)**
- 5. Is this marker implicated in any other disorders (use PubMed)?**

Post-Lab Deliverables (This is a group assignment/group turn in):

1. Update your google doc with research about your assigned protein marker.
2. Poster Update to include Abstract. Your poster needs to have the author line, the title and an abstract. Your abstract must include your hypothesis. At this point, you can say that you observed changes (you don’t have to say that there was an increase or decrease in protein levels).

The following about abstracts is directly from : <https://writing.wisc.edu/handbook/assignments/writing-an-abstract-for-your-research-paper/>

The Contents of an Abstract

Abstracts contain most of the following kinds of information in brief form. The body of your paper will, of course, develop and explain these ideas much more fully. As you will see in the samples below, the proportion of your abstract that you devote to each kind of information—and the sequence of that information—will vary, depending on the nature and genre of the paper that you are summarizing in your abstract. And in some cases, some of this information is implied, rather than stated explicitly. The Publication Manual of the American Psychological Association, which is widely used in the social sciences, gives specific guidelines for what to include in the abstract for different kinds of papers—for empirical studies, literature reviews or meta-analyses, theoretical papers, methodological papers, and case studies.

Here are the typical kinds of information found in most abstracts:

- the context or background information for your research; the general topic under study; the specific topic of your research
- the central questions or statement of the problem your research addresses
- what's already known about this question, what previous research has done or shown
- the main reason(s), the exigency, the rationale, the goals for your research—Why is it important to address these questions? Are you, for example, examining a new topic? Why is that topic worth examining? Are you filling a gap in previous research? Applying new methods to take a fresh look at existing ideas or data? Resolving a dispute within the literature in your field? . . .
- your research and/or analytical methods
- your main findings, results, or arguments
- the significance or implications of your findings or arguments.

Your abstract should be intelligible on its own, without a reader having to read your entire paper. And in an abstract, you usually do not cite references—most of your abstract will describe what you have studied in your research and what you have found and what you argue in your paper. In the body of your paper, you will cite the specific literature that informs your research.

Molecular Biology Lab #3- generate an introduction and a materials and methods section for your poster.

Lab "To Dos":

- (1) Using your lab manual, your textbook, and the google doc of notes, you will generate an introduction for your poster.
- (2) You will also generate a materials and methods section for your poster.

Pre -Lab Assignment (This is an individual assignment):

Answer the questions below in the space below. Then, cut and paste the questions and answers to the text box provided on Canvas for this lab. These must be submitted before lab, otherwise you will not be allowed to attend lab.

- 1. What information should be in an introduction on a poster? What are possible formats? How is this different from a manuscript/published paper?**
- 2. What information should be in a materials and methods section on a poster? What are possible formats? How is this different from a manuscript/published paper?**
- 3.**

Post-Lab Deliverables (This is a group assignment/group turn in):

1. The Poster with the Introduction and Materials and Methods section added.

Introduction section: give background information to help our class understand your topic.

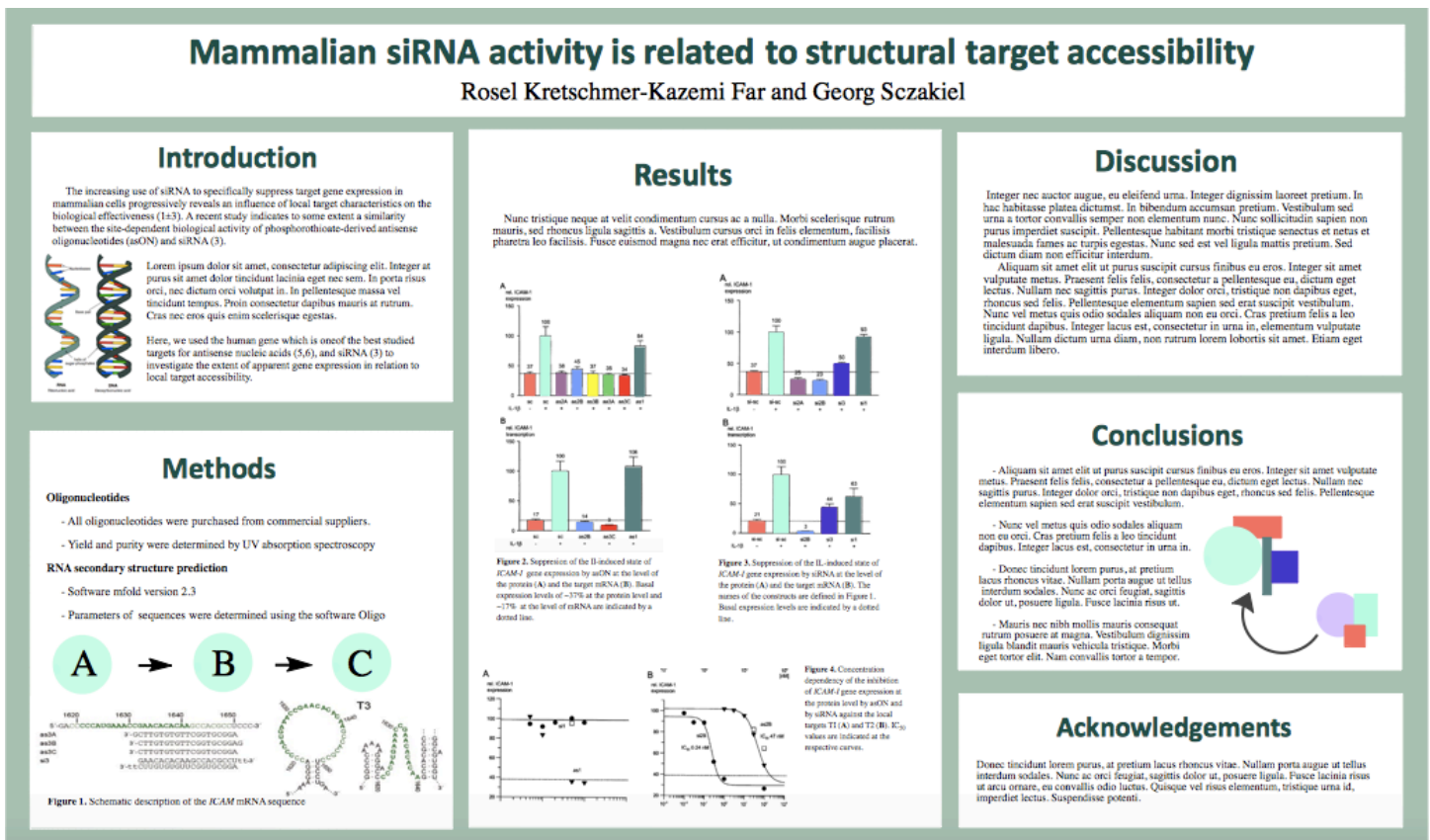
Why is this experiment important?

What background information will help us understand what was done? State the hypothesis.

****If you can give this information in a figure or a flow chart, it is way more appealing to people looking at your poster than a bunch of words! You can cite figures from previous papers if that helps. Infographics are great.**

Methods section: highlight the techniques used to gather the data. Explain what we are measuring and what we used for those measurements.

You don't have to give step by step on how to do it, just what these are and why we are using them for our experiment. The underlying concept to cover is why were these techniques used and how will they address the question that is stated in the hypothesis? If you can do this with a flow chart, it looks much better than a bunch of words!



Molecular Biology Lab #4 - Prepare WB samples for analysis

The overall purpose of this series is to be able to run a western blot, which will allow us to compare protein levels of interest between samples. Part 1 in a WB – Run an SDS-PAGE gel. Part 2 – transfer to nitrocellulose or PVDF membrane to be able to probe with antibodies (AB).

Remember there are several controls.

First, we have a control samples (no genetic alterations = C57/B6) for the WB and then we have the Autism Mouse samples (SHANK3B mutants) for the WB. That is so we can figure out if your protein is altered in any way in autism.

AND we have to have in-experiment controls to make sure that the WB was loaded evenly. In the WB, that is actin, which we will detect using the actin antibody. Your graphs - you are reporting the ratio of your protein density to the in-experiment control protein density.

You report the ratio of your protein to actin – the bar graph should be an average of all the control cell ratios compared to the average of the SH3B KO cells. That is the y axis is the ratio of your protein to actin. The x axis would the 2 bars for the graph – the types of cells they represent.

The t-test - you compare the individual samples ratio of the protein variable/control protein of the control cells (that is data set 1) to the individual samples ratio of the protein variable/control protein of the experimental cells (data set 2). This is an unpaired test and uneven samples.

Lab “To Dos”:

(1) Prepare WB samples

Pre -Lab Assignment (This is an individual assignment):

Answer the questions below in the space below. Then, cut and paste the questions and answers to the text box provided on Canvas for this lab. These must be submitted before lab, otherwise you will not be allowed to attend lab.

1. **Why do we measure the protein levels of the samples?**
2. **Explain what is represented in each variable for our western blots in the $C_1V_1 = C_2V_2$ calculation.**
3. **What are the 2 controls and why are they important?**
4. **How does we calculate the amount of lysis buffer we will need in our final volume of WB samples?**
5. **Why is this lab necessary?**

Post-Lab Deliverables (This is a group assignment/group turn in):

1. Upload your notes from this lab (your notes = any of the spaces that you had to record math or sample measurements). The notes can be an image of your group’s handwritten notes or a cut and paste from notes in a google doc version of the manual.

PREPARING THE SAMPLES:

When we load our samples into our gel, we need to load equal volume as well as equal concentration. Why is that important?

Because we want to be able to compare the bands on our gel to see if we have more or less of certain proteins in our different samples.

To do that, we need to load the *same volume* and *the same concentration*. It is not enough to load the same volume. We have to load the same volume and the same concentration.

You are provided with whole brain lysates from mice. You will need to ensure that the samples are the same concentration per volume added to the gel.

Measuring the Samples

1. The nanodrop lite will measure the absorbance of the samples and compare that to a standard curve of known protein concentrations and their absorbance reading to give us the protein concentration in our samples.
2. Under the home menu, select protein.
3. Then select BSA.
4. The machine will ask you to blank – in other words, you are telling the machine what no protein looks like.
5. For the blank, use water. Place 2 μ L of water on the small silver disc and close the arm.
6. Push the ‘blank’ button.
7. It will ask you to confirm the blank.
8. Blot the silver disc with a kimwipe and put 2 μ L of water on again.
9. Once the machine is blanked, it is ready to read the samples.
10. To do measure the samples after the machine has been properly blanked, clean the small silver disc with a kimwipe. Place 2 μ L of your sample on the small silver disc of the nanodrop.
11. Measure your samples absorbance in triplicate, writing down each of the absorbance readings in the space below or in google sheets.
12. Change out your tip between each pipette and use a kimwipe to blot the silver disc in between each read.
13. Take an average of all 3 readings for each sample.



Sample	Absorbance #1	Absorbance #2	Absorbance #3	Average Absorbance
Control #1				
Control #2				
Control #3				
Experiment #1				
Experiment #2				
Experiment #3				

RECORD YOUR ABSORBANCE READINGS HERE OR IN A GOOGLE SHEETS

Measuring sample NOTES:

Any changes should be noted here.

Special reagents/equipment used? Manufacturer?

Gel Sample Preparation

14. Now that you know the concentration of your proteins, we need all samples to be at a concentration of $2\mu\text{g}/\mu\text{L}$.
15. Reminder:
When we load our samples into our gel, we need to load equal volume as well as equal concentration. Why is that important? Because we want to be able to compare the bands on our gel to see if we have more or less of certain proteins in our different samples. To do that, we need to load the same volume and the same concentration.

16. Remember $(C_1)(V_1) = (C_2)(V_2)$ from chemistry.
17. C_1 is your starting concentration – what you just measured on the spec. – the average absorbance reading.
 V_1 – will be what we solve for – the volume of your sample that you will need to add to lysis buffer.
 C_2 is $2\mu\text{g}/\mu\text{L}$
 V_2 is $500\mu\text{L}$ of sample

Sample	C_1 (concentration 1) - the average absorbance reading	V_1 - what we are solving for	$C_2 = 2\mu\text{g}/\mu\text{L}$	V_2 - the volume we will need for all of our blots	Lysis Buffer Volume	Loading Buffer Volume
Control #1			2	500		
Control #2			2	500		
Control #3			2	500		
Experiment #1			2	500		
Experiment #2			2	500		
Experiment #3			2	500		

So you solve for V_1 . $C_1 \cdot v_1 = C_2 \cdot V_2$. Or $V_1 = (C_2 \cdot V_2) / C_1$

18. Use this formula and fill in the table above.
19. That gives you the amount of **your sample** you need in a clean eppendorf tube. Do this for each of your samples. Make sure your tubes are labeled with your groups initials.
20. We are using a 3X loading dye, so divide 500 by 3 and add that to the “Loading Buffer Volume”
21. To calculate the amount of lysis buffer needed in your samples see below.
22. $(\text{Total Volume}) - (\text{Loading Dye Volume}) - (V_1 \text{ for the sample}) = \text{Volume of lysis buffer to add to that specific sample. Add this math to the table above}$
23. Mix the lysis buffer, the sample, and loading dye together by vortexing for 20 seconds.
24. Take samples to the 75°C heat block and submerge in the silver beads for 5 minutes. With membrane proteins you do not want to boil as most labs do. Boiling will lead to aggregates, especially in transmembrane proteins.
25. Take your samples back to the eppendorf tube rack for your lab section.

Sample Preparation NOTES:

Any changes should be noted here.

Special reagents/equipment used? Manufacturer?

Molecular Biology Lab #5 - Run the WB

Lab "To Dos":

- (1) Run the WB
- (2) While the gel is running, the group will incorporate any feedback on the poster.

Pre -Lab Assignment (This is an individual assignment):

Answer the questions below in the space below. Then, cut and paste the questions and answers to the text box provided on Canvas for this lab. These must be submitted before lab, otherwise you will not be allowed to attend lab.

- 1. What is a western blot gel and how does it work?**
- 2. Why do we have to transfer the gel to the membrane?**
- 3. Describe how we will use primary antibodies and secondary antibodies.**
- 4. Why does the host species matter?**
- 5. What are the steps in running a western blot (use a flow chart or list steps)?**

Post-Lab Deliverables (This is a group assignment/group turn in):

1. Upload your notes from this lab (your notes = any of the spaces that you had to record math or sample measurements). The notes can be an image of your group's handwritten notes or a cut and paste from notes in a google doc version of the manual.

Part 1 – Load and Run Criterion Gel

1. The gels are stored at 4°C.
2. Remove from protective wrapper
3. Remove green tape at bottom of the gel. If you do not remove this, the gel will not run.
 4. Set in electrode cassette (shown below) being sure that the gel is all the way at the bottom of the apparatus. If you are only running one gel, you will need to add a buffer dam. The gel should be loaded so that the numbers are not visible from the outside of the apparatus. The buffer dam is labeled with “this side towards the gasket”. The gasket is the green rubber.
 5. Once both gels or a gel and buffer dam are loaded into the electrode cassette, clamp it shut and place it in the WB tank without the lid (shown below with the lid on).



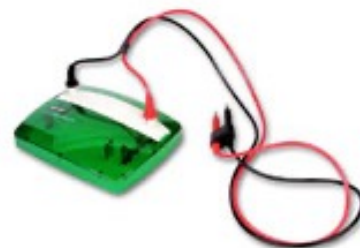
6. Make running buffer. This is stored in a box from BIO-RAD and is a 10X solution – so 100mL of the 10X stock in a graduated cylinder with 900mL of ddH₂O. Use the entire 1L for your tank.
7. Add running buffer to the center of the electrode cassette ONLY for a few minutes. This allows you to determine if any running buffer is leaking outside the cassette.
8. If the running buffer is leaking, you need to open the cassette and re-set your gel and buffer dam. Once you have a tight seal and no buffer is leaking out, then you can pour the remaining running buffer in the entire tank.
9. Set the WB tank on the lab bench near to the edge to make loading easier.
10. Make sure that the gel you are loading is closest to you to make it easier to load.
11. Take out the green comb slowly and evenly. If you pull too fast or pull on one side more than the other, it will destroy the wells the samples are supposed to be loaded into.
12. Using gel loading tips, insert the tip into the well. The tip should be pressed up against the plastic edge of the gel closest to you. This lets you know that you will actually be in the well. Follow the tip and line it up with the bottom of the number of the well. If you go much further, you can puncture the well, which means the sample will not run. Finally, release the sample slowly. If you release too fast, the sample will splash out of the well.
12. The ladder is a mixture of known sample proteins that will indicate which molecular weight we can expect our antibodies to show up at.
13. Load 15µL of the ladder and 25µL of the sample.
14. Record what sample you load in which lane.



NOTES:

Sample in Gel	Lane 6
Lane 1	Lane 7
Lane 2	Lane 8
Lane 3	Lane 9
Lane 4	Lane 10
Lane 5	

15. Put the lid (shown right) on the WB tank – red on red and black on black.
16. Connect electrodes and run gel at around 60V until front line blue loading dye) moves into the gel (out of the wells)
17. ***during the run, make up the blocking solution** (4g powdered milk in 40mL TBST)*
18. Up the voltage to around 100V once the samples and the ladder are inside the gel.
19. Once the front line is half way down the gel, up the voltage to 150V.
20. Once the front line is out of the gel, can stop the run or you can go longer if you want to. Just make sure you don't let your size of interest proteins run off the gel.
21. When the run is stopped, take the gel plates out and place them on a paper towel.
22. Rinse the WB container with ddH₂O ONLY, buffer dam and the apparatus with water only. Hang them up to dry. Be sure the apparatus is not hanging on the wire, but hanging by plastic only.
23. Crack the gels apart with metal spatula (shown right). Remove the plastic that the gel isn't on and throw it away.
24. Continue on to transferring to membrane.



Load and Run Gel NOTES:

Any changes should be noted here.

Special reagents/equipment used? Manufacturer?

Part 2: Transferring sample protein from the gel to a membrane.



Transferring Gel to Membrane

1. You will need a package of turbo transfer membrane blot which is stored at 4°C.
2. Membrane is very sensitive. Do not fold, bend, or crinkle. **DO NOT TOUCH!!!**
3. Remove a cassette from the turbo transfer unit. (shown left) and open the cassette.
4. Place the entire bottom half of the WB sandwich (Labeled “bottom” in the TurboTransfer packet) on the bottom of the turbo cassette (shown left). Use the roller to flatten



- 5.
6. Put the gel on top of the “bottom” part of the sandwich. Use the roller to flatten (shown right).
7. Place the top half of the WB sandwich (labeled “top” in the TurboTransfer packet) on top of the gel. Use the roller to flatten.
8. Align the lid and put it down. Once it is down, you cannot lift or move until the run is complete. Lock the cassette. And slide it into the space on the unit.
9. Select List
10. Select “Bio Rad Defined”
11. Select number of gels.
12. Select the second program option – 1.5mm, 10 minutes
13. Select the cassette and press either “A run or B run” – whichever slot you placed the gel cassette into.
14. When the run is over, pull the cassette out and open it by lifting the lid straight up.
15. Orient yourself to which side of the membrane contains your protein. This is the side that is next to your gel.
16. The side of the membrane that has the protein on it needs to “protein side up” when placed into the blocking solution.

Transfer NOTES:

Any changes should be noted here.

Special reagents/equipment used? Manufacturer?

Blocking

This step “cleans” the membrane so that there are no non-specific protein-protein interactions when we probe with an antibody.

1. After the run, the membranes are transferred, they will be placed in blocking solution for 20 minutes at room temp on a shaker or overnight. The blocking solution is pre-made
2. Pour some milk in the bottom of Tupperware that is labeled for blocking.
3. Place the blot in the tub containing the 5% milk with the protein side up.
4. Rock at room temp for 20min to 1/2 hour. The membranes can rock overnight in 4°fridge overnight.

Blocking NOTES:

Any changes should be noted here.

Special reagents/equipment used? Manufacturer?

Antibodies (Primary and Secondary)

We separated ALL the proteins present in the sample on the gel and transferred those proteins to a membrane. We can now apply primary antibodies, which will detect ONLY our proteins of interest.

To that, we will incubate in a 2°Ab, which will allow us to detect our primary antibody. We can then rinse in a solution which will enhance the signal and allow us to detect our proteins as bands.

To make an antibody, you inject part of your protein of interest into a host animal (usually mice, rabbits, goats, or chickens). For this example, we will use the protein, actin. A company will inject actin protein into a host species, say a mouse. The mouse will mount an immune response to the injected protein and generate antibodies to the actin protein that was injected. So then, serum can be collected from the mouse that are primary antibodies against the actin protein. And because they came from a mouse, the IgG on the antibody is mouse specific. This primary is mouse anti-actin. Mouse is the host species and the mouse antibody detects only actin.

But that primary does not have a tag on it. To detect it, we need a secondary. Secondary antibodies have a tag. And they are generated to detect the IgG. They are usually made in host species that are not used for primaries – typically secondaries are made in goats or donkeys. So. For this example, we could use donkey anti-mouse to detect our anti-actin antibody.

1°AB for WB

For WB, we run a control AB or a reference protein. For this lab, we will use actin. This reference protein or control AB is a protein that does not change among the samples. It tells us that we loaded the samples evenly. To compare our samples, we will eventually make a ratio using this control AB. To do this, we will cut your membranes into 2 pieces using the ladder and the molecular weight.

We will cut near the actin weight of 52 KD. The piece of the membrane containing the 52 KD section will incubate in actin and the piece of the membrane containing the KD section of your experimental AB will incubate in your experimental antibody.

1. When your membrane is done with its incubation in block, rinse with distilled water ~15 times (or until water runs clear) to remove the milk solution thoroughly from the membrane.
2. Transfer the blot out of the blocking Tupperware to a 1°Ab Tupperware that has TBST in it and shake for 2minutes.
3. Dump the TBST in the sink.
4. Add the Ab you want to use to the tupperware from the conical tubes.
5. Incubate for overnight at 4°C**
6. To maintain the Ab, return the empty conical to the fridge.
7. For reference - Primary Antibodies are diluted in : 3% BSA weight /volume 0.2% sodium azide in PBS

Primary NOTES: RECORD PRIMARIES USED HERE:

Which antibodies? Which Manufacturer? Which host species?

Any changes should be noted here.

Special reagents/equipment used? Manufacturer?

FRIDAY: 2°AB for WB

You will need to discuss with your group who is doing this. It will take about 20 minutes.

6. Drain as much of the primary AB BACK into the conical tube that is labeled with that 1°AB type. Because primary antibodies are expensive, we can reuse them.
7. Wash w/ TBST 3x for 5 min each. The 5 minute incubation should be on the room temperature plate shaker.
9. Add the pre-made, labeled 2° Ab to membrane and rock until Monday in 4° fridge.

Monday

You will need to discuss with your group who is doing this. It will take about 20 minutes.

10. After the overnight incubation, dump the secondary down the drain.
11. Wash the membrane 3x with TBST 5 min each.
12. Store in TBS at 4°C until lab on Thursday.

Secondary NOTES: RECORD SECONDARIES USED HERE:

Which antibodies? Which Manufacturer? Which host species?

Any changes should be noted here.

Special reagents/equipment used? Manufacturer?

Molecular Biology Lab #6 - Visualize our WB data and produce a figure for the poster.

Lab “To Dos”:

(1) use web browser Image J to produce a figure of the WB

<https://imagej.nih.gov/ij/>

Pre -Lab Assignment (This is an individual assignment):

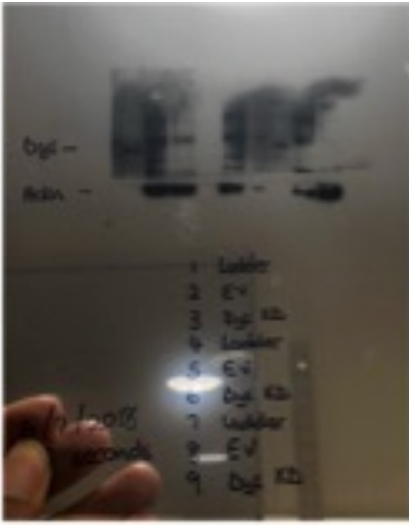
Answer the questions below in the space below. Then, cut and paste the questions and answers to the text box provided on Canvas for this lab. These must be submitted before lab, otherwise you will not be allowed to attend lab.

- 1. Why are we using film? What will detect on the film?**
- 2. Why are we quantifying the blots?**
- 3. How will we quantify the blots? Explain in detail what the graph at the end of lab will look like.**
- 4. What is a t-test? What will it tell us?**
- 5. How does this experiment connect with your hypothesis?**

Post-Lab Deliverables (This is a group assignment/group turn in):

1. Upload the poster with all the previous parts and a WB figure added in the results.

Visualizing – Film Developing using ECL to detect the HRP-conjugated 2^oAB



- 1.
2. Use the Thermo ECL kit to further detect our 2^oAb, this is to enhance the 2^oAB signal. To do this, the SuperSignal Dura West is a 1:1 ratio, so 200 μ L of the clear bottle and 200 μ L of the brown bottle per membrane. Each full membrane gets 400 μ L total of ECL
3. Cut a page protector so that only 1 side of the page protector is connected. Tape the page protector into the film developing cassette and place a piece of lab tape across the connected edge.
4. Open up the page protector.
5. Transfer the membrane to a page protector inside the film developing cassette. As you lift the membrane out of the TBST, gently shake off any loose drops of the buffer.
6. Using a P1000, slowly distribute the 400 μ L per full membrane of ECL on the membrane and allow it to incubate for 5 minutes in the page protector.
7. Carry the film cassette downstairs (Ground level) to develop the film.
8. As the film is developed, for each film - label with a sharpie - the exposure time the film had to the membrane in seconds or minutes, the date, your initials, what sample is in which lane, what sample set the samples are from, which band area is which antibody, and draw the ladder onto the ladder lane.
9. Take a picture of the film and label the file name. Store the image in a digital lab notebook.

Developing the Gel NOTES:

Any changes should be noted here.

Special reagents/equipment used? Manufacturer?

Image J – Quantifying the Protein Bands on the Film Image

We will examine our images. Using a free software program, Image J, you can analyze your western blot and quantify each of the protein bands. **Before Lab: Download Image J from the NIH PRIOR to coming to lab – google NIH Image J.**

Remember there are several controls.

First, we have a control cells (no genetic alterations) for the WB and then we have the Autism Mouse cells for the WB. That is so we can figure out if your protein is altered in any way in autism.

And we have to have in-experiment controls to make sure that the WB was loaded evenly. In the WB,

that is actin.

Your graphs -

you are reporting the AVERAGE ratio of your protein of interest density to the in-experiment control protein density (usually actin or synaptophysin).

So for each well of the WB - you have a ratio of your protein divided by actin - your protein ratio.

For all the controls, you average that ratio to create one bar on your bar graph.

For all the Sh3B wells, you average that ratio to create the other bar on your bar graph.

That is the y axis is the ratio of your protein to actin. The x axis would be the 2 bars for the graph – the types of cells they represent.

The t-test - you compare the individual samples ratio of the protein variable/control protein of the control cells (that is data set 1) to the individual samples ratio of the protein variable/control protein of the experimental cells (data set 2). This is an unpaired test and uneven samples.

Here is how to do a t-test in google sheets : <https://support.google.com/docs/answer/6055837?hl=en>

This is a 2 tailed, unpaired (unequal variance) test.

Here is an example of a figure from a previous class:

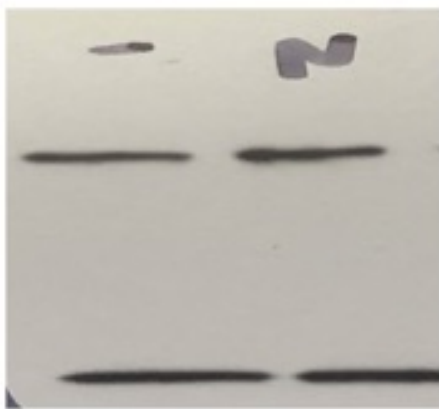


Fig. 7A: Western Blotting



Fig. 7B: Graph

Fig.7.

The comparison of levels of PSD-95 and actin found in the mice models. (a) The Western Blot Image shows 2 bands of PSD-95 (top) and actin (bottom) found in the two different models. (b) Quantification of PSD-85 from control of mice models in average ratio. The average ratio of the control was 0.6526895544 and the average ratio of the experimental was 0.7536030643. The standard deviation was 0.2089397986. ($p < 0.5$)

Quantification

1. Go to NIH Image J
2. Go to File → Open → (wherever your data is).
3. Select the dotted line box (the select tool) to select the first band on the gel. It should be a tall, rectangular box.
4. Go to “Analyze, Gel, select first lane”..
5. Move the box over by using the hand tool and dragging the box from the center of the box to the next band. Go to Analyze, gel, select next lane.”
6. Repeat until you have selected all the bands.
7. Go to analyze, gel, plot lanes. This tells the computer you are done.
8. A new window will open up with curves to show where the heaviest area is. Peak = band.
9. Use the line tool to close off the bottom of the curve. Start where the background is and go across. Be generous, completely closing off the band.
10. Use the hand tool to move the window if you need.
11. Use the wand tool to select the area inside of a peak. IF you select it more than once, the data will be recorded twice and you will have to start over. Use the hand tool if needed to scroll through the peaks.
12. The window that opens has your results in it. Copy and paste to an excel spreadsheet.

In Excel....

1. Each sample should have its own row. The experimental antibody should have its own column for each sample. And the control antibody (usually actin) should have its own column for each sample.
2. Create a ratio of the control antibody to the experimental antibody (Divide the experimental antibody by the control antibody) in a third column. Do this for EACH data set. A data set is the experimental AB and control AB for each well. You CANNOT average the experimental antibody and the control antibody to make a ratio – each control antibody is specific for that well – so you make individual ratios for EACH of your data sets.

Sample	Date imaged	Experiment Ab	Control Ab name	
	05/31	AP3	Actin	
		Density from Image	Density from	Ratio of AP3/
m1 dg		57.503	54.047	1.063944345
m1 dg		99.757	88.986	1.121041512

After you graph it, make a figure legend that describes what the samples are, the number of samples that are represented in the graph.

T-Test in Excel

(or instructions for a T-test in google sheets here: <https://support.google.com/docs/answer/6055837?hl=en>)

Step 1: Enter your data in an organized fashion.

Be sure to label what units were used to measure and all details. The more you label, the less confusion there is.

After the data is entered, you can pick any empty cell to enter in the formula.

For the T-Test, let's compare the males:

In an empty cell, enter the "=" sign (shown in A15 in the screen shot). Then, select the stats test in the function box shown on the top left just before the function formula bar.

For Array 1, click in the array 1 box on the right, then highlight the cells with data for all the control males.

For array 2, click in the array 2 box on the right, then highlight the cells with data for all the variable males.

Then fill in the data for the tails and type of test using the definitions given and what you know about your data.

Finally, click done. In the cell that you originally put the "=" sign, the p value will come up. Label this!

Additional Data that is useful for reporting: the average and the Standard deviation of the group.

Again, create some space. Insert rows if you need to. So you can label your data.

To determine the average, place the "=" under a set of data. Select the function "Average" and in the space to the right, click in the number 1 box and then highlight the data you want to average – all of one particular group.

To determine the standard deviation, place the "=" under a set of data. Select the function "STDEV" and in the space to the right, click in the number 1 box and then highlight the data you want the standard deviation of – all of one particular group.

Molecular Lab #7 - Study your protein of interest by IF

Lab “To Dos”:

(1) Use the protocol below to stain your slides with primary antibodies.

Pre -Lab Assignment (This is an individual assignment):

Answer the questions below in the space below. Then, cut and paste the questions and answers to the text box provided on Canvas for this lab. These must be submitted before lab, otherwise you will not be allowed to attend lab.

- 1. What is IF? What type of data does it generate?**
- 2. What controls are we using? How are they important?**
- 3. What is the mixture/recipe for each of the primary antibodies you will use?**
- 4. What is the mixture/recipe for each of the secondary antibodies you will use?**
- 5. How will you use this to support your hypothesis?**

Post-Lab Deliverables (This is a group assignment/group turn in):

- 1. Upload your notes from this lab (your notes = any of the spaces that you had to record math or sample measurements). The notes can be an image of your group’s handwritten notes or a cut and paste from notes in a google doc version of the manual.**

Immunofluorescence (IF) of Brain Slices

Primaries : 1 hour 20 minutes total

1. 60 micron sections of mouse brain tissue are removed from -20 storage and transferred to PBS. Below, label which sample is located in which position of the mesh circle:

Position 1

Position 4

Position 2

Position 5

Position 3

Position 6

2. Tissues are incubated (in fume hood) in 1% sodium borohydride (1g in 100mL PBS) for 15 minutes. (DURING THIS TIME MAKE BLOCK)
3. Wash with 1XxPBS until no bubbles are present (at least 4 times). To do this, remove the mesh circle, dump the sodium borohydride in the waste container and return the mesh circle to the Pyrex. Add PBS. Gently shake. Then remove the PBS and repeat this at least 4 times.
4. Tissues are incubated for 45 minutes at 37°C on shaker in block (5% serum, 1% BSA, 0.3% triton in PBS). Put your initials next to each reagent after you added it.
 1. 30mL PBS
 2. 1.5mL NHS
 3. 0.3g BSA
 4. 450µL of 20% Triton
5. During the block, make your primary antibodies.
6. Calculate how much antibody base you need to make: (each brain slice gets its own well and each well needs the final volume of 500µL/well)

Antibody Base Recipe (1% serum, 1% BSA in PBS):

Make the antibody base in a 50mL conical tube and label it antibody base. Put your initials next to each reagent after you added it. (recipe below is for 10 samples or less)

20mL

200µL NHS

0.2g of BSA

Calculate how much antibody base you need:

each brain slice gets its own well and each well needs the final volume of 500µL/ well

Transfer the amount of antibody base you need to a clean conical and label it - primary antibodies.

The volume of antibody base you need – convert that to µL.

Then, divide that antibody base needed µL volume by the dilution ratio for that antibody. As all of our antibodies are new to us, we are going to dilute them all at 1:100.

Say you are staining 3 control brains and 3 experimental brains. 6 brains, so 6 wells.

So you need 6mL of antibody base for 6 brains
That is 6000µL total volume.

For the synaptophysin antibody we use, SY38 from Millipore, the dilution ratio is 1:500. (we will use SY38 as our control for IF staining, so you need this math to figure out the primary dilution for this antibody)

Total volume / dilution ratio = volume of antibody to add to the antibody base

$6000 / 500 = 12\mu\text{L}$ of synaptophysin antibody in your 6mL of antibody base.

$6000 / 100 = 60\mu\text{L}$ of your antibody of interest in your 6mL.

****Remember, you need 2 antibodies – a control AB that does not change between the control sample and the experimental sample and your experimental stain. For the control AB, we will use either synaptophysin or VAMP2. Use the internet to determine what those proteins do. And because primaries are not tagged, we will need to use secondaries to detect them. And secondaries recognize the IgG of the host species in the primary. Therefore, your primaries MUST be generated in different host species. ****

To make an antibody, you inject part of your protein of interest into a host animal (usually mice, rabbits, goats, or chickens). For this example, we will use the protein, actin. A company will inject actin protein into a host species, say a mouse. The mouse will mount an immune response to the injected protein and generate antibodies to the actin protein that was injected. So then, serum can be collected from the mouse that are primary antibodies against the actin protein. And because they came from a mouse, the IgG on the antibody is mouse specific. This primary is mouse anti-actin. Mouse is the host species and the mouse antibody detects only actin.

But that primary does not have a tag on it. To detect it, we need a secondary. Secondary antibodies have a tag. And they are generated to detect the IgG. They are usually made in host species that are not used for primaries – typically secondaries are made in goats or donkeys. So. For this example, we could use donkey anti-mouse to detect our anti-actin antibody.

Calculate your Primary Antibody Volumes here:

Control AB: Total volume / dilution ratio = volume of antibody to add to the antibody base
Experimental AB: Total volume / dilution ratio = volume of antibody to add to the antibody base

7. Add primary antibodies to the conical labeled primary antibodies.
8. Add the primary antibody mixture to each well (500µL per well) into the labeled plate.

Primary IF NOTES:

Any changes should be noted here.

Special reagents/equipment used? Manufacturer

FRIDAY: Secondaries :1 hour 30 minutes

You will need to discuss with your group who is doing this. It will take about 20 minutes in person.

10. Wash tissue 3 X 2 minutes with 1X PBS. To do this, use a transfer pipette to remove the liquid in the well and place it in the waste container. Using a clean transfer pipette, add PBS to the well.
11. Add secondary antibodies to the conical labeled primary antibodies.
12. Incubate secondaries for 60 minutes at 37°C , covered in tin foil so that the light in the room does not cause any fading of your samples. During this incubation, make the cupric sulfate if it hasn't been made already. Also, label your slides with the date, your initials, the sample type, what is in the green channel and what is in the red channel.
Example:
4-16-2020 JL
Control Mouse
G = AP3
R = SY38
13. Washed tissue 3 x 3 minutes 1X PBS. To do this, use a transfer pipette to remove the liquid in the well and place it in the waste container. Using a clean transfer pipette, add PBS to the well.
14. Tissue is incubated in cupric sulfate for 15 minutes at 37°C under tin foil.
15. Wash tissue 3 X 3 minutes with 1X PBS. To do this, use a transfer pipette to remove the liquid in the well and place it in the waste container. Using a clean transfer pipette, add PBS to the well.
16. Mount onto glass slides in PBS glass by floating the tissue in the Pyrex with PBS.
17. Add vectashield mounting media on the brains and remove air bubbles
18. Vacuum seal
19. Seal with nail polish
20. Store at 4°C

Secondary IF NOTES:

Any changes should be noted here.

Special reagents/equipment used? Manufacturer?

Molecular Lab #8 - Image your slides using IF scopes

Lab “To Dos” :

View the YouTube Video here: <https://www.labtube.tv/video/MTAoODUx>

Pre -Lab Assignment (This is an individual assignment):

Answer the questions below in the space below. Then, cut and paste the questions and answers to the text box provided on Canvas for this lab. These must be submitted before lab, otherwise you will not be allowed to attend lab.

- 1. What is the BIORad Zoe?**
- 2. What kind of data will we examine with the BIORad Zoe?**
- 3. Did you watch the video?**
- 4. Why is this experiment important?**
- 5. How does this experiment support your hypothesis?**

Post-Lab Deliverables (This is a group assignment/group turn in):

1. Upload your notes from this lab (your notes = any of the spaces that you had to record math or sample measurements). The notes can be an image of your group’s handwritten notes or a cut and paste from notes in a google doc version of the manual.

Molecular Lab #9- Analyze your IF data

Lab “To Dos” :

- (1) Create a figure with data analysis.
- (2) Poster with conclusion section

Pre -Lab Assignment (This is an individual assignment):

Answer the questions below in the space below. Then, cut and paste the questions and answers to the text box provided on Canvas for this lab. These must be submitted before lab, otherwise you will not be allowed to attend lab.

- 1. Why are we quantifying the images?**
- 2. How will we quantify the images?**
- 3. Explain what the graph at the end of lab will look like.**
- 4. How does this experiment connect with your hypothesis?**
- 5. Do you expect this data graph to look similar to your western blot data – why or why not**

Post-Lab Deliverables (This is a group assignment/group turn in):

1. Turn in your poster with the IF figure
2. IF poster with Conclusion Draft

Conclusion: simple statements (bullet points work) to describe the key, take-away points from the experiment and their importance in the field of study. How did the study contribute to the hypothesis? Also, this should include future studies – what would propel this study if there was something more to be added to it.

Remember there are several controls.

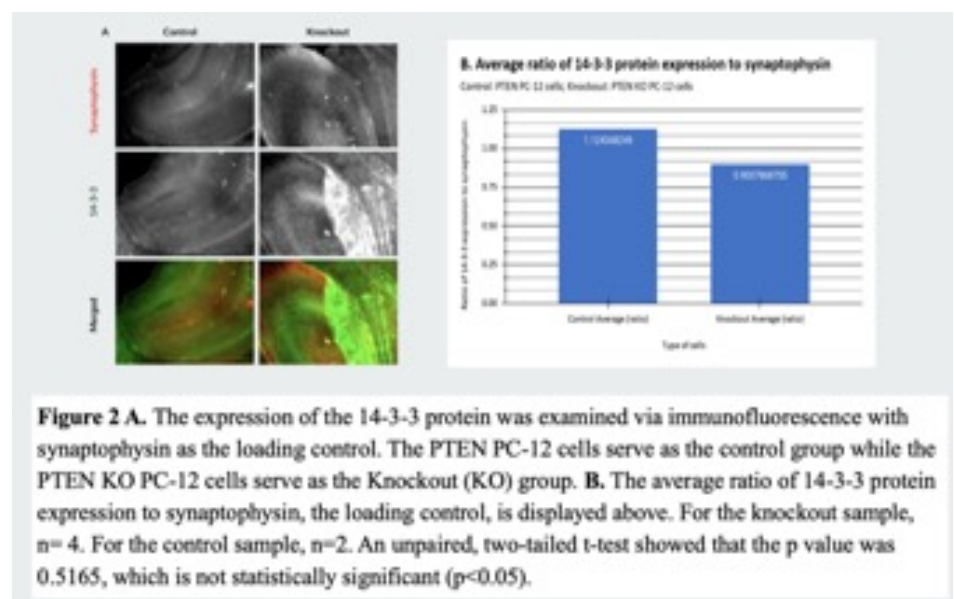
First, we have a control mouse (C57 Black/6) and then we have the Autism Mouse. That is so we can figure out if your protein is altered in any way in autism.

And we have to have in-experiment controls to make sure that the IF was loaded/stained evenly. In the IF, it was synaptophysin (showing in red).

Your graphs - you are reporting the ratio of your protein of interest to the in-experiment control (synaptophysin). For the IF - it is average ratio of all the control mouse ratios of your protein to synaptophysin (that is the y axis) compared to the average ratio of all the experimental mouse avg ratios of your protein to synaptophysin.

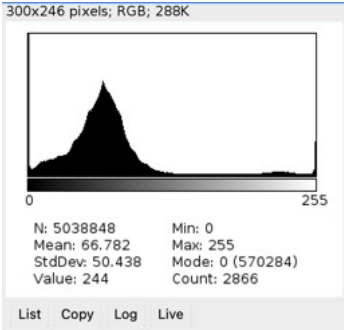
The t-test - you compare the individual samples ratio of the protein variable/control protein of the control tissue (C57B6)(that is data set 1) to the individual samples ratio of the protein variable/control protein (synaptophysin) of the experimental tissue (data set 2). This is an unpaired test and uneven samples.

The purpose of IF is to identify the location and/or protein levels (by comparing IF levels in control and variable brains). IF in brain sections allows us to compare proteins in sub-regions of the brain that we cannot dissect out – or compare cell bodies to dendrite regions, etc. It gives more information than a western blot in the level of overall detail. Both should be used to compliment each other when possible.



Quantification of IF

1. Go to NIH Image J
2. Go to File → Open → (wherever your data is). The file you open should be the individual image of your red and green channels.



3. Select Histogram – record the mean value for each channel.
4. Copy and paste to an excel spreadsheet.
In Excel....
5. Each sample should have its own row. The experimental antibody should have its own column for each sample. And the control antibody (usually actin) should have its own column for each sample.
6. Create a ratio of the control antibody to the experimental antibody (Divide the experimental antibody by the control antibody) in a third column. Do this for EACH data set. A data set is the experimental AB and control AB for each well. You CANNOT average the experimental antibody and the control antibody to make a ratio – each control antibody is specific for that well – so you make individual ratios for EACH of your data sets.

Sample ID	Date imaged	Experiment Ab name (green)	Control Ab name (red)	
	05/31	AP3	Actin	Ratio of green/
		Density from Image J	Density from Image J	Ratio of AP3/ACTIN
m1 dg		57.503	54.047	1.063944345
m1 dg		99.757	88.986	1.121041512

To graph this – you should have three brain sections with 2 dentate gyri each for the control and the same for the experimental brains. So 6 data points each.

Before you take the average, run a T- test to compare the 6 data points of the control sample and the 6 data points for your experimental sample.

Are the data significant?

Average the ratio of the control to the experimental ratio.

Control Sample Average –

Experimental Sample Average –

Now to graph it – you report on the X – axis that sample group, and on the Y axis – ratio of experimental AB/Control AB. The graph is a simple bar graph of the average ratio for each sample.

After you graph it, make a figure legend that describes what the samples are, the number of samples that are represented in the graph.

T-Test in Excel

(or instructions for a T-test in google sheets here: <https://support.google.com/docs/answer/6055837?hl=en>)

Step 1: Enter your data in an organized fashion.

Be sure to label what units were used to measure and all details. The more you label, the less confusion there is.

After the data is entered, you can pick any empty cell to enter in the formula.

For the T-Test, let's compare the males:

In an empty cell, enter the "=" sign (shown in A15 in the screen shot). Then, select the stats test in the function box shown on the top left just before the function formula bar.

When you select t test, the screen should look like this:

For Array 1, click in the array 1 box on the right, then highlight the cells with data for all the control males.

For array 2, click in the array 2 box on the right, then highlight the cells with data for all the variable males.

Then fill in the data for the tails and type of test using the definitions given and what you know about your data.

Finally, click done. In the cell that you originally put the "=" sign, the p value will come up. Label this!

Additional Data that is useful for reporting: the average and the Standard deviation of the group.

Again, create some space. Insert rows if you need to. So you can label your data.

To determine the average, place the "=" under a set of data. Select the function "Average" and in the space to the right, click in the number 1 box and then highlight the data you want to average – all of one particular group.

To determine the standard deviation, place the "=" under a set of data. Select the function "STDEV" and in the space to the right, click in the number 1 box and then highlight the data you want the standard deviation of – all of one particular group.

Molecular Biology Lab #10 - Poster Presentations

Lab “to Dos”

To polish your poster

Pre -Lab Assignment (This is an individual assignment):

Answer the questions below in the space below. Then, cut and paste the questions and answers to the text box provided on Canvas for this lab. These must be submitted before lab, otherwise you will not be allowed to attend lab.

1. How many times did you practice your poster presentation section? Practice your poster presentation at least 4-6 times prior to coming to lab.

2. Complete your peer evaluation for lab member #1.

Group member's name _____

This member contributed to the project.	1	2	3	4	5
This member made an effort to work with the other members.	1	2	3	4	5
This member came to lab time prepared and on time.	1	2	3	4	5
This member was a valuable member to our group.	1	2	3	4	5

Would you work with this member on another project? Yes No

Why or why not? _____

3. Complete your peer evaluation for lab member #2.

Group member's name _____

This member contributed to the project.	1	2	3	4	5
This member made an effort to work with the other members.	1	2	3	4	5
This member came to lab time prepared and on time.	1	2	3	4	5
This member was a valuable member to our group.	1	2	3	4	5

Would you work with this member on another project? Yes No

Why or why not? _____

4. Complete your peer evaluation for lab member #3.

Group member's name _____

This member contributed to the project.	1	2	3	4	5
This member made an effort to work with the other members.	1	2	3	4	5
This member came to lab time prepared and on time.	1	2	3	4	5
This member was a valuable member to our group.	1	2	3	4	5

Would you work with this member on another project? Yes No

Why or why not? _____

5. Complete your self evaluation for your participation in the lab.

I contributed to the project.	1	2	3	4	5
I made an effort to work with the other members.	1	2	3	4	5
I came to lab time prepared and on time.	1	2	3	4	5
I was a valuable member to our group.	1	2	3	4	5

Did you do your best to ensure the group work was even and efficient throughout the semester?

Post-Lab Deliverables (This is a group assignment/group turn in):

All feedback should be incorporated into the into a final draft of the poster that will be turned in at the end of lab.