

# *Immunodetection methods in biochemistry*

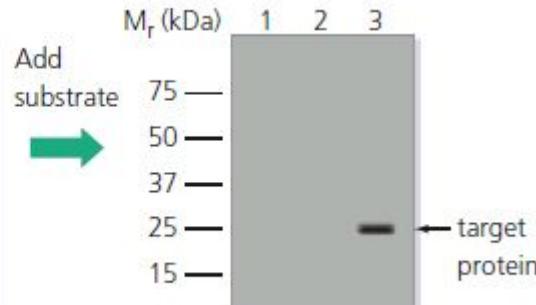
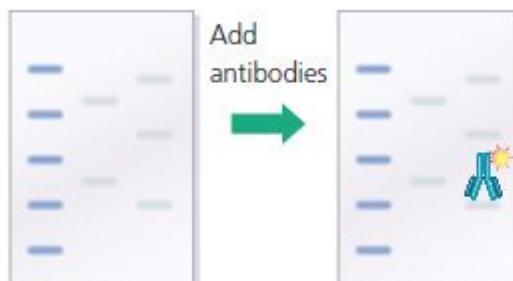
*Heather MacGregor*

# **Overview**

- A. Rationale and overview of available methods**
- B. Practical aspects to consider in experimental design**
- C. Results and Discussion**
- D. Possible improvements to the experiment**
- E. Bonus:**  
**Summer 2019 Research Project in the Maribel Rios Lab**

# Immunodetection

## General protocol



Source: [Bio-Rad Laboratories, Inc.](#)

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# ***Immunodetection Methods: Blocking***

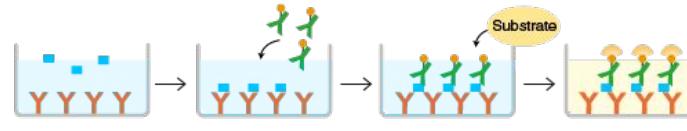
<b>Blocking Reagent</b>	<b>Membrane Compatibility</b>	<b>Recommended Concentration</b>	<b>Notes</b>
Gelatin	Nitrocellulose	1–3%	Requires heat to solubilize
Nonfat dry milk, BLOTTO, blotting-grade blocker	Nitrocellulose, PVDF	0.5–5%	PVDF requires higher concentrations of nonfat milk than nitrocellulose
Bovine serum albumin (BSA)	Nitrocellulose, PVDF	1–5%	PVDF requires higher concentrations of BSA than nitrocellulose
Tween 20	Nitrocellulose	0.05–0.3%	May strip some proteins from the blot

# Immunodetection Methods: ELISA

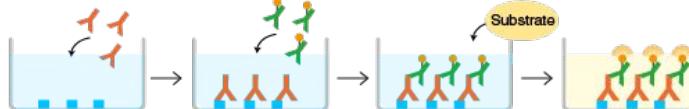
Direct



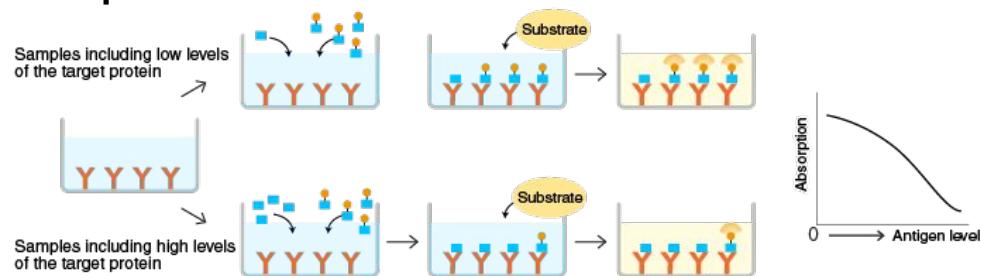
Sandwich



Indirect



Competitive



Antibody

Target protein



Enzyme-labeled antigen

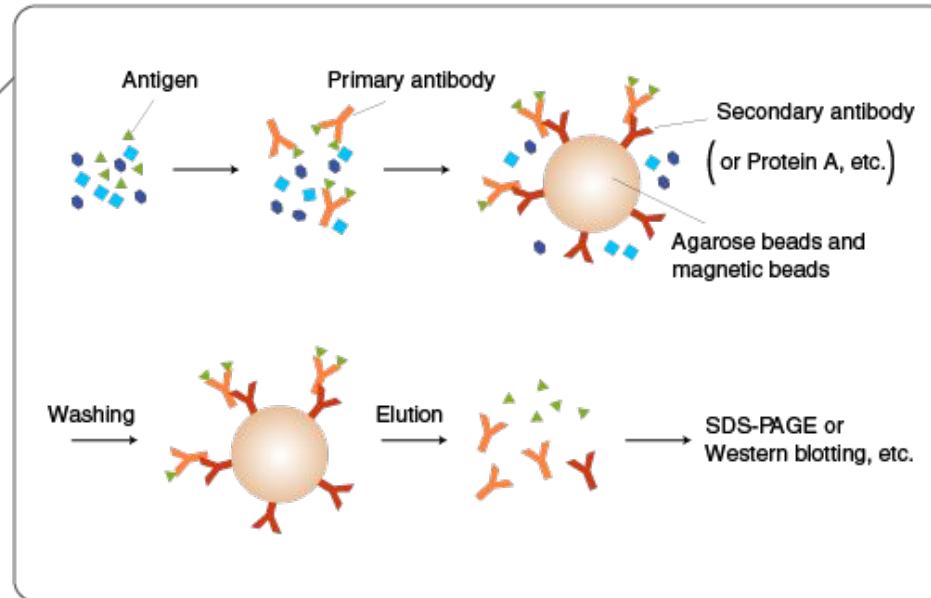


Enzyme reaction

Source: [MBL Life Science](#)

# *Immunodetection*

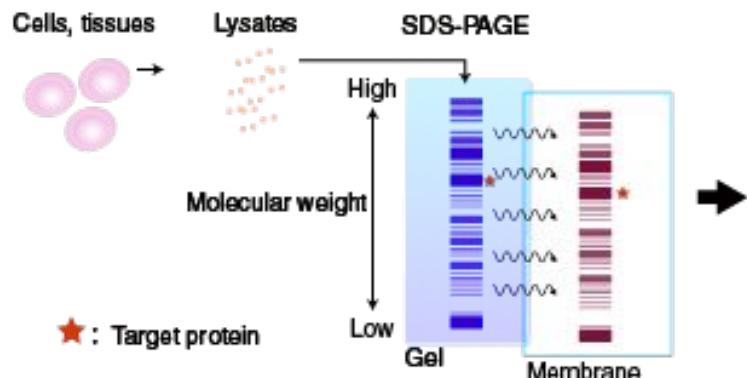
## *Methods: Immunoprecipitation and Coimmunoprecipitation*



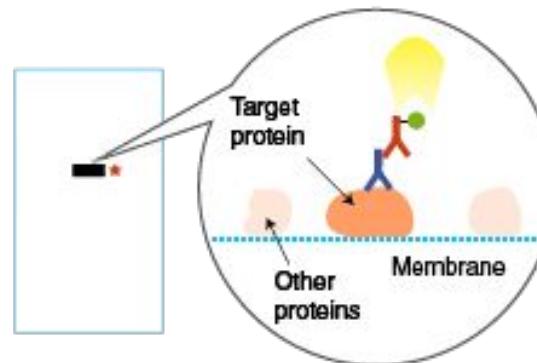
Source: [MBL Life Science](#)

# **Immunodetection Methods: Western Blot**

Proteins are separated by electrophoresis and transferred to a membrane.



Probing with antibodies, and detection of the target protein by an enzyme reaction.

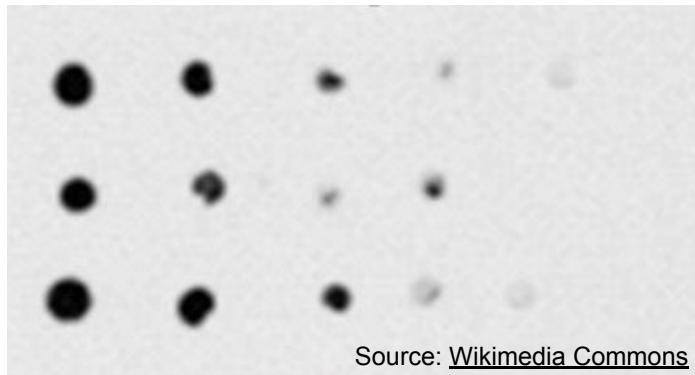


- Y : Primary antibody
- Y : Secondary antibody (enzyme-labeled antibody)
- Yellow : Light emission or colorimetric change caused by an enzyme reaction

Source: [MBL Life Science](#)

# **Immunodetection**

## **Methods: Immunoblot or “Dot Blot”**



Source: [Wikimedia Commons](#)

### **Advantages:**

- Doesn't require prior separation by gel electrophoresis
- Short and simple procedure

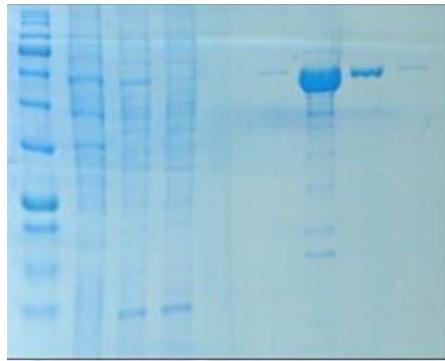
### **Disadvantages:**

- Provides no information about:
  - Molecular weight
  - Enzyme activity
- False positives are difficult to detect
- Potential routes for contamination

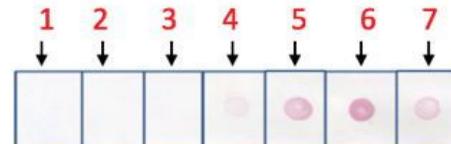
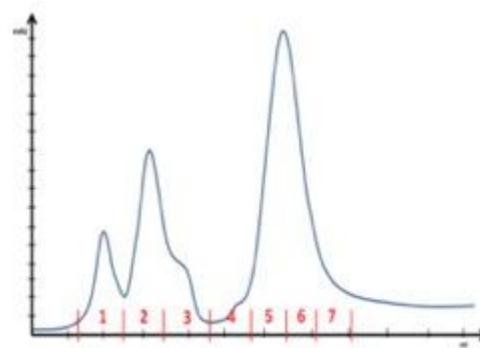
# **Immunodetection**

## **Methods: Immunoblot or “Dot Blot”**

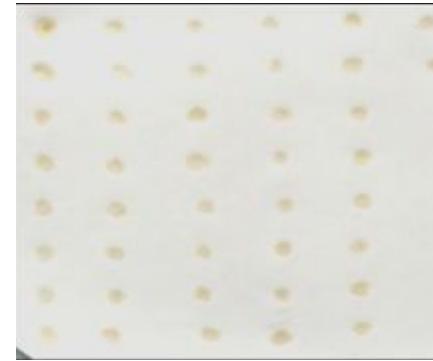
Bands on SDS-PAGE gels



Chromatography fractions



Expression level of colonies



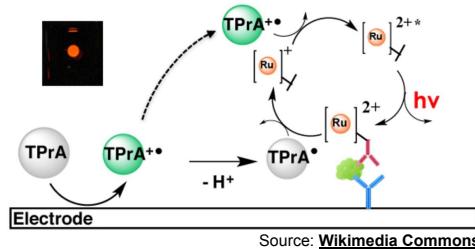
Source: [Jena Biosciences](#)

# ***Immunodetection Methods: And so on...***

- **Radioimmunoassay**
- **Real-time immuno quantitative PCR (iqPCR)**
- **Electrochemiluminescence**
- **Lateral flow tests or immunochromatographic assays**
- **And more!**

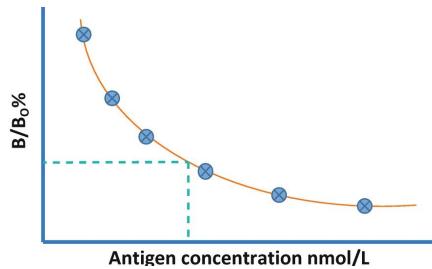
# Immunodetection Methods: Labels

## Electrochemiluminescent tags

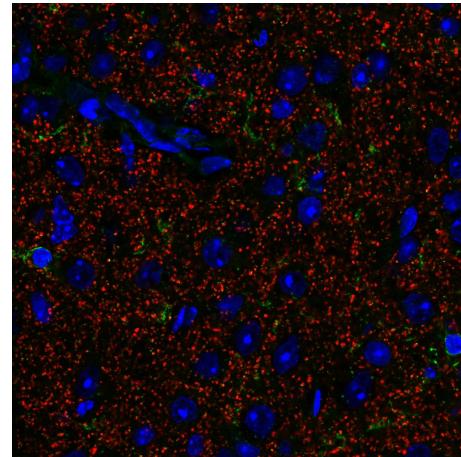


Source: [Wikimedia Commons](#)

## Radioactive isotopes



Source: [SpringerLink](#)



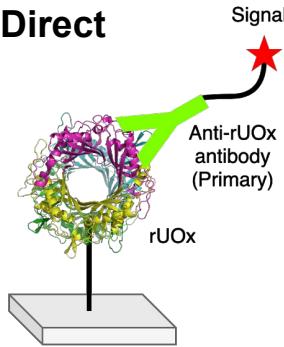
## Fluorescent reporters

## Conjugated Enzymes

# ***Practical Aspects***

***How can we detect our protein of interest?***

## **Direct**



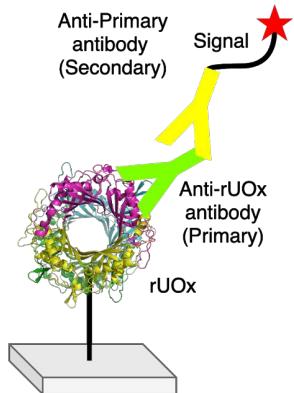
## **Advantages**

- Simple and fast
- Lower non-specific binding and cross-reactivity

## **Disadvantages**

- Less sensitive
- Requires more 1' ABs (\$\$\$)

## **Indirect**



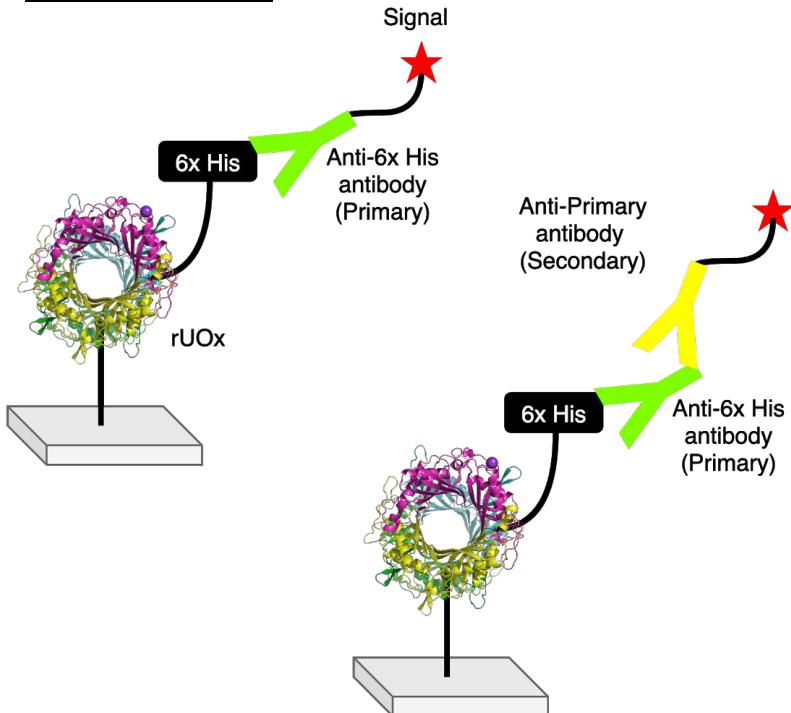
- Stronger signal (multiple 2' ABs bind to one 1' AB)
- More flexible
- 2' ABs are relatively less expensive

- Longer, more complex protocol
- 2' ABs bind less specifically, which can result in high background

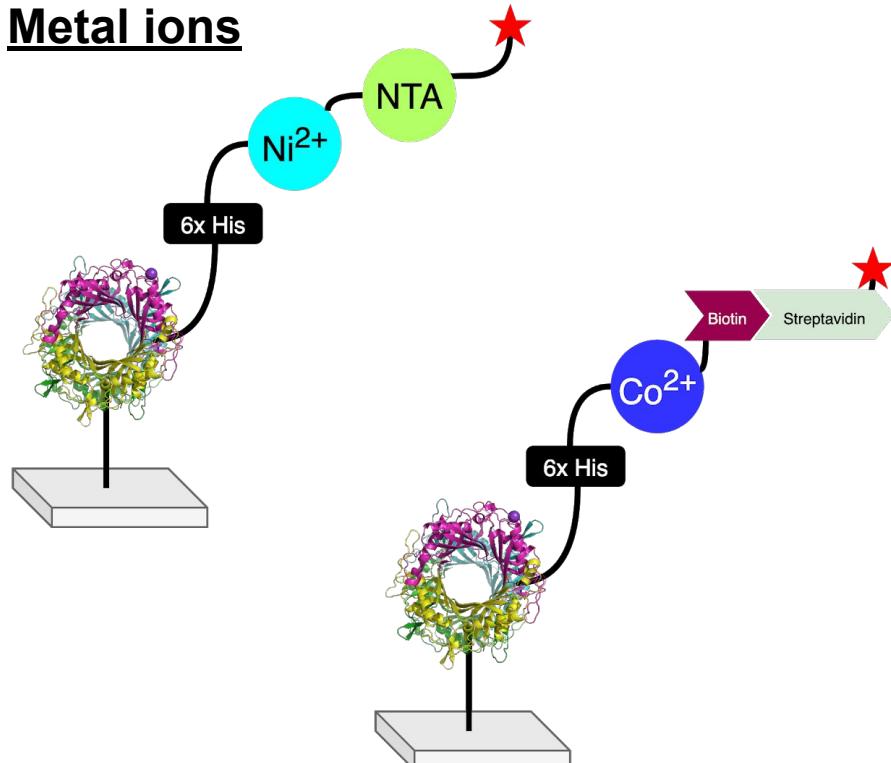
# *Practical Aspects*

~~How can we detect our protein of interest?  
the 6x-His moiety?~~

## Antibodies



## Metal ions

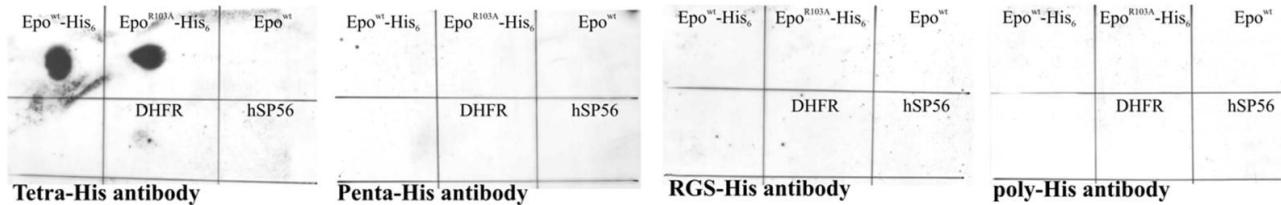


# *Practical Aspects*

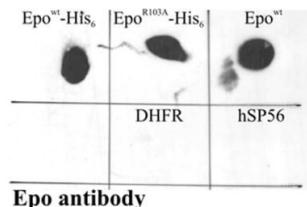
~~How can we detect our protein of interest?  
the 6x-His moiety?~~

## Antibodies

### anti-His



### anti-POI



+ Cheaper than using specific antibodies

- Nonspecific binding to other proteins with consecutive His residues
- Nonspecific binding to fragments of the protein containing consecutive His residues
- Poor signal

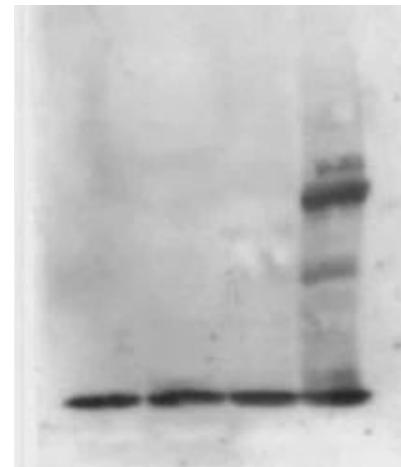
# *Practical Aspects*

*How can we detect our protein of interest?  
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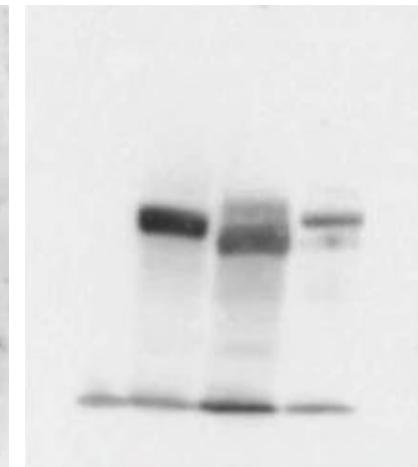
## Metal ions

- + Cheaper than specific and anti-His antibodies
- + Faster (reduces the number of steps)
- Nonspecific binding to other proteins with consecutive His residues
- Nonspecific binding to fragments of the protein containing consecutive His residues

Anti-6xHis antibody



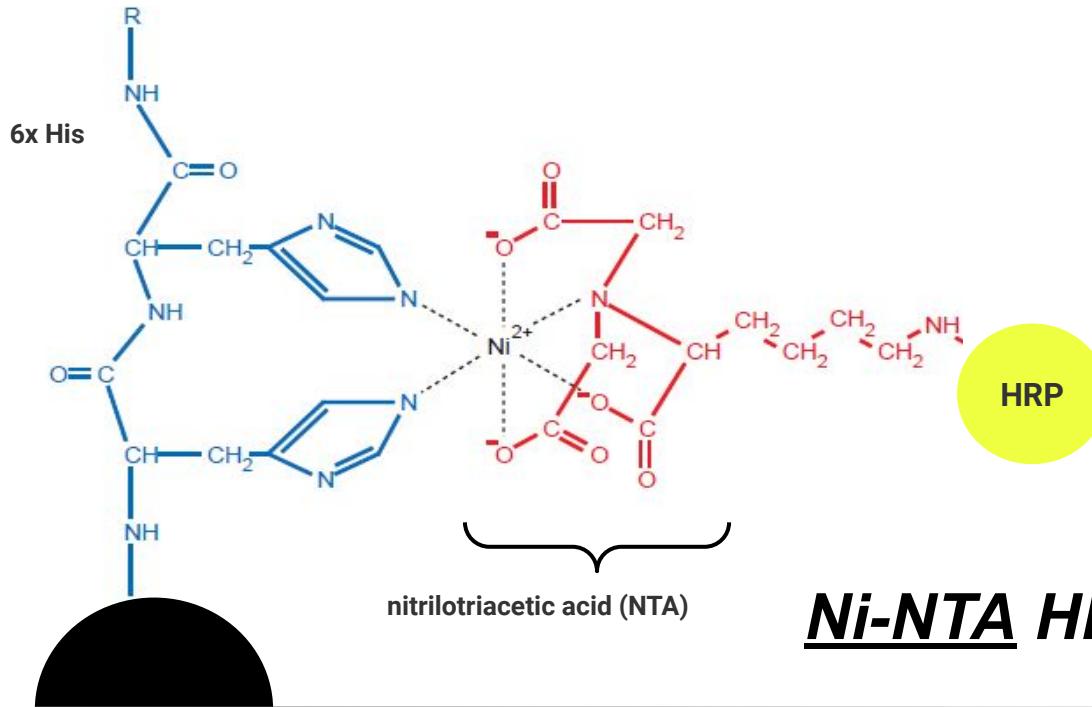
HisProbe-HRP Conjugate



Source: Thermo Scientific™ HisProbe™-HRP Conjugate

# *Practical Aspects*

~~How can we label our protein of interest?  
the 6x-His moiety?~~



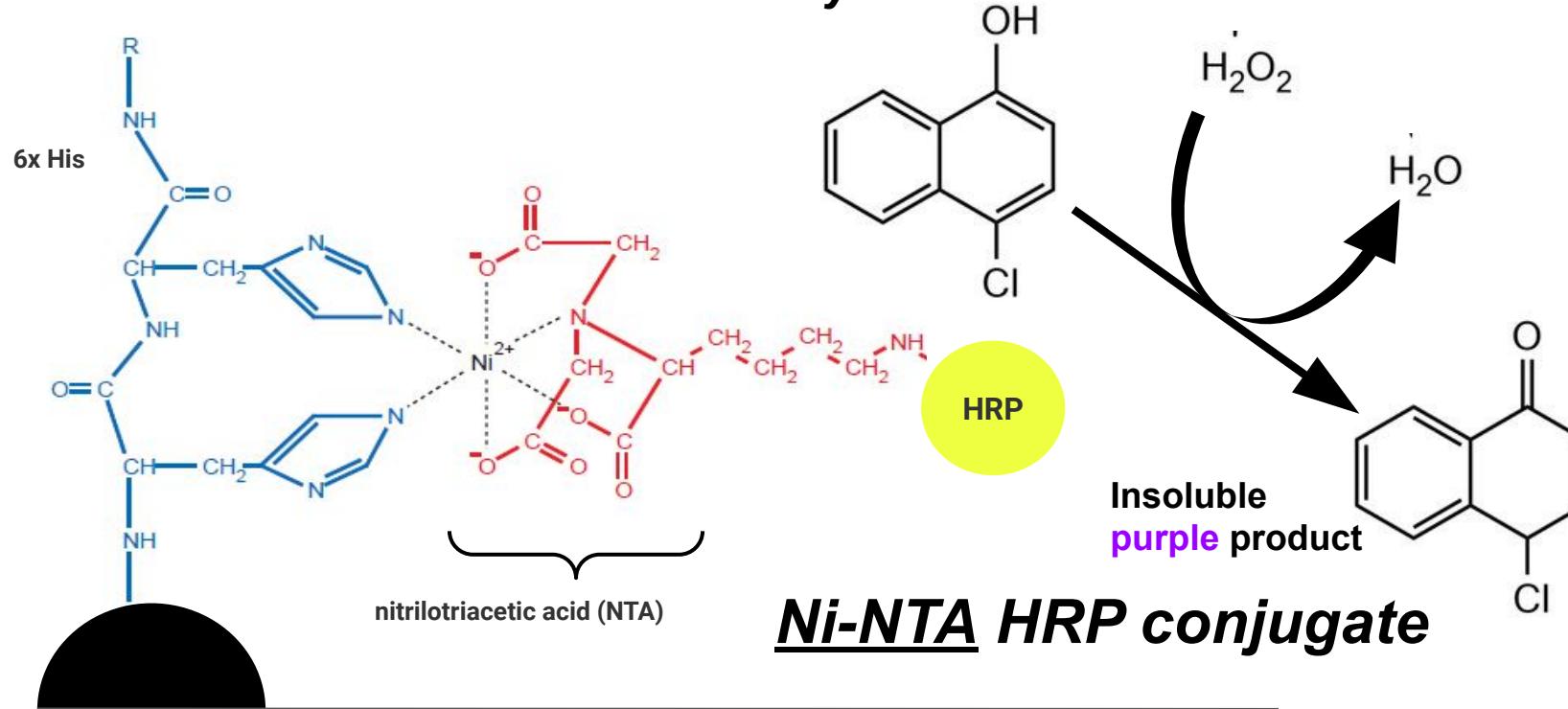
## **Horseradish peroxidase:**

- Produces a brown (DAB) or purple/black (4CN, TMB, NBT/BCIP) stain or chemiluminescent signal (luminol)
- Detection limit in the low nanogram range
- Colorimetric detection reaction proceeds until stopped (risk of overdevelopment)
- Stain fades over time

**Ni-NTA HRP conjugate**

# Practical Aspects

~~How can we label our protein of interest?  
the 6x-His moiety?~~



doi: 10.13140/RG.2.2.21964.62088

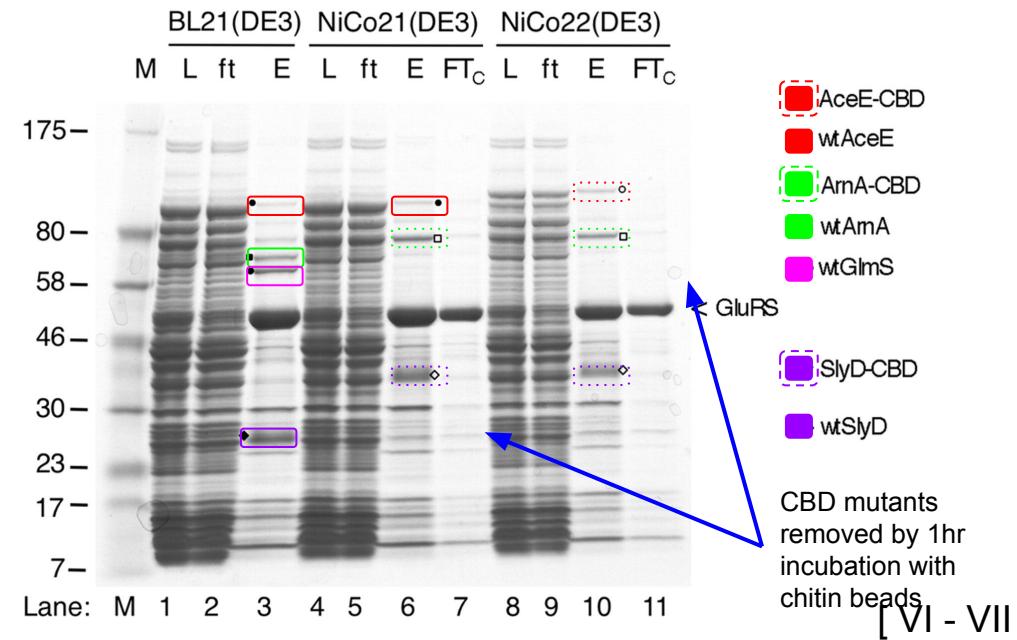
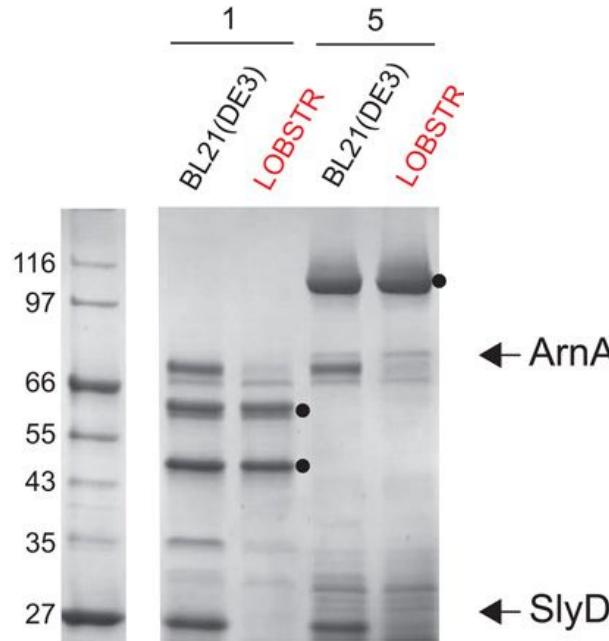
Source: Farka et al., 2016.

# Practical Aspects

## Issues with a 6x-His tag target

**Issue:** His-tag affinity chromatography can result in the co-purification of contaminating histidine-rich E. coli proteins

**Solution:** Optimized E. coli strains (Ex. LOBSTR (Kerafast), NiCo21, and NiCo22 (NEB))



# ***Practical Aspects***

## ***Issues with a 6x-His tag target***

**Issue:** His-tag affinity chromatography can result in the co-purification of contaminating histidine-rich E. coli proteins

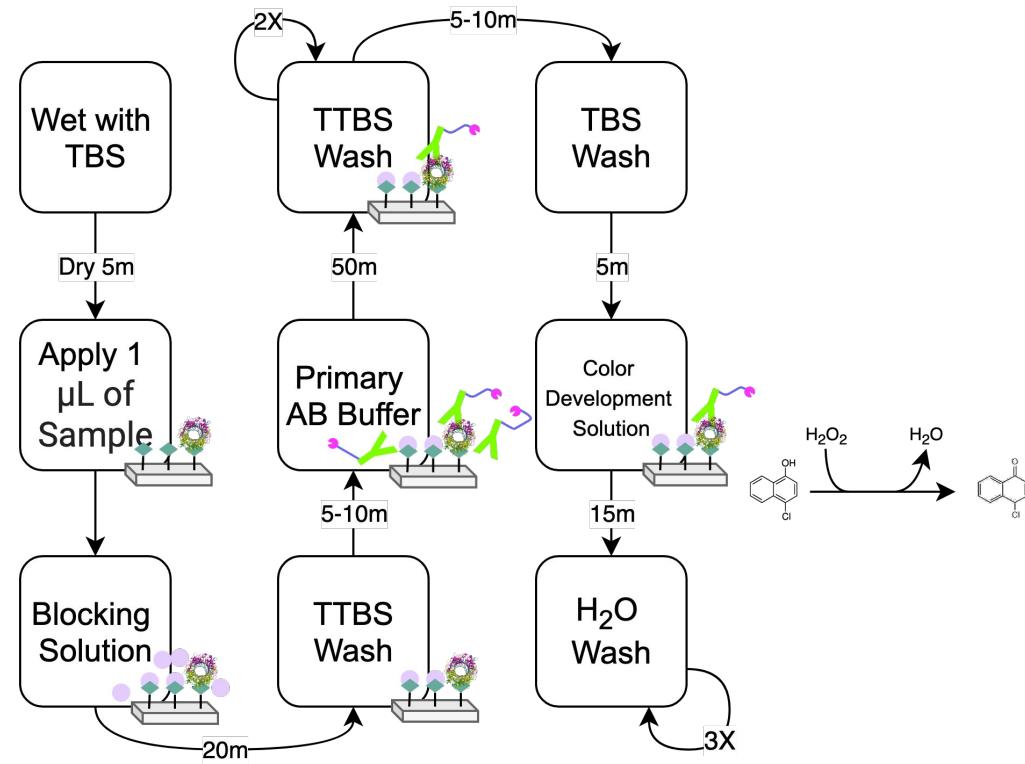
**Solution:** ~~Optimized E. coli strains (Ex. LOBSTR (Kerafast), NiCo21, and NiCo22 (NEB))~~

### ***What this could mean for our experiment:***

If these contaminants were co-purified from the Ni-NTA column along with rUOx, they will also be recognized by the Ni-NTA HRP conjugate and increase the amount of signal observed

# *Practical Aspects*

## *Experimental Protocol*



# *Practical Aspects*

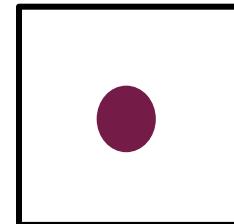
## *Positive and Negative Controls*

### **Positive control:**

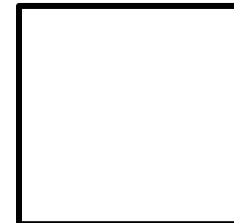
- Sample containing purified 6x-His tagged UOx

### **Negative control:**

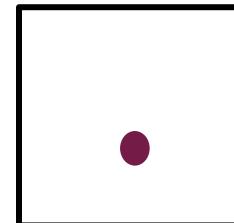
- Cell lysate of induced BL21 *E. coli* that have been transfected with pET14b, but not UOx



Cell Lysate (+)



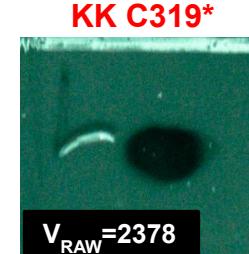
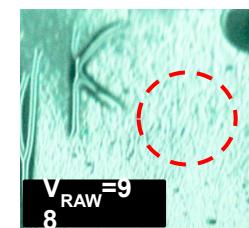
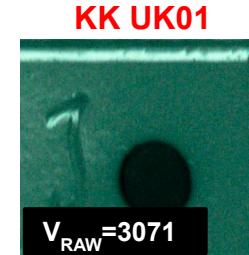
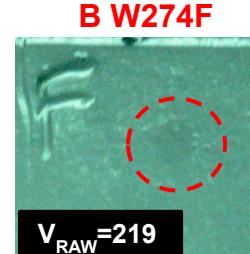
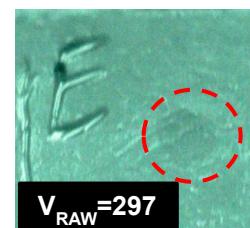
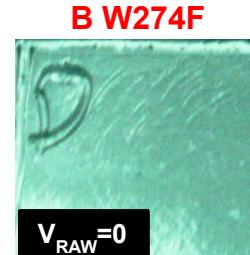
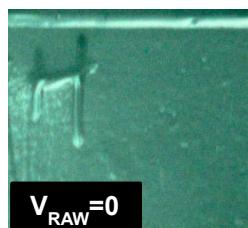
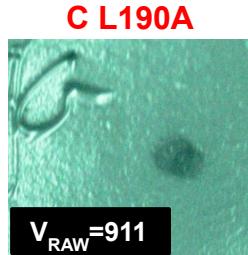
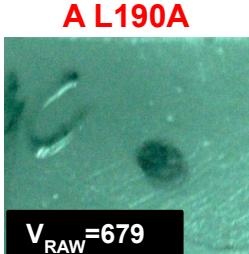
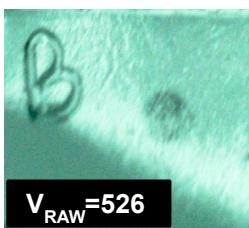
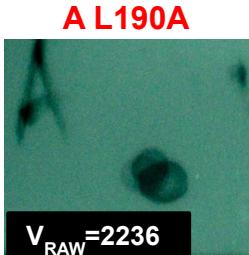
Column Flowthrough (-)\*\*



Final fraction eluted

*\*\*The column flowthrough isn't a good negative control, but would theoretically work as one if the experiment had worked perfectly.*

# Results



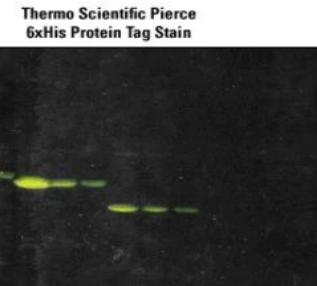
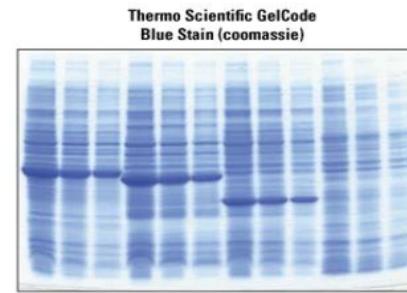
# Troubleshooting low signal

Possible Cause	Solution	Possible Cause	Solution
Improper blocking reagent	<i>If the blocking agent has an affinity for the protein of interest, it can prevent its detection.</i> Try a different blocking agent and/or reduce both the amount or exposure time of the blocking agent.	Use of tap water	<i>Tap water inactivates chromogenic detection reagents.</i> Try using nanopure water.
Insufficient reaction time	Increase the incubation time.	Azide inhibits HRP	Do not use azide in the blotting solutions.
Antibody concentration is too low or antibody is inactive	<i>Multiple freeze-thaw cycles, bacterial contamination, or repeated use of antibody solution can change antibody titer or activity.</i> Increase antibody concentration or prepare it fresh.	Antigen concentration is too low	<i>Load more antigen on the gel prior to the blotting.</i>
		Detergents (e.g., SDS)	<i>Detergents may inhibit lower molecular weight proteins from binding to the membrane.</i>

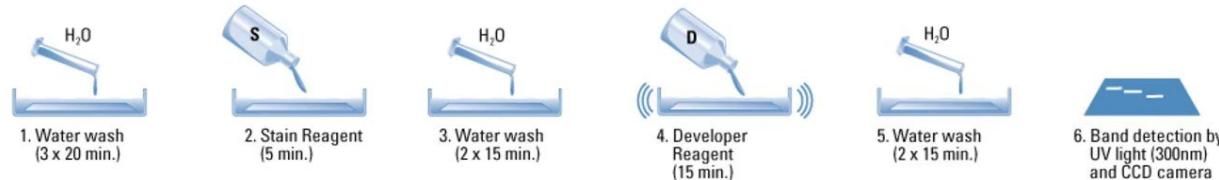
# Potential alternatives

## Thermo Scientific Pierce 6xHis Protein Tag Stain Reagent Set

- Specifically detects histidine-tagged fusion proteins that have been electrophoresed in polyacrylamide gels
- Works in-gel (no need to transfer and perform WB)
- Compatible with subsequent staining with coomassie dye
- UV lamp detection (300 nm)

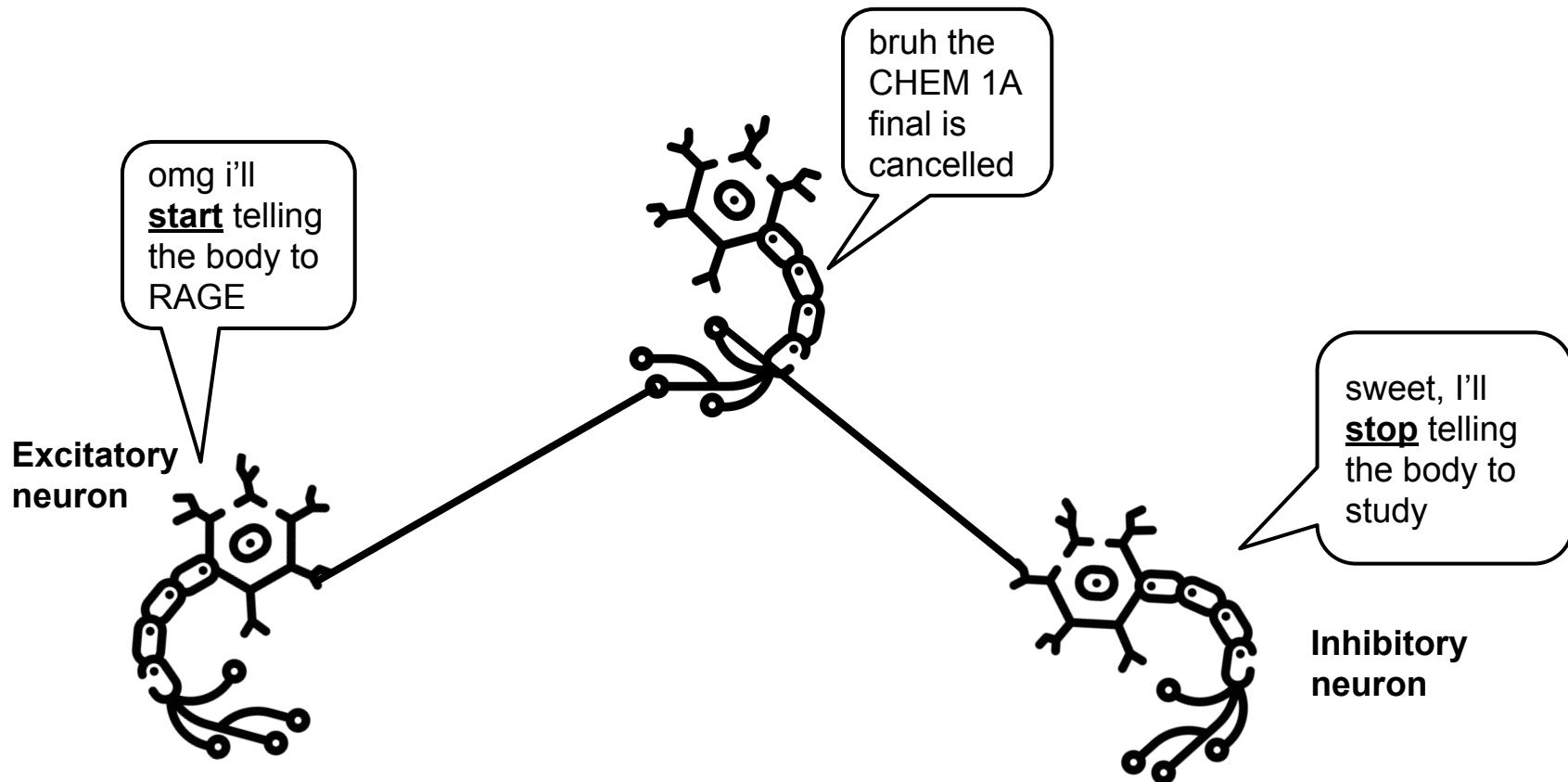


Western blot of the SDS-PAGE gels (time permitting)



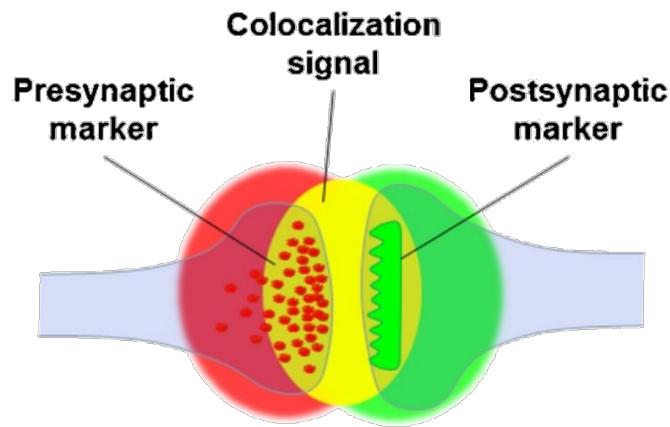
# *Summer 2019 Research Project*

## *Excitatory and inhibitory neurotransmission*

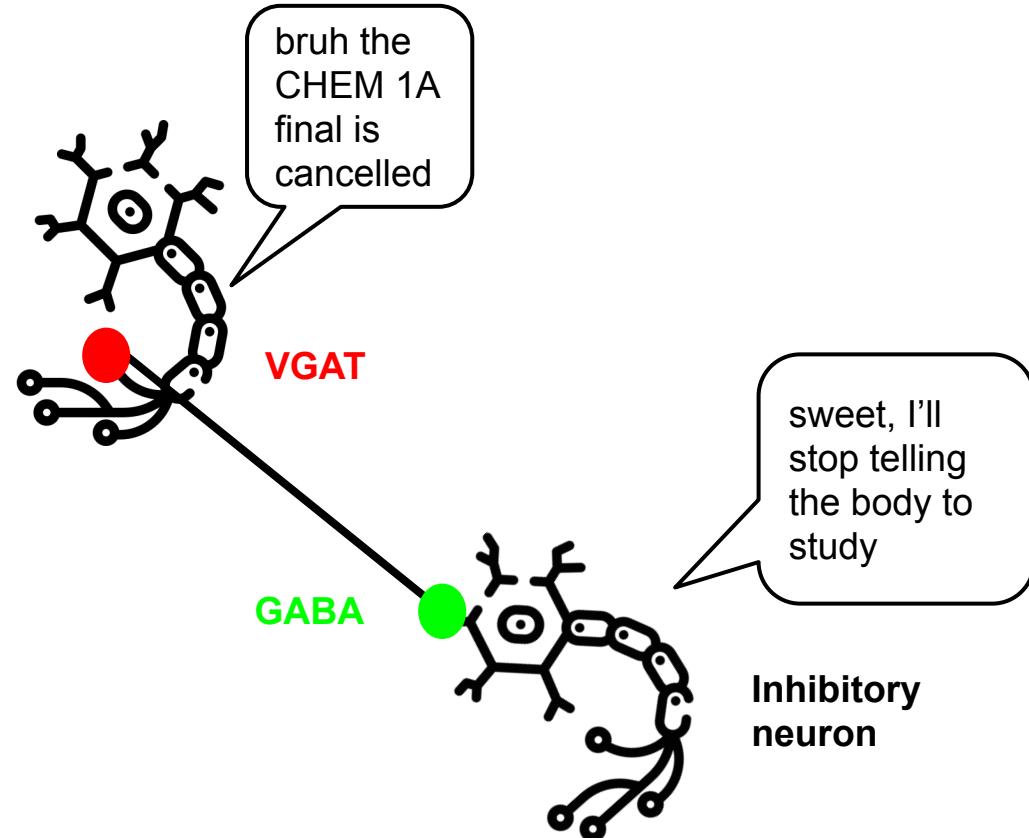


# *Summer 2019 Research Project*

## *Excitatory and inhibitory neurotransmission*



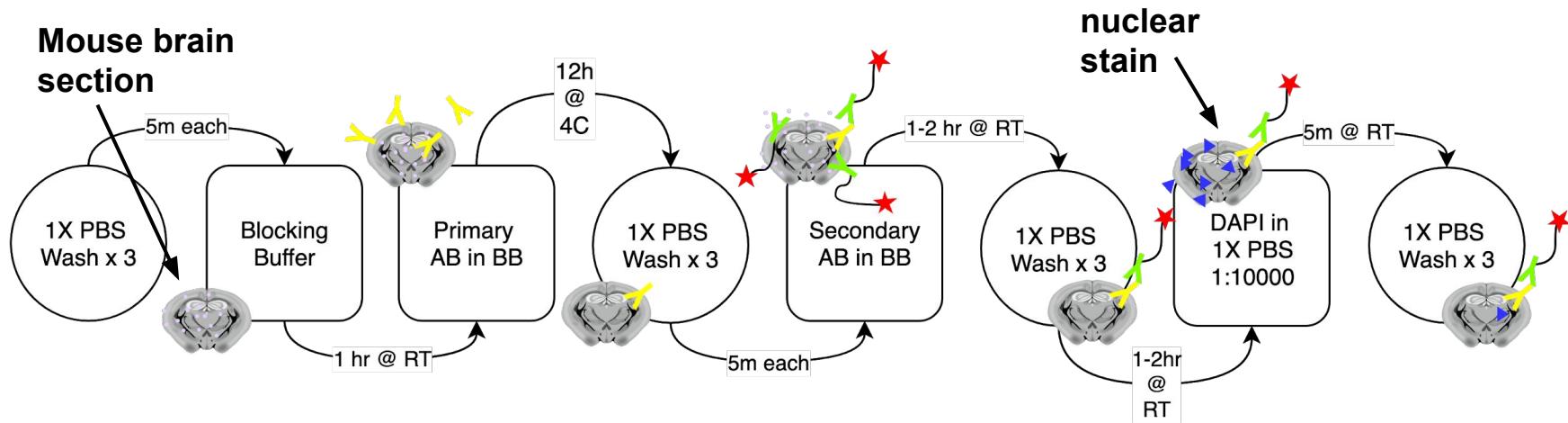
Colocalization reveals structurally  
accomplished synapses



[ X ]

# *Summer 2019 Research Project*

## *Experimental Design*

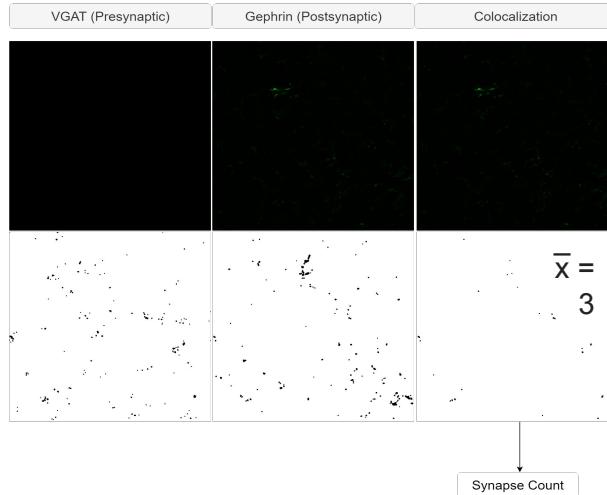


The amount of colocalized synapses in each image (63X magnification on a confocal microscope) of the region of interest was automatically processed by the Synapse Counter plug-in for ImageJ.

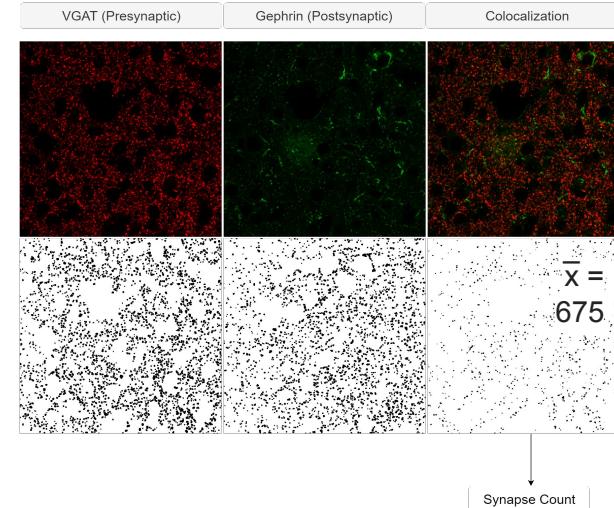
# *Summer 2019 Research Project*

## *Experimental Design*

**Negative control: NO 1' AB**



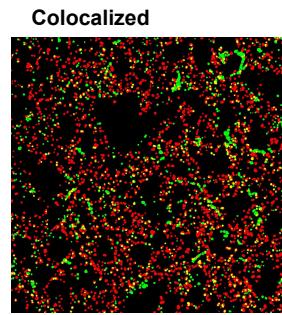
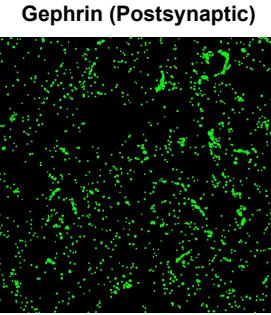
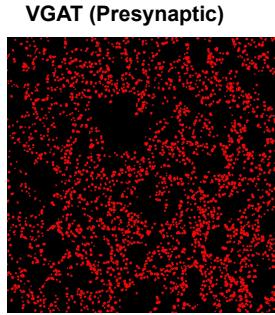
**Positive control:** sample known to express both proteins of interest



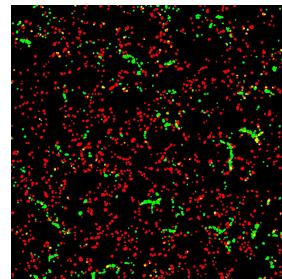
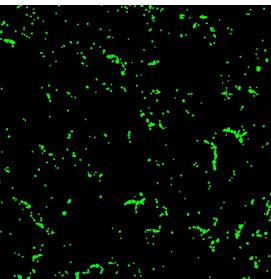
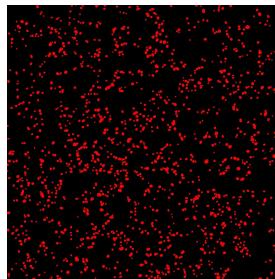
**Combinations of specific antibodies were screened for the best signal-to-noise ratio.**

# *Summer 2019 Research Project Results*

■ FED

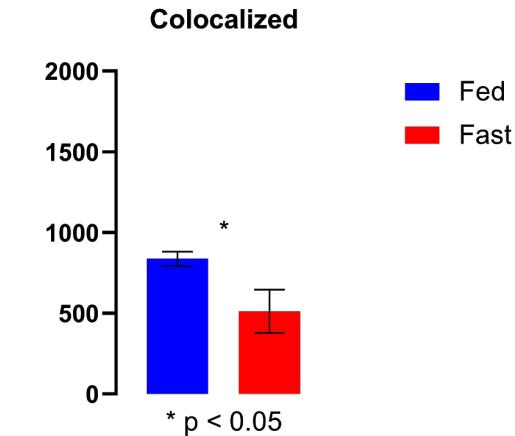
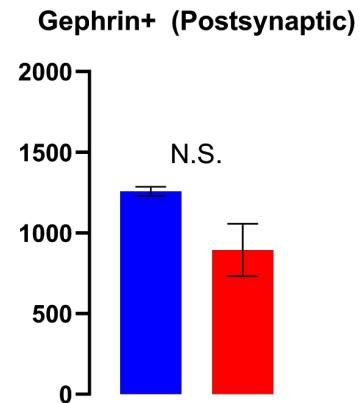
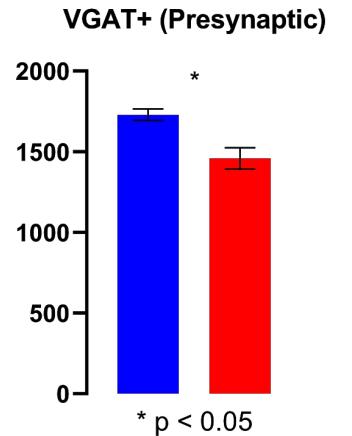


■ FAST



# *Summer 2019 Research Project*

## *Results*



**Fed animals have greater inhibitory synaptic transmission than fasted animals.**

# References

- I. **Introduction to Immunodetection.** 2020; Bio-Rad Laboratories, Inc.
- II. Kahn K. **The Operations Manual for Component 2.** 2020, Santa Barbara, CA.
- III. Debeljak N, Feldman L, Davis KL, Komel R, Sytkowski AJ. **Variability in the immunodetection of His-tagged recombinant proteins.** *Analytical Biochemistry.* 2006;359(2):216-223. doi: 10.1016/j.ab.2006.09.017.
- IV. **Western Blot: Test Blots, Slot Blots, and Dot Blots.** 2020; Bio-Rad Laboratories, Inc.
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- VI. Andersen KR, Leksa NC, Schwartz TU. **Optimized E. coli expression strain LOBSTR eliminates common contaminants from His-tag purification.** *Proteins.* 2013;81(11):1857–1861. doi:10.1002/prot.24364
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- VIII. **Dot/Slot (Filtration) Blotting.** 2020; Millipore Sigma.
- IX. Park, J. **Immunodetection: Winning Westerns Webinar.** 2020; Millipore Sigma.
- X. Macgregor H, Onder Y, Rios M. **Feeding state dependency of inhibitory synaptic dynamics in the ventromedial hypothalamus.** *Annual Biomedical Research Conference for Minority Students.* 2019;E(Neuroscience); doi: 10.13140/RG.2.2.11525.50403