

Lab Protocol 2

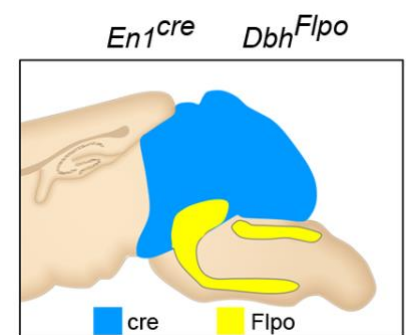
Mapping norepinephrine neurons and their projections using fluorescent microscopy

Background

A fundamental challenge of modern neuroscience is to map the connections and functions of all neurons in the brain. Map building may seem mundane, but the intrigue and challenge of building a map of the nervous system lies in the sheer numbers. The human brain is composed of billions of neurons that communicate through trillions of synaptic connections that define who we are and how we interact with the world. Our research in this course will focus on a tiny sliver of this complexity, the central norepinephrine (NE) system. Despite the small number of neurons in the system (relative to the billions in the brain), norepinephrine neurons modulate a diverse array of essential processes including attention, stress, learning, memory and the perception of pain. And in disease states, where these behaviors are disrupted (Alzheimer's disease, Parkinson's disease, etc.) specific subsets of NE neurons are often compromised. The mechanisms that generate such functional diversity and differential susceptibility in the NE system are unknown. In this course, we will work collaboratively with researchers on the long-term research goal to construct a functional map of the diverse subsets of NE neurons and their connections in the brain. Specifically this semester, our class will explore how *in vivo* manipulation of a single **genetically defined subpopulation** of NE neurons affects animal behavior. With the power to manipulate genetically defined subsets of NE neurons in living mice, we hope to uncover how NE signaling contributes to various behaviors and disease states.

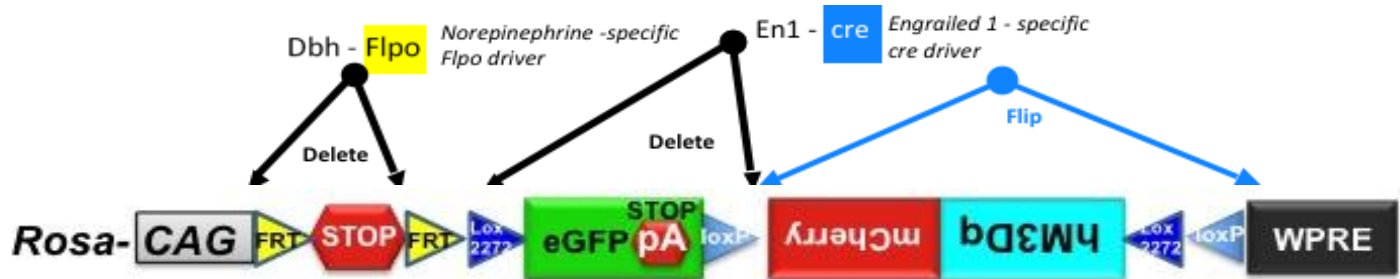
To explore the function and connections of a genetically defined subpopulation of NE neurons, we will work with tissue from newly generated genetically modified mice. Through the application of a **recombinase based intersectional genetic approach**, our mice are designed to express the **mCherry tagged hM3Dq DREADD receptor** in a genetically defined subset of norepinephrine neurons while all other NE neurons express only eGFP. The hM3Dq DREADD (Designer Receptor Exclusively Activated by Designer Drugs) receptor is a mutant human muscarinic receptor **engineered** by scientists (Bryan Roth's group at UNC) to activate only in response to the otherwise **inert ligand clozapine-N-oxide (CNO)**. Expression of the hM3Dq receptor in a genetically defined subset of neurons will enable us to study the behavioral affects of "turning on" (i.e. stimulation of action potentials) a specific subset of central NE neurons. This "remote control" of neuronal activity is incredibly powerful and will help us build a functional map of the heterogeneous central NE system.

Since we are working with a newly generated animal strain, it is crucial that we assess the efficacy of our genetically modified alleles. In other words, we must determine if our genetic approach works. Do we see mCherry-hM3Dq and eGFP expression in the appropriate NE neuron subpopulations? To understand where we expect to see mCherry-hM3Dq and eGFP expression, you need to consider our **recombinase based intersectional genetic approach**. Our mice are triple transgenic animals that contain three genetically modified alleles: two recombinase **driver alleles** (a NE specific *Dbh^{Flpo}* allele and an *Engrailed 1* specific *En1^{cre}*) and a dual recombinase responsive **effector allele** *RC::hM3Dq*. *Dbh* (dopamine beta hydroxylase) is an enzyme expressed specifically in neurons that synthesize and release norepinephrine because it is required to convert dopamine into norepinephrine within neurotransmitter filled vesicles. *En1* (*Engrailed 1*) is a transcription factor that is expressed early in development. The picture to the right depicts a schematic of neurons that have a history of *Dbh^{Flpo}* and *En1^{cre}* expression (right). When these recombinase driver alleles are expressed in combination with the effector allele *RC::hM3Dq* neurons that have a history of *En1* and *Dbh* expression should express mCherry-hM3Dq and all other NE neurons with a history of only *Dbh* expression should express eGFP. This is called an intersectional genetic approach because only neurons with a history of intersecting *Dbh* and *En1* expression will express the mCherry-hM3Dq DREADD receptor (i.e. the effector).



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How does this **recombinase based intersectional genetic approach** work? Our effector allele, which is knocked into a ubiquitously expressed locus in the mouse genome contains *eGFP* and the *mCherry-hM3Dq* DREADD following two transcriptional **STOP** cassettes. These transcriptional stop signals must be removed to turn *eGFP* and *mCherry* expression on. Removal of the stop cassettes is facilitated by two site-specific recombinases, cre and Flpo, which recognize loxP and FRT sites, respectively, and catalyze DNA exchange reactions. DNA between two loxP or FRT sites oriented in the same direction will be excised while DNA between two loxP sites arranged in an opposing orientation will be inverted. Therefore, transcription will stop prior to eGFP in cells that do not express Flpo or cre. Cells that express *Dbh* and therefore Flpo will have the first stop cassette removed and express eGFP (all NE neurons). Cells that express *En1*, which turns on early in development (before *Dbh*), will express Cre which deletes the loxP2722 flanked *eGFP* and inverts the *mCherry-hM3Dq* DREADD sequence so that it is in the proper orientation for transcription. In these cells with only a history of *En1* expression however, the first stop cassette remains (i.e. no eGFP or hM3Dq expression)! It is only in neurons that express **both** *Dbh^{Flpo}* and *En1^{Cre}* that *mCherry-hM3Dq* DREADD expression should occur. In this lab, we will immunostain coronal sections from mice expressing all three alleles (*En1^{Cre};Dbh^{Flpo};RC::hM3Dq*) and investigate eGFP and mCherry-hM3Dq expression patterns to determine which NE neurons express the DREADD receptor and assess how well our genetic approach restricts expression of the DREADD receptor to the population of neurons we intend.



Day1

Purpose: to acquire 40µm coronal sections of the whole mouse brain using a cryostat.

Materials:

- Frozen mouse brains
- Cryostat
- Forceps, 2x sweeping brush, 2x tweezers
- TFM
- Paintbrush
- 12 well plate
- Freezing solution

Schedule a time with your instructor to section your brain tissue (1-2 hours outside of class).

Each pair of students will be assigned a brain, either vehicle or CNO treated. Number the wells “1, 2, 3, 4” across the first two rows, the 1st row as FB or forebrain and the 2nd row as HB. Label with your animal’s number and your initials in the bottom corner. Fill each of the eight wells 2/3rd full with PBS. Set the cryostat to take 40µm sections, and carefully start to section until you hit the olfactory bulbs. Take this moment to double-check that you are taking evenly sliced sections (both bulbs should be equal sized as you progress). Harvest your sections using the tweezers, placing each new section in the next well, repeating 1-4. This will create four equally distributed samples of both forebrain and hindbrain to use for four unique experiments.

Tip: take 4 sections at a time, but be sure not to forget which well to place your next section in. Be very gentle when handling sections! They tear very easily.

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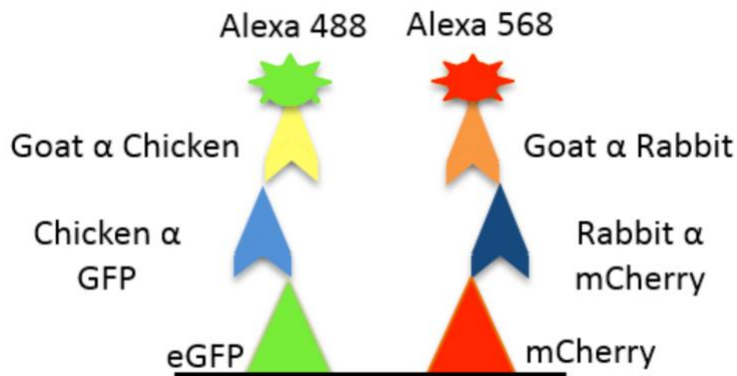
Once the whole brain is sectioned, label eight Eppendorph tubes corresponding to the wells, and fill them halfway with freezing solution. Transfer your samples into the appropriate tubes using a paintbrush, and fill the remainder with freezing solution. Place the samples in the -20C freezer.

Day2

Purpose: Start immunohistochemistry-wash sections and bind primary antibodies.

Immunohistochemistry summary

Immunohistochemistry refers to the detection of proteins **in tissues** using their binding properties to antibodies. Think back to your immunoblot in BIT410/510. We will be using primary and secondary antibodies to confirm the presence of our fluorescent proteins eGFP and mCherry-hM3Dq. Do we see eGFP and mCherry-hM3Dq in **only** the NE neurons we expect to?



Materials:

- Brain sections
- Platform shaker
- Sharpies
- Paintbrush
- 2 Eppendorph tubes, tube racks
- Bench top mat
- Eppendorph tube rotator (shared)
- 15 mL conical tubes
- PBS + PBST squirt bottles
- Graduated cylinder for rinsing runoff
- New 12 well plate
- Stock PBST
- NGS (350 μ L aliquots per lab group)
- Rabbit α mCherry (AB Cam Cat# AB167453; 3 μ L aliquots per lab group)
- Chicken α GFP (AB Cam Cat # AB13970; 2 μ L aliquots per lab group)

Please review the video of how to wash your sections carefully. Remember your tissue is fragile!

Whenever you are moving the brain sections, use the paintbrush. Your tissue is fragile and will tear easily. ALL rinsing steps should be performed in the net well using a squirt bottle over a graduated cylinder. Use a very weak stream to wash as there is potential to tear the sections. Do not spray the solution directly on the sections but instead on the side of the net well. After EVERY round of rinsing, place wells on orbital shaker (set to ~3, low enough so the sections don't spill over) for the prescribed amount of time. Look ahead to the next step as you wait so you can prepare in advance.

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1. Allow 1FB and 1HB tube come to room temperature. Transfer samples to a new 12 well plate with net wells for your samples.
2. Rinse sections in 0.1M PBS, shake for 1 min.
3. Rinse sections in 0.1% Triton-PBS (PBST), shake for 5 min.
 - a. During the 5 minute incubation prepare solutions for **step 4 and step 5**
4. Prepare **4mL** of a 5% normal goat serum (NGS) in 0.1% PBST in a conical tube. Fill Eppendorph tubes (label!) halfway with the NGS solution (1mL), place sections into the tubes, and fill the rest with the solution (~1.5mL). Place tubes on the rotator at room temperature for 1 hour.

Rinsing the sections in PBS and PBST will wash away any remaining freezing solution and the detergent (Triton) will help prepare the tissue for antibody penetration. The Triton-X100 component of PBST also acts as a non-ionic detergent, preventing non-specific binding with any hydrophobic components of proteins. To further prevent non-specific binding, we also incubate the sections in Normal Goat Serum, which will prevent our secondary (goat-based) antibody from binding to any non-specific sites in the tissue.

Double-check your math with a buddy for Step 5! Make 10mLs of antibody solution (contains a,b,c from below). Make sure you have enough total solution before moving on (each Eppendorph tube contains 1.5mL).

5. Prepare **10 mL** primary antibodies in 0.1% PBST in a conical tube. Using new Eppendorph tubes (label, sample #, name!), incubate the sections in primary antibodies. Place on the rotator in the 4C fridge. The samples will rotate overnight and then remain in the fridge until Tuesday.
 - a. Rabbit anti-mCherry at [1:5000]
 - b. Chicken anti-GFP at [1:10,000]
 - c. 1% NGS

Day3

Purpose: to incubate with secondary antibodies and mount sections onto slides

Materials:

- PBST + PBS bottles
- PBS baths
- Paintbrush (2 per group)
- Bench mats
- Graduated cylinder
- Secondary antibodies
 - Goat anti-rabbit alexa 568 (Life Technologies, Molecular Probes; Cat # A-11036; 5 μ L aliquots/group)
 - Goat anti-chicken alexa 488 (Life Technologies, Molecular Probes; Cat # A-11039; 5 μ L aliquots/group)
- Aluminum foil
- 600 μ L aliquot per group of Mounting Medium with DAPI
- Mounting slides + slips (6 slides/slips per group)
- Mounting chambers (blue, 2 per group)
- 12 well plate and nets (1 plate and 2 nets)
- 6 well plate (2 per group)
- Eppendorph tubes

Please review the video of how to mount your brain tissue sections. Remember your tissue is fragile and that mounting tissue sections and adding a cover slip to sections takes time and patience!

1. Using a 12 well plate, rinse sections 3 times in 0.1% PBST, shaking for 10 minute periods.
 - a. During your 3 washes prepare your solution and tubes for **step 2 and step 3**

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2. Prepare 4mLs of secondary antibody in 0.1% PBST in a conical tube (double check your math).
 - a. Goat anti-rabbit alexa 568 [1:1000]
 - b. Goat anti-chicken alexa 488 [1:1000]
 - c. 1% NGS
3. Label new Eppendorph tubes, and **wrap sample tubes in aluminum foil** before adding sections to the antibody solution. Incubate the sections, and place tubes on the rotator at room temperature for two hours.

Our secondary antibodies contain the fluorophores alexa 568 and alexa 488. The number here specifies what wavelength these fluorophores will excite at (allowing us to visualize the eGFP and mCherry-hM3Dq) and emit fluorescence. Because they are fluorescent, it is important to keep them hidden from light as their fluorescence will diminish when continually activated.

4. Move samples to the 12 well plate in net wells, and rinse 3 times in PBS, shaking for 10 minute periods.
 - a. **Be sure to keep 12 well plate covered in foil between rinses.**

Mounting

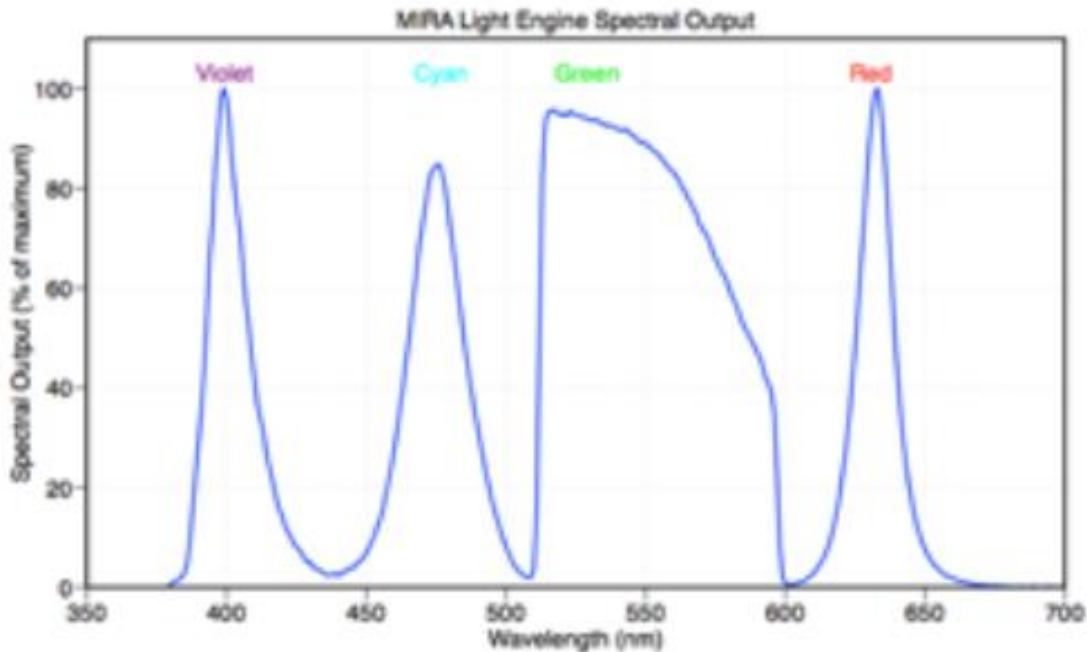
5. You will need 2-3 slides per brain sample to mount every section. Label each one in **pencil** with your animal number, group initials and brain sample # and the antibodies used (ck α GFP and rabbit α mCherry).
6. Fill a 6 well plate with PBS, and transfer one section from the net wells into the 6 well plate with a paintbrush.
7. Using the Mouse Brain Atlas as your guide, divide the sections from rostral to caudal through the 6 wells. This will make it easier to mount.
8. Starting with the most rostral sections, mount each section so that two columns are formed down the slide. Stop about an inch from the bottom as the cover slips 2/3rd the length of the slide itself.
9. Once a slide is finished, allow the sections to adhere to the slide in a drawer, away from activity and dust for about <15 minutes.
10. Once a slide is finished, your partner can mount the cover slip. Using 90uL of mounting solution, distribute the solution along the bottom edge of the slide. Line the edge of the slip with the edge, and (gently!) press down until the slip lays flat on the slide. These are made of glass, and they will shatter if you press too hard. Allow the slide to dry for another 15 minutes.

Day4

Purpose: To assess mCherry-hM3Dq and eGFP expression in *En1^{Cre};Dbh^{Flo};RC::hM3Dq* animals. Remember, we are working with a newly generated animal strain, and it is crucial that we analyze the efficacy of our chemogenetic approach.

We will be using fluorescence microscopy to explore the expression of fluorescent molecules (eGFP and mCherry) in our brain tissue that has also been immunostained with fluorescent secondary antibodies (alexa fluor 488 and 568). We will be using an inverted Nikon microscope (TS100 Eclipse) to visualize NE neurons expressing these fluorophores. These microscopes use an LED illumination system, the MIRA light engine as an excitation light source. This light source provides four possible outputs (violet, cyan, green and red), which are optimized for efficient excitation of DAPI, FITC, TRITC, Cy5 and other similar fluorophores. You will use our microscopy set up to visualize both mCherry and eGFP fluorescence in your brain sections.

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1. Use your atlas! Locate all of the NE nuclei in your atlas (LC, SubCD, SubCV, A5, A2, and A1). Remind yourself from our class discussions, in which NE nuclei do you expect to see eGFP and mCherry-hM3Dq? Fill in the table below with your **expectations**. You may use a computer to fill out the table below so you can edit it as you make your observations (some NE nuclei will appear on multiple tissue sections).
2. Start with exploring eGFP expression in your tissue with the 10x objective. The eGFP signal is very bright and will be easier to see and by using the 10x objective you can see more anatomical landmarks to help determine where you are at in the brain. Use the cyan "C" excitation on the dial of the MIRA light source to turn on the 488nm excitation (remember eGFP and alexa 488nm excitation provides ~500 nm green emission). Be sure the intensity dial is at 100%. Be sure to use the **silver objective** (i.e. the Plan Fluor® objective optimized for fluorescence).
3. Look for the anatomical nuclei where you expect to see eGFP expression. Can you find eGFP neurons? How many do you see? Look at the neurons under higher magnification using the 20x objective.
4. Do you see any mCherry-hM3Dq neurons in the same anatomical nucleus? Switch to the green "G" excitation on the dial of the MIRA light source to turn on the 568nm excitation (remember mCherry and alexa 568nm excitation provides ~600nm red emission).
5. Record your observations in the table below. Find a minimum of 2 sections that contain the anatomical nucleus and write down the number of neurons. In the LC and other domains where there are too many neurons to count write down an estimate. **It is critical that you write down the slide and section number for the various nuclei and your observations.** These notes will help you re-locate interesting areas more easily without having to look through all of your tissue sections again.
6. Look for any unexpected expression of eGFP and mCherry-hM3DQ. Look at your forebrain and hindbrain sections. If you find unexpected expression add it to your table (i.e. add an anatomical nucleus).

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Slide and Section #	Anatomical Nucleus	# of mCherry-hM3Dq neurons	# of eGFP neurons	Expectations
	A7			
	LC			
	SubCV			
	SubCD			
	A5			
	C2/A2			
	C1/A1			
	Expression outside of the NE system? Write the anatomical region here:			
	Expression outside of the NE system? Write the anatomical region here:			

- Once you have **thoroughly** assessed eGFP and mCherry-hM3Dq expression in all of your tissue sections (forebrain and hindbrain), begin acquiring **ONE picture of each anatomical nucleus**. If you have to wait for a turn on the camera, move on to look for some NE neuron projections. **Save the files on your jump drive they will be required for your lab report!**

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Exploring neuronal projections:

8. eGFP fills the entire neuron including the axon terminals. On the other hand mCherry-hM3Dq expression is largely restricted to the dendrites and cell body. Therefore, we will look for projections from the eGFP expressing NE neuron subpopulation. The bed nucleus of the stria terminalis (BNST) receives the largest NE neuron input in the brain. Locate the BNST on your atlas and then find a forebrain tissue section with the BNST. Do you see NE projections? Describe your observations in the table.
9. Find the other 3 anatomical regions listed and record your observations about the presence, absence or intensity of NE projections there.

Slide and Section #	Anatomical Nucleus	Neuronal Projection Expectations	Neuronal Projection Observations
	STMV and STLV (Bed Nucleus of the Stria Terminalis-BNST) FIG 30	Do you expect to see projections in the region from non-locus coeruleus neurons?	How dense are the NE projections in the region? How does the colorimetric staining of projections compare with the fluorescent staining in the region? Can you see more projections? Or less?
	PaDC, PaLM, PaMM, PaV, PaMP (Paraventricular Hypothalamic Nucleus) FIG 38		
	DI, AID, AIV (Insular Cortex) FIG 16		
	Cortex FIG 5-70	If you are unsure about the expectations here talk with your instructor	Remember this is a new tool for exploring projections for exploring the projections of a unique subset of NE neurons (non-locus coeruleus neurons), which means this is an opportunity for YOU to discover something new!!

10. ****Homework step****: The images that we can obtain on our scope do not have sufficient resolution or quality for what you would need for a research publication. Contact Dr. Eva Johannes from the Cellular and Molecular Imaging Facility here at NC State (4115 Gardner Hall) to schedule an appointment to **take a single confocal image of one of your NE anatomical nuclei**. Your confocal appointment should take 1 to 1.5 hours. Find a time both you and your lab partner can attend. Your picture is not due until your final lab report (October 13th), **but you need to make your appointment TODAY**. The confocal microscope is heavily used by researchers, and you need to get your appointment on the calendar ASAP!
http://www.ncsu.edu/project/cmif-micro/CMIF_pages/eva.html

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Discussion Questions

1. Do you see mCherry-hM3Dq and eGFP expression in the appropriate NE neuron subpopulations?
2. Do you see mCherry-hM3Dq and eGFP expression in any brain regions that you do not expect? What group of neurons do you think this expression is in?
3. It is possible that we could observe mCherry-hM3Dq and eGFP in non-NE neurons. Why might this happen?
4. Do you think our recombinase based intersectional genetic approach is working?
5. We cannot see projections from the mCherry-hM3Dq neurons because mCherry-hM3Dq expression is restricted to the dendrites and cell body. Why do you think mCherry-hM3Dq expression is restricted to the dendrites and cell body?
6. Do you expect to see eGFP projections in the motor cortex? (*Hint*: Look through the scientific literature. Which NE neurons project to the cortex?)