Development of Second-Generation Brainbow Adeno-Associated Virus for Increased Labeling Brightness and Color Diversity in the Mouse Brain

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Abstract

Revealing structure-function relationships of neurons in the brain is one of the primary goals in Neuroscience. While function is not always easily defined from structure alone, it is limited by what structures are physically present. Thus our goal is to show what is present via fluorescent membrane labeling of individual neurons using Brainbow technology in the mouse brain. We have created several labeling constructs, each of which use Cre/lox recombination to stochastically express either a dark, non-fluorescent protein or a spectrally distinct fluorescent protein to label individual neurons and their dendritic and axonal projections. When co-transfected *in vitro*, these constructs created distinct composite colors in different N2A cells due to expression of random ratios of different fluorescent protein species. Encouraged by the cell culture results we will package them into Adeno-Associated Virus vectors for injection into the mouse brain. We have not only increased the amount of fluorescent colors available but have also made each fluorescent protein brighter by expressing tandem copies in a single polypeptide chain.

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Introduction

One of the main goals of neuroscience research is to relate the structure of a neuron or tissue or system to its function. The length and morphology of an axonal projection, for example, constrains with which neurons it can form synapses. Before being able to discuss the projections of a single neuron, the sheer number of neurons, and the trillions of synapses in the brain, must be understood and reduced to single-cell studies (Young, 2008). There are billions of cells, tightly packed together and interacting trillions of times via synapses. Thus the goal must always be three-fold; (1) to study complete functional neuronal circuits at (2) cellular resolution in a (3) single animal or sample. It is our goal to visualize these individual neurons, the dendritic and axonal projections that make up the neuronal circuits, and the synapses formed between them and their somas with fluorescent labeling in the Brainbow mouse by increased labeling brightness and color diversity in the mouse brain.

A. *What:* Brainbow through the Generations

In order to visualize neurons and their projections, fluorescent color labeling tools have been developed and are constantly being improved upon. Brainbow is a multispectral labeling tool that uses Cre recombination to create random expression ratios of three or more spectrally-distinct fluorescent proteins (FP) in a cell, therefore creating distinct colors in different neurons in the mouse brain.

Brian Sauer and Nancy Henderson developed the Cre/lox recombination system from the bacteriophage P1 (Sauer, 1987; Sauer, Henderson 1988). This system allows for cell type-specific sequence excision using two identical lox sites to flank the specific

sequence (flanked by lox, “floxed”) (Gu, 1994). The lox sites are activated for excision when the Cre recombinase gene in a transgenic mouse is crossed with the floxed mice (Akagi, 1997). A cell-specific promoter upstream of the Cre recombinase gene allows for targeting recombination in particular cell types. This excision has been proven in various cell types including brain, liver, spleen, and kidney (Kühn, 1995).

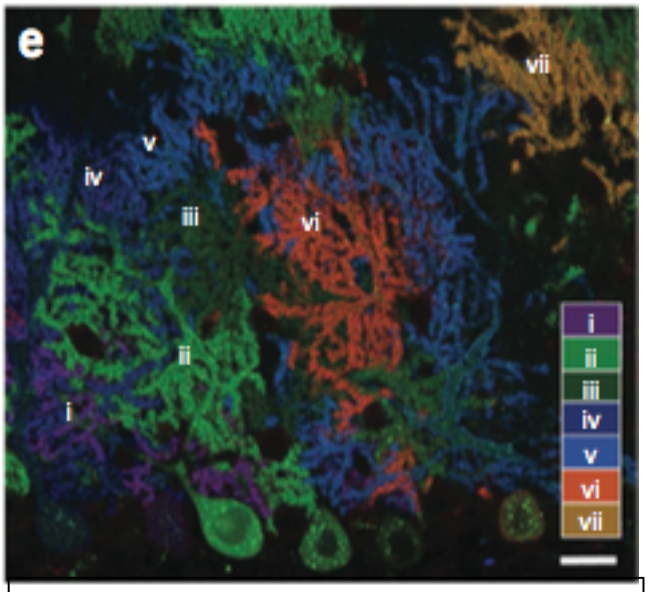
Brainbow is an improvement of previously used single-color labeling tools, as multiple colors allow for distinction between neurons. The first generation of Brainbow, Brainbow 1.0, used a construct that allowed for default red FP expression and a stochastic choice of yellow or Cyan upon Cre recombination (Livet, 2007). Using two variant pairs of lox sites, *loxP* and *lox2272,* Cre recombination allowed for two outcomes. Choosing one pair of lox sites prevented the second pair from further recombination, giving stable expression of color. A third paired lox site, *loxN,* was added in *Brainbow-1.1* for a third recombination option (Livet, 2007).

While this strategy of variant lox pair sites allowed for both a stochastic and stable choice between FPs, the default color expression presented a problem. Since FPs, RFP or OFP, were used in its default, non-Cre expression, the cells were flooded with fluorescence in the case of non-Cre expression (Livet, 2007). Reduced diversity due to expression in a majority of cells remained a problem of distinction even though multiple lines can be screened without a Cre reporter (Cai, 2013; Livet, 2007).

To address this problem of flooded fluorescence in non-Cre induced background expression, Brainbow 3.1 included the insertion of a third non-compatible *loxP* site pair as a ‘stop’ cassette in the first position for default output (Cai, 2013). The ‘stop’ cassette

has a non-fluorescent Phi YFP inserted to indicate the default in neurons with no Cre recombination. The pre-Cre recombination segments can then be screened via rat antibodies for Phi-YFP (Cai, 2013).

Brainbow 3.1 used three XFPs (mO2f, EGFPf, mK2f) in each construct along with a nuclear-targeted non-fluorescent XFP in the 'stop' cassette of the first position (Cai, 2013). Cre recombination could render four options; any of the XFPs or the 'stop'/dark option. A poly-A tail after each XFP stops any expression of fluorescent colors after the chosen color in the construct, post-recombination or, in the case of no recombination, just the default is expressed. Each XFP in the construct was a farnesylated derivative, to target the XFPs to the membranes of all neurites (Cai, 2013) (see section IB).

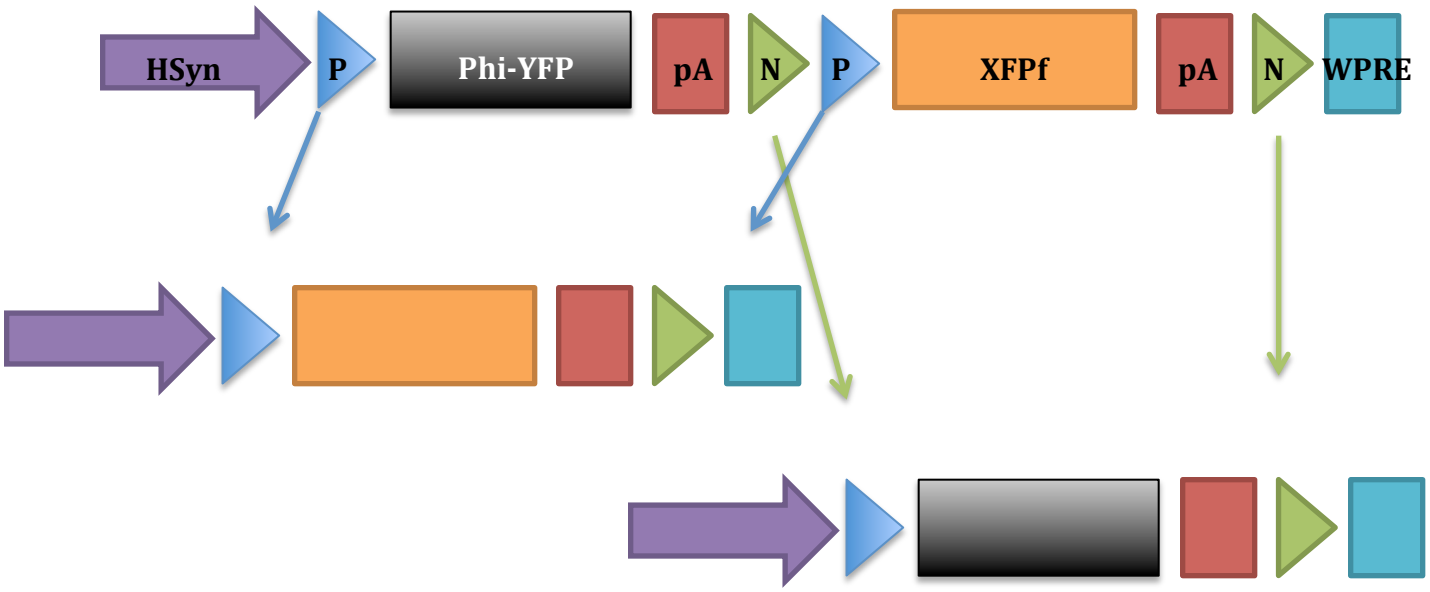


Adapted from Figure 1e; Cai, 2013
A minimum of seven different colors in
10 Purkinje cells of the l7-Cre mouse Cerebellum.

Brainbow 3.1 showed exciting improvements for color distinction and background silencing. However, with the construct incorporating three XFPs in one,

giving four recombination options, too much simultaneous fluorescent expression and thus overlapping labeling could occur. With various colors expressed simultaneously in the same region, images rendered white spots especially in the soma that prevented distinction. For our most recent construct, and the highlight of this thesis project, we wanted to improve upon labeling intensity and color diversity to increase distinction between cells, and thus eliminating over-expression and indistinguishable white spots.

We created an ON/OFF construct that narrowed the cassette to a non-fluorescent STOP and a single XFPf (farnesylated). With two incompatible Cre-lox pair sites in the cassette, a stochastic choice of fluorescent color (ON) or dark STOP (OFF) is available with recombination. The non-fluorescent STOP works as default output to prevent non-Cre induced background expression. Upon Cre recombination, excision between LoxP sites will result in XFPf expression (ON status) and excision between LoxN sites will keep the construct in non-fluorescent STOP (OFF status). Once again, using farnesylated derivatives of each XFP (XFPf), allows for membrane targeting. A polyA tail after the STOP prevents leaking of the XFPf insert.



Goal Construct. The non-fluorescent phi-YFP insert will be the default in cases where Cre is turned off. With simple cut and paste constructs, the XFPf can be replaced with a variety of fluorescent colors. Blue and green arrows indicate loxP and loxN post-recombination construct schemes, respectively.

B. *Where:* Cytoplasm versus Membrane Labeling

Previous attempts at neuronal tracing have been made by anterograde or retrograde uptake of hydrophilic dyes. These methods are highly cumbersome, as they require precise in-vivo injections with tracers that have slow transportation times in fixed tissues (Badaloni, 2007).

Alternatively, genetic techniques have been developed to express green fluorescent protein (and other proteins with distinct spectra) to selectively label neurons (Tsien, 1998). Upon labeling individual, specific neurons, it is important to label all parts of the cell evenly, with an equal concentration of fluorescence. When XFPs go

unmodified they diffuse in the cytoplasm, label the soma with a high concentration of fluorescence, but fail to label nearby processes such as dendritic spines or axonal projections (Cai, 2013). With a large volume, there is great space to incorporate a high amount of fluorescence. As the diameter and volume of the neuron largely decreases leaving the soma, the high fluorescent concentration is unable to flow into the tiny projections.

Farnesylation of XFPs targets them to the cell membrane and gives stable fluorescence to the projections. This is especially important when distinguishing specific neuronal cell types and lineages. If the neuronal projections can be traced for long distances, synaptic connections onto other neurons can be visualized, quantified, analyzed, and distinguished from other neurons. The challenge of membrane labeling is the smaller volume and so fluorescent concentration will be lower compared to the somata cytoplasm.

There are two ways of improving membrane fluorescence. One way is to have stronger and/or longer periods of expression so that more FPs can be inserted into the membrane. There is a limitation however to the amount of saturation of FPs, where an increased time will not render brighter colors in the saturated membrane. Another way is to increase the intensity of each fluorophore so that at the same expression level the overall intensity increases. To do this we propose to create tandem copies (2X or 3X) of FPs expressed as one polypeptide chain. Each molecule, as a result, would have a higher intensity of fluorescence compared to its single copy counterpart.

C. *How:* Adeno-Associated Virus (AAV) vectors

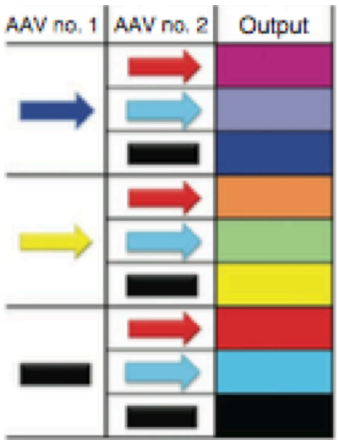
Adeno-Associated Virus (AAV) vectors are the preferred method for transducing Brainbow constructs into the mouse brain. AAV provides long-term expression that is both safe and stable (Allen, 2004). Administering AAVs via stereotaxic injections, has allowed precise spatial and temporal control over Brainbow expression in the mouse brain. In addition, Brainbow AAVs can be administered to label other mammalian brains that currently do not have many transgenic animals available.

While current Brainbow AAV label neuron morphology very well, the labeling intensity and color diversity is unsatisfactory. We are looking for bright, intense colors that can clearly show neuronal projections and that we would be able to simultaneously distinguish between the colors of the FPs in the neuronal membranes. In determining where one neuronal projection ends and the next one begins, we can decipher synapses between them; where and how often different neurons connect and communicate. As explained above, there are a few ways to manipulate both the intensity of the fluorophore and the time periods of FP expression to increase overall fluorescent brightness in the membrane.

Aside from an increase in brightness, we are increasing the number of colors available to minimize the labeling redundancy problem; i.e. multiple neurons labeled in the same color. To do this in the past, we have used a set of two AAVs that each included an AAV vector with two XFPs. The XFPs had the two lox sites that could render either of the two fluorescent colors or a dark option, thus creating eight color outputs (3x3-1) (Cai, 2013).

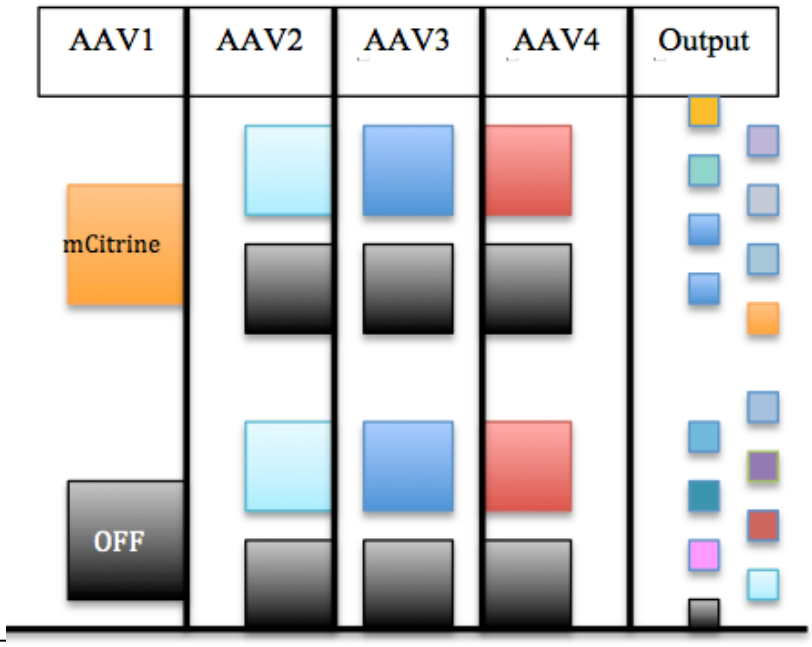
To increase the color number, we will create a set of four AAVs, each one having the newest created ON/OFF expression construct (see section IA) with Cre recombination. A set of four AAVs with four distinct FPs can create fifteen color outputs (2^4- 1 (all dark, all OFF option)).

To extend this idea even further, multiple copies of each of the AAVs can be injected for more color variation. For example, if Cre recombination rendered two blue FPs from two separate AAVs, it would show a brighter blue than recombination of a single blue FP. The distinction in brightness between the singular and multiple FP copies, reads as separate colors and thus more options for color labeling.



Adapted from Figure 5C ; Cai, 2013

With co-infection of both viruses, paired recombination gives a mix of colors (Figure 5C, Cai 2013). A combination of an XFP and the dark option yields pure expression of that XFP.



Four separate AAVs, each with a NF phi-YFP and possible XFP insert. With co-infection and Cre recombination,
2^4- 1 (all dark, all OFF option) = **15 minimum colors**. Multiple copies of each specific virus would create even more colors.

D. *Why:* It Matters

Fluorescent tracings of individual neurons, from the soma membrane all the way to its detailed projections, give an exciting view into the future of neuroscience. We are not sacrificing detail or color distinction for a sense of the larger picture of the brain and its firing and wiring in synaptic communications. In fact, we do both. We give detail down to the individual neuron and then are able to trace its projections, visualize its connections, mark its synapses, and then repeat for hundreds of neurons, giving us a larger picture of how these cells communicate. It is critical to emphasize that this can all be done in a single mouse brain. Previous anatomical reconstruction strategies rely on sparsely labeled samples with measurements averaged across many samples, greatly diminishing confidence.

The human connectome, first named by Olaf Sporns at Indiana University in 2005, captures the essence of this project and its importance. Inspired by the Human Genome Project, also thought to be an impossible feat at one time, the connectome aims to create a structural, 3D blueprint of the human brain (Nair, 2013; Sporns, 2005). Modern imaging techniques allow the intense network of billions of neurons and trillions of connections to be visualized. Sidney Brenner first completed this network mapping in *Caenorhabdidtis elegans* at a much smaller scale of 300 total neurons and 5,000 synapses (Nair, 2013). Jeff Lichtman, at Harvard University, focuses his work on constructing the mouse connectome. Brainbow is utilized for the finest, most-detailed level of this mouse connectome; single neurons and their synaptic connections (Livet, 2007; Sporns, 2011).

This structural blueprint of the networking in the brain will be invaluable to studies in human development, aging, and disease. It could provide the link between

mental illness and structural anomalies in neurosurgery. As disease and learning can simultaneously shift these networks, the connectome could also give insights into the plasticity of the brain; the wiring and rewiring that occurs in individuals over time (Nair, 2013).

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Methods

A) DNA Cloning

i. Mini Prep + Nano Drop

For the Mini Prep I used the Promega Wizard® *Plus* SV Minipreps DNA Purification System protocol with the Mini Prep kit (Cat # A1460, Wisconsin, USA). The DNA colony was put in a four mL Lysogeny Broth (LB) solution and placed in a 37-degree shaker overnight. To produce the cleared lysate, 3 mL of the solution were centrifuged at 5XG for one minute at room temperature. The pellet was resuspended with 250ul of Cell Resuspension Solution. 250ul of Cell lysis solution was added and inverted four times. 10ul of Alkaline Protease Solution was added, inverted 4 times and incubated for five minutes at room temperature. 350ul of Neutralization Solution was added and inverted four times. The solution was centrifuged (at 21.1 XG for 10 minutes at room temperature.

For binding of plasmid DNA, the cleared lysate was placed into the manifold vacuum and the liquid was pulled through the column for two minutes. For washing of the liquid, 750ul of Wash solution (with ethanol) was added and pulled through. This step was repeated with 250ul of the Wash Solution. The column was dried for three minutes and centrifuged at 21.1XG for two minutes. For elution, the column was transferred to a sterile 1.5ml microcentrifuge tube, 100ul of Nuclease-Free Water was added to the column, and centrifuged for 1 minute at 21.1 XG at room temperature. The column was discarded.

For DNA concentration assessment, I used the NanoDrop 2000C Spectrophotometer (Thermo Scientific, Serial #M860). I added 1.5ul of Nuclease-Free water for the blank and then 1.5ul of each DNA sample following.

ii. Digestion

	30ul	50ul
DNA	1ug (~6 ul)	1ug (~5ul)
Enzymes (2)	1ul each	1-2 ul each
Buffer	3ul	5ul
Milli-Q Water	to 30ul	to 50ul

For a double digest we often used the CutSmart® Buffer (Cat#B7204S, NEB) for highest percent activity (100%, double digest finder, neb.com). Based on DNA concentrations derived from the NanoDrop, we calculated the amount of DNA needed for the digestion for 1ug. Based on single or double digestions, we used one or two enzymes simultaneously and then used Milli-Q water to bring the solution up to thirty or fifty microliters.

The Milli-Q water, Buffer, DNA, and enzymes were combined in a .6mL microcentrifuge tube and placed in the 37-degree incubator for four hours.

iii. PCR (Phusion®, EconoTaq® PLUS)

For PCR Phusion® we used the New England BioLabs® Inc. PCR Protocol for Phusion® High-Fidelity DNA Polymerase (MO530).

Component	20 µl Reaction	50 µl Reaction	Final Concentration
Nuclease-free water	to 20 µl	to 50 µl	
5X Phusion HF or GC Buffer	4 µl	10 µl	1X
10 mM dNTPs	0.4 µl	1 µl	200 µM
10 µM Forward Primer	1 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 250 ng
DMSO (optional)	(0.6 µl)	(1.5 µl)	3%
Phusion DNA Polymerase	0.2 µl	0.5 µl	1.0 units/50 µl PCR

Thermocycling conditions for a routine PCR:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25-35 Cycles	98°C 45-72°C 72°C	5-10 seconds 10-30 seconds 15-30 seconds per kb
Final Extension	72°C	5-10 minutes
Hold	4-10°C	

New England BioLabs® Inc.

For a PCR Screen, we used the EconoTaq PLUS Green 2X Master Mix (Cat#30033-2, Lucigen®).

PCR Screen (10ul)	Primer 1	Primer 2	Milli-Q Water	2X Econotaq Master Mix
Amount	.5ul	.5ul	4ul	5ul

Cycling step	Temperature	Time	# of Cycles
Initial Denaturation*	94°C	2 min	1
Denaturation*	94°C	15 - 30 sec	25 - 35
Annealing**	50 - 65°C	15 - 30 sec	
Extension	72°C	1 min/kb	
Final Extension	72°C	5 - 10 min	1
Hold	4°C	Indefinitely	1

MAO37Rev.B, Lucigen®

iv. Agarose Gel + Purification

We used agarose gels to verify PCR, digestion, and ligation results. For six and ten-combed wells, .4 grams of agarose was combined with 50ml of 1X TAE and heated in the microwave for 1:45 minutes. For a 30-well comb we used .8 grams of agarose and 100mL of 1X TAE, heated for 2 minutes. The gel was poured into the well and solidified over twenty minutes. We used the 1KB ladder from New England BioLabs®Inc. (Cat#N3232L). Gels were run for different amounts of time (15-30 minutes) depending on DNA size.

Gels were purified using the Promega's Wizard® SV Gel and PCR Clean-Up System Kit (Cat#A9282, USA). Cut gel slices were weighed and 10ul of Membrane Binding Solution was added for every 10mg of gel slice. The gel was dissolved using a 65-degree Celsius heat block over twenty minutes. The SV Minicolumn was inserted into the Adapter of the manifold vacuum and the gel mixture was transferred to the Minicolumn. After one minute of incubation at room temperature, the liquid was pulled through the vacuum. I added 700ul Membrane Wash Solution (ethanol added) and the vacuum pulled it through. This step was repeated with 500ul Membrane Wash solution.

The Minicolumn was transferred to a Collection tube and centrifuged at 16XG for five minutes. The Minicolumn was transferred to a 1.5ml microcentrifuge tube and 50ul of Nuclease-Free Water was added. After one-minute incubation at room temperature, the Minicolumn in the 1.5ml microcentrifuge was centrifuged at 16XG for one minute. The Minicolumn was discarded and the solution was prepared for NanoDrop concentration assessment.

v. Ligation

	Volumn (ul)	Mass (ng)	Mass Conc. (ng/ul)	Size (nt)
Insert	4.83	48.30	10	200
Backbone	12.17	304.26	25	6300
T4 Ligase	1.00			
10x T4 Ligase Buffer	2.00			
Total	20.00			

Cai Lab

We used our lab's protocol for ligation, based on NanoDrop DNA concentrations and the size of the DNA insert and backbone in nucleotides, for a 20ul total solution. We used T4 DNA ligase (Thermo Scientific, Cat#M02025) and 10x T4 DNA Ligase Buffer (Thermo Scientific Cat#B02025). The DNA insert, DNA backbone, T4 Ligase, and 10x T4 DNA Ligase Buffer were combined in a 1.5ml microcentrifuge tube and were incubated at room temperature overnight.

v) Transformation

For our transformation protocol we used both Mach1™ (Cat#C8620-03, Invitrogen™) and Stbl3™ (Cat#C7373-03, Invitrogen™) competent cells. We transferred 10ul of transformed ligation reaction into 100ul of these cells. After 20 minutes of incubation on ice, the cells were heat shocked for one minute at 42 degrees Celsius. We

added 500ul of LB solution and placed the cells in the 37-degrees Celsius shaker for one hour. LB-Agar plates, antibody-specific, were pre-warmed in a 37-degrees Celsius incubator. The LB cell solution was distributed out onto the plate using glass beads (Cat#11-312B, Fisher Scientific, Germany) and placed in the 37-degree Celsius incubator overnight.

vi. Sequencing

The DNA Sequencing Core at the University of Michigan Medical School conducted all DNA sequencing.

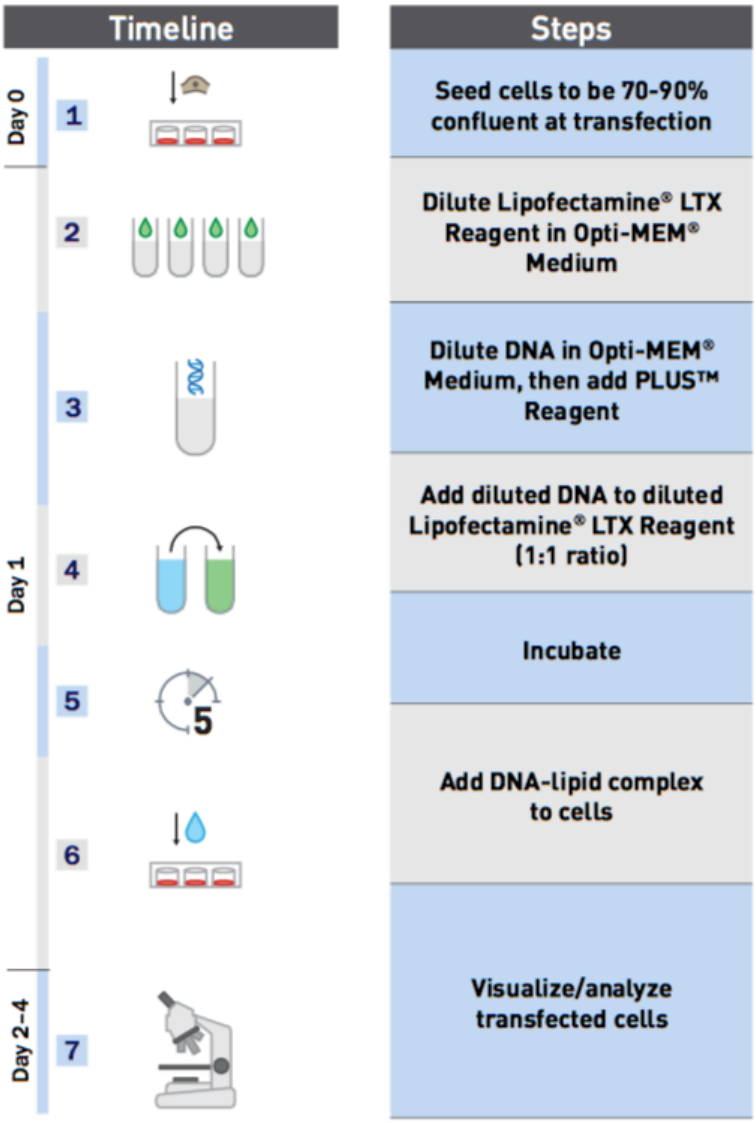
B) Cell Culture

For the N2A medium, we used 10% FBS (Cat#10437-028), which was placed in a 50-degree Celsius water bath for one hour. We added 1% GlutaMAX-I supplement (Cat#35050-061) and .1% MEM Non-Essential Amino Acids Solution (100X) (Cat#11140-050).

For cell passaging, the medium was aspirated and one milliliter of Trypsin was added. We let the plate rest for thirty seconds to allow clumping of cells before removing the Trypsin and adding 1 milliliter of N2A medium. In a new 6cm plate, we added four milliliters of N2A medium. After pipetting the N2A cells up and down in the original plate to ensure singular cells, 200ul of cells were removed and evenly distributed in the new plate (1:5 dilution). The plate was checked under the microscope for sufficient spacing between cells. The plate was placed in a 5% CO_2 Incubator.
Cell passaging was repeated every four days.

C) Transfection

We used the Lipofectamine® LTX & PLUS™ Reagent Protocol 2013 (Invitrogen™ by Life Technologies™) for 6-well N2A cell transfections. The DNA was diluted in Opti-MEM® Medium and PLUS™ reagent was added. The Lipofectamine® LTX reagent was diluted in Opti-MEM® medium and combined with the DNA solution. The mix was incubated at room temperature for twenty minutes. Two hundred fifty microliters of the mix was added to each of the six wells.



Procedure Details			
Component	96-well	24-well	6-well
Adherent cells	1–4 × 10^4	0.5–2 × 10^5	0.25–1 × 10^6
Opti-MEM® Medium	25 µL × 4	50 µL × 4	150 µL × 4
Lipofectamine® LTX Reagent	1, 1.5, 2, 2.5 µL	2, 3, 4, 5 µL	6, 9, 12, 15 µL
Opti-MEM® Medium	125 µL	250 µL	700 µL
DNA (0.5–5 µg/µL)	2.5 µg	5 µg	14 µg
PLUS™ Reagent	2.5 µL	5 µL	14 µL
Diluted DNA (with PLUS™ Reagent) Total	25 µL	50 µL	150 µL
Diluted Lipofectamine® LTX Reagent	25 µL	50 µL	150 µL
Incubate for 5 minutes at room temperature.			
Component	96-well	24-well	6-well
DNA-lipid complex per well	10 µL	50 µL	250 µL
Final DNA used per well	100 ng	500 ng	2500 ng
Final Lipofectamine® LTX Reagent used per well	0.2–0.5 µL	1.0–2.5 µL	5.0–12.5 µL
Incubate cells for 1–3 days at 37°C. Then, analyze transfected cells.			

Invitrogen™ 2013

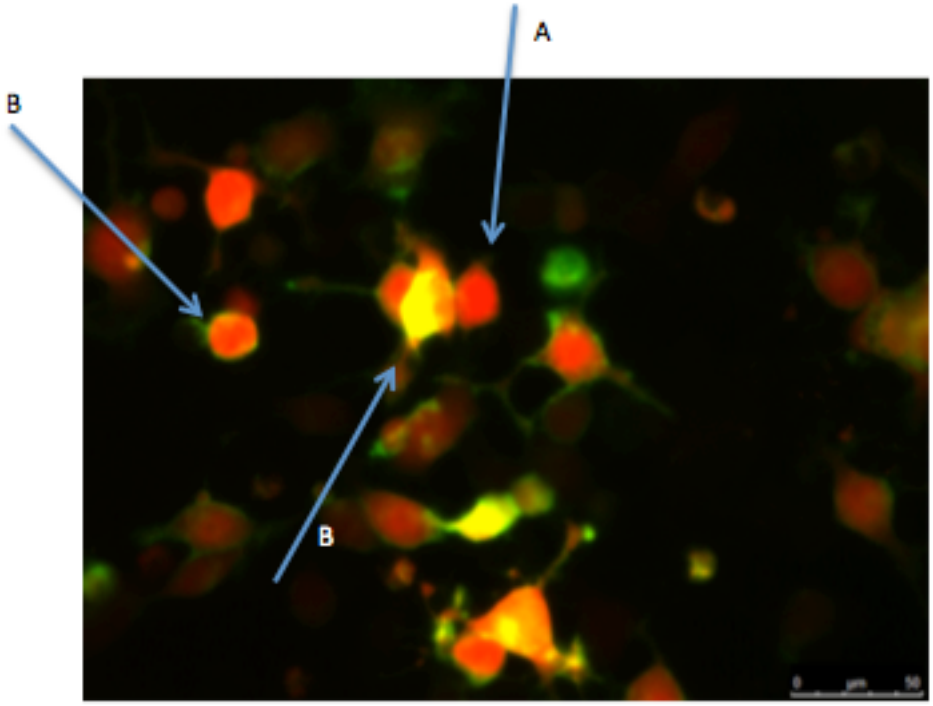
Results

A) The Constructs

To commence building an ON/OFF construct, both inserts had to be tested to check for individual viability. Since recombination has to be both stochastic and mutually exclusive (Livet, 2007), we wanted to make sure that both inserts were expressed in equal amounts, post-Cre recombination. To do so, we used two fluorescent colors, flanked by incompatible loxP and loxN sites respectively. When Cre is not turned on, cytoplasmic mCherry (Shaner, 2004) is expressed as the default color. Once Cre is turned on, it can randomly recombine between the loxN or loxP sites, resulting in stable cytoplasmic mCherry or membrane monomeric Citrine (mCitrine) (Griesbeck, 2001) labeling, respectively.

Figure 1A: First construct with loxP and loxN sites, using mCherry and mCitrine-farn fluorescent markers. mCherry is the default mode when Cre is turned off. Recombination of loxP sites gives mCitrine-farn membrane expression. Recombination of loxN sites gives mCherry cytoplasmic expression.

We inserted this construct into the Rosa 26 plasmid and transfected it into N2A cells. Fluorescence was checked under a microscope to determine equal expression of both mCherry and mCitrine. Since mCherry is a cytoplasm-labeling fluorescent protein, its red color is seen in the soma, while the mCitrine-farn is in the membrane, showing recombination.



Fujia Lu

Figure 1B: Fluorescent image of N2A mouse cells under microscope, post-transfection. (A) Cytoplasmic mCherry expression (B) mCitrine-farn membrane expression due to Cre recombination

Since both mCherry and mCitrine-farn were roughly expressed in equal proportions, we replaced the mCherry insert with the intended OFF non-phi YFP insert.

After determining equal expression of both inserts in the construct, we wanted to make sure that in cases of no Cre expression, there would be no leakage of the fluorescent protein, i.e. that the default would be truly dark/OFF. We compared the non-phi YFP insert to the Small Ubiquitin-related Modifier (SUMO) and the Nuclear-Localization Signal (NLS) SUMO in a N2A transfection and checked for leakage of fluorescence when Cre is turned off. SUMO is smaller in size compared to the Phi-YFP insert and thus proves useful for the limited space in AAV packaging later on.

Figure 1C: Three separate constructs to determine the best OFF insert in cases of no Cre/default expression. Goal is to minimize leakage of fluorescent protein downstream of the OFF insert to obtain a truly dark background.

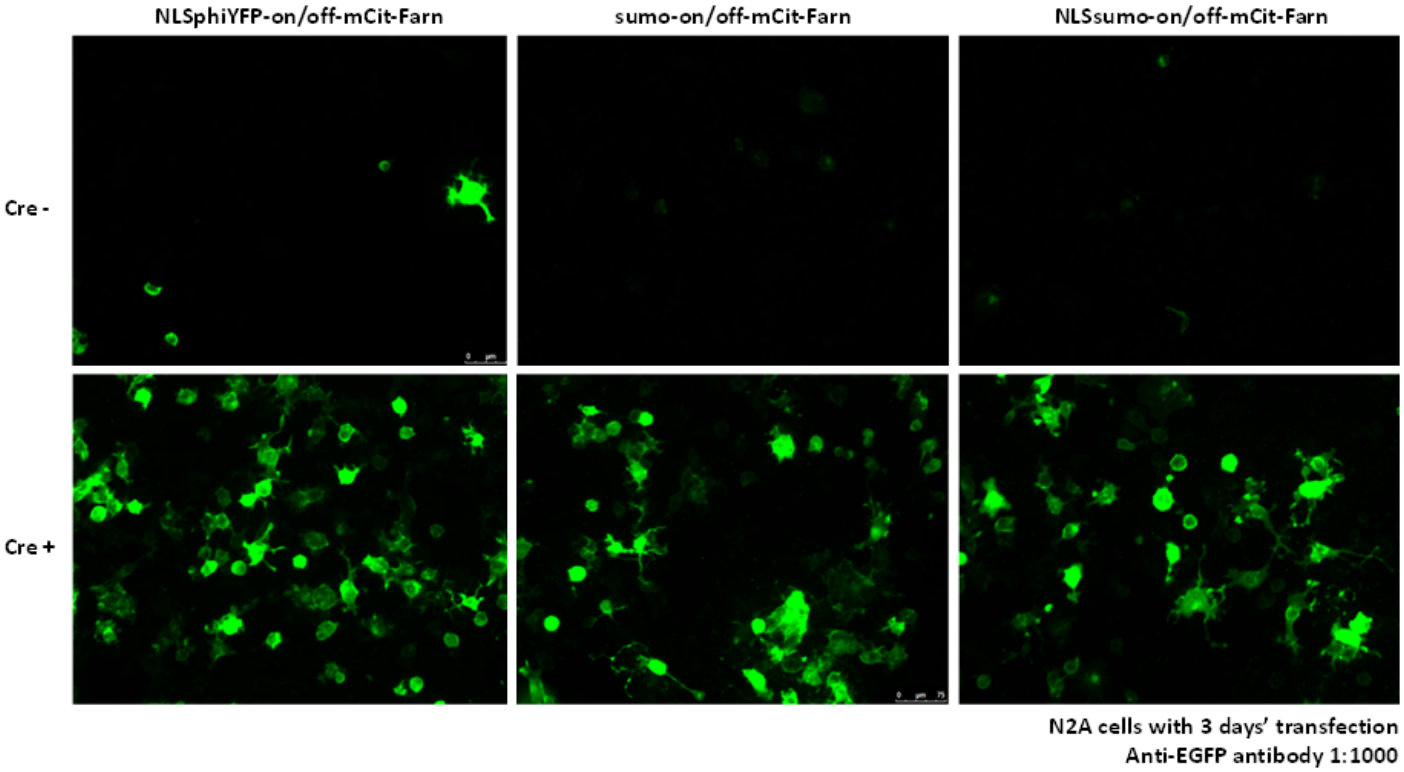


Figure 1D: Comparison of three separate OFF inserts; NLSphiYFP, SUMO, NLSsumo, 3 days post-transfection into N2A cells. When Cre expression is turned on, (Cre+) mCit-farn is clearly visible. When Cre expression is turned off (Cre-), leakage of mCit-farn is seen most strongly with the NLSphiYFP insert.

Both the SUMO and NLS SUMO constructs showed less leakage of mCitrine-farn in default, non-Cre expression. Therefore, we chose to move forward with the SUMO insert, more specifically the NLS SUMO construct because of the preferred nuclear localization over the cytoplasmic localization with SUMO.

B) Brightness Comparisons

To show the improved brightness of the fluorescent inserts in our constructs, I prepared a transfection of both mKusabiraOrange2 1X and mKusabiraOrange2 2X into N2A cells for a side-by-side comparison (Figure 2 A-B). Monomeric Kusabira Orange2 (mKO2) (Kaida, 2011) is an orange fluorescent protein. The 2X insert has tandem copies of the fluorescent protein mKO2 expressed as a single polypeptide chain. To quantify the difference in fluorescent brightness, we measured the intensity of specific cell membranes and calculated the mean intensity in Arbitrary Fluorescent Unites (AFUs) and the standard deviation between the samples. With a mean intensity of 184 AFUs for the mKO2 2X cells and a mean intensity of 90 AFUs for the mKO2 1X cells, the 2X cells have roughly twice the fluorescent intensity (Figure 2C).

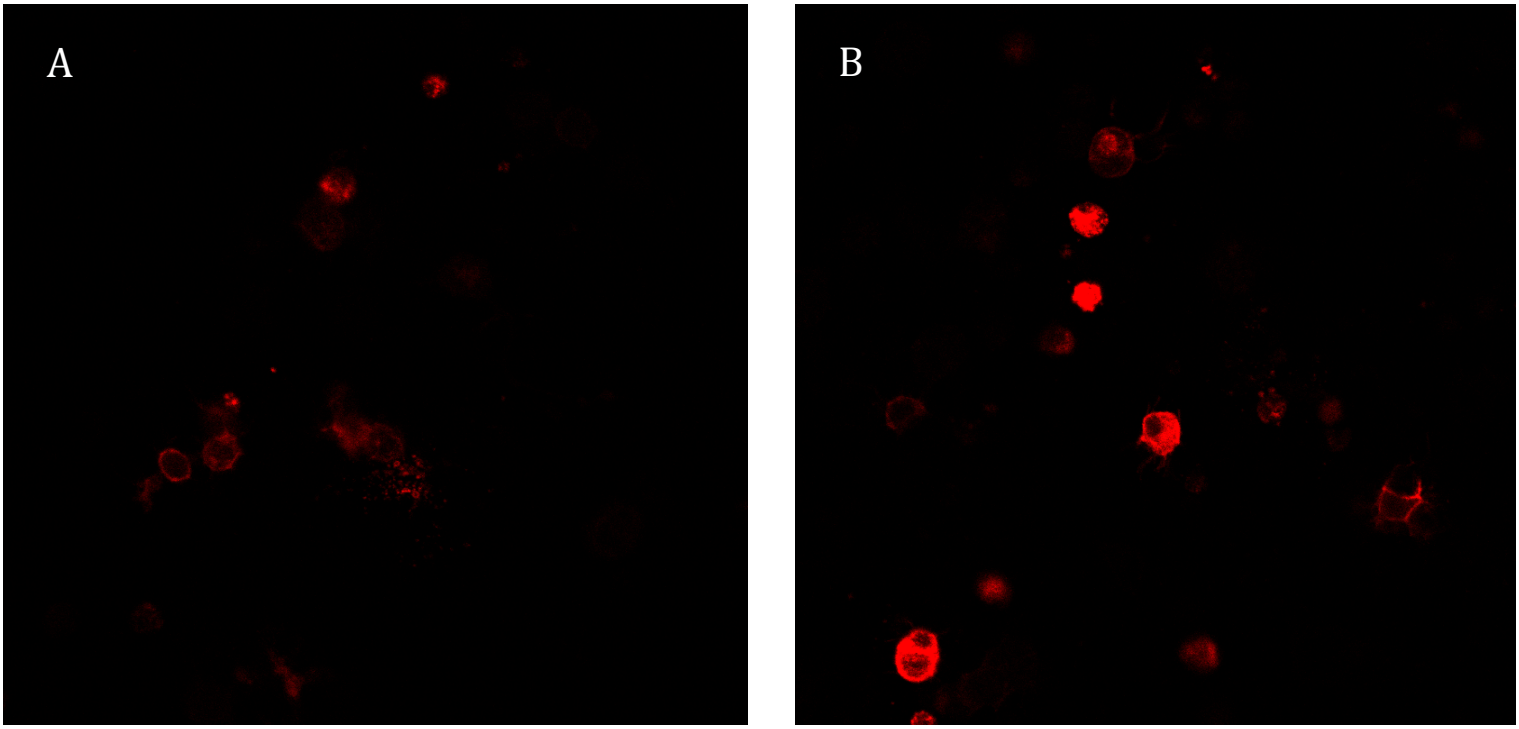


Figure 2A-B: N2A cells, 6 days post-transfection. **A)** 1X mKO2 **B)** 2X mKO2. A and B were imaged sequentially at 10X magnification under identical imaging settings.

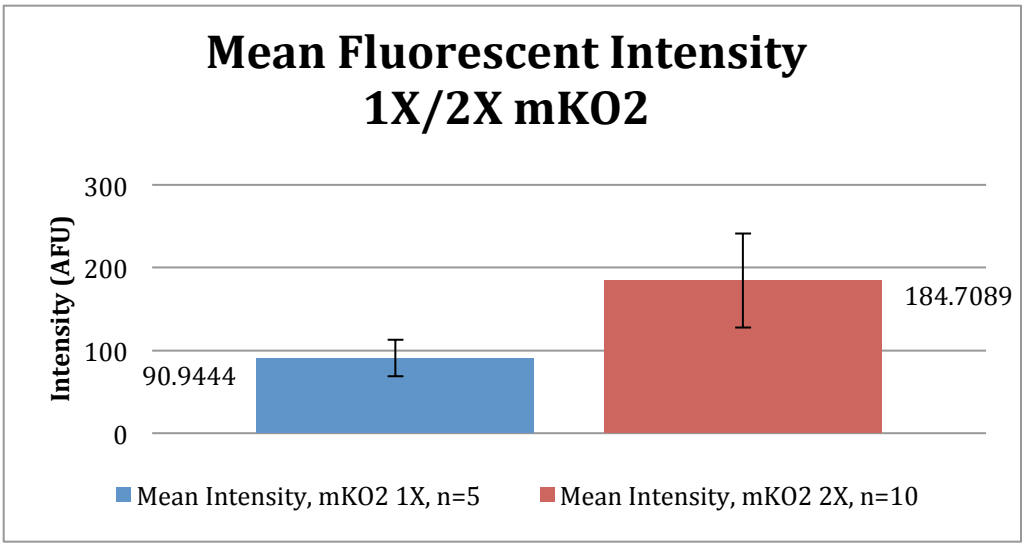


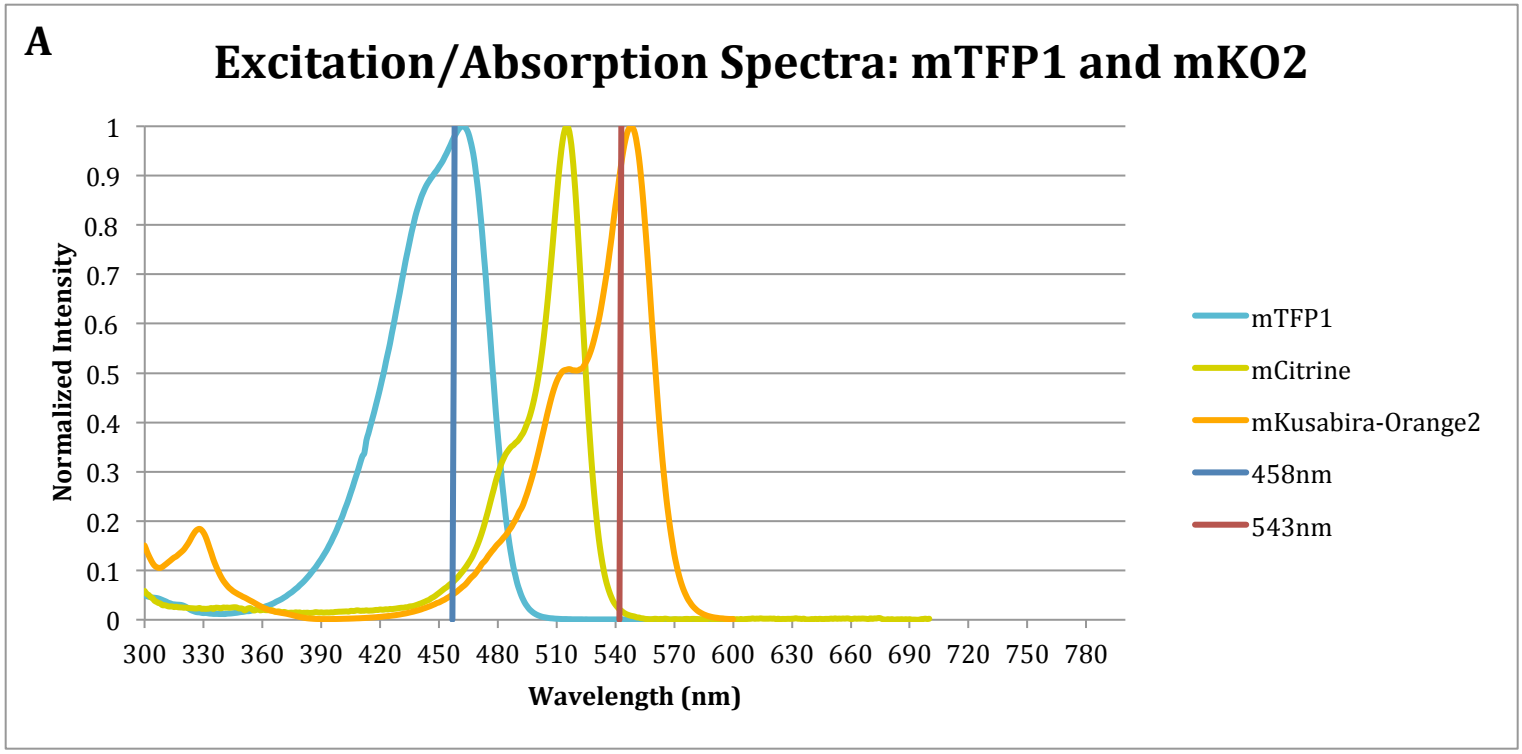
Figure 2C: Mean Intensities of N2A cells measured in Arbitrary Fluorescent Units (AFUs). 5 cells were assessed from mKO2 1X for a mean intensity of 90.944 AFU, with a Standard Deviation of 21.95. 10 mKO2 2X cells were measured with a mean intensity of 184.7089 AFU, with a Standard Deviation of 56.913.

C) Excitation/Emission Spectra

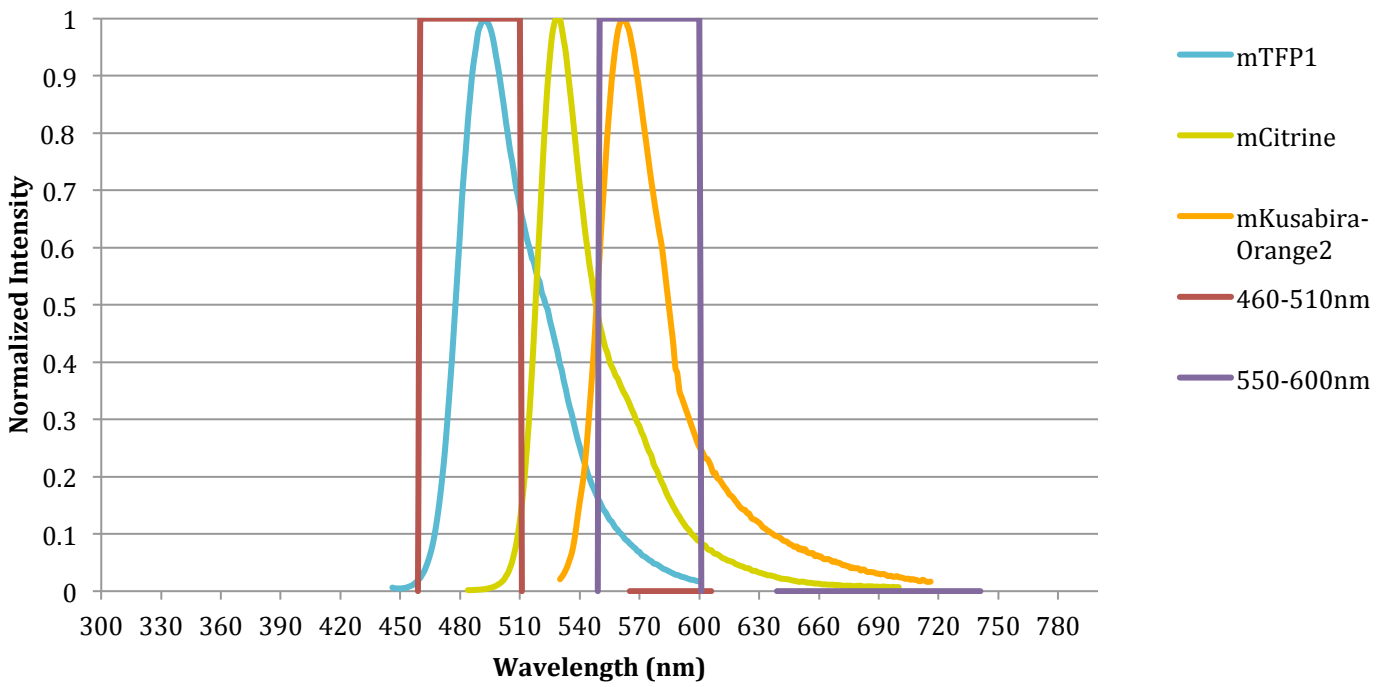
When a fluorescent material, such as a fluorescent protein (FP) absorbs light, it does so at a specific range of excitation wavelengths. As the excitation levels fall back to baseline, the sample emits certain photons at the range of the "emission wavelength" as an intrinsic property of the molecule. In confocal microscopy, the excitation photons are introduced at a very specific wavelength via lasers (Kinsey, 1977). Different lasers at different wavelengths are used for specific FPs.

For our project, we used mCitrine, mKusabira-Orange2 and mTFP1 (Griesbeck, 2001; Kaida, 2011; Ai, 2006)FPs. Because of the different emission wavelengths of each of those FPs, we can visualize all three FPs simultaneously in one image. The excitation/absorption spectra in the figures below (Figure 3 A,C) show the specific laser that was used and the absorption wavelength of each FP. For mTFP1 we used the 458nm

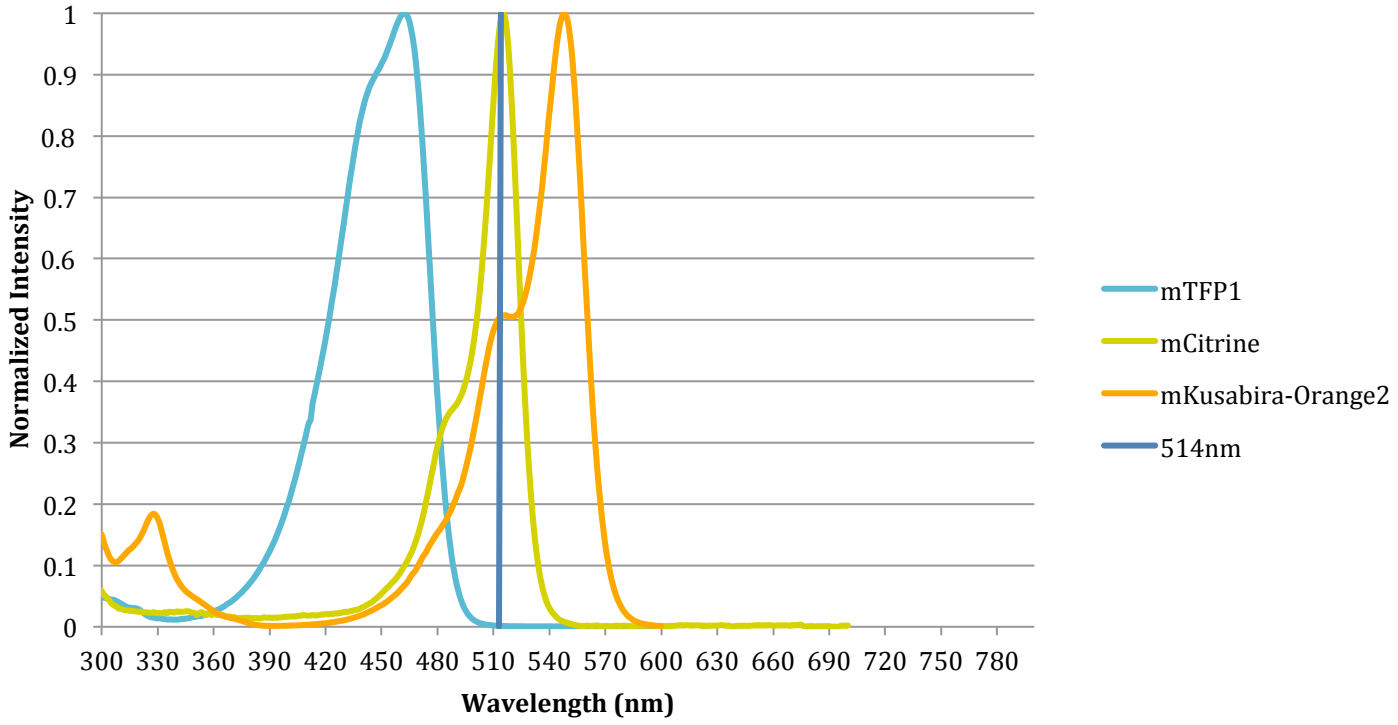
laser, for mKO2 we used the 543nm laser and for mCitrine we used the 514nm laser. In Figure 3B and 3D, we show the emission spectra of each of the FPs and the collection windows. The collection windows specify the range of wavelengths allowed to pass through for best imaging.



B


Emission Spectra: mTFP1 and mKO2
Normalized Intensity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
300 330 360 390 420 450 480 510 540 570 600 630 660 690 720 750 780
Wavelength (nm)
mTFP1
mCitrine
mKusabira-Orange2
460-510nm
550-600nm

C


mCitrine Excitation/Absorption Spectrum
Normalized Intensity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
300 330 360 390 420 450 480 510 540 570 600 630 660 690 720 750 780
Wavelength (nm)
mTFP1
mCitrine
mKusabira-Orange2
514nm

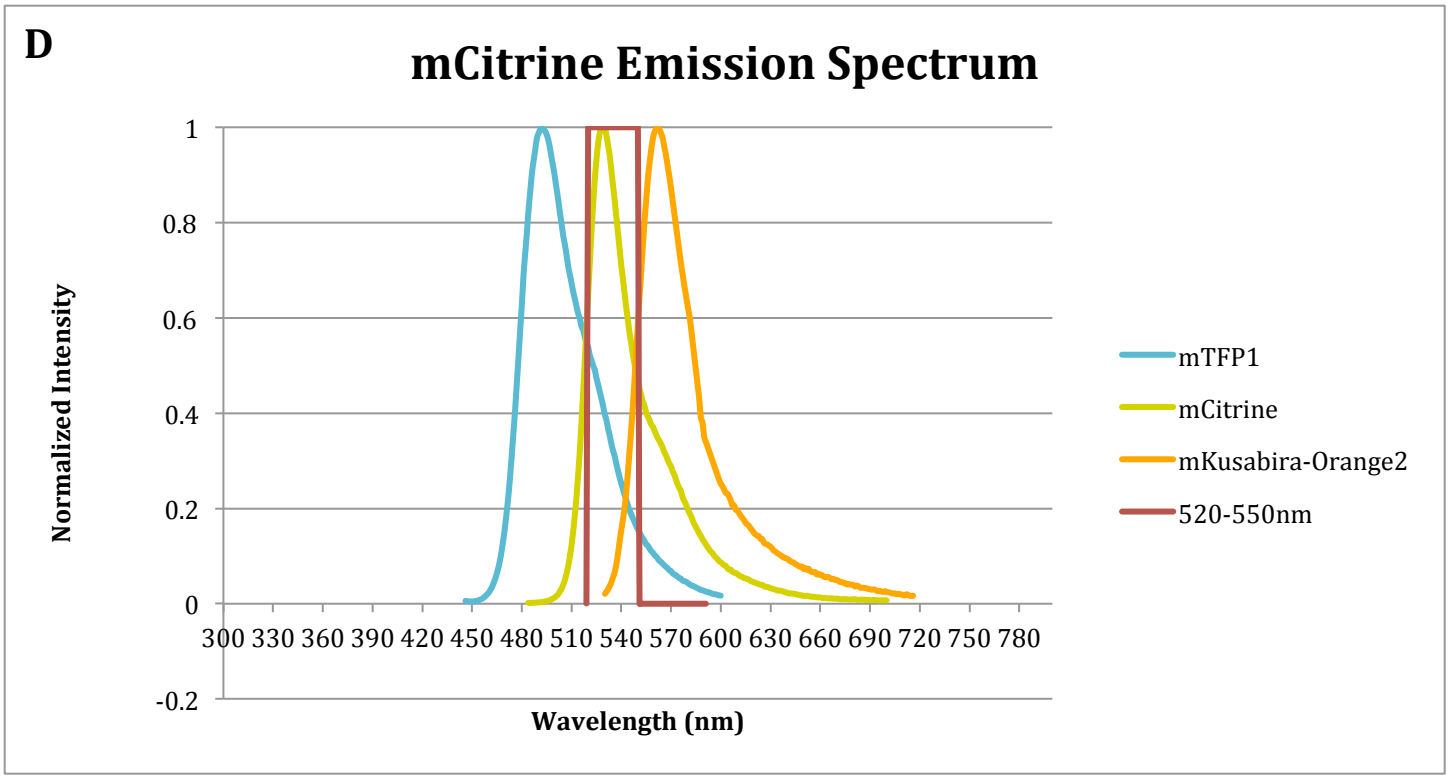


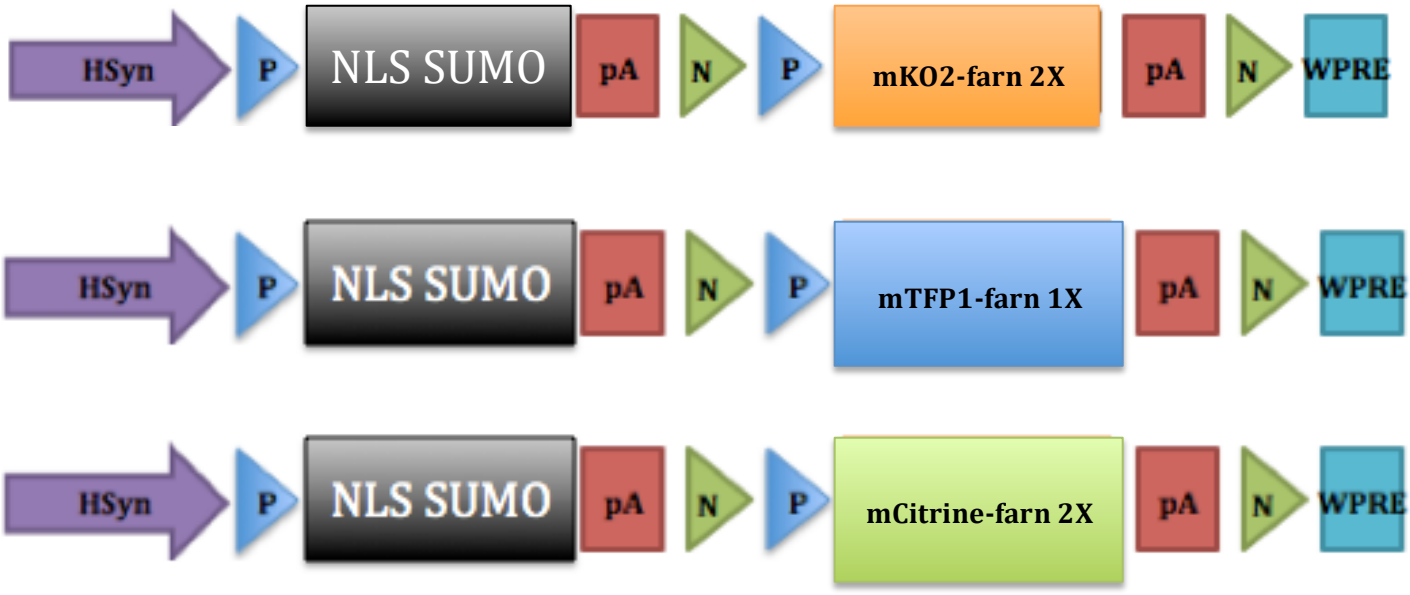
Figure 3: Spectra based on confocal microscopy imaging. **A)** Excitation/Absorption Spectra of mTFP1 and mKO2 using 458nm and 543nm lasers, respectively. **B)** Emission Spectra of mTFP1 and mKO2 including collection windows of 460-510nm and 550-600nm, respectively . **C)** Excitation/Absorption Spectrum of mCitrine using 514nm laser. **D)** Emission Spectrum of mCitrine including a collection window of 520-550nm.

D) Co-transfection into N2A cells

For our final figure, we completed a co-transfection into N2A cells of all three of our ON/OFF constructs with Cre. The fluorescent proteins we used were mCitrine 2X, mKusabira-Orange2 (mKO2) 2X, and mTFP1 1X. All of these FPs were modified with farnesyl tags for membrane targeting. The specific constructs are displayed in Figure 4A.

With Cre expressed, there is a stochastic recombination choice between the incompatible LoxP and LoxN sites (see page 5).

A



B

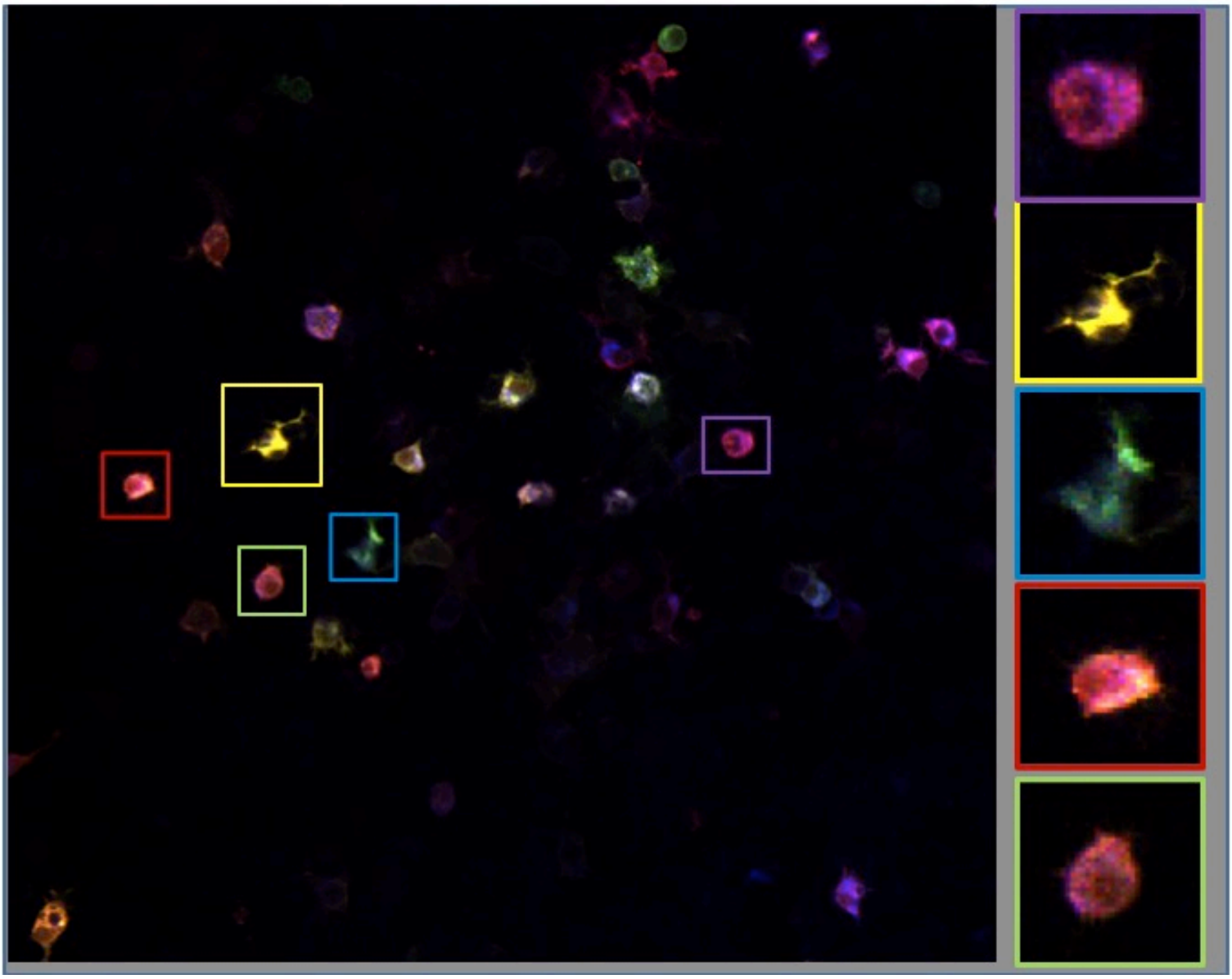


Figure 4: 3 days post-co-transfection of three On/Off constructs with Cre into N2A cells. **A)** The three On/Off constructs with three FPs (mKO2 2X, mTFP1 1X, and mCitrine 2X). **B)** Confocal microscopy imaging results at 10X magnification, 3 days post-co-transfection. Imaging done according to spectra in Figure 3. Right panel shows specific cells, color-coded with the image on the left, and overall color diversity.

Discussion

We validated a novel Brainbow design, which utilized a binary ON/OFF output to increase color diversity and utilized tandem fluorescent proteins to increase labeling intensity. In using a non-fluorescent insert as the default, when Cre is not expressed, the background is dark, which serves for better distinction. Using NLS SUMO proved to be the best option for this non-fluorescent insert, as it showed the least amount of leakage of the downstream FP.

The ON/OFF construct with its stochastic choice in Cre/lox recombination also improves on distinction between neurons as the choice between fluorescent or non-fluorescent inserts limits the over-labeling problem. Previously, with multiple FPs in a single construct, multiple colors would label the same neuron, marking it an indistinguishable white. The labeling-redundancy problem, where multiple neurons are labeled in the same color, has also been reduced with more color options. With every ON/OFF construct that is inserted individually into an AAV vector, the color options increase. With four separate ON/OFF constructs inserted into four AAV vectors, there are a minimum of 15 different colors (2x2x2x2-1(all-dark option).

We have shown improved labeling brightness by inserting tandem copies of FPs as a single polypeptide chain. The comparison between mKO2 1X versus mKO2 2X showed roughly double the intensity in N2A cells, post-transfection. This will contribute to being able to distinguish between neurons and neuronal projections in dense tissue samples. Also, when multiple neurites synapse onto dendrites or a soma, they can be separated out by their fluorescence and then quantified to determine how many neurites are present. This gives information on the strength or importance of a synapse and could

lead to further inquiries on why some neurons have significantly more synaptic connections than others.

While we did not have time to finish packaging the AAV constructs for this thesis, we are confident of the work we have completed thus far in preparing them. So far we have packaged the ON/OFF mCitrine 2X construct into the AAV and will image the results in a few weeks.

For the future of this project, we will continue to improve both the number of fluorescent colors available in a variety of ON/OFF constructs, and the brightness of each FP. While the AAV vector has a limited amount of space, using the smaller NLS SUMO non-fluorescent OFF insert will give us space to include at least two tandem copies of the FP (2X).

The defining of individual neurons and their dendritic and axonal projections via fluorescent membrane labeling has immeasurable benefits. The human connectome project, the driving force behind the mouse connectome and Brainbow, aims to create a structural blueprint of the human brain and thus make advances in brain development, aging, and disease. Even providing neurosurgeons with such a map could aid in linking mental illnesses to specific brain lesions or connectivity incongruences (Nair, 2013). While this connectome project is being completed, Brainbow by itself is already proving to be independently useful. Logistically, a single Brainbow mouse provides hundreds of data points that previously required many mice. Gathering information from a single source improves confidence in the overall relatedness of the neurons and their circuits.

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