S. BAMJI LAB

Our Guide to Basic Lab Technique & Rationale

Synapses of the central nervous system are highly specialized regions of cell-cell contact designed to rapidly and efficiently relay signals from one neuron to another. By establishing a dynamic yet precise network of synaptic connections, the brain is able to attain a level of functional complexity that enables not only simple motor tasks, but also sophisticated emotional and cognitive behaviour. The study of how synapses form and function is therefore essential to our ultimate understanding of higher brain functions such as learning and memory.

Synapses of the CNS are continuously formed, remodeled and eliminated over the life of an organism. It has been suggested that perturbation of these processes may contribute to the etiology of neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease, as well as developmental and psychiatric diseases including autism and schizophrenia.

- Shernaz Bamji
www.bamjilab.com

Welcome to the lab!

Where do we get our neurons?

Embryonic day 18 (E18) Sprague-Dawley rats are extracted from pregnant dams. Their embryonic brains are then collected and the hippocampus is dissected out. Hippocampal E18 neurons are then disaggregated and plated sparsely onto circular glass coverslips forming what is known as a primary culture. These coverslips are then placed into the wells of a plate and given nutrient media to sustain the cells, before being moved to an incubator to regulate temperature and CO_2 levels. These *in vitro* primary cultures of hippocampal neurons could be used for a variety of purposes, but in the Bamji lab, our primary focus is on synapses.

Pictures on the right give an indication of the steps involved. Images of an adult Sprague-Dawley rat, an adult rat brain, x-gal staining of the hippocampal region of an adult rat brain, the morphology of suspended neurons at 1 day *in vitro* (DIV), a coverslip with a 12-well plate and the morphology of hippocampal neurons cultured on a coverslip at >20DIV are shown, respectively.
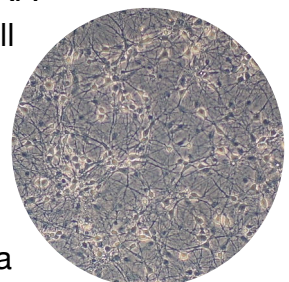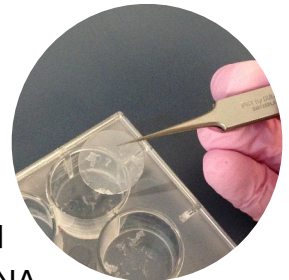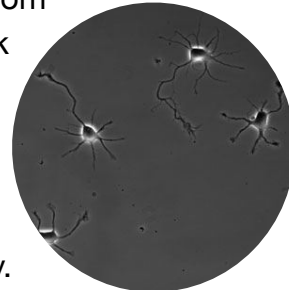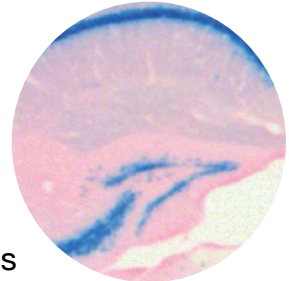
What is a synapse?

Synapses of the central nervous system are highly specialised regions of cell-cell contact designed to rapidly and efficiently relay signals from one neuron to another. By establishing a dynamic yet precise network of synaptic connections, the brain is able to attain a level of functional complexity that enables not only simple motor tasks, but also sophisticated emotional and cognitive behaviour. The study of how synapses form and function is therefore essential to our ultimate understanding of higher brain functions, such as learning and memory.

What is a transfection?

Transfection is a process by which particular nucleic acids are introduced to cells in the form of a plasmid. This technique enables researchers to selectively introduce the expression of a gene and therefore its associated protein. One may wish to perform a DNA/RNA transfection for a range of reasons, including the visualisation of cell morphology (e.g. a green fluorescent protein or GFP cell fill), the fluorescent tagging of a protein (e.g. PSD-95-GFP) and the cellular consequences of overexpression of a particular protein. In some instances, the transfection may involve the use of short RNAs that prevent the cell from expressing a certain protein, then referred to as a knockdown experiment.
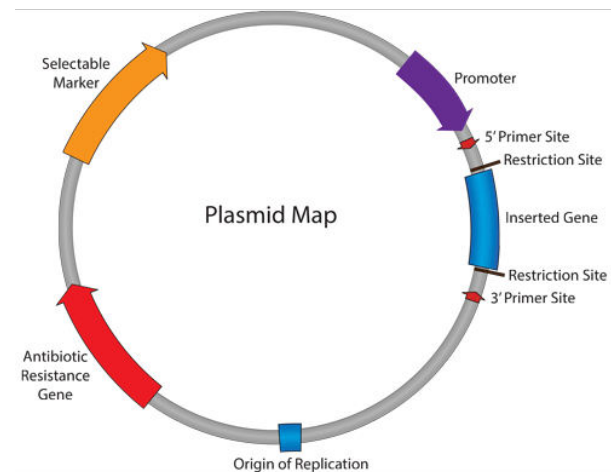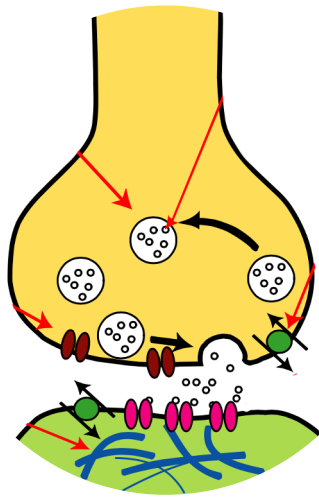
Why might we perform a transfection?

Let's say protein X is known to be expressed in neurons and decreases in its expression have been reported in the post-mortem brains of autism patients compared to healthy controls. However, the localisation of this protein within neurons and the trafficking of this protein remains unknown. One way to approach this problem is to fluorescently tag the protein of interest (e.g. X-BFP) in our transfection plasmid, meaning that our introduced nucleic acid produces a protein that contains both our molecule of interest (X) and a region that emits light when stimulated at a given wavelength (e.g. BFP). Then, using a fluorescence imaging technique known as confocal microscopy, images can be taken of these transfected neurons. When our introduced X-BFP protein is present, the BFP region shall emit a small amount of light that can be detected by the imaging software. However, just visualising the presence of our transfected protein tells us little about the localisation of that protein within the neuron. To do that, we first need to simultaneously visualise the cell morphology. In order to achieve this, we co-transfect a fluorescent protein expressed at high levels capable of filling the entire cell body. It is important to remember that this protein must fluoresce at a different light wavelength, such that two separate images can be taken of the same cell: one showing the X-BFP protein and the other displaying the cell morphology. In the Bamji lab, we are primarily concerned with molecules present at synapses either basally or as a result of recruitment. However, synapses are difficult to establish or categorise from cell morphology alone. Instead, we commonly transfect fluorescently-tagged synaptic proteins, such as PSD95. PSD95 is present on the post-synaptic region of the synapse and thus indicates incoming connections to the neuron being imaged. Again, if we fluorescently tag our synaptic protein (e.g. PSD95-RFP), the tag must fluoresce at a different wavelength to the cell fill and our X protein. If our protein of interest co-localises (overlaps) with a synaptic protein, we can assume our protein is therefore present at a synapse. This imaging can be performed in both fixed or live cells. In the latter case, the trafficking of a given protein to and/or from the synapse can be observed over time, under different conditions or both.
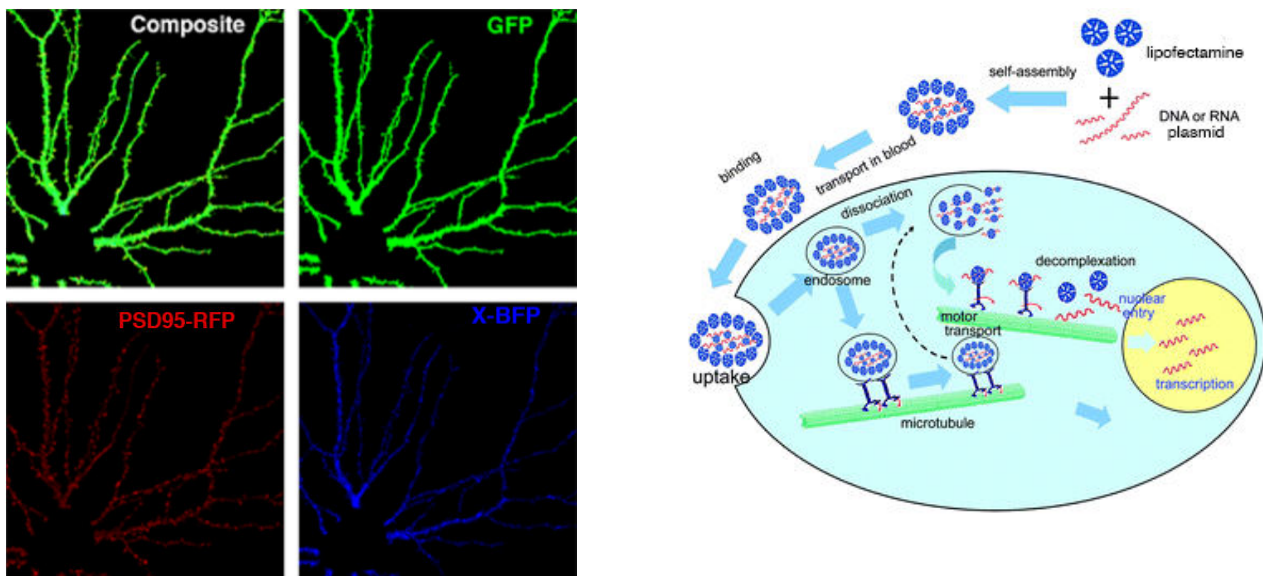
Pictures to the left show the kind of images produced by fluorescent transfection and confocal microscopy post-processing to remove the cell body and axon. Displayed is a composite image of the three pictures, a GFP image of cell morphology, a PSD95-RFP image of postsynaptic regions and an X-BFP image of our protein of interest. These three proteins are suitable for co-transfection as BFP fluoresces under the 405nm laser, GFP under the 488nm laser and RFP under the 543nm laser. The schematic to the right outlines the lipofectamine transfection process and broadly how it leads from nucleic acid plasmids to transcription.

How do I perform a transfection?

1. Prior to transfection, calculate the desired concentrations for your constructs. Frequently used constructs in the lab will have an established weight known to work well (e.g. 0.5µg for GFP). Be sure to check with fellow lab members, but if you're using a newly acquired construct, 0.5-2µg is the normal range of individual construct weights used in the lab. Occasionally, you will have to compare coverslips transfected with different constructs. Even though the constructs may differ, you must ensure that the total plasmid weight being applied to each coverslip overall is the same. If the weight of your test constructs cannot be altered to match across your conditions, you will need to use an empty vector such as pDisplay. Each construct preparation will also differ in concentration, so be sure to check your aliquot every time for the µg/µl. An example of how to perform this calculation and write it in your lab book is displayed below;

2. Next, locate your plate in the incubator. At any given time, three weeks of plates are present in the incubator. The dates on the inner door correspond to the date the cells were plated. Transfection should usually occur at day *in vitro* (DIV) 11 and thus the plates you are transfecting should have a date 10 days prior to the day of transfection;

3. Take your plate out of the incubator and check your coverslips under the light microscope. Some of them may contain fasciculated neurons or have only sparse coverage. If you must use them, try to spread them out across your conditions as

they may not produce the best images. Be sure to label which coverslips fall into each condition on the lid of your plate. Try to keep this step fast as the cells dislike being out of the incubator and once complete, place your plates back into the incubator;

4. Fill two aliquots with 25µl of Opti-MEM media for every coverslip of neurons being transfected;

5. Again, for every coverslip, add the DNA plasmids you are transfecting at the relevant concentrations for the corresponding coverslip to one of the two aliquots;

6. To the second aliquot for each coverslip, add 1µl of lipofectamine and allow it to sit for 5 minutes. Lipofectamine produces complexes and allows your DNA constructs to enter the cell;

7. Pipette the lipofectamine aliquot into the DNA aliquot and mix thoroughly for every coverslip. Once complete, allow the mixtures to sit for 20 minutes before proceeding;

8. Add your mixtures drop wise to their corresponding coverslip and place your plate back in the incubator on the top transfected shelf.

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(1) All of the <u>constructs</u> being transfected in the <u>number of coverslips</u>
—> <u>Construct name</u> (<u>desired weight</u>) from <u>aliquot concentration</u>: <u>desired weight</u> divided by <u>aliquot concentration</u> = <u>volume</u> of construct preparation required per coverslip
—> Second construct…and so on.

For example:
(1) GFP + PSD95-RFP + X-BFP in 10 coverslips
—> GFP (0.5µg) from 1.15µg/µl: 0.5/1.15 = 0.43µl per coverslip
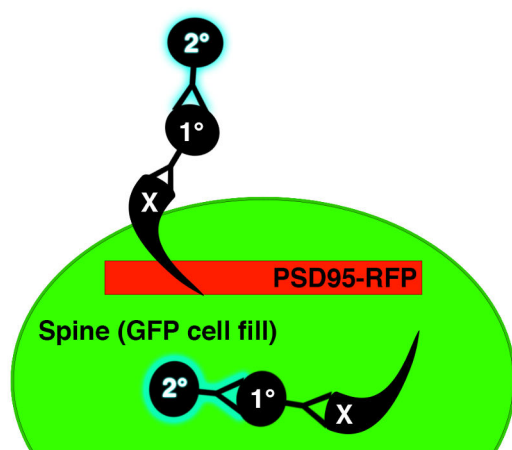—> PSD95-RFP (1µg) from 2.15µg/µl: 1/2.15 = 0.47µl per coverslip
—> X-BFP (1µg) from 1.6µg/µl: 1/1.6 = 0.63µl per coverslip

Model lab book entry for your construct calculations, which should be written in preparation.

What is immunocytochemistry?

Immunocytochemistry (colloquially referred to as 'immunos') is the staining or labelling of proteins in cell cultures using antibodies. In the Bamji lab, we most frequently use fluorescent immunocytochemistry. In this technique, a primary (1°) antibody is targeted against a protein of interest within our neurons, e.g. the aforementioned PSD95 present post-synaptically. A secondary (2°) antibody is then targeted against the primary antibody, creating a complex. However, this secondary antibody is tagged with a fluorescent molecule (or conjugated fluorophore) such that imaging the fluorescence of this antibody provides a rough estimate of the location of the protein of interest. In the below figure, a primary antibody is shown targeting a particular region of the X protein and a secondary antibody in turn is targeting the primary antibody. To enable this kind of binding, the first antibody is produced by exposing an animal (e.g. a rabbit) to the

protein of interest intravenously. Eventually, certain animals will develop antibodies against a particular epitope or region present on your protein of interest, as it's perceived by the host immune system as non-self. These antibodies can then be purified from the animal serum or manufactured by culturing extracted B-cells. Polyclonal antibodies capable of binding to several epitopes present on your protein are extracted from animal serum, while monoclonal antibodies that bind to a single protein epitope typically arise from cultured B cell-myeloma hybridoma lines. The secondary antibody, however, is slightly different. It targets the conserved portion of all antibodies from a certain animal (e.g. a rabbit) and is thus nonspecific. As you can then imagine, if you need to immunostain for several proteins, it is essential to use primary antibodies from different animals, so that each secondary antibody is only targeting one primary antibody. However, all secondary antibodies must be raised in goats, as we use goat serum to prevent nonspecific binding of the secondary fluorescent antibodies (and thus false positives in our immunostain). As with transfections, when fluorescently immunostaining for several proteins simultaneously, secondary antibodies that fluoresce at different wavelengths must be used, such that each wavelength represents only a single type of protein. The excitation wavelength of a secondary antibody is typically represented in its name, e.g. Alexa Fluor 633 which has an optimal excitation wavelength of 633nm. We often perform immunocytochemistry in conjunction with transfection.

Why might we use immunocytochemistry?

Immunocytochemistry is primarily concerned with visualising proteins in our neurons under various conditions. Transfections introduce nucleic acids that are not endogenous to our neurons, creating a range of considerations. These include an increase in intracellular protein concentrations, unknown cellular effects and atypical treatment of the exogenous protein by the cell, among others. Further, a tagged protein (e.g. PSD95-RFP) may have a different biochemistry to endogenous, untagged proteins (e.g. PSD95) due to alterations in protein folding or interactions. In many cases, immunocytochemistry is therefore the preferred method for looking at endogenous trafficking or expression of proteins in fixed cells. However, it relies on the existence of a specific antibody for your protein of interest, which may not exist, and it cannot be performed in live cells. Should you wish to visualise proteins in live neurons or you lack an antibody for your protein of interest, the transfection of a tagged (e.g. fluorescent) protein is the preferred method. Transfection can also be used to indirectly enable immunocytochemistry. For example, fluorescent tags are often large molecules, increasing the likelihood of upsetting protein biochemistries. By utilising both

techniques, a plasmid can be designed to express a protein with a small, non-fluorescent tag (e.g. the human influenza hemagglutinin or HA tag of PSD95-HA), which can then be transfected and immunocytochemistry targeting this small tag can be performed. Certain experiments seek to observe the cellular effects of over-expressing a particular protein. For these experiments, an antibody may exist for your protein of interest, but you will still want to increase protein expression through transfection. These two techniques therefore have a range of applications and their use will depend upon the intention of the study at hand and the availability of relevant antibodies/plasmids.

1 = Eppendorf tubes
2 = 1X PBS
3 = 0.1% Triton-X in 1X PBS
4 = Microscope slides
5 = Tube rack
6 = 10% goat serum in a 15ml Falcon tube
7 = 1% goat serum in a 15ml Falcon tube
8 = 1-200µl pipette tips
9 = 100-1,250µl pipette tips
10 = 0.1-10µl pipette tips
11 = twelve-well plate
12 = 2-20µl pipette (P20)

13 = 100-1,000µl pipette (P1000)
14 = 0.2-2µl pipette (P2)
15 = 20-200µl pipette (P200)
16 = Fine forceps
17 = ProLong
18 = Aspirator
19 = Suction tube of aspirator
20 = Kimwipes tissue
21 = Timer
22 = Dark box
23 = Condensed immunocytochemistry protocol

Left: A picture of the immuno bench in the Bamji lab. As it can appear a little daunting, the schematic shown on the *right* in the above figure highlights the key elements.

How do I perform immunocytochemistry?

Before starting your immunocytochemistry procedure, you shall want to make sure that you have first:

- *Performed your antibody calculations* — for every large coverslip, you require 120µl of 1% goat serum. Check the dilution of your different antibodies; for example, one antibody may be 1:500, while the other is 1:200. This refers to their dilution in 120µl of goat serum. So, for a 1:500 antibody, 120µl/500 = 0.24µl of antibody per coverslip.

The recommended format for your lab book is displayed below. In order to find the dilution factor, you can consult the Bamji lab antibody list or ask a fellow lab member;

- *Pre-warmed enough 4% PFA/4% sucrose solution and goat serum* — use the 37°c water bath in the lab for ~20 minutes. This step is important as both of these solutions are kept in the freezer and cold solutions can shock the cells, leading to undesirable neurons later when imaging your coverslips. Even if you do not use all of the PFA/sucrose or goat serum preparations that you thaw, please discard the excess as it cannot be reused. As such, be sure to warm just what you need: one tube of 4% PFA/4% sucrose and one aliquot of goat serum is enough for 18 large coverslips;

- *Diluted your goat serum* — every aliquot of goat serum is ~1ml. You will need to make two dilutions in 1X PBS: 10% and 1%. Take two of the skinny 15ml Falcon tubes and label them correspondingly with their composition (goat serum in PBS), their dilution factor (1%, 10%, etc.), the date and your name. For every coverslip, you require 500µl of 10% goat serum and 240µl of 1% goat serum. Always make 100-200µl more than the total required to make pipetting a little easier and to avoid transferring bubbles. Once complete, place your goat serum dilutions back into the 37°c water bath until you're ready to use them.

Blocking	1°ABs	Animal	2°ABs	Animal
10% goat serum	PSD95	Rabbit	anti-rabbit-633	Goat
0.5ml/coverslip	VGAT	Guinea Pig	anti-gp-568	Goat

1°ABs	Concentration	Per coverslip	No. of coverslips	Total
PSD95	1:200	0.6µl	10	6µl
VGAT	1:500	0.24µl	10	2.4µl
Goat serum	1%	120µl	10	1,200µl

2°ABs	Concentration	Per coverslip	No. of coverslips	Total
anti-rabbit-633	1:500	0.24µl	10	2.4µl
anti-gp-568	1:500	0.24µl	10	2.4µl
Goat serum	1%	120µl	10	1,200µl

Model lab book entry for your antibody calculations, which should be written in preparation.

Once you've finished your preparations, you're ready to begin:

1. Aspirate all of the maintenance media from every well;
2. Using a P1000 pipette, add 4% PFA/4% sucrose solution to each of your wells (0.5ml/well). Remember to pipette slowly so that it trickles down the side of the well and is not ejected directly onto the coverslip. This solution fixes your cells, thus

preserving the present state of the neurons by halting ongoing biochemical reactions and increasing the stability of cellular structures;

3. Using a lab timer, incubate the cells for exactly 10 minutes at room temperature in a dark box;

4. Aspirate the PFA/sucrose mixture and then wash the coverslips with 1X PBS at 1ml/ well (or mount them if you are not immunostaining and are just fixing). Remember to pipette your wash slowly and to the side of the well;

5. Aspirate your 1X PBS wash and then add 0.1% Triton-X (in PBS) at 0.5ml/well to permeabilise cellular membranes. This step allows the antibodies you later apply to enter the cell and target intracellular proteins or protein regions;

6. Incubate for exactly 10 minutes at room temperature in a dark box;

7. Slowly aspirate the 0.1% Triton-X solution and then wash your coverslips with 1X PBS at 1ml/well;

8. Aspirate your 1X PBS wash and then add 0.5ml of 10% goat serum (in PBS) to each well. This step prevents non-specific binding of your antibodies;

9. Cover your plate and incubate for 1 hour in a dark box at room temperature. In the meantime, ~10 minutes before finishing, make up your previously calculated 1°AB solution(s) in Eppendorf tubes and place on ice. Each Eppendorf tube can hold enough antibody solution for 10 coverslips, so consider this when doing your calculations. When removed from the fridge, antibodies should be placed on ice as quickly as possible. Ice can be collected from a room down the hall and should be placed into one of the red tubs or in a styrofoam box;

10. Cover the plate lid in a 3 square high, 2 square wide piece of Parafilm. Using fine forceps, remove your coverslips from their wells and place them on the Parafilm above the well from which they came. Once complete, put your lid with your coverslips back on top of the plate. As quickly as possible, to prevent your coverslip from drying out, add 111µl of your primary antibody solution(s) to the corresponding coverslips;

11. Make a humidity chamber to prevent evaporation by dampening some tissue and placing it at the bottom of a plastic container. Put your plate inside this humidity chamber, put the lid on and leave it overnight in the 4°c cold room.

The following day, you are ready to:

12. Aspirate the 10% goat serum from the wells and add 1ml of 1X PBS into each well before using fine forceps to place your coverslips back into their corresponding wells;

13. Aspirate and wash the coverslips three times with 1ml/well of 1X PBS to rid your coverslips of excess, unbound antibody. Leave 10 minutes between each wash and be sure to store your plates in a dark box during this time - <u>washing is critical</u>! Remember to pipette slowly and trickling down the side of the well. In the meantime, make up your 2°AB solution(s) in an Eppendorf tube on ice;

14. Cover your plate lid with the same amount of Parafilm as before, remove your coverslips and place them above their corresponding wells. This time, add 111µl of your secondary antibody solution(s) to the appropriate coverslips;

15. Incubate your cells for 1 hour at room temperature in a humidity chamber. At this stage, be sure to incubate your humidity chamber in the dark as your secondary antibodies are fluorescent and therefore affected by the bright lab lights;

16. Again, wash your coverslips three times with 1ml of 1X PBS (storing plates in the dark for 10 minutes between each wash) - washing is critical! After your third PBS wash, use the 10 minute wait time to label glass slides with your name, the date, any transfected constructs, the antibodies used and any additional conditions (control, +cLTD, etc.);

17. Mount your coverslips onto your labelled slides using around 8µl or one drop of Molecular Probes ProLong Anti-fade mounting media, remembering to flip the coverslip as you do. All fluorescent signals fade with time as they are exposed to their excitation light wavelength and emit photons. This step reduces the amount of fade that occurs in this fluorescence signal from neurons on your coverslip and allows us to store them for up to two weeks. If using a pipette, take up 10µl of ProLong to avoid creating bubbles;

18. Place your slides in a flat slide box in the 4°c fridge overnight to dry. Make sure you label your slide box with some tape and your name so that it's easier to locate later.

On the third and final day:

19. Add clear nail varnish to the perimeter of your mounted coverslips to seal them. Once dry, they're ready to be imaged!

What is a confocal microscope?

In the Bamji lab, we primarily use our confocal for fluorescence microscopy. It works by beaming a specific wavelength of light onto a given specimen, causing responsive elements (e.g. fluorescent molecules excited by that wavelength) in your specimen to reflect light. This reflected light is then focused by mirrors towards the camera. However, the majority of the camera is obstructed and only a pinhole-sized opening permits the entry of light. The only light capable of entering the camera must then have travelled a direct path known as the focal plane. Thus, only in-focus light reaches the camera and becomes reproduced in the final image by the imaging software (FluoView). Other types of microscope can be used for fluorescence microscopy. However, all microscopes produce a degree of light scatter, which creates fuzzy, out-of-focus regions in your images. The confocal microscope is a popular piece of equipment as it limits the amount of scatter, producing crisper images and it is from this quality that it derives its name. An additional benefit to confocals is that microscopy can be performed on live cells, as it uses light to image (not electrons) and it is unaffected by single-cell depths.

Why might we use confocal microscopy?

Confocal microscopy permits the imaging of fluorescence within your specimen. In untreated wild-type rodent neurons, fluorescent molecules are not endogenously expressed and thus imaging them on a confocal would result in blank images. However, when a fluorescent construct has been transfected or a fluorescent antibody used to immunostain, the presence of the associated protein will create a small area of light within the image. The relative amount of a fluorescent molecule present in a given space will create a range of intensities in the resulting image. The relative intensity is therefore a crude measure of protein concentration in that region when using fluorescent protein constructs or monoclonal antibodies. This phenomenon can be utilised for a range of purposes, including the visualisation, trafficking and colocalisation of proteins. For example, if GFP, PSD95-RFP and X-BFP constructs were transfected into our neurons, a myriad of data could be extracted from confocal images of these cells. The cell fill (in this case GFP) allows us to visualise the cell morphology and localise the rest of our proteins therein using a specific wavelength laser as the light source (which for GFP is 488nm). The PSD95-RFP protein indicates postsynaptic regions in your transfected neuron excited instead by a different wavelength of light and thus a different laser. Similarly, the X-BFP protein is excited by another wavelength and thus three different images of the cell can be taken using different wavelengths of light, each displaying a single protein. These images can then be superimposed. In the case of our example, the X-BFP protein can be localised either within the dendrites, spines or cell body by overlaying the GFP cell fill. PSD95-RFP, however, can be used to test whether the X-BFP protein is localised specifically to post-synaptic regions or is recruited to post-synaptic regions under certain conditions.

How do I use the confocal microscope?

This lab guide has been created as a reference for you. As with all of these techniques, you will be trained by a lab member on how to perform them, but when you're new to the lab, we understand that it's easy to become swamped by all this new information. However, the confocal microscope is an expensive piece of equipment that facilitates a lot of our research. It is therefore imperative that you have received one-on-one training on how to use the microscope before attempting it. Do not use these guidelines in lieu of in-person training!

Important: On any given day, it is rare to find the microscope unoccupied. A calendar (accessible here — user: Bamjilab pass: synapse) exists for the microscope, which you can use to book yourself time. Do not assume that you will be able to get time for the following day. Instead, try to book at least a week in advance.

1. To turn on the microscope, flip the switches for the HG power (1), microscope (2) and confocal PSU (3) in that order. You'll also need to turn on the lasers you require. The 543nm and 633nm are operated by turning a key, while the 405nm and 488nm laser have both a power switch and then an emission key;

2. Load up the FluoView software (user: BamjiLab pass: synapse) on the microscopy room computer (user: Administrator pass: fluoview). If FluoView is already open when you enter the room, we recommended closing it and reloading it, as the software is prone to crashing. Make sure the epi-lamp filter is open;

3. If the microscope is properly turned on, an 'Image Acquisition Control' interface will open in FluoView upon loading. Click the dye list icon and select the dyes you are imaging (e.g. eGFP, Alexa Fluor 568, etc.);

4. Using the 'Acquisition Setting' interface, set the objective lens you require for your images by selecting it from the drop down list under Microscope;

5. Spray some glass cleaner onto a tissue and gently clean the top of your coverslip;

6. If you're using an oil immersion objective (please check this with a lab member), add a drop of immersion oil to the lens, which lies beneath the stage;

7. Ensure that the lens is centrally positioned beneath the stage and then place the slide facedown on the microscope stage so that your coverslip is facing the lens;

8. Press the epi-lamp button on the 'Image Acquisition Control' interface to begin beaming light onto your specimen. Use the keypad to select the relevant filter (wavelength stimulation) for your cell fill;

9. Look down the eyepiece and use the focus knob to adjust the field of view until a cell is clearly visible. It will likely be easiest to find a cell to focus on in the middle of your coverslip. Unlike traditional microscopes, our confocal uses a button to switch the focus knob between coarse and fine adjustments;

10. On the 'Image Acquisition Control' interface, press the Focus x4 button. A live image of the neuron should appear on the screen. You will have to adjust the focus again. To the left hand side of this live image is a series of numbers referring to the order of your different channels and thus the images that are being superimposed. By clicking a number, you can remove the corresponding channel from the display;

11. This next step we recommend you perform on a control coverslip, though this may not be sensible for all experiments. Excluding your cell fill channel where the aim is to capture the cell morphology and not to perform quantitative analysis, you need to adjust the intensity levels of each of your channels. To do this, remove the other channels from the live image view and press Ctrl + H to switch to an intensity map view. Your intention is to prevent all of the red maxxed out intensity and to set the background of your image as the blue minimal intensity. In order to do this, use the HV and offset columns in the 'Image Acquisition Control' interface for each of your channels. As you adjust the settings in these columns, your live image will update. You want to bring the HV up to the point that red intensity just starts to appear by hovering over the column and scrolling up on the mouse. Once maxxed intensity first starts to appear, bring the HV down a little, so that no areas of red intensity are

present. For the same channel, you need to bring offset down until no blue minimal intensity is present in your live image. Then, bring it up until blue patches first start to appear in your image. Repeat this step for all of your channels, excluding your cell fill. For the cell fill, the intensity can be adjusted to produce the clearest depiction of cell morphology, but the amount of red maxxed out intensity is irrelevant. The background, however, should contain some patches of blue minimal intensity;

12. Now that you have set your intensity levels, you must not alter them. All of your images for an experiment must be taken at the same settings. If you are returning to the microscope to take more images, you can reload your settings by opening one of your previously taken .oif files for that experiment in Fluoview, pressing the ⏮ button to open the control panel and then reloading the image settings using the 🛠 button.

13. Begin imaging the coverslip from the upper leftmost portion and scan it using the path shown in the diagram to the right. When you encounter a cell you would like to image, readjust the focus on the software using the Focus x4 button and the focus knob. When you feel that you are ready to take the picture, use the ◻️ button. The software will take a moment to take the image, but it will pop up once complete. If this is your first time imaging, create a folder with your name on the desktop of the microscope computer. Inside this folder, create a dated folder giving the date the coverslips were mounted on the glass slides. Inside, create a series of conditional folders (e.g. control, knockdown, rescue, etc.). If you are imaging coverslips from more than one experiment, you may want to add additional folders to distinguish between them. Open a condition folder (e.g. control) and create a folder that starts with the condition and ends with the number of the cell for that condition. For example, let's say you are saving the third cell from your control condition, the folder should be called 'Con_N003'. Inside this folder, you can save the image of your cell. Make sure you are saving the file as a .oif file type as this will give you both the original FluoView image and additional .tif files for each channel that you need to analyse later;
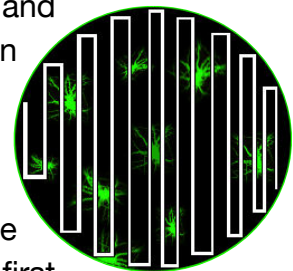
14. Once you have imaged a coverslip, remove your slide from the stage. Try to find at least 10 neurons per large coverslip. If you are using immersion oil, clean the coverslip with a tissue and the objective using a lens cleaning wipe. Label the imaged coverslip with an indelible dot and, if using it, replace the oil before moving on to image the next coverslip, starting again at step 13 of this protocol;

15. If the microscope will sit unused for several hours after you've finished, we recommend that you turn it off. Use the confocal calendar and consider asking fellow lab members before doing so. Start by closing the Fluoview software. Then, turn off your lasers. For those lasers with power switches (405nm and 488nm), turn off the emission key and allow them to sit for 10 minutes before returning to switch it

off. Finally, turn off the confocal PSU (3), microscope (2) and HG power (1) in that order.

How do I analyse my confocal images?

There are four major elements to analysing the images you obtain from the confocal: Photoshop, ImageJ, Excel and GraphPad Prism. Not all of these steps will be relevant or correct for any given experiment, but the common sequence of events shall be outlined in this lab guide. Instructional videos of these steps are available from the desktop of the lab computers in the 'Tutorials' folder.

Photoshop

Open the associated .tif image files for a cell in Photoshop. On the cell fill image, press Cmd + L (🍎) or Ctrl + L (⊞) to bring up the levels command and change the last 255 value to around 15. Press 'OK'. Next, select the paint brush tool, ✏️ make the active colour absolute black (#000000) and alter the paint brush size as necessary. Begin to mask the cell fill by blacking out parts of the image that are out of focus or broken and cellular parts you are not sure belong to your cell, e.g. they enter the image from off-screen or seem to stem from an adjacent cell. Lastly, you want to remove the cell body or soma, leaving you with the dendrites and the axon. If you have lower magnification images than x60, the axon can also be removed if need be. Once complete, use the magic wand tool 🪄 to select the black background ensuring that the tolerance is set to 20 and contiguous is unticked. Create a new layer 🗔 and then use the paint bucket tool 🪣 to fill the selection in with black. The image visually shouldn't change, but you should notice in the layers section that you now have one image of your cell and one layer containing just the black regions. Make sure that your newly created layer is active, select all and then copy and paste this layer onto the other .tif images for that cell. In doing so, you have excluded any fluorescent signal (and thus protein) that does not arise from your cell of interest. Merge these two layers together by pressing Cmd + E (🍎) or Ctrl + E (⊞). Convert every image to 8-bit by going to Image > Mode > 8-bit, then you are ready to save. In the folder containing your conditions, create a new folder called 'Masks'. Then reproduce the folder layout for the conditions and cell number, e.g. Control > Con_N003. Save all three of your masked .tif images for the cell into the cell name folder, being sure to create a new one for each cell in that condition.

ImageJ and Excel

ImageJ is an open access image processing software developed by the National Institutes of Health. You can download the basic program free of cost here. A number of

plugins exist to adapt the software for more specific purposes. One we frequently use in our lab is NeuronJ, which you can download here.

Setting the scale

Whenever you reopen ImageJ, you need to set the scale for your images. We most frequently work in µm, while the default settings are typically either pixels or cm. In order to change these settings, open one of your unedited .tif images for that experiment in ImageJ. Go to Analyze > Set Scale… You'll notice that the default 'unit of length' is cm. Change this to um and then move the decimal point in the 'distance in pixels' up four numbers (as 1cm = 10,000µm). Make sure you tick 'Global' to maintain this setting for all of the images you open until ImageJ is closed, then hit OK and close the unedited image.

Tracing your neuron

As the cells you are comparing will differ in size, you cannot directly compare the number of proteins present in your images. One way to account for these size difference is to take a measure of cellular length and divide the amount of protein by the length. Equally, you may wish to look at changes in neuronal length under various conditions. In order to perform these analyses, you must trace your neuron using the NeuronJ plugin. In ImageJ, go to Plugins > NeuronJ. You should notice that the ImageJ interface changes. Load your masked cell fill image in NeuronJ using the load button 📂 a n d then click the add tracing button 🖊 to start measuring length. You'll notice that your cursor forms a + sign. Left click on a dendrite to start tracing it and use the mouse to follow its length. The program will try to predict the trajectory of the dendrite, but if you find it is making incorrect predictions, you can left click again to set a fixed point along the trajectory. When you've finished tracing a single dendrite, left click and then press the spacebar. The program should stop tracking the movement of your mouse and the tracing should end at the last fixed point you set at the end of your dendrite. Simply left click again in another area of the image to start another tracing. If you ever make a mistake in a tracing, end the tracing and select the remove tracing button 🖊. Click the tracing you'd like to delete and press 'Yes'. Once you've traced the entirety of your neuron, you can now measure the length using the measure tracings button, which 🔁 will create a popup. Typically, you will not need the tracing or vertex measurements. Click 'Run' and ImageJ will present you with the data for your cell. Copy and paste this data into a number processor (e.g. Excel) sheet titled 'Tracings' using the below format:

DD/MM/YYYY	Cluster	Type	Count	Sum Len (um)	Mean Len (um)	SD Len (um)	Min Len (um)	Max Len (um)	Mean Val [au]	SD Val [au]	Min Val [au]	Max Val [au]
Con_N001	##	##	##	##	##	##	##	##	##	##	##	##
Con_N002	##	##	##	##	##	##	##	##	##	##	##	##
And so on…	##	##	##	##	##	##	##	##	##	##	##	##

Once you've copied the data into your number processor, save your tracings into the same folder as your masked .tif images for that cell and close the image. You can now repeat this process with the rest of your cells.

Running a macro

A macroinstruction or macro is a piece of coding that defines a number of software functions. When you run a macro, the coding therefore instructs the software on the steps it should perform. It allows us to standardise analysis and to code conserved sequences of image processing, rather than manually performing it over and over for every cell. As such, there is no standard protocol that applies to all macros, so instead I shall take you through a commonly used macro in the Bamji lab — the colocalisation macro. This macro takes two confocal images and quantifies the amount of light patches or puncta present in each of the images. It also tells you how many of those puncta would overlap if you were to superimpose the images. As you can imagine, if your first image is an immunostain for a ubiquitously present synaptic protein and the second image is an immunostain for your protein of interest, when any points overlap, you can assume that puncta of labelled protein is present at a synapse. To run this macro, two of your non-cell fill layers must be open in ImageJ. A threshold must be set for each of these images. To do so, click on one of your images and go to Image > Adjust > Threshold. A popup with two sliding bars should appear. Move the bottom bar all the way to the right and you should notice that your image turns entirely red. Slowly move the top bar to the right and you will start noticing that red spots of reducing size appear in your image. You want to adjust this top bar until all the light spots in your image and as little of the background as possible are captured by the red. When you are comfortable you have set the appropriate threshold, take note of the number of the top bar. Repeat this step for the other channel. You may want to do this for several cells (~10), keeping note of the channel you are thresholding. You can then produce an average threshold for each of the channels and use it to edit the threshold settings in the macro code, which you must not alter between cells. When you have added the threshold average to your macro for both channels, save it and close any images open in ImageJ.

Now, open two non-cell fill images of a cell you wish to analyse and go to Plugins > Macro > Run…, which you should use to find the macro file. The program will then begin performing the encoded functions. At the end, only two screens should be open on ImageJ: One providing you with data output and the other depicting a colour coded superimposed image of the puncta detected by the software at your set threshold. Copy and paste the data output into the same number processor file as your tracings, though preferably into a new sheet labelled 'Colocalisation'. Use the format displayed below.

| DD/MM/YYYY	Channel	Count	Total area	Average size	% Area	Mean
Con_N001						
	s_C002.tif	171	246.000	1.439	0.023	142.730
	s_C003.tif	109	144.000	1.321	0.014	127.859
	Colocalizated points (8-bit)	0	0.000	NaN	0.000	NaN

Close any open windows and then repeat the steps described in the second paragraph of this section for all of the cells you wish to analyse. Once you have all of the data in your number processor, you can use it to calculate any descriptive values you might need (e.g. number of PSD95-RFP puncta/length in µm) in additional sheets. For example, you may need to calculate the density of the PSD95-RFP puncta, so a sheet named 'PSD95-RFP density' can be produced. In which, every cell separated by condition would have its PSD95-RFP puncta count divided by total cell length. Then, the mean and standard error of the mean (SEM) of these values can be calculated and graphed (bars representing the mean and error bars representing the SEM) to give you an overall representation of your data.

Obtaining statistical significance with GraphPad Prism

In science it is not enough to simply graph data. Our data must be analysed using statistics to discern the level of mathematical confidence we have that the null hypothesis for our experiment can indeed be rejected. The specific statistical test you need to run will differ from experiment to experiment, depending on the type/quantity of data you've collected and whether you're looking for a difference or a relationship. Make sure you speak to fellow lab members if you are unsure which test you need to run for your data. The majority of our cellular experiments look for differences between the means of three or more conditions and thus the appropriate stats test is an analysis of variance (ANOVA). This guide shall therefore take you through how to use GraphPad Prism to perform an ANOVA with a Tukey post-hoc test. A post-hoc test is necessary as an ANOVA will only tell you if at least one of your means significantly differs from at least one other mean. However, an ANOVA alone will not tell you where this significance lies (e.g. between control and knockdown, but not between control and rescue), which the post-hoc test will.

In the Bamji lab student office (Room 2.453), load GraphPad Prism on Zeus. A 'Welcome to GraphPad Prism' interface will appear. Click Column under the New table & graph section, then select the vertical column bar graph. Under plot, select the Mean with SEM and click Create. Separate your conditions on the resulting spreadsheet by naming each column. Consider the row number to be the cell number for each condition. Copy each data point (e.g. for PSD95-RFP density/µm) from your number processor into the corresponding cell on Prism. Next, click the Analyze button under analysis. On the resulting popup, select 'One-way ANOVA (and nonparametric)' under Column analyses

and click OK. Another popup shall appear for the ANOVA. As previously stated, many of these options may be pertinent to your specific analysis, but for the purpose of this booklet we shall not be using the vast majority of them. Click the Post test drop down list and select 'Tukey: Compare all pairs of columns'. Click OK. The program shall then give you an output for both the ANOVA and the Tukey post-hoc test, detailing where any statistical significance lies. On the sidebar to the left,
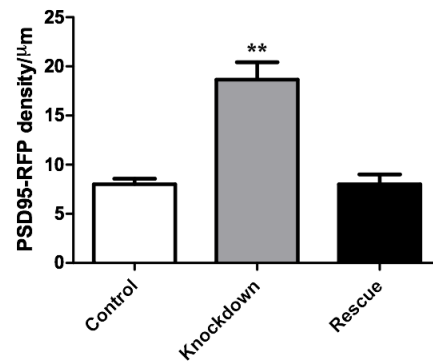
N = 28-31 per group, 3 cultures; p <0.05

different steps in your GraphPad Prism analysis can be seen, including Data Tables for the raw data, Results for the output of statistics and Graphs for the graphical representation of your data. You will need to combine the info from the Results section with the graph to indicate which bars of the graph significantly differ from each other. Once you've edited the graph and titled your axes, the graph can be copied, e.g. into Powerpoint, Word or Photoshop. The figure to the right gives an idea of the expected format of any graphs you produce.