Identifying the precise dendritic location of distal synaptic connections

Many efforts are currently being devoted to “connectomics” of various forms, however there is a crucial piece if information that is missing from most attempts: Where on the dendritic arbor the identified synapse from different presynaptic cell types reside. This information is particularly lacking for distal connections that cannot easily be contained in the same high resolution volume. Multiple lines of evidence, including dendritic nonlinearities, heterosynaptic plasticity, and functional clustering of inputs, suggest that the location of synapses constrains the computation of a single neuron. In our eyes, understanding which cells are connected is only the beginning of the project for connectomics, and the enterprise will necessarily be incomplete until we also know where in the dendritic arbor those synapses occur and their proximity to other synapses from similar or different presynaptic cell types.

Specific aim 1
We will leverage graded, cortex wide florescent labeling to identify approximate spatial identity of each unique pre-synaptic neuron for all synapses on a post synaptic neurons dendritic arbor.
We will do this by taking advantage of proteins that are expressed in a graded fashion across the brain, such as Pax6, to create a “coordinate frame,-like” presynaptic label. Expressing a synaptically targeted florescent label, such as syn-tdTomato, under the same promoter as Pax6 will associate the strength of td-Tomato expression with the anterior-posterior location of the soma. High resolution imaging of sparsely labeled post synaptic cells will allow us to map approximately where in the anterior-posterior axis the impinging afferent originated. One advantage of this technique over Aim 2 is that it can be easily applied to multiple post-synaptic neurons of interest in the same mouse, and can be done in-vivo, enabling exciting experimental measurements or interventions using other common techniques like calcium indicators or optogenetics to be performed AFTER the approximate mapping is established.

Specific aim 2
We will use synaptically trafficked RNA barcoding combined with single cell targeted rabies virus to identify a one to one mapping from each dendritically located synapse to a single pre-synaptic partner neuron.
The previous aim will yield a course understanding of how presynaptic soma location corresponds to post synaptic synapse location. This aim seeks to develop methods that will generate a one to one mapping between presynaptic cell and postsynaptic synapse location. First, single cell in-vivo electroporation targeted rabies virus will be used to deliver cre to the post-synaptic target neurons presynaptic partners. Cre dependent TVA will be expressed in the presynaptic cells, and will be used to target delivery of virus providing synaptically trafficked barcodes. Finally single cell transcriptomics can be done on presynaptic neurons to identify both location and transcriptomic profile of the neurons expressing a specific barcode, and spatial transcriptomics can be done on tissue sections containing the postsynaptic target neuron to identify which barcodes are present in each presynaptic bouton.

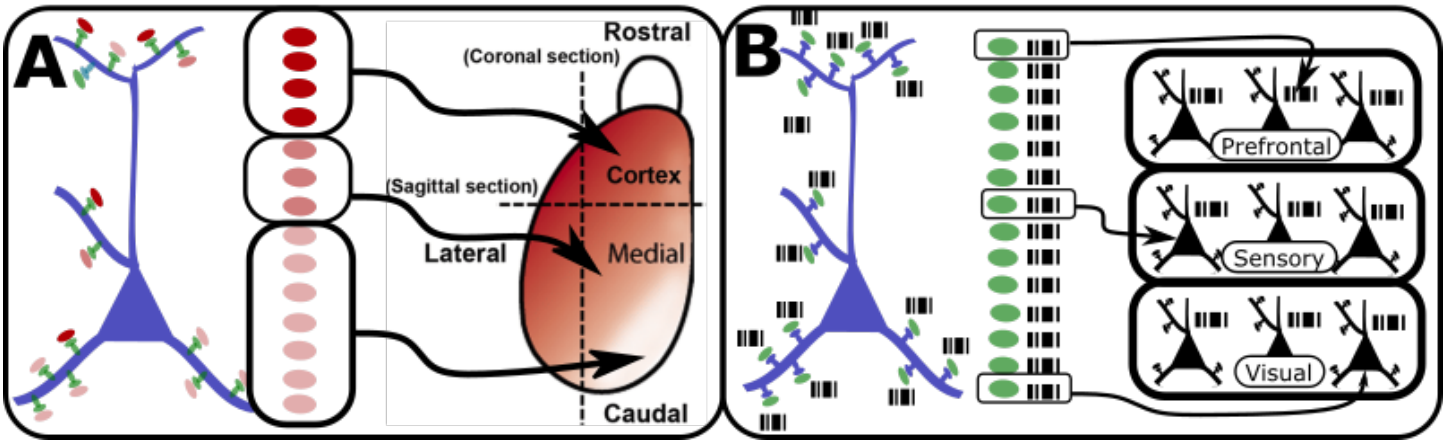

Figure 1: Schematics of the specific aims. A: Schematic of specific aim 1. Boutons are labeled in shades of red, cell fill is BFP and spines containing PSD95 are green (GFP). Afferent boutons can be mapped back to the approximate region of cortex where they originated. Right half of graphic is taken directly from 21. B: Schematic of specific aim 2. Boutons are labeled in green (GFP) with the cell fill blue (BFP). Boutons can be mapped back to the specific cell where they originated using RNA barcodes.

Research Strategy

(A) Significance

We believe this work serves two important purposes 1. to validate and further constrain the connectivity information gathered using other techniques and 2. to give unique insight into the computations that might actually be performed by cortical neurons.

Constraining large scale, connectivity based models

Current efforts to map the connectome promise that by generating a detailed wiring diagram for the brain we will finally crack the puzzle of how the mammalian brain works. Indeed, partial or complete wiring diagrams for *C.Elegans* (1, 2) and more recently *Drosophila* (3) have generated or confirmed theories about the circuit mechanisms that enable specific behaviors (2,4). However, when scaling up these approaches, there are reasons to believe that the weight matrix alone will be insufficient to gain similar insights in mice.

Dendritic nonlinearities have been repeatedly confirmed in in-vitro experiments (5 and MANY others). The presence of these non-linearities can drastically change the effect of a synapse depending on its proximity to the soma and how it interacts with its neighbors. Current state of the art models are not able to realistically constrain dendritic targeting because very little information is currently available (6, 7). What is currently available is limited to coarse rules about connectivity between broad classes (PV cells target the soma, SST/ HTR3 the dendrites), with no information available about the dendritic targeting of distinct sources of excitatory input. While it is possible that these connections are largely unstructured, evidence of heterosynaptic plasticity (8) and functional clustering of inputs in-vivo (9) both indicate that that dendritic targeting may be highly structured. Determining the rules that constrain dendritic targeting between specific cell types will be an important step to further constrain large scale connectivity based models.

Focus on understanding single neurons

Many computational theorists propose that the dendritic nonlinearities may enable single neurons to perform a vast array complex computations (10 and many others). Despite the many papers proposing what operations or functions a single neuron MIGHT be capable of computing, there have been surprisingly few attempts to constrain these possibilities based on biological realities like where synapses near enough to interact originate, or the actual nature of the activity patterns produced there. For example, cortical and thalamic inputs intermixed on the same dendritic segment enable very different computations than if they tend to be segregated to different branches. We see these aims as a significant first step towards understanding what single neurons actually compute, rather than what they MIGHT be capable of.

Lastly, we think that our Aims have significance because they offer a largely unique perspective to understanding the brain by focusing on single neurons as the brains building blocks. Many techniques employed in modern neuroscience (especially the alternatives assessed in our innovation section) are forced

to deal with incomplete information. Activity or connectivity is assessed from a small subsample of the full population of interest. A single behavior, stimulus, brain area, cell type or protein of interest is selected from the many possibilities. By focusing on individual post-synaptic target neurons we constrain the problem so that the information gathered is complete (from the target neurons perspective), and the question becomes much more tractable. We can place firm limits on what information that neuron has access to, and what it could compute from that information.

(B) Innovation

Current methods trade off between proximity and spatial resolution

To our knowledge, none of the existing methods to identify connectivity in the brain capture both the precise dendritic location of distal synaptic connections and the detailed identity of the presynaptic partner neurons. We assess these alternatives (ordered from low to high resolution) and highlight how they are insufficient to to answer the question at hand.

Distal connections are restricted to low resolution

Multiple methods can be used to identify distal connections between areas at a coarse resolution. Bulk viral injections (11), “projectomes” based on axonal tracing (12), transcriptomic barcoding (SYNseq(13) or BARseq(14)) or slice physiology (sCRACM) (15) are capable of identifying approximately where synapses occur by identifying what parts of the cortical volume contain the axons of interest, and can identify which layers are targeted most heavily. While this restricts which dendritic compartments can contain these synapses, that is the limit of its resolution. Except in the case of sCRACM these methods also provide little information about which post-synaptic cell types receive the synapses. For example it is always possible that axons targeting L4 actually synapse predominantly onto the basal dendrites of L2/3 neurons or the oblique dendrites/apical trunk of L5/6 neurons. None of these techniques can identify exactly where on the postsynaptic dendrite a synapse occurs, or the identity of dendritically-neighboring synapses.

Electrical connections are local and very sparse

Local connections can be mapped with paired patch experiments (16) and subsequent tracing of both the presynaptic axon and the postsynaptic dendrite can confirm the precise location of that particular synapse. This method is not only restricted to local connections due to the necessity of slicing the brain, but is also incredibly low throughput (lots of effort for individual pairs) and sparse (only a few pairs at most per target neuron). While still valuable, we pursue a more efficient method that also includes afferent identity for many synapses onto the same dendritic arbor to assess relationships between neighboring synapses.

High resolution structural connectomics are local

Electron microscopy methods have the spatial resolution to identify not only which neurons are connected, but also the precise location of the connecting synapses (17). However, this comes at the expense of long range connections. Thus far EM is limited to identifying connections between local neurons within the imaged volume which is at most (to date) 1 cubic millimeter. These methods also do not provide any transcription information about the presynaptic cells. While others are pursuing lower resolution axonal connectomes that might enable more tracing of more distant connections, these methods will always have trade offs and we choose to pursue an alternative approaches here that capture non-structural information.

Multicolor imaging of co-localized pre and post-synaptic proteins

To date, this technique has yielded the most detailed dendritic targeting information from non-local sources (in review from the Nedivi lab, MIT). It is also the only technique that provides this information in-vivo. Briefly, synapses of a post-synaptic target neuron are identified by florescent labeling of post-synaptic proteins. Pre-synaptic populations of interest are labeled with a synaptically targeted florescent protein (like syn-tdTomato). Co-localization of the presynaptic and postsynaptic colors indicates the identity of the synapse in question, and full dendritic arbors can be imaged and traced to produce precise and complete dendritic targeting information we desire. However, this technique is limited to binary classification - labeled synapses are either from the labeled pre-synaptic population or they are not. With multi-color labeling this technique could be scaled to more populations, but it will never yield single cell transcriptomic information about the pre-synaptic partner, only broader population labels.

Transsynaptic viral transfection

Transsynaptic viral techniques can identify that two populations or even two single cells are synaptic partners (18), but have not, to our knowledge, been used to determine dendritic targeting because this would require full

tracing of both the pre-synaptic axon and the postsynaptic dendrite which is currently not possible because labeling of all presynaptic axons will be too dense for axon tracing.

Our Innovation

We propose to extend existing methods by enhancing multicolor co-localization techniques with graded cortical expression patters (Aim 1) or by combining single cell, mono-synaptic rabies labeling with transcriptomic barcoding techniques (Aim 2). This will obtain both precise dendritic localization and the identity of the presynaptic neuron without being constrained to small local volumes. We will do this at two different resolutions of presynaptic identity - a course resolution that can be used in-vivo to guide targeted imaging, stimulation or other interventions (Aim 1) and at single cell resolution where each dendritically localized synapse can be mapped to the specific presynaptic neuron of origin, and its location and transcriptomic identity. Notably, we propose to do this in a manner that will make it possible to map every synapse on a single post-synaptic neuron enabling a complete picture of that neurons afferents.

(C) Research Plan

Specific Aim 1 Research Plan

Aim 1: We will leverage graded, cortex wide florescent labeling to identify approximate spatial identity of each unique pre-synaptic neuron for all synapses on a post synaptic neurons dendritic arbor.

Rationale for Aim 1

The goal of this aim is to develop techniques to capture coarse information about the nature of presynaptic afferents, while retaining precise information about their dendritic targeting. To give this aim the greatest chance of success, we chose to rely on well established techniques and pipelines that are not heavily dependent on axon tracing to limit the labor requirements. None of the techniques proposed are completely novel, merely novel combinations of techniques that have already been successfully implemented. We also chose to rely on basic florescent microscopy so that this Aim could be applied preceding in-vivo experiments, as opposed to most connectomics techniques that can only be mapped to in-vivo experiments post hoc.

We chose to perform this aim in mouse primary visual cortex (V1) because this area is currently being focused on by other connectomic (19), cell type (20) and modeling (6) efforts that will make our research the most mutually beneficial to the community.

We chose to begin with Pax6 because it has graded expression (21) that expresses most strongly in lateral rostral parts of cortex, distal to visual cortex. V1 receives most of its cortical input from itself or other nearby visual areas, but it also receives input from non-visual sensory areas and higher cortical areas (and of course substantial subcortical input from thalamus)(11, 24). This will ensure that the bouton labeling remains sparse enough near the target neuron to be useful for primarily distal connections.

Experimental Approach for Aim 1

First we must generate a mouse with a synaptic florescent label that varies with Pax6 expression. Transcription of a flourophore linked with a synaptic protein will result in anterograde trafficking of the flourophore to the presynaptic boutons (22). Placing this construct under the same promoter as Pax6 should result in florescence that is highly correlated with Pax6 expression, which we can confirm using single cell transcriptomics or standard immunohistochemistry.

Once we have successfully generated the labeled mouse, we will follow the technique outlined in (23). For this aim we will express a structural marker (BFP) and a post-synaptic label (PSD95-GFP) in an ultra sparse population of the neurons of interest using sparse cre-dependent AAVs, or sparse in-utero electroporation of the constructs (23). Finally the target postsynaptic neurons will be imaged at high resolution (either in-vivo 2 photon, or ex-vivo fmost or confocal microscopy) in 3 colors. After demixing, the images can be analyzed for co-localization of the pre and pos synaptic labels.

Expected Results and Interpretation for Aim 1

Pax6 expression levels should allow us to distinguish between at there major sources of cortical input (prefrontal, visual and non-visual sensory), as well as some stratification within those regions (ie we may be

able to distinguish inputs closer to RL from inputs closer to PM, or inputs nearer the frontal pole from inputs nearer ACA). Using Pax6 as a proxy for distance from lambda as a source of cell type identification will necessarily be somewhat messy, and will be better at identifying where inputs are NOT from rather than pinpointing specifically where they are from. However this is the highest resolution non-binary form of cell soma location that can be performed in-vivo with simple, already available techniques.

We expect these results will be able to distinguish between two specific hypothesis. If excitatory afferents are largely unstructured and distributed randomly we should find that the florescent strength of boutons is similarly unstructured. However if we find that the inputs are structured, i.e. that strongly labeled boutons are more likely to be on the same branch as other strongly labeled boutons, or are more likely to be found on distal or proximal portions of the dendrite this would confirm the hypothesis that excitatory afferents target specific functionally or structurally distinct portions of the dendritic arbor.

Potential Pitfalls for Aim 1

The major pitfalls that we anticipate are potential difficulties titrating of the florescent bouton label so that there is neither too little or too much florescence in the boutons in order to provide valid spatial information about the afferents. It is also possible that the florescence in the individual boutons will be decoupled from the overall label present in the cell, further adding noise to our already coarse label. We will perform a separate set of experiments to ensure that the florescence observed at the boutons can adequately distinguish between somas in different parts of cortex, and quantify the precision of our method.

Alternative Approaches for Aim 1

Alternative approaches include using alternative mouse lines or injection strategies to identify the origin of afferents. A mouse line has already been utilized to identify the location of all synaptic inputs from thalamus onto unique post synaptic cells (Under Review, Elly Nedivi's lab at MIT). The difficulty is that scaling up to additional population would require not only complex line crosses to generate multicolored mice, but additional color channels in the microscope which is typically limited to 4. Using multiple injections avoids needing a complex mouse line cross, but adds complexity of multiple injections that can fail, and introduces significant ambiguity if you fail to fully label an area of interest. We intend to use this technique in conjunction with Aim 1 primarily to confirm the validity of our results.

Specific Aim 2 Research Plan

Aim 2: We will use synaptically trafficked RNA barcoding combined with single cell targeted rabies virus to identify a one to one mapping from each dendritically located synapse to a single pre-synaptic partner neuron.

Rationale for Aim 2

The goal of this aim is to develop techniques to capture precise transcriptomic information about each presynaptic afferent mapped to their precise dendritic targeting. Since cortical neurons can receive inputs from over 1000 presynaptic neurons we needed a technique that was capable of resolving this many distinct cells. Using multiplexed color combinations (such as brainbow (25)) can greatly increase the number of distinct objects, however these methods are still limited to multiple dozens, perhaps up to hundreds of distinct colors (25, 26) which still falls well short of our needs. Thus we turned to RNA barcoding techniques that can easily handle thousands of distinct labels (13, 14).

We have chosen to combine barcoding based connectomics with a monosynaptic rabies (31) in order to limit the total number of barcodes necessary, to make our signal more sparse so individual boutons are more easily separable, and to help ensure we acquire the identity of nearly all presynaptic neurons, rather than those constrained to a certain region. This technique has been shown to target as high as 80% of all presynaptic neurons (27)

One of the issues with second generation, non-toxic double deleted rabies is that presynaptic neurons only receive a very small amount of transcribable genetic material, resulting in very low presynaptic expression levels of the delivered construct (28). This is ideal for cre-dependent labels because only a small amount of cre (potentially a single protein) is necessary to flip the reporter gene which is then be continually transcribed (28, 32). However to our knowledge, there is no cre dependent mouse line that could express unique barcodes in each expressing neuron. Rather than using monosynaptic rabies to express the barcodes directly, we chose to use it to express the avian receptor TVA in all presynaptic neurons.

These neurons can then be targeted by a second virus to deliver unique barcodes, a florescent label, and a modified presynaptic protein MAPP-nlambda (29). The MAPP protein is necessary to ensure that the RNA barcodes are trafficked to the synapses when they normally would remain in the soma (29). We chose to use a retrograde transfection strategy for the second virus so that it can be easily injected near the starter neuron, and retrogradely transported to somata throughout the brain. The florescent protein, while not strictly necessary is useful so that the presynaptic somata can subsequently be selected using FACS sorting for single cell transcriptomics (30).

We will have to determine which virus is most appropriate to use - current barcoding techniques rely on Sindbis virus to obtain rabid and high expression of the barcodes and trafficking protein (29, 33), however we do not know of a TVA dependent variant of Sindbis virus, and the retrograde transport efficacy of Sindbis is unclear. Rabies seems a likely candidate since it has efficient retrograde transport, and TVA dependent variants already exist (34). However, it would be necessary to integrate the unique barcodes and the MAPP protein into the rabies genome. Finally lentivirus also exists in TVA dependent variant (34), and has been modified for efficient retrograde transport (35), with the obvious issue being that lentivirus is a DNA based virus instead of RNA based that will require additional accommodations. We will first attempt to work with rabies to generate the necessary barcode library as the path of least resistance. If that proves difficult we will turn to other routes.

Spatial transcriptomics is typically not suitable for barcode based connectomics because it can resolve a limited number of genes that is far beneath the number of barcodes (14). However, we find spatial transcriptomics appealing because it will allow us to obtain cell type information about local presynaptic neurons that are contained in the volume of interest. Two aspects of our method make it compatible with spatial transcriptomics - out focus on a single neuron and its ~10^3 presynaptic partners limits the number of necessary barcodes to that resolvable by spatial transcriptomics. Furthermore, by performing single cell transcriptomics on the presynaptic cells we can identify barcodes of interest and choose a primer library to selectively identify these barcodes (36).

Experimental Approach for Aim 2

First, we will use second generation, non-toxic double deletion rabies to infect all presynaptic cells for a single postsynaptic target neuron. In brief, in a TVA-Cre mouse line (37) single cell in-vivo electroporation is used to introduce genetic material to the target neuron (31). We will electroporate plasmids for BFP as a structural marker, an avian virus receptor TVA and the rabies glycolprotein G. 1-3 days later the area will be targeted with the double deletion rabies expressing cre(31). As described in (31), this allows for monosynaptic retrograde infection of the presynaptic neurons and minimal expression of cre. Presence of cre will lead to cure dependent expression of TVA which is then trafficked throughout the cell membrane.

A second TVA dependent viral injection at the location of the starter cell will then selectively infect the presynaptic afferent fibers. Retrograde transport to the soma will result in expression of 3 proteins - a different colored structural marker (GFP), one or multiple unique barcodes, and the MAPP protein (as in 14). After allowing sufficient time for expression and trafficking of the barcodes mice will be euthanized and tissue prepared for subsequent analysis. Slices containing distal afferent regions of interest (thalamus, prefrontal cortex, distal portions of higher visual cortex (outside the target cells dendritic arbor) auditory cortex, somatasensory cortex) will be blunt dissected to isolate approximate spatial location for presynaptic neurons, dissociated into single cells, and FACS sorted to select only the infected presynaptic neurons. Single cell transcriptomics will be used to recover the transcriptomic profile of the presynaptic neurons, as well as their associated RNA barcodes. Determining which barcodes were successfully expressed will allow us to select the primer library for high resolution spatial transcriptomics (38) on the slices containing the starter neuron to identify which synapses are associated with each barcode.

Expected Results and Interpretation for Aim 2

Our expected results are a one to one mapping between each presynaptic neurons transcriptomic profile and its dendritic target location on an individual post synaptic target neuron.

One of the primary results from this subset of experiments will be to confirm our results from Aim 1 with increased resolution. The presynaptic transcriptomic profile contains much more detailed cell type information than what was available in Aim2, and clustering these profiles or aligning them to existing datasets will allow us to assess smaller more specific subgroups for patterns and trends in their corresponding dendritic targets. Examples of some of the questions that could be answered are: are inputs from prefrontal cortex concentrated

on specific neurons in visual cortex or randomly distributed? Do synapses from prefrontal cortex cluster with eachother on certain dendritic segments or are they distributed across the dendritic arbor? Do certain types of local excitatory synapses cluster more with each other than would be expected by chance? Which synapses tend to be the most proximal and which tend to be the most distal?

Even this limited initial application of the technique may prove useful for constraining both the proportion and location of synaptic connections between cell types, depending on how strong the biological trends are. This proof of concept will pave the way to using this technique to build up a library of single cell “synaptomes” that will help validate the results observed in both EM and paired patch datasets, with additional insights into the proportion, variability and location of both local and distal inputs.

Potential Pitfalls for Aim 2

One major pitfall will be the ability to express barcodes in the presynaptic population. As discussed in the “approach” section, there are a number of viral options to pursue. If none of these are successful, or prove too intensive for preliminary results we will take a simpler approach. We can use the initial round of monosynaptic rabies to express GFP in the presynaptic population instead of TVA. Then we can infect many cells with the barcodes using already established Sindbis viral tools. This may require multiple injections at different locations, and will undoubtedly miss some of the presynaptic neurons. We then can still FACS sort to identify the relevant barcodes for subsequent spatial transcriptomics. Although some neurons will be missed, this is a sure fire way to deliver barcodes in order to validate the rest of the technique.

Another pitfall is that insufficient co-localization between barcodes and the postsynaptic neuron could make the dendritic target ambiguous. We doubt that this will be the case, but if it is we will develop analytical methods to predict the most likely dendritic targets based on the precise locations all presynaptic axons, minimizing the amount of missing axon necessary to explain the observations.

It may be difficult to image the entire dendritic arbor using spatial transcriptomics. If this is the case we will focus on single dendritic branches, until spatial transcriptomic techniques develop sufficiently.

If spatial transcriptomics with selected appropriate primers doesn’t work, we can also use the in-situ sequencing technique that has already been validated (14). This should still allow us to identify the dendritic target of both distant and local afferents, but will not provide transcriptomic information for local afferents within the sequenced tissue.

Finally, if FACS sorting loses too many of our presynaptic cells we would be forced to sequence entire presynaptic populations to pick out the barcoded afferents which is not ideal but clearly feasible, especially as technologies rapidly improve to easily and cheaply sequence larger quantities of single cells.

Alternative Approaches for Aim 2

An alternative approach would be to use the single cell targeted rabies to deliver cre to presynaptic cells in a brainbow mouse (25), and then trace the afferent axons back to their origins. This has 3 main disadvantages. First, tracing is not fully automated, and while tracing the full axon would not be necessary this still requires significant labor for each postsynaptic target neuron (even large projectors trace only a few thousand axons (12). Second, because we are specifically selecting axons that densely innervate a specific region (i.e. the area around the target cell) our subpopulation may not be sparse enough for even brainbow to disentangle all axons. Third, axon tracing techniques are not compatible with single cell transcriptomics to get the transcriptomic identity of all the presynaptic neurons.

Another alternative approach would involve trying to use functional in-vivo imaging to try to connect activity at the boutons to activity in the presynaptic somata. One can imagine using optogenetics to silence specific presynaptic populations while observing activity in the boutons/spines of the target neuron. This approach is fraught with complications including methods development for targeted cortex wide light delivery, limitations of current in-vivo microscopy to simultaneously monitor all the relevant synapses, ambiguity of calcium dynamics, and potential network effects from optogenetics. While a best case scenario of this will yield higher resolution than Aim2, ultimately it is constrained to a many to many mapping without detailed transcriptomic profiles which, in our eyes, is not worth pursuing given the complications.

References

1. Thomas, J. H. (1994). The mind of a worm. *Science (New York, N.Y.)*, *264*(5166), 1698–1699. https://doi.org/10.1126/SCIENCE.7911601

2. Cook, S. J., Jarrell, T. A., Brittin, C. A., Wang, Y., Bloniarz, A. E., Yakovlev, M. A., Nguyen, K. C. Q., Tang, L. T. H., Bayer, E. A., Duerr, J. S., Bülow, H. E., Hobert, O., Hall, D. H., & Emmons, S. W. (2019). Whole-animal connectomes of both Caenorhabditis elegans sexes. *Nature 2019 571:7763*, *571*(7763), 63–71. https://doi.org/10.1038/s41586-019-1352-7

3. Zheng, Z., Lauritzen, J. S., Perlman, E., Robinson, C. G., Nichols, M., Milkie, D., Torrens, O., Price, J., Fisher, C. B., Sharifi, N., Calle-Schuler, S. A., Kmecova, L., Ali, I. J., Karsh, B., Trautman, E. T., Bogovic, J. A., Hanslovsky, P., Jefferis, G. S. X. E., Kazhdan, M., … Bock, D. D. (2018). A Complete Electron Microscopy Volume of the Brain of Adult Drosophila melanogaster. *Cell*, *174*(3), 730. https://doi.org/10.1016/J.CELL.2018.06.019

4. Scheffer, L. K., Xu, C. S., Januszewski, M., Lu, Z., Takemura, S. Y., Hayworth, K. J., Huang, G. B., Shinomiya, K., Maitin-Shepard, J., Berg, S., Clements, J., Hubbard, P. M., Katz, W. T., Umayam, L., Zhao, T., Ackerman, D., Blakely, T., Bogovic, J., Dolafi, T., … Plaza, S. M. (2020). A connectome and analysis of the adult drosophila central brain. *ELife*, *9*, 1–74. https://doi.org/10.7554/ELIFE.57443

5. Lafourcade, M., Goes, M.-S. H. van der, Vardalaki, D., Brown, N. J., Voigts, J., Yun, D. H., Kim, M. E., Ku, T., & Harnett, M. T. (2022). Differential dendritic integration of long-range inputs in association cortex via subcellular changes in synaptic AMPA-to-NMDA receptor ratio. *Neuron*, *110*(9), 1532-1546.e4. https://doi.org/10.1016/J.NEURON.2022.01.025

6. Billeh, Y. N., Cai, B., Gratiy, S. L., Dai, K., Iyer, R., Gouwens, N. W., Abbasi-Asl, R., Jia, X., Siegle, J. H., Olsen, S. R., Koch, C., Mihalas, S., & Arkhipov, A. (2020). Systematic Integration of Structural and Functional Data into Multi-scale Models of Mouse Primary Visual Cortex. *Neuron*, *106*(3), 388-403.e18. https://doi.org/10.1016/J.NEURON.2020.01.040

7. Thomson, A. M., & Lamy, C. (2007). Functional Maps of Neocortical Local Circuitry. *Frontiers in Neuroscience*, *1*(1), 19–42. https://doi.org/10.3389/NEURO.01.1.1.002.2007/BIBTEX

8. Chistiakova, M., Bannon, N. M., Bazhenov, M., & Volgushev, M. (2014). Heterosynaptic Plasticity: Multiple Mechanisms and Multiple Roles. *The Neuroscientist: A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, *20*(5), 483. https://doi.org/10.1177/1073858414529829

9. Kerlin, A., Boaz, M., Flickinger, D., Maclennan, B. J., Dean, M. B., Davis, C., Spruston, N., & Svoboda, K. (2019). Functional clustering of dendritic activity during decision-making. *ELife*, *8*. https://doi.org/10.7554/ELIFE.46966

10. Gidon, A., Zolnik, T. A., Fidzinski, P., Bolduan, F., Papoutsi, A., Poirazi, P., Holtkamp, M., Vida, I., & Larkum, M. E. (2020). Dendritic action potentials and computation in human layer 2/3 cortical neurons. *Science*, *367*(6473), 83–87. https://doi.org/10.1126/SCIENCE.AAX6239/SUPPL_FILE/AAX6239_GIDON_SM.PDF

11. Harris, J. A., Mihalas, S., Hirokawa, K. E., Whitesell, J. D., Choi, H., Bernard, A., Bohn, P., Caldejon, S., Casal, L., Cho, A., Feiner, A., Feng, D., Gaudreault, N., Gerfen, C. R., Graddis, N., Groblewski, P. A.,

Henry, A. M., Ho, A., Howard, R., … Zeng, H. (2019). Hierarchical organization of cortical and thalamic connectivity. *Nature 2019 575:7781*, *575*(7781), 195–202. https://doi.org/10.1038/s41586-019-1716-z

12. Gao, L., Liu, S., Gou, L., Hu, Y., Liu, Y., Deng, L., Ma, D., Wang, H., Yang, Q., Chen, Z., Liu, D., Qiu, S., Wang, X., Wang, D., Wang, X., Ren, B., Liu, Q., Chen, T., Shi, X., … Yan, J. (2022). Single-neuron projectome of mouse prefrontal cortex. *Nature Neuroscience 2022 25:4*, *25*(4), 515–529. https://doi.org/10.1038/s41593-022-01041-5

13. Peikon, I. D., Kebschull, J. M., Vagin, V. v., Ravens, D. I., Sun, Y. C., Brouzes, E., Corrêa, I. R., Bressan, D., & Zador, A. M. (2017). Using high-throughput barcode sequencing to efficiently map connectomes. *Nucleic Acids Research*, *45*(12), e115. https://doi.org/10.1093/NAR/GKX292

14. Chen, X., Sun, Y. C., Zhan, H., Kebschull, J. M., Fischer, S., Matho, K., Huang, Z. J., Gillis, J., & Zador, A. M. (2019). High-Throughput Mapping of Long-Range Neuronal Projection Using In Situ Sequencing. *Cell*, *179*(3), 772-786.e19. https://doi.org/10.1016/J.CELL.2019.09.023

15. Petreanu, L., Mao, T., Sternson, S. M., & Svoboda, K. (2009). The subcellular organization of neocortical excitatory connections. *Nature*, *457*(7233), 1142. https://doi.org/10.1038/NATURE07709

16. Seeman, S. C., Campagnola, L., Davoudian, P. A., Hoggarth, A., Hage, T. A., Bosma-Moody, A., Baker, C. A., Lee, J. H., Mihalas, S., Teeter, C., Ko, A. L., Ojemann, J. G., Gwinn, R. P., Silbergeld, D. L., Cobbs, C., Phillips, J., Lein, E., Murphy, G., Koch, C., … Jarsky, T. (2018). Sparse recurrent excitatory connectivity in the microcircuit of the adult mouse and human cortex. *ELife*, *7*. https://doi.org/10.7554/ELIFE.37349

17. Abbott, L. F., Bock, D. D., Callaway, E. M., Denk, W., Dulac, C., Fairhall, A. L., Fiete, I., Harris, K. M., Helmstaedter, M., Jain, V., Kasthuri, N., LeCun, Y., Lichtman, J. W., Littlewood, P. B., Luo, L., Maunsell, J. H. R., Reid, R. C., Rosen, B. R., Rubin, G. M., … van Essen, D. C. (2020). The Mind of a Mouse. *Cell*, *182*(6), 1372–1376. https://doi.org/10.1016/J.CELL.2020.08.010

18. Suzuki, T., Morimoto, N., Akaike, A., & Osakada, F. (2020). Multiplex Neural Circuit Tracing With G-Deleted Rabies Viral Vectors. *Frontiers in Neural Circuits*, *13*, 77. https://doi.org/10.3389/FNCIR.2019.00077/BIBTEX

19. *IARPA - MICrONS*. (n.d.). Retrieved May 19, 2022, from https://www.iarpa.gov/index.php/research-programs/microns

20. Tasic, B., Yao, Z., Graybuck, L. T., Smith, K. A., Nguyen, T. N., Bertagnolli, D., Goldy, J., Garren, E., Economo, M. N., Viswanathan, S., Penn, O., Bakken, T., Menon, V., Miller, J., Fong, O., Hirokawa, K. E., Lathia, K., Rimorin, C., Tieu, M., … Zeng, H. (2018). Shared and distinct transcriptomic cell types across neocortical areas. *Nature 2018 563:7729*, *563*(7729), 72–78. https://doi.org/10.1038/s41586-018-0654-5

21. Manuel, M. N., Mi, D., Masonand, J. O., & Price, D. J. (2015). Regulation of cerebral cortical neurogenesis by the Pax6 transcription factor. *Frontiers in Cellular Neuroscience*, *9*. https://doi.org/10.3389/FNCEL.2015.00070

22. Vue, T. Y., Lee, M., Tan, Y. E., Werkhoven, Z., Wang, L., & Nakagawa, Y. (2013). Thalamic Control of Neocortical Area Formation in Mice. *Journal of Neuroscience*, *33*(19), 8442–8453. https://doi.org/10.1523/JNEUROSCI.5786-12.2013

23. Villa, K. L., Berry, K. P., Subramanian, J., Cha, J. W., Oh, W. C., Kwon, H. B., Kubota, Y., So, P. T. C., & Nedivi, E. (2016). Inhibitory Synapses Are Repeatedly Assembled and Removed at Persistent Sites In Vivo. *Neuron*, *89*(4), 756–769. https://doi.org/10.1016/J.NEURON.2016.01.010

24. Morimoto, M. M., Uchishiba, E., & Saleem, A. B. (2021). Organization of feedback projections to mouse primary visual cortex. *IScience*, *24*(5), 102450. https://doi.org/10.1016/J.ISCI.2021.102450

25. Weissman, T. A., & Pan, Y. A. (2015). Brainbow: New Resources and Emerging Biological Applications for Multicolor Genetic Labeling and Analysis. *Genetics*, *199*(2), 293. https://doi.org/10.1534/GENETICS.114.172510

26. Loulier, K., Barry, R., Mahou, P., Franc, Y. le, Supatto, W., Matho, K. S., Ieng, S., Fouquet, S., Dupin, E., Benosman, R., Chédotal, A., Beaurepaire, E., Morin, X., & Livet, J. (2014). Multiplex Cell and Lineage Tracking with Combinatorial Labels. *Neuron*, *81*(3), 505–520. https://doi.org/10.1016/J.NEURON.2013.12.016

27. Kim, E. J., Jacobs, M. W., Ito-Cole, T., & Callaway, E. M. (2016). Improved Monosynaptic Neural Circuit Tracing Using Engineered Rabies Virus Glycoproteins. *Cell Reports*, *15*(4), 692. https://doi.org/10.1016/J.CELREP.2016.03.067

28. Chatterjee, S., Sullivan, H. A., MacLennan, B. J., Xu, R., Hou, Y. Y., Lavin, T. K., Lea, N. E., Michalski, J. E., Babcock, K. R., Dietrich, S., Matthews, G. A., Beyeler, A., Calhoon, G. G., Glober, G., Whitesell, J. D., Yao, S., Cetin, A., Harris, J. A., Zeng, H., … Wickersham, I. R. (2018). Nontoxic, double-deletion-mutant rabies viral vectors for retrograde targeting of projection neurons. *Nature Neuroscience 2018 21:4*, *21*(4), 638–646. https://doi.org/10.1038/s41593-018-0091-7

29. Kebschull, J. M., Garcia da Silva, P., Reid, A. P., Peikon, I. D., Albeanu, D. F., & Zador, A. M. (2016). High-Throughput Mapping of Single-Neuron Projections by Sequencing of Barcoded RNA. *Neuron*, *91*(5), 975–987. https://doi.org/10.1016/J.NEURON.2016.07.036

30. Liao, X., Makris, M., & Luo, X. M. (2016). Fluorescence-activated Cell Sorting for Purification of Plasmacytoid Dendritic Cells from the Mouse Bone Marrow. *Journal of Visualized Experiments: JoVE*, *2016*(117), 54641. https://doi.org/10.3791/54641

31. Marshel, J. H., Mori, T., Nielsen, K. J., & Callaway, E. M. (2010). Targeting Single Neuronal Networks for Gene Expression and Cell Labeling In Vivo. *Neuron*, *67*(4), 562–574. https://doi.org/10.1016/J.NEURON.2010.08.001

32. Jin, L., Sullivan, H. A., Zhu, M., Lavin, T. K., Matsuyama, M., Lea, N. E., Xu, R., Hou, Y., Rutigliani, L., Pruner, M., Babcock, K. R., Pak, J., Ip, K., Hu, M., Daigle, T. L., Zeng, H., Sur, M., & Wickersham, I. R. (2021). Long-term labeling and imaging of synaptically-connected neuronal networks in vivo using nontoxic, double-deletion-mutant rabies viruses. *BioRxiv*, 2021.12.04.471186. https://doi.org/10.1101/2021.12.04.471186

33. Ehrengruber, M. U. (2002). Alphaviral gene transfer in neurobiology. *Brain Research Bulletin*, *59*(1), 13–22. https://doi.org/10.1016/S0361-9230(02)00858-4

34. Sakurai, K., Zhao, S., Takatoh, J., Rodriguez, E., Lu, J., Leavitt, A. D., Fu, M., Han, B. X., & Wang, F. (2016). Capturing and Manipulating Activated Neuronal Ensembles with CANE Delineates A Hypothalamic Social Fear Circuit. *Neuron*, *92*(4), 739. https://doi.org/10.1016/J.NEURON.2016.10.015

35. Kato, S., Kobayashi, K., Inoue, K. I., Kuramochi, M., Okada, T., Yaginuma, H., Morimoto, K., Shimada, T., Takada, M., & Kobayashi, K. (2011). A lentiviral strategy for highly efficient retrograde gene transfer by pseudotyping with fusion envelope glycoprotein. *Human Gene Therapy*, *22*(2), 197–206. https://doi.org/10.1089/HUM.2009.179

36. Wang, X., Allen, W. E., Wright, M. A., Sylwestrak, E. L., Samusik, N., Vesuna, S., Evans, K., Liu, C., Ramakrishnan, C., Liu, J., Nolan, G. P., Bava, F. A., & Deisseroth, K. (2018). Three-dimensional intact-tissue sequencing of single-cell transcriptional states. *Science*, *361*(6400). https://doi.org/10.1126/SCIENCE.AAT5691/SUPPL_FILE/AAT5691_WANG_SM_TABLE-S2.XLSX

37. Beier, K. T., Samson, M. E. S., Matsuda, T., & Cepko, C. L. (2011). Conditional Expression of the TVA Receptor Allows Clonal Analysis of Descendents from Cre-expressing Progenitor Cells. *Developmental Biology*, *353*(2), 309. https://doi.org/10.1016/J.YDBIO.2011.03.004

38. Sun, Y.-C., Chen, X., Fischer, S., Lu, S., Gillis, J., & Zador, A. M. (2020). Integrating barcoded neuroanatomy with spatial transcriptional profiling reveals cadherin correlates of projections shared across the cortex. *BioRxiv*, 2020.08.25.266460. https://doi.org/10.1101/2020.08.25.266460