**RESOURCE CORE: Registration And Histology**

Brain functions arise from the coordinated activity of many brain regions which communicate by anatomically specific pathways. In this way local circuit processing by multiple far-flung areas, communicating with one another, lead in the aggregate to the processing of events and the generation of behavior. A full understanding that process requires levels of explanation at multiple levels: neural processing in any particular region, and how that region fits into brain-wide networks.

In the study of working memory, animal models are valuable because they allow both function and anatomy to be probed with high resolution. Human studies provide the clinical relevance and identify key questions and phenomena, but only in nonhuman animals can activity and connectivity be probed routinely at a cellular level.

This core facility will serve several essential functions in building a broad integrative framework for the program project. First, the facility will support the registration of observed areas in an anatomical framework that includes connectivity and functional significance. Second, the facility will support long-distance, transsynaptic tracing to identify paths of connectivity through which information is conveyed between distant brain regions that are involved in evidence accumulation. Third, the facility will organize this information in a relational database that links all experiments done by the collaborating laboratories, and in a format that can be coordinated with in the entire neuroscience research community. Taken together, these functions are essential in placing recorded and perturbed neural activity into a brainwide anatomical context.

The Registration and Histology resource core will rely on a combination of classical and newer technologies, which will be organized according to the following three major goals:

**Goal 1: Register brains and automate cell recognition.** Using methods for nonlinear stretching of images of brain sections, all recorded and perturbed brain regions in this U19 project will be placed in anatomical context. Automated cell recognition methods will be used to survey both directly imaged regions and indirectly connected regions, and classify neurons and other objects of interest. These methods will be applied to images gathered using light-sheet microscopy and two-photon tomography.

**Goal 2: Identify tracts and pathways using virus-based methods.** We will build the capability to trace pathways using viruses whose tracing properties are directional and controlled. Using a viral core facility that is already in house, we will use viruses that are retrograde single-step (weakened rabies, retro-AAV) and multi-step (pseudorabies) as well as anterograde (herpes simplex virus strain H129). These viruses will drive the expression of labels that can be visualized and quantified.

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**Goal 3: Build anatomical datasets and integrate them with functional in vivo experiments.** Light-sheet microscopic capabilities will be combined with clearing methods such as iDISCO and enhanced to allow rapid-throughput scanning of entire brains. Automated cell counting methods will be used to quantify the strength and location of pathways that are connected to the site of recording or perturbation.

As technologies for registration and tracing advance over time, this core facility will keep up by acquiring the new technology. The capabilities of this Core require the services of two full-time personnel, one to perform labeling and imaging, and one to perform alignment, registration, and database construction and management.

The ultimate service of this Core will be to reveal where the one brain region's activity is projected in distal sites, as well as which cell types and distal sites provide influence that impacts the region. This service will be used on a routine basis in experiments done by all the collaborators.

**Background and Significance**

Our initial approach to locating brain regions for recording and perturbation has been to use stereotaxic coordinates and functional optical imaging. This approach does not take full advantage of the tremendous effort that has been put into mapping the connectivity of brain regions. The combination of the Allen Brain Atlas and long-distance connectivity mapping has provided a recent expansion in the amount of detailed anatomical information available for registering our physiological data.

We will use classical and modern methods of characterizing regions and pathways to place our obsevations in a brain-wide functional context. Consider the following example: Our observations of cerebellar requirements for evidence accumulation are based on lobule-specific perturbations and imaging. Yet it is known that the cerebellar cortex is divided not just into lobules, but parasagittal slabs of circuitry known as microzones. These microzones, which were defined first according to their alternating antigenicity for the protein zebrin, are important units of function because they tend to be associated with specific parts of the inferior olive, a principal source of instructive signals; and deep nuclei, the output of the cerebellum. The deep nuclei project in turn to specific regions of midbrain and neocortex. For these reasons, cerebellum appears to be parcellated in a manner that maps to the parcellation of neocortex. And in crus I, where we have seen reward error-related signals, one of those microzones strongly and specifically influences prefrontal cortex. It would be invaluable to know whether reward signals in cerebellum reach the prefrontal cortex.

Such orderly maps have emerged throughout the brain, and are starting to be mapped in detail. The discovery and characterization of these long-distance mappings has been hampered in the past by an inability to trace a neuron beyond its axonal or dendritic terminations. However, this difficulty has been overcome in recent years thanks to the advent of virus-based directional and transsynaptic tracers. Viruses have now entered common use that are specifically taken up by axon terminals and transported retrogradely within a single neuron (retro-adeno-associated-virus, or retro-AAV), across one synapse ("Callaway" rabies virus), or across multiple synapses (pseudorabies virus, PRV); or anterogradely across multiple synapses (herpes simplex virus variant H129, or HSV-H129). These viruses are currently in use at Princeton for the purpose of identifying targets and partners of forebrain, midbrain, and cerebellar regions of interest.

A second wave of mapping-enabling technology consists of microscopies that can scan large volumes of brain tissue without need for aligning sections to recover the volume of interest. One such technology is light-sheet microscopy, which can image fluorescence from tissue samples that rendered near-transparent by processes such as iDISCO. Another technology is fluorescence two-photon tomography, in which successive block faces are imaged after removal of one section at a time, thus eliminating the alignment problem and allowing automated volumetric recovery. In both cases, a roughly-aligned whole brain volume can be obtained in under a day, several orders of magnitude faster than sectioning-based techniques.

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**Figure X, Registration procedure.** Original stacks of cleared brain tissue (*left)* will be brought into register with an Allen Brain Atlas reference frame through a series of rigid, affine, and B-spline stretches. The resulting alignment (*right*) has a median registration error of less than 0.2 mm.

The combination of viral tracing and high-throughput volume microscopy creates a considerable data analysis challenge. This analysis challenge can be met by methods for large-scale cell recognition, tracing, and data handling, all of which have been made possible by advances in desktop computing and machine vision [Sebastian, correct usage?].

We propose to create a Core Facility at Princeton that spans all three of these domains. Our facility will use viral tracing, volume microscopy, and automated data analysis to map connectivity in every brain region that we image or perturb. These powerful anatomical approaches will add enormous depth to the interpretation of our in vivo experiments.

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**Figure XX. Cell recognition and placement in aligned brain.** *Left*, a current approach being tested in which training examples are used to train a convolutional neural net to classify putative neurons, which are then compared with human-annotated images. *Right*, placement of detected cells in an aligned whole brain.

**Specific Goals**

**Goal 1: Register brains and automate cell recognition.** We are building a pipeline to register image stacks to a common reference frame. In all experiments, a key step is to register individual cleared brains to Allen Brain Institute coordinates. This step will be carried out by rigid and affine stretching, followed by B-spline nonuniform stretching. The result of these steps is a transformation that is accurate to within ~0.2 mm (**Figure X**). To obtain reference data for registration, a broadly available signal such as autofluorescence will be taken during the acquisition. This volume will be used to register the experimenter's sample with a common coordinate system, the Allen Brain's mouse reference atlas. Custom software incorporated into our analysis pipeline leverages alignment software built on top of the National Library of Medicine's Insight Segmentation and Registration Toolkit (ITK). This allows for unbiased determination of anatomical location within each brain.

We are also addressing the challenge of automated cell recognition. Large-scale cell detection from volumetric data carries challenges that are reminiscent of other datasets such as those arising from cellular calcium imaging. We are currently leveraging convolutional neural net (CNN; **Figure XX**) software from the Seung laboratory [REF [https://arxiv.org/abs/1606.07372](https://owa.princeton.edu/owa/redir.aspx?C=fy_D3SLVyN1GOKV26GDuNM_696R9dbogp0vM0LGp6MeI_OFv_kfUCA..&URL=https%3a%2f%2farxiv.org%2fabs%2f1606.07372)] for calcium imaging by treating the third "z" dimension in a manner analogous to time. This method may be refined using nonnegative matrix factorization methods (collaboration with Eftychios Pnevmatikakis and Andrea Giovannucci, Flatiron Institute/Simons Foundation) As an alternative, we are pursuing an iterative cell-recognition/border-erosion method in collaboration with Partha Mitra (Cold Spring Harbor Laboratory). In both cases our goal is to attain 95 percent sensitivity (i.e. true positive rate) and specificity (i.e. positives as a total of true plus false positives) relative to ground truth as defined by human annotators.

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**Figure XXX, Anterograde tracing of posterior cerebellum to thalamic nuclei.** *Left*, injection of HSV-H129 into cerebellum (green, bottom) leads to successive expression in deep nuclei and thalamus. *Right*, darker rectangles indicate stronger levels of expression in specific clusters of thalamic nuclei (outlined in red). Of particular interest is dorsomedial thalamus, which projects to mPFC and other associational regions).

**Goal 2: Identify tracts and pathways using virus-based methods.** We will establish a facility that allows the tracing of pathways from sites of imaging or perturbation. A necessary step in building this capacity is the recovery of virus-specific signal from cells of interest.

In the case of light-sheet microscopy, brains will be cleared using the iDISCO method, After transcardial perfusion and fixation, animal tissue will be permeabilized through a series of methanol dehydration steps, bleaching, and exposure to nonpolar solvents. Brains will then then passed through a typical immunostaining protocol of blocking, primary staining, and secondary staining, all steps occurring for longer-than-usual time periods because of the longer diffusion time into specimens. Next, using an organic based solvent approach the brain will be delipidized leaving a cleared brain of mostly crosslinked proteins. This approach is superior to aqueous based solutions which we have found produce less consistent clearing, and preserve less well the morphology of the tissue.

We have successfully combined iDISCO clearing with pseudorabies virus (PRV) and anterogradely transported viruses (herpes simplex virus variant H129, or HSV-H129). In preliminary results, we have begun to create a map of posterior cerebellum to thalamus with nucleus-level resolution (**Figure XXX**). This map identifies thalamic nuclei that project to regions with high potential relevance to evidence accumulation (PPC, PFC, M1, M2, and VTA). These preliminary results demonstrate the power of transsynaptic tracing to contribute to an integrative model for evidence accumulation.

**Goal 3: Build anatomical datasets and integrate them with functional in vivo experiments.**

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**Figure XXXX, Alignment of two-photon imaged regions with cleared light-sheet volumetric reconstructions.**

To place calcium imaging experiments into full anatomical context, imaged regions will be registered with cleared brains. The pattern of fluorescent cells and artificially-placed fiduciary marks will be used to align two-photon image stacks with cleared brain tissue (**Figure XXXX**). This alignment will be a routine part of every imaging experiment. In some experiments, at the end of in vivo imaging, tracing virus will be injected at the imaging site to obtain detailed projection patterns at distal sites.

Brains will be imaged in up to four spectral channels using a light-sheet microscope (LaVision BioTec). This imaging approach optimizes speed, allowing for high throughput acquisition, and allows whole-brain imaging at a resolution of 1.63 µm/pixel in X-Y directions and 5 µm/pixel in the Z direction, without the need for physical sectioning. A tile scan at 4x is over 150,000 images. In experiments requiring increased resolution, volumes will be acquired using fluorescence two-photon tomography. Custom python code will be used acquisition, stitching and image blending into a single volume. The data will then be compressed for long-term storage.

**Resource Strategy**

The Registration and Histology core facility will have two full-time staff and be housed in the Bezos Center for Systems Neuroscience. The staff members will have the following duties:

1) **Technician for staining, clearing, and imaging.** This wet-bench technician will process brains for clearing and light-sheet microscopy, or serial two-photon tomography (STP). He/she will also perform imaging.

2) **Data analyst.** This data analyst will align images, register brains with the Allen Brain Atlas, perform initial data analysis, and establish and maintain the anatomical component of the project database. He/she will manage data storage and provide advice to postdocs and students on data storage and management. These functions will be coordinated with the data core [insert NAME OF THAT CORE HERE].

**Example Projects**

This core facility will provide a uniform source of high-quality reconstruction for all members of the U19 collaboration. This will result in cost-effectiveness and increased efficiency for anatomical and histological work.

**Example project 1: tracing of in vivo imaging experimental animals**. Any time an in vivo imaging experiment is done, tracer will be injected as a routine final step in the live animal. Projections to and from an imaged site will be characterized in anterograde (HSV-H129) or retrograde (retro-AAV, PRV) directions. The PFC, ACC, and parietal cortex are polysynaptic target regions of ventral tegmental and cerebellum and thus will be injected with the retrograde viral tracer PRV.

**Example project 2: Long-distance polysynaptic mapping between neocortex and cerebellum.** Most existing anatomical knowledge of long-distance projections focuses on the direct terminations of axons. Virus-based methods will allow the identification of targets which are joined by disynaptic or longer paths of connectivity. Prominent examples include neocortex, striatum, and cerebellum, which are joined in an orderly manner that has been difficult to trace because of the presence of intermediate sites such as the ventral tegmental area and the deep cerebellar nuclei.

We will start with two major brain divisions where we expect spatial resolution to be good, the neocortex and cerebellum. We will test the hypothesis that cerebellar lobules VI and VII, and crus I and II, are polysynaptically connected with specific nonmotor forebrain targets through thalamic and VTA intermediates. Cerebellar perinatal damage leads to a decrease in contralateral PFC volume relative to the ipsilateral PFC by the age of 2 (32, 46). Furthermore, a neuroimaging meta-analysis identified lobules VI, VII and Crus I to be associated with language, working memory, spatial tasks, executive function and emotional functioning in humans (38). Finally, several studies have indicated that lobules VI, VII and crus I and II are associated with nonmotor function (39-45). Anterograde tracing from these lobules and crura will be done using HSV-H129.

**Significance.** This core facility's services will be of extremely high significance. To date, nearly all studies of systems neuroscience function have encompassed imaging of one or a few small regions of tissue, on a local basis. This facility will add the capability to identify the precise region imaged in functional terms, both locally and in relation to the brainwide set of regions that are to be studied in this U19 proposal. Neocortex, striatum, cerebellum, and other subcortical areas are joined by precisely mapped relationships, and this Core will provide detailed information on the connectivity between the parts of these brain divisions which support evidence accumulation.

**Innovation.** This core will be highly innovative. It will use advanced tissue-clearing technologies on a brainwide basis, a scale that requires automated cell identification and counting. In addition to these methods, it will be necessary to trace connectivity with directional and pathway-specific manner. The technologies to be used (iDISCO, cell recognition, viral tracing) are innovative both individually and in combination.

In addition to the high innovation of this project, our approach to data archival will place the information from this collaboration in a context that is useful to neuroscientists worldwide. Our data will be tagged as to location, as well as to connectivity to distant brain regions. The functional connectivity BLAH BLAH BLAH

**Approach.** These technologies rely on close communication with leaders in the field. We have light-sheet microscopy in-house. We are also in touch with innovators who either invented or polished methods to high reliability, such as iDISCO's Nicolas Renier (formerly with Marc Tessier-Lavigne, now at XXXX Paris); automated cell recognition and data analysis (Partha Mitra, Cold Spring Harbor, and S. Seung, co-PI on this U19); virus construction (Lynn Enquist at Princeton, and various collaborators at other institutions).

**Budget**

**Staff.** *(two salaries totaling $120,000 DC per year, plus benefits rate)*

**Equipment.** The Princeton Neuroscience Institute already has a LaVision Biotec light-sheet microscope. This microscope will be supplemented with an objective to allow wider field-of-view image acquisition. In addition, we will acquire a custom-built serial two-photon tomographic microscope based on a recent design at the Howard Hughes Medical Institute (Janelia Farm). Other equipment to be obtained includes nutators, incubators, and refrigerators, and a standard histology benchtop suite for sample processing and handling.

*(total $80,000 – if we really get the microscope, add $300,000 for that)*

**Supplies.** Reagents are needed for approximately 10 users, at ~100 brains per year. The primary expense is antibodies, whose cost varies greatly depending on the particular study and cell type(s) to be recovered. Additional costs include clearing reagents and other chemicals, as well as portable data storage.

*($30,000 DC per year)*

**Computational Resources.** For dedicated use in data analysis, the Registration and Histology Core will require two computers equipped with graphic acceleration and computational capabilities for [GIVE SPECIFICATIONS HERE]. Estimated storage requirements are 1 PB based on an acquisition rate of 2 TB/brain for the period of the grant. Beyond that time, per-brain costs may decrease as storage becomes less expensive. Storage costs will be coordinated with the data core [insert NAME OF THAT CORE HERE].

*(not sure…$20,000?)*

**Literature Cited (pardon numbering – will fix once we are near the end on this one)**

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**6 pages.** as a reference manager we’ll be using the Google Docs add-on [Paperpile](https://paperpile.com).

Instructions from [RFA](https://grants.nih.gov/grants/guide/rfa-files/RFA-NS-17-018.html#_Section_IV._Application_1):

**Specific Aims:**  Provide a concise description of the goals of the Resource Core. Explain how the Resource Core will contribute to individual Research Projects.

**Resource Strategy:** A Resource Core can be a laboratory, a facility, a service, or other shared resource that supports at least two Research Project components. Descriptions for each Resource Core should include a brief overview and a description of the services and resources to be provided to other components. This section should address how the Resource Core will contribute to the overall goals of the program as well as which research projects will be supported by the Resource Core and the manner in which that support will be rendered. The description of each Resource Core should clearly indicate the facilities, resources, services, and professional skills that the facility will provide. Issues to be addressed can include: quality control, special expertise, cost effectiveness, and increased efficiency.

*Significance:* Describe overall goals and the impact of the science proposed in relation to the state of the field. This section should also explain the contribution of the core to the overall goals of the program and how the component will interact with and benefit from other components.

*Innovation:* Describe the unique and innovative contributions that will be made by this component. Explain how these contributions will be made possible by team synergy beyond the otherwise independent research projects.

*Approach:* Describe and offer evidence for the feasibility of the proposed experiments, the advantages of any new methodologies, the potential pitfalls and alternative approaches for the project and how these might impact overall progress.