

## Video Article

# Multi-layer Cortical Ca<sup>2+</sup> Imaging in Freely Moving Mice with Prism Probes and Miniaturized Fluorescence Microscopy

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## Abstract

*In vivo* circuit and cellular level functional imaging is a critical tool for understanding the brain in action. High resolution imaging of mouse cortical neurons with two-photon microscopy has provided unique insights into cortical structure, function and plasticity. However, these studies are limited to head fixed animals, greatly reducing the behavioral complexity available for study. In this paper, we describe a procedure for performing chronic fluorescence microscopy with cellular-resolution across multiple cortical layers in freely behaving mice. We used an integrated miniaturized fluorescence microscope paired with an implanted prism probe to simultaneously visualize and record the calcium dynamics of hundreds of neurons across multiple layers of the somatosensory cortex as the mouse engaged in a novel object exploration task, over several days. This technique can be adapted to other brain regions in different animal species for other behavioral paradigms.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/55579/>

## Introduction

The cortex is an essential player in many complex mental and behavioral functions, from attention, sensory perception and top-down cognitive control<sup>1,2,3</sup> to motivational, reward, and addiction pathways<sup>4,5</sup>. Understanding the computational processes that underlie its function is an important goal to drive a better clinical understanding of many mental and behavioral disorders.

Many current theories of psychiatric disease center around the idea that cortical neural circuit dysfunction or discoordination may underlie cognitive and behavioral abnormalities that are the hallmarks of conditions such as schizophrenia<sup>6</sup>, autism<sup>7</sup> or obsessive-compulsive disorder<sup>8</sup>. Thus, obtaining population level neural activity data from cortical circuits within the proper context of simultaneous behavioral information is of great importance, and ideally can be targeted to specific cell types for finer neural circuit dissection.

Miniaturized microscopes in conjunction with implantable gradient refractive index (GRIN) microlenses enable optical access to neuronal ensembles under freely moving conditions from a diversity of possible brain regions<sup>9,10,11,12,13</sup>, including the cortex<sup>14,15,16</sup>. Using a mobile microscopy system coupled with genetically encoded calcium indicators allows for consistent imaging of the same cellular population encompassing hundreds of neurons over days to weeks in many brain regions<sup>9</sup>, and can be genetically targeted to specific cell types using viral vector or transgenic techniques.

As the cortex is known to support different functions and connect to different brain regions depending on the location of the cells within the cortical lamina<sup>17,18,19</sup>, we are interested in obtaining simultaneous multi-layer neural activity in awake behaving subjects. Here we demonstrate how to image hundreds of fluorescently labeled neurons in freely behaving mice over days, using the miniaturized fluorescence microscope<sup>20</sup> paired with an implanted prism probe, which offers a multi-layer view of the cortex (**Figure 1**).

The prism probe used here is composed of two separate GRIN lenses: a prism and a cylindrical relay lens (**Figure 1**). The light from the microscope excites the fluorescently labeled cells located along the imaging face of the prism probe, after being reflected off the hypotenuse of the prism portion of the probe. The emitted light from the cells also reflects off the hypotenuse of the prism, is collected through the objective of the microscope and reaches the sensor in the microscope. The prism probe used in this procedure is adapted for easy use with standard stereotaxic equipment.

The miniaturized fluorescence microscope<sup>20</sup> detects action potential-evoked Ca<sup>2+</sup> transients in neuronal populations with single cell resolution, after those cells have been specifically labeled with Ca<sup>2+</sup>-sensitive genetically encoded fluorescent indicators. In this protocol, we inject Ca<sup>2+</sup> indicator encoded in a viral vector (AAV1.CaMKII.GCaMP6f.WPRE.SV40), implant a prism probe, install the microscope, then obtain multiple days of somatosensory (S1 hind limb) neural activity data from an animal exposed to novel object surfaces during free exploration (**Figure 2**).

## Protocol

Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at LifeSource Biomedical Services, NASA Ames Research Center, California.

### 1. Pre-operative Preparation

1. Sterilize the tools to be used in surgical procedures in a hot bead sterilizer and wipe the surgery area with 70% ethanol. Turn on the heating pad placed over the stereotaxic stage and maintain it at 37 °C.
2. Anesthetize the animal using isoflurane (5% for induction, and 1-2% for maintenance, 0.6-0.8 L/min O<sub>2</sub>). Check for the absence of a toe-pinch reflex to assess depth of anesthesia.
3. Mount the animal in a stereotaxic frame fitted with ear and teeth bars.
4. Apply ophthalmic ointment on the animal's eyes and cover them with a piece of dark paper to protect them from drying and intense surgical lights.
5. Subcutaneously inject the animal with ketoprofen (2.5 mg/kg) or carprofen (2.5 mg/kg)

### 2. Virus Injection Surgery

1. Trim and shave the scalp between the eyes and ears and disinfect the skin with 3 alternate swabs of 70% ethanol and betadine.
2. Expose the skull by making an incision in the scalp, starting between the eyes and extending 1.5 cm rostrocaudal with a sterile surgical blade. Open the skin to expose the skull, and remove the periosteum around the desired injection site using cotton swabs and a scalpel. Rinse the skull with sterile PBS. Clean and polish the skull with cotton swabs.
3. Level the skull, and with a marker, mark the stereotaxic coordinates for virus injection. Using a 0.5 mm burr on a high-speed microdrill (set to around 7,000-10,000 rpm), create a small hole in the skull. Apply slight pressure while drilling and intermittently clean the bone dust and moisten the area with sterile PBS to prevent brain tissue from overheating, until the brain surface is reached. Keep the brain moist where the hole is drilled.
4. Use a 26 G needle for picking up virus (e.g. AAV1.CaMKII.GCaMP6f.WPRE.SV40) in the microsyringe, and then replace it with a 35 G needle for injection. Attach the microsyringe loaded with virus to the manipulator arm of the stereotaxic apparatus.
5. Bring the syringe close to the injection site hole and adjust the angle of the needle so it enters at a 90° angle to the brain surface. Lower the needle until it touches pia mater and pierce through the dura. Begin lowering the needle in increments of 10 µm/s until it reaches the desired depth (z). Fix the needle location there using the stereotaxic arm.
6. **Set the microsyringe pump to inject 250 nL of virus at 25 nL/min.**
  1. Critical step: Since the virus volume to be injected depends on the titer and dilution, run dilution experiments beforehand, to establish optimum volume and concentration criteria for imaging cells in the experiment.
7. If some virus is seen exuding out of the injection site, pause the injection and wait until the brain tissue absorbs the virus drop. Wait for 5-7 min after the total volume has been injected before retracting the needle. Dyes such as fast green could also be added to the virus solution to help control the rate of injection in the event the virus is seen exuding out of the brain surface.
8. For labeling multiple layers in cortex, use multiple injections if necessary. Start at the ventral-most site first, waiting for 5-7 min after the injection, and pulling up the needle to the next most dorsal point for injection (e.g. inject at -1.0 mm AP, 1.5 mm ± ML and 400 and 600 µm DV). Wait 10 min after the final injection before pulling out the needle and removing it from the stereotaxic setup.
9. Mix a small quantity of an *in vivo* biocompatible transparent elastomer adhesive from the double barrel syringe (e.g. Kwik-Sil) and cover the hole in the skull with it. Apply cyanoacrylate adhesive on top of the layer of elastomer adhesive and let it cure.
10. Suture the scalp and allow the animal to recover from anesthesia in a warm recovery cage until it is ambulatory. Administer ketoprofen (2.5 mg/kg) or carprofen (2.5 mg/kg) subcutaneously before returning the animal to its home cage. Singly house post-surgery subjects to protect the surgery site and repeat the dose 24 h later.
11. After removing the microsyringe, flush both 26 G and 35 G needles 7-10 times with distilled water to clean before storage.

### 3. Prism Probe Implant Surgery

1. 1-2 weeks post virus injection, prepare for the prism probe implant surgery. Disinfect the prism probe in 70% ethanol and clean it with lens paper. Insert the prism probe in the lens holder tool and tighten the hex screw with the screwdriver. Seat the microscope in the base holder (the magnets will hold it in place).
2. Prepare the animal as outlined under the Pre-operative Preparation section.
3. Trim and shave animal's head between the eyes and ears and disinfect the skin with alternate swabs of 70% ethanol and betadine.
4. Expose the skull by incising the skin with a pair of sterile scissors and remove the skin flap and underlying periosteum. Dry and polish the skull with cotton swabs. Ensure adequate removal of surrounding muscle tissue to create a clean, dry, wide bone foundation in preparation for the following steps.
5. Implant skull screws in the contralateral hemisphere to make the implant stable and secure. These may also be useful if choosing to implant a headbar for awake head fixing to ready the animal for experimental imaging sessions in Section 5.
6. **Level the skull and with a marker mark the AP and ML co-ordinates for lens insertion. Using a 0.5 mm burr on a microdrill open a round craniotomy, ensuring the craniotomy diameter is just larger than the prism diameter i.e. 1.0 mm in this case. Drill gently while pausing intermittently to flush the skull with sterile PBS and suction it away with cotton swabs. Remove the bone dust that is generated.**

1. Critical step: Place the craniotomy such that when the prism is inserted in the cortex, its flat edge (imaging surface) is facing the virus injection site and is within a 150-200  $\mu\text{m}$  radius.
7. Stop drilling right before the skull is completely thinned. Blood vessels should be visible through the thinned bone. Remove the bone plug gently with fine 45° forceps.
8. **Remove the dura with #5 forceps.**
  1. Critical step: Once the brain tissue is exposed, always keep the tissue moist. Place a cotton swab dipped in sterile saline over the craniotomy. This will also maintain pressure on the tissue.
9. To alleviate pressure in the brain tissue during insertion of the prism probe, create an insertion tract ahead of time. Attach a straight-edged dissection knife to the electrode holder arm of the stereotaxic apparatus and mount it on the stereotaxic apparatus at an angle such that the knife blade is perpendicular to the curvature of the skull (10° angle in this case) and in a plane parallel to the virus injection column.
10. Carefully position the knife above the craniotomy along its anterior medial edge in this case and ~200  $\mu\text{m}$  lateral to the virus injection site with the cutting edge facing posteriorly (**Figure 1**). Zero out the Z-axis when the knife tip touches the pia and lower it gradually (in 10  $\mu\text{m}$  /s increments) to a depth at which the prism probe will be inserted. Then move the knife 1 mm posteriorly to create a path for the prism's leading edge. Pause and control for any bleeding that may happen while making the incision with a pre-sterile saline soaked piece of gelfoam.
11. Once the knife is in this position, flush the site with sterile saline and wait until any bleeding subsides. Then slowly retract the knife using the stereotaxic arm micromanipulator in 10  $\mu\text{m}$ /s increments, and place a piece of gelfoam sponge soaked in sterile saline over the incision.
12. Attach the lens holder (with the prism probe and microscope) to the stereotaxic manipulator arm at the same angle as the knife in the previous step. Align the prism such that the flat side of the prism is over the incision and parallel to the virus injection column. This step might require some fine correction of the stereotaxic arm position. Adjust the alignment by staying close to the skull for faster results.
13. Once the prism is at the correct angle, gradually lower it in the brain in 10  $\mu\text{m}$  increments to a final z of 1.1 mm for this probe, starting from the brain surface. The brain tissue will expand around the prism and any pressure that is created is behind the visualization plane and will not affect the field of view. Plug in the microscope to a computer with acquisition software installed via a USB3 port and visualize field fluorescence by turning on the LED.
14. Cover any exposed tissue around the prism in the craniotomy in a very thin protective layer of elastomer adhesive using a 25G needle.
15. After the elastomer adhesive is cured (usually in ~ 3-5 min) use a 25G needle to apply some cyanoacrylate adhesive to attach the glass of the prism lens to the adjoining skull over the layer of elastomer adhesive, to prevent the lens from moving inside the craniotomy. Include the edges of the prism probe cuff for better adhesion. Do not get any adhesive on the top face of the implanted prism probe. Once the cyanoacrylate adhesive is cured unscrew the lens holder and carefully remove the microscope. Then slowly retract the stereotaxic manipulator arm to leave the prism probe securely implanted.
16. **Apply a layer of dental acrylic or cyanoacrylate adhesive around the implant to cover all exposed skull surface, up to but not touching the surrounding retracted muscle tissue. Covering a large area of skull with this cranial cap will later help in baseplate attachment. The skin around the implant site should heal on its own around the cranial cap.**
  1. Critical step: Do not let adhesive touch any of the surrounding skin or muscle tissue, and do not engulf any skin in the cranial cap. Doing so will irritate the skin, and may result in excessive scratching and potential damage to the implant.
17. Optional: If wishing to use an awake head-fixed setup to attach and detach the microscope to an animal's baseplate in experimental imaging sessions rather than briefly anesthetizing or scruffing the animal, implant a headbar in the cranial cap that is compatible with an awake head-fixed setup of choice (not demonstrated in this protocol).
18. Mix the catalyst and base from a silicone adhesive syringe and put a drop of the elastomer inside the prism probe cuff to cover the probe lens top to prevent any damage and dust from settling.
19. Remove the animal from the stereotaxic frame and allow for recovery from anesthesia in a warm chamber. Administer ketoprofen (2.5 mg/kg) or carprofen (2.5 mg/kg) subcutaneously, and return the animal to a clean home cage once it is ambulatory. Singly house all subjects to protect the implant and repeat the dose 24 h later.

#### 4. Baseplate Attachment for Miniature Microscope Installation

1. One week to 10 days after implanting the prism probe, check for virus expression in the tissue through the implanted prism probe, and to attach a baseplate on the skull if the preparation shows cell activity. The microscope will dock on the baseplate during live imaging.
2. Follow the steps outlined in the pre-operative procedure for preparing the animal for baseplate attachment.
3. Remove the silicone adhesive cap over the surface of the implanted prism probe lens top. Examine the lens probe surface, and clean off any debris gently with lens paper and 70% ethanol to ensure the imaging surface is clean.
4. Plug the microscope to its DAQ box and connect it through the USB3 port to the PC.
5. Open the acquisition software on the computer and connect the microscope via USB3 port. Use the acquisition software for checking neural activity, and for measuring and documenting the field of view settings for future recordings in this subject.
6. Attach a baseplate to the microscope and fasten the baseplate set screw to hold the baseplate in position, and secure the microscope into the microscope gripper on the stereotaxic micromanipulator arm by the body of the microscope. Attach the gripper to a Newport rod, which can be mounted on the stereotaxic micromanipulator arm.
7. Position the microscope above the prism probe lens using the stereotaxic micromanipulator arm. Visually inspect the orientation by viewing the prism lens from the side and back of the animal stage. The optical axes of both the microscope objective and prism probe lens must be aligned.
8. Turn on the microscope LED through the software. Evaluate the quality of the microscope alignment by focusing on the top face of the implanted prism probe lens in the acquisition software. When properly aligned, the edges of the prism probe lens top face should be sharp.
9. Adjust the microscope's physical distance above the implanted prism probe using the stereotaxic manipulator arm to obtain the desired focal plane inside the tissue. The optically optimized distance between the microscope objective and implanted GRIN lens is ~500  $\mu\text{m}$ .
10. Save a reference fluorescence image once the desired imaging plane is captured.  
Critical point: From this point on, do not adjust the position of the microscope, as this will change the location of the imaging plane in the tissue.

NOTE: Apply adhesive in the next step to permanently fix the position of the baseplate in relation to the cranial cap. The adhesive may experience some volume shrinkage in the following day or two, which can change the focal plane in the tissue. Preemptively account for this by measuring the amount of shrinkage for your adhesive mix and distance *ex vivo*, then backing the final Z position of the microscope + baseplate by that amount before progressing to the adhesive application step.

11. **Use dental acrylic or cyanoacrylate to permanently attach the baseplate to the acrylic cap covering animal's skull, bridging the gap with the acrylic or adhesive. Applying the dental acrylic/ cyanoacrylate gradually and curing in multiple stages may minimize the effect of the previously mentioned shrinkage on the final position of the microscope's image plane.**
  1. Critical step: Take care while applying dental acrylic/ cyanoacrylate to prevent any material from contacting the microscope's objective lens, the set screw, or the microscope body, which will prevent proper operation of the instrumentation later on.
  2. Critical step: While applying the adhesive, do not push on the microscope. Pressure on the microscope or baseplate can cause movement of the microscope objective relative to the prism probe lens, which could result in misalignment or a change of the focal plane in the tissue that would require prompt re-adjustment.
12. Verify that the dental acrylic/ cyanoacrylate has cured and hardened by tapping the acrylic with a pair of forceps or syringe tip. Acquire a final reference fluorescence image with the acquisition software.
13. Release the microscope from the gripper and retract the gripper from the microscope. If cyanoacrylate or another transparent adhesive was used, cover it with black nail polish or a layer of black dental cement to prevent ambient light leakage into the head cap, which can contaminate future images acquired during experiments.
14. At this point, remove the microscope if needed. For separating the microscope from the baseplate, release the baseplate set screw by turning the set screw approximately ½ turn counter-clockwise. Pinch the microscope body while supporting the baseplate and acrylic cap with the other hand, and pull the microscope straight up. Replace it in its storage container.
15. Protect the implanted prism probe with a baseplate cover. This will prevent any dust particles from settling on the lens surface, which can be tricky to clean after the baseplate has been installed.
16. Attach the baseplate cover on the baseplate and advance the set screw by an approximate ½ turn clockwise or until the set screw is flush with the baseplate cover. Do not overtighten.
17. Remove the animal from anesthesia and monitor in a warm recovery chamber until ambulatory. Return the animal to its home cage. Singly house all animals with implanted baseplates to protect the implant.

## 5. Imaging Multiple Cortical Layers in a Freely Moving Mouse

1. Prepare the behavioral apparatus, (e.g. Phenotyper, Noldus) by cleaning and disinfecting it and wiping down with 10% bleach solution.
2. Plug the microscope to its DAQ box, and connect it to the computer and launch the acquisition software.
3. Check for ample file storage space on the acquisition computer and make room for the calcium imaging movies. Save directly from the software to the local hard disk, rather than writing to an external hard drive, to accommodate the high data transfer rate between the microscope and the computer and prevent data loss during recordings.
4. Anesthetize the animal with isoflurane (5% in oxygen) in an induction chamber to attach the microscope. Alternatively, gently scruff the animal or use an awake head-fixed setup with a headbar if anesthesia is known to interfere with the behavioral paradigm of choice.
5. Remove the baseplate cover by turning the baseplate setscrew counter clockwise and lifting out the baseplate cover.
6. **Seat the microscope in the baseplate on the animal. The microscope should snap into place with the aid of the magnets on the baseplate. Advance the baseplate set screw until slight resistance is felt.**
  1. Critical Step: Do not over tighten the baseplate set screw to prevent damage to the microscope housing.
7. **Check the imaging plane in the tissue by acquiring a fluorescence snapshot in the software, and if necessary adjust the focal plane in the tissue by loosening the microscope turret set screw, rotating the microscope turret to adjust the fine focus, then re-tightening the turret housing set screw.**
  1. Critical Step: Never force the turret to turn without first loosening the set screw, and do not over tighten the turret set screw.
8. If conducting a longitudinal study, return to the physical turret position to capture the same field of view. In the hardware, note the number of turret turns, or the physical position of the turret, for each animal imaged with the same microscope for a quick return to the same field of view.
9. **Release the animal carrying the microscope into its home cage or behavioral chamber for acclimation, and to await the wearing off of anesthesia if applicable.**
  1. Critical Step: Train the animals to carry the weight of the microscope using a dummy microscope for several sessions until ensured that wearing the microscope does not interfere with their normal behavior, before starting experimental sessions. Regular handling and training for awake restraint will prevent undue stress to the animals.
10. Select the acquisition settings to be used to gather data. This includes the frame rate for capturing data (e.g. 20 fps, Gain of 1, and LED power of 50%). Check the image histogram when selecting the settings to ensure good SNR.  
NOTE: The numerical aperture for fluorescence collection is 0.35 for the 1 mm prism probe compared to 0.5 for the 1 mm straight probe.
11. Launch the behavioral software and program it to trigger the microscope at the desired imaging recording cycle (e.g. a 4X 5 min ON 2 min OFF). Connect the TTL port on the Noldus IO box to the TRIG port on the DAQ box via a RJ45 to BNC cable.
12. Place the animal in the behavioral arena if not already there, and start the experiment.
13. After acquiring desired data, re-anesthetize the animal with isoflurane (5% in oxygen) in an induction chamber, or gently awake-restrain the animal.
14. Loosen the baseplate set screw and detach the microscope from the baseplate by gently pulling up the microscope. Replace the baseplate cover and gently tighten the baseplate set screw.
15. Return the animal to the home cage until the next recording session. Use the reference fluorescence images as a guide for subsequent imaging sessions to return to the same field of view.

## 6. Evaluating Large-Scale Ca<sup>2+</sup> Imaging Data

1. To extract cell location and Ca<sup>2+</sup> dynamics within the field of view from data, different data analysis platforms can be used. Mosaic, a data analysis platform designed specifically to process large-scale Ca<sup>2+</sup> imaging movies has been used for this study here.
2. Rectify defective pixels and interpolate any individual dropped frames in the raw movies in the preprocessing step. Bin the images in space from the full 1,440 x 1,080 pixel field of view to 720 x 540 pixels to reduce the data footprint.
3. To correct for motion artifacts in the brain with respect to the microscope image sensor, register the movies using a rigorous ImageJ based image registration algorithm (TurboReg).
4. To identify individual neurons, re-express the images as relative changes in fluorescence  $\Delta F/F_0 = F - F_0/F_0$  where  $F_0$  is the mean image obtained by averaging the entire movie.
5. Identify the spatial filters corresponding to individual cells using an established cell-sorting algorithm. Here we used principal and individual component analysis<sup>21</sup> to identify individual neurons.

NOTE: An event was specified when peak amplitude of an event in a trace was more than 8 standard deviations from trace's baseline in our data set and the cell location and Ca<sup>2+</sup> dynamics data was exported for further analysis.

### Representative Results

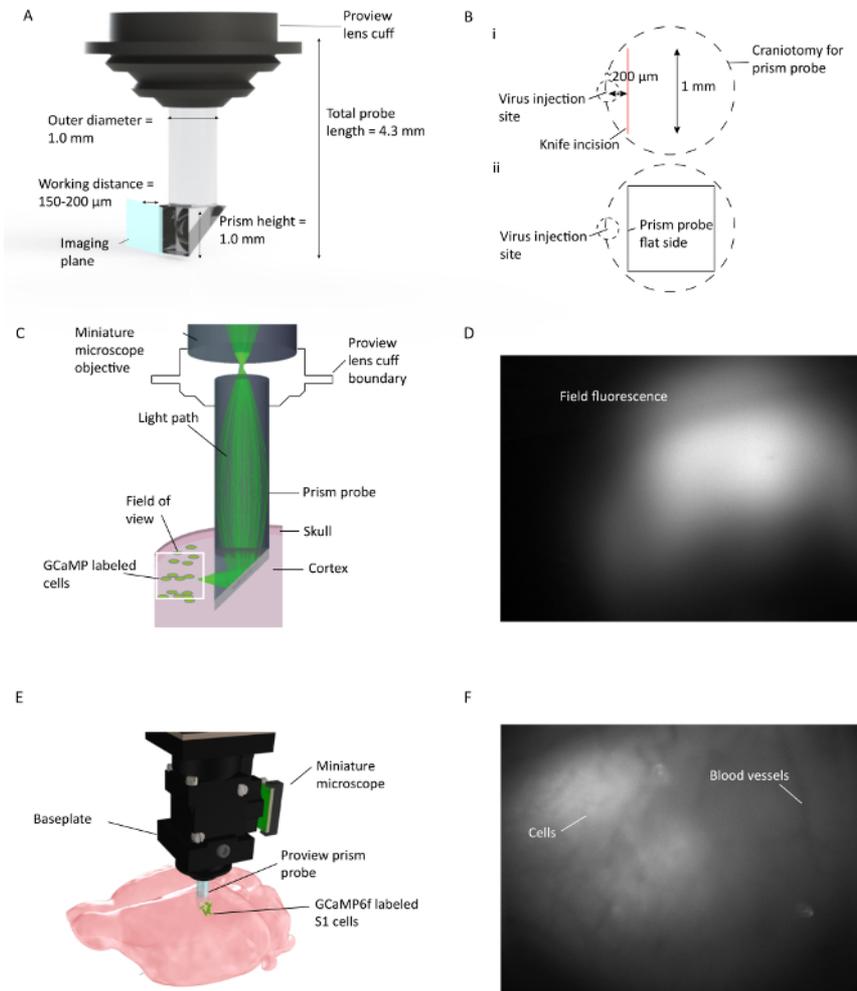
The protocol outlined here describes an effective and efficient way to perform longitudinal multi-layer Ca<sup>2+</sup> imaging from hundreds of cortical neurons in freely behaving mice using prism probes (**Figure 1**). Previous approaches towards multi-layer cortical imaging have primarily been restricted to head fixed animals<sup>22,23,24,25,26,27</sup>. In order to acquire this level of data in a freely behaving context, a miniaturized microscope platform was used for behavioral flexibility; a genetically encoded calcium indicator (GCaMP6f) was used to target a specific cell population (CAMKII+ cells in the cortex); and a prism probe was chosen to provide a chronic, multi-layer field of view.

We demonstrated the workflow for preparing the animal for imaging. A viral vector encoding an appropriate calcium indicator was injected into the cortex (**Figure 2**, Step 1), before chronically implanting a prism probe to enable optical access to the labeled cells (**Figure 2**, Step 2). A baseplate that serves as a secure, temporary dock for positioning of the microscope during imaging sessions was then installed over the head of the animal (**Figure 2**, Step 3), enabling the visualization of cortical activity across multiple cell layers in an awake behaving experimental setup (**Figure 2**, Step 4).

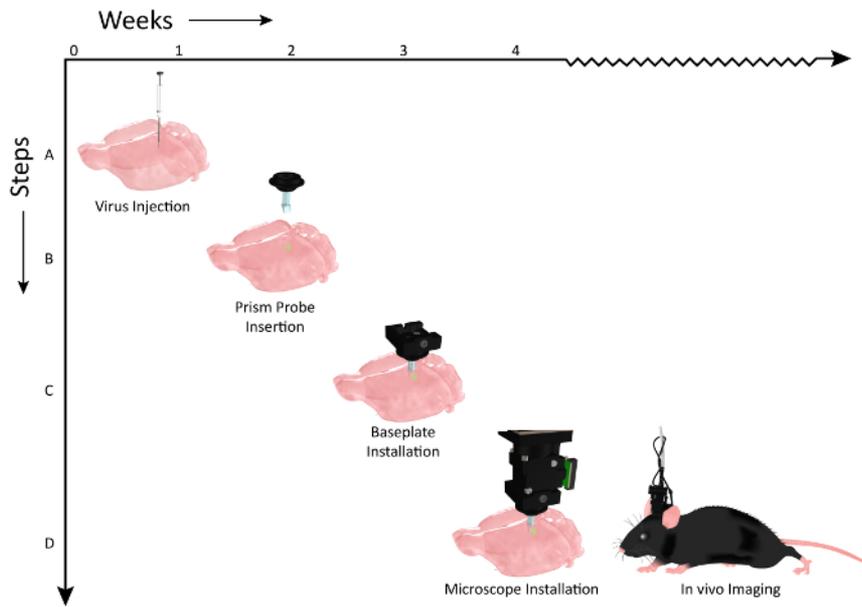
To ensure that the desired cellular population was being targeted, a post- mortem coronal brain section from a representative mouse is shown in **Figure 3** with the prism probe tract and field of view marked relative to the GCaMP6f labeled neurons in Layers 2/3 and 5 of the somatosensory cortex.

During awake behaving imaging with the system, the activity of the somatosensory cortical neurons was recorded when the mouse was exposed to three different environments- Open Field (Day 1), Object Familiarization (Day 2-4) and Novel Object (Day 5) (**Figure 4**). On Day 1 the mouse was placed in a behavioral arena devoid of any objects. On Day 2-4 the mouse was placed in the arena with the same two texturally different objects (a gel pad and a wood block). On Day 5, one of the objects was replaced with a novel object. The animal was imaged across 5 days for 20 min each day.

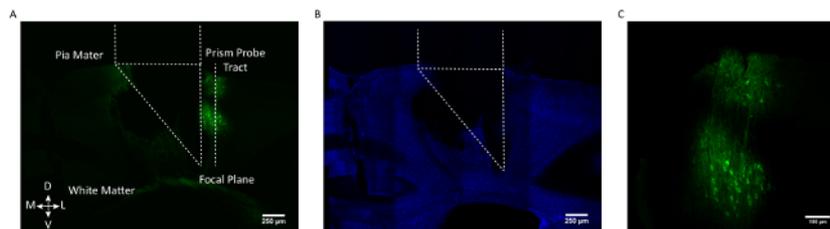
Following cell extraction using the Ca<sup>2+</sup> image data analysis software, spatial filters corresponding to cell locations were overlaid on the mean fluorescent intensity projection of the microscope recording data (**Figure 5**). A white dashed line separates layers 2/3 and 5 cells. Corresponding Ca<sup>2+</sup> traces from 5 cells from each of the layers show the firing pattern of the cells in two different behavioral contexts- Object Familiarization and Novel Object Exposure. Layer 2/3 cells were more active compared to layer 5 cells on the day when the mouse was exposed to a novel object. This is also evident from the raster plots that show the thresholded firing activity of all imaged cells on days 1, 4 and 5.



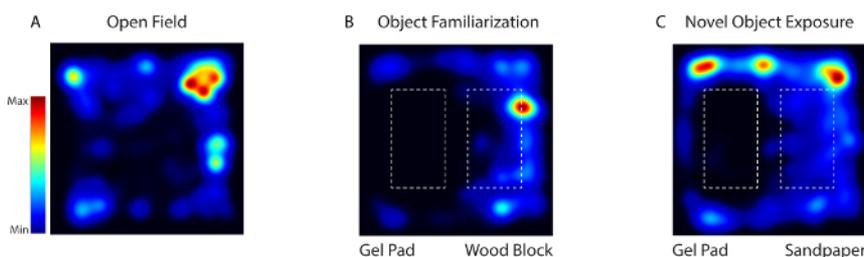
**Figure 1: *In Vivo* Ca<sup>2+</sup> Imaging Across Multiple Cortical Layers in Freely Moving Mice.** (A) Prism probe specifications and depiction of imaging plane. The reflective coating on the inside of the hypotenuse allows for imaging 90° from the insertion plane of the prism probe. The lens cuff integrates with the lens holder, which streamlines the implantation procedure and allows for potential viewing of ambient tissue fluorescence during implantation (B) (i). Illustration of placement of prism probe craniotomy and knife incision relative to the virus injection site, and (ii) illustration of location of prism probe flat side relative to the knife incision and virus injection site. (C) Illustration of *in-vivo* Ca<sup>2+</sup> imaging setup showing the light path for a small area within the full field of view through prism probe implanted in mouse cortex. (D) Example field of view during prism probe installation. Miniature microscope is attached to the lens holder, which holds the prism probe allowing for checking the virus expression during prism probe installation. (E) Integration of microscope with prism probe for multi-layer cortical imaging of GCaMP6f labeled S1 cells. F Example field of view during baseplate installation. Clear blood vessel pattern is visible at the time of baseplate install with some cells in raw image. More cells are clearly visible when DF/F is turned on in the acquisition software window. [Please click here to view a larger version of this figure.](#)



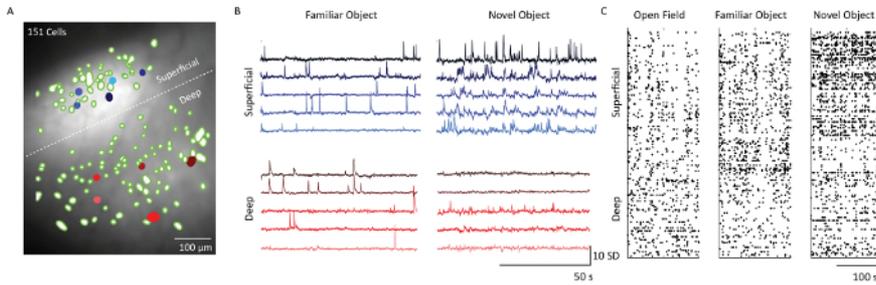
**Figure 2: Schematic Showing the Timeline of Workflow Events for Prism Probe Implantation and Microscope Installation.** Number of weeks is represented on the X-axis and the workflow steps of the procedures along the Y axis. **(A)** Graphic illustrating viral injection (AAV1.CaMKII.GCaMP6f.WPRE.SV40) along the same dorso-ventral axis, to label multiple layers of the mouse somatosensory cortex. **(B)** 2 weeks post virus injections, a prism probe is implanted at an axis that is parallel to the virus injection sites. **(C)** Approximately one week after the prism probe implantation, the animal is checked for expression with the microscope and a baseplate is mounted on the head if a population of cells is visible. **(D)** The animal is then ready for chronic imaging during relevant behavioral tasks (Mouse clip art modified after permission from-UW-Madison Biochemistry MediaLab). [Please click here to view a larger version of this figure.](#)



**Figure 3: Postmortem Histological Validation of Prism Probe Location and GCaMP Expression.** **(A)** Coronal section from a representative mouse brain showing the prism probe tract and with its imaging side facing the GCaMP6f expressing cells (AAV1.CaMKII.GCaMP6f expressed in neurons in layers 2/3 and 5). **(B)** Same coronal brain section following staining for DAPI. Scale bar = 250  $\mu$ m **(C)** Zoomed in view of GCaMP6f expressing cells in somatosensory cortex. Scale bar = 100  $\mu$ m. [Please click here to view a larger version of this figure.](#)



**Figure 4: Mouse Activity During Habituation, Familiarization, and Novel Object Exposure Testing was Video-tracked Using Video Software.** **(A)** On Day 1, the animal was placed in a behavioral arena devoid of any objects (Open Field). **(B)** On days 2-4, the same two texturally different objects (gel pad and wood block) were placed in the arena (Object Familiarization). **(C)** On Day 5, one of the objects was replaced with a novel object (wood block with sand paper) (Novel Object Exposure). [Please click here to view a larger version of this figure.](#)



**Figure 5: Calcium Dynamics from Superficial and Deep Layers of Somatosensory Cortex of a Representative Mouse Imaged with the Microscope.** (A) Merged image of neuronal spatial filters (green blobs) and mean fluorescence intensity projection of the microscope recording through prism probe field of view. Border between supragranular and infragranular layers indicated by a white dashed line. Scale bar = 100  $\mu\text{m}$ . (B) Calcium traces from five example superficial and deep layer cells (filled blue and red cells in panel A), indicating units of standard deviation of fluorescence following principal and independent component analysis. Horizontal scale bar 50 s and vertical scale bar 10 S.D. (C) Raster plot of cells from superficial (layers 2/3) and deep layers (layer 5) shown over Open field, Object Familiarization and Novel object exploration. Scale bar = 100 s. [Please click here to view a larger version of this figure.](#)

## Discussion

Understanding neural circuit activity during awake behavior is a vital level of neuroscientific investigation needed to effectively dissect brain function in health and disease. The cortex is a particularly important region to study in the context of awake behavior, as it plays an important role in many vital sensory, cognitive and executive functions<sup>28,29</sup>.

The cortical column is thought to be the basic functional unit in the cortex and population-level activity of cortical cells is known to differ based on their physical location within the column. For example, excitatory neurons in layers 2/3 in the somatosensory cortex project primarily to other neocortical regions and modulate other cortical networks<sup>30</sup>, while cells in deeper layers project primarily to subcortical regions like the thalamus<sup>31</sup>. Recording the activity of hundreds of pre-specified cortical cells simultaneously and reliably over time across different lamina in freely behaving subjects would greatly advance our understanding of cortical information flow, allowing for finer functional dissection of cortical columns informed by real-time behavioral information and task-relevant time-scales.

Collecting this level of neural circuit data is made possible with the use of an efficient and streamlined miniaturized microscopy platform to conduct large-scale  $\text{Ca}^{2+}$  imaging in freely behaving subjects (or head-fixed subjects as desired). Used with genetically encoded calcium indicators to enable cell-type specific targeting, and imaging a multi-layer field of view provided by a chronically implanted prism probe, this protocol explored one case among many possible applications: observing laminar differences in somatosensory cortical processing when mice physically engaged with a novel object (Figure 5). This is the first procedural illustration of this kind of cell-type specific, *in vivo* approach to study multiple cortical layers in awake, freely behaving animals, and broadens the spectrum of experimental methods available to understand laminar structures in the active brain.

The periscopic field of view enabled by the prism probe in this technique can feasibly be applied to other brain structures when preservation of the tissue directly dorsal to a region of interest is desired; for example, CA3 imaging could be achieved without disruption of hippocampal function.

Prism probe based approach for imaging  $\text{Ca}^{2+}$  activity requires the physical insertion and permanent implantation of a microprism into the cortex, which equates to the creation of a cortical lesion where the lens probe is inserted. This may result in disruptions to the local neural circuitry, including the severing of apical dendrites and processes. This procedure will also cause an initial activation of glial cells in the region, though this is expected to be localized to the tissue about 150  $\mu\text{m}$  from the prism face, and to subside after the brain has healed<sup>22</sup>. It is very important to consider if this technique will affect the normal circuit anatomy and/or behavior of the animals when planning experiments. Behavioral control groups should always be conducted to ensure that there are no significant alterations in baseline behaviors that could produce confounding experimental results.

Using this miniaturized, mobile  $\text{Ca}^{2+}$  imaging technique with neuropharmacological manipulation, various cognitive, social, motor or intrinsic behavioral paradigms, and combining it with other physiological metrics can deepen and enrich studies focused on understanding the functional roles of neural circuits in behavior and signal processing<sup>32</sup>. Suppression or activation of certain pathways modulated by drugs can influence associated behaviors, which can be easily studied using this technology<sup>33</sup>. Branching out into different cell types by modifying the targeting of the calcium indicator is another powerful and useful application, and enables many creative combinations of experimental tools to address diverse neural circuit questions.

## Disclosures

The authors have read the journal's policy and have the following competing interests: SG, SO and VC are paid employees at Inscopix.

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