

Video Article

# Measuring the Calcium Dynamics of Individual, Genetically-labeled Neurons of the Developing Mouse Neocortex

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## Abstract

Spontaneous activity in the developing mammalian cortex is necessary for proper network formation. Such activity may be intrinsic to individual cells or driven by network interactions, and different types of activity may affect distinct components of development. A striking feature of cortical development is the propagating waves of activity that cause simultaneous action potential firing in neurons across broad cortical regions. Waves have been proposed to play roles in patterning connections, such as those between the cortex and thalamus, as well as in placing inhibitory interneurons into the correct cortical layers. Calcium signaling induced by waves is likely to mediate these effects on development. Calcium imaging techniques in brain slice preparations may be used to visualize wave activity propagating between brain structures and to examine the contribution of individual cells to population activity. Slices have an advantage over dissociated cultures because of the ability to examine cellular activity in a setting with preserved network features, such as cortical layering. However, slice preparation for the physiological examination of developing cells can be difficult. The slicing process reduces network connectivity and injures cells. High potassium ringer solutions are often necessary to produce the synchronous activity that is normally present *in vivo*. This work describes a set of methods for brain slice preparation that allow for the measurement of the physiological patterns of synchronous activity without increasing potassium by using short-term organotypic slice cultures to increase cell health. Methods to identify genetically-labeled neuronal subpopulations in the cortical plate of these slices while conducting calcium imaging of heterogeneous neurons in the cortical network are presented. An overview of the slice preparation and imaging techniques of the developing cortex, which are useful for assaying both single-cell and population-level activity patterns, are presented. These methods may be adapted to many different neuronal subtypes and anatomical regions.

## Introduction

Spontaneous physiological activity regulates brain development via the actions of calcium, which serves as a second messenger to affect processes such as neurogenesis and cellular migration<sup>1</sup>. Physiological activity may elicit changes in cellular calcium levels in different ways. For example, during the first postnatal week of mouse development, neurons of the cerebral cortex exhibit spontaneous action potential firing, which propagates as a wave through a large subset of cortical neurons<sup>2,3</sup>. Calcium influx occurs through voltage and ligand-gated ion channels and triggers further calcium release from internal stores. At the same time, neurons of the same network may exhibit other types of calcium activity. For example, individual neurons may exhibit calcium spikes, which are not synchronous across multiple cells and are driven largely by L-type calcium channels<sup>4,5</sup>. Such channels may be activated by subthreshold spontaneous depolarizations resultant from stochastic ion channel opening or ligand binding. Different types of calcium signaling may elicit distinct effects on development. Waves have been proposed to play roles in patterning cortical connections<sup>4,6</sup>, while L-type calcium channels are important for neuronal migration<sup>5</sup>.

Calcium imaging techniques are used to describe the activity of calcium *in vivo* or in brain slice preparations. Imaging calcium dynamics rather than electrical signals in development has several benefits. Measurements of the properties of the calcium signal, such as the frequency and duration of calcium events or the correlation of activity in one cell to the activity of another cell, inform experiments that examine how calcium activity affects development. Importantly, calcium imaging allows for the simultaneous measurement of the activity of the hundreds of cells in a neural network. Additionally, calcium spikes may be used as a proxy for electrical activity, as population calcium events in the developing neocortex correlate 1:1 with simultaneously recorded local field potentials<sup>2</sup>. Brain slices have been useful for examining spontaneous activity in cortical development, although synchronous wave activity can be difficult to record in slices due to its dependence on exceptional slice health. Nonetheless, calcium imaging in brain slice studies has shown that cortical waves initiate in discrete pacemaker regions<sup>7,8,9</sup> and are driven by two neurotransmitter systems<sup>2,10</sup>. Recent technical innovations have allowed the measurement of calcium dynamics during wave activity in individual genetically-labeled inhibitory interneurons<sup>4</sup>.

The preparation described here is similar to that of Elias and Kriegstein, 2007<sup>11</sup>. However, the recording of physiological activity from individual cells requires increased attention to slice health during the culturing and imaging process. These methods allow for activity generation with more physiological levels of potassium and magnesium than are often used in experiments on brain slices. Increasing potassium may cause regions of slices that do not normally initiate wave activity to become pacemakers and can change the duration and number of wave events, in part by increasing the participation of NMDA(n-methyl-D-aspartate)-type glutamate receptors. While the anatomy of the slice may change in culture over time, short-term cultures preserve slice anatomy while allowing for the recovery of slice viability. The addition of antibiotics to the culture medium could be predicted to affect the network, but it has previously been shown that ion channel properties develop normally in culture<sup>12</sup> and that inhibitory interneurons terminate their migration in culture on a normal timeline<sup>4</sup>. While special cutting solutions<sup>13</sup> and recovery procedures<sup>14</sup>

may be used to improve the viability of acute slices, short-term cultures provide the best opportunity for cell recovery after slicing, allowing for the clearance of dead tissue at the surface of the slice, which might impair the imaging of deeper, healthier cells. Cultured slices are necessary to study early postnatal activity in the cortex of genetic mutants that do not survive past birth<sup>4,2</sup>. Chronic pharmacological manipulations can be conducted to study the effects of activity on network development<sup>12</sup>.

This work also describes methods of calcium imaging in organotypic brain slices. Brain slices have been used to measure population activity propagating across large regions of the cortex<sup>2,8,10</sup>, as well as single-cell activity when higher microscope magnifications are used<sup>4,9</sup>. Propagation patterns are measured with low-magnification microscope objectives, whereas single-cell activity is measured with higher-powered objectives. The methods presented here focus on higher-magnification imaging experiments that use spinning-disk confocal microscopy for the acquisition of confocal images of transgenically labeled cell populations, as well as wide-field epifluorescence imaging of calcium activity. This system allows for the fast acquisition of calcium recordings with a high signal-to-noise ratio and a low bleaching rate, and it is more cost effective than laser-scanning confocal systems. These techniques allow for the analysis of cellular-level studies of the mechanisms and functions of spontaneous physiological activity in development by enabling the simultaneous measurement of calcium transients in genetically labeled and non-labeled neuronal subpopulations.

## Protocol

All steps described below are in compliance with the animal use and regulation policies of the University of Washington and IACUC.

### 1. Advanced Preparations

1. Prepare 5% agar in 100 mL of water and autoclave for 20 min at 120 °C to dissolve the agar. Immediately remove the agar from the autoclave to prevent the solution from solidifying. Portion it into 5-mL petri dishes and cover them with lids. Place the dishes in a refrigerator.
2. Make 1 L of artificial cerebrospinal fluid (ACSF) by mixing the following (mM): 140 NaCl, 3 KCl, 2 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 1.25 NaHPO<sub>4</sub>, 26.5 NaHCO<sub>3</sub>, and 20 D-glucose.
3. Add 1 mL of penicillin-streptomycin (100x) per 1 L of ACSF.
4. In a sterile hood, remove the cap from an autoclaved 500-mL bottle and place a vacuum filter on the bottle. Filter the ACSF using a 0.2-µm sterilizing filter and place the sterile ACSF in the refrigerator to chill overnight.
5. Prepare the culture medium by combining the following: 75% sterile cell culture basal medium (1X), 25% horse serum, penicillin (100 IU/mL), streptomycin (0.1 mg/mL), and 2 mM L-glutamine.
6. Place all tools (*i.e.*, 2 forceps, 1 pair of vanna scissors, 1 pair of large scissors, glass petri dishes, 2 capillary tubes for bubbling, 2 spatulas, 2 blunt forceps, 1 razor blade, 1 glass bubbler, and 1 slicing stage) in autoclave bags and autoclave them to sterilize.

### 2. Day-of Preparation

1. Add 1 mL of culture medium to a 5-mL petri dish. Place a sterile culture membrane (0.4 µm, 30 mm diameter) on the culture medium so that the top of the membrane is dry and no air bubbles form beneath the membrane.
2. Repeat step 2.1 for as many dishes as necessary. Each membrane can hold 1-3 slices, depending on the section and age of the tissue.
3. Put the petri dishes in a water-jacketed incubator at 37 °C, 5% CO<sub>2</sub> and allow them to equilibrate for the duration of the dissection.
4. Freeze ACSF at -80 °C until ice chunks form (approximately 30-40 min).
5. While the ACSF is cooling, thoroughly clean all work surfaces and tools with 70% ethanol.
6. Once the ACSF is partially frozen, oxygenate it for at least 5 min by bubbling with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Continue the oxygenation of this solution throughout the dissection.
7. Fill two small petri dishes with ACSF. Chill with an ice tower or occasionally add ACSF ice chunks to keep the ACSF cold.

### 3. Surgery

1. Decapitate an E17-P7 mouse so that the head falls directly into the cold, oxygenated ACSF.  
NOTE: Due to age of the mouse, no anesthetic is required. Euthanasia is confirmed via direct decapitation. For embryonic-stage mice, the mother is euthanized via CO<sub>2</sub> and secondary euthanasia is performed by cervical dislocation.
2. Using forceps, remove the skin from the skull, starting from the posterior end and working towards the nose. Be careful to keep the forceps tangential to the skull and avoid damage to the brain.
3. Once the skin is removed, position the skull with its ventral side down.
4. Insert the ends of the forceps through the eye sockets at a roughly 45° angle from horizontal to hold the brain steady. Do not pinch the forceps together.
5. Using the vanna scissors, make a vertical cut through the part of the skull covering the cerebellum.
6. Continue to cut down the midline of the skull from the posterior to the anterior, up to the eye sockets. Keep the scissors as superficial as possible, hooking under the skull and pulling lightly outwards to cut.
7. Use the forceps to grip the skull near the eye sockets, without touching the brain. Pull away from the brain to remove the skull.  
NOTE: The brain should come off largely in one piece per hemisphere.
8. Using a spatula, carefully scoop the brain out of the ventral skull and place it into the cold, oxygenated ACSF petri dish (step 2.7).

### 4. Slicing the Brain Tissue

1. Turn one glass petri dish upside down and use a spatula to place the brain on top of the overturned dish, ventral side down.
2. Prepare the brain slices, depending on the desired plane.
  1. For sagittal sections, cut directly down the midline with a razor blade, separating the two hemispheres of the brain.

2. For coronal sections, using either a razor blade or vanna scissors, make a cut to remove the cerebellum. Make the cut 90° to midline to ensure that the left and right hemispheres are at the same rostral-caudal level in the individual slices.
3. Gently push the brain into the petri dish containing oxygenated, ice-cold ACSF.
4. Use a razor blade to cut a small strip (2 cm long, 0.5 cm wide, 1 cm tall) of the autoclaved agar (step 1.1) and glue it onto the slicing plate. Complete this step in the absence of ACSF.  
NOTE: Place a small amount of glue directly in front of the agar on the slicing plate. Do not let excess glue stick to the agar, as this will cause the slices to stick and will interfere with slicing.
5. Position the brain.
  1. Remove excess ACSF from the brain using lab tissue before slicing. Use a spatula to position the brain so that the cut side of the brain is on the surface of the slicing plate (where the glue was placed) and the ventral side is up against the agar.  
NOTE: Multiple brains or hemispheres may be sliced at one time.
6. Put the slicing plate into the slicing stage and quickly add oxygenated, ice-cold ACSF. Place ice chunks in the slicing chamber so that it stays cold for the duration of the slicing.
7. Position the plate so that the slicing blade cuts first through the dorsal side of the brain (for sagittal and coronal slices) and then into the agar.
8. Calibrate the vibratome to cut 300-350  $\mu$ M-thick slices. Do not exceed 0.1 mm/s while cutting through the tissue.  
NOTE: Once the blade is through the tissue, the slice should rest freely on top of the razor blade, while the sharp edge is embedded in agar.
9. Pause the vibratome. Use a broad-tipped pipette to gently suction the tissue slice into the pipette. Transfer them to a petri dish with oxygenated, ice-cold ACSF.

## 5. Incubation

1. Remove the prepared culture membranes (steps 2.1-2.3) from the incubator and place them in a sterile hood.
2. Bring the cold, oxygenated slices in ACSF to the hood.
3. Using a broad-tipped pipette, gently suction up one of the brain slices; use plenty of liquid to pick it up. If the slice seems to stick to the pipette, gently rock the ACSF until the slice comes free. Keeping the pipette vertical, allow the slice to sink to the open end of the pipette.  
NOTE: The ACSF should make a slight convex curve extending out the broad end of the pipet. Let the slice rest in this meniscus before gently touching it to the membrane.
4. Touch the meniscus to the membrane to release the unfolded slice onto the culture dish with some extra ACSF. If the slice does not lay flat, pick it up with plenty of ACSF and reposition it.
5. Using a fine-tipped pipette, remove the excess ACSF surrounding the tissue, keeping the slice a safe distance from other slices and from the edge of the dish.  
NOTE: Residual ACSF can prevent the slice from adhering to the membrane.
6. Repeat the procedure for each slice to be cultured (1-3 slices per membrane).
7. Return the culture membranes with the slices to the incubator (37 °C, 5% CO<sub>2</sub>). Replace the culture medium (step 1.5) every 1-2 days.
8. Incubate the slices for 2 days in the incubator (recommended).  
NOTE: This will provide enough time for the slices to recover from the slicing process and will preserve the anatomy of the slice. Longer culture times may be used to study the effects, for example, of drugs.

## 6. Resting the Slices

1. Prepare a resting chamber for the slices at 28 °C (the chamber should allow for movement of the solution and oxygenation of the slices). Oxygenate the ACSF with carbogen for 10 min before moving the slices to the chamber.  
NOTE: The resting chamber may consist of a mesh to suspend the slices in ACSF, with carbogen bubbling up from beneath the slices.
2. Remove the culture plates from the incubator.
3. Use a razor blade to cut the slices from the membrane using a light but steady touch.  
NOTE: Pulling the membrane will cause distortion that negatively affects slice health. Cutting perpendicularly into another long cut is the best way to create clean corners.
4. Gently pull the corners of the membrane to drag the slice onto a spatula.
5. Position the slice so that the spatula is in contact with ACSF in the resting chamber and so that the tissue is facing upwards and not touching the ACSF.
6. Use a pipette to administer a few drops of ACSF onto the tissue/membrane while inserting it into the bath to prevent tearing due to surface tension; the slice will sink to the bottom of the resting chamber. Ensure that the membrane side is down and that tissue is facing up to prevent damage to the slice and to allow proper gas exchange.
7. Allow the slice to rest for approximately 1 h in the resting chamber. Maintain the temperature at 28 °C.

## 7. Loading the Slices

1. While the slices are resting, prepare a petri dish to load the slices with fluorescent dye.
2. Add 4 mL of oxygenated ACSF to a 5-mL petri dish. Add 70  $\mu$ L of poloxomer (stock: 2 g of poloxomer in 10 mL of DMSO) and 20  $\mu$ L of Fluo-4 AM dye (stock: 50  $\mu$ g of dye in 91  $\mu$ L of DMSO) to a bath concentration of 0.07% poloxomer and 2.5  $\mu$ M dye.
3. Mix thoroughly with a transfer pipette; the poloxomer acid will not evenly distribute on its own.
4. Transfer the slices from the bath to the loading dish using a transfer pipette for acute slices and a spatula for cultured slices. Ensure that the slice is on top of the membrane when placed into the bath and not upside-down, as this will hamper loading.
5. Bubble the loading dish using a bent glass pipette. Monitor the loading dish to ensure that the bubbles from the pluronic do not overflow.  
NOTE: Briefly touching the bubbles will cause them to recede. Also, carefully monitor oxygenation so that the flow does not stop or cause excessive bubbling.

6. Leave the slice in the loading dish for 30-40 min and then return it to the resting chamber at 28 °C. Use an intermediate ACSF chamber during the transfer to reduce the poloxamer contamination of the resting bath. Limit light exposure to slices before imaging.

## 8. Imaging

1. To obtain the most complete cortical anatomy, identify the cultured slices that are rostral-side up and use these for the experiments.  
NOTE: In this orientation, the rostral-most cut will be visible just inside the dorsal edge of the slice. The caudal cut tends to have a more unpredictable morphology.
2. Place a slice into an imaging chamber perfused with oxygenated, 32 °C ACSF and let it equilibrate for 30 min before beginning the experiments.
3. Hold the slice in place by pinning down with a harp. Use harps with strings that do not press into the tissue.  
NOTE: For imaging using an upright microscope, a harp may be used to hold a culture membrane down without touching the slice. On an inverted scope, the culture membrane will protect the slice from the harp strings.
4. Measure the calcium dynamics.
  1. Use an epifluorescent microscope with a scientific digital camera for image acquisition. To prevent bleaching, set the intensity of the excitation light to the lowest possible levels that still allow good signal-to-noise ratios in the acquired images.
  2. For long-duration experiments (e.g., washing drugs in and out to measure their effects on activity), use a slow sampling rate to measure the activity over time. For example, use 1-Hz acquisition of 300-ms images or 2-Hz acquisition of 200-ms images.
    1. For experiments to determine the precise propagation patterns of activity, either between brain regions at low magnification or brain cells at high magnification, use a faster acquisition. For example, use 20-Hz acquisition rates to resolve developmental wave activity propagation; waves propagate from 1-10 mm/s, depending on the region.  
NOTE: The sampling rate of the camera used, as well as its sensitivity, will determine the fastest rate at which images can be acquired and the calcium signal detected. Lowering the resolution of the sampled images by camera binning will allow faster collection of images by increasing both the rate at which the camera may sample, and decreasing the amount of light needed to detect the calcium signal.

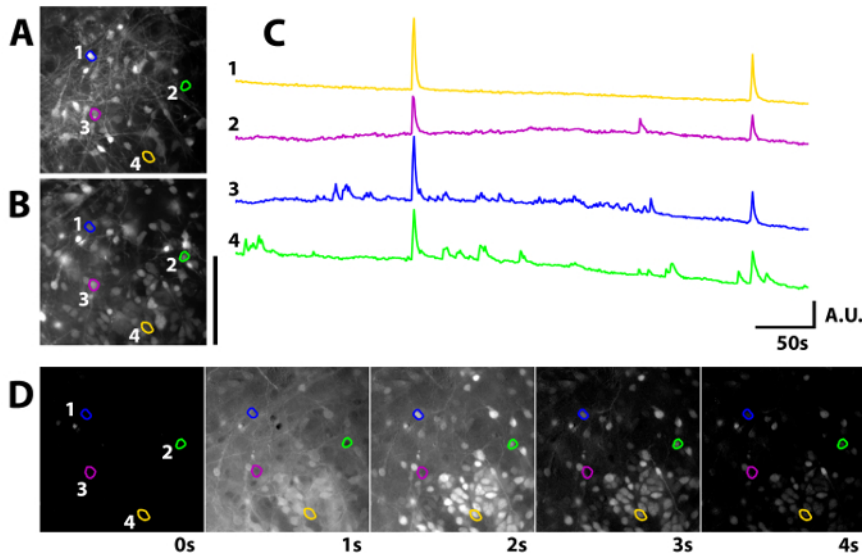
## 9. Identify Cell Types

1. Use a red fluorescent label to mark the cell types.  
NOTE: The preservation of the green channel for calcium imaging will allow the use of high signal-to-noise calcium indicators, such as fluo4.
  1. Use a Cre-dependent tdTomato transgenic mouse paired with a cell-type specific Cre transgenic mouse to label specific cell types with red fluorescent protein (RFP).  
NOTE: For example, Dlx5/6 Cre will label inhibitory interneurons. Slices from transgenic animals can be prepared with no changes to the described protocol.
  2. Alternatively, use a dye such as SR101 to label glial cells.  
NOTE: This requires the addition of SR101 to the bath during the application of the calcium indicator dye<sup>8</sup>.
2. Using an epifluorescent microscope with a spinning disk attachment, use widefield mode to find the best plane of focus in the calcium indicator channel; this plane is just below the top layer of visible cells.
3. Turn on spinning-disk confocality and take an image of the red fluorescence of the slice.  
NOTE: This will identify genetically labeled cells. It may be necessary to take a small z-series to identify all genetically labeled cells that are visible in the widefield calcium record.
4. Revert back to widefield imaging mode and obtain a time-lapse image series of calcium indicator fluorescence in the slice. If the slice shifts during imaging, repeat step 9.3 after the calcium imaging run to ensure that all imaged cells are identified.  
NOTE: Post-imaging, ROIs can be created over the red channel. The ROIs can then be applied to the calcium record to obtain fluorescence records of the identified cell population over time.

## Representative Results

**Figure 1** shows components of the raw data that may be used to determine the activity of individual, transgenically labeled cells. To measure activity, regions of interest (ROIs) are drawn onto cell bodies in a confocal image of RFP-labeled cells (**Figure 1A**). These ROIs are then applied to the calcium record associated with the confocal image. In **Figure 1B** shows a single image from the calcium record associated with confocal image 1A, with the ROIs applied. **Figure 1C** shows the average pixel intensity from each ROI over time in the calcium fluorescence record. Calcium events are detected by calculating the derivative of the fluorescence record and noting instances where the derivative crosses a threshold<sup>15</sup>. This threshold is set to separate physiological spikes in the calcium record from fluctuations in the noise of the recordings. Such automated processes usually require manual or visual error-checking and calibration. Also, ROIs over cells that clearly do not participate in a wave of activity (i.e., cell 3 in **Figure 1D**) may still show a small spike in the calcium record associated with out-of-focus cells and a neuropil. This spike must be excluded when auto-detecting cell bodies participating in population activity. Easton *et al.*<sup>4</sup> addressed this issue by creating background ROIs over areas of the slice that contain no active cell bodies, determined through the visual inspection of the calcium image series, and subtracting an average of these records from all other ROIs before automatic event detection.

Healthy slices should have a clear anatomy, with no folds or tears in the tissue that negatively affect network connectivity. Visual inspection of the slices under a microscope should reveal no bacteria moving on the surface of the slice, and individual cells should be clearly visible in the green channel, loaded with a calcium indicatory dye. If cells are not clearly visible in this channel, this indicates a problem with the slice preparation or loading process. For the clearest identification of slice anatomy in coronal sections, ensure that the rostral slice edge faces away from the culture membrane. Image registration techniques may be used to correct small translational shifts in tissue position over the course of calcium image acquisition.



**Figure 1. Representative data showing calcium signals in genetically labeled inhibitory interneurons.** (A) Confocal image of tdTomato expression showing labeled inhibitory interneurons in the cortical plate of a P2 (E18 + 3 days *in vitro*) brain slice. (B) Widefield epifluorescence image of Fluo-4 AM expression in the same plane of focus as A. In both A and B, four ROIs mark individual cells. Cells 1 and 2 are inhibitory interneurons, as they express tdTomato. Cells 3 and 4 are not labeled with tdTomato. (C) Mean fluorescence over time from these regions, with each cell expressing a distinct pattern of activity. Arbitrary units are shown on the y-axis. Measurements of amplitude should not be compared between cells for non-ratiometric calcium imaging. (D) A series of images during a 4-s window spanning the second large spike visible in all ROIs in C. These images represent the raw fluorescence images during this time period, minus a baseline image of the slice when little spontaneous activity is present. The calcium level of individual cells is shown, with white representing a high change in fluorescence and black showing no change. Scale bars: 100 µm. [Please click here to view a larger version of this figure.](#)

## Discussion

The combination of confocal imaging of fluorescent proteins to label individual cells with widefield epifluorescent calcium imaging uses important features of each technique. Epifluorescence imaging provides several benefits over other fluorescence imaging methods. Compared to confocal imaging, it allows for fast image acquisition at low light levels, as removal of out-of-focus light reduces the overall fluorescence signal. The use of high signal-to-noise fluorescent indicators (such as Fluo4 AM) allows for the resolution of very fine processes without the need for the removal of out-of-focus light. Thus, epifluorescence imaging more efficiently translates excitation light into signal at the detectors, which allows for better signal-to-noise ratios at higher acquisition speeds and less dye bleaching at the same acquisition rate. The use of inverted microscopes allows an even plane of focus to be obtained in widefield imaging across large brain areas. A spinning-disk confocal adapter was used here to obtain images of RFP-labeled neuronal subtypes in the same preparation used for the epifluorescence imaging of calcium dynamics. Spinning-disk confocal microscopy allows for quick switching between epifluorescent and confocal modes. This confocality is sufficient to obtain clear images of neurons labeled with variants of GFP, which are less sensitive than calcium indicators in regard to dye bleaching. With this system, it is possible to measure calcium activity in both genetically labeled and non-labeled neuronal populations at the same time.

Organotypic slice cultures have been used to study proteins, which are important in early development but difficult to study at later stages because they cause the death of newborn pups. For example, organotypic slice cultures were used to study spontaneous activity in the first postnatal week of brains lacking either GAD67<sup>2</sup>, the primary enzyme creating GABA in the brain, or Tbr1, a transcription factor important for cortical layering<sup>4</sup>. In both cases, insights into the role of these neonate-lethal genes in patterning the newborn cortex were achieved by extending the age of assayable tissue using organotypic slice cultures.

This preparation provides access to easily assayed, healthy brain slices. Slice preparations allow access to cell populations deep in the brain that would be difficult to measure with *in vivo* techniques. Epifluorescence imaging works well in slices and is used for surface imaging *in vivo*, such as for cells in cortical layers I-III. However, it is ill-suited to imaging deep cell populations. Additionally, while the fluorescence imaging methods described here may be used on acute slices of adult tissue, organotypic slice cultures typically only work well in development and are not easily adapted to adult preparations. Finally, the bath application of calcium indicator dyes is much less effective in adult brain slices; thus, genetically-encoded calcium indicators are preferable for calcium imaging in adult slices.

The calcium imaging methods presented here are easily adapted to a variety of cell types in different brain regions. The bath loading of calcium indicator dyes works well across brain regions during development. To achieve the labeling of cell types besides inhibitory interneurons, it is necessary to cross the Ai14, Cre-dependent tdTomato transgenic mouse with a transgenic mouse expressing Cre under the control of a different



promotor region. For example, Chat-Cre would drive the expression of tdTomato in cholinergic neurons. Future studies can take advantage of this ability to describe the physiological patterns of activity in defined cell populations during development. This can help to explain how these cell types contribute to the generation of activity and what effect such activity has on brain development.

## Disclosures

The authors have no competing financial interests.

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