Video Article

Targeted Labeling of Neurons in a Specific Functional Micro-domain of the Neocortex by Combining Intrinsic Signal and Two-photon Imaging

Philip O'Herron[1], Zhiming Shen[1], Zhongyang Lu[1], Adrien E. Schramm[1], Manuel Levy[1], Prakash Kara[1]

[1]Department of Neuroscience, Medical University of South Carolina

Correspondence to: Prakash Kara at kara@musc.edu

URL: https://www.jove.com/video/50025
DOI: doi:10.3791/50025

Keywords: Neuroscience, Issue 70, Molecular Biology, Cellular Biology, Anatomy, Physiology, Two-photon imaging, non-rodent, cortical maps, functional architecture, orientation pinwheel singularity, optical imaging, calcium-sensitive dye, bulk loading, single-cell electroporation

Date Published: 12/12/2012

Citation: O'Herron, P., Shen, Z., Lu, Z., Schramm, A.E., Levy, M., Kara, P. Targeted Labeling of Neurons in a Specific Functional Micro-domain of the Neocortex by Combining Intrinsic Signal and Two-photon Imaging. *J. Vis. Exp.* (70), e50025, doi:10.3791/50025 (2012).

Abstract

In the primary visual cortex of non-rodent mammals, neurons are clustered according to their preference for stimulus features such as orientation[1-4], direction[5-7], ocular dominance[8,9] and binocular disparity[9]. Orientation selectivity is the most widely studied feature and a continuous map with a quasi-periodic layout for preferred orientation is present across the entire primary visual cortex[10,11]. Integrating the synaptic, cellular and network contributions that lead to stimulus selective responses in these functional maps requires the hybridization of imaging techniques that span sub-micron to millimeter spatial scales. With conventional intrinsic signal optical imaging, the overall layout of functional maps across the entire surface of the visual cortex can be determined[12]. The development of *in vivo* two-photon microscopy using calcium sensitive dyes enables one to determine the synaptic input arriving at individual dendritic spines[13] or record activity simultaneously from hundreds of individual neuronal cell bodies[6,14]. Consequently, combining intrinsic signal imaging with the sub-micron spatial resolution of two-photon microscopy offers the possibility of determining exactly which dendritic segments and cells contribute to the micro-domain of any functional map in the neocortex. Here we demonstrate a high-yield method for rapidly obtaining a cortical orientation map and targeting a specific micro-domain in this functional map for labeling neurons with fluorescent dyes in a non-rodent mammal. With the same microscope used for two-photon imaging, we first generate an orientation map using intrinsic signal optical imaging. Then we show how to target a micro-domain of interest using a micropipette loaded with dye to either label a population of neuronal cell bodies or label a single neuron such that dendrites, spines and axons are visible *in vivo*. Our refinements over previous methods facilitate an examination of neuronal structure-function relationships with sub-cellular resolution in the framework of neocortical functional architectures.

Video Link

The video component of this article can be found at https://www.jove.com/video/50025/

Protocol

1. Surgical Preparation

1. Induce anesthesia and continuously monitor heart rate, end tidal CO_2, EEG, and temperature. All procedures were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and were based on those we previously published[9,15].
2. Expose the dorsal surface of the skull by cutting the skin with a scalpel blade. Dissect the connective tissues overlying the bone using a Brudon curette. Clean the bone using cotton tipped applicators and cotton gauze. Apply bone wax (as needed) to the skull, to stop occasional bleeding through small emissary veins.
3. Attach a titanium or stainless steel head plate to the skull (over the region of interest where the craniotomy will be performed) by using dental cement mixed with black paint (see *Materials*). A rectangular opening in the center of the head plate provides access for the craniotomy and optical imaging. Attach a metal chamber on top of the head plate. This chamber will serve as a fluid reservoir for imaging with the water immersion objective lens.
4. Secure the head plate to an *xy* stage using metal posts.
5. Thin a large area of the bone within the rectangular opening of the headplate by using a dental drill. The area to be thinned must extend well beyond the boundaries of the planned craniotomy. The thick skull of non-rodent mammals must be thinned if high-resolution images are to be acquired from the neocortex because at the craniotomy site, the thickness of bone will dictate the thickness of the agarose between the coverglass and neocortical surface (see below).
6. Drill a 2 x 2 mm square outline in the bone for the craniotomy. Rinse the chamber periodically with fresh artificial cerebrospinal fluid (ACSF) to remove bone shards and minimize heat dissipation to the underlying cortex. Apply bone wax as needed, to stop occasional bleeding.
7. Lift the bone flap with a #3 forceps.
8. Remove all but the bottom layer of dura using a fine forceps (#5CO) and Vannas spring scissors. The dura in non-rodent mammals is thick and opaque but can be removed in distinct layers. Bleeding is rare but can be stopped with Gelfoam and any blood residue should be gently

removed from the remaining layer of dura using forceps and rinsing with ACSF. The final layer of dura is transparent and pial vessels should be clearly visible through it. Leaving this final layer of dura intact during intrinsic signal imaging will increase the success rate of bulk loading of fluorescent calcium indicators for the subsequent two-photon imaging phase of the experiment (see Discussion).

9. Apply a drop of warm 2% agarose (dissolved in ACSF) and immediately place a coverglass over the craniotomy. Check the craniotomy to ensure that respiratory and cardiovascular pulsations are minimal by using a surgical dissecting microscope or the oculars of the two-photon microscope in bright-field mode.

2. Intrinsic Signal Optical Imaging

1. Add gel-foam soaked with ACSF around the outside of the coverglass to prevent the agarose from drying during intrinsic signal imaging.
2. Attach the intrinsic signal imaging CCD camera to the standard C-mount port on top of the two-photon microscope (**Figure 1**). Place the illuminating light source on the air table near the *xy* stage (**Figure 1A**). Position and secure the light guides over the craniotomy and headplate/chamber (**Figure 1B**). Retract the primary dichroic and the mirror below the C-mount port so that the reflected red light needed for intrinsic signals will pass directly from the craniotomy to the CCD camera (**Figure 1C-D**). Using the 4x air objective (0.13 NA, 17 mm WD), a 2 x 2 mm field of view is available for intrinsic signal imaging.
3. Insert a heat filter in the light path to prevent the cortex from heating. Use a filter that passes green light (546 nm center λ) and record a digital reference image of the cortical surface blood vessels.
4. Move the *z* focus to 500 µm below the cortical surface. Use a red filter (630 nm center λ) to illuminate the cortex for measuring intrinsic signals. Position the light guides such that the cortical surface is uniformly illuminated. Shield the craniotomy from the light produced by the visual stimulus display. Present a sequence of visual stimuli and record reflectance data images. To generate an orientation map within 10 min, collect data corresponding to 4 repetitions of a sequence of 8 stimuli (4 orientations and two directions for each orientation).
5. Analyze the intrinsic signal data to generate a false color orientation map. Select potential target regions for two-photon labeling of neurons, *e.g.,* orientation pinwheel singularities - points in the map where all preferred orientations converge (**Figure 2A**). Overlay the orientation map and the image of the cortical surface vasculature (**Figure 2B-C**). Use the layout of functional domains and the location of large surface vessels to select a target, avoiding locations with large blood vessels and arterioles[15].

3. Bulk Loading of Fluorescent Calcium Indicators

1. Prepare a solution of 20% Pluronic/DMSO by adding 1 g Pluronic in 5 µl DMSO. Heat the mixture at 60 °C for 10 min to completely dissolve the Pluronic. Allow the mixture to cool to room temperature. Add 4 µl of the Pluronic/DMSO mixture to a 50 µg vial of the fluorescent calcium indicator Oregon Green 488 Bapta-1 AM (OGB-1 AM). Then add 1 µl of a red dye (Alexa Fluor 594, 2 mM stock solution) and 35 µl of pipette solution (see *Materials*) for a final concentration of 1 mM OGB-1 AM. Sonicate the solution for ½ hr in a chilled water bath and then centrifuge with a 0.45 µm filter to remove impurities.
2. Pull a long taper pipette with an outer tip diameter of 2.0-2.5 µm. Load 5 µl of the dye mixture into the pipette.
3. Remove the final layer of dura to facilitate pipette insertion and obtain the highest quality two-photon images.
4. Determine the exact location on the cortical surface that the pipette must be placed in order to reach its target in cortical layer 2/3. Our predetermined cortical target is a pinwheel singularity in the orientation map (**Figure 2**). The pipette must be driven into the cortex at a 30° angle to pass between the chamber wall and the objective. Thus, to label the target region 200 µm below the cortical surface, the pipette entry position on the cortical surface will be approximately 350 µm lateral to the target position (**Figure 3 A-B**).
5. Move the *xy* stage (on which the animal and the pipette micromanipulator are placed) to center the cortical surface entry position under the objective (**Figure 3C**). Use a 20x or 40x objective with a working distance (WD) of at least 3 mm (see *Materials*) to ensure that the pipette will easily enter the cortex without touching the objective or the bone along the wall of the craniotomy. Once the entry position is centered under the objective, raise the Z position of the objective by 2 mm (**Figure 3D**). With the green epi-fluorescence light turned on to visualize the red Alexa dye in the pipette, use the diagonal axis of the micromanipulator to move the pipette until its tip is centered (and focused) under the objective directly above the cortical surface entry position (**Figure 3E**).
6. Lower the pipette vertically (along the *z* axis) until the pipette reaches the cortical surface (**Figure 3F**), while continuously viewing the pipette tip through the oculars of the microscope. Using bright-field illumination, the cortical surface blood vessels and remaining meningeal membranes (pia and arachnoid) are easily visible as the pipette approaches the cortical surface. If the pipette does not reach the desired entry position, raise the pipette and recalibrate targeting based on the vascular landmarks on the cortical surface.
7. Remove the objective, wick excess cerebrospinal fluid from the craniotomy and dry the adjacent bone using lint-free absorption spears. Apply a drop of warm 3% agarose (dissolved in ACSF) to stabilize the cortex that is visible through the craniotomy (**Figure 3G**). In non-rodents, 3% agarose is necessary to dampen pulsations for successful uptake of fluorescent dyes by neurons. For intrinsic signal optical imaging only 2% agarose is needed because the combination of 2% agarose and the coverglass provides the necessary stability. Here, no coverglass is used for the dye-loading step so 3% agarose is necessary. After the agarose has solidified, insert a 40x objective (0.8 NA, 3.3 mm WD) and re-fill the chamber with ACSF.
8. Switch from bright-field to two-photon viewing and locate the pipette tip. Using the diagonal axis of the micromanipulator, move the pipette until its tip reaches the desired depth in the cortex (**Figure 3H**). Occasional pressure ejection puffs of the dye mixture (a few psi, each pulse 0.1 sec) will help prevent the pipette tip from clogging. Because OGB-1 AM only becomes visible upon entering cells, the red Alexa dye is critical for visualization of the pipette tip inside the cortex.
9. Before attempting to load neurons in cortical layer 2/3 with the full dose of dye, re-focus the objective on the cortical surface to confirm the depth of the pipette tip and ensure that the tip is in the target location based on the layout of surface blood vessels. If the pipette is in layer 2/3, small puffs of the dye mixture should illuminate the extracellular space and reveal a dense assembly of circular cell bodies as dark shadows.
10. For loading neuronal cell bodies in a sphere of cortex 300-600 µm diameter, inject the dye mixture into the extracellular space using 30-90 pulses, each pulse 1.0 sec, 2-10 psi. The pulses of pressure should not be so strong that the tissue moves. After the injection is complete, wait a few min, then remove the pipette and objective. Wait for 1 hr to allow the OGB-1 AM to be fully taken-up by neurons.
11. Remove the agarose used for the dye loading and rinse the recording chamber. Put a small drop of 2-3% agarose on the surface and quickly place a coverglass over the craniotomy. Because the fluorescent dyes are light-sensitive, avoid over-exposing the cortex to bright-field or epi-

fluorescent light. Insert a high NA (1.0) 20x objective into the objective holder and re-center the labeled cortical region under the objective using the *xy* stage. Shield the craniotomy from extraneous light sources, *e.g.*, visual stimulus display, using multiple layers of blackout material.

4. Single-cell Electroporation of Fluorescent Dyes

1. For studying dendritic morphology, prepare a solution of 100 µM Alexa Fluor 594 (1:20 dilution in ACSF of 2 mM Alexa Fluor 594 stock). For functional imaging of dendrites an OGB salt may be used[16,17].
2. Pull a long taper pipette with a tip size of 1 µm and load 5 µl of dye into the pipette. All other steps of targeting a functional domain are identical to the bulk loading method (**Figures 1-3**). To position the pipette tip adjacent to a neuronal cell body membrane, monitor the increase in electrode impedance and apply pressure puffs of dye to illuminate the extracellular space and see cell bodies as shadows during two-photon imaging.
3. When the pipette tip is adjacent to a neuronal cell body membrane, use the Axoporator 800A to apply 1-3 pulses (-8 to -10 V, 10 msec pulse). The immediate filling of the neuron with dye is readily visualized with two-photon microscopy. Remove the pipette and collect high-resolution images of dendrites and axons *in vivo*.

Representative Results

To illustrate the precision of our dye labeling methods, we targeted the smallest micro-domain of any known functional map in the non-rodent neocortex. Sparsely punctuated throughout the orientation map in the primary visual cortex are singularities. These occur at points where all preferred orientations converge such that in false color maps of preferred orientation, the regions around the singularity look like "pinwheels" (**Figure 2A-B**). One pinwheel per craniotomy is selected for dye labeling (green circle in **Figure 2C**), being sure to avoid large vessels and arterioles in particular[15]. With bulk-loading of OGB-1 AM, tracking changes in neuronal calcium indicator fluorescence to a sequence of oriented grating visual stimuli reveals single-cell resolution functional maps around pinwheel singularities (**Figure 4**). With single-cell electroporation, the spatial organization of dendrites and axons from a single neuron located at a pinwheel singularity is determined *in vivo* (**Figure 5**).

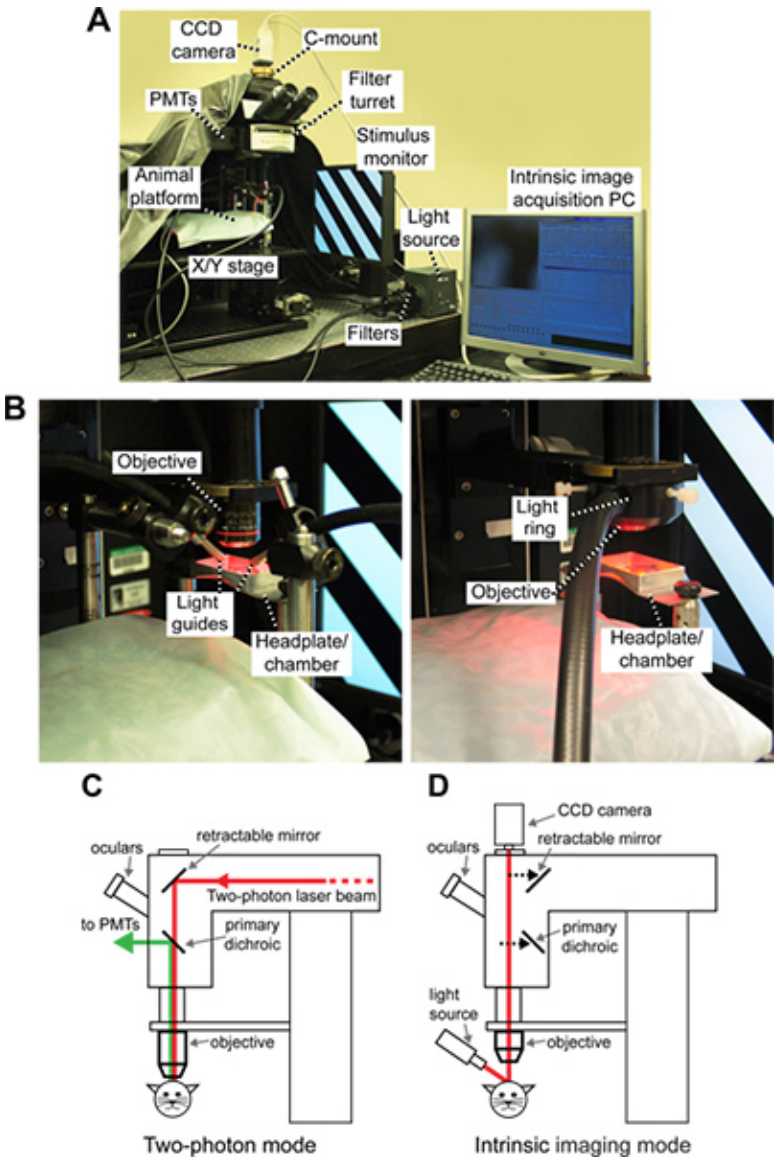

Figure 1. Intrinsic signal optical imaging setup. (A) The animal is positioned on the *xy* stage and intrinsic signal imaging is performed via a CCD camera mounted on the two-photon microscope. The light source and computer used to control the imaging session are brought over to the setup for intrinsic imaging and moved away before the two-photon imaging phase of the experiment begins. (B) Close up view of the 4x objective and the 630 nm illumination through liquid light guides. Either two light guides attached to metal posts (left), or a single illuminating ring attached to directly the objective (right) can be used. Blackout material used to shield the objective from external sources of illumination, *e.g.*, visual stimulus display, during optical imaging sessions, is not shown. (C) Schematic of the microscope and light path in the conventional two-photon imaging configuration. See Supplementary Fig. 20 in Shen *et al.* 2012[15] for a description of the complete two-photon light path. The retractable mirror is positioned to deflect the light from the laser through the dichroic and onto the cortex. The primary dichroic, which passes the laser beam and reflects the emitted light from the cortex to the PMT's, is moved into the light path by turning the filter turret. (D) For intrinsic imaging the mirror is retracted and the filter turret is turned to move the primary dichroic out of the way, allowing a direct light path from the cortex to the CCD camera. Click here to view larger figure.

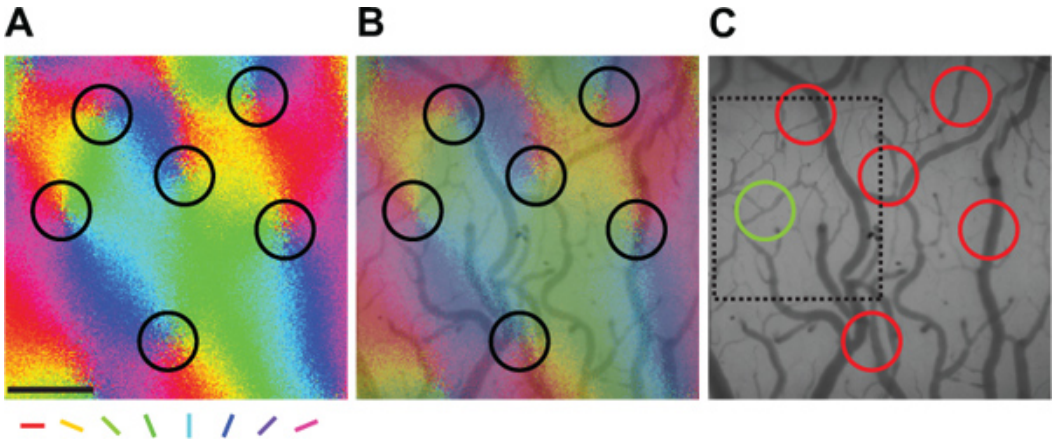

Figure 2. Using functional maps obtained with intrinsic signal imaging to select a micro-domain for labeling neurons with fluorescent dyes. (A) Orientation map from cat primary visual cortex acquired via intrinsic signal optical imaging. The color of each pixel represents the preferred orientation at that location. Potential orientation pinwheel singularities suitable for labeling neurons with fluorescent dyes are shown within black circles. (B) The map of cortical surface blood vessels overlaid with the orientation map. (C) Pinwheels within red circles are near large blood vessels or arterioles and so the pinwheel site within the green circle is chosen as the target for dye labeling. The dashed rectangle corresponds to the region shown in **Figure 3B**. Scale bar in (A) 500 μm.

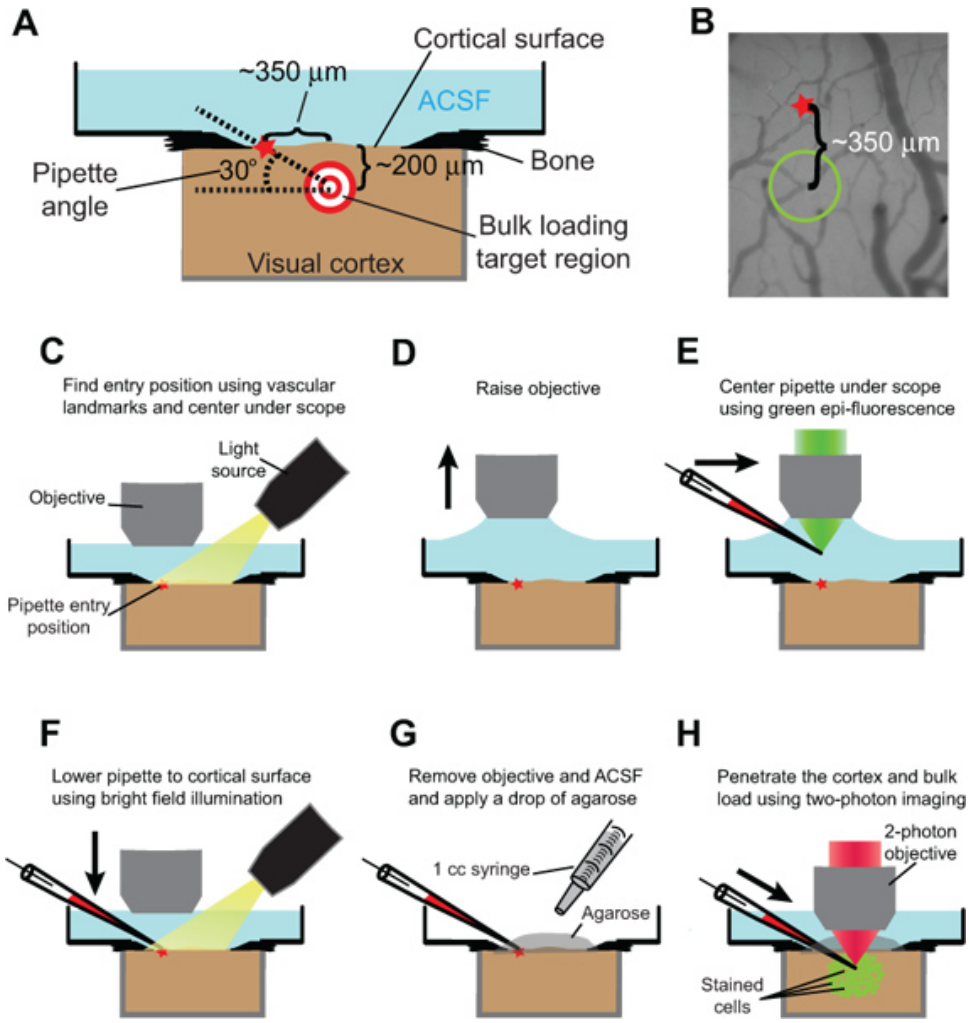

Figure 3. Optimizing the pipette placement for loading neurons with fluorescent dyes. (A) Schematic side-view of the recording chamber shows that the dye loading site to be targeted in visual cortex (red-and-white bull's eye) is ~200 µm below the cortical surface. The *xy* coordinate of the target corresponds to the location of an orientation pinwheel singularity determined from intrinsic signal imaging. The pipette will enter the cortex at a 30° angle. If the cortical surface is parallel to the *xy* stage surface, the pipette tip will move laterally approximately 350 µm before reaching the pinwheel singularity. (B) Top view of recording chamber shows the cortical surface vasculature and the pipette entry position (red star) that is 350 µm from the pinwheel singularity. (C) The *xy* stage position is adjusted such that the pipette entry position is centered under the 20x or 40x objective (3.3-3.5 mm WD). (D) The objective is raised ~2 mm. (E) The pipette is brought over the craniotomy and its tip is positioned directly above the entry position on the cortical surface using green epi-fluorescent light. (F) The pipette and objective are lowered concurrently using bright-field illumination until the cortical surface blood vessels come into focus. (G) The objective and ACSF are removed and a drop of 3% agarose is applied over the craniotomy. (H) Two-photon visualization with a 40x (0.8 NA, 3.3 mm WD) objective is used to follow the pipette down to the chosen cortical depth and inject the dye. After confirming that neurons are successfully labeled with fluorescent dye, typically, a higher NA 20x objective (1.0 NA, 2.0 mm WD) is used to collect two-photon *t*- and *z*-series data. Schematics shown in A, C-H are not drawn to scale.

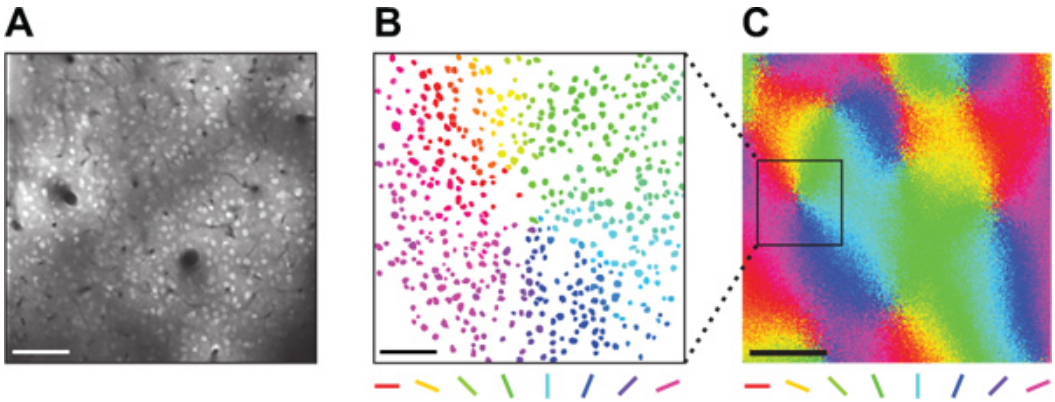

Figure 4. Correspondence of orientation preference in two-photon and intrinsic signal imaging functional maps. (A) Two-photon imaged area showing cell bodies labeled with OGB-1 AM in visual cortex layer 2/3. (B) Orientation preference of individual neuronal cell bodies from the same site shown in (A). Around the pinwheel singularity that was centered in the imaged region is an organized map of preferred stimulus orientation. (C) The orientation map obtained with intrinsic signal imaging. The black square corresponds to the region shown in (B). These data were obtained from the same experiment shown in **Figures 2A-C and 3B**. Scale bar in (A-B) 100 µm and (C) 500 µm.

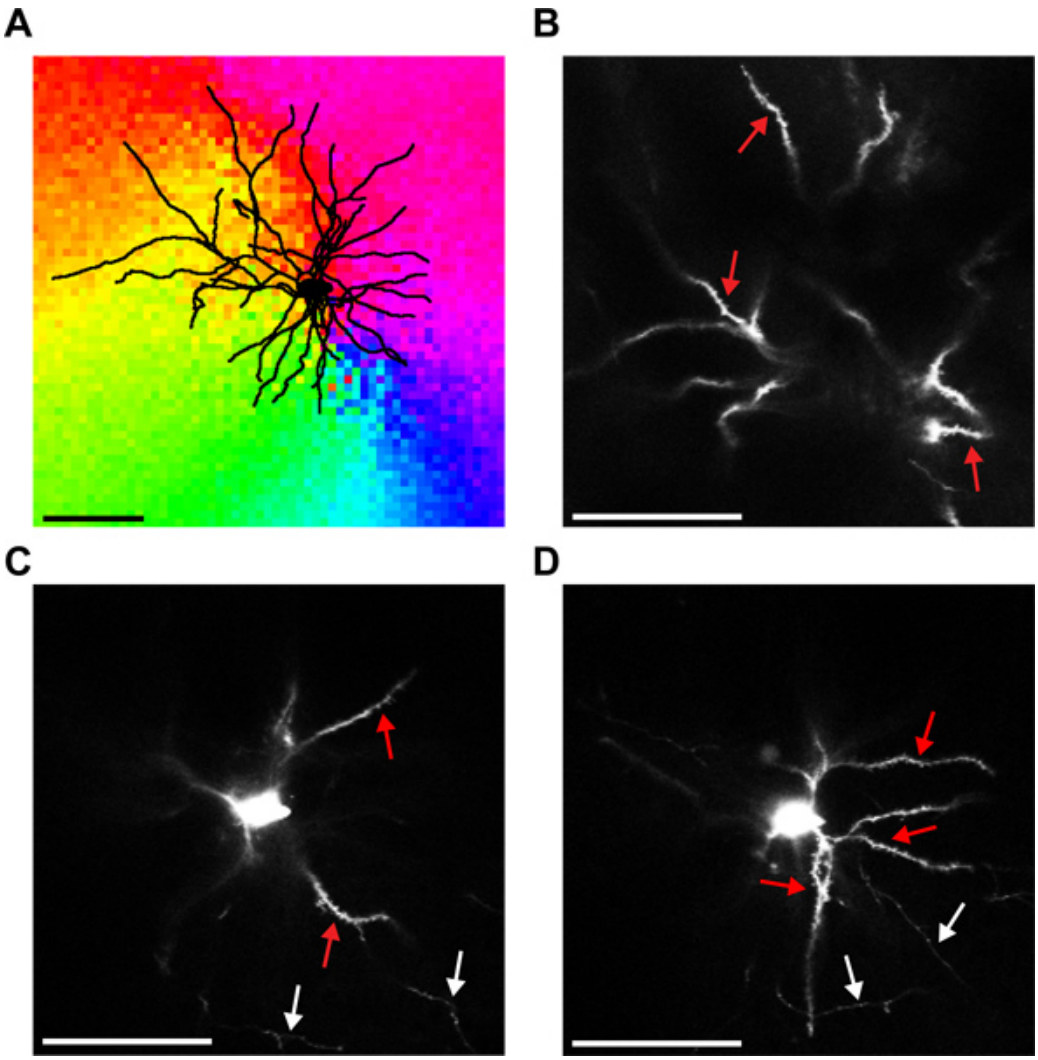

Figure 5. *In vivo* morphology of a single neuron at an orientation pinwheel singularity. (A) The dendrites and cell body of a single neuron overlaid with the orientation map from the same visual cortical site. The neuron was electroporated with Alexa Fluor 594 under two-photon guidance, a *z*-stack was collected *in vivo*, and dendritic processes were traced offline using Neurolucida software. The orientation map was obtained using intrinsic signal imaging before single-cell electroporation. (B) Average intensity 10-µm-*z*-projection from cortical layer 1 shows apical dendrites. (C-D) Average intensity 10-µm-*z*-projections from cortical layer 2/3 show basal dendrites and axons. Red arrows show spines along dendrites and white arrows point to individual axons. Scale bars in (A-D) 100 µm.

Discussion

We present a method to target the labeling of neuronal cell bodies (or dendrites and axons) in pre-determined functional micro-domains of the neocortex. Merging intrinsic signal optical imaging with two-photon microscopy offers the possibility of determining which synapses and cells contribute to the micro-domain of any functional map, whether neuronal selectivity correlates with the location of the neuron in a functional map, and the neuronal circuit components that change with visual experience[7] or the application of clinically therapeutic drugs[18].

No maps for stimulus orientation, direction, ocular dominance, or binocular disparity have been reported in the visual cortex of adult wild-type rodents[6,19-21]. However, the ease of loading neurons with fluorescent dyes and attaining imaging stability, together with the powerful genetic tools available for mice has resulted in the vast majority of studies using two-photon imaging of the cerebral cortex being performed in rodents instead of ferrets, cats, and monkeys.

High-resolution two-photon imaging of neurons in non-rodent mammals can be very challenging because of long surgery times, thicker bone and dura, and extra steps needed to keep respiratory and cardiovascular mediated pulsations of the brain to a minimum[15]. Transitions from surgery to intrinsic signal imaging and intrinsic to two-photon imaging need to be efficient, requiring timely preparation of reagents, dyes and the execution of micro-surgical procedures. Using the two-photon microscope for intrinsic signal imaging obviates the need for much of the equipment used in conventional intrinsic signal optical imaging rigs[22] and reduces the time required to setup intrinsic imaging and transition to subsequent two-photon imaging.

Our approach is specifically designed to provide high-resolution two-photon imaging in pre-determined functional domains. This provides minimal cross talk of functional signals between neuronal cell bodies, and individual dendritic spines and axons can be resolved *in vivo*. Using a high numerical aperture (NA = 1.0) objective and filling the back aperture of the objective with beam expansion optics (see Supplementary Fig. 20 in Shen *et al.*, 2012[15]) are critical for high-resolution imaging. Depending on the speed of imaging in XY and/or Z, respiratory and cardiovascular pulsations may reduce the effective imaging resolution no matter how good the NA and optical light path are. By limiting the craniotomy size in non-rodents to 2 x 2 mm, using the relatively higher concentration of agarose with a coverglass, and performing a pneumothorax and lumbar suspension when necessary[15], brain pulsations are limited to 1-2 µm. Minimizing respiratory and cardiovascular induced pulsations of the neocortex to 1-2 µm during bulk loading of calcium indicators also ensures that the cortex forms a tight seal around the inserted pipette and that no dye tracks up along the shank of pipette to the cortical surface. Selecting dye injection and imaging sites away from large arterioles is also critical for high quality functional imaging because relatively fast sensory-evoked hyperemia (hemodynamic) artifacts will be excluded[15].

At the craniotomy site, leaving the final layer of dura intact throughout the intrinsic signal imaging and fluorescent dye preparation phases - thus removing it just before the pipette insertion into the cortex for dye loading - increases the success rate and quality of neuronal labeling in the following ways: (1) keeps the cortical surface clean and/or prevents dural tissue regrowth so that the pipette tip is less likely to get clogged and the transparency of the tissue is maintained, (2) avoids having the agarose used for intrinsic imaging touch the cortical surface blood vessels which may get damaged when the agarose is removed, (3) prevents the agarose used during intrinsic imaging from entering between the dura and cortex underneath the skull around the edges of the craniotomy. Agarose in these regions will increase the distance between the coverglass and the cortical surface which makes the insertion of the pipette more difficult, leads to compression of the cortical tissue and reduces the transparency for imaging.

When preparing the AM fluorescent dyes for bulk loading, the 20% Pluronic/DMSO mixture should never be used beyond 1 week from its initial preparation - old Pluronic/DMSO quenches fluorescence. For optimal bulk loading of many adjacent cell bodies with a functional indicator, *e.g.*, OGB-1 AM, the dye mixture is stored on ice, loaded into the pipette as needed but always injected into the brain within 2 hr of its preparation.

During the bulk loading phase of the experiment, repeated 1 sec pulses of pressure rather than a continuous injection of 1 min allows for iterative calibration of ejection parameters, which ensures that excessive dye or pressure is not applied. Because of the potential tissue damage from multiple pipette insertions needed to label very large regions of tissue and potential toxicity from large volumes of Pluronic/DMSO from such a series of many injections, we prefer one 300-600 µm dye injection site per 2 x 2 mm craniotomy. Compared to blind injections[14], two-photon visually guided navigation around blood vessels, use of negative contrast of neuronal cell bodies to determine lamina position, and gauging the spread of the dye upon pressure ejection as an index of the volume of cells that will be labeled with OGB-1 AM, improves the quality of dye loading in non-rodent preparations.

The protocol we describe here leads to successful labeling of neurons with fluorescent dyes in targeted micro-domains in every animal attempted. For bulk loading with OGB-1 AM, we consistently obtain visually-evoked responses in 75-100% of simultaneously imaged neurons[9,15] and the technique is sensitive to the detection of single action potentials *in vivo*[23,24].

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was supported by grants from the National Eye Institute R01EY017925 and R21EY020985 and funding from the Dana & Whitehall Foundations to P.K. We also thank Matthew Petrella for assistance with surgical procedures; Grace Dion for tracing the dendrites shown in **Figure 5A**; and Pratik Chhatbar for comments on the manuscript.

References

1. Blasdel, G.G. & Salama, G. Voltage-sensitive dyes reveal a modular organization in monkey striate cortex. *Nature.* **321**, 579-585 (1986).
2. Grinvald, A., Lieke, E., Frostig, R.D., Gilbert, C.D., & Wiesel, T.N. Functional architecture of cortex revealed by optical imaging of intrinsic signals. *Nature.* **324**, 361-364 (1986).
3. Bonhoeffer, T. & Grinvald, A. Iso-orientation domains in cat visual cortex are arranged in pinwheel-like patterns. *Nature.* **353**, 429-431 (1991).
4. Ohki, K., *et al.* Highly ordered arrangement of single neurons in orientation pinwheels. *Nature.* **442**, 925-928 (2006).
5. Shmuel, A. & Grinvald, A. Functional organization for direction of motion and its relationship to orientation maps in cat area 18. *J. Neurosci.* **16**, 6945-6964 (1996).
6. Ohki, K., Chung, S., Ch'ng, Y.H., Kara, P., & Reid, R.C. Functional imaging with cellular resolution reveals precise micro-architecture in visual cortex. *Nature.* **433**, 597-603 (2005).
7. Li, Y., Van Hooser, S.D., Mazurek, M., White, L.E., & Fitzpatrick, D. Experience with moving visual stimuli drives the early development of cortical direction selectivity. *Nature.* **456**, 952-956 (2008).
8. Bonhoeffer, T., Kim, D.S., Malonek, D., Shoham, D., & Grinvald, A. Optical imaging of the layout of functional domains in area 17 and across the area 17/18 border in cat visual cortex. *Eur. J. Neurosci.* **7**, 1973-1988 (1995).
9. Kara, P. & Boyd, J.D. A micro-architecture for binocular disparity and ocular dominance in visual cortex. *Nature.* **458**, 627-631 (2009).
10. da Costa, N.M. & Martin, K.A. Whose Cortical Column Would that Be? *Frontiers in Neuroanatomy.* **4**, 16 (2010).
11. Kaschube, M., *et al.* Universality in the evolution of orientation columns in the visual cortex. *Science.* **330**, 1113-1116 (2010).
12. Villeneuve, M.Y., Vanni, M.P., & Casanova, C. Modular organization in area 21a of the cat revealed by optical imaging: comparison with the primary visual cortex. *Neuroscience.* **164**, 1320-1333 (2009).
13. Chen, X., Leischner, U., Rochefort, N.L., Nelken, I., & Konnerth, A. Functional mapping of single spines in cortical neurons *in vivo*. *Nature.* **475**, 501-505 (2011).
14. Stosiek, C., Garaschuk, O., Holthoff, K., & Konnerth, A. *In vivo* two-photon calcium imaging of neuronal networks. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 7319-7324 (2003).
15. Shen, Z., Lu, Z., Chhatbar, P.Y., O'Herron, P., & Kara, P. An artery-specific fluorescent dye for studying neurovascular coupling. *Nat. Methods.* **9**, 273-276 (2012).
16. Nevian, T. & Helmchen, F. Calcium indicator loading of neurons using single-cell electroporation. *Pflugers Archiv.* **454**, 675-688 (2007).
17. Kitamura, K., Judkewitz, B., Kano, M., Denk, W., & Hausser, M. Targeted patch-clamp recordings and single-cell electroporation of unlabeled neurons *in vivo*. *Nat. Methods.* **5**, 61-67 (2008).
18. Pohl-Guimaraes, F., Krahe, T.E., & Medina, A.E. Early valproic acid exposure alters functional organization in the primary visual cortex. *Exp. Neurol.* **228**, 138-148 (2011).
19. Bock, D.D., *et al.* Network anatomy and *in vivo* physiology of visual cortical neurons. *Nature.* **471**, 177-182 (2011).
20. Rochefort, N.L., *et al.* Development of direction selectivity in mouse cortical neurons. *Neuron.* **71**, 425-432 (2011).
21. Mrsic-Flogel, T.D., *et al.* Homeostatic regulation of eye-specific responses in visual cortex during ocular dominance plasticity. *Neuron.* **54**, 961-972 (2007).
22. Bonhoeffer, T. & Grinvald, A. Optical Imaging Based on Intrinsic Signals. In *Brain mapping: The Methods.*, Toga, A.W. & Mazziotta, J.C., eds., Academic Press, San Diego, 55-97 (1996).
23. Kerr, J.N., Greenberg, D., & Helmchen, F. Imaging input and output of neocortical networks *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 14063-14068 (2005).
24. Hofer, S.B., *et al.* Differential connectivity and response dynamics of excitatory and inhibitory neurons in visual cortex. *Nat. Neurosci.* **14**, 1045-1052 (2011).

