Large-scale imaging of cortical dynamics during sensory perception and behavior

*Joseph B. Wekselblatt, *Erik D. Flister, Denise M. Piscopo, and Cristopher M. Niell

Institute of Neuroscience and Department of Biology, University of Oregon, Eugene OR, 97403, USA

*these authors contributed equally

Running title: Imaging cortical dynamics during perception and behavior

Correspondence
Cristopher Niell
Institute of Neuroscience
222 Huestis Bldg
1254 University of Oregon
Eugene OR 97405
cniell@uoregon.edu
Abstract
Sensory-driven behaviors engage a cascade of cortical regions to process sensory input and generate motor output. To investigate the temporal dynamics of neural activity at this global scale, we have improved and integrated tools to perform functional imaging across large areas of cortex using a transgenic mouse expressing GCaMP6s, together with a head-fixed visual discrimination behavior. This technique allows imaging of activity across the dorsal surface of cortex with spatial resolution adequate to detect differential activity in local regions at least as small as 100um. Imaging during an orientation discrimination task reveals a progression of activity in different cortical regions associated with different phases of the task. After cortex-wide patterns of activity are determined, we demonstrate the ability to select a region that displayed conspicuous responses for two-photon microscopy, and find that activity in populations of individual neurons in that region correlates with locomotion in trained mice. We expect that this paradigm will be a useful probe of information flow and network processing in brain-wide circuits involved in many sensory and cognitive processes.
**Introduction**

One of the central challenges in neuroscience is to understand how sensory input is processed and used to guide behavior. This involves the flow of neural activity and transformation of encoded information across multiple brain regions. In vision (Felleman and Van Essen 1991), these include primary visual cortex and extrastriate regions to extract information from the visual scene, higher-order association areas involved in decision-making, and motor regions to generate appropriate behavior.

Electrophysiological studies of single unit activity underlying sensory-driven behavior have generally been limited to recording either one or a small number of regions at a time. For example, one may target primary visual cortex in studying sensory encoding, or frontal cortical areas for decision-making. In addition to limiting the understanding of temporal dynamics and interactions across areas, such an approach makes it likely that one might miss a previously unknown region that might be involved in processing, due to the infeasibility of simultaneously recording individual neurons across the expanse of cortex with electrodes.

Recently, imaging methods have been developed for species with smaller nervous systems, including nematodes and zebrafish larvae, to allow imaging of activity across nearly complete neural populations (Ahrens et al. 2012; Prevedel et al. 2014). However, these species lack cortex. At the other end of the complexity spectrum, functional imaging and EEG methods in humans allow approximate localization of task-specific activity to cortical areas, but lack high spatial resolution and the capacity for follow-up causal studies that are available in genetic model systems.

Here we present a toolbox of methods to bridge these domains in the mouse, using widefield imaging to measure dynamic patterns of activity across broad regions of cortex during behavior, then targeting areas of interest for two-photon imaging. The mouse is a prime model system for understanding cortical circuits due to the genetic tools available (Luo et al. 2008; O'Connor et al. 2009), as well as the development of behavioral paradigms to probe sensory and cognitive function (Carandini and Churchland 2013). While recent studies have used both intrinsic signal imaging and GCaMP3 to generate static maps of sensory topography in the mouse (Andermann et al. 2011; Issa et al. 2014;
Marshel et al. 2011; Tohmi et al. 2014), these methods have not shown the sensitivity and temporal resolution necessary to track activity at the timescale of behavior.

We achieve this goal using a new transgenic mouse line, which expresses the fluorescent calcium indicator GCaMP6s in excitatory neurons throughout cortex under the control of a tTA driver line. GCaMP6s is an ultra-sensitive indicator of neuronal activity (Chen et al. 2013) that allows detection of single action potentials in individual neurons with two-photon imaging. Using a transgenic line allows broad expression of the indicator and provides a global view of cortical activity. By imaging GCaMP6s mice with widefield tandem-lens optics similar to that used in intrinsic imaging (Ratzlaff and Grinvald 1991), we observe activity across the dorsal surface of cortex with adequate signal-to-noise and spatio-temporal resolution to track local activation during single trials within many cortical areas simultaneously at the timescale of behavioral events.

We demonstrate the utility of this method by 1) mapping multiple sensory/motor modalities across the cortical surface, 2) rapidly generating maps of extrastriate visual areas, which can be aligned across sessions and subjects, 3) imaging the dynamics of activity across cortical areas during the performance of a visual discrimination task, and 4) performing targeted two-photon imaging to measure the response properties of ensembles of neurons within functionally localized regions, including an association area between visual, somatosensory, and auditory cortex that was identified in the behavioral task. Thus, using a combination of widefield and targeted two-photon imaging, we are able to span the scale of the brain from global patterns of activity across multiple areas, down to cellular resolution in local networks.
Methods

DNA Constructs

Plasmid encoding the DNA sequence for GCaMP6s (pGP-syn-GCaMP-nls-mCherry-WPRE) was obtained from D. Kim (Janelia Farm). To generate the tetO-GCaMP6s transgenic mouse, the coding sequences for GCaMP6s-nls-mCherry-WPRE were subcloned into a pTRE-tight plasmid (Clontech), placing GCaMP6s expression under the control of the tetracycline response element (TRE; tetO). All constructs were verified by sequencing.

Transgenic Mice

Animals were maintained in the animal facility at University of Oregon and used in accordance with protocols approved by the University of Oregon Institutional Animal Care and Use Committee. tetO-GCaMP6s mice were generated by injection of a gel-purified linear DNA fragment into fertilized oocytes. Embryos for injection were obtained by mating (C57BL/6J and CBA) F1 hybrids. Transgenic founders were crossed to mice expressing tTA under the control of the CaMK2a promoter on the C57BL/6J background (JAX stock number 007004). Primers for genotyping are 5'-GGGATCTGTACGACGATGACG-3' (forward) and 5'-CTCGATGTTGTGGCGGATGT-3' (reverse). This mouse strain has been deposited at The Jackson Laboratory (stock number 024742).

Although our expression construct included a nuclear-localized mCherry reporter, we did not detect noticeable red fluorescence in cortex, perhaps due to poor IRES-mediated downstream gene expression. However, lack of a co-label did not impact the methods described here.

Histology

Animals were euthanized under deep anesthesia by cervical dislocation. Animals were either perfused (for ISH) or the brain was removed immediately and fixed by immersion in 4% paraformaldehyde in PBS at 4°C overnight, after which 30-70um coronal sections were cut with a vibratome. The sections were mounted using Vectashield with DAPI.
Co-localization of CaMKII and GCaMP6 expression by nonradioactive ISH was performed as described previously (Wehr et al. 2009). For GCaMP6, we used a digoxygenin (DIG)-labeled pWPRE riboprobe (1:500), visualized by anti-digoxygenin sheep Fab fragments conjugated to horseradish peroxidase (Roche No. 11207733907). For CaMKII, we used a FITC- riboprobe (1:1000). Following overnight incubation at room temperature, 30µm thaw-mounted sections were washed at room temperature with MABT buffer 5 × 5', and then AP staining buffer 2 × 10', after which 3.5µl/ml NBT and 2.6µl/ml BCIP, and 80µl/ml levamisole were added and the colorimetric reaction was allowed to develop for 3 hours at 37°C under agitation. The reaction was stopped by two washes with PBS (0.1% Tween-20), then two washes in DI H₂O.

Animal use

All procedures were conducted in accordance with the ethical guidelines of the National Institutes of Health and were approved by the IACUC at University of Oregon. Adult mice 2–8 months old, both male and female, were used in this study. Animals were maintained on a 12hr light / 12hr dark reverse light cycle. Training and experiments were performed during the dark phase of the cycle.

The spherical treadmill was based on Dombeck et al (2007) and Niell and Stryker (2010), with modifications by L. Fischer (mousevr.blogspot.com). Briefly, Tygon E-3603 tubing supplied pressurized air through the bottom of a hollow polystyrene hemisphere (OD 250mm, ID 200mm) (Graham Sweet studios). A 200mm hollow polystyrene ball placed inside the hemisphere provided a freely rotating surface on which the mouse stood. Air flow was set to just enough to allow free rotation using a rotameter with valve (mcmaster.com 41945K77) connected with a hose fitting (mcmaster.com 5346K18). This configuration significantly reduced noise relative to previous implementations, especially by also replacing standard lab air nozzles/valves with large diameter hose fittings (mcmaster.com 5346K19) and valves (mcmaster.com 4082T43) set to fully open. The animal’s head was fixed via a surgically attached headplate that could be screwed into a
rigid crossbar above the floating ball. Headplates were manufactured from titanium for biocompatibility, by emachineshop.com. Designs are available upon request.

Surgical Procedures

The cranial window implant procedure was based on Holtmaat et al. (2009) with modifications as described below. First, a titanium headplate was cemented to the skull to allow head fixation. Animals were anesthetized using isoflurane (3% induction; 1.5%–2% maintenance), in 100% O2 (0.8 – 1.0 L/min), and positioned in a stereotax using earbars placed just below the ear canal for stability. The animals were given subcutaneous injections of the analgesic carprofen (5mg/kg) and 0.2ml saline to prevent post-operative dehydration. Body temperature was maintained at 37.5°C by a feedback-controlled heating pad; temperature and breathing were monitored throughout surgery. Sterilized instruments and aseptic technique were used throughout. Sterile ocular lubricant (Puralube) was applied at the beginning of each surgical procedure. Scalp hair was removed using an electric shaver, and the surgical site was cleaned using 70% isopropanol applied with cotton tip applicators. Topical lidocaine hydrochloride jelly (2%, Akorn) was applied and left for 1-2 minutes before being cleared with isopropanol. Betadine was then applied to surgical site with cotton tipped applicators for final sterilization.

Following a scalp incision, the periosteum was cleared from the surface of the skull using #5 forceps. Once the skull was completely cleared of connective tissue, sutures were marked using a surgical skin marker (Securline), along with the center position for window implantation (3.5mm lateral, 1.5mm anterior of Bregma for visual cortex). Next, the surface of the skull was lightly scored and a thin layer of cyanoacrylate (VetBond, WPI Inc.) was applied, to provide a substrate to which the dental acrylic could adhere. The headplate was then attached to the skull with black (for light block) dental acrylic (Ortho-jet) and the opening in the headplate was filled with a silicone elastomer (Kwik-Sil, WPI Inc.) to protect the skull. The animal was allowed to recover for several days with free access to food and water before subsequent cranial window surgery.
Following recovery from the headplate surgery, a 5-8mm diameter cranial window (No. 1 coverglass, Warner Instruments) was implanted. Surgical preparation was as above, with the addition of dexamethasone sodium phosphate (2 mg/kg subcutaneously) ~3 hr prior to surgery to reduce brain edema. The anesthetized animal was stabilized by screwing the headplate to a custom machined fork. The silicone plug was removed from the well of the headplate and the skull was cleaned with isopropanol and rinsed with saline. A craniotomy matching the diameter of the window was made with a pneumatic dental drill (Western Tradition) with carbide burs (FG 1/4). To prevent heating of cortex, the drilling of the craniotomy was done slowly, using compressed air to blow off bone chips with a cold sterile saline rinse between short periods of drilling. When drilling across the sutures, care was taken to avoid damaging the underlying sinuses. Once the inner portion of the craniotomy moved somewhat freely when touched, angled forceps were used to carefully remove the center piece of bone, lifting vertically while the skull was immersed in saline. A thin layer of transparent polymer (part no. 3-4680, Dow Corning), used to seal craniotomies in primates (Jackson and Muthuswamy 2008), was applied to the dura surface, just enough to cover the craniotomy, for protection and stability. The coverslip was secured in place using cyanoacrylate applied around the edges of the window followed by dental acrylic. Because the polymer fills the gap between the curved cortical surface and the coverslip, the cortex was not flattened by pressing the coverslip against it. The mice were allowed to recover for at least 4 days before beginning habituation and training.

**Imaging**

Our custom macroscope was based on the tandem lens design used for intrinsic signal imaging (Kalatsky and Stryker 2003; Ratzlaff and Grinvald 1991). Two camera lenses (Nikon 50mm f/1.2 and 105mm f/1.8) were interfaced with a dichroic filter cube (Thorlabs). Blue and green illumination were provided by LEDs (Luxeon Rebel 470nm 70lm and 530nm 161lm) with custom driver hardware using feedback stabilization to control light levels to <0.1% variability during rapid switching, since LEDs are subject to fast current-temperature-brightness dynamics. Blue light illumination was supplied in epifluorescence configuration through the filter cube housing a HQ470/40x excitation
filter, T495lxr dichroic mirror, and HQ525/50m emission filter (Chroma Technology). Green light was supplied directly through a fiber placed obliquely above the brain. The total power of blue light delivered to the specimen was 5-10mW, and because this is spread over a large area it results in illumination intensity less than 0.1mW/mm². At these levels we did not observe bleaching over imaging sessions of multiple hours.

Green fluorescence or reflected light was collected and passed through the filter cube, then focused onto a pco.edge sCMOS detector (PCO Corporation). Images were acquired at 10Hz with 4x spatial binning using Camware software (PCO Corporation), with frame acquisition and LED illumination triggered by TTL pulses from the stimulus presentation computer to synchronize with visual stimulus frames. Illumination intensity, exposure duration, and data storage rate should be balanced to achieve high framerates and fill the dynamic range of the sensor.

Camera lenses allow a relatively high numerical aperture (NA) for light collection, which can also be adjusted easily using the f-stop setting in order to restrict the NA. This permits a flexible trade-off between sensitivity and depth of field, especially as increased depth of field is useful given the curvature of the cortical surface. Imaging was generally performed at an f-stop of 5.6. The ratio of the focal lengths of the two lenses determines image magnification. To map 1cm of cortex across the 2cm detector (6.5um pixels), we chose 50mm and 105mm lenses, yielding magnification of 2.1x and 3.1um specimen pixels. In practice, we find an effective spatial resolution of ~25um, based on the highest spatial frequencies present in non-binned images of vascular structure. Binning across spatially oversampled pixels can reduce shot noise by allowing more total photons to be detected with increased illumination or NA. This is a standard practice in intrinsic signal imaging (Kalatsky and Stryker 2003) and is generally applicable at high light levels where readout noise is negligible compared to photon count noise.

Two-photon imaging was performed using a MOM moveable objective microscope (Sutter Instruments, Inc.) coupled to a Mai-Tai HP Ti-Sapph pulsed laser, with a 16x/0.8NA objective (Nikon). ScanImage software (Pologruto et al. 2003) in Matlab was used for data acquisition, with a custom user function to record TTL output from the stimulus/behavior system on the imaging timebase for later synchronization. Images were
acquired at 3.7fps and 256x256 pixels over a ~400x400um field of view, using 35-50mW illumination power as measured at the front aperture of the objective.

**Stimulus delivery and behavior control**

Visual stimuli were presented on a Viewsonic VA2342 LCD monitor (28 x 50cm, linearized by eye to correct for gamma (mean luminance 35cd/m²), oriented tangentially 25 cm from the mouse’s right eye (see Wiesenfeld and Branchek (1976) for optimal distance in rat vision) in portrait configuration, covering ~60x90° of visual space. Stimuli were generated with custom software using the Psychtoolbox extension for Matlab (Brainard 1997; Pelli 1997). For passive mapping of visual responses, we binarized a 1/f noise stimulus described previously (Niell and Stryker 2008) with spatial frequency corner of 0.05cpd and cutoff of 0.12cpd, and temporal frequency cutoff of 5Hz. This stimulus was binarized to black/white instead of grayscale to increase contrast and generate edges. Note that the hard edges imposed by binarization caused higher spatial frequency overtones.

For mapping of total visual responsiveness, this noise stimulus was presented in blocks of 5secs, with 5secs of gray screen intervening. For retinotopic mapping, this noise stimulus was masked to create a 20deg wide bar that moved across the visual display with a 10sec period (topographic noise stimulus, Figure 4A). In order to define auditory responsive regions, white noise bursts of 50ms duration were presented at 2Hz (450msec inter-stimulus interval) for 5 sec, followed by 5 sec of silence.

Movement of the mouse on the spherical treadmill was measured with a single optical USB computer mouse positioned on the Styrofoam ball, acquired once per stimulus frame (60Hz) in Matlab. Note that because we only used one USB computer mouse to measure locomotion, we do not completely specify the motion of the ball. In order to maintain sensitivity to forward locomotion while maximizing sensitivity to lateral motion (used for behavioral response) the optical mouse was positioned laterally to the subject, midway between the vertical and horizontal axes. Therefore, one coordinate of the mouse’s readout represents direct lateral movement of the subject, and the other coordinate represents a mixing of forward locomotion and rotation around the vertical axis. Therefore, while we can detect forward locomotion (moving vs stationary), there is...
a scale factor in the absolute speed of forward motion, which could be computed based on precise geometry but was not necessary for the current study.

The behavioral control system was based on Meier et al. (2011) with modifications to allow optical mouse input for behavioral report of responses and TTL control of the LEDs and sCMOS camera exposures. Visual stimuli for behavior consisted of 45° diameter circular patches of square wave gratings at random spatial phase with spatial frequency 0.16cpd. Visual stimuli for two-photon assessment of orientation selectivity consisted of full-display drifting square wave gratings of eight evenly-spaced directions of motion, at 2Hz temporal frequency and both 0.02cpd and 0.08cpd spatial frequency. These were presented in randomly interleaved order, along with a gray (linearized mean luminance) blank stimulus, for 4sec, with 4sec gray inter-stimulus interval.

**Behavioral training**

Prior to beginning behavior, mice were handled for several days until they were comfortable with the experimenter. Once water scheduling was begun, animals received water only during and immediately after head-fixed training on the ball. Training sessions increased in duration over the course of 1–2 weeks, from 20 minutes to 2 hours per day. All training was performed with a mostly automated system (Meier et al. 2011). First, mice learned the simple visual task of discriminating the location of a luminance stimulus. Animals were required to request a trial by stopping spontaneous locomotion for 1 second to receive a water reward. Upon requesting a trial, a stimulus was presented with dark on either the top or bottom 2/3 of the screen, and light on the other 1/3. The animal was rewarded with a second water drop for moving right (left) on the ball if dark was on the top (bottom). When an animal could reliably request trials, the water reward for stopping to initiate trials was eliminated. This task took about one week to learn and established trial structure and attention to the visual stimulus.

Once a mouse could perform a significant number of trials and reached ~75% accuracy on the luminance task, they were graduated to the orientation discrimination task. This task followed a similar structure, but a circular grating patch (either horizontal or vertical) was presented in the middle of the screen and the mouse was rewarded for moving left (right) for horizontal (vertical). The stimulus remained on the screen for one second after
correct responses. Incorrect responses triggered a 3.5 sec timeout with potentially aversive flashing error stimulus and 50% probability of a correction trial, where the previous stimulus was repeated until answered correctly, to prevent development of a side bias.

During training, water rewards were calibrated by the experimenter to maintain consistent weights (>80% of baseline), corresponding to ~1.5 ml of water through the course of a session. Mice were trained 7 days per week for 1-2 hours per day, with a session ending when the subject stopped initiating trials. We found that it was important to avoid letting the animals continue for extended periods after satiation, as this reduces motivation and slows training progress.

Imaging during behavior was performed after an animal reliably performed the orientation discrimination at >80% correct. The imaging behavioral configuration was identical to training, except the stimulus remained on the screen for one second even after incorrect responses, replacing the flashing error stimulus used during training, to avoid differences in visual input between correct and error trials.

Data analysis – widefield imaging

To analyze widefield images, 3:1 alternating blue and green frames were separately interpolated to produce a continuous image series at 10 Hz. To account for differences across pixels in expression, illumination, and detection, following standard practice the fractional fluorescence change (dF/F) relative to the mean over the recording period (an approximation of the baseline) was calculated for each pixel in each channel. The apparent baseline showed some non-stationarity, including a decrease over the first few seconds after stimulus onset, likely due to adaptation. If such effects have spatial structure, such as being specific to visual areas, they could potentially distort phase maps, but in our data these non-stationarities were much smaller than visually evoked signals.

The green reflectance image was used to normalize the fluorescence image to compensate for changes in absorbance due to hemodynamics. This can be performed by dividing the normalized total signals (F/Fmean) or approximated by subtracting the fractional changes (dF/Fmean). Division of total signals and subtraction of fractional fluorescence are mathematically identical to first order in the Taylor expansion, and give experimentally
indistinguishable results. Note that because signals at each pixel are normalized to Fmean, this correction does not depend on constant illumination across the field of view. Temporal deconvolution was performed using the Lucy-Richardson non-negative deconvolution function in Matlab, using a bi-exponential convolution function with 180msec rise time and 550msec decay time, based on measured values (Chen et al. 2013). More advanced models of the mapping between neural activity and fluorescence changes could further improve deconvolution (Vogelstein et al. 2009). All widefield data presented was deconvolved except where noted in several examples comparing raw and deconvolved timecourses.

For analysis of block stimuli (Figures 2 and 4), we computed the mean dF/F during stimulus periods minus the mean dF/F during non-stimulus periods to obtain the stimulus-evoked response. For periodic stimuli (Figure 5), we computed the amplitude and phase of the Fourier component of the dF/F signal at the stimulus frequency (0.1Hz). Periodic mapping was also performed immediately before behavioral sessions to allow spatial registration in subsequent analyses.

To generate retinotopic maps from periodic stimuli, we computed the spatial gradient of the phase value for both azimuth and elevation maps, giving the Jacobian, a 4-dimensional measure of relative topography at each cortical location (abstracting the absolute retinotopic location of the stimulus, which can vary between sessions and individuals). The azimuth and elevation components of the Jacobian were individually normalized to unit length, to avoid over-weighting pixels with artifactsually large gradients, such as poorly responsive regions or craniotomy boundaries. The 4-dimensional representation was aligned across imaging sessions (x-y translation and rotation) by maximization of their cross-correlation. While the full 4-dimensional gradient completely describes the topography, it may also be possible to use the visual field sign alone to perform alignment.

To the extent that a given area’s retinotopy is not highly warped, the azimuth and elevation gradients are roughly orthogonal and equal magnitude, which implies its topography can be summarized by a single angle of orientation and a single binary chirality (excluding within-map variations in “cortical magnification”). Chirality can be
quantified by the “visual field sign” (Sereno et al. 1994), defined as the sine of the angle between the azimuth and elevation gradients, equivalent to the z-component of the cross-product between unit vectors along the gradients of the two maps. It is therefore a binary (for orthogonal components) function of the Jacobian whose sign indicates mirror reflected topography. It does not preserve information within region interiors, eliminating both sensitivity to potentially differential “cortical magnification” within regions and the opportunity for non-rigid within-region alignment across subjects or development.

Two methods were used for determining boundaries between retinotopic regions. In an automated partitioning approach (Fig 5E), we use a watershed transform of retinotopic position, supplemented by visual field sign boundaries. The zero contour of visual field sign identifies most region boundaries, but misses those across saddle points such as between V1 and AM. We therefore performed a watershed transform of the retinotopic distance from the centroid of locations represented by the 30% most responsive pixels, followed by hmin smoothing of 1.22% of the standard deviation of distances, which delineated most region boundaries, including those that visual field sign missed. To generate a final partitioning, we took the union of the watershed and visual field sign boundaries and labeled areas that corresponded to published extrastriate maps. We note that using a lower hmin smoothing factor allowed all known boundaries to be identified without using visual sign, but led to additional subdivision within regions.

Alternately, distinct retinotopic areas were delineated for presentation by mapping the visual field sign and one axis of the azimuth gradient into RGB space. Regions where the visual field sign was negative were labeled red, and positive were labeled green. Because this does not unambiguously describe the local gradient, we also mapped the sign of the x-component of the azimuth gradient into the blue channel. Finally, the absolute amplitude of the local gradient was mapped into saturation of the RGB signal, so that regions with minimal gradient, representing poor topography, appeared white.

Behavioral data was analyzed by aligning imaging frames to stimulus onset, and computing the median dF/F across trials at each timepoint. The resulting average timecourse was then temporally deconvolved as described above. Performing
deconvolution prior to averaging gives nearly identical results, but is far more computationally demanding.

Data analysis – two-photon imaging

Two-photon image data was first spatially aligned, using phase correlation to estimate x-y translation (courtesy of D. Ringach). Similar results could be obtained using imregcorr in Matlab. Cell body ROIs were extracted semi-automatically, by first manually selecting a pixel in the cell body from the mean fluorescence image and then automatically extracting all pixels within a 20um window that had >0.8 correlation coefficient in fluorescence timecourse with the selected pixel. This provided a clear outline of the cell body in nearly all locations selected that corresponded to a labeled neuron in the fluorescence images. The final signal for each cell was computed by taking the median dF/F timecourse across selected pixels, although this doesn’t account for differences in SNR across pixels. Neuropil fluorescence was computed by extracting the mean dF/F in a region around the cell, with identified cell bodies removed. This signal was multiplied by a correction factor (ranging from 0.6 to 0.9) based on the fluorescence intensity in blood vessels, and subtracted from the cellular dF/F to give the corrected timecourse of activity. Deconvolution was not applied to two-photon data.

For analysis of drifting gratings, the mean dF/F during the second half of the preceding inter-stimulus interval was subtracted from the mean dF/F during stimulus presentation, to compute the evoked response. Visually responsive units were defined as those with a significantly different response to at least one grating stimulus relative to the gray blank condition (students t-test, p<0.05 with correction for multiple comparisons). Although this is a standard measure, response distributions are not Gaussian, violating the preconditions for Student’s t-test; the lower power Komolgorov-Smirnof may be more appropriate for future studies. Orientation tuning curves were computed at the spatial frequency that gave the strongest response for each cell, by computing the mean over the seven repeated presentations of each stimulus condition. The orientation selectivity index (OSI) was computed from the tuning curve as in Niell and Stryker (2008) and Chen et al (2013) as (R_{pref}-R_{orth}) / (R_{pref}+R_{orth}), from a fit to a sum of two wrapped Gaussians. Pixel-
wise maps of orientation selectivity were computed in a similar manner, but based on the fluorescence trace at each pixel rather than summed over cell body ROIs.

Analysis of responses to locomotion was performed by first calculating the fractional fluorescence change for each cell (or individual pixel for pixel-wise maps) as described above, and then calculating the correlation coefficient between the fluorescence trace and running speed as measured by the optical mouse. Direct observation ruled out the possibility that low speeds corresponded to locomotion around the axis to which our single optical mouse was insensitive. Statistical significance was determined by comparing the measured correlation coefficient with the distribution of correlation coefficients computed for all possible temporal shifts. Units with a z-score of >2.6 (corresponding to p<0.01) relative to the shuffled distribution were considered significant. Data was pooled across the subjects, disregarding potential correlations of neurons within subjects. Although this is typically ignored, it may be significant when neural activity is largely influenced by subject-specific factors, as in spontaneous locomotion.

Unless otherwise noted, summary statistics are presented as medians with error bars representing bootstrapped confidence intervals.

**Results**

*A transgenic mouse expressing an ultra-sensitive reporter of neural activity throughout cortex*

With the goal of obtaining robust expression of GCaMP6s across the cortical surface, we generated a transgenic mouse expressing GCaMP6s under the control of a tetracycline-responsive promoter element (TRE; tetO). (Fig 1A). Following confirmation of transgene incorporation by PCR, we screened for GCaMP6s expression after crossing to a CaMK2-tTA driver line (Mayford et al. 1996). CaMK2 is expressed in most cortical excitatory cells, but not inhibitory GABAergic cells (Benson et al. 1992). In one founder line, we found strong expression throughout cortex, with a distribution that approximates the expression of the tTA driver. No fluorescence was observed in mice that were not crossed to the tTA driver line, confirming that expression is dependent upon the driver.
Expression was found across the extent of cortex, in a large fraction of cells in all cortical layers (Fig 1 B-D). *In situ* mRNA hybridization showed that GCaMP6s was co-expressed in most CaMK2-positive neurons (212/247 cells, 86 +/- 4% s.d., across three cortical locations). Furthermore, cells that expressed GCaMP6s always expressed CaMK2 indicating that expression is in excitatory neurons, consistent with the driver. The reliability of co-expression suggests that the observed variability in label density across cortical layers reflects the density of CaMK2-positive neurons. Importantly, we did not find evidence of fluorescence filling neuronal nuclei (Fig 1E), which has previously been correlated with cellular toxicity resulting from GCaMP over-expression (Tian et al. 2009). Although our expression construct included a nuclear-localized mCherry reporter, we did not detect red fluorescence above wild-type background, perhaps due to poor IRES-mediated downstream gene expression.

**Widefield mapping of neural activity across the dorsal surface of cortex**

In order to image fluorescence signals across broad regions of cortex with adequate spatial and temporal resolution to isolate region-specific activation during behavior, we implemented a widefield epifluorescence system based on the tandem lens configuration commonly used for intrinsic signal imaging (Ratzlaff and Grinvald 1991). This consists of two 35mm camera lenses with an epifluorescence filter cube in between, projecting onto an sCMOS camera detector (Figure 2A). To image brain regions over multiple sessions, we implanted a metal headplate for head-fixation and performed a craniotomy up to 8mm in diameter, covered with a transparent polymer and a glass coverslip, to create a chronic cranial window. Ideal surgical outcomes result in a window that remains viable for 6 months or more. Head-fixed animals were placed on a spherical treadmill allowing locomotor behavior during imaging (Dombeck et al. 2007).

Illumination of cortex with blue light, in the transgenic GCaMP6s mouse line, results in bright fluorescence across the cortical surface (Fig 2B). In order to assess visual responses across areas of potentially dissimilar sensitivities, we presented binary spatiotemporal noise approximately distributed over the sensitivity range of reported mouse visual cortical areas (Marshel et al. 2011; Niell and Stryker 2008), which appears...
as full contrast moving edges (Movie S1 inset). To maximize responses, this was presented as a periodic stimulus (5 sec on, 5 sec off).

Since blood absorbs light through a wide band including the GCaMP excitation (blue) and fluorescence emission (green) wavelengths, changes in blood volume significantly distort single photon \textit{in vivo} GCaMP measurements (Fig 3B,E). This effect needs to be corrected, as it is much larger and less directly coupled to local neural activity than the intrinsic signal under red illumination, which arises from changes in hemoglobin oxygenation. Cortical reflectance in the green wavelengths that do not excite GCaMP provides a relatively isolated measure of the fraction of signal lost to hemodynamic absorbance. Normalizing the blue signal to this amount provides an estimate of the corrected fluorescence signal.

To perform the correction, on every 4\textsuperscript{th} frame we interleaved an image acquired with green, rather than blue illumination. Compared to the mixed signal under blue illumination, the green signal varies more slowly, with more delay from stimulus onset, and is strongest around vasculature (Fig 3A,D; B,E), where it approaches the scale of fractional change in GCaMP fluorescence under blue illumination.

We computed the corrected signal based on interpolated fractional changes, giving a result that was closely locked to the visual stimulus (Fig 2C,D, green). Finally, temporal deconvolution using the empirically measured GCaMP6s time constants (Chen et al. 2013) provided even greater temporal resolution (Fig 2D, black) and a close match to the temporal pattern of multi-unit neural activity recorded in V1 (figure 2D, blue).

Although the green reflectance subtraction cannot provide a complete correction, particularly since hemoglobin absorbance is slightly higher for blue than green light (Horecker 1943), it clearly provides a significant reduction in hemodynamic signals, especially around vasculature (Figure 3C). Future studies may improve on this correction method using measurements at multiple wavelengths to more accurately estimate the hemodynamic signal (Yoshida et al. 2015).

We note that this correction is not simply masking blood vessels, but preserves the underlying change in fluorescence, as seen in Figure 3F. Because regions with vessels are
darker, they emit fewer photons which will result in higher shot noise and slightly lower SNR around vessels, although this was not apparent in resulting maps.

This visual stimulus resulted in evoked activity in posterior areas, corresponding to the known location of visual cortex (Figure 4A,B). The individual frames in Fig 4A, as well as Supplemental Movie 1, demonstrate that activation was clearly visible on individual cycles without averaging. Averaging across cycles allowed evoked activity to be isolated from ongoing endogenous activity (Figure 4B).

We compared the spatial distribution of visually evoked activity with two other modalities. Vibrissal stimulation evoked activity in lateral cortex (Figure 4C), the known location of primary barrel somatosensory cortex, as well as midline regions corresponding to vibrissal motor cortex. Spontaneous locomotion (Figure 4D) activated regions of motor cortex and limb sensory cortex, together with weaker activation in posterior visual areas consistent with previously described locomotion signals (Andermann et al. 2013; Keller et al. 2012; Saleem et al. 2013). Figure 4E shows maps from the three modalities overlaid.

**Retinotopic mapping to identify cortical areas across subjects**

To map retinotopy in visually responsive areas, we used the periodic encoding method employed in both human fMRI and mouse intrinsic signal imaging (Engel et al. 1997; Kalatsky and Stryker 2003). In this approach, a stimulus periodically sweeps across the display. Assuming eye movements are unrelated to stimulus location, the amplitude of the Fourier component of the activity time series for each pixel at the sweep frequency represents how strongly activity at each cortical location depends on stimulus location, while the phase corresponds to the stimulus location in retinotopic space. Although we did not measure eye movements, the consistent maps we recorded suggest eye movements were substantially unrelated to the stimulus under these conditions.

In order to evoke stronger visual responses than the typical, luminance bar or checkerboard, we used a vertical or horizontal bar of the binary spatiotemporal broadband noise described above (Figure 5A inset and Supplemental Movie 2). The sweep (10sec period) elicited corresponding waves of activity across the visual cortex (Figure 5A, Supplemental movies 2, and 3). Separate bands moving across primary visual
cortex and smaller patches corresponding to extrastriate areas are easily distinguished. Indeed, clear retinotopic responses could be observed in single trials with no averaging (Supplemental movie 2), although averaging over stimulus cycles removed background cortical activity to isolate the stimulus-locked signal (Figure 5A, Supplemental Movie 3).

Both the azimuth and elevation topographic noise stimuli elicited strong periodic responses, with fluorescence changes up to 25% (mean = 19.3 +/- 5.8% s.d., n=15 sessions in 5 mice). These resulted in clear topographic maps revealing a number of retinotopic areas with 5 minutes or less of imaging time (Figure 5B-D). Analyzing the correlation between maps obtained from shorter intervals of the data demonstrates that a single 10sec trial shows 85% correlation with the final map, and that one minute of data provides a nearly identical map (Figure 5B). Because the timecourse of the mapping stimulus (10sec) is slow relative to the GCaMP6s time constant, deconvolution did not have a large impact on the results, although it can be seen that the raw signal is slightly delayed due to temporal summation and the slow decay (Figure 5E).

In order to align maps across sessions and identify extrastriate areas across individuals, we computed the Jacobian (the concatenation of gradients in each direction) of retinotopic position, confirming that it is roughly constant within a given visual area, but unique across neighboring areas, as gradients are naturally sensitive to reversals. The Jacobian represents the progression of retinotopic position at each cortical location relative to neighboring cortex, while discarding differences in absolute positioning across sessions and individuals, eliminating error that would arise from attempting to align position directly.

The population we studied (male and female mice, age 2-8 months) was sufficiently uniform that rigid alignment by maximizing cross-correlation of the Jacobian yielded aligned topographic maps with an average pairwise correlation of 0.90 +/- 0.05 s.d. across 15 sessions in 5 mice. Averaging across sessions and subjects yields the pooled retinotopic maps shown (Figure 5F,G).

In order to define the boundaries of retinotopic areas, we used two approaches that both included the chirality of the azimuth and elevation maps, known as the visual field sign, developed by Sereno et al. (1994) and recently applied to mouse cortex by Garrett et al.
A watershed transform of retinotopic position, together with visual field sign boundaries, clearly isolates V1 plus 6 additional extrastriate areas (Figure 5H). The areas can also be directly visualized by mapping the visual field sign and one specific component of the Jacobian into RGB space (Figure 5I). Both methods generate a layout of extrastriate areas consistent with recent anatomical and functional studies (Garrett et al. 2014; Wang and Burkhalter 2007), including the visual field biases present in some regions such as the over-representation of the medial visual field in RL and over-representation of lateral visual field in AM and PM (Garrett et al. 2014).

**Imaging activity across cortex during a visual discrimination task**

We next measured task-specific activity across these demarcated cortical areas while mice perform a visual discrimination task. We trained mice to indicate the orientation of a grating (horizontal or vertical) by moving a threshold distance either left or right on the spherical treadmill for water reward (Figure 6A). Mice initiated trials by remaining stationary for 1 second and were free to decide when to respond. Mice learned this task to high accuracy (>90%) in 3-4 weeks and performed hundreds of trials in each 1-2 hour session (mean = 245 +/- 90 s.d.). On typical trials, mice begin movement 0.25-0.35 secs after stimulus onset and cross threshold between 0.3-0.7 secs (Figure 6B). Accuracy was high within this window, whereas earlier and later responses drop to chance (Figure 6C). These tended to occur at the end of sessions (Figure 6D), presumably due to mice disengaging from the task structure during periods when they are too frantic, satiated, fatigued, or distracted. In human behavioral studies, trials with long reaction times also show poorer performance (Weissman et al. 2006).

Aligning the deconvolved neural activity signal to the stimulus onset reveals a progression of activity across cortical areas in the range of hundreds of milliseconds, during which the animal makes the perceptual judgment and performs the motor output to report a decision and receive the reward (Figure 7A,B). Average activation maps for three individual subjects show similar, though not identical, patterns of activity (Figure 7B). In order to align across subjects, behavior sessions included 5 minutes each of mapping stimuli along azimuth and elevation. By averaging all correct trials that crossed...
response threshold between 0.4-0.6 sec, we observed the timecourse of activity in each
cortical area (Figure 7C,D). The locus of early activity is in extratiate regions lateral to
V1 that are proposed to be members of a “dorsal” stream homologous to that in primates
(Wang et al. 2012). V1 is most active at intermediate times. In the last few hundred
milliseconds, as expected during locomotor response, activity was strongest in the same
area that was most active during spontaneous locomotion (Figure 4D) that likely includes
hindlimb S1 (Fig 7C,D red). Notably, the same temporal pattern of activation was present
in the raw data before deconvolution, although the dynamics are greatly broadened and
less tightly locked to behavioral epochs, due to the relatively slow dynamics of GCaMP6s
(Figure 7E).

Curiously, a small region between lateral extrastriate, barrel, and auditory cortex is active
throughout the trial (Fig 7C arrow). The next section will return to this poorly understood
area, which we refer to as area 39 based on Krieg (1946), although subsequent studies
have assigned other designations. Lip and tongue representations in S1, which would
presumably be activated by licking and reward consumption, are beyond the anterior
lateral extent of the window.

Two-photon imaging of individual neuronal responses in behaviorally identified networks
Although the macroscopic scale examined up to this point provides a global network
view of cortical processing, ultimately understanding the computations being performed
requires access to activity in populations of individual neurons. Therefore, we conclude
by examining the activity of individual neurons in area 39, the region identified at the
macroscopic scale as active throughout the behavior.

First, to confirm that GCaMP6s expression in this mouse line does not disrupt response
properties and that the expression is adequate for two-photon measurements at
illumination levels low enough to avoid gross damage, we measured orientation
selectivity in V1 neurons, which has been well-characterized in previous studies (Chen et
al. 2013; Niell and Stryker 2008). Supplemental Movie 5 shows the robust V1 response
to drifting square-wave gratings at two spatial frequencies (0.02 and 0.08cpd) and eight
evenly-spaced orientations/directions of motion. Figure 8A shows baseline fluorescence
of a typical field of view, confirming clear exclusion of GCaMP6s in nuclei in vivo.
Figure 8B shows the pixel-wise responses, collapsing across both spatial frequencies and opposite directions of motion, showing a large fraction of cells with strong responses and high orientation selectivity. 75% of cells (357/479 cells; 3 mice) were responsive to this stimulus set, and 98% of responsive cells (349/357 cells; 3 mice) had an orientation selectivity index (OSI) greater than 0.33, corresponding to a 2:1 preference for optimal versus orthogonal stimuli. The median dF/F for responsive cells was 0.46±0.25 s.d (Figure 8C). The distribution of OSI across the responsive cells (Figure 8D) was similar to that measured previously in layer 2/3, both by electrophysiology (Niell and Stryker 2008) and two-photon imaging (Chen et al. 2013), although it is important to keep in mind that estimates of tuning curves with calcium imaging are susceptible to distortion due to nonlinearities of the indicator (Nauhaus et al. 2012). Notably, two-photon data were acquired using illumination intensities between 35-50mW at the specimen, a typical level for in vivo studies that does not cause significant cortical damage over multiple imaging sessions.

We next used widefield imaging to test whether we could elicit activity in area 39 in simpler contexts than the visual orientation discrimination task, and determine its precise location relative to other sensory cortices. Although the area was not strongly driven by visual or auditory input sufficient to drive other areas, it was highly active during spontaneous locomotion in mice trained on the orientation discrimination (Figure 9A), but not as clearly activated in untrained mice (data not shown). Without specific visual stimulation (dimly lit laboratory surroundings), two-photon imaging at the location targeted on vasculature (Figure 9B) revealed activity in many area 39 neurons to be either positively (45 +/- 6%) or negatively (18 +/- 5%) correlated with spontaneous locomotion (Figure 9C,D,E,F; 284 cells; 3 mice, see methods for caveats on pooling data). This was in contrast to V1 where, consistent with previous studies (Andermann et al. 2013; Erisken et al. 2014), we found a much smaller fraction of cells modulated by running, 8 +/- 2% positively and 9 +/- 2% negatively (489 cells in 3 mice). This also indicates that the strong correlations in area 39 were likely not due to motion artifacts coupled to locomotion.

These results establish a potential connection with the locomotor component of the discrimination task, which required the subject to first remain stationary to request a trial, and then locomote to indicate response. While additional functional properties of this...
region remain to be investigated, these data provide a direct demonstration of the capacity
to identify cortical areas active during behavior and probe their properties at the cellular
level.

Discussion

Widefield imaging of neural activity across cortex
The widefield imaging approach and transgenic GCaMP6s mouse line described here
address the need for a high spatial and temporal resolution method to map the dynamics
of neural activity across cortical areas during behavior. As we demonstrate, this enables
the direct visualization of multiple regions involved in stages of a task, allowing
identification and characterization of structures that might not be expected to be involved.
Paired with subsequent two-photon imaging, this integrates studies of the temporal
dynamics of brain function from the global level to local networks.

Previous studies have used intrinsic hemodynamic signals (Kalatsky and Stryker 2003;
Schuett et al. 2002), metabolic autofluorescence (Andermann et al. 2011; Tohmi et al.
2014), or GCaMP3 (Issa et al. 2014; Marshel et al. 2011) to demarcate cortical regions
based on sensory stimuli. However, these studies did not demonstrate the temporal
resolution and sensitivity that are needed to analyze activity on the timescale of
perception and behavior. Widefield imaging of voltage signals in mouse cortex, either
with voltage-sensitive dyes or fluorescent proteins (Akemann et al. 2012; Ferezou et al.
2006; Mohajerani et al. 2013), has much higher temporal resolution than calcium
imaging, but generally has smaller fractional changes with activity and has yet to be
paired with controlled behavioral tasks.

The method we describe here tracks the dynamics of activity across functionally defined
regions, with different areas showing distinct profiles of activation relative to the task
(Figure 7). Importantly, we show that behavioral imaging data can be aligned with
retinotopically mapped areas, and employ a quantitative method to assist in demarcation
of discrete extrastriate regions. Furthermore, this method extends to imaging over large
regions of the dorsal surface of cortex, enabling study of the relation between multiple
modalities (Figures 4 and 9A and see Glickfeld et al. (2014)).

Broad and robust expression of GCaMP6s using a transgenic mouse line
Imaging large brain areas simultaneously requires broad labeling with a fluorescent indicator. In contrast to viral transduction or *in utero* electroporation, both of which are limited to relatively local labeling, the extent of expression using a transgenic mouse line can be determined primarily by the selected driver line. Here we show strong expression in a high proportion of excitatory cortical neurons by using a CaMK2-tTA driver with a tetO-responsive GCaMP6s line. Furthermore, using viral delivery or electroporation can result in inconsistent targeting and expression levels, whereas expression in the transgenic line does not require an invasive step and is consistent within strain.

Expression level is particularly important with genetically encoded calcium indicators, as expression that is too low prevents accurate measurements of activity, whereas expression that is too high is detrimental to the health of neurons (Tian et al. 2009). Fortunately, the level of expression in this line, when crossed to the CaMK2a-tTA driver, falls within the usable range for both widefield imaging and extended 2-photon imaging, without exhibiting the filled nuclei indicative of cellular toxicity. In addition, because transcription from the tetO promoter can be controlled by doxycycline, expression can be silenced until shortly before the experimental period. This may be particularly useful for driver lines active early in development, when high levels of an exogenous calcium buffer such as GCaMP6 might be deleterious, or if driver lines for other cell types result in unhealthy levels of expression.

Importantly, the imaging paradigm presented here should be broadly applicable to other GCaMP6 mouse lines that are developed, such as those that do not require a separate driver line (Dana et al. 2014) or those where expression is dependent on Cre recombinase (Madisen et al. 2015). In addition, because this line is driven by the tetO system, it provides an orthogonal targeting scheme to Cre control that may be useful for other imaging approaches.

In this study, although GCaMP6s was expressed in excitatory cells across cortical layers using a CaMK2-tTA driver, widefield signals likely originated mostly in layers 1 and 2/3, due to the high attenuation of blue light in tissue. In addition to neurons in these layers, processes from neurons in deeper layers together with projections from other cortical areas likely contribute to the activity observed. Also, although previous studies with
GCaMP6 focus on action potential responses, it is possible that subthreshold calcium
signals are present as well.

Driver lines that target more specific cell types could help isolate activity in particular
populations. It will be especially interesting to determine the activation of different
inhibitory cell types during behavior, where they may control the level of activation or
synchrony based on behavioral state and task demands (Fu et al. 2014; Pi et al. 2013;
Tiesinga et al. 2008). This imaging approach could also be applied with other optical
measures of neural activity, such as voltage indicators, which might allow higher
temporal resolution.

Imaging and analysis considerations

The signal-to-noise ratio provided by the combination of the ultra-sensitive GCaMP6s
indicator, the sCMOS detector, and high numerical aperture lenses provided high spatial
resolution, sufficient to detect activity structure within extrastriate areas. The stimuli we
used generated retinotopic maps with features as small as 50um. This was also the
smallest observed scale of activity correlations during behavior. However, this is coarser
than the spatial resolution measured by surface vasculature, presumably because
widefield fluorescence imaging samples a large volume in depth outside of the focal
plane.

The high signal-noise ratio also facilitates temporal deconvolution, which can be
susceptible to noise. Although the decay time constant of the GCaMP6s response is
roughly a half second, high frequency information is available in the faster rising edge,
and deconvolution revealed activity changes at least as fast as our 100 ms imaging rate.
Even faster imaging is possible, but high frequency information is ultimately swamped
by shot noise and detector noise. Faster GCaMP6 variants exist, but have lower signal
amplitude, so it remains to be determined whether deconvolution of signals from these
indicators would net an improvement. In general, the large responses of the slower
GCaMP6s used here may be preferable in many cases when measuring amplitude of
responses over slower timescales (such as retinotopic mapping), whereas faster indicators
may be preferable when the highest temporal resolution is essential.
Although deconvolution improves the interpretability of calcium imaging data in some respects, principally by reducing temporal summation and the long decay time, resulting in a potentially closer match to neural activity, it does have its own caveats. In particular, inaccurate fitting of the temporal response kernel can cause responses to shift forward in time excessively, although this is less likely due to the asymmetric nature of the measured kernel. Also, deconvolution assumes a linear response, which may result in distortion of large signals and inaccurate summation of temporally overlapping signals such as sensory stimuli and motor outputs. Thus, although the deconvolved signal should generally be closer to neural activity, it will not be perfect and should be interpreted carefully.

One notable limitation to the widefield imaging approach is that not all cortical areas are readily accessible for imaging, even in a lissencephalic species such as the mouse. Those that are buried in the midline or require difficult surgical approaches may not be conducive to recording in alert animals. Systems involving prisms or endoscopes (Andermann et al. 2013; Ziv et al. 2013) may permit imaging of deeper structures, but would likely not allow the large field of view available on the dorsal surface.

_Imaging multiple visual areas during perception and behavior_

The mouse has emerged as an important model system for visual function, as it shares many aspects of both local visual processing (Hubener 2003; Huberman and Niell 2011) and organization of higher visual areas (Glickfeld et al. 2014) with cats and primates including humans. Notably, in addition to primary visual cortex, there are at least 7 retinotopically mapped extrastriate visual areas in posterior mouse cortex, and both connectivity and functional properties suggest an organization into two streams analogous to the dorsal and ventral streams of primates (Andermann et al. 2011; Marshel et al. 2011; Wang et al. 2011). Although differential tuning for stimulus properties such as spatial and temporal frequency have been described, specific roles in visual behavior for each area remain to be determined.

Here we have demonstrated an integrated approach to studying the functional role of the constellation of cortical visual areas. We begin by mapping the visually responsive regions with a high signal-to-noise ratio that allows us to align across sessions and across
animals, which enabled differentiation of activity in identified extrastriate areas during visual behavior. New statistical methods for delineating retinotopic areas (Garrett et al. 2014) may benefit from the higher SNR provided by GCaMP6s imaging over intrinsic signal imaging, helping to solidify the demarcation of mouse extrastriate cortex. We also expect the higher SNR to allow efficient retinotopic mapping using non-periodic stimuli, thereby avoiding potential confounds due to spatiotemporal correlations in sweeping stimuli such as anticipation, adaptation, and pursuit-type eye movements.

For the orientation discrimination task presented here, our data reveal a distinct pattern of activation following stimulus onset, with an extrastriate region reaching its peak most rapidly, followed by V1 and another set of extrastriate areas, and finally somatosensory areas that are coupled to the locomotor response (Figure 7). The continuing rise in V1 response may be due to sustained visual input, locomotor signals, recurrent local activity, or top-down feedback from extrastriate and/or cognitive areas. On the other hand, the fast activation in extrastriate areas may also represent anticipatory potentiation, other task-specific signals (Cardoso et al. 2012), or activity in intracortical axonal projections into the region. We bear in mind that the dynamics of each cortical area may not be mutually comparable – even if initial activation progresses feed-forward from V1, rise-times and peak activations could differ simply due to differing internal network structure. This finding thereby opens up future studies of the intracortical circuits that determine the dynamics of neural activity specific to a perceptual task. It will also be informative to measure how activity profiles change over the course of training, beginning with naïve subjects.

In the final phase of this study, we used two-photon imaging to target networks of neurons in a functionally defined region. In this way, it is possible to bridge global dynamics down to ensembles of individual neurons within subject. Specifically, we measured the activity during locomotion in a parietal association area between visual, auditory, and somatosensory cortex. Interestingly, this area is adjacent to AL, which has been shown to respond to high speeds of visual motion as might be encountered during locomotion (Andermann et al. 2011), as well as to extrastriate visual areas that receive input via an extrageniculate pathway through lateral posterior nucleus of thalamus (Tohmi et al. 2014).
Krieg identified this region in rat as receiving projections from the three surrounding secondary sensory cortices, naming it “area 39” in homology with Brodmann's name for the inferior part of posterior parietal association cortex in primates, an area known for sensory integration that had been found to perform executive functions after lesions to premotor frontal cortex (Krieg 1947; 1946). Caviness confirmed the area was cytoarchitecturally distinct in mouse, but renamed it “area 2”, implying a predominantly somatosensory function and arguing that "it is doubtful that this cortical region of the rodent will prove to subserve complex functions comparable to those associated with field 39 in the primate brain" (Caviness 1975). Later studies in rats and mice support Krieg over Caviness, showing these higher-order regions between the primary sensory cortices to exhibit polymodal responses (Olcese et al. 2013), potentially co-registered feature maps (Brett-Green et al. 2003), connectivity with multiple sensory and executive areas (Hishida et al. 2014), recognition of stimuli as behaviorally significant (Geissler and Ehret 2004), and involvement in navigation and intentional movements (Whitlock 2014). The activity we observed in this area may correspond with recognizing the behavioral relevance of the visual discriminandum and/or the correlated visual, somatosensory, and auditory sensations involved in the learned skills of controlling the treadmill and collecting the reward.

Beyond the demonstrated application to visual discrimination, we anticipate that the methods we describe will be broadly applicable to other sensorimotor and cognitive tasks. More generally, techniques such as this may enable linking features of brain-wide networks to those of cellular ensembles, which will likely be essential to understand the neural basis of perception and action.
Acknowledgements

We would like to thank K. Gebreab, E. Fenstermacher, and R. DiRicco for assistance with behavioral training; A. Mosman for providing histology; U. Hostick and the UO Transgenic Mouse Facility for assistance in generating mouse lines; H. Wu and C. Kentros for assistance with in situ analysis; Dr. D. Kim and the GENIE Project for providing GCaMP6 plasmids; Dr. D Kleinfeld for suggesting the method to correct for hemodynamic signals; and Dr. M. Wehr, Dr. M. Smear, and Dr. S. Gandhi for comments on the manuscript.

Grants

This work was supported by NIH T32 HD007348 and F31 EY025459 (J.W.), UO Cognitive & Systems Neuroscience Graduate Training Grant (E.D.F.), NIH DP2 EY023190, NIH R01 EY023337, and the Searle Foundation (C.M.N.).
Figure Legends

Figure 1. A transgenic GCaMP6s reporter mouse.
A) tetO construct used to generate transgenic mouse line. B) Isolated brain from GCaMP6s mouse crossed to CaMK2 driver, viewed under fluorescence dissecting scope, demonstrating strong expression restricted to cortex. Scalebar 5mm C) Low magnification coronal view, showing broad expression throughout cortex. Scalebar 2mm. D) High magnification coronal view, showing expression throughout the layers of cortex. Scalebar 500um. E) Confocal section showing that majority of neurons express GCaMP6s, with negligible filling of cell nuclei. Blue = DAPI counterstain. Scalebar 50um.

Figure 2. Widefield imaging of neural activity
A) Diagram of imaging and head-fixation setup. B) Widefield fluorescence image of GCaMP6s fluorescence in a cranial window. Inset shows positioning of cranial window. C) Fluorescence trace from a single 26um pixel, after blue-green subtraction, showing large step increases in fluorescence locked to timing of a 5sec on / 5 sec off noise stimulus. D) Cycle-averaged traces from the data in C, before (green) and after (black) temporal deconvolution, showing agreement with multi-unit electrophysiological recording (blue). Stimulus duration shown in grey.

Figure 3. Correction for hemodynamic signals. A-C) Fourier phase maps in response to a 5sec on, 5sec off binary noise stimulus (as in Figure 2), showing significant vascular artifact present in green (A) and blue (B) illumination, but greatly reduced in the subtraction (C). D-F) Time course of signals for point shown by circle in A-C, demonstrating that blue-green subtraction eliminates a delayed, slow dip evident in the green reflectance signal. Stimulus duration shown in gray.

Figure 4. Cortex-wide mapping of sensorimotor modalities.
A) Individual frames from two cycles (top and bottom rows) of a block-presentation visual noise stimulus, showing visually evoked activity superimposed on spontaneous activity. White arrowhead denotes location of visual cortex. B) Average response to presentation of visual noise stimulus. C) Average response to tactile whisker stimulation.
D) Average activity during periods of locomotion. E) Overlay of responses during visual stimulus, whisker stimulus, and locomotion, demonstrating partitioning of cortex by functional properties. Average maps are based on 5 minute imaging sessions. Scalebar 2mm.

**Figure 5. Mapping and alignment of retinotopically defined areas.** A) Individual frames from the cycle-averaged response to the topographic noise stimulus, showing that the moving stimulus evokes corresponding moving bands of neural activity in multiple cortical areas. See supplementary Movie 3 for full movie. B) Reliability of maps from short subsets of data, measured by correlation with map obtained from full 5 minutes of data. C,D) Phase maps from one session in one mouse, demonstrating retinotopy in azimuth (C) and elevation (D). Color represents spatial position in degrees, based on phase of the Fourier response and the position and size of the monitor. Brightness represents Fourier amplitude. E) Timecourse for an individual pixel in (C) before and after deconvolution. F,G) Average retinotopic maps (azimuth and elevation, respectively) resulting from alignment across subjects and sessions, with correspondence to known extrastriate areas based on demarcation in I. H) Overlay of visual field sign boundaries (black) with watershed transform of retinotopic location (gray) delineates known retinotopic regions. I) Color-coded map shows patches of consistent gradient and sign, representing discrete retinotopic regions. Assignment of cortical area identity in H, I and wireframe boundaries in I are based on previous anatomical and imaging studies. Putative assignment to processing streams, based on Wang et al. (2012) is shown in blue (ventral) and magenta (dorsal). F, G, H, I are averaged across 15 sessions in 5 mice. Scalebar in all panels is 500um.

**Figure 6. Head-fixed visual discrimination task.** A) Schematic of head-fixed behavior paradigm, where mouse must run on the ball left or right depending on orientation of the stimulus. B) Distribution of response times from 15 sessions in 5 mice. C) Performance as a function of response time for sessions in B, showing high performance during an optimal response window. D) Median response time and percent correct through session duration, showing drop in performance toward the end of session.
Figure 7. Imaging spatiotemporal dynamics of cortical activity during a visual discrimination task.

A) Tracks of locomotor response during behavior for trials where the animal responded left versus right. Correct trials are labeled green, incorrect trials are labeled magenta. B) Average neural activity in individual subjects averaged across multiple imaging sessions, for trials when the animal responded between 400 and 600msec after stimulus onset, showing distinct patterns of activation across cortical areas at 100msec intervals. Each row represents an individual subject. C) Average neural activity aligned across 15 sessions in 5 subjects, with overlay of putative area boundaries from corresponding retinotopic mapping, showing temporally specific activation of restricted cortical areas that is consistent across subjects. Scalebar 1mm. D) Average timecourse at three cortical locations from data shown in C), at locations marked by colored squares in the right panel. Response is normalized to maximum for each location to demonstrate differences in temporal activation. Gray box shows response window.

Figure 8. Two-photon recording of passive visual responses in V1.

A) Baseline fluorescence image of a typical field of view in upper layer 2/3 of primary visual cortex. B) Pixel-wise map of orientation selectivity as measured with drifting gratings of two spatial frequencies and eight orientations, showing a large number of responsive cells with nearly all showing orientation selectivity. C) Histogram of visually-evoked dF/F for all cells recorded (n= 479 cells in 3 mice). D) Histogram of orientation selectivity for all visually responsive cells (n= 353 cells in 3 mice). Scale bar in A, B is 50um. Error bars represent bootstrapped confidence intervals.

Figure 9. Targeted two-photon recording in area 39 during spontaneous locomotion.

A) Overlaid widefield mapping of response to visual white noise stimulus. Scalebar 1mm. B) Widefield mapping of activity during spontaneous locomotion superimposed on an image of the vasculature, used to identify region for 2-photon recording. Scalebar 1mm. C) Pixel-wise correlation map between fluorescence traces and locomotion, showing neurons positively correlated with locomotion (red) and negatively correlated (blue). Scalebar 250um. D, E) Example fluorescence and locomotion traces from two neurons in C, showing activation correlated with locomotion (D) and activation correlated with stationary periods (E). F) Histogram of correlation between fluorescence...
and locomotion for individual cells, with green representing cells that are significantly
positively correlated and red representing cells that are significantly negatively correlated
(n=284 cells in 3 animals).
**Supplemental movies**

**Movie 1.** Neural activity across multiple cortical regions during block presentation of a binary noise visual stimulus (upper left). In addition to clear stimulus-locked activation of visual areas, background activity in multiple areas is also evident. Movie is played at 4x actual speed.

**Movie 2.** Neural activity in visual areas during presentation of the topographic noise stimulus (upper left). The moving stimulus elicits moving waves of activity in multiple retinotopic visual areas, in addition to ongoing background activity. Movie is played at 4x actual speed.

**Movie 3.** Cycle-averaged activity during the topographic noise stimulus. Averaging over repeated presentations isolates stimulus-locked activity, showing multiple retinotopic areas. Movie is played at 4x actual speed, and repeats four times.

**Movie 4.** Timelapse movie of two-photon imaging in primary visual cortex, in response to drifting gratings, demonstrating large responses in a majority of neurons across a wide field of view. Movie is played at 4x actual speed.
References


Figure 1

A

Ptight  GCaMP6s IRES nls-mCherry WPRE SV40 PolyA

B

C

D

E

Figure 1
Figure 2

(A) Diagram showing the setup with a sCMOS camera, a 105mm camera lens, a 470nm LED fiber delivery, a 50mm camera lens, and a 510nm LED fiber delivery.

(B) Image of an optical mouse.

(C) Graph showing dF/F over time with three lines: raw, deconv, and spikes.

(D) Graph showing dF/F over time with three lines: raw, deconv, and spikes.
Figure 3

Green illumination  Blue illumination  Blue minus green

D  E  F

\( \frac{df}{F} \) vs. secs

2π phase

0  2  4  6  8  10  0  2  4  6  8  10  0  2  4  6  8  10

0.2  0.15  0.1  0.05  0  0.2  0.15  0.1  0.05  0  0.2  0.15  0.1  0.05  0

0  2  4  6  8  10  0  2  4  6  8  10  0  2  4  6  8  10
Figure 4
Figure 6

A

B

C

D

% of trials

% correct

session completion

0 0.25 0.5 0.75 1 1.25 >1.5

0 0.25 0.5 0.75 1 1.25 >1.5

0 0.2 0.4 0.6 0.8 1

0 0.2 0.4 0.6 0.8 1

response time (secs)

response time (secs)

resp time (secs)

resp time (secs)

fraction correct

correct

DB
Figure 8

A

B

C

D

Figure 8
Figure 9

A

Auditory  Locomotion  Visual

B

C

D

E

F

normalized speed / dF/F

time (secs)

normalized speed / dF/F

time (secs)

fraction

correlation

Auditory  Locomotion  Visual

0
60
120
180
240
300

0
60
120
180
240
300

0
0.2
0.4
0.6
0.8
1

0
0.1
0.2
0.3
0.4
0.5

-0.5
0
0.5

n.s.

positive

negative