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Topography and Functional Specialization of Mouse Higher Visual Areas

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Topography and Functional Specialization of Mouse Higher Visual Areas

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Neurosciences

by

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2014
The Dissertation of Marina Elizabeth Garrett is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

Chair

University of California, San Diego

2014
DEDICATION

In recognition of my foundation

Mary, Mark, Monique, Michael, Rita, Jerry, Jo, Harold

Any success I have is because of you. Thank you for a lifetime of unconditional love and support.

In recognition of my fellows

All past and present Callaway lab members

It has been a privilege to be part of such an extraordinary group of people. I truly respect, admire, and appreciate each and every one of you. Thank you for making the lab such an enjoyable place.

In recognition of my future

Ronnie Lewis

You continue to amaze and inspire me every day with your kindness and ingenuity. Thank you for filling my heart and my life with happiness.
The mind is everything
What you think, you become.

Siddhartha Gautama Buddha
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Chapter 2 consists of material as it appears in: Marshel, James H*; Garrett, Marina E*; Nauhaus, Ian; Callaway, Edward M. "Functional Specialization of Seven Mouse Visual Cortical Areas" Neuron 72(6): 1040-54, 2010 *equal contributions. The dissertation author was the primary co-author of this material, performing experiments and analysis, in collaboration with James H. Marshel, Ian Nauhaus, and Edward Callaway. Permission for reprint of material is granted by all original authors.
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ABSTRACT OF THE DISSERTATION

Topography and Functional Specialization of Mouse Higher Visual Areas

by

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Doctor of Philosophy in Neurosciences
University of California, San Diego, 2014
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The senses are the interface between an organism and its environment. To interact with the world, animals must detect external stimuli, interpret sensory signals to encode relevant information, and make appropriate decisions based on previous experience and behavioral goals. All of these abilities depend on the organization and activity of neural circuits.

To piece together the puzzle of how circuits produce perception and behavior, it is necessary to label, monitor and manipulate specific network
components to assess their role in information processing. The recent development of molecular, genetic, and optical techniques enables this type of circuit dissection with unprecedented precision in the mouse model. While the mouse uses sensory information to navigate its environment and make decisions, as in other mammals, relatively little is known about processing beyond primary sensory areas in this species. Anatomical studies have predicted the existence of multiple higher visual areas in the mouse (Wang & Burkhalter 2007), yet their functional and behavioral roles remain unknown.

The aim of this dissertation is to begin adding pieces to the puzzle by examining the information represented by higher visual areas, in terms of visual field topography and encoding for basic visual features. First, the representation of the visual field across the cortical surface was mapped using intrinsic signal imaging. As each distinct area contains its own independent map of visual space, analysis of visual field gradients allowed precise identification of 11 distinct higher areas and their boundaries, including two previously undescribed regions in mice. Characterization of visual coverage across areas demonstrated that higher areas often have incomplete or biased representations of visual space, suggesting an emphasis on processing particular types of visual information.

Next, areas identified using intrinsic imaging were selectively targeted for higher resolution imaging using two-photon microscopy to measure the sensory evoked response properties across large populations from seven different visual areas. We found that neurons in higher visual areas respond
selectively to basic visual features including orientation, direction, spatial frequency and temporal frequency, and that different areas could be distinguished based on encoding for specific combinations of spatiotemporal features. These results demonstrate the unique functional specializations of different mouse higher visual areas, and suggest specific hypothesis about their behavioral roles.

Together, this work establishes the basic topographic and functional organization of nearly a dozen distinct visual areas across the mouse cortex, providing a foundation for future studies examining the circuit and cellular mechanisms of information processing in this system. Future work examining more complex functional properties, the circuitry that links areas across the network, and the relationships between specific circuit components and behavior, will be essential to create a more complete picture of sensory processing in the mouse.
INTRODUCTION

Our experience of reality is determined by the operations of billions of tiny biological machines that reside within the intricate networks of the brain. Every interaction with the external world is mediated by the senses, every perception is a product of neural activity, and every decision is fueled by memories stored in the ever-changing pattern of synaptic connections. The mysterious mechanisms by which cognitive phenomena are produced by the physiology of the brain comprise nature’s most fascinating puzzle.

How is sensory information encoded by the activity of neurons? How do specialized brain areas construct representations of a dynamic environment? How do neural networks transform information to facilitate behavior? How does the brain change with experience? To answer these questions, it is necessary to analyze the structure and function of neural circuits, and relate these properties to sensory perception and behavior in a defined experimental system.

The functional organization of the visual system has been a primary focus of scientific investigation since the pioneering work of Hubel & Wiesel in the early 1960’s. Their discovery that neurons in the primary visual cortex respond selectively to oriented edges, in contrast with the circular receptive fields of the retina, demonstrated a clear transformation of information between the eye and the brain (Hubel & Wiesel, 1959). This finding has become the
quintessential example of cortical computation, setting the stage for the subsequent 50 years of research into the properties of cortical circuits.

The visual system is a particularly advantageous model of brain function for a number of reasons. First, the visual system is highly organized, making it possible to define specific areas and circuit components based on their anatomical properties, including cytoarchitecture, cellular morphology, expression of molecular markers, topography, and connectivity (Van Essen, 2004). Second, visual responses can be probed with highly controlled stimuli in order to associate neural activity with specific features of visual information. This is essential to determining the function of a given neuron, circuit, or area. Finally, psychophysics can be used to relate the properties of visual stimuli to signal detection and discrimination, providing a quantitative readout of sensory perception and behavior. These characteristics have facilitated many discoveries about the fundamental principles of visual information processing through decades of research primarily in primates and carnivores (Gattass et al., 2005; Maunsell & Newsome, 1987; Orban, 2008; Payne, 1993; Van Essen & Gallant, 1994; Van Essen et al., 1992).

Importantly, the visual system employs fundamental processing strategies that are shared by diverse other regions throughout the brain. Sensory inputs detected at the periphery flow into the brain through parallel pathways that convey specific types of information, allowing efficient processing through relatively independent circuits (Nassi & Callaway, 2009). This organization begins in the retina, where dozens of distinct retinal ganglion
cell types, each having a characteristic morphology and pattern of connectivity, form unique populations that evenly tile the retina to selectively collect information about particular visual attributes (Wassle, 2004; Masland, 2001, 2012). For instance, parasol retinal ganglion cells have large receptive fields that are sensitive to high contrast and rapid motion, while the midget ganglion cells encode distinct visual features, responding at lower contrasts, to slower moving stimuli with high spatial frequencies, in smaller regions of space (Dacey, 2004). These parallel streams of information continue into the brain, remaining segregated through the magno and parvocellular layers of the lateral geniculate nucleus (LGN), which in turn provide input to distinct targets in layer 4 of the primary visual cortex (V1) (Nassi & Callaway, 2009). This computational strategy is employed by essentially every sensory system, with specific tastes, tones, smells, and types of touch encoded by dedicated sensory receptor neurons and conveyed to the brain in parallel (Kaas & Garraghty, 1991).

Yet, sensory percepts do not consist of discrete, simple features; instead, we perceive a coherent, unified experience of the external world (Treisman, 1996). The circuits of the brain transform the simple spots of light cast onto the retina into complex representations of entire objects and their trajectories through space. This is accomplished through a series of hierarchical processing stages that extract and elaborate behaviorally relevant information, combining information across channels to produce novel representations. The visual cortex is composed of multiple areas, each
containing its own topographically organized map of visual space to represent specific features about the environment (Wandell et al., 2007; Gattass et al., 2005; Maunsell & Newsome, 1987). These distinct regions are interconnected by feedforward and feedback pathways that define hierarchical relationships and guide the flow of information (Felleman & Van Essen, 1991; Van Essen, 2005). As information is passed between cortical layers and areas, specific computations modify and elaborate sensory responses, revealing the transformations that ultimately lead to unified perception and goal directed behavior. For instance, while neurons in the primary visual cortex are selective for oriented edges of light (Hubel & Wiesel, 1959), the secondary visual area (V2) shows enhanced responses to naturalistic textures (Freeman et al., 2013) with a subset of neurons that respond to combinations of orientations (Anzai et al., 2007). Neurons in an even higher stage (V4) are tuned to object curvature and shape (Pasupathy & Connor, 2002; Roe et al., 2012). At the highest level of processing, in the inferotemporal cortex (IT), neurons can be found that are strongly and selectively activated by entire objects, such as faces (Bell et al., 2009; Kanwisher & Yovel, 2006; Tanaka et al., 1991). An additional hallmark of higher order processing is invariance - that a neuron will represent a particular object or feature regardless of its size, luminance, or viewing angle (DiCarlo et al, 2012). The information processing strategies that underlie these increasingly complex computations have not been clearly defined.

While we have learned a great deal about the functional responses of a variety of visual areas, and about the patterns of connections between them,
we are still lacking in our understanding of the precise circuit mechanisms that operate within and across defined networks to perform specific computations. For instance, how does a neuron become selective for a particular angle of curvature? How do invariant representations arise? While we can speculate, model, and hypothesize, the answers to these and many other similar questions are unknown. To precisely determine how neural circuits function, it is necessary to examine function and connectivity simultaneously, at high resolution, with as much information as possible about the involved neuron types, their location, morphology, biophysics, synaptic connections, gene expression, and sensory evoked activity. These types of detailed, high-yield measurements must be obtained from different layers and areas, as well as during different behavioral and cognitive states, to assess the changes in representations as information flows through the system. Importantly, the activity of defined network components must be selectively and reversibly manipulated in order to establish causality in circuit relationships and conclusively demonstrate the functional roles of specific cells, circuits, and areas. Only then will we be able to reveal how the intricate connections of the brain produce functional specialization and transform representations to ultimately facilitate the range of experiences that contribute to dynamic, flexible behavior.

Fortunately, the last few decades have seen a revolution in the methods of molecular biology and optical engineering, permitting dissection of neural circuits with unprecedented levels of detail (Luo et al., 2008). It is now possible
to image the responses of hundreds of individual neurons simultaneously using fluorescent calcium indicators and two-photon microscopy (Grienberger & Konnerth, 2012; Helmchen & Denk, 2005; Svoboda & Yasuda, 2006). The generation of transgenic animals allows specific cell types to identified and labeled, so that their function and connectivity can be assessed and manipulated (Madisen et al., 2010; O'Connor et al., 2009). Viral vectors can be used to introduce specific genes into defined brain areas, trace neural pathways, or to target specific cell types or circuits (Callaway, 2008; Davidson & Breakfield, 2003; Dum & Strick, 2013; Osakada et al., 2011). Light activated ion channels allow selective and reversible activation or inactivation of any cell population of interest, and can be combined with any of the above methods for gene delivery (Bernstein & Boyden, 2001; Peron & Svoboda, 2001; Yizhar et al., 2001). All of these techniques are beginning to be used in concert with behavioral tasks in mice to link function and connectivity to sensory perception and decision making (Carandini & Churchland, 2013; Dombeck et al., 2007; O'Connor et al., 2009). These innovations have revolutionized neuroscience, enabling scientists to ask questions that were previously impossible or prohibitively difficult. Currently, these methods are primarily available and most conveniently applied in the mouse model, due to its genetic and experimental accessibility. Accordingly, studies of circuit computation are becoming increasingly reliant on the mouse to employ these powerful molecular and genetic tools. However, relatively little is known about the circuitry and
functional organization of the mouse visual cortex, compared to more traditionally studied species.

The aim of this dissertation is to help establish the mouse visual system as a genetically and experimentally tractable model of cortical information processing by characterizing the organization and function of higher visual areas in the mouse. The ability to identify, label, monitor and manipulate specific circuit components using the techniques of molecular biology will allow mechanistic questions of cortical computation to be investigated in unprecedented levels of detail. However, before these powerful new techniques can be applied, a basic understanding of the organization of mouse visual cortex is required. Further, it is important to determine the similarities and differences between the visual system of the mouse and other species, so that appropriate comparisons can be made. Importantly, neurons in mouse primary visual cortex can be highly selective for fundamental visual features, including orientation, direction, spatial frequency and temporal frequency (Gao et al., 2010; Niell & Stryker, 2008), as in other species. In addition, anatomical evidence demonstrates that the primary visual cortex sends topographic projections to multiple cortical targets, predicting the existence of at least 9 higher visual cortical areas (Wang & Burkhalter, 2007). While these findings indicate that the mouse shares basic characteristics with other mammalian species, the organizational principles governing information processing in mouse higher visual areas remain unknown.
In chapter 1, we characterized the representation of visual space across the mouse cortex to precisely identify distinct higher visual areas based on their topographic organization. Retinotopic maps obtained with intrinsic signal imaging were used to compute local visual field sign, revealing discrete patches of areas with reversals in retinotopic gradients at the borders between areas. Determining precise boundaries facilitated a characterization of the visual field representations of each area, demonstrating that higher visual areas often have incomplete, distorted, or biased representations of space. This preferential processing for specific regions in the visual world has important implications for the function of each area. In primates, cortical regions emphasizing the central visual field tend to be involved in the ventral processing stream, encoding features relevant to recognizing objects and their identify, ultimately facilitating visual perception (Kravitz et al., 2013; Mishkin et al., 1983; Ungerleider & Mishkin, 1982; Van Essen & Gallant, 1994). Areas with representations biased towards the peripheral visual field participate in the dorsal stream of processing, focused on computing object motion and trajectory through space, to permit action and behavior (Kravitz et al., 2011; Mishkin et al., 1983; Ungerleider & Mishkin, 1982; Van Essen & Gallant, 1994). We find that a similar organization is seen in the mouse, with more anterior and medial areas biased towards the peripheral visual field, and lateral posterior areas focused on the central part of space.

To begin to explore the type of visual information encoded by these defined areas, we examined the sensory response properties of neurons in
seven visual cortical areas in Chapter 2. Specific visual areas were targeted in each individual animal using intrinsic signal imaging to map retinotopy and locate distinct visual field representations. Next, in vivo two-photon calcium imaging was performed to assess the responses of neural populations with single cell resolution, as the change in the fluorescence of the calcium indicator dye Oregon Green Bapta in response to neural activity. Visual stimuli consisting of drifting sinusoidal gratings that varied in orientation, direction, spatial frequency and temporal frequency were used to demonstrate that neurons in different visual areas are selective for distinct combinations of these spatiotemporal features. Importantly, we identified a subset of visual areas that was highly direction selective and tuned to high temporal frequencies, indicating a role in the processing of visual motion. The areas with these properties were located in the analogous cortical position to the posterior parietal cortex, a major target of the dorsal processing stream in primates (Kravitz et al., 2011). These results suggest that the mouse visual cortex is similarly organized into parallel processing streams with distinct functional specializations that likely serve to facilitate distinct behavioral goals.

Together, this work provides a foundation for the use of the mouse as a model of visual information processing. We have begun to define the fundamental characteristics of unique visual areas - structured topographic organization and representation of visual space, and functional specialization for specific visual features. These results demonstrate that the mouse visual system shares essential organizing principles with other mammalian species,
with a network of defined cortical regions that interact to produce the representations necessary to engage with a dynamic environment. Future studies will be crucial to continue to understand the structure and function of higher visual areas in the mouse, and ultimately relate these properties back to perception and behavior.
References


CHAPTER 1. TOPOGRAPHY AND AREAL ORGANIZATION OF MOUSE VISUAL CORTEX

Abstract

In order to guide future experiments aimed at understanding the mouse visual system, it is essential that we have a solid handle on the global topography of visual cortical areas. Ideally, the method used to measure topography is robust and simple enough to guide subsequent targeting of visual areas in each subject. We generated retinotopic maps in mouse visual cortex using intrinsic signal imaging (Kalatsky & Stryker, 2003; Marshel et al., 2011; Schuett et al., 2002). We then applied an algorithm to automatically identify cortical regions that satisfy a set of quantifiable criteria for what constitutes a visual area, beginning with visual field sign analysis to identify area borders (Sereno et al., 1994, 1995). Our approach facilitated detailed parcellation of mouse visual cortex, delineating 9 known areas, and revealing two additional areas that have not been previously described as visuotopically mapped in mice. We quantified multiple features of each identified visual area across animals: variability in area position, area size, visual field coverage, and cortical magnification. We demonstrate that higher areas in mice often have representations that are incomplete, distorted, or biased toward particular regions of visual space, suggestive of specializations for processing specific types of information about the environment. This work provides a comprehensive description of mouse cortical areas and describes essential tools for accurate functional localization of visual areas.
Introduction

Visual information is processed by a series of discrete cortical areas, each of which has a unique representation of the visual scene. Uncovering the mechanisms behind this flow of information is a demanding problem, yet one that is fundamental to sensory systems as a whole. A necessary step toward the detailed study of the visual cortex hierarchy is a thorough characterization of the spatial layout of visual areas on the cortical surface, along with each area’s visual field representation. For instance, numerous primate studies have relied on the established visual field maps as a framework for further investigation of different stages of the hierarchy (Gattass et al., 2005; Rosa & Tweedale, 2005; Van Essen, 2004; Wandell et al., 2007). In the visual cortex of mice, it is apparent that many of the established fundamental questions can be addressed with far greater precision, thanks to powerful new experimental tools (Huberman & Niell, 2011; Luo et al., 2008; Madisen et al., 2010; Marshel et al., 2010). However, a rigorous quantitative characterization of the topography of mouse visual cortex is still needed to best guide the study of cortical hierarchies in this species.

Both functional and anatomical studies have clearly demonstrated the existence of multiple visual areas in the mouse. The retinotopic organization of the mouse visual cortex has been described using electrophysiology (Drager, 1975; Wagor et al, 1980), anatomical methods (Wang & Burkhalter, 2007), intrinsic signal imaging (Schuett et al., 2002; Kalatsky & Stryker, 2003), voltage
sensitive dye imaging (Pollack & Contreras, 2012), and two-photon calcium imaging (Marshel et al., 2011; Andermann et al., 2011). Taken together, these studies have shown up to ten different retinotopic maps, thus establishing the existence of at least 10 visual cortical areas. Response properties have been investigated in seven of these areas, demonstrating encoding for distinct combinations of spatiotemporal features, and supporting their functional specialization (Marshel et al., 2011; Andermann et al., 2011; Roth et al., 2012). Mouse visual areas also have specific cortical and subcortical projections, suggesting that they are organized into parallel processing pathways analogous to the dorsal and ventral streams observed in other species (Wang et al., 2011; Wang et al., 2012; Wang & Burkhalter, 2013). While these and other advances have shed light on the basic organization of the mouse visual system, further work is needed to more clearly establish the hierarchical relationships, behavioral roles, and precise circuit mechanisms that operate across cortical areas.

In order to accurately target specific visual areas for study, it is necessary to identify their precise location and boundaries. While it may be possible to take advantage of anatomical coordinates to target large visual areas (such as the primary visual cortex), functional methods provide the most reliable means of identifying small areas in individual animals. As each visual area contains its own unique map of visual space, it is possible to identify different areas from functionally measured retinotopic representations (Sereno et al., 1994; Wandell et al., 2007; Van Essen, 2004). Here, we provide an
automated method that uses specific criteria to compute area boundaries in mouse visual cortex, via retinotopic maps obtained with intrinsic signal imaging. The processing steps first segment the visual cortex of each animal into a set of discrete regions based on reversals in retinotopy and coverage of visual space. Each region was then classified as a visual area if reliably detected across experiments. This method rigorously outlined the location of nine known areas, and also revealed two additional areas that have not been previously described as visuotopically mapped in mice. The 11 identified areas were then assessed for cortical coverage, visual field coverage, and cortical magnification. In general, the visual field representation of higher areas was shown to have consistent biases toward localized regions of visual space, suggestive of functional specialization. This work provides a novel characterization of visual field map organization, and describes essential tools for accurate functional targeting of visual areas in the mouse brain.
Results

Automatic identification of visual areas from retinotopic maps

The goal of this study was to identify the spatial layout and boundaries of mouse visual cortical areas and characterize the visual field representation of each identified area. A visual area is defined as having a single orderly representation of space that can be consistently identified across individuals (Wandell et al., 2007). We designed an algorithm to identify regions that meet this definition by applying a series of automated processing steps to maps of visual topography. The analyses all rely on retinotopic maps obtained with intrinsic signal imaging, representing the right visual hemifield in spherical coordinates (Marshel et al., 2011). Visual maps were segmented according to four specific criteria that quantify features of visuotopic organization and compute areal boundaries. The computational details of the algorithm are given in Methods. A general description of the rationale behind each step follows, with the results of a single experiment used as a guide (Figure 1.1).

The first criterion that a visual area must satisfy is having a consistent visual field sign within its borders (Sereno et al., 1994, 1995). At each cortical location, taking the sine of the angle between the gradients of vertical and horizontal retinotopy yields a map of visual field sign (Figure 1.1A-C). The absolute value of this metric indicates the degree of orthogonality between the map gradients, and the sign indicates whether the representation of visual space across the cortical surface is a reflection of the visual field. Negative
regions, such as the large blue patch in the middle of Fig 1.1C (i.e. V1), are mirror image transformations of the visual field. Positive regions, such as the smaller extrastriate patches in red surrounding V1, are non-mirror image transformations of the visual field. Transitions between positive and negative values in the map indicate the locations of reversals in the retinotopic gradients, and provide a robust way to find borders between different visual field representations (Sereno et al., 1994; Warnking et al., 2002). Thresholding the map of visual field sign results in a set of discrete patches (Fig. 1.1D). For pairs of adjacent visual areas that are of the opposite field sign, the visual field sign analysis correctly delineates their borders. However, the possibility remains that two distinct visual field representations could have the same visual field sign and share a border, as is the case for some adjacent regions in humans (Larsson and Heeger, 2006; Wandell et al., 2007). The second criterion addresses this issue.

The second criterion a visual area must meet is that it cannot have a redundant representation of visual space. For instance, if a patch identified in the previous step contains multiple pixels that represent the same visual field location, this would indicate that it contains more than one visual area. To check each patch for redundancy, we compared the integral of visual field coverage over all pixels within a patch to the union of coverage by the patch (Methods Eq 4). Redundancy would yield an overestimate of coverage by the integral measure compared to the union, resulting from a duplication in the representation by multiple pixels. Fig. 1.1E is a scatter plot comparing the
integral to the union for the patches in Fig 1.1D. All patches sit along the unity line, with the exception of patch no. 11. Redundant patches such as no. 11 were segmented automatically by applying the marker-controlled watershed algorithm to a map of visual eccentricity (Methods Eq 5). The watershed segmentation creates a border at the "ridge line" that separates local minima in the visual eccentricity map. This method takes into account the relative size and rate of change of the map gradients to create accurate borders.

The third criterion requires that all adjacent areas of the same visual field sign have overlapping representations of visual space. This largely serves to check that the processing steps under Criterion 1 did not create inappropriate boundaries. For instance, if a single visual map were improperly split into separate patches, combining the two would produce a more complete, yet still non-redundant representation of space, satisfying our definition of a visual area. In turn, we merged each pair of neighboring same sign patches, assessed for redundancy as done for Criterion 2, and simply left them fused if their visual representations were non-overlapping. Figures 1.1F-I show the visual field coverage for pairs of neighboring patches that were split by the preceding steps of the algorithm. In the first three examples (Figure 1.1F-H), despite being adjacent on the cortex with the same visual field sign, each pair has overlapping visual field coverage and was therefore appropriately segmented by the algorithm. In contrast, the example in Figure 1.1I shows essentially no overlap, so the two patches were fused and considered as a single area.
The final set of boundaries (Fig. 1.1J) computed from the retinotopic maps in this experiment contains individual visual field maps fulfilling the first three requirements in our definition of a visual area. A clear correspondence between the computed borders and reversals in retinotopy can be observed in the contour plots of altitude and azimuth (Figs. 1.1K-L). Additional examples of retinotopic maps and the resulting visual field sign maps and associated borders are shown in Figure 1.2. Upon visual inspection, some patches are consistently identified at the same relative cortical location in all experiments. However, others, particularly those along the posterior edge of the visual cortex, display more variability across cases.

The fourth and final criterion is that a visual area must be reliably identified at the same location in different individuals. Whereas the preceding steps compute borders for each independent experiment, the final step looks across experiments to determine whether the identified patches have a statistically significant mean location. The position of visual cortex within the camera’s field-of-view for each experiment was variable, so the maps were aligned using an easily identified landmark - the largest, most reliably identified central patch, corresponding to V1. The map from each experiment was first shifted to align V1’s center-of-mass, and then rotated to align the direction of V1’s horizontal retinotopic gradient. Following this alignment, we overlaid the centers-of-mass of all patches in the 14 experiments, shown separately for mirror and non-mirror image representations in Figures 1.3A-B. At first glance, there is visible structure in the arrangement of the patch centers. To evaluate
whether patch centers fell into clusters that could be associated with particular visual areas, K-means clustering was performed on the mirror and non-mirror scatter plots. The Voronoi lines in black segregate each identified cluster, along with a black dot for each cluster mean (Figs. 1.3C,D). As our definition of a visual area states that regions should be reliably identified across individuals, we performed an additional analysis to remove patch centers that were not associated with significant clusters. A shuffling analysis (Methods) produced 11 consistently separated clusters of patch centers, shown in Figures 1.3E,F. Patch centers that did not belong to significant clusters were excluded from further analysis.

The spatial arrangement of the clusters closely matched previous descriptions of the layout of visual areas in the mouse. Nine of the definitively classified visual areas corresponded to previously described visual areas: V1, LM, LI, AL, RL, AM, PM, P, & POR (Olavarria et al., 1982; Olavarria and Montero, 1989; Wang and Burkhalter, 2007). In addition, we were surprised to find two clusters of patch centers that did not map on to any known mouse visual areas. While unexpected, these two regions met all conditions of our analysis and were robustly identified across cases (Figure 1.3), warranting their classification as independent, well-defined visual areas. The first belonged to the set of non-mirror patches and was located on the far lateral side of the visual cortex, neighboring to area AL. The second unexpected cluster belonged to the set of mirror image patches and was located medial to V1 and just posterior to area PM. Although not previously reported in mice,
visual regions in corresponding locations have been described in other rodents, including the rat, and we labeled these analogous regions accordingly as the laterolateral-anterior (LLA) and medial (M) visual areas (Olavarria and Montero, 1984; Malach, 1989; Sereno and Allman, 1991; Spatz et al., 1991; Espinoza et al., 1992). In contrast, another area predicted by anatomy to be located anterior to V1, between RL and AM - the anterior area, A (Wang and Burkhalter, 2007) - was not reliably detected by our analysis procedure, although a small patch in this region was observed in 2 out of 15 cases (Fig 1.3C upper right cluster). However, the field sign of this patch was the opposite of that predicted for A based on topography of cortico-cortical projections. (Compare Fig 1.1D patch 13 to Wang and Burkhalter, 2007). Several other variable patch centers were observed at the posterior edge of the cortex, on both the medial and lateral sides of V1, but were not deemed significant by the cluster analysis, and remain unclassified (seen in Figure 1.3A-D but not 1.3E-F). It is important to note that the classification of visual areas reported here is undoubtedly limited by the resolution of our imaging method. Although the identified areas should be robustly detected with more sensitive methods, such as two-photon imaging, there may be additional small visual areas that were missed.

Uncertainty in the position of visual areas

While the arrangement of visual areas was fairly stereotyped across animals, evident from the overlay of area borders (Fig 1.4a) and the overlay of centers-of-mass (CoMs) after alignment (Fig 1.4b), the relative location of
different higher visual areas had varying degrees of consistency. Quantifying an extrastriate area’s positional uncertainty across multiple animals can be an important guide for their targeting in future experiments. Because V1 is the most reliably and clearly identified area, with intrinsic imaging as well as other localization methods, a measurement of variability within this reference frame serves as an informative baseline. As described above, the maps from each experiment were independently shifted and rotated to align V1 for subsequent analysis.

To measure the positional uncertainty for each visual area, we first quantified each area’s size (Fig 1.4C) along with the standard deviation of CoM scatter (Fig 1.4D). Higher visual areas were all significantly smaller than V1 (~4mm²), with the next largest area being LM (1mm²), followed by RL (0.9mm²), and all other areas falling between 0.1 and 0.3 mm² (Fig 1.4C). The CoMs of the extrastriate visual areas had different degrees of variability – the standard deviation of all areas fell between 0.09 and 0.22 mm, with LM and AL having the lowest (0.09 mm, 0.09 mm), and P and POR having the highest (0.22 mm, 0.20 mm) (Fig 1.4D). The values from Figs 1.4C,D were combined to compute the ratio of positional standard deviation to the square-root of area size, shown in Fig 1.4E. This normalized metric more directly indicates the ability to accurately identify the position of extrastriate areas relative to V1. For instance, one of the larger cortical areas with relatively low variability, LM, shows a distribution of area centers that is only 10% of the size of the entire area, meaning that it could be reasonably targeted using the position of V1.
alone. In contrast, the positional scatter of POR is more than 50% of its overall size, making it difficult to identify accurately without a direct measurement of POR’s borders in a given individual. These differences are important to consider when targeting specific higher visual areas using localization methods that rely on generalized coordinates or frames of reference, as well as when interpreting results of studies that are highly dependent on area identification.

Average retinotopic organization of visual cortex

To further assess the architecture of mouse visual cortex we computed the average horizontal and vertical retinotopic map after alignment (Figure 1.5). Specifically, the average map of vertical retinotopy was taken to be $\mathcal{a}Maei\mathcal{V}a$, where $\mathcal{M}a\mathcal{M}a$ is the aligned map of response magnitude (i.e. the F1 amplitude) and $\mathcal{V}a$ is the aligned map of vertical retinotopy, for animal $a$. To define area borders in the average map, we used the segmentation algorithm as before (Criteria 1-3).

In the map of horizontal retinotopy (Fig 1.5C), the vertical meridian occurs at the nasal most representation in the map of horizontal retinotopy (in purple) and is the most prominent feature separating V1 from lateral extrastriate areas. The progression of visual space in areas LM, AL, RL, and P going from medial to lateral and away from V1 moves from the nasal visual field, out to temporal. Areas LI and POR share the lateral and posterior borders of LM and have a reflection of this topography across their borders, reversing from temporal to nasal as they move away from LM towards the lateral and
posterior edges of the brain. In contrast, the map of horizontal retinotopy in area P shares LM’s organization, appearing almost continuous along V1’s posterior edge. The horizontal map in area LLA is a reflection of that of LI along the anterior-posterior axis, with nasal visual field represented more posteriorly, and the temporal fields at its most anterior point. RL is medial to AL and LLA, along V1’s rostral edge, and also moves from nasal to temporal as it reflects away from V1. However, the visual maps observed in RL are often complex and sometimes show a warping of visual space in the center of the area, creating a ring like structure. This strange feature resembles the observed pattern of callosal connections in the corresponding region (Olavarria and Montero, 1989; Wang and Burkhalter, 2007; Wang et al., 2011). Finally, areas AM, PM and M are contained within a long strip of cortex on the medial edge of V1. The maps of horizontal retinotopy in AM and PM are reflections of each other, with the vertical meridian running along the border between them. The map in M is a reversal of that in V1, moving from temporal to nasal as it extends medially.

In the map of vertical retinotopy (Fig. 1.5D), the horizontal meridian (blue) typically runs through the middle of visual areas, with peripheral representations corresponding to the boundaries between distinct visual field maps. The horizontal meridian extending through the middle of V1 diverges at the LM-AL-RL border, splitting into three separate arms, one running through each area. LM’s horizontal meridian extends laterally into LI, then curls around towards the posterior edge of the cortex through POR and P. The upper visual
field is represented by the caudal portion of V1, and along the boundary between LM, LI, P and POR. While the most posterior edge of the cortex can be difficult to image, a transition towards lower field representations is typically observed at the caudal boundary of P and POR. A reversal in the vertical gradients at the lower visual field marks the intersection between LM, LI, AL, LLA. Moving more anterior, AL and LLA are bounded by a reversal at the upper fields, transitioning into RL. The vertical gradients of medial areas AM, PM and M, are aligned with the horizontal meridian running through the center of all three areas, moving towards the lower fields as they approach V1.

**Characterizing visual field coverage**

The region of visual space covered by a cortical area has important implications for its overall function. For instance, V4 in primates primarily represents the central 30-40 degrees of visual space, enabling processing of more fine detail near the fovea, whereas parietal visual areas tend to over represent the periphery (see Gattass et al., 2005 for review). Our measurements show that mouse extrastriate visual areas have consistent biases in visual field coverage as well.

To measure the average visual field coverage by a given cortical area, offline steps were taken to ensure that the stimulated eye of all subjects was registered to a common point of visual space. Although efforts were made during the experiment to have the visual stimulus at the same location in all subjects, small differences in head and/or eye alignment were nonetheless evident in the retinotopic maps. To correct for these differences, we registered
the maps with an easily identified anatomical location: the intersection of areas V1, LM, and RL. That is, the [0 0] point of altitude and azimuth was shifted to the V1-LM-RL point of intersection. In addition to being easily defined anatomically, this point is a natural reference for assessing visual coverage, as it coincides with the center of visual space and the location of the binocular zone in mice. Accordingly, shifting the origin of the maps to this location on the brain produces maps of eccentricity (Fig. 1.6A) and polar angle.

Each panel of Figure 1.6B shows the visual field coverage by the designated visual area. The cardinal axes are altitude and azimuth coordinates, and contours illustrate iso-eccentricity lines at 30° intervals. The origin of the coordinate system sits at the intersection of the vertical and horizontal meridians. While rodents do not have a clear fovea, the approximate center of the retina corresponds to around 60° azimuth and 0° degrees altitude (see Methods). Regions colored in black indicate points in visual space that were represented every time the area was found, whereas regions with white were never represented by the area. This depiction is considered the “coverage profile” for each area, and demonstrates the reliability of having coverage at each receptive field location across animals. The black contour overlaid on each plot represents the coverage profile outline obtained from the area in the average map (Fig. 1.5).

Comparison of coverage profiles between visual areas presents several interesting observations. First, it is apparent that the region of space covered by V1 is significantly larger than that of all other visual areas, indicating that
higher areas typically have less than complete representations of space. The average visual field coverage (deg$^2$) is shown in Figure 1.6C, with PM, LM, and RL having the next highest degrees of coverage, after V1. The second distinction is that many higher visual areas show a significant bias in the region of space they represent. While V1’s coverage profile is centered near the midpoint of the mouse’s eye (~0° altitude, ~50° azimuth), the coverage of higher areas is clearly skewed away from this midpoint. For example, RL’s representation of space is clearly biased towards the lower nasal visual field, below the horizontal meridian and towards the vertical meridian. In contrast, PM preferentially represents the temporal visual field, and area P primarily covers the upper visual field. LM’s coverage profile is the closest in appearance to that of V1, although it still has a significantly reduced representation. These biases are summarized in Figure 1.6D, which plots the center-of-mass of each area’s coverage profile. Figure 1.6E is also a summary of the biases in coverage, showing the proportion of each area’s coverage profile within four different quadrants of visual space.

Finally, we characterized the degree of coverage overlap between all pairs of areas, shown as a matrix in Figure 1.6F. The value for each position in the matrix was computed from the sum of the coverage profiles from the visual areas on the x and y axes, normalized by the profile of the area on the y-axis (Methods). Consequently, pixel values near one (red) indicate that the coverage profile of the visual area on the y-axis is contained within the coverage profile of the area on the x-axis. Pixel values near zero (blue)
represent coverage profiles that are largely exclusive. Pixels with a black dot indicate pairs of areas that are neighboring and have the same visual field sign.

**Cortical magnification emphasizes the central visual field**

The cortical magnification factor is the amount of cortical territory devoted to processing a given unit of visual space. The overlay of altitude and azimuth gradients from the average map can be used to illustrate these relationships (Fig. 1.7A). The contour lines for both gradients are equally spaced at 5 degree intervals, meaning that each square created by their intersection corresponds to a 25 deg² region of visual space. The cortical area (mm²) within each 25 deg² region is proportional to the magnification factor (mm²/deg²) at that cortical location. Cortical magnification is also represented by the color map in Figure 1.7B. The colors represent the Jacobian determinant computed at each pixel from the maps of retinotopy. We quantified each area’s average magnification factor by taking the average value across all pixels within each of the visual area boundaries of the map in Figure 1.7B. These mean values are indicated by red dots in the plot of average magnification across experiments in Figure 1.7C. We also performed the same analysis on each of the 14 individual experiments to determine the average areal magnification across cases, represented in Figure 1.7C. A logical expectation would be that magnification was proportional to visual areas size. For instance, area PM has the lowest average magnification, in accordance with it being a small area with a relatively larger coverage profile.
However, this trend did not generalize across areas because of the variability in visual field coverage. For instance, RL has a higher mean magnification factor than V1, a result of its emphasis on the nasal visual field. The limited visual field representation of many smaller areas also creates a much higher magnification than what might be expected based on V1’s magnification.

From the map in Figure 1.7B, it appears that magnification factor is not constant across each area of visual cortex, as may otherwise be expected by the relatively homogenous density of photoreceptors in the mouse retina (Drager and Olsen, 1981; Jeon et al., 1998). Instead, an increase in magnification can be seen at the V1-LM-RL intersection, which corresponds to the binocular zone at the center of visual space. To directly examine the relationship between magnification factor and eccentricity, we computed the mean magnification within 20° intervals of eccentricity. The black curves in Figure 1.7D show the average magnification across experiments, within each eccentricity interval. The red dots are from the average map alone (Fig. 1.7B).

For the largest areas, V1, LM, RL, PM and AL, there is an increase in magnification at 0 degrees eccentricity, which is likely accounted for by the binocularity at this location. Greater magnification at the binocular zones of the cortex is to be expected, as they likely receive more inputs per degree of visual field. For areas such as LI, ALL and M, no relationship is observed. However, for the two posterior lateral areas, P and POR, an interesting trend occurs where magnification is increased in the middle range of eccentricities, but not at the center of space or extreme periphery.
Cortical magnification is not necessarily an isotropic quantity, meaning that the visual field representation may be stretched along a particular direction of the cortex. This is clearly the case for many of the areas in the mouse. The regions enclosed by the contour intersections (Fig. 1.7a) are not perfect squares, but are often elongated. This corresponds to the principal axis of magnification and can be quantified at each pixel using the Jacobian matrix from the retinotopic maps (Methods). The black lines overlaid on the map of Figure 1.7B represent the principal axes of magnification sampled every 250 µm. For the map of V1, it appears that the elongation occurs parallel to the altitude gradient, indicating an expanded representation along the vertical dimension of visual space.
Discussion

In this study, we analyzed topographic maps in the mouse visual cortex to determine the boundaries of distinct visual field representations and classify them as reliably identified areas. This investigation builds on previous work by providing a quantitative method for parceling distinct areas based on their visuotopic organization, clarifying the spatial layout of 9 known visual areas, and revealing two additional retinotopically mapped cortical areas for the first time. Using the area boundaries delineated based on defined criteria, we were able to assess the representation of visual space for each identified area. We found that higher visual areas often have partial or incomplete representations of space that can be biased towards particular regions of the visual field, suggesting specializations in information processing. Finally, we found relationships between cortical magnification and eccentricity, suggesting an overall emphasis on the representation of the vertical meridian at the region of binocular overlap in mice. These results provide a resource for the identification of distinct visual cortical areas, and describe essential features of map organization that serve as a foundation for future studies of visual information processing in the mouse.

Identifying visual cortical areas

In order to correlate the results of functional, anatomical and behavioral studies with well-defined visual cortical areas, a straightforward and reliable method for identifying clear area boundaries is needed. While anatomical
techniques can be used to estimate area boundaries in fixed tissue, the ideal method for area localization should be minimally invasive and readily applied in vivo to allow direct targeting of areas in the same animal. Functional mapping of retinotopy is a robust method to define area boundaries based on reversals in retinotopic gradients. In humans and primates, fMRI is routinely used to non-invasively map the organization of the visual cortex using periodic visual stimulation (Sereno et al., 1995; Warnking et al., 2002; Dumoulin et al., 2003; Wandell et al., 2007). Electrophysiological experiments have charted visual topography in mice, revealing retinotopic maps in both medial and lateral extrastriate cortex (Dräger, 1975; Wagor et al., 1980; Wang and Burkhalter, 2007). Two-photon calcium imaging allows high-resolution measurements of the receptive field position of individual cells (Smith and Häusser, 2010; Bonin et al., 2011) and across populations (Andermann et al., 2011; Marshel et al., 2011; Glickfeld et al., 2013), but can currently only sample from ~1mm wide fields of view and typically requires invasive surgery. In contrast, intrinsic signal imaging allows functional mapping across the entire extent of the visual cortex, through the intact skull (Schuett et al., 2002; Kalatsky and Stryker, 2003).

While a few studies have targeted recordings to specific higher areas using retinotopic mapping with intrinsic signal imaging (Andermann et al., 2011; Marshel et al., 2011; Glickfeld et al., 2013), none have quantitatively determined the precise borders between areas, or analyzed the features of map organization. Interpreting areal organization from retinotopy is especially challenging in the mouse where maps can be small, clustered, or distorted,
emphasizing the need for a rigorous, unbiased approach. Fortunately, an analytical method for identifying areas and their boundaries based on visual field sign has been developed and used in combination with fMRI and electrophysiological mapping in humans and primates (Sereno et al., 1994, 1995; Warnking et al., 2002; Dumoulin et al., 2003). Visual field sign is a local measure that yields clear boundaries between areas according to reversals in retinotopic map gradients. It is invariant to the orientation of the maps and to the absolute receptive field coordinates of the reversals, reducing retinotopy into a single map that clearly disambiguates visual areas from each other and the background.

Our approach combined the virtues of retinotopic mapping using intrinsic signal imaging, periodic visual stimulation, and visual field sign analysis with a novel algorithm for area classification to more precisely determine the location and boundaries of mouse visual cortical areas compared to previous studies. Several key improvements, including using a full-field spherically corrected visual stimulus, increased stimulus duration, and development of a paradigm for unsupervised quantitative analysis, allowed us to resolve the number, layout, and variability of visuotopically defined areas. Our description revealed 11 unique representations of the visual field that were reliably identified across animals, including V1, LM, LI, AL, RL, AM, PM, M, P, PL and LLA. Visual field maps for areas LLA and M were detected here for the first time in mice, demonstrating the utility of our analysis.
Some parcellation schemes have proposed that the visual field representations along the entire lateral V1 border comprise a unified map and should be considered as a single area V2 (Wagor et al., 1980; Malach, 1989). Recent work has demonstrated topography more consistent with the presence of multiple distinct areas within this region (Wang and Burkhalter, 2007; Andermann et al., 2011; Marshel et al., 2011; Polack and Contreras, 2012). Our results confirm that the region of cortex lateral to V1 contains multiple distinct visual areas with overlapping representations of space. In particular, the characterization of lateral extrastriate cortex as one large V2 would require considering areas LM and RL as belonging to a single region. Based on our analysis of visual field representations in these regions, merging them would result in a duplication of visual space, providing a clear indication that LM and RL are distinct areas that cannot be grouped into a single V2. Differences in functional properties and patterns of connectivity also argue that LM and RL are independent areas (Marshel et al., 2011; Wang et al., 2012). Furthermore, only LM shares the vertical meridian representation with V1, and receives the strongest projections from V1, making it the most likely candidate for a V2-like area (Wang and Burkhalter, 2007; Wang et al., 2011, 2012). Similarities in response properties between V1 and LM strengthen this argument (Andermann et al., 2011; Marshel et al., 2011; Glickfeld et al., 2013). In contrast, response characteristics in RL are more consistent with belonging to the dorsal stream, as it is implicated in the processing of visual motion and visual-somatosensory integration (Marshel et al., 2011; Olcese et al., 2013).
We were surprised to find an additional visuotopic map just lateral to area AL, that was not previously identified based on projections from V1 (Olavarria and Montero, 1989; Wang and Burkhalter, 2007). A region in the corresponding location has been described in rats as the laterolateral anterior area, LLA, and we have named this patch accordingly (Olavarria et al., 1982; Olavarria and Montero, 1984; Thomas and Espinoza, 1987; Malach, 1989). Given its proximity to auditory cortex, it is possible that LLA is involved in audiovisual integration, and may correspond to area DP of Wang and Burkhalter, 2012a,b. The lack of direct input from V1 to LLA indicates that this area may be relatively higher in the visual system hierarchy, and explains why it was not identified in previous studies based on projections from V1 (Wang and Burkhalter, 2007). The retinotopic structure of area LLA was present in some previous map descriptions in the mouse, but was not identified as a distinct area, presumably due to the difficulty in identifying reversals and borders subjectively. Still, our algorithm reliably identified this region in nearly every case, demonstrating the utility and necessity of automated, more quantitative approaches.

We were also surprised to find a reversal in visual field sign just posterior to PM, revealing an additional visuotopic map for area M. The border between M and PM may have been difficult to detect in previous studies because of the way that the retinotopic gradients change in both scale and direction. However, when analyzed with the visual field sign method, area M and its borders are robustly identified. While this region has not been shown to
receive topographic input from V1 in the mouse (Wang and Burkhalter, 2007), it is a projection target of V1 and lateral extrastriate cortex in the rat (Olavarria and Montero, 1984; Malach, 1989). The narrow strip between AM, PM, M and retrosplenial cortex, cytoarchitecturally defined as V2MM, receives weak projections from V1 but was not demonstrated to be clearly visuotopically organized in this study (Wang and Burkhalter, 2007; Paxinos & Franklin, 2008).

Area A, proposed to be just anterior to V1, between RL and AM, was not readily evident in the visual field sign maps obtained with this method. A likely explanation for this discrepancy is that area A does not have a clear retinotopic structure, consistent with the degree of overlap in topographic projections from V1 (Wang and Burkhalter, 2007). Additional possibilities include that this area is too small to detect with intrinsic imaging, or that stimulus parameters, including stimulus size and/or speed, were not matched to the preferences of this area. Nevertheless, it is likely that a portion of the territory previously ascribed to area A (Wang and Burkhalter, 2007) actually belongs to RL, given the size and extent of RL’s functionally identified borders.

Lateral-temporal areas LI, P, POR are small and had more positional variability than most other areas. It is likely that this inconsistency can be partially accounted for by the difficulty of detecting such a tight cluster of areal borders with intrinsic imaging. Nonetheless, area LI was evident in every case. Posterior to LM and LI, we observed several small visuotopic maps of alternating field sign, consistent with previous studies demonstrating multiple distinct projection fields of V1 in this region in both mice (Olavarria and
Montero, 1981; Olavarria et al., 1982) and rats (Olavarria and Montero, 1981; Montero, 1993). While some studies have labeled this region as a single area P (Wang and Burkhalter, 2007), other anatomical experiments have recognized multiple distinct fields in this location (Olavarria and Montero, 1981, 1989; Olavarria et al., 1982; Montero, 1993). Here, we identified two patches of opposing visual field sign in the region of area P, just posterior to LM. Just lateral to region P and posterior to LI, we found another small region of non-mirror, red field sign, likely corresponding to area PL defined in previous studies (Olavarria and Montero, 1989). Areas P, and PL were initially identified in the cluster analysis, but only area P was deemed significant. This may be a result of the small size and variable nature of the PL patch. We observed an additional visuotopic map lateral to P and LI in a location corresponding to the postrhinal area, POR (Burwell and Amaral, 1998; Burwell, 2001; Wang and Burkhalter, 2007). In a few cases, we found additional visuotopic maps lateral to LI and POR, potentially corresponding to areas 36p or TEp as described in Wang and Burkhalter, 2012.

The ability to detect visual field representations of higher visual areas using a simple drifting bar stimulus is highly beneficial for mapping and localization studies, and the methods described here should prove useful for areal identification. However, it is possible that many extrastriate regions have nonlinear receptive field properties and are more effectively driven under natural conditions, with representations that are increasingly dependent on behavioral state and context. Experiments taking advantage of more complex
stimuli and higher resolution imaging methods in awake animals will be essential to provide a more detailed view of visual representations in extrastriate cortex.

**Representations of visual space in extrastriate areas**

We found that many mouse extrastriate visual areas consistently overrepresented a particular subsection of the visual space covered by V1. Incomplete representations of space have also been observed in extrastriate areas of primates, with larger reductions in coverage associated with increasing hierarchical position (Gattass et al., 2005). However, the difference in total coverage between V1 and higher areas appears more extreme in mice than in other species (Gattass et al., 2005; Orban, 2008). The strong coverage biases in the mouse may be driven by the need for functionally specific encoding of localized regions of the visual field with limited cortical territory. For example, RL devotes a relatively large region of cortex to the nasal visual field, implying that behaviors depending on this area require detailed information from this region of space. A potential role for RL is the combined coordination of vision and whisking (Olcese et al., 2013), consistent with the fact that the nasal visual field coincides with the location of whisker stimuli. Another indication of this role for RL in multimodal integration is its physical proximity to somatosensory cortex and associated connectivity (Wang and Burkhalter, 2007). We may find similar clues in determining the behavioral roles of other extrastriate areas. It has been shown that a moving stimulus positioned above a mouse elicits a strong escape response, while the same stimulus displayed
in the lower visual field does not show this effect (Wallace et al., 2013). To guide this behavior, a population of visual neurons would ideally respond to stimuli over a broad expanse of the upper visual field, as do multiple dorsal-medial areas (such as PM and M), due to their low cortical magnification. Furthermore, the outputs of such areas should be strongly directed towards motor cortex to create a more reflexive behavioral response, which may be the case for some dorsal-medial visual cortical areas (Wang et al., 2012).

It is likely that the extents of the visual field representations we have observed are artificially reduced by the limited resolution of intrinsic signal imaging - blurring of the retinotopic maps could clip the visual field representations around their edges. For instance, our measurement of V1 coverage is about 100° in azimuth and about 60° of altitude (Figure 1.6), whereas an electrode study measured these to be 140° and 100°, respectively (Wagor et al., 1980). We would predict similar discrepancies for the higher visual areas. However, it seems highly unlikely that this would significantly alter our conclusions about relative biases in coverage between areas.

**Higher cortical magnification near the central visual field**

Changes in magnification factor reflect a visual area’s division of labor across the visual field. In general, if a particular sub-region within a visual area receives more inputs per degree of visual field, then it will require more neurons to encode the extra information. The most common example of this architecture is the dramatic increase in magnification near the foveal representation of the primate visual cortex (Van Essen et al., 1984; Perry and
Cowey, 1985). However, the mouse retina does not have a fovea, which was reflected in our maps of cortical magnification. No increase in magnification was observed near the center of the right eye’s visual field (i.e. right hemifield), which is found near the center of V1. However, consistent with previous reports (Wagor et al., 1980; Schuett et al., 2002), there was an increase in magnification at the representation of the central/nasal visual field of V1, corresponding to the binocular zone. Furthermore, we showed that extrastriate areas with coverage of the central visual field also showed a similar increase in magnification factor (LM, AL, RL, PM). The higher magnification in the binocular zone, combined with the recent observation that neurons in this region are tuned to binocular disparity (Scholl et al., 2013) suggests that mice utilize binocular information to guide behavior.

A recently observed sampling bias in the retina may also contribute to the higher cortical magnification in the central visual field. While most reports have indicated a relatively homogenous distribution of retinal ganglion cells across the retina (Drager and Olsen, 1981; Jeon et al., 1998), Bleckert et al. demonstrated that a specific subpopulation of Alpha-On RGCs, thought to subserve higher resolution vision, displays an increased density at the temporal retina (Bleckert et al., 2014). It would be interesting to investigate whether there is a corresponding increase in the peak spatial frequency of cortical neurons in binocular regions. Taken together, the spatial coincidence of disparity tuning (Scholl et al., 2013) and higher sampling density of Alpha-
On RGCs (Bleckert et al., 2014) could yield enhanced stereoacuity at the central visual field in the mouse.

Alpha-like RGCs have been implicated in chromatic processing specifically at the location of the opsin transition zone along the horizontal meridian (Chang et al., 2013). Accordingly, we observed a slight increase in magnification factor along the horizontal meridian of V1 (Fig. 1.7b), which may correlate with the increased information provided by color opponent responses in this region of the retina. Also in support of the increased magnification at the horizontal meridian is the observation that the density of RGCs along the central retina is slightly elongated in the corresponding dimension, in mice as well as other rodents, suggestive of a structure similar to the visual streak in carnivores (Drager and Olsen, 1981; Picanço-Diniz et al., 2011).

Concluding remarks

A hallmark of sensory cortex is the grouping of neurons with similar function, particularly through unique inputs from parallel pathways. This architecture allows for a powerful experimental paradigm, whereby the locations of homogenous compartments are first identified noninvasively, followed by targeted injections or recordings for subsequent manipulation or characterization. We show that retinotopic mapping with intrinsic signal imaging combined with objective analytical tools provides a means for targeting mouse visual areas even when they are too small or too variable in their location to be targeted by approaches such as stereotaxic coordinates. Furthermore, rodent visual areas contain significant biases in both response
properties (Andermann et al., 2011; Marshel et al., 2011; Tohmi et al., 2014) and receptive field position (this study). This overall functional specialization is likely to be governed by parallel circuits (Glickfeld et al., 2013) and also to help guide parallel behaviors. In turn, there is great promise in establishing a link between circuits and behavior by exploiting both the architecture of mouse visual cortex and genetic tools. Our objective, quantitative approach to characterizing the layout of mouse visual areas is an important element of this approach.

**Experimental Procedures**

**Animal Preparation and Surgery**

All experiments involving living animals were approved by the Salk Institute's Institutional Animal Care and Use Committee. C57BL/6 mice (n = 15) between 2 and 3 months old were anesthetized with isoflurane (2% induction, 1%–1.5% surgery) and implanted with a custom made metal frame over the visual cortex for head fixation. Carprofen (5 mg/kg) was administered subcutaneously prior to surgery, and ibuprofen (30 mg/kg) was given in drinking water for up to 1 week after implanting the recording chamber. Mice were allowed to recover for 1–2 days following head frame implantation. For intrinsic imaging experiments, mice were again anesthetized with isoflurane (2% induction) and head fixed in a custom holder. Chlorprothixene (1.25 mg/kg) was administered intramuscularly and isoflurane was reduced to
0.25%–0.8% (typically ~0.5%) for visual stimulation. Anesthesia levels were titrated to maintain a lightly anesthetized state. Imaging was performed either through the intact skull or through a thinned skull. The right eye was coated with a thin layer of silicone oil to ensure optical clarity during visual stimulation. Following experiments, animals were recovered overnight on a heating pad.

**Measuring Retinotopic Maps**

To perform intrinsic imaging, we used the camera, frame grabber, illumination, and filters described in Nauhaus & Ringach, 2007. Retinotopic maps were measured by taking the temporal phase of response to a periodic drifting bar (Kalatsky & Stryker, 2003). We adapted this general paradigm to keep the size (deg) and speed (deg/sec) of the bar constant relative to the mouse’s perspective. More specifically, the temporal phase of the horizontal and vertical drifting bar directly encodes iso-azimuth and iso-altitude coordinates on a globe, respectively. From the mouse’s perspective, azimuth and altitude lines are perpendicular at all locations of the monitor. A contrast-reversing checkerboard was also presented within the boundaries of the drifting bar to more effectively drive the cortex. The mathematical details of this stimulus were described previously (Marshel et al. 2011). Each trial consisted of ten sweeps of the bar in one of the four cardinal directions (up/down altitude, left/right azimuth), with a temporal period of 18.3 s. Six-to-ten trials were presented for each of the four directions.

A 55” 1080p LED TV (Samsung) was placed in the right hemifield, 30° from the mouse’s midline, approximately parallel to the right eye
(Oommen and Stahl, 2008). The perpendicular bisector of the monitor from the eye was placed at the origin of the vertical and horizontal retinotopy coordinates (Fig. 1.1A,B). Taken together, this puts the vertical meridian of the mouse’s visual field at approximately 60° (nasal direction) from the origin of the stimulus coordinates. The horizontal meridian was approximately centered on the origin.

**Identifying Visual Areas**

The processing steps outlined below were designed to satisfy our four criteria for identifying visual areas. The steps associated with Criteria 1-3 compute borders between visual areas in each experiment. The final steps satisfy Criterion 4 to determine which of the regions found in the previous steps are reliably found across experiments.

**Criterion 1:** Each area must contain the same visual field sign at all locations within the area.

Our first step was to create segregated patches of visual cortex from the visual field sign map (S):

$$S = \sin(4\nabla H - 4\nabla V), \quad Eq\ 1$$

H and V are gradients of the maps of horizontal and vertical retinotopy, respectively. Values of S closer to -1 or 1 indicate regions that have more orthogonal intersection. Positive and negative values of S indicate a non-mirror and mirror representation of the visual field, respectively. The S map is shown in Figure 1.1C. Because adjacent visual areas tend to have opposite
visual field sign this operation is quite useful as a first step in delineating areas (Sereno et al., 1994). Next, we created discrete patches on the cortex by thresholding the S map at +/-1.5 times its standard deviation. That is, we compute:

\[
S_{\text{Thresh}} = \begin{cases} 
+1; & S > +1.5\sigma \\
-1; & S < -1.5\sigma \\
0; & \text{otherwise}
\end{cases}, \text{Eq 2}
\]

A map of \(S_{\text{Thresh}}\) is shown in Figure 1.1D. A threshold of +/-1.5 is quite liberal in the sense that it often leaves separate areas fused together as one patch. However, the low threshold ensured that we don’t miss actual visual areas in this step. The processing steps of Criterion 2 split patches that are identified as being multiple areas. The low threshold also tends to let through many pixels that are from the background noise. Many of these were eliminated by simply performing morphological opening on \(S_{\text{Thresh}}\). Others were cleared in the following step.

Before moving on to the processing steps for Criterion 2, we recomputed patch borders so that they are all connected. This step and others make extensive use of a class of image processing operations called morphological filters. Morphological filters are nonlinear operations that are especially useful for processing binary images (Serra, 1982). To compute the border of visual cortex, we first perform morphological “closing” on \(S_{\text{Thresh}}\), followed by “opening”, followed by “dilation”,

\[
\text{VisualCortexBoundary} = \text{Dilate}(\text{Open}(\text{Close}(|S_{\text{Thresh}}|))), \text{ Eq 3}
\]
This invariably yields one large patch at the center of the field-of-view, corresponding to patch 11 in Figure 1.1D. By assuming that visual cortex is one connected set of areas we are able to automatically remove many of the unwanted regions contained in $S_{\text{Thresh}}$.

Next, we recomputed areal borders using morphological “thinning”, iterating to infinity (Lam et al., 1992). This operation takes the regions between all the patches and thins it to boundaries that are 1 pixel wide.

Although computing $S_{\text{Thresh}}$ alone is efficient at delineating most of the visual area borders (Sereno et al., 1994), it is nonetheless far from perfect in mice. The following steps were designed to identify borders that were missed (Criterion 2) or borders that should not have been made (Criterion 3).

**Criterion 2:** Each visual area cannot have a redundant representation of visual space.

If two visual areas have adjacent borders and are of opposite visual field sign, then the steps under Criterion 1 do the job of splitting the two neighboring areas. However, two areas of the same visual field sign often have neighboring borders, which will leave them fused in $S_{\text{Thresh}}$. Our criteria for determining if a patch contains more than one visual area was based on whether or not it contains a redundant representation of visual space. To determine if a patch contains a redundant representation, and should thus be segmented, we compared two different measurements of the visual field coverage. The first measurement is the area in visual space covered by the “union” of all pixels in the patch, which we denote as $A_{U}$. For example, the
outline of pixels from a patch in cortical space corresponds to an outline of pixels in visual space. The area encompassed by the outline in visual space is $A_U$. Next, we computed the total areal coverage by integrating over pixels within the patch, which we denote as:

$$A_{EU} = \sum_{i \in \text{patch}} \left| \begin{array}{cc} \Delta H_i / \Delta x & \Delta H_i / \Delta y \\ \Delta V_i / \Delta x & \Delta V_i / \Delta y \end{array} \right|$$  \hspace{1cm} \text{Eq. 4}

The summation is over all pixels within the patch. The top and bottom rows of the 2x2 matrix (i.e. “Jacobian”) are the gradients of the horizontal and vertical retinotopy ($\degree$/mm), respectively, and the brackets indicate a determinant. If $A_{EU} / A_U > 1$, the patch contains a redundant representation. We found it best to set the decision boundary to be slightly above unity (e.g. $\approx 1.1$) because under cases that the patch’s representation is not redundant, the ratio is almost exactly unity. The ratio of patch 11 in Figure 1.1D is $>1$ and should therefore be segmented.

Next, we split patches with a redundant representation of visual space using the eccentricity map. Segmenting redundant patches entails determining the areal borders. The first step in this process was to compute the visual eccentricity map for each area independently, defined as

$$E_i = \tan^{-1} \sqrt{\tan^2(H_i - \langle H_i \rangle) + \frac{\tan^2(V_i - \langle V_i \rangle)}{\cos^2(H_i - \langle H_i \rangle)}}, \hspace{1cm} \text{Eq. 5}$$

This equation is derived based on the fact that the horizontal ($H$) and vertical ($V$) retinotopy were generated on the monitor as azimuth and altitude in
spherical coordinates (Marshel et al., 2011), respectively. Note that the center of visual space is made relative to the average of $H$ and $V$ within the patch. Next, we found the local minima of $E$ within the patch. To robustly determine local minima of $E$, we first discretize it at $5^\circ$ intervals. Next, local minima were determined as groups of pixels completely surrounded by pixels with larger eccentricity values. Most often, this would yield two local minima, but occasionally three. Lastly, to segment $E$, we used the watershed transform with local minima in the ecc map as markers (Hamdi, 2001). The watershed transform is a typical approach to image segmentation that considers the image as topographic structure to identify “catchment basins” and “watershed ridge lines”. Using markers (the minima) makes the algorithm more robust because it forces the location of the catchment basins and number of segmented regions. Appropriately, the borders were determined to be along the “watersheds” of the eccentricity map.

**Criterion 3:** Adjacent areas of the same visual field sign must have a redundant representation.

The goal of this step is to determine if a single area was improperly split when computing $S_{\text{Thresh}}$. The cause of an erroneous border is often from noise in the image, such as a blood vessel artifact, or from noisy maps in less well organized or more variable areas. A pair of same-sign patches is identified as adjacent if they overlap after morphological dilation. Next, if the patches are adjacent, they are fused together using morphological closing. Last, we use
the same criteria defined in step 3 to determine if the fused patch has a redundant representation. That is, if ~1 when fused, then they are left fused.

In summary, the above steps ensure that all of the defined patches have orthogonal representations of horizontal and vertical retinotopy (A, step 1), they are all connected (A, step 2), they are not actually two areas combined into one (B, steps 1,2), and that two adjacent patches are not actually a single area (C).

Criterion 4: An area’s location must be consistently identifiable across experiments.

The steps above identify a series of patches for each experiment that presumably correspond to the mouse’s predefined cortical architecture and are thus predictable across the different experiments. The goal of this last section is to identify cortical locations that are likely to contain the center-of-mass (CoM) of a patch. To summarize, we first aligned all the experiments using the location of V1, and then pooled all of the patch CoMs to search for patch clusters. Significant clusters were then identified and patches associated with each cluster were given a common visual area name.

First, we aligned visual cortex across experiments. To do so, we identified a common origin and rotation axis for each field of view. We used the V1 patch because it is always the largest patch in the field-of-view, which makes it easy to disambiguate from the others. For the origin, we chose V1’s center-of-mass (“CoM”). For the axis we chose the average direction of V1’s
horizontal retinotopy gradient. All experiments were then shifted and rotated accordingly.

Next, we identified clusters within two different scatter plots of the patch CoM positions – one for all the non-mirror patches in our data set (Figure 1.4B), and one for all the mirror patches (Figure 1.4A). We then performed “k-means clustering” on both the mirror and non-mirror scatter plots. The number of k-means in our search was determined as the maximum number of mirror or non-mirror patches in a given experiment ($k_{\text{non-mirror}} = 10; k_{\text{mirror}} = 8$). These values of $k$ gave an intuitive segregation of the clusters, although it clearly yielded some k-means that did not contain significantly clustered CoMs relative to the overall distribution of CoMs. The patches associated with these k-means may be a consequence of measurement noise, or possibly they are areas that are more difficult to detect with intrinsic signal imaging. To determine which k-means were not actually associated with a cluster we computed a normalized metric for the degree of clustering associated with each k-mean.

$$\text{Clustering } C_k = \frac{\frac{1}{N_k} \sum_{\text{neighbours of patch } p} |M_p - M_{n(p)}| / (S_p + S_{n(p)})}{\frac{1}{N} \sum_{\text{all patches}} |M'_p - M'_{n(p)}| / (S_p + S_{n(p)})} \quad \text{Eq. 6}$$

where $M_p$ is a two element vector for the CoM of patch $p$, $M_n$ is the patch CoM that is the nearest neighbor to $n$. Each nearest neighbor distance linked to patch $p$, is then normalized by the sum of the pair of patch sizes, $S_p$. Specifically, $N$ is the square root of the patch’s area. The numerator sum is over the patches associated with a given k-mean. The sum in the denominator, however, is over all patches in all experiments. The CoM
locations in the denominator were computed after randomly shuffling each experiment. To shuffle the CoMs, we independently rotated each experiment around the V1 CoM by a random value between $0^\circ$ and $360^\circ$. This helped to maintain most of the maps’ inherent spatial statistics. The brackets around the denominator indicate that the average was computed across (100) trials of shuffling. A group was deemed significantly clustered if $>1$. Seven of the k-mean groups did not pass this criteria and are absent in Figure 1.3E-F. The remaining 11 k-mean groups were subsequently labeled as visual areas.

**Quantifying Scatter in Areal Position**

For an intuitive quantification of the amount of intra-areal overlap across the experiments, we computed the following metric for each area (Figure 1.4):

$$\% \text{ Scatter} = 100 \times \frac{\langle |M_p - \langle M_p \rangle| \rangle}{\langle S_p \rangle} \quad \text{Eq. 7}$$

The numerator is the average absolute distance between the patch CoMs and the mean CoM of the patch (mm). The denominator is the average size of the cortical area (mm).

**Coverage overlap**

Each pixel in Figure 1.7E was computed as

$$\text{Coverage overlap}_{x,y} = \frac{[D_x^\top - 0.3]^+[D_y - 0.3]^+}{\|[D_y - 0.3]^+\|} \quad \text{Eq. 8}$$

where $D_x$ and $D_y$ are the visual field coverage profiles from the visual area labeled on the x and y axis, and thresholds at zero. Specifically, $D_x$ equals
one when $> 0.3$, and it equals 0 otherwise. Pixel values near one indicate that the coverage profile of the visual area on the y-axis is contained within the coverage profile of the visual area on the x-axis. Pixel values near zero indicate that the two coverage profiles are largely exclusive.

**Cortical Magnification**

Magnification maps (Figure 1.7B) were computed using the Jacobian determinant of the horizontal and vertical retinotopy, at each pixel. Variables in the equation below are identical to those in Eq. 4.

$$ Magnification\ map_i = \begin{vmatrix} \frac{\Delta H_i}{\Delta x} & \frac{\Delta H_i}{\Delta y} \\ \frac{\Delta V_i}{\Delta x} & \frac{\Delta V_i}{\Delta y} \end{vmatrix} \quad Eq. 9 $$
Figure 1.1 Segmentation procedure for delineating visual area patches from retinotopic maps

(A) Example map of horizontal retinotopy, in degrees of visual space. The nasal visual field is represented in purple, transitioning through the center of space in blue, and out to the peripheral fields in yellow. (B) Map of vertical retinotopy from the same mouse, in degrees of visual space. The upper field representation is indicated in purple, through the center of space corresponding to the horizontal meridian in blue, and the lower fields in yellow. (C) Map of visual field sign computed as the sine of the difference in the angle between the horizontal and vertical map gradients on a pixel-by-pixel basis. Regions having a positive field sign, in red, represent non-mirror image transformations of the visual field. A negative field sign, shown in blue, indicates a mirror image representation. Regions with values close to zero lack defined topographic structure. Transitions in field sign between positive and negative values correspond to reversals in the organization of the visual map gradients. (D) Thresholded map of visual field sign, revealing discrete patches corresponding to organized topographic maps. (E) The ratio between the integral of coverage and outline of coverage for each patch identified in D identifies areas with redundant representations as values above the unity line, with a ratio of $A\Sigma/AU$ greater than 1. (F-I) Assessment of overlap in the visual coverage of each pair of neighboring patches of the same field sign. Retinotopic maps for the two segmented patches are shown in the bottom of the panel. The region of visual space covered by each patch is plotted above, with the value for degree of overlap. Regions showing zero overlap in coverage, such as in I, were considered to belong to a single area and were fused into a single patch. Overlap greater than zero indicates that the patches were appropriately segmented, and that their union would create a redundant representation. (J) Final set of patch boundaries with the center of mass of each region labeled according to visual field sign (blue = mirror, red = non-mirror). (K) Patch boundaries overlayed with contour plot of azimuth, corresponding to horizontal retinotopy as in A, with the associated colormap. Contour lines for altitude are shown in black to illustrate the degree of orthogonality in the maps across patches. (L) Contour plot of altitude lines, corresponding to the map values for vertical retinotopy in B, with patch boundaries overlayed in gray, and azimuth contours in black.
Figure 1.1 Segmentation procedure for delineating visual area patches from retinotopic maps, continued

(A) Example map of horizontal retinotopy, in degrees of visual space. The nasal visual field is represented in purple, transitioning through the center of space in blue, and out to the peripheral fields in yellow. (B) Map of vertical retinotopy from the same mouse, in degrees of visual space. The upper field representation is indicated in purple, through the center of space corresponding to the horizontal meridian in blue, and the lower fields in yellow. (C) Map of visual field sign computed as the sine of the difference in the angle between the horizontal and vertical map gradients on a pixel-by-pixel basis. Regions having a positive field sign, in red, represent non-mirror image transformations of the visual field. A negative field sign, shown in blue, indicates a mirror image representation. Regions with values close to zero lack defined topographic structure. Transitions in field sign between positive and negative values correspond to reversals in the organization of the visual map gradients. (D) Thresholded map of visual field sign, revealing discrete patches corresponding to organized topographic maps. (E) The ratio between the integral of coverage and outline of coverage for each patch identified in D identifies areas with redundant representations as values above the unity line, with a ratio of AΣ/AU greater than 1. (F-I) Assessment of overlap in the visual coverage of each pair of neighboring patches of the same field sign. Retinotopic maps for the two segmented patches are shown in the bottom of the panel. The region of visual space covered by each patch is plotted above, with the value for degree of overlap. Regions showing zero overlap in coverage, such as in I, were considered to belong to a single area and were fused into a single patch. Overlap greater than zero indicates that the patches were appropriately segmented, and that their union would create a redundant representation. (J) Final set of patch boundaries with the center of mass of each region labeled according to visual field sign (blue = mirror, red = non-mirror). (K) Patch boundaries overlayed with contour plot of azimuth, corresponding to horizontal retinotopy as in A, with the associated colormap. Contour lines for altitude are shown in black to illustrate the degree of orthogonality in the maps across patches. (L) Contour plot of altitude lines, corresponding to the map values for vertical retinotopy in B, with patch boundaries overlayed in gray, and azimuth contours in black.
Figure 1.2 Topography and patch boundaries for individual examples.

Each row corresponds to an individual mouse. (A) Borders identified by the segmentation algorithm for 5 mice. The visual field sign of each patch is indicated by the color of the point corresponding to the center of mass for each area (blue = mirror, red=non-mirror). (B) Horizontal retinotopy, shown as azimuth contours, are shown in color with values indicated by the associated colormap. Altitude lines are overlayed in black to illustrate the relative organization of the map gradients across areas. Identified patch boundaries are shown in gray. (C) Vertical retinotopy, shown as altitude contours, with corresponding colormap. Azimuth lines are overlayed in black and boundaries in gray. Scale for all panels is shown in the bottom row of A.
Figure 1.3 Classification of visual areas based on clustering of patch centers after alignment across animals.

(A,B) Scatter plot of mirror and non-mirror patch centers from each animal, after alignment of the maps across cases using the center of mass of the largest patch and the direction of its horizontal gradient. Some patch centers fall into visible spatial clusters, while others appear more variable in their distribution. (C,D) K-means clustering reveals grouping of patches for mirror (red) and non-mirror (blue) scatter plots, with associated means shown in black, and distinct clusters separated by Voronoi lines. (E,F) Significant clusters remaining after shuffling analysis (see Methods). Variable patches that were not associated with significant clusters were removed from further analysis. Remaining patch centers are considered as reliably identified visual areas, found in a consistent spatial position across animals, labeled and color coded according to their grouping and anatomical position relative to V1. For the set of mirror image areas: LM, lateromedial area; RL, rostrolateral area; PM, posterior-medial area; P, posterior area; POR, postrhinal area; LLA, laterolateral-anterior area. For the set of non-mirror image areas: V1, primary visual cortex; LI, lateral-intermediate area; AL, anterior-lateral area; AM, anterior-medial area; MM, mediomedial area.
Figure 1.4 Cortical coverage and variability in area position across animals

(A) Overlay of all area boundaries across experiments, aligned using V1’s center of mass and horizontal gradient direction. Areal identity of patch borders was determined according to the classification procedure described in Figure 1.3. (B) Combined scatter plot of all area centers, with cluster mean in black, demonstrating the differences in area distributions relative to V1. (C) Average size of areas across cases. Red dots indicate values from the average map (shown in Figure 1.5). (D) Scatter in area position taken as the standard deviation of the cluster distribution for each area. (E) Variability in area position normalized by area size provides an estimate of how consistently a given area can be found in the same cortical location across animals.
Figure 1.5 Average topographic maps demonstrate defining features of mouse visual cortical organization

(A) Visual field sign map produced by a weighted average of all experiments, emphasizing those with stronger responses. The segmentation algorithm was used to identify area boundaries based on visual gradients, as with individual cases. Areal organization of the average map closely corresponds to the layout observed in individual cases, with all major areas identified. (B) Map of the center of the right visual hemifield, produced by combining information from the vertical and horizontal maps into a single representation. The origin of the center map corresponds to the zero point of both altitude and azimuth, corresponding to the approximate center of the mouse’s eye, showing radial distance away from the center as increasingly positive values. This discrete retinotopic position can be used to distinguish different areas according to their independent representation of eye-centered coordinates. (C) Average azimuth contours showing progression of the horizontal gradient from temporal fields in yellow to the nasal field in purple. Altitude contours are overlayed in black to illustrate relationships between the gradients, and area boundaries are shown in gray. (D) Average altitude contours showing progression of vertical retinotopy from the lower fields in green to the upper field in purple. Azimuth contours are overlayed in black and area boundaries in gray. (E) Map of visual eccentricity from the junction of V1, LM and RL, corresponding to the intersection of the vertical and horizontal meridians at the center of space. (F) Map of polar angle in the same coordinate system, demonstrating progression of radial visual angle from the center of space.
Figure 1.6 Visual coverage of higher areas is often incomplete or biased in space

(A) Coverage of visual space measured as the normalized sum of coverage at each visual field position across animals. Black lines indicate the coverage profile of the area (see methods). Maps are aligned in a common anatomical reference frame at a point corresponding to the intersection of the vertical and horizontal meridians at the center of space. Accordingly the nasal most representation at the vertical meridian corresponds to zero degrees azimuth, with increasing values indicating more peripheral fields, with positive values of altitude representing the upper field and negative values indicating the lower field. Comparison of visual representations across areas demonstrates differences in the extent and region of visual coverage. (B) Center of mass of visual field coverage profile for each area reveals biases in visual representations towards particular parts of space. (C) Average total visual coverage for each identified region. V1 covers a large extent of the visual hemifield, higher areas represent significantly smaller portions of space. (D) Proportion of total coverage contributed by each visual field quadrant quantifying the bias in representation for different areas. (E) Matrix of visual field overlap between each pair of areas. Coverage overlap was determined as the sum of the coverage profiles of the pair of areas on the x- and y-axes, normalized by the coverage of the area on the y-axis. Black dots indicate pairs of neighboring areas that have the same visual field sign.
Figure 1.7 Differences in cortical magnification between areas and across visual eccentricity

(A) Overlay of altitude and azimuth contours for the average map illustrating the relationships between vertical and horizontal gradients across cortical space. Contour lines are spaced at 5 degree intervals. (B) Cortical magnification computed for each pixel of the average map, in mm²/deg². An increase in magnification is observed at the lateral V1 border, at the intersection with LM and RL, corresponding to the center of visual space and the binocular zone. (C) Magnification averaged across all pixels for each area across cases, with the values for the average map in red. While the majority of areas have lower magnification compared to V1, as would be expected from having smaller total cortical coverage, a few, notably RL, have similarly high magnification, perhaps associated with their incomplete coverage of visual space, or an emphasis on the nasal visual field. (D) Relationship between magnification and visual eccentricity determined for each visual area across cases, shown in black, and in the average map, in red. A trend towards higher magnification at central representations is observed for several areas. However, others either lack coverage of the central visual field, or show no trend.
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References


CHAPTER 2. FUNCTIONAL SPECIALIZATION OF SEVEN MOUSE VISUAL CORTICAL AREAS

Abstract

To establish the mouse as a genetically-tractable model for high-order visual processing, we characterized fine-scale retinotopic organization of visual cortex, and determined functional specialization of layer 2/3 neuronal populations in seven retinotopically-identified areas. Each area contains a distinct visuotopic representation and encodes a unique combination of spatiotemporal features. Areas LM, AL, RL, and AM prefer up to three times faster temporal frequencies and significantly lower spatial frequencies than V1, while V1 and PM prefer high spatial and low temporal frequencies. LI prefers both high spatial and temporal frequencies. All extrastriate areas except LI increase orientation selectivity compared to V1, and three areas are significantly more direction selective (AL, RL, AM). Specific combinations of spatiotemporal representations further distinguish areas. These results reveal that mouse higher visual areas are functionally distinct, and separate groups of areas may be specialized for motion-related versus pattern-related computations perhaps forming pathways analogous to dorsal and ventral streams in other species.
Introduction

Specialized neural circuits process visual information in parallel hierarchical streams, leading to complex visual perception and behavior. Distinct channels of visual information begin in the retina and synapse through the lateral geniculate nucleus to primary visual cortex (V1), forming the building blocks for visual perception (Nassi and Callaway, 2006). In primates, these information channels are transformed and integrated multiple times over, through increasingly higher order computations at each stage in a complex hierarchy of extrastriate visual areas that each contain a discrete visuotopic representation of space (Felleman and Van Essen, 1991; Orban, 2008; Van Essen, 2003).

Strikingly, neurons in each of these areas are selective for specific features of a visual stimulus within their receptive fields. For example neurons may respond with a barrage of action potentials to a narrow range of directions of motion, orientations of a bar of light, wavelengths of light, depths in the field, and/or complex shapes or patterns defining particular objects. In most cases, visual areas represent at least some information along basic feature dimensions such as direction, orientation, spatial frequency and temporal frequency (Felleman and Van Essen, 1987; Orban, 2008). Differences in the ranges of parameters represented by each population, and/or the fraction of neurons selective for particular stimulus attributes functionally distinguish different areas (Baker et al., 1981; Felleman and Van Essen, 1987; Foster et
Selective feedforward and feedback projections link together areas with related feature selectivities to form parallel processing streams and define hierarchical relationships (Felleman and Van Essen, 1991). Two major parallel processing pathways have been defined based on functional specializations, patterns of connections, and associations with different behaviors. The dorsal pathway is specialized to process motion and spatial relationships and is related to behaviors involving visually guided actions. The ventral pathway is specialized to process fine-scale detail, shapes and patterns in an image to support object recognition and is associated with visual perception (Desimone et al., 1985; Maunsell and Newsome, 1987; Ungerleider and Mishkin, 1982; Van Essen and Gallant, 1994).

This wealth of information about the visual system has resulted from decades of research primarily in primate and carnivore species. However, large gaps in understanding remain, most notably relating circuit-level mechanisms and gene expression to specific neuron response characteristics and high-order extrastriate computations. The main limitation preventing this level of understanding is the inaccessibility of these species to large-scale, high-throughput studies relating response characteristics to specific circuit elements or circuit development to specific genes.

The last decade has seen enormous advances along this front in terms of molecular and genetic methods available to understand circuit structure and function at the level of specific genes, well-defined neuronal populations, specific cell types, and single neurons in the mouse (Arenkiel and Ehlers,
2009; Luo et al., 2008). These include methods for identifying connectivity and manipulating or monitoring activity or gene expression across all of these levels. By combining methods for targeting defined cell populations and networks with techniques for monitoring or manipulating activity, it is possible to identify the functions of particular circuit components or specific genes and directly test causal relationships on circuit function and behavior. While these approaches have allowed important insight into numerous neural systems, their use in studies of the mouse visual system has been limited primarily to the mechanisms that generate orientation selectivity in V1 (Huberman and Niell, 2011). This is due to both a historical reliance on species other than mice, technical limitations, and a lack of knowledge about fundamental properties of mouse visual areas beyond V1.

In order to combine the power of mouse genetics with the advantages of the visual system as a model for understanding mechanisms of brain function, we must obtain an understanding of the mouse visual system that rivals that of the more traditional primate and carnivore models. Recent observations indicate that the mouse visual system is surprisingly sophisticated. Behavioral studies indicate that mice can perform complex, visually guided behaviors (Prusky and Douglas, 2004). Functional studies demonstrate that neurons in mouse V1 can be highly tuned for visual features such as orientation and spatial frequency, despite the overall lower spatial resolution of the system (Drager, 1975; Gao et al., 2010; Niell and Stryker, 2008). Furthermore, anatomical experiments reveal that mouse V1 is surrounded by at least nine
other visual cortical regions that receive topographically organized input from V1 (Wang and Burkhalter, 2007). However, despite some preliminary work (Tohmi et al., 2009; Van den Bergh et al., 2010), the functions of mouse extrastriate visual areas are largely unidentified. As a result, answers to the most basic and fundamental questions about mouse visual cortical organization remain unknown. Is each cortical area specialized for extracting information about particular types of features in the visual world? Are increasingly complex representations built up within a hierarchy of visual areas? Are there relatively independent sets of visual areas comprising distinct pathways that carry information related to processing motion versus shape, or specialized for behavioral action versus perception as in the primate visual system?

To establish the mouse as a model for visual information processing, we sought to assess the functional organization of mouse visual cortex. We developed a high-throughput method for characterization of response properties from large populations of neurons in well-defined visual cortical areas. First, we determined the fine-scale retinotopic structure of ten visual cortical areas using novel, high-resolution mapping methods to outline precise area boundaries. We then targeted seven of these visual areas (V1, LM, LI, AL, RL, AM, and PM) for in vivo two-photon population calcium imaging to characterize functional responses of hundreds to thousands of neurons in each area. This allowed us to determine selectivity for fundamental visual features including orientation, direction, spatial frequency and temporal frequency of
defined neural populations in each area, under the same carefully controlled conditions. Comparison of these tuning properties across areas revealed that higher visual areas in the mouse encode unique combinations of spatiotemporal information that are distinct from V1. Furthermore, we found that each extrastriate area could be distinguished from every other visual area based on specific combinations of visual feature representations. Together with anatomical information (Berezovskii et al., 2011; Coogan and Burkhalter, 1990, 1993; Wang and Burkhalter, 2007), these results suggest that mouse visual cortical areas may comprise hierarchically-organized parallel pathways perhaps similar to the dorsal and ventral streams suggested in other species. This study provides a fundamental understanding of the basic tuning properties of the majority of mouse visual cortical areas using novel high-throughput methods, laying a foundation for the use of the mouse as a genetically-tractable model of visual information processing.
Results

We devised an experimental strategy to efficiently map the precise locations of mouse striate and extrastriate visual areas using a combination of intrinsic signal imaging (Figure 2.1) and high-resolution, wide-field two-photon calcium imaging (Figure 2.2). We then performed two-photon calcium imaging of populations of neurons in ~1-3 visual areas in each animal, and in 7 visual areas total across our dataset: the primary visual cortex (V1), lateromedial area (LM), laterointermediate area (LI), anterolateral area (AL), rostrolateral area (RL), anteromedial area (AM), and posteromedial area (PM). Two experiments were performed for each sample of neurons in a given two-photon field of view: a spatial frequency (SF) experiment and a temporal frequency (TF) experiment. In the SF experiment, drifting sine-wave gratings were presented to the animal, varying across 5 spatial frequencies and 8 directions, at a single temporal frequency (~1 Hz). In the TF experiment, the gratings varied across 5 temporal frequencies and 8 directions, at a single spatial frequency (~0.04 cpd). This design permitted us to generate SF, TF, orientation and direction tuning curves for each neuron (Shapley and Lennie, 1985; Figure 2.3). We analyzed these tuning curves to determine the preferred SF and TF, orientation and direction-selectivity indices (OSI, DSI), and tuning bandwidth metrics for each neuron. We then compared population statistics across areas to determine whether mouse visual cortical areas are specialized to process
different information (Figure 2.4-2.6). We found that higher visual areas represent different combinations of spatiotemporal information (Fig. 2.7), bearing on potential pathways of information processing within the mouse visual cortical system.

Fine-scale retinotopic mapping of mouse visual cortex reveals nine mouse visual areas

Our first goal was to efficiently and precisely map the retinotopic organization of mouse striate and extrastriate visual cortex in order to rapidly target distinct visual areas for population imaging and analysis. Previous anatomical work in mice predicts the existence of at least 9 extrastriate visual cortical areas, based on topographic projections from V1 (Olavarria and Montero, 1989; Wang and Burkhalter, 2007). However, functional studies have failed to identify several detailed features of the retinotopic maps predicted by anatomy, resulting in significant variation between proposed schemes for the areal organization of mouse visual cortex (Kalatsky and Stryker, 2003; Schuett et al., 2002; Wagor et al., 1980; Wang and Burkhalter, 2007). Given the extremely small size of some proposed extrastriate visual areas (≤ 500 µm), we reasoned that insufficient resolution of previous recording methods, in combination with stimulation of only portions of the visual field in some studies resulted in incomplete functional retinotopic maps. Thus, to rapidly and reliably target any given visual area in each animal, we developed a fast, sensitive,
high resolution functional recording method to map the retinotopic organization of cortex corresponding to the complete visual field.

We adopted a two-step approach that provided sufficient resolution to reliably define the extent and organization of each cortical visual area rapidly in each animal. First, we used intrinsic signal imaging to measure the hemodynamic response across the visual cortex to drifting bar stimuli at moderate resolution (estimated previously to be on the order of 200 µm by (Polimeni et al., 2005). This was adapted from a sensitive method used previously (Kalatsky and Stryker, 2003), with the key difference that we used an improved, larger stimulus that mapped the entire known retinotopic visual hemifield of the mouse (Wagor et al., 1980) in spherical coordinates (Supplemental Experimental Procedures). Second, we used wide-field two-photon calcium imaging to measure the retinotopic organization of visual cortex at high resolution (~3 µm/pixel; 16X objective). This resulted in a retinotopic map which was continuous within the extent of visual cortex and allowed us to precisely define borders between several areas based on visual field sign reversals at peripheral representations (Sereno et al., 1995; Figure 2.2).

An example intrinsic imaging map from one animal is shown in Figure 2.1. Several features of previous map schema are present in the map (for a direct comparison, see Wagor et al., 1980, Figure 4 and Wang and Burkhalter, 2007, Figure 10). Our data are most consistent with the map predicted primarily from anatomy by Wang and Burkhalter, 2007, and thus all further analyses and discussion are made in reference to their schema and area
names. Intrinsic imaging maps were sufficient to detect activation in V1, LM, LI, AL, RL, A, AM, PM, P and POR, but often could not resolve fine-scale details in the maps of relatively small areas (such as LI, RL, A, AM and PM) that were necessary to precisely define area boundaries.

Using the intrinsic imaging maps as a guide, several calcium dye loadings were performed to load a volume of cortex spanning several millimeters and encompassing several visual areas (Experimental Procedures). We then systematically imaged the extent of the loaded area by moving the objective in ~500-700 µm steps in order to ultimately tile the whole loaded region. At each position, we displayed the retinotopic mapping stimulus (similar to that used for intrinsic imaging) to the animal, and mapped the retinotopy of that ~800-1000 µm² patch of cortex. Mosaics of these individual maps resulted in a complete high-resolution map of the region, often spanning several millimeters and encompassing the full visuotopic extent of several extrastriate visual areas (Figure 2.2 and Figure 2.2A). At this resolution, we observed several features in the maps that were not seen with intrinsic imaging, revealing the fine-scale organization of each of 8 extrastriate visual areas predicted previously (LM, LI, AL, RL, AM, PM, P and POR; Figure 2.2 and Figure 2.2A). We observed some retinotopic structure in the putative location of area A, but did not target this area for population analysis because its retinotopic map was ambiguous in relation to its predicted organization (Wang and Burkhalter, 2007; Figure 2.2 and Figure 2.2A). It was also difficult to obtain complete maps of areas P and POR given their cortical location (Figure 2.2A).
Using this method, we located the precise region of cortex representing the central visual field within each confidently identified area (~0 degrees azimuth, ~20 degrees altitude) for further analysis. We then converted the microscope to higher-resolution imaging mode (~1 μm/pixel; 40X objective) to characterize visual response properties of populations of neurons in each identified visual area.

Response characterization and population analysis of neural populations in seven visual areas

In all, we recorded from well over 4,000 neurons, with populations ranging from hundreds to thousands of neurons for each of 7 visual areas (V1, LM, LI, AL, RL, AM, PM; Table 1). Two-photon calcium imaging permits recording of neural activity, with single cell resolution, simultaneously from populations of hundreds of neurons in a given field of view (Figure 2.3, panel A, left). After confirming the precise eccentricity represented by each neuron (retinotopy stimulus), drifting grating stimuli that varied across 5 spatial frequencies, ranging from 0.01-0.16 cycles per degree (cpd), and 8 directions (SF experiment), or 5 temporal frequencies, ranging from 0.05-8Hz, and 8 directions (TF experiment), were displayed to the animal. Responses were measured as the average change in the fluorescence of the calcium dye during the stimulus period across multiple trials, relative to the baseline fluorescence during the prestimulus period (Figure 2.3, panel A and Figure 2.3A).

Two-photon calcium imaging provides the unique advantage of being able to quantify the fraction neurons in a cortical region that reliably respond
under a given stimulus condition. Across the entire population of cells from all visual areas, 39% (n = 1,811/4,609) of neurons in the spatial frequency experiments, and 27% (n = 1,195/4,449) of neurons in the temporal frequency experiments were reliably responsive to at least one stimulus condition (see Supplemental Experimental Procedures). Areas differed in the proportion of neurons that responded robustly and reliably to at least one stimulus condition (Table 1). Areas V1, LM and AL had the highest proportions of reliably responsive neurons (51%, 51%, 37% respectively for SF, 42%, 29%, 28% for TF). Areas RL, LI and PM had moderate proportions of reliably responsive neurons (33%, 23%, 29% respectively for SF, 17%, 12%, 10% respectively for TF), and area AM demonstrated the lowest fraction of reliably responsive neurons (16% for SF, 4% for TF). Intriguingly, in areas with lower proportions of responsive cells (such as AM), responsive neurons were generally extremely robust and selective (Figure 2.3, panel B and Figure 2.3A, panel F). This demonstrates that neurons in extrastriate visual areas are highly selective for the appropriate stimulus, and suggests that the neurons which did not respond likely require stimuli or other conditions not explored in this study. That a higher fraction of neurons responded during the spatial frequency experiment suggests that neurons may be more selective to the appropriate spatial frequency than they are to temporal frequency within the ranges we tested. Indeed, spatial frequency bandwidth tuning was generally sharper than temporal frequency bandwidth tuning over the four octaves we sampled in each domain (Figure 2.4-2.5).
For each reliably responsive neuron (Table 1.1, Experimental Procedures), we computed a tuning curve for spatial frequency, temporal frequency, direction and orientation. For spatial and temporal frequency, the tuning curves were taken at the direction that gave the maximal response (orange and magenta boxes Figure 2.3, panel A, and Figure 2.3), and for orientation and direction, the tuning curves were taken at the spatial frequency that gave the maximal response (yellow boxes Figure 2.3, panel A, and Figure 2.3A). From these tuning curves, we determined tuning and selectivity metrics including: the preferred spatial or temporal frequency yielding the maximal response (pref. SF, pref. TF), spatial and temporal frequency selectivity bandwidth (BW) and low and high cutoffs (LC and HC), orientation and direction selectivity indices (OSI and DSI; text above tuning curves in Figure 2.3, panel A, and Figure 2.3A).

We compared the population distributions of these tuning metrics between areas to determine whether mouse visual areas encode distinct combinations of visual features. We found that overall, there was a main effect of area on our four primary dependent variables: preferred spatial frequency, preferred temporal frequency, orientation selectivity index (OSI) and direction selectivity index (DSI), meaning that at least one visual area could be distinguished from another based on scores on these metrics (one-way MANOVA, independent variable: Area, $F_{(24, 2537)} = 18.021$, $p < 0.0005$, Wilk’s $\lambda = 0.577$, $\varepsilon^2 = 0.128$). We followed up this multivariate test with both parametric and non-parametric univariate tests (both one-way ANOVA and Kruskal-Wallis.
tests) comparing the scores on each dependent variable as a function of area to determine whether the mean and/or medians could be distinguished statistically in each comparison. Both parametric and non-parametric one-way tests gave comparable results in all instances, and we have shown the results of the ANOVA tests here. We followed up each significant one-way test with the appropriate post-hoc test (Tukey-Kramer Honestly Significant Difference (HSD) method) in order to determine which pairs of areas differed significantly from each other for each parameter. This statistical design accounted for the familywise error rate in the MANOVA test and for multiple comparisons in each one-way test and post-hoc tests.

By characterizing responses from large populations of neurons across seven visual areas under the same carefully controlled conditions, we were able to directly compare the statistics of each area’s population. The statistical power of this experimental design provides confidence in comparisons made between areas based on combinations of features encoded in each area. As the results presented below indicate, this establishes the basis for the identification of functional specialization of each area investigated.

**Extrastriate visual areas encode high temporal frequency information**

The geometric means and distributions of preferred temporal frequency tuning for each area’s population revealed two groups of areas: one representing low temporal frequencies and one representing higher temporal frequencies (Figure 2.4, panel A). The cumulative distributions of preferred temporal frequency show that the majority of layer 2/3 neurons in V1 (60%)
and PM (54%) responded maximally to the lowest temporal frequency we presented (0.5 Hz) and tended to prefer slower frequencies in general, while the populations for all other extrastriate areas were shifted towards faster frequencies (Figure 2.4, panel A). We compared the geometric mean preferred temporal frequency across all areas (Figure 2.4, panel B), and found a main effect of visual area on preferred temporal frequency (one-way ANOVA $F_{(6,1180)} = 49.958$, $p < 0.0005$). We followed up with post-hoc multiple comparisons tests to determine which areas were different from each other in terms of preferred temporal frequency. All extrastriate visual areas investigated except area PM had higher preferred temporal frequency tuning than V1 (LM, LI, AL, RL, AM; $p < 0.05$, Tukey-Kramer HSD; Figure 2.4, panel B inset). The mean preferred temporal frequency tuning for area PM was slightly higher than V1 (Figure 2.4, panel B), but this difference was not statistically significant when accounting for multiple comparisons. We also found differences between several extrastriate areas, and these results are summarized in Figure 2.4, panel B (inset). Area LM had the highest mean preferred temporal frequency tuning (significantly higher than areas V1, PM, AL and RL, $p < 0.05$, Tukey-Kramer HSD).

The preferred temporal frequency indicates the temporal frequency that drove the maximal response in each neuron. However, most neurons responded robustly to surrounding temporal frequencies as well, and thus convey that information, albeit less strongly, to neurons downstream. First, neurons were characterized as lowpass, highpass or bandpass (Figure 2.4,
panel C, see Supplemental Experimental Procedures). Not surprisingly, the
great majority of V1 neurons were lowpass for temporal frequency, indicating
that they responded higher than 50% maximal to the lowest frequency. All
other areas had larger fractions of bandpass and highpass cells, suggesting
that the neurons’ tuning curves were shifted to higher temporal frequencies
compared to V1. To determine the range of temporal frequencies represented
by neurons in each population from each area, we examined temporal
frequency cutoffs, the stimulus frequency at which the response reached half
the maximal response, for each neuron (Heimel et al., 2005). Mean low cutoffs
were similar across areas, with only areas LM and surprisingly PM having
statistically higher low cutoff frequencies compared to V1 (Figure 2.4, panel D,
one-way ANOVA, $F_{(6,251)} = 2.89$, $p < 0.01$; post-hoc comparisons $p < 0.05$,
Tukey-Kramer HSD). High cutoff frequencies were far more variable across
areas (one-way ANOVA, $F_{(6,1013)} = 45.36$, $p < 0.0005$), with areas LM, AL and
RL demonstrating higher high cutoff values than V1. Similar to the
comparisons for the mean preferred temporal frequency values, area LM had
the highest high cutoff value (significantly higher than V1, LI, AL and PM, $p <
0.05$, Tukey-Kramer HSD) and PM had the lowest high cutoff after V1
(significantly lower than LM, AL and RL, $p < 0.05$, Tukey-Kramer HSD).

Given the substantially higher preferred temporal frequency tuning of
extrastriate visual areas (up to three times the mean tuning of V1), we asked
whether temporal frequency encoding in the V1 layer 2/3 population
overlapped with extrastriate areas to determine whether V1 could provide a
source of fast frequency information to extrastriate areas. We compared the high cutoff temporal frequencies of V1 to the low cutoff frequencies of all the extrastriate visual areas investigated. We found that V1 neurons had a mean high cutoff which was significantly higher than the mean low cutoff frequencies for all of the extrastriate areas except LI and AM (Figure 2.4, panel D, p < 0.05 indicated on graph). These results suggest that, while its mean preferred temporal frequency is relatively low, V1 does encode temporal frequency information that overlaps with the information encoded in areas LM, AL, RL and PM, and thus could potentially supply high temporal frequency information to these visual areas. Still, this does not fully explain how populations in LM, AL and RL derive higher high cutoff temporal frequencies. The lack of significance for the comparisons between V1 high cutoff with LI and AM low cutoff frequencies may reflect insufficient statistical power, or slightly higher low cutoff frequencies indicative of higher-order processing of extrastriate inputs.

To address the sharpness of temporal frequency tuning across areas, we examined tuning bandwidth. A bandwidth value was computed for bandpass cells as the half width at half max in octaves, in other words, the difference between the low and high cutoff frequencies (Heimel et al., 2005; Figure 2.4). All extrastriate areas had higher mean temporal frequency bandwidth values than V1. This effect was significant for areas LM, AL and RL (Figure 2.4A, one-way ANOVA $F_{(6,191)} = 5.2$, $p < 0.005$; post-hoc comparisons
p < 0.05, Tukey-Kramer HSD), and indicates that neurons in these areas tend to respond to a broader range of temporal frequencies than those in V1.

A subset of extrastriate areas encode high spatial frequencies

The cumulative distributions of preferred spatial frequency for each area’s population of neurons showed that all of the visual areas had populations encoding the spectrum of spatial frequencies tested. One group of areas—AL, RL and LM—consisted of neurons preferring relatively low spatial frequencies (Figure 2.5, panel A). Area AM contained neurons which preferred intermediate spatial frequencies, and areas V1, LI and PM all showed high spatial frequency tuning. Areas LI and PM show particularly interesting distributions. LI contains a relatively large subset of neurons that prefer the lowest spatial frequency, similar to area AL. However, the remaining distribution deviates towards high spatial frequencies, suggesting the presence of separate populations of neurons in LI, preferring distinct ranges of spatial frequencies (Figure 2.5, panel A, and Figure 2.6). Area PM’s distribution also has an interesting pattern, with a small population of neurons preferring low spatial frequencies which deviates rapidly towards a larger population preferring high spatial frequencies (Figure 2.5, panel A, and Figure 2.6).

We compared the geometric mean preferred spatial frequency of each population of neurons from each visual area (Figure 2.5, panel B), and found a main effect of visual area on preferred spatial frequency (one-way ANOVA, $F_{(6,1783)} = 59.7576$, p < 0.0005). Post-hoc multiple comparisons tests revealed that areas LM, AL, RL and AM all have lower preferred spatial frequency tuning
than V1 (Figure 2.5, panel B, p < 0.05, Tukey-Kramer HSD), while areas LI and PM cannot be distinguished from V1 based on mean preferred tuning. Area AL had the lowest preferred spatial frequency tuning, significantly lower than areas V1, LM, LI, AM and PM (Figure 2.5, panel B, p < 0.05, Tukey-Kramer HSD). Only area RL showed comparably low tuning. Areas LM and AM showed similar, intermediate preferred spatial frequency tuning (Figure 2.5, panel B).

In the same manner as for temporal frequency tuning, we characterized neurons as lowpass, highpass or bandpass for spatial frequency (Figure 2.5, panel C). Areas LM, LI, AL, and RL all had relatively high proportions of neurons which were lowpass, however the populations of neurons from these areas differed in other respects. While areas AL and RL had populations which otherwise consisted of bandpass cells, indicating that we had fully sampled the spatial frequency range of the population with our stimuli, areas AM, PM and most notably LI had relatively large fractions of neurons which were highpass, indicating that they theoretically would have responded robustly to even higher spatial frequencies than we presented. This is particularly interesting for area LI since its fraction of highpass cells essentially matches V1’s population, while a larger subset of neurons within LI appear to be lowpass for spatial frequency. That LI contains two distinct populations of either highpass or lowpass cells, with relatively few bandpass cells, may help explain the similarity in the high and low cutoff spatial frequencies observed for this area (Figure 2.5, panel D). All areas except area AM have significantly different low cutoff values than V1. This effect was toward lower values in all areas except areas LI and PM, which
had a higher mean low cutoff spatial frequency tuning than V1 (Figure 2.5, panel D, one-way ANOVA, $F_{(6,1205)} = 9.91, p < 0.0005$; post-hoc comparisons $p < 0.05$, Tukey-Kramer HSD). Several differences distinguished between extrastriate areas in terms of low cutoff spatial frequencies (Figure 2.5, panel D). All extrastriate areas had significantly lower mean high cutoff spatial frequency tuning than V1, with the exception of PM, which had a slightly higher mean cutoff than V1, but this effect was not found to be significant. Comparing across extrastriate areas showed that high cutoff spatial frequencies were similar for all areas, except LM, which had a significantly higher mean high cutoff than area AL (Figure 2.5, panel D, one-way ANOVA, $F_{(6,1445)} = 27.55, p < 0.0005$; post-hoc comparisons $p < 0.05$, Tukey-Kramer HSD).

Spatial frequency tuning bandwidth was sharper in all extrastriate areas compared to V1, except area LI (Figure 2.5, one-way ANOVA $F_{(6,903)} = 15.23, p < 0.0005$; post-hoc comparisons $p < 0.05$, Tukey-Kramer HSD). Area LM had significantly broader spatial frequency tuning than extrastriate areas AL, RL and AM (Figure 2.5, $p < 0.05$, Tukey-Kramer HSD). Area AM had the sharpest spatial frequency tuning bandwidth. These results demonstrate that extrastriate visual areas are more selective for the spatial frequency of the stimulus than V1.

**Extrastriate visual areas are highly selective for orientation and a subset are highly selective for direction**

To examine selectivity for orientation, we calculated the orientation selectivity index (OSI) for each neuron (Experimental Procedures). The OSI
was computed from tuning curves taken at the optimal spatial frequency for each neuron (SF experiment). OSI is a measure of the response to the preferred orientation relative to the orthogonal orientation, with a value of 1 indicating perfect orientation selectivity. A clear separation could be seen between the cumulative distributions of area V1 compared to all other areas, with the distributions of all extrastriate areas shifted toward higher OSI values (Figure 2.6, panel A). Population distributions in areas PM, AL, RL and especially AM stand out as particularly well tuned for orientation relative to the other areas (Figure 2.6, panel A).

All areas except area LI had significantly higher mean OSI values than V1 (Figure 2.6, panel B, one-way ANOVA $F_{(6,1783)} = 41.74, p < 0.0005$; post-hoc comparisons $p < 0.05$, Tukey-Kramer HSD). A subset of areas stood out above the rest: areas AL, RL and especially area AM had higher mean OSI values than all other areas, except area PM, which was only significantly lower than area AM (Figure 2.6, panel B, $p < 0.05$, Tukey-Kramer HSD). AM showed the highest OSI of any of the areas, with significantly higher tuning than all areas except AL (Figure 2.6, panel B, $p < 0.05$, Tukey-Kramer HSD). These results were also reflected in the proportion of cells that were highly orientation selective (OSI > 0.5, indicating a 3:1 selectivity for the preferred to the orthogonal orientation, Figure 2.6, panel C). All extrastriate areas had a higher proportion of highly orientation selective cells than V1, with AL, RL, AM and PM having the highest proportions of highly selective cells.
We calculated the direction selectivity index (DSI) for each neuron, using the tuning curve taken at the optimal spatial frequency for each neuron. DSI compares a neuron’s response to its preferred stimulus direction to the opposite direction of movement, with 1 representing perfect direction selectivity (zero response to the opposite direction). Two groups of areas are apparent in the cumulative distributions across areas (Figure 2.6, panel D). Areas LM, LI and PM’s distributions closely overlap with V1’s distribution, while areas AL, RL and AM overlap each other and are shifted towards higher direction tuning (Figure 2.6, panel D). This distinction is well demonstrated by the mean DSI of each area. Areas AL, RL and AM had significantly higher mean DSI than areas V1 and LM (Figure 2.6, panel E, one-way ANOVA, $F_{(6,1783)} = 10.45$, $p < 0.0005$; post-hoc comparisons $p < 0.05$, Tukey-Kramer HSD). Similarly, this group of areas had a higher proportion of highly direction selective neurons with DSI $> 0.5$ (Figure 2.6, panel F).

Mouse visual areas encode distinct combinations of spatiotemporal information

The statistics comparing areas along each tuning metric can be evaluated between pairs of metrics to reveal different combinations of features encoded across areas and to investigate correlations in the coding of pairs of features. We present each combination of preferred spatial frequency, preferred temporal frequency, OSI and DSI in Figure 2.7 as the mean and standard error of each tuning metric versus another for each area. Direct statistical comparisons between areas for each metric are described above and shown in Figures 2.4-2.6. In Figure 2.6A we perform formal correlation
analyses between each pair of metrics on a cell-by-cell population basis to determine whether linear relationships exist between pairs of stimulus parameters on the level of encoding in single neurons. In Figure 2.8 we summarize the mean tuning metrics for each area, intended as a synopsis of the main findings of the paper.

Two main questions about the data can be addressed with these analyses: 1) do combinations of feature representations further distinguish areas from each other, beyond the tuning for any one metric, and 2) do relationships exist between the tuning for particular stimulus parameters?

In reference to the first question, differences between areas are revealed by sensory coding across multiple stimulus parameters. For instance, while areas LM, LI and AM all had statistically similar preferred temporal frequency tuning (Figure 2.4, panel B), area AM can be distinguished from the other two areas as having higher orientation and direction selectivity tuning (Figure 2.7, panels C-D and 2.6, panels B,E). It is also apparent that V1 can be distinguished from extrastriate areas based on several parameters. In terms of temporal frequency tuning, V1 is distinguishable from all areas except PM (Figure 2.7, panel A, Figure 2.4, panel B and Figure 2.5, panel B). V1’s high spatial frequency tuning and relatively low orientation selectivity separate it from all areas except LI (Figure 2.7, panel C, Figure 2.4, panel B and Figure 2.6, panel B). Areas AL, RL and AM are distinguishable from V1 across all stimulus dimensions, having higher temporal frequency tuning, lower spatial frequency tuning and higher orientation and direction selectivity (Figure 2.7,
panels A-F, Figure 2.4, panel B, Figure 2.5, panel B, and Figure 2.6, panels B,E). These relationships also distinguish LM from V1, except in terms of direction selectivity for which they are indistinguishable (Figure 2.7, panels A-F, Figure 2.4B, 1.5B and 1.6B). With few exceptions, each extrastriate area could be distinguished from all other extrastriate areas based on mean preferred SF, preferred TF, OSI and/or DSI. PM and LM were distinguishable from LI only in terms of mean spatial and/or temporal frequency cutoffs (Figure 2.4, panel D and 2.5, panel D). Only areas AL and RL were statistically indistinguishable from each other across all mean tuning metrics. A formal comparison of the proportion of responsive cells in each area revealed statistical differences between AL and RL ($\chi^2 = 31.535$, 1 degree of freedom, $p < 0.0001$ for TF proportion, $\chi^2 = 5.047$, 1 degree of freedom, $p < 0.05$ for SF proportion). These results demonstrate that mouse visual areas (V1, LM, LI, AL, RL, AM and PM) are functionally distinct and are specialized to represent different spatiotemporal information.

In terms of general trends in encoding combinations of visual features, some relationships were evident in the mean tuning across visual areas and in cell-by-cell population correlations of each visual area. In most cases, significant correlations in the populations of individual neurons were generally low (generally less than or equal to $R = 0.3$, Figure 2.6). This seemed to indicate that populations of neurons in each area were more or less evenly distributed in terms of tuning for pairs of stimulus parameters. Still, some trends were observed, and they may be informative in understanding
relationships between tuning for different stimulus parameters. For example, orientation and direction selectivity appear closely related across areas in terms of mean OSI and DSI, and are positively correlated in terms of cell-by-cell correlations in areas V1 and LM (Figure 2.7, panel B and Figure 2.6, panel B). Areas with mean high spatial frequency tuning tend to have low temporal frequency tuning except for areas AM and especially LI which have particularly high mean tuning for both (Figure 2.7, panel A). Unlike any of the other areas which show no correlation between spatial and temporal frequency tuning on a population level, LI shows a strong negative correlation (R = -0.77, p < 0.05, Bonferroni corrected), suggesting that neurons in this area tend to either encode high temporal or high spatial frequencies, but not the combination of both (Figure 2.6A). Areas with mean high preferred temporal frequency tend to have higher mean orientation and direction selectivity (Figure 2.7, panels C-D). Positive correlations between these metrics were found for areas V1 and AL for orientation selectivity and areas V1, LM, AL and RL for direction selectivity across each population of neurons (Figure 2.6, panels C-D). The relationships between spatial frequency tuning and orientation and direction selectivity are most apparent in cell-by-cell correlations, which show positive correlations between the spatial frequency tuning and orientation selectivity in areas V1, LM and AL (Figure 2.6, panel E). Spatial frequency and direction selectivity are negatively correlated in areas AL, RL and PM and weakly positively correlated in V1 (Figure 2.6, panel F).
Discussion

In the present study, we found that mouse visual cortex contains a highly organized arrangement of several visual areas, which each encode unique combinations of spatiotemporal features. Our nearly complete, high-resolution retinotopic maps reveal a continuous fine-scale organization across mouse visual cortex, comprising at least 9 independent representations of the contralateral visual field. As was previously shown for mouse V1 (Niell and Stryker, 2008) and similar to what has been found in other species (Felleman and Van Essen, 1987; Orban, 2008; Payne, 1993), each of the six mouse extrastriate visual areas we investigated contains neurons that are highly selective for fundamental visual features. All extrastriate areas investigated, with the exception of PM, encode faster temporal frequencies than V1, suggesting a role for these higher visual areas in the processing of visual motion. For a subset of areas, AL, RL and AM, this potential role is further supported by a significant increase in direction selectivity in each population. Another subset of areas, LI and PM, show high spatial frequency tuning, suggesting a role in the processing of structural detail in an image. Nearly all higher visual areas improve selectivity for orientation compared to V1.

Every visual area could be distinguished from every other visual area statistically by comparing scores on multiple tuning metrics, indicating functional specialization of spatiotemporal information processing across mouse visual areas. This rule is violated only by areas AL and RL, which are
each distinct from all other visual areas based on one or more tuning metrics, but are only distinguished from each other based on differences in the proportions of responsive neurons between the two areas, as well as their independent visuotopic representations. The combination of distinct retinotopic representations and functionally specialized neuronal populations establish that mouse visual cortex is composed of several discrete visual areas that each encode unique combinations of visual features. These findings reveal that the mouse visual system shares fundamental organizational principles with other species and is more highly developed than expected from previous work focusing almost exclusively on V1.

Striking similarities are evident among subsets of extrastriate areas along specific feature dimensions. These complex relationships likely reflect underlying rules of connectivity which link processing between certain areas, and may relate to the grouping of areas into hierarchically organized parallel pathways. Areas AL, RL and AM are all highly direction selective and respond to high temporal frequencies and low spatial frequencies. These properties have served as hallmarks of the dorsal pathway in other species (Maunsell and Newsome, 1987; Nassi and Callaway, 2009; Van Essen and Gallant, 1994), and suggest that AL, RL and AM perform computations related to the analysis of visual motion. This role is further supported by the anatomical position of these areas in the posterior parietal cortex, which corresponds to the location of dorsal stream areas in other species and is closely related to neural systems for spatial navigation and motor output across species (Baizer et al., 1991;
Kaas et al., 2011; Pinto-Hamuy et al., 2004; Ungerleider and Mishkin, 1982; Whitlock et al. 2008). In contrast, areas LI and PM respond to high spatial frequencies, and PM is highly orientation selective, suggesting a role in the analysis of structural detail and form in an image (Desimone et al., 1985; Maunsell and Newsome, 1987). These results suggest that the mouse visual cortex may be organized into groups of areas that process information related to motion and behavioral actions versus image detail and object perception, analogous to the dorsal and ventral streams described in other species (Maunsell and Newsome, 1987; Ungerleider and Mishkin, 1982; Van Essen and Gallant, 1994).

Defining hierarchical relationships in mouse visual cortex, and conclusively relating specific areas to dorsal and ventral streams will require significant future behavioral, anatomical and functional work. Rodents can perform spatial and pattern discrimination tasks (Douglas et al., 2006; Espinoza et al., 1999; Pinto-Hamuy et al., 2004; Prusky and Douglas, 2004; Prusky et al., 2000; Sanchez et al., 1997; Wong and Brown, 2006), similar to those shown to depend on dorsal and ventral pathways in higher species (Mishkin et al., 1983). However, little is known about how specific mouse visual areas or pathways relate to these behaviors. Recently, it was found that AL and LM afferents differentially target brain regions typically associated with the dorsal and ventral pathways in other species (Wang et al., 2011). These distinctions led to the suggestion that LM and AL are the “gateways” to the ventral and dorsal streams respectively. The results of the current study
support the role of areas AL, RL and AM in dorsal-like motion computations and of LI and PM in ventral-like spatial computations. However, our results are less conclusive for area LM’s role in ventral-like computations. It encodes the highest temporal frequencies in our dataset and prefers moderate spatial frequencies—properties typically associated with the dorsal stream in other species (Van Essen and Gallant, 1994). In addition to behavioral and anatomical data, determining the selectivity of neurons in higher visual areas to more complex stimuli will further illuminate the higher order computations performed by different areas.

While our data indicate that mouse visual cortex shares general organizational principles with other species, several important distinctions can be made. One major difference between the rodent visual cortex and primate visual cortex is the existence of direct V1 input to essentially all extrastriate visual areas in the mouse and rat (Coogan and Burkhalter, 1993; Olavarria and Montero, 1989; Wang and Burkhalter, 2007), whereas only areas V2, V3, V4 and MT are known to receive substantial direct V1 input in the primate brain (Felleman and Van Essen, 1991). Differences in the function and organization of visual areas between mice and other species are likely related to specializations resulting from species-specific behavioral adaptations. While multi-modal interactions are typically associated with select higher-level areas in primates (Felleman and Van Essen, 1991; Ungerleider and Mishkin, 1982), there is evidence that several rodent extrastriate areas process information related to other sensory modalities (Miller and Vogt, 1984; Sanderson et al.,
1991; Wagor et al., 1980). This may indicate fewer hierarchical stages in the rodent, relating visual information more readily to multimodal interactions or complex behaviors such as spatial navigation. The extent to which mouse visual pathways resemble dorsal and ventral streams and are organized into hierarchical pathways, as well as understanding the role of specific areas in perception and behavior, form the basis for useful, testable hypotheses for future investigation.

Implications for motion processing in mouse extrastriate cortex

Our population analyses revealed prominent differences in the tuning for motion related visual features between several extrastriate areas and V1. V1 neurons generally prefer low temporal frequencies, theoretically making it difficult for V1 neurons to resolve stimulus motion beyond low velocities (Chichilnisky and Kalmar, 2003). On the other hand, all mouse extrastriate visual areas prefer high temporal frequencies relative V1, and this relationship was significant for all areas except PM. Some extrastriate areas, notably areas LM, AM and LI prefer temporal frequencies two to three times the rate of V1 on average, and areas AL and RL prefer frequencies almost double that of V1. Areas LM, AL and RL had higher mean high temporal frequency cutoff than V1 indicating further that neurons in those areas encoded faster frequencies not represented in V1. Furthermore, areas AL, RL and AM contain a larger proportion of highly direction selective neurons, and are significantly more direction selective across the population on average compared to V1 and LM. These findings demonstrate that mouse extrastriate visual cortex, and
especially areas AL, RL and AM, are better suited to process motion information than V1. Intriguingly, these areas compose part of the posterior parietal cortex which has been implicated in spatial discrimination and navigation tasks in rats and is involved in similar behaviors and composes part of the dorsal pathway in primates (Ungerleider and Mishkin, 1982; Whitlock et al., 2008). The ability of neurons in these areas to encode changes in a stimulus at fast frequencies suggests that they can follow movement of stimuli at relatively high velocities through their receptive fields. Determining whether each extrastriate area we examined encodes motion information per se, or rather encodes high temporal resolution to serve higher-order motion computations in other areas, requires future studies. For example, in addition to having high direction selectivity, neurons in the motion-selective, middle temporal area (MT) in primates can be distinguished from neurons in many other areas based on speed tuning and/or pattern motion detection (Maunsell and Newsome, 1987).

**Encoding spatial detail in mouse visual cortex**

The mouse visual system, while modest in acuity compared to primates and many carnivore species, is capable of spatial discrimination across several orders of spatial magnitude (Prusky and Douglas, 2004), and is known to contain neurons that are highly selective for spatial frequency and spatial details such as orientation in primary visual cortex and to some extent subcortical structures (Grubb and Thompson, 2003; Niell and Stryker, 2008; Wang et al., 2010). In the present study we found that extrastriate areas LI and
PM prefer spatial frequencies comparable to the relatively high frequencies represented in V1. Additionally, all extrastriate visual areas except perhaps LI are more sharply tuned for spatial frequency and are more selective for orientation than V1. These findings imply that a subset of extrastriate visual areas, including LI and PM, are capable of conveying high spatial resolution details in an image, and in the case of area PM, actually improve the fidelity of these responses in terms of spatial frequency bandwidth and relatively high selectivity for orientation (higher than both V1 and LM). Tuning for high spatial frequencies, and good orientation selectivity are attributed to the ventral pathway in primates, ultimately leading to object perception (Maunsell and Newsome, 1987; Van Essen and Gallant, 1994). This suggests that area PM, and to some extent LI, may perform similar computations within the mouse visual system.

**Implications for future studies**

The circuit mechanisms which facilitate computation of fast frequency information, increased direction selectivity and high spatial frequency preference in different subsets of extrastriate visual areas remain unclear. Selective response properties in extrastriate visual areas could be inherited from lower areas (e.g., V1) based on selective connectivity. Higher-order computations performed across hierarchical levels via specific connections could also help explain the observed patterns of selectivity. Additionally, local computations within each area could sharpen orientation selectivity (Liu et al., 2011) or spatial frequency bandwidth tuning via local circuit interneurons.
Extrastriate areas could also receive selective information through alternate pathways, such as via projections from the superior colliculus, a subcortical structure important for visually guided orienting behaviors (Gross, 1991; Rodman et al., 1990), through the lateral posterior nucleus of the thalamus bypassing V1 entirely (Sanderson et al., 1991; Simmons et al., 1982). A similar pathway exists between the analogous pulvinar nucleus and extrastriate areas in the primate (Lyon et al., 2010). Finally, given that we sampled exclusively from layer 2/3 neurons, the possibility remains that information is conveyed via deeper layers in V1 (and perhaps other areas), bypassing the typical layer 4 → layer 2/3 cortical circuit. Indeed, such circuitry has been demonstrated anatomically in the primate between V1 deep layers and area MT (Nassi et al., 2006; Nhan and Callaway, 2011). Future studies directly examining the relationships between function and connectivity are necessary to understand how visual areas derive their response properties. The mouse model provides powerful tools to address these issues.

Understanding the mechanisms by which information is routed in the cortex requires methods to simultaneously examine both the functional roles of specific cells, circuits and areas and their patterns of connections with each of these component levels of the network. Further, in order to obtain a complete picture of these interactions and establish causal relationships, techniques allowing controlled, reversible activation and inactivation of targeted circuit elements are necessary. By combining molecular, genetic and viral methods for identifying, targeting and manipulating specific genes, cell types and
connections with advanced recording and imaging technologies will make these types of experiments possible. Studies utilizing these technologies have already contributed to an increased understanding of network function in many systems. Currently, these tools are most readily applicable in the mouse, due to its genetic accessibility and small size. Importantly, the small lissencephalic cortex of the mouse permits access to all cortical regions exposed on its flat surface, and deep cortical layers to be analyzed with two-photon imaging (Osakada et al., 2011). While these methods may eventually be applied in other, larger species such as the primate, large-scale studies involving many animals will remain difficult. As such, the mouse will prove an invaluable system for the study of cortical information processing.

The present study provides the first thorough characterization of the function of the majority of mouse extrastriate visual areas, demonstrating specialized information processing in seven, retinotopically-identified visual areas. These results suggest that several high-order computations may occur in mouse extrastriate cortex, and that the mouse visual system shares many of the complexities of the primate system, including well organized, retinotopically-defined visual areas and highly selective, specialized neuronal populations, perhaps organized into specific parallel pathways. Furthermore, this study develops and demonstrates several methodological approaches to efficiently investigate several visual areas in the same animal, and across multiple animals in a high-throughput fashion. The results and implications of the current study, as well as the development and application of novel
technologies, lay the foundation for future studies investigating the complexities of the mouse cortical system to reveal circuit-level mechanisms driving high order computations.
Experimental Procedures

Animal Preparation and Surgery

All experiments involving living animals were approved by the Salk Institute’s Institutional Animal Care and Use Committee. C57BL/6 mice (n = 28) between 2 and 3 months were anesthetized with isoflurane (2-2.5% induction, 1-1.25% surgery). Dexamethasone and carprofen were administered subcutaneously (2 mg/kg and 5 mg/kg respectively), and ibuprofen (30 mg/kg) was administered post-operatively in the drinking water for the duration of the experiment (typically 1-2 days). A custom-made metal frame was mounted to the skull and the bone was thinned over visual cortex for intrinsic imaging, and a craniotomy was made for calcium imaging. After surgery, chloprothixene (2.5 mg/kg) was administered intramuscularly and isoflurane was reduced to 0.25-0.8% for visual stimulation and recording experiments. See Supplementary Experimental Procedures for details.

Intrinsic Signal Optical Imaging

The intrinsic signal imaging protocol was adapted from previous studies (Kalatsky and Stryker, 2003; Nauhaus and Ringach, 2007). See Supplemental Experimental Procedures for details.

Dye Loading and Two-Photon Imaging

The retinotopic map from the intrinsic signal imaging experiment, aligned and overlaid on an image of the cortical blood vessels, was used as a reference for targeting the locations of calcium-sensitive dye loading. Oregon
Green Bapta-1 AM (OGB-1AM) and sulforhodamine-101 were loaded into cortex (Nimmerjahn et al., 2004; Ohki et al., 2005; Stosiek et al., 2003) using the intrinsic imaging retinotopy map as a reference. The pipette was lowered to 225 µm below the dura surface (layer 2/3). A single pulse of 1 min duration and 10 PSI was applied for the loading protocol resulting in ~0.5-1 mm² of loading. Several locations were injected in the same animal resulting in an area of loading spanning up to several millimeters. At least one hour after loading the two-photon imaging began. See Supplementary Experimental Procedures for details and two-photon imaging parameters.

**Visual Stimulation**

Drifting bar and drifting grating stimuli were displayed on a gamma-corrected, large LCD display (68 cm width x 121 cm height, 120 Hz monitor, stimulus presented at 60Hz). The screen was tilted and placed 10 cm from the animal so as to stimulate the entire known visual hemifield of the mouse (-40° to 70° lower to upper and -70° to 70° nasal to temporal (Wagor et al., 1980). Furthermore, this placement was beyond the theoretical point of ‘infinite focus’ of the mouse eye (de la Cera et al., 2006; Green et al., 1980; Remtulla and Hallett, 1985) leading to similar focus at all points on the screen.

For retinotopy experiments, a periodic drifting bar stimulus was used. The bar was 20° wide and subtended the whole visual hemifield along either the vertical or horizontal axis. The bar was drifted 10 times in each of the four cardinal directions. A counter-phase checkerboard pattern was flashed on the bar, alternating between black and white (25° squares with 166 ms period).
Azimuth correction was applied to bars drifting along the horizontal axis and altitude correction was applied bars drifting along the vertical axis (Figure 2.1, Supplementary Experimental Procedures), in order to define eccentricity using standard spherical coordinates. The bar drifted at 8.5-9.5°/s for intrinsic imaging experiments and at 12-14°/s for two-photon imaging experiments.

For drifting grating experiments, sinusoidal drifting gratings were used. Spherical altitude correction was used to hold spatial and temporal frequencies constant throughout the visual field (Figure 2.1, Supplemental Experimental Procedures). For each simultaneously-recorded population of neurons (a single 40X imaging plane), we almost always presented four sets of stimuli: a temporal frequency varying experiment (0.5, 1, 2, 4 and 8 Hz, 8 directions plus blank, ~0.04 cpd, 5 repeats pseudorandomized for each parameter combination), a spatial frequency varying experiment (0.01, 0.02, 0.04, .08 and 0.16 cpd, 8 directions plus blank, ~1 Hz, 5 repeats pseudorandomized for each parameter combination), a 12 direction orientation tuning experiment (~1 Hz, ~0.04 cpd, 5 repeats pseudorandomized for each parameter combination and a blank condition), and a drifting bar retinotopy experiment (same as for the large-field 16X retinotopy experiments) to map the precise eccentricity of each neuron in the field of view. A gray screen (mean luminance of grating stimuli) was shown between trials and during the prestimulus baseline period. Stimulus durations were 4 sec for the temporal frequency experiment and 2 sec for the spatial frequency and 12 direction experiments. Note: data are not presented
for the 12 direction experiments. See Supplementary Experimental Procedures for details.

Data Analysis

Retinotopic maps from intrinsic signal imaging were computed as previously described (Kalatsky and Stryker, 2003). A comparable approach was used to compute retinotopy in \( \text{Ca}^{2+} \) imaging experiments. Data in terms of phase were converted to eccentricity by dividing the known angle covered by the stimulus by the total degrees of phase. See Supplementary Experimental Procedures for details.

For population analysis, movement correction was applied to time-lapse movies and regions of interest (ROIs) were drawn around each cell in the field of view. Glia cells were removed from the analysis using sulforhodamine staining (Nimmerjahn et al., 2004). Pixels were averaged within each ROI for each time point (each single image) to reflect the raw time course of a given neuron. Baseline calcium fluorescence was computed for each trial as the average response during the prestimulus period (1 sec, mean luminance gray screen). Then, the entire time course for each trial was converted from absolute fluorescence values to percent change above baseline according to the following equation: 

\[
\frac{\Delta F}{F} = \frac{F_i - F}{F},
\]

where \( F_i \) is the instantaneous fluorescence signal and \( F \) is the baseline fluorescence. The \( \frac{\Delta F}{F} \) response for each cell was averaged during a two-second window following stimulus onset for each trial, and the mean and standard deviation across trials for each stimulus and blank condition were computed for each neuron.
We applied several criteria to include neurons in further population analyses. Neurons were deemed visually responsive if they gave a mean response above 6% $\Delta F/F$ to any stimulus. A response reliability metric ($\delta$) was computed for each neuron as follows:

$$\delta = \frac{\mu_{\text{max}} - \mu_{\text{blank}}}{\sigma_{\text{max}} + \sigma_{\text{blank}}}$$

where $\mu_{\text{max}}$ and $\sigma_{\text{max}}$ are the mean and standard deviations of the response to the preferred stimulus respectively, and $\mu_{\text{blank}}$ and $\sigma_{\text{blank}}$ are the mean and standard deviations of the response to the blank stimulus respectively. Neurons were deemed reliable for $\delta > 1$. Finally, neuron eccentricity mapped with the retinotopy stimulus was used to restrict our population analyses to eccentricity-matched neurons within the central 100° of space in each area (see Table S1). The numbers of neurons which met all of these criteria were used as the denominator in all subsequent analyses of fraction of neurons exhibiting a particular score on a given parameter. See Supplementary Experimental Procedures for further details.

**Tuning Metrics**

Temporal and spatial frequency tuning curves were taken at the optimal orientation and direction for each neuron, using the average $\Delta F/F$ response for each condition across trials. These tuning curves were fit with a Difference of Gaussians function (Sceniak et al., 2002). A log$_2$ transform was performed on the x-axis of the curves, converting it to octaves. The preferred frequency was
taken at the peak (maximum) location of this curve. See Supplemental Experimental Procedures for explanation of bandwidth criteria.

The orientation and direction tuning curves were taken at the optimal spatial frequency for each neuron, using the average ∆F/F response for each condition across trials. The orientation selectivity index (OSI) was computed as follows:

$$OSI = \frac{\mu_{max} - \mu_{orth}}{\mu_{max} + \mu_{orth}}$$

where $\mu_{max}$ is the mean response to the preferred orientation and $\mu_{orth}$ is the mean response to the orthogonal orientation (average of both directions). The direction selectivity index was computed as follows:

$$DSI = \frac{\mu_{max} - \mu_{opp}}{\mu_{max} + \mu_{opp}}$$

where $\mu_{max}$ is the mean response to the preferred direction and $\mu_{opp}$ is the mean response to the opposite direction. See Supplementary Experimental Procedures for details.

Statistics

Statistical procedures are described in the text and in detail in Supplementary Experimental Procedures.
Table 2.1 Numbers of neurons sampled by cortical area.

Total neurons indicates number (N) of neurons recorded from in the spatial frequency (SF) and temporal frequency (TF) experiments for each cortical area. Number (N) and percent of total of responsive (ΔF/F > 6%) and reliable neurons (δ > 1, Experimental Procedures) for each area. Neurons which were responsive and reliable, as well as representing eccentricities within 50° of the center of space (over 99% of responsive and reliable neurons, Table S1) were included in subsequent population analyses. "N Expts" refers to the number of fields of view (samples of neurons) recorded from each area. Table S1 further separates inclusion criteria.

<table>
<thead>
<tr>
<th>Area</th>
<th>Total Neurons</th>
<th>Responsive &amp; Reliable</th>
<th>N Expts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SF</td>
<td>TF</td>
<td>N (% Total)</td>
</tr>
<tr>
<td>V1</td>
<td>1419</td>
<td>1382</td>
<td>728 (51%)</td>
</tr>
<tr>
<td>LM</td>
<td>590</td>
<td>584</td>
<td>300 (51%)</td>
</tr>
<tr>
<td>LI</td>
<td>182</td>
<td>193</td>
<td>42 (23%)</td>
</tr>
<tr>
<td>AL</td>
<td>890</td>
<td>918</td>
<td>330 (37%)</td>
</tr>
<tr>
<td>RL</td>
<td>616</td>
<td>555</td>
<td>201 (33%)</td>
</tr>
<tr>
<td>AM</td>
<td>404</td>
<td>311</td>
<td>63 (16%)</td>
</tr>
<tr>
<td>PM</td>
<td>508</td>
<td>506</td>
<td>147 (29%)</td>
</tr>
<tr>
<td>Total</td>
<td>4609</td>
<td>4449</td>
<td>1811 (39%)</td>
</tr>
</tbody>
</table>
Figure 2.1 Retinotopic organization of mouse visual cortex measured with intrinsic signal imaging.

Retinotopic organization of cortex representing the entire contralateral visual hemifield, mapped in spherical coordinates. Colors indicate angular position in degrees of a drifting bar stimulus that elicited a hemodynamic response at each cortical location (Figure S1 and Movie S1 and S2). For altitude maps of vertical retinotopy (A-C, G), positive values indicate the upper visual field and negative values the lower visual field. For azimuth maps of horizontal retinotopy (D-F, H), positive values indicate the nasal visual field and negative values the temporal visual field. (A-C) Vertical retinotopy. (A) Continuous vertical retinotopy map. The horizontal meridian is located at approximately 20 degrees altitude (blue). (B) Contour lines of vertical eccentricity, with 10 degree spacing. (C) Overlay of continuous vertical retinotopy map (as in (A)) with altitude contours (solid lines, as in (B)) and azimuth contours (dashed lines, as in (E)). (D-F) Horizontal retinotopy. (D) Continuous horizontal retinotopy map. The vertical meridian representing the nasal periphery is located at approximately 60 degrees azimuth (purple-red). (E) Contour lines of horizontal eccentricity with 20 degree spacing. (F) Overlay of continuous horizontal retinotopy map (as in (D)) with azimuth contours (solid lines, as in (E)) and altitude contours (dashed lines, as in (B)). (G) Overlay of vertical retinotopy contour lines (as in (B)) with area borders (black lines, as in (I)). (H) Overlay of horizontal retinotopy contour lines (as in (E)) with area borders (black lines, as in (I)). V1 borders extrastriate areas LM, AL, and RL laterally at a reversal at the vertical meridian (purple-red). Li borders LM laterally at a reversal at the temporal periphery (green-yellow). Areas AM and PM border V1 medially at a reversal at the temporal periphery (green-yellow). (I) Area border diagram of mouse visual cortex. Borders determined based on reversals at peripheries in the vertical and horizontal maps, taking into account high-resolution mapping data (Figure 2.2 and Figure 2.2A), and correspondence with previous descriptions of areal organization in mouse visual cortex (Wagor et al., 1980; Wang and Burkhalter, 2007). All panels same scale; scale bars 500 µm.
Figure 2.2 Fine-scale retinotopic organization of mouse visual cortical areas, measured with wide-field two-photon calcium imaging.

High-resolution retinotopic maps of mouse visual cortex (A-L). Area borders and names correspond to diagram in Figure 2.1l. Colors represent retinotopic eccentricity. Panels A, E & I show continuous vertical retinotopy maps, in altitude coordinates. Positive values indicate the upper field and negative values the lower field. Panels B, F and J show the corresponding contour plots of vertical retinotopy with area borders outlined in black. Panels C, G and K show continuous horizontal retinotopy maps, in azimuth coordinates, with positive values representing the nasal visual field and negative values representing the temporal visual field. Panels D, H and L show contour plots of horizontal retinotopy, overlaid with area borders. (A-D) Retinotopic organization of lateral extrastriate areas and their borders with V1. Area borders shown in (B, D) correspond to V1, LM, LI, AL, and RL (starting at bottom right, moving clockwise). A reversal at the vertical meridian (nasal periphery, purple-red in (C, D)) delineates the border between V1 and extrastriate areas LM, AL and RL. A reversal at the temporal periphery (yellow, (C, D)) marks the border between LM and LI. The border between LM and AL is identified at the reversal near the horizontal meridian (blue in (A, B)). AL is separated from RL by a representation of the vertical meridian, extending lateral and anterior from the vertical meridian in V1 (purple-blue, (C,D), see also Supplementary Figure 2.2A, panels E-H). (E-H) Retinotopic organization of anterior and medial extrastriate areas and their borders with V1. Area borders shown in (F, H) correspond to V1, LM, AL, RL, A, AM, and PM (starting at lower left, moving clockwise). The maps of vertical eccentricity in areas AM and PM are compressed mirror image representations of the altitude map in V1, rotated ~90 degrees relative to V1 (E, F). The border between V1 and PM is identified by a reversal at the temporal periphery (green-yellow, (E, F)). A representation near the vertical meridian marks the border between AM and PM (blue-purple, (G, H)). (I-L) Retinotopic organization of 7 visual areas along the nearly entire medial-lateral extent of the visual cortex. Areas shown include LM, AL, RL, A, AM, PM, and V1 (starting from lower left, moving clockwise). See also Figure 2.2A for high resolution maps of P, POR and the ring-like structure of area RL. All scale bars 500 µm.
Figure 2.2 Fine-scale retinotopic organization of mouse visual cortical areas, measured with wide-field two-photon calcium imaging, continued

High-resolution retinotopic maps of mouse visual cortex (A-L). Area borders and names correspond to diagram in Figure 2.1l. Colors represent retinotopic eccentricity. Panels A, E & I show continuous vertical retinotopy maps, in altitude coordinates. Positive values indicate the upper field and negative values the lower field. Panels B, F and J show the corresponding contour plots of vertical retinotopy with area borders outlined in black. Panels C, G and K show continuous horizontal retinotopy maps, in azimuth coordinates, with positive values representing the nasal visual field and negative values representing the temporal visual field. Panels D, H and L show contour plots of horizontal retinotopy, overlaid with area borders. (A-D) Retinotopic organization of lateral extrastriate areas and their borders with V1. Area borders shown in (B, D) correspond to V1, LM, LI, AL, and RL (starting at bottom right, moving clockwise). A reversal at the vertical meridian (nasal periphery, purple-red in (C, D)) delineates the border between V1 and extrastriate areas LM, AL and RL. A reversal at the temporal periphery (yellow, (C, D)) marks the border between LM and LI. The border between LM and AL is identified at the reversal near the horizontal meridian (blue in (A, B)). AL is separated from RL by a representation of the vertical meridian, extending lateral and anterior from the vertical meridian in V1 (purple-blue, (C,D), see also Supplementary Figure 2.2A, panels E-H). (E-H) Retinotopic organization of anterior and medial extrastriate areas and their borders with V1. Area borders shown in (F, H) correspond to V1, LM, AL, RL, A, AM, and PM (starting at lower left, moving clockwise). The maps of vertical eccentricity in areas AM and PM are compressed mirror image representations of the altitude map in V1, rotated ~90 degrees relative to V1 (E, F). The border between V1 and PM is identified by a reversal at the temporal periphery (green-yellow, (E, F)). A representation near the vertical meridian marks the border between AM and PM (blue-purple, (G, H)). (I-L) Retinotopic organization of 7 visual areas along the nearly entire medial-lateral extent of the visual cortex. Areas shown include LM, AL, RL, A, AM, PM, and V1 (starting from lower left, moving clockwise). See also Figure 2.2A for high resolution maps of P, POR and the ring-like structure of area RL. All scale bars 500 µm.
Figure 2.3 Neurons in higher visual areas are selective for particular stimulus features.

(A) Example calcium imaging experiment in area AL. Left panel, example two-photon field of view of OGB-1AM loaded neurons (green) and astrocytes stained with sulforhodamine-101 (red). Right panels show responses of 3 example neurons (corresponding to cell numbers indicated in left panel), displayed as a matrix of all stimulus conditions for a given experiment. Columns indicate the direction/orientation of the drifting grating, and rows designate the spatial frequency (upper matrices) or temporal frequency (lower matrices) of the stimulus. Gray boxes illustrate the duration of the stimulus, 2 seconds for SF experiments and 4 seconds for TF experiments. The average response across all trials of a given stimulus condition is shown in black, with the responses to each trial in gray. Scale bar to lower right of each response matrix indicates 40% $\Delta F/F$. Tuning curves were generated from response matrices by computing the average $\Delta F/F$ over the two second window following the stimulus onset for each condition along the relevant stimulus dimension. Orientation tuning curves (outlined in yellow) are generated by taking the response to each direction, at the optimal spatial frequency. Spatial frequency (orange box) and temporal frequency (magenta box) tuning curves show the response to each SF or TF at the preferred orientation. Values describing selectivity metrics (OSI, DSI), the preferred spatial and temporal frequency (pref. SF, pref. TF), as well as low cutoff (LC) and high cutoff (HC) frequencies, are listed above the corresponding tuning curves. Scale bars indicate 20% $\Delta F/F$, and correspond to all tuning curves for a given cell. (B) Example tuning curves from each visual area demonstrate the diversity and selectivity of receptive field properties across the populations. Horizontal axes are identical to corresponding tuning curves shown in (A) for orientation/direction (yellow boxes in (A), first column for each area in (B)), spatial frequency (orange boxes in (A), second column for each area in (B)) and temporal frequency (magenta boxes in (A), third column for each area in (B)). Scale bars represent 20% $\Delta F/F$ for the vertical axis and correspond to the set of tuning curves for a given cell. Rows with one scale bar show Dir, SF and TF tuning curves taken from the same neuron, as in (A). However, in some cases, the Dir and SF tuning curves are from one neuron, but the TF tuning curve is from a different neuron, indicated by the presence of a scale bar between the SF and TF curves. Example responses from neurons in each area are displayed in Figure 2.3A.
Figure 2.3 Neurons in higher visual areas are selective for particular stimulus features, continued

(A) Example calcium imaging experiment in area AL. Left panel, example two-photon field of view of OGB-1AM loaded neurons (green) and astrocytes stained with sulforhodamine-101 (red). Right panels show responses of 3 example neurons (corresponding to cell numbers indicated in left panel), displayed as a matrix of all stimulus conditions for a given experiment. Columns indicate the direction/orientation of the drifting grating, and rows designate the spatial frequency (upper matrices) or temporal frequency (lower matrices) of the stimulus. Gray boxes illustrate the duration of the stimulus, 2 seconds for SF experiments and 4 seconds for TF experiments. The average response across all trials of a given stimulus condition is shown in black, with the responses to each trial in gray. Scale bar to lower right of each response matrix indicates 40% $\Delta F/F$. Tuning curves were generated from response matrices by computing the average $\Delta F/F$ over the two second window following the stimulus onset for each condition along the relevant stimulus dimension. Orientation tuning curves (outlined in yellow) are generated by taking the response to each direction, at the optimal spatial frequency. Spatial frequency (orange box) and temporal frequency (magenta box) tuning curves show the response to each SF or TF at the preferred orientation. Values describing selectivity metrics (OSI, DSI), the preferred spatial and temporal frequency (pref. SF, pref. TF), as well as low cutoff (LC) and high cutoff (HC) frequencies, are listed above the corresponding tuning curves. Scale bars indicate 20% $\Delta F/F$, and correspond to all tuning curves for a given cell. (B) Example tuning curves from each visual area demonstrate the diversity and selectivity of receptive field properties across the populations. Horizontal axes are identical to corresponding tuning curves shown in (A) for orientation/direction (yellow boxes in (A), first column for each area in (B)), spatial frequency (orange boxes in (A), second column for each area in (B)) and temporal frequency (magenta boxes in (A), third column for each area in (B)). Scale bars represent 20% $\Delta F/F$ for the vertical axis and correspond to the set of tuning curves for a given cell. Rows with one scale bar show Dir, SF and TF tuning curves taken from the same neuron, as in (A). However, in some cases, the Dir and SF tuning curves are from one neuron, but the TF tuning curve is from a different neuron, indicated by the presence of a scale bar between the SF and TF curves. Example responses from neurons in each area are displayed in Figure 2.3A.
Figure 2.4 Encoding for temporal frequency information differs across visual areas.

(A) Cumulative distributions of preferred temporal frequency for each visual area (inset indicates color-coding for each area and corresponds to the whole figure). 
(B) Geometric mean preferred temporal frequency for each visual area. 
(C) Proportions of highpass, bandpass and lowpass neurons for each area. 
(D) Geometric mean temporal frequency cutoffs show the range of temporal frequencies encoded by each population. For each area, the left bar indicates the low cutoff temporal frequency and the right bar indicates the high cutoff frequency. Asterisks and lines above plot indicate significant differences (p < 0.05) between V1’s high cutoff frequency and the low cutoff frequency of extrastriate areas. Insets in (B) and (D) show statistical significance (p < 0.05) of pair-wise comparisons between areas, corrected for multiple comparisons using the Tukey-Kramer method. Error bars are standard error of the mean (S.E.M.) in (B, D). Temporal frequency bandwidth comparisons are in Figure 2.4A.
Figure 2.5 Encoding for spatial frequency information differs across visual areas.

(A) Cumulative distributions of preferred spatial frequency for each visual area (inset indicates color-coding for each area and corresponds to the whole figure). (B) Geometric mean preferred spatial frequency for each area. (C) Visual areas differ in the proportions of highpass, bandpass and lowpass neurons across each population. (D) Geometric mean spatial frequency cutoffs across visual areas. For each area, left bar indicates the low cutoff spatial frequency and the right bar indicates the high cutoff spatial frequency. Insets in (B) and (D) show statistical significance (p < 0.05) of pair-wise comparisons between areas, corrected for multiple comparisons using the Tukey-Kramer method. Error bars are standard error of the mean (S.E.M.) in (B, D). Spatial frequency bandwidth comparisons are in Figure 2.5A.
Figure 2.6 Encoding for orientation and direction information differs across visual areas.

(A) Cumulative distributions of orientation selectivity index for each area (inset indicates color-coding for each area and corresponds to the whole figure). (B) Mean orientation selectivity index for each visual area. (C) Percent of highly orientation selective neurons (OSI > 0.5) across the population for each area. (D) Cumulative distributions of direction selectivity index for the population of neurons in each visual area. (E) Mean direction selectivity index for each visual area. (F) Percent of highly direction selective neurons (DSI > 0.5) across the population for each visual area. Insets in (B) and (E) show statistical significance (p < 0.05) of pair-wise comparisons between areas, corrected for multiple comparisons using the Tukey-Kramer method. Error bars are standard error of the mean (S.E.M.) in (B, D).
Figure 2.7 Mouse cortical visual areas encode unique combinations of spatiotemporal information.

(A-F) Pair-wise combinations between mean preferred temporal frequency, preferred spatial frequency, OSI and DSI as a function of visual area. For all plots, each point represents the population means of a single visual area for the combination of tuning metrics indicated on the respective axes. Lines extending from each point define an area’s standard error of the mean (S.E.M.) for the tuning metric defined on the parallel axis (point and line colors correspond to area name indicated by inset in A). Statistical comparisons between areas for each metric are same as insets in Figures 2.4, panel B, 2.5, panel B, 2.6, panel B and 2.6, panel E. See cell-by-cell population corselations for each metric for each area in Figure 2.6A.
Figure 2.8 Summary of feature selectivities across seven mouse visual areas.

Mean values for orientation selectivity (OSI), direction selectivity (DSI), preferred spatial frequency (SF) and preferred temporal frequency (TF) for each area correspond to results depicted in Figures 2.4-2.7, and are summarized together here to illustrate the specific combinations of spatial and temporal features encoded by each area. Color intensity represents the magnitude of each parameter relative to the lowest and highest values across all areas, with higher values indicated by darker colors. Combinations of feature representations reveal similarities and differences among areas. Areas AL, RL and AM all display high orientation selectivity, high direction selectivity, and prefer low spatial and high temporal frequencies. V1 shows the lowest orientation and direction selectivity, combined with high spatial and low temporal frequency preference. Area LI is unique in that it encodes both high spatial and high temporal frequency information. While it may not be possible to distinguish between some areas based on encoding for any one feature alone, comparison of tuning across multiple stimulus parameters clearly distinguishes each area from the others and reveals the unique combinations of spatiotemporal information represented by each area.

<table>
<thead>
<tr>
<th>Area</th>
<th>OSI</th>
<th>DSI</th>
<th>Pref. SF (cpd)</th>
<th>Pref. TF (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>0.5</td>
<td>0.26</td>
<td>0.045</td>
<td>0.69</td>
</tr>
<tr>
<td>LM</td>
<td>0.55</td>
<td>0.27</td>
<td>0.028</td>
<td>1.8</td>
</tr>
<tr>
<td>LI</td>
<td>0.56</td>
<td>0.28</td>
<td>0.04</td>
<td>1.5</td>
</tr>
<tr>
<td>AL</td>
<td>0.72</td>
<td>0.35</td>
<td>0.022</td>
<td>1.2</td>
</tr>
<tr>
<td>RL</td>
<td>0.69</td>
<td>0.35</td>
<td>0.025</td>
<td>1.3</td>
</tr>
<tr>
<td>AM</td>
<td>0.81</td>
<td>0.38</td>
<td>0.033</td>
<td>1.6</td>
</tr>
<tr>
<td>PM</td>
<td>0.67</td>
<td>0.29</td>
<td>0.046</td>
<td>0.87</td>
</tr>
</tbody>
</table>
Table 2.1A Further tabulation of numbers of neurons sampled by cortical area.

Total neurons indicates number \(N\) of neurons recorded in the spatial frequency (SF) and temporal frequency (TF) experiments for each area. Number \(N\) and percent of total of responsive \((\Delta F/F > 6\%)\) and reliable neurons \(\delta > 1\), Experimental Procedures\) for each area. Neurons which were responsive and reliable, as well as representing eccentricities within 50° of the center of space (over 99% of responsive and reliable neurons) were included in subsequent population analyses. “\(N\) Expts” refers to the number of fields of view (samples of neurons) recorded from for each area. This table (compared to Table 1) separates responsive from reliable criteria, and shows number of neurons passing eccentricity criteria for inclusion in our analysis.

<table>
<thead>
<tr>
<th>Area</th>
<th>Total Neurons</th>
<th>Responsive Neurons</th>
<th>Reliable Neurons</th>
<th>Responsive &amp; Reliable</th>
<th>Included Neurons</th>
<th>(N) Expts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SF TF</td>
<td>SF TF</td>
<td>SF TF</td>
<td>SF TF</td>
<td>SF TF</td>
<td>SF TF</td>
</tr>
<tr>
<td>V1</td>
<td>1419 1382</td>
<td>826 (58%) 718 (52%)</td>
<td>1063 (75%) 984 (71%)</td>
<td>728 (51%) 588 (42%)</td>
<td>728 (51%) 584 (42%)</td>
<td>11 10</td>
</tr>
<tr>
<td>LM</td>
<td>590 584</td>
<td>328 (56%) 198 (34%)</td>
<td>495 (84%) 404 (69%)</td>
<td>300 (51%) 171 (29%)</td>
<td>295 (50%) 171 (29%)</td>
<td>7 7</td>
</tr>
<tr>
<td>LI</td>
<td>182 193</td>
<td>51 (28%) 35 (18%)</td>
<td>85 (47%) 56 (29%)</td>
<td>42 (23%) 23 (12%)</td>
<td>40 (22%) 23 (12%)</td>
<td>2 2</td>
</tr>
<tr>
<td>AL</td>
<td>890 918</td>
<td>418 (47%) 343 (37%)</td>
<td>541 (61%) 542 (59%)</td>
<td>330 (37%) 257 (26%)</td>
<td>323 (36%) 253 (26%)</td>
<td>6 6</td>
</tr>
<tr>
<td>RL</td>
<td>616 555</td>
<td>232 (38%) 124 (22%)</td>
<td>382 (62%) 295 (53%)</td>
<td>201 (33%) 96 (17%)</td>
<td>194 (31%) 95 (17%)</td>
<td>6 6</td>
</tr>
<tr>
<td>AM</td>
<td>404 311</td>
<td>80 (20%) 25 (6%)</td>
<td>203 (50%) 70 (23%)</td>
<td>63 (16%) 12 (4%)</td>
<td>63 (16%) 11 (4%)</td>
<td>6 6</td>
</tr>
<tr>
<td>PM</td>
<td>508 506</td>
<td>172 (34%) 78 (15%)</td>
<td>332 (65%) 230 (45%)</td>
<td>147 (29%) 50 (10%)</td>
<td>147 (29%) 50 (10%)</td>
<td>3 3</td>
</tr>
<tr>
<td>Total</td>
<td>4609 4449</td>
<td>2107 (46%) 1521 (34%)</td>
<td>3101 (67%) 2561 (58%)</td>
<td>1611 (39%) 1195 (27%)</td>
<td>1790 (39%) 1157 (27%)</td>
<td>41 40</td>
</tr>
</tbody>
</table>
A mathematical transformation (Supplemental Experimental Procedures) was applied to all stimuli so that they were displayed in spherical coordinates on a flat monitor (tangent to the hemisphere). (A) Altitude coordinates. Colors represent isoaltitude lines. This transformation was applied to drifting sine wave grating stimuli in order to keep spatial and temporal frequencies constant throughout the hemifield. It was also applied to vertical retinotopy stimuli (Movie S1). (B) Azimuth coordinates. Colors represent isoazimuth lines. This transformation was applied to horizontal retinotopy stimuli (Movie S2). The combination of altitude and azimuth-corrected retinotopy stimuli mapped retinotopic eccentricity in spherical coordinates using a flat monitor.

Figure 2.1A  Spherical coordinates transformed to a flat surface.
Figure 2.2A  High-resolution retinotopic maps measured with wide-field two-photon calcium imaging reveal structural details of small visual areas.

Fine-scale retinotopic organization of mouse visual cortex (A-H). Colors represent retinotopic eccentricity, as in Figure 2.2. Panels A & E show continuous vertical retinotopy maps, in altitude coordinates. Positive values indicate the upper field and negative values the lower field. Panels B & F show the corresponding contour plots of vertical eccentricity with area borders outlined in black. Panels C & G show continuous horizontal retinotopy maps, in azimuth coordinates, with positive values representing the nasal visual field and negative values representing the temporal visual field. Panels D & H show contour plots of horizontal retinotopy, overlaid with area borders. Area borders correspond to area diagram in Figure 2.1I. (A-D) Retinotopic organization of posterior-lateral extrastriate areas. The retinotopic structures of areas P and POR, often difficult to resolve using intrinsic imaging, are revealed by mapping with wide-field calcium imaging. Areas shown in (B, D) correspond to V1, AL, LI, POR, and P surrounding area LM counterclockwise. The border between V1 and LM and AL is marked by a reversal at the vertical meridian (blue-purple, (C, D)). LM and LI are separated by a reversal at the temporal periphery (yellow-green, (C,D)). AL is separated from LM by a reversal near the horizontal meridian (purple-blue (A, B)). A reversal in horizontal retinotopy separates both P and POR from areas LM and LI (yellow-red, (C,D)). (E-H) Retinotopic organization of anterior extrastriate areas, located in the posterior parietal cortex. Areas shown in (F, H) include LM, AL, RL, A, AM, PM, V1 (starting at lower left, moving clockwise). The retinotopic structure of all areas is consistent with maps displayed in Figure 2.2. Fine-scale features resolved by calcium imaging include a ring like structure in the representation of the vertical meridian in area RL (purple, (G, H)), as well as a complete, organized representation of the visual field in both the horizontal and vertical dimension for areas AM and PM (right, (F, H)). Here, visual activation is observed in the expected location of area A, however, a clear retinotopic structure is not apparent. This area receives less-organized (less topographic) input from V1 than the other areas (Wang and Burkhalter 2007). All scale bars 500 µm.
Figure 2.2A  High-resolution retinotopic maps measured with wide-field two-photon calcium imaging reveal structural details of small visual areas, continued

Fine-scale retinotopic organization of mouse visual cortex (A-H). Colors represent retinotopic eccentricity, as in Figure 2.2. Panels A & E show continuous vertical retinotopy maps, in altitude coordinates. Positive values indicate the upper field and negative values the lower field. Panels B & F show the corresponding contour plots of vertical eccentricity with area borders outlined in black. Panels C & G show continuous horizontal retinotopy maps, in azimuth coordinates, with positive values representing the nasal visual field and negative values representing the temporal visual field. Panels D & H show contour plots of horizontal retinotopy, overlaid with area borders. Area borders correspond to area diagram in Figure 2.1I. (A-D) Retinotopic organization of posterior-lateral extrastriate areas. The retinotopic structures of areas P and POR, often difficult to resolve using intrinsic imaging, are revealed by mapping with wide-field calcium imaging. Areas shown in (B, D) correspond to V1, AL, LI, POR, and P surrounding area LM counterclockwise. The border between V1 and LM and AL is marked by a reversal at the vertical meridian (blue-purple, (C, D)). LM and LI are separated by a reversal at the temporal periphery (yellow-green, (C,D)). AL is separated from LM by a reversal near the horizontal meridian (purple-blue (A, B)). A reversal in horizontal retinotopy separates both P and POR from areas LM and LI (yellow-red, (C,D)). The border between P and POR occurs at the representation of the upper periphery (purple (A, B)). (E-H) Retinotopic organization of anterior extrastriate areas, located in the posterior parietal cortex. Areas shown in (F, H) include LM, AL, RL, A, AM, PM, V1 (starting at lower left, moving clockwise). The retinotopic structure of all areas is consistent with maps displayed in Figure 2.2. Fine-scale features resolved by calcium imaging include a ring like structure in the representation of the vertical meridian in area RL (purple, (G, H)), as well as a complete, organized representation of the visual field in both the horizontal and vertical dimension for areas AM and PM (right, (F, H)). Here, visual activation is observed in the expected location of area A, however, a clear retinotopic structure is not apparent. This area receives less-organized (less topographic) input from V1 than the other areas (Wang and Burkhalter 2007). All scale bars 500 µm.
Figure 2.3A Responses are robust and selective across visual areas.

Calcium responses and tuning curves for example cells from each visual area. Response matrices (upper plots) show the change in fluorescence over baseline for each stimulus condition, for the spatial frequency experiment (top of each panel) and temporal frequency experiment (middle of each panel). The average response across all trials of a given condition is shown in black, with the responses to each trial in gray. Gray boxes indicate the duration of the stimulus, 2 seconds for SF experiments, 4 seconds for TF experiments. Scale bars to lower right of matrices correspond to 40% ΔF/F. Tuning curves (bottom of each panel) were generated by taking the average ΔF/F for each stimulus condition, at the intersection of the matrix that gave the peak response. Orientation tuning curves (yellow boxes) are taken across all orientations at the preferred spatial frequency, spatial frequency tuning curves (orange boxes) are taken across all spatial frequencies at the preferred orientation, and temporal frequency tuning curves (magenta boxes) are taken across all temporal frequencies at the preferred orientation. Values for preferred stimuli and selectivity metrics are listed above each tuning curve. OSI = orientation selectivity index, DSI = direction selectivity index, LC = low cutoff frequency, HC = high cutoff frequency, BW = bandwidth. (A) Example cell from V1. This cell is highly orientation selective, is bandpass for spatial frequency and lowpass for temporal frequency, preferring the lowest TF tested (0.5 Hz). (B) Example cell from LM. This cell is perfectly orientation selective (OSI = 1), bandpass for spatial frequency with broad tuning (BW = 3.5 octaves), and highpass for temporal frequency, preferring the highest temporal frequency tested (8 Hz). (C) Example cell from LI. This cell is broadly tuned for orientation, responding similarly to all orientations across all stimulus conditions (OSI = 0.18) and prefers high spatial frequencies (highpass, pref. SF = 0.16 cpd), and low temporal frequencies (1 Hz). (D) Example cell from AL. This cell is highly orientation selective, bandpass for spatial frequency with moderately sharp tuning (BW = 2.3 octaves) and broadly tuned for temporal frequency (BW= 3.5 octaves). (E) Example cell from RL. This cell is highly selective for direction, responding primarily to only one direction of motion for the same orientation (DSI = 0.65), and is highly selective for spatial frequency, with a bandwidth of 1.5 octaves. (F) Example cell from AM. This cell is orientation selective, has moderate spatial frequency selectivity (pref. SF = 0.4cpd, BW = 2 octaves), and responds similarly to all temporal frequencies. (G) Example cell from PM. This cell is orientation selective, is highpass for spatial frequency, and responds weakly across temporal frequencies.
Figure 2.3A Responses are robust and selective across visual areas, continued.

Calcium responses and tuning curves for example cells from each visual area. Response matrices (upper plots) show the change in fluorescence over baseline for each stimulus condition, for the spatial frequency experiment (top of each panel) and temporal frequency experiment (middle of each panel). The average response across all trials of a given condition is shown in black, with the responses to each trial in gray. Gray boxes indicate the duration of the stimulus, 2 seconds for SF experiments, 4 seconds for TF experiments. Scale bars to lower right of matrices correspond to 40% ΔF/F. Tuning curves (bottom of each panel) were generated by taking the average ΔF/F for each stimulus condition, at the intersection of the matrix that gave the peak response. Orientation tuning curves (yellow boxes) are taken across all orientations at the preferred spatial frequency, spatial frequency tuning curves (orange boxes) are taken across all spatial frequencies at the preferred orientation, and temporal frequency tuning curves (magenta boxes) are taken across all temporal frequencies at the preferred orientation. Values for preferred stimuli and selectivity metrics are listed above each tuning curve. OSI = orientation selectivity index, DSI = direction selectivity index, LC = low cutoff frequency, HC = high cutoff frequency, BW = bandwidth. (A) Example cell from V1. This cell is highly orientation selective, is bandpass for spatial frequency and lowpass for temporal frequency, preferring the lowest TF tested (0.5 Hz). (B) Example cell from LM. This cell is perfectly orientation selective (OSI = 1), bandpass for spatial frequency with broad tuning (BW = 3.5 octaves), and highpass for temporal frequency, preferring the highest temporal frequency tested (8 Hz). (C) Example cell from LI. This cell is broadly tuned for orientation, responding similarly to all orientations across all stimulus conditions (OSI = 0.18) and prefers high spatial frequencies (highpass, pref. SF = 0.16 cpd), and low temporal frequencies (1 Hz). (D) Example cell from AL. This cell is highly orientation selective, bandpass for spatial frequency with moderately sharp tuning (BW = 2.3 octaves) and broadly tuned for temporal frequency (BW= 3.5 octaves). (E) Example cell from RL. This cell is highly selective for direction, responding primarily to only one direction of motion for the same orientation (DSI = 0.65), and is highly selective for spatial frequency, with a bandwidth of 1.5 octaves. (F) Example cell from AM. This cell is orientation selective, has moderate spatial frequency selectivity (pref. SF = 0.4cpd, BW = 2 octaves), and responds similarly to all temporal frequencies. (G) Example cell from PM. This cell is orientation selective, is highpass for spatial frequency, and responds weakly across temporal frequencies.
**Figure 2.4A Mean temporal frequency bandwidth across visual areas.**

Mean temporal frequency bandwidth (in octaves) is higher in several extrastriate areas (LM, AL, RL) compared to V1, indicating that neurons in these areas respond to a broader range of temporal frequencies on average. Inset, statistical significance (p < 0.05) of pair-wise comparisons between areas, corrected for multiple comparisons by the Tukey-Kramer method. Error bars are standard error of the mean (S.E.M.).
Mean spatial frequency bandwidth (in octaves) is sharper in all extrastriate areas except LI, indicating that higher visual areas are more selective for spatial frequency than V1. Further, some extrastriate areas (AL, RL, AM) have significantly sharper bandwidth values than LM. Inset, statistical significance ($p < 0.05$) of pair-wise comparisons between areas, corrected for multiple comparisons by the Tukey-Kramer method. Error bars are standard error of the mean (S.E.M.).
Figure 2.6A  Correlation analyses between each pair of metrics on a cell-by-cell basis for each area.

Scatter plots of neuron scores from each area for each combination of selectivity metrics. Area names at top of figure correspond to the entire column of scatter plots. Pearson’s correlation coefficients (R) are listed above each plot for each area, along with the p value corresponding to a significance test against zero correlation (α < 5% for p < 0.007 after accounting for multiple comparisons with the Bonferroni method). (A) The preferred spatial frequency of each neuron is plotted as a function of preferred temporal frequency of each neuron for each area. Note: not all neurons in our dataset received scores for both spatial and temporal frequencies (e.g., we did not record from them in both experiments), thus the populations in the plots in (A) are a subset of the populations used in other analyses of SF and TF experiments alone. (B) The orientation selectivity index (OSI) of each neuron is plotted as a function of direction selectivity index (DSI) of each neuron for each area. (C) Preferred temporal frequency of each neuron is plotted as a function of OSI of each neuron for each area. (D) Preferred temporal frequency of each neuron plotted as a function of DSI of each neuron for each area. (E) Preferred spatial frequency of each neuron plotted as a function of OSI of each neuron for each area. (F) Preferred spatial frequency of each neuron as a function of DSI of each neuron for each area.
Supplemental Experimental Procedures

Animal Preparation and Surgery

All experiments involving living animals were approved by the Salk Institute’s Institutional Animal Care and Use Committee. All experiments were performed using C57BL/6 mice \((n = 28)\) between 2 and 3 months of age. Surgical plane anesthesia was induced and maintained with isoflurane (2-2.5% induction, 1-1.25% surgery). Dexamethasone and carprofen were administered subcutaneously (2 mg/kg and 5 mg/kg respectively), and ibuprofen (30 mg/kg) was administered post-operatively in the drinking water for the duration of the experiment (typically 1-2 days). A custom-made metal frame was mounted to the skull, centered on the visual cortex of the left hemisphere using stereotaxic coordinates. The frame was mounted so that it was as close to the skull as possible, tilted to be tangent to the brain surface. The angle of tilt was recorded using a digital protractor by measuring the tilt of the stereotaxic frame (Narishige) used to parallelize the frame relative to the table. The skull overlying visual cortex was carefully thinned with a dental drill, stopping often and rinsing with chilled artificial cerebral spinal fluid (ACSF, in mM: 125 NaCl, 10 d-glucose, 10 HEPES, 3.1 CaCl\(_2\), 1.3 MgCl\(_2\); or 150 NaCl, 2.5 KCl, 10 HEPES, 2 CaCl\(_2\), 1 MgCl\(_2\)). Great care was taken to prevent overheating of the brain or subdural bleeding. The bone was thinned just passed the spongy middle layer which often contained blood vessels. The resultant preparation was a thin layer of semi-flexible bone, free of blood vessels. This was especially relevant for alignment of cortical blood vessel landmarks and functional retinotopic maps. After the surgery, chloprothixene (2.5 mg/kg) was administered intramuscularly and isoflurane was reduced to 0.25-0.8%, for visual stimulation and recording experiments. The most robust visual responses coincided with higher respiration rates when the animal appeared to be lightly anesthetized.
Intrinsic Signal Optical Imaging

The intrinsic signal imaging protocol was adapted from previous studies (Kalatsky and Stryker, 2003; Nauhaus and Ringach, 2007). Imaging setup is as described in Nauhaus and Ringach, 2007. All other relevant modifications are described elsewhere in the text.

Dye Loading and Two-Photon Imaging

The retinotopic map from the intrinsic signal imaging experiment aligned and overlayed on an image of the cortical blood vessels was used as a reference for targeting the locations of calcium-sensitive dye loading. The dye solution contained 1mM Oregon Green Bapta-1 AM (OGB, Invitrogen) with 10% DMSO and Pluronic F-127 in ACSF (Stosiek et al., 2003; Ohki et al., 2005). The solution also included 50 µM sulforhodamine-101 to selectively label astrocytes (Nimmerjahn et al., 2004). The pipette (3-5 µm outer diameter tip) was lowered diagonally (30° from horizontal) to 225 µm below the dura surface (layer 2/3) while short pulses of pressure were applied to the back of the pipette to keep the tip clear of debris. Then, a single pulse of 1 min duration and 10 PSI was applied for the loading protocol. This typically resulted in approximately 0.5-1 mm² of OGB loading. The loading was observed under epifluorescence to confirm sufficient dye injection. In most cases, several locations were injected in the same animal often using the same pipette multiple times, resulting in an area of OGB loading spanning up to several millimeters. Each loading site was separated by approximately 0.5-1mm. At least one hour after the last loading the two-photon imaging began.

A custom-built Movable Objective Microscope (Sutter) coupled to a Chameleon Ultra II mode-locked Ti:Sapphire laser (Coherent) was used for two-photon imaging. Standard wavelength for two-photon microscopy was 920-950 nm. A waveplate (Newport) was used in conjunction with a beamsplitter cube (Newport) to modulate the power of the excitation laser light. A 16X water-immersion objective (Nikon) was used for large-field retinotopy experiments, and a 40X water-immersion objective (Olympus) was used to
measure cellular responses. Fluorescent light was detected by photomultiplier tubes (Hamamatsu), after being filtered by one of two emission filter sets: HQ535/50, and HQ610/75 (Chroma). A third acquisition channel was used to record the output of a photodiode to detect a brief pulse of light at the corner of the screen, which precisely indicated the phase of the periodic visual stimulus (e.g., drifting bar retinotopy stimulus), in addition to the stimulus beginning and end. ScanImage software (Pologruto et al., 2003) was used for image acquisition. Images and time lapse videos were processed offline in Matlab (MathWorks) and ImageJ (http://rsbweb.nih.gov/ij/). Imaging experiments were performed at ~130-180 µm below the dura surface (layer 2/3). For retinotopy experiments, images were typically acquired at approximately 2 Hz (256 x 256 pixel frames, unidirectional scanning, ~800 x 800 µm field of view for wide-field imaging). For image cellular responses, images were typically acquired at approximately 8 Hz (256 x 256 pixel frames, bidirectional scanning, either ~150 x 150 µm or ~300 x 300 µm field of view). After each experiment in a given location, a stack of images (5 µm z step) was taken starting at the experiment image plane and ending above the dura in order to determine the precise depth of the experiment offline. Images were taken of the aligned overlying surface blood vessel pattern in order to register images taken during experiments with the 40X objective with those with the 16X objective, and with the intrinsic imaging experiments. High-resolution images (1024 x 1024 pixel frames) were often taken of the experiment imaging plane as an anatomical reference of the cells recorded.

Visual Stimulation

Drifting bar and drifting grating stimuli were displayed on a gamma-corrected, large LCD display (68 x 121 cm, 120 Hz monitor, stimulus presented at 60Hz), rotated in 'portrait' mode such that the monitor was taller than it was wide. The screen was placed so as to stimulate the entire known visual hemifield of the mouse (at most -40° to 70° lower to upper and -70° to 70° nasal to temporal (Wagor et al., 1980), when defining 0° altitude as a plane.
passing through the center of the eyes parallel to the ground, and defining $0^\circ$ azimuth as the coronal plane passing through the center of the eye). The screen was tilted to match the tilt of the animal relative to its normal position around its anteroposterior axis. The screen was placed at an angle of $25^\circ$ relative to the animal around its dorsoventral axis such that the screen was pointed slightly inward toward the animal’s nose (i.e., to stimulate the nasal periphery). A perpendicular bisector was defined as the origin of the line perpendicular to the screen that intersected with the animal’s eye. This point on the screen defined the center of the stimulus and was slightly anterior to the $0^\circ$ azimuth plane. The screen was raised so that the perpendicular bisector was 28 centimeters from the bottom of the screen, centered on the screen left to right, and 10 cm from the animal. This positioning of the monitor, in conjunction with the spherical stimulus correction described below, accurately simulated a hemispherical display around the mouse (the screen covered $>140^\circ$ of the nasal to temporal field and $>110^\circ$ lower to upper field). Furthermore, it was beyond the theoretical point of ‘infinite focus’ of the mouse eye (Remtulla and Hallett, 1985; Green et al., 1980; de la Cera et al., 2006) leading to similar focus at all points on the screen. Absolute eccentricity coordinates were rotated relative to stimulus coordinates as described in the Data Analysis section in order to follow the convention for the mouse visual hemifield described above (i.e., azimuth was rotated slightly from the plane through the perpendicular bisector that defined the center of the stimulus to accurately define $0^\circ$ azimuth).

For retinotopy experiments, a periodic drifting bar stimulus was used (Kalatsky and Stryker, 2003). The bar was $20^\circ$ wide and subtended the whole visual hemifield along either the vertical or horizontal axis. The bar was drifted 10 times in each of the four cardinal directions. Azimuth correction was used for the bar drifting along the horizontal axis and altitude correction was used for the bar drifting along the vertical axis (see below), in order to define eccentricity using standard spherical coordinates. By drifting the bar in both
directions along an axis it was possible to subtract the delay in the intrinsic signal relative to neural activity (Kalatsky and Stryker, 2003; see Data Analysis). The bar drifted at 8.5-9.5°/s for intrinsic imaging experiments and at 12-14°/s for two-photon imaging experiments. The stimulus was generated to be larger than the display (155° wide x 173° tall), preventing the periodic drifting bar stimulus from stimulating two points in space simultaneously.

For drifting grating experiments, sinusoidal drifting gratings were used. For each population of neurons imaged (a single 40X imaging plane), we presented with rare exceptions four sets of stimuli: a temporal frequency varying experiment (0.5, 1, 2, 4 and 8 Hz, 8 directions plus blank, ~0.04 cpd, 5 repeats pseudorandomized for each parameter combination), a spatial frequency varying experiment (0.01, 0.02, 0.04, .08 and 0.16 cpd, 8 directions plus blank, ~1 Hz, 5 repeats pseudorandomized for each parameter combination), a 12 direction orientation tuning experiment (~1 Hz, ~0.04 cpd, 5 repeats pseudorandomized for each parameter combination and a blank condition), and a drifting bar retinotopy experiment (same as for the large-field 16X retinotopy experiments) to map the precise eccentricity of each neuron in the field of view. Stimulus durations were 4 sec for the temporal frequency experiment and 2 sec for the spatial frequency and 12 direction experiments. Population data from previous experiments in each area and from published data from V1 (e.g., Niell and Stryker, 2008) were used to determine the value for the parameters held constant in each experiment in an effort to strongly stimulate as large a population as possible, and keep parameters consistent across visual areas. An altitude correction was used to account for the spherical distortion of the flat monitor. The isoluminance lines of this stimulus are analogous to the altitude lines of a globe. That is, they form parallel rings that are evenly spaced in eccentricity. This correction held the spatial frequency and temporal frequency constant throughout the wide visual field.

Spherical corrections were applied to all stimuli in order to account for the distortions created by displaying stimuli to the animal on a flat monitor.
(Figure 2.1A, Movie S1 and S2). Without correction, the spatial frequency and temporal frequency of a drifting grating shrink rapidly with eccentricity—a problem present in any study using a flat screen, but especially pronounced without appropriate correction in our study which used a very large screen. Drifting bar and grating stimuli were generated through custom routines in Psychtoolbox and Matlab and then transformed with spherical projection, as described below.

Spherical Stimulus Correction

Retinotopic coordinates are often defined in units of degrees of visual field, a spherical parameter. However, monitors are flat, which means that spatial frequency (cyc/deg) and speed (cyc/sec) become gradually reduced for larger eccentricities when they are kept constant in cycles/centimeter. When the eccentricities are kept to reasonably low values (e.g. <30°), this distortion is small. However, our experiments call for stimulation over a large enough region of visual space that this distortion becomes quite substantial. For example, a drifting bar that is kept constant in its width (cm) and speed (cm/sec) will become dramatically smaller (degrees) and slower (deg/sec) at the largest eccentricities of our stimulus. We thus defined our stimuli such that the angular variables in spherical coordinates (altitude or azimuth) are the independent variables. For example, our sine wave gratings were defined as a function of spherical variables, and these values were then projected onto a flat surface.

In our 3-dimensional coordinate system, we defined the eye as the origin. For the reasons described above, our desired visual stimuli are initially described as a function of altitude, θ, and azimuth, φ: S(θ,φ). The spherical coordinate space is oriented such that the perpendicular bisector from the eye to the monitor is the (0°,0°) axis of the altitude/azimuth coordinates, altitude increases above the mouse, and azimuth increases posterior to the mouse. Next, we convert these spherical coordinates to Cartesian coordinates so that they can be drawn on the monitor. To do so, we orient the Cartesian space so
that the perpendicular bisector between the eye and the monitor is the x-axis. The y and z axes are defined as the horizontal and vertical dimensions on the monitor, with the x-dimension a constant on the monitor surface (x₀). We can now define the stimulus as a function of the horizontal (y) and vertical (z) pixel locations:

Next, we provide an example of a drifting grating that is a function of azimuth, ϕ, and constant in θ. We start with \( S = \cos(2\pi f_s \phi - tf_t) \), where ‘f_s’ is the spatial frequency (cycles/radian), ‘f_t’ is the temporal frequency (radians/second), and ‘t’ is time. We then apply the polar-to-Cartesian transformation, which gives

To change the ‘orientation’ of the drifting grating, we rotate around the x-axis. This is done by applying a rotation of the y/z plane. We now have

We have thus defined a drifting grating with contours along azimuth lines that have been oriented by ‘ϕ’. The stimulus is drawn on the monitor using the y and z domains.

Data Analysis

Intrinsic optical imaging retinotopy data were analyzed as previously described (Kalatsky and Stryker, 2003). Briefly, data were acquired in a continuous block during stimulus presentation of a bar drifting in one direction periodically. The phase of response relative to the stimulus was computed for each pixel by computing the phase of the Fourier component at the same frequency as the stimulus. Contrasting this phase difference with the data from the bar drifting in the opposite direction along the same axis made it possible to estimate the center point between them, accounting for the hemodynamic delay, and thus determining the best estimate of the position in space that the cortical location responded to. A comparable approach was used to analyze Ca²⁺ imaging retinotopy experiments.
Eccentricity values were rotated to follow the convention described in Visual Stimulation by first transforming the data into Cartesian coordinates, rotating the data using standard matrix rotation, and then converting the data back to spherical coordinates. This transformation does not warp the data in any way, and any arbitrary transformation/rotation can be accomplished using this method.

For population analyses of cellular responses to drifting gratings, data were preprocessed in the following ways. Each experiment (e.g., temporal frequency varying experiment) typically lasted approximately 30-45 minutes. To account for drifts in the image over time, we applied a movement correction algorithm that aligned each trial of the experiment to the average image of the first trial by determining the highest cross-correlation between the images. Then regions of interest (ROIs) around each cell in the image were created using a semi-automated procedure, separately for the OGB channel (neurons and glia) and the sulfarhodamine-101 channel (glia). Candidate ROIs were determined by thresholding an image of the field of view that had first been processed with a local Z-score to normalize the local statistics. Then, ROIs were carefully edited manually for both channels. Glia cells were removed from the analysis of the OGB channel by removing any ROIs in the OGB channel that overlapped with ROIs marked in the glia channel. The remaining ROIs in the OGB channel were neurons. The pixels were averaged within each of these ROIs for each time point (each single image) to reflect the raw time course of a given neuron.

In order to link neuron identities across experiments (e.g., to perform spatial and temporal frequency cell-by-cell correlations), we found the relative position between the ROI maps for each experiment that maximized the cross-correlation. Then we determined the cell ROIs which overlapped across the ROI maps for each experiment by at least 3 pixels (the great majority of neurons were overlapping) and created a new ROI map with unique identifiers of each common neuron. Inverse transformations were applied to each ROI
map so that each map realigned with each original experiment. The end result was a common ROI map that identified neurons across multiple experiments within the same field of view that accounted for any drifts in the imaging field of view over the course of the imaging session.

The baseline calcium fluorescence signal was averaged within each cell ROI for each trial during a prestimulus period during which a gray screen was shown (1 sec). Then, the entire time course was converted from absolute fluorescence values to the percent change relative to baseline by computing the following: $\Delta F/F = (F_I - F)/F$, where $F_I$ is the instantaneous fluorescence signal and $F$ is the baseline fluorescence. The $\Delta F/F$ response for each cell was averaged during the stimulus time window (2 sec) for each trial, and the mean and standard deviation across trials for each stimulus and blank condition were computed for each neuron. We applied several criteria to include neurons in further population analyses. Neurons were deemed visually responsive if they gave a mean response above 6% $\Delta F/F$ to any of the stimuli. A response reliability metric ($\delta$) was computed for each neuron as follows:

$$\delta = \frac{\mu_{\text{max}} - \mu_{\text{blank}}}{\sigma_{\text{max}} + \sigma_{\text{blank}}}$$

where $\mu_{\text{max}}$ and $\sigma_{\text{max}}$ are the mean and standard deviations of the response to the preferred stimulus respectively, and $\mu_{\text{blank}}$ and $\sigma_{\text{blank}}$ are the mean and standard deviations of the response to the blank stimulus respectively. Neurons were deemed reliable for $\delta > 1$. Finally, neuron eccentricity mapped with the retinotopy stimulus was used to restrict our population analyses to eccentricity-matched neurons within the central 100° of space (see Table 1). The numbers of neurons which met all of these criteria were used as the denominator in all subsequent analyses of fraction of neurons exhibiting a particular score on a given parameter.

**Tuning Metrics**

Temporal and spatial frequency tuning curves were taken at the optimal orientation and direction for each neuron, using the average $\Delta F/F$ response for
each condition across trials. These tuning curves were fit with a Difference of Gaussians function (Sceniak et al., 2002). A log₂ transform was performed on the x-axis of the curves, converting it to octaves. The preferred frequency was taken at the peak (maximum) location of this curve. For bandwidth and low and high cutoff measurements, each cell was characterized as either bandpass, highpass or lowpass for temporal and spatial frequency based on whether its minimum and/or maximum frequency response passed below the half max for the tuning curve. The high cutoff was computed for bandpass and lowpass cells as the highest frequency which drove a half-max response. Low cutoff was computed for bandpass and highpass cells as the lowest frequency which drove a half-max response. Bandwidth was measured for bandpass cells as the difference between high cutoff and low cutoff.

The orientation and direction tuning curves were taken at the optimal spatial frequency for each neuron, using the average ΔF/F response for each condition across trials. The orientation selectivity index (OSI) was computed as follows:

\[ OSI = \frac{\mu_{max} - \mu_{orth}}{\mu_{max} + \mu_{orth}} \]

where \( \mu_{max} \) is the mean response to the preferred orientation and \( \mu_{orth} \) is the mean response to the orthogonal orientation (average of both directions). The direction selectivity index was computed as follows:

\[ DSI = \frac{\mu_{max} - \mu_{opp}}{\mu_{max} + \mu_{opp}} \]

where \( \mu_{max} \) is the mean response to the preferred direction and \( \mu_{opp} \) is the mean response to the opposite direction. The half-width at half-maximum (HWHM) was computed for each neuron orientation and direction tuning curves but our results suggested that we did not adequately sample the direction domain to make comparisons based on these measurements (i.e., neurons may have had sharper tuning widths than our sampling of the direction domain could detect).
Statistics

We started with the most basic question: are mouse cortical visual areas distinguishable based on population tuning metrics? We asked this question statistically using a multivariate analysis of variance (MANOVA) on the four values we computed for every neuron in our sample from each area: preferred temporal frequency, preferred spatial frequency, orientation selectivity index and direction selectivity index. We also computed bandwidth and high and low cutoff metrics for each population, however not every neuron received a value for all of these metrics and thus we excluded these metrics from the multivariate analysis. We followed up this analysis with individual univariate analyses to confirm whether there was a main effect of area on each of our dependent variables (both one-way ANOVA and one-way Kruskal-Wallis tests). Finally, for each significant one-way test, we performed post-hoc pairwise comparisons to determine which areas were different from each other in terms of each metric, correcting the p value for multiple comparisons using the Tukey-Kramer Honestly Significant Difference (HSD). Pearson correlation coefficients and statistical significance compared against no correlation were computed using Matlab routines, and the p value was corrected for multiple comparisons using the Bonferroni method (Figure 2.6A).

Supplemental References

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