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Layer-specific BOLD activation in awake monkey V1 revealed by ultra-high spatial resolution functional magnetic resonance imaging

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Abstract

The laminar structure of the cortex has previously been explored both in non-human primates and human subjects using high-resolution functional magnetic resonance imaging (fMRI). However, whether the spatial specificity of the blood-oxygenation-level-dependent (BOLD) fMRI is sufficiently high to reveal lamina specific organization in the cortex reliably is still unclear. In this study we demonstrate for the first time the detection of such layer-specific activation in awake monkeys at the spatial resolution of $200 \times 200 \times 1000 \mu\text{m}^3$ in a vertical 4.7T scanner. Results collected in trained monkeys are high in contrast-to-noise ratio and low in motion artifacts. Isolation of laminar activation was aided by choosing the optimal slice orientation and thickness using a novel pial vein pattern analysis derived from optical imaging. We found the percent change of GE-BOLD signal is the highest at a depth corresponding to layer IV. Changes in the middle layers (layer IV) were 30% greater than changes in the top layers (layers I–III), and 32% greater than the bottom layers (layers V/VI). The laminar distribution of BOLD signal correlates well with neural activity reported in the literature. Our results suggest the high intrinsic spatial resolution of GE-BOLD signal is sufficient for mapping sub-millimeter functional structures in awake monkeys. This degree of spatial specificity will be useful for mapping both laminar activations and columnar structures in the cerebral cortex.

Keywords

BOLD spatial resolution; Cortical layers; Functional MRI; Non-human primate; Visual cortex

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Disclosure/conflict of interest

The authors declare no conflict of interest.

1. Introduction

The ability to identify laminar-specific functional activation with fMRI methods would add considerably to our abilities to study cortical function. For example, the signature for feed forward activation is greater activation in the middle layers, while that for feedback activation is marked by greater activation in superficial and deep layers. Superficial, middle, and deep cortical layers are also believed to have different extents and patterns of lateral connectivity and therefore different degrees of lateral integration. Improved knowledge on laminar activation patterns would be important for understanding both normal cortical processes as well as changes that occur in developmental, neurological and mental disease.

Non-human primates, in particular Macaque monkeys, have been highly instructive for our understanding of human cortical function. Similar to the human, cells in primary visual cortex (V1) of monkey are arranged into six layers; cortical thickness tends to be thinner in sulci and thicker on gyri. The thickness of V1 in monkeys, which spans roughly 1.5 mm (Chen et al., 2012b; Herculano-Houzel et al., 2008; Rockel et al., 1980), is thinner than humans and much less convoluted. Inputs to V1 terminate mainly layer IV (Hubel and Wiesel, 1972), the middle layer which is roughly 400 – 500 μm in thickness. Layer IV is further divided into several sub-layers, marked by dense intra- and inter- sublaminar connections (Callaway, 1998). In response to visual stimulation, neural circuits within layer IV tend to consume more energy compared to other layers, reflecting laminar differences in glucose uptake (Tootell et al., 1988), and long-term changes in neuronal activity related to metabolic activity (Horton, 1984; Wong-Riley, 1979). This differential energy consumption and oxygen demand lead to laminar differences in BOLD signal distribution and reflects local synaptic activity related to both the input and intracortical connections (Logothetis et al., 2001; Smirnakis et al., 2005). Taken together, the above-mentioned studies suggest that lamina-specific activation in V1 of monkeys is a good test bed for assessing the functional specificity of the BOLD signal. As part of this test, given previous studies, we expect a particularly robust increase in BOLD signal in layer IV in response to visual stimulation.

To date, studies acquired with gradient-echo (GE) sequences, the most popular sequences used in fMRI, have found the strongest BOLD responses at the surface of the brain in V1. This is true both for anesthetized monkeys (Goense and Logothetis, 2006; Goense et al., 2007; Smirnakis et al., 2007; Zappe et al., 2008) and cats (Harel et al., 2006; Zhao et al., 2004). Other reports in humans find a localized increase in the middle layer of V1, which is relatively small compared to the large peak close to the cortical surface (Koopmans et al., 2010; Polimeni et al., 2010; Ress et al., 2007). This raises the possibility that some degree of laminar differentiation is possible and that the failure to reveal laminar organization in V1 may, in fact, be due to the high density of large pial veins at the cortical surface. In comparison to capillaries, GE-BOLD is known to be more sensitive to large veins, vessels which contain greater deoxyhemoglobin content (Frahm et al., 1994; Turner, 2002). Because of the relatively low spatial resolution of fMRI compared to the size of pial veins, previous methods to eliminate the contribution of pial veins by spatially masking voxels associated with large veins (Cheng et al., 2001; Koopmans et al., 2010; Shmuel et al., 2007) are likely to underestimate the influence of large veins on the cortical surface due to partial volume effects. Lamina-specific activations can also be strongly affected by anesthesia (Duong, 2007; Peeters et al., 2001) so it would be preferable to study such signals in awake subjects.

Here, we have addressed these issues by: 1) using optical imaging to map pial veins for the purpose of determining optimal fMRI slice orientation and thickness and thereby eliminating pial vein contribution, 2) using smaller in-plane voxel sizes to minimize the influence from partial volume effects, and 3) conducting fMRI in awake, behaving monkeys to reduce

complications associated with anesthesia. Using these methods, we find that, as predicted, the percentage change of GE-BOLD signal is highest at a depth corresponding to layer IV, and are able to demonstrate layer-specific hemodynamic changes.

2. Materials and methods

2.1. Animal preparation and surgical procedures

Four Macaque monkeys (*Macaca mulatta*) were used in this research. All procedures conformed to the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Vanderbilt University. Detailed procedures for preparing animals for MRI experiments may be found in Chen et al. (2012a), and those for awake optical imaging experiments could be found in Lu et al. (2010) and Tanigawa et al. (2010).

Two monkeys were used in MRI experiments. Each of them was implanted with a plastic headpost secured by ceramic screws (Thomas Recording GmbH, Geissen, German) and dental cement under general anesthesia (1~2% isoflurane). Monkeys were trained to sit in MR-compatible primate chairs constructed from high density polycarbonate. A secure frame, adjustable to accommodate different animals, was used to mount the head-post to the chair. Frame, head-post and head-post mount were made by ULTEM (GE Polymers, Huntsville, AL). Plastic, fiber-optic binocular glasses, independently adjusted for each monkey, mounted to the frame were used to present visual stimuli. A plastic mouth piece was rigidly attached to the head piece to deliver fluid reward. Both the head-post mount and the eyepiece-mount were designed to be installed from the opening at the top of the chair.

The other two animals participated in optical imaging experiments only. To facilitate optical imaging, monkeys were implanted with an MRI-compatible nylon chamber (Chen et al., 2002) over V1. A craniotomy and durotomy was performed to expose visual cortex within the chamber. An artificial dura (Tecoflex) was used to protect the exposed brain. Animals were given at least two weeks to recover from surgeries.

2.2. Stimulus presentation and animal training

For monkeys involved in MRI experiments, visual stimuli were presented binocularly via a pair of plastic, fiber-optic glasses (Avotec, Stuart, FL). The field of view of the system was 24.5° horizontal by 17° vertical visual angle. Eye locations were tracked by a Real Eye RE-4601 eye monitor system (Avotec, Stuart, FL) and iView eye tracking system (SensoMotoric Instruments GmbH, Teltow, Germany). Visual stimuli consisted of monochromatic counter-phasing checkerboard patterns (6 Hz visual modulation at 90% contrast) alternating with periods of gray background of equal mean luminance (50 cd/m²) and diameter of 16°. The stimulations were divided into blocks with duration of 20 s each. The visual stimuli were the same within a block. In optical imaging experiments, visual stimulation was presented on a CRT monitor (Sony Trinitron GDM F500R). The eye position was tracked by a high speed non-human primate eye tracking system (SensoMotoric Instruments GmbH, Teltow, Germany). Monkeys were trained to perform a fixation task and were required to maintain fixation continuously within 1.5° radius of a centrally presented fixation spot (0.2° × 0.2°) to receive fluid rewards.

2.3. Optical imaging and data analysis

Images of primary visual cortex with in-plane resolution of 16 × 16 μm² were acquired by an IMAGER 3001 system (Optical Imaging, Germantown, NY) with 570 nm or 630 nm wavelength illumination. Collected images were high-pass filtered to release the potential influence from uneven illumination. As veins have a high concentration of deoxygenated

blood, they are easily identified at 630 nm illumination, a wavelength at which absorbance of deoxyhemoglobin is dominant (Malonek and Grinvald, 1996). Only large veins (radius greater than 30 μm) and principal veins (radius greater than 50 μm) (Duvernoy et al., 1981) were included in the pial vein pattern analysis. Based on the fact that 2% percent of blood volume in a voxel is contained in capillaries (Pawlik et al., 1980), a voxel was marked as contaminated by pial veins if its vein-coverage was greater than 2%.

2.4. MRI acquisition and data analysis

Functional scans were acquired on a Varian 4.7T vertical MR scanner (Varian Inc., Palo Alto, CA) using a 2-cm transmit and receive coil. Three voxel sizes ($200 \times 200 \times 1000 \mu\text{m}^3$, $500 \times 500 \times 2000 \mu\text{m}^3$, and $1500 \times 1500 \times 2000 \mu\text{m}^3$) were compared to estimate the best voxel size. The field of view was placed to cover the cortical area of V1 close to the lunate sulcus. We used a four-shot or eight-shot T_2^* -weighted gradient-echo echo-planar imaging (EPI) sequence to collect BOLD signal. The echo time was 10 ms, the flip angle was between 30° and 45° , and the repetition time was 750 ms (four-shot) or 500 ms (eight-shot). Each scanning session consisted of 3–5 functional runs. Each run lasted up to 2400 s. T_2^* -weighted gradient-echo structural images were acquired in the same session with the slices of identical orientation and thickness (echo time = 10 ms, repetition time = 600 ms) as anatomical references. Seven sessions in which monkey maintained fixation exceeded 90% of the time were included for data analysis.

Data were analyzed using code written in Matlab (Mathworks, Natick, MA). Functional data were not smoothed in the space domain. The initial ten volumes of each run were discarded from data analysis so that only steady state signals were included. After filtering to remove low-frequency temporal fluctuations, the correlation of each functional time course to a reference waveform was calculated (Chen et al., 2007), and functional maps were generated at a threshold of $p < 0.01$ (uncorrected for multiple comparisons) with a cluster criterion of at least five activated voxels.

Data stability was estimated by an in-plane rigid body model with three degrees of freedom (two translational movements $Tran_x$, $Tran_y$, and one rotational movement) or a 3D rigid body model with six degrees of freedom (three translational movements and three rotational movements). All images within a run were aligned to the first image of each run. The index of motion *Displacement* is defined as:

$$Displacement = \sqrt{Tran_x^2 + Tran_y^2} \quad (1)$$

The temporal *SNR* was defined as the time-averaged value of 200 volumes divided by their temporal standard deviation, and the image *SNR* was calculated as the signal in each voxel divided by the standard deviation of voxel intensities within a relatively dark and featureless region of the image. Only significantly activated voxels were included in the *SNR* analysis. The thermal noise and the physiological noise are the two dominant sources of noise in the image time-course. The ratio of physiological noise to thermal noise can be determined if the temporal *SNR* and the image *SNR* are known.

$$\frac{noise_{Physiology}}{noise_{Thermal}} = \sqrt{\left(\frac{SNR_{Image}}{SNR_{Temporal}}\right)^2 - 1} \quad (2)$$

The functional contrast-to-noise ratios were obtained by calculating the difference between the conditions with and without stimulation divided by the standard deviation of the difference.

3. Results

3.1. The optimal voxel size in awake monkey fMRI

The smallest voxel size that can be used in fMRI is limited by both the image *SNR* (Edelstein et al., 1986) and temporal *SNR* (Bellgowan et al., 2006; Parrish et al., 2000). We measured both indices in awake monkeys at three voxel sizes ranging from 0.04 mm³ to 4.5 mm³. Figure 1A shows the temporal *SNR* as a function of image *SNR*. Results from the different voxel sizes are indicated by color. We found a close to linear relationship between the voxel size and the image *SNR* (Fig. 2A inset, slope of 100/mm³). With the largest voxel size (in-plane resolution of 1500×1500 μm², blue dots), the highest image *SNR* was more than 1000. In comparison, temporal *SNR* (plotted on Y axis), which is the amplitude of image intensity fluctuations in the fMRI time-course, tended to reach a stable level around 100. The nonlinear relationship (note log-log plot) between temporal *SNR* and image *SNR* reflects the different contributions of noise sources. Thermal noise and physiological noise are the dominant noise source for image *SNR* and temporal *SNR*, respectively. Increases in image *SNR* will have diminishing gains in temporal *SNR* when the contribution of the physiological noise is dominant (Kruger et al., 2001; Triantafyllou et al., 2005).

To determine the relative contribution of physiological noise, we calculated the ratio of the physiological over thermal noise determined from Eq. 2. It is usual to accept that at the optimal voxel size for fMRI, the contribution of physiological noise and the contribution of thermal noise will be equal (Bodurka et al., 2007). The distributions of ratios at three voxel sizes are plotted in Fig. 1B. The mean ratios of the physiological and thermal noise were 0.92 (red), 3.19 (green), and 4.69 (blue) for voxel size of 0.04 mm³, 0.5 mm³, and 4.5 mm³, respectively. Thus, our results suggest that the smallest voxel size we used (200×200×1000 μm³) is the one closest to the optimal voxel size. fMRI results obtained at this resolution will have a balance between detection of activation induced signal changes and minimization of partial volume averaging. All subsequent functional data analysis is based on data acquired at this voxel size.

3.2. Minimization of large vein influence in BOLD signals

Changes in tissue concentrations of deoxyhemoglobin is the basis for BOLD contrast, so large veins may potentially produce larger BOLD signal changes. However, because BOLD signals from large veins have low spatial specificity (Frahm et al., 1994; Yu et al., 2012), achieving high spatial resolution functional imaging can be aided by minimizing contributions from large veins. Our approach is to minimize large vessel contributions by using optical imaging to determine the large vein patterns on the surface of V1 (pial veins) in awake monkeys. The difference between blood vessel patterns in the awake monkey obtained at wavelengths of 570 nm (Fig. 2A) and 630 nm (Fig. 2B) were used to distinguish veins (black) from arteries (red) (Fig. 2C). Principal veins over the visual operculum near the lunate sulcus, a region typically overlying V1 in the Macaque monkeys (orange arrows in Fig. 2D), were found to course roughly perpendicular to the lunate sulcus over the cortical surface. The typical distance between two principal veins was about 3 mm (Fig. 2C). This perpendicular orientation and spacing is typical of veins arising from the lunate sulcus (Chen et al., 2008; Lu et al., 2010; Ts'o et al., 1990).

We then examined the effect of taking slices oriented parallel and perpendicular to the lunate sulcus on pial vein content. Figure 2 illustrates our strategy. We selected a cortical slice in V1 between two principal veins five millimeters in length and 2 mm in width. The location of a slice (slice #1) parallel to the lunate sulcus is shown in Fig. 2D (blue rectangle, shown enlarged in the top panel of Fig. 2E). With an in-plane resolution of 200 μm, only 4 voxels appeared uncontaminated by pial veins (Fig. 2E, white squares). Choosing an

orthogonal slice orientation (slice #2, gray rectangle in Fig. 2D, shown enlarged in middle panel of Fig. 2E) resulted in a two-fold increase (to 40%) in vein-free voxels (Fig. 2E, middle panel, white squares). Further decreasing the slice thickness from 2 mm to 1 mm (slice #3, pink rectangle in Fig. 2D, shown enlarged in Fig. 2E bottom) led to a further 50% increase (to 64%) of vein-free voxels (Fig. 2E). Figure 2F summarizes the amount of vein coverage for all slices examined in two awake monkeys in which we did optical imaging. The mean vein-free coverage was 15%, 21%, and 40% in V1 of two monkeys for slice#1 type (110 locations), slice#2 type (104 locations), and slice#3 type (135 locations), respectively. For slices of slice #1 type, a large proportion of the slices was contaminated by large pial veins. 74% of these slices had vein coverage greater than 80%. Only 7% of slices had a vein-free coverage more than 30%, and no slice of this type had vein-free region more than 50%. In comparison, close to 40% of the slice #3 type slices had a vein-free coverage of more than 50%. In 22% of #3 type slices without principal veins, more than 60% had vein-free regions without large pial veins.

In summary, both slice orientation and thickness influence large pial vein content. Slices with thickness of 1 mm oriented perpendicular to the lunate sulcus are least affected by veins on the cortical surface. As in the macaque monkey the lunate sulcus runs roughly mediolaterally, the optimal slice orientation for minimizing pial vein content is roughly sagittal. Theoretically, if large principal veins can be avoided, vein-free coverage could be as high as 60%. However, large veins contaminate at least 30% of the cortical surface even in the best cases.

3.3. Image quality of high resolution fMRI in awake monkeys

We trained monkeys with a paradigm that achieves extremely low head motion. Briefly, as reward-related body motion is one of the major sources of motion artifacts in awake monkeys (Pfeuffer et al., 2007), increasing the duration between rewards can greatly reduce the body motion (Chen et al., 2012a). We trained monkeys to perform continuously for a few hours on a visual fixation task with 20 second intervals between fluid rewards (Fig. 3A). To evaluate the image quality, we then estimated the in-plane displacements of functional images using a rigid in-plane body registration algorithm. The index *In-plane Displacement* takes both directions of movement into consideration (Eq. 1). Figure 3B shows the displacements from sessions we collected at the optimal voxel size ($200 \times 200 \times 1000 \mu\text{m}^3$). The mean displacement was $51 \mu\text{m}$, or about a quarter of the in-plane voxel size. The displacements were less than $100 \mu\text{m}$ in more than 90% of volumes, and greater than $200 \mu\text{m}$ in only 2% of volumes. Similar results were obtained when the head motion was estimated with a 3D rigid alignment algorithm (Supplementary Fig. 2). The mean translations were $34 \mu\text{m}$ (along x-axis), $72 \mu\text{m}$ (along y-axis), and $76 \mu\text{m}$ (along z-axis), and the mean rotations were 0.069° (pitch), 0.018° (yaw), and 0.027° (roll).

Another way to evaluate the quality of functional imaging is to estimate the contrast-to-noise ratio (*CNR*) which is calculated by obtaining the difference between the activation with (red square in Fig. 3C) and without (green square) visual stimuli, divided by the standard deviation of activation without stimulation. The averaged BOLD signals from one session are plotted relative to the stimulus presentation period (yellow shading) in Fig. 3C. The mean *CNR* of functional data from seven sessions was 3.0 ± 1.1 , a value similar ($p = 0.23$, Student's *t* test) to the *CNR* of data acquired with a volume coil at an in-plane resolution of $1500 \times 1500 \mu\text{m}^2$ (Chen et al., 2012a).

Thus, our methods are accompanied by a negligible amount of motion artifact and high contrast-to-noise ratio of MR signals. This suggests the functional image quality acquired in trained monkeys at optimal voxel size in trained monkeys can be very good.

3.4. Layer-specific cortical activation in V1

Figure 4 presents high-resolution anatomical and functional images from awake monkey V1 with a GE sequence. Slices were 1-mm in thickness and were oriented perpendicular both to the surface of the cortex and the lunate sulcus (roughly sagittal). From the T_2^* -weighted anatomical image, the cortical gray matter and the location of the lunate sulcus (white arrow) are clearly visible (Fig. 4A). A darker laminar structure in the middle of the gray matter (marked by a green arrow) reflects the location of cortical layer IV, a layer marked by dense myelination (cf. Chen et al., 2012b). The red arrows indicate the region chosen for analysis, a relatively flat area with relatively constant cortical thickness.

Figure 4B shows the functional activation map acquired at in-plane resolution of $200 \times 200 \mu\text{m}^2$ in response to a 16 degree checkerboard stimulus. We observe no significant MR signal dropout on the surface of the cortex, suggesting an absence of principal veins within the field of view (cf. Figure 2 in Chen et al., 2012b). The areas in this image with the strongest activation are within the lunate sulcus (green arrows), reflecting the high density of large veins in the lunate sulcus. Besides the large activation at the surface, elevated activation in the middle of the gray matter can be seen (white arrow). In Fig. 4C, the average percentage change (average of 7 sessions acquired at the spatial resolution of $0.04 \mu\text{L}$) is plotted as a function of cortical depth. Approximate boundaries of superficial, middle, and deep cortical layers are indicated with vertical lines based on laminar MR microscopy methods (Chen et al., 2012b). Besides the large activation in the superficial layer, a second peak in the middle layers can be seen (black star).

To compare relative BOLD changes across different layers, we divided visual cortex into three activation regions of roughly equal size. The first region included only top layers (layers I–III, cortical depth 0 to $300 \mu\text{m}$). The second region contained the middle layers (layer IV, cortical depth 500 to $900 \mu\text{m}$). The third region contained the deep layers (layers V and VI, cortical depth 1100 to $1400 \mu\text{m}$). These three regions are indicated in red, green and blue, respectively, in Fig. 5A. We found the averaged activations to the checkerboard visual stimulation (across 7 sessions) within top, middle, and bottom zones were 0.9%, 0.8%, and 0.6%, respectively. The activations from top layers were not significantly greater than middle layers (Fig. 4C, $p = 0.07$).

Our pial vein pattern analysis above suggests that, in slices with roughly sagittal orientation and 1 mm thickness, pial veins will contaminate at least 30% of the voxels on the cortical surface. Large veins may have larger BOLD changes due to their greater volume (Yu et al., 2012). Because veins greater than $30 \mu\text{m}$ radius dive down through all six cellular layers (Duvernoy et al., 1981), the percentage of voxels within the gray matter at different cortical depths influenced by large intracortical draining veins may be similar to the pial-vein coverage. Therefore, we reasoned that at each cortical depth the voxels with large veins (approximately 30%) will have the strongest BOLD activations and be the dominant component in the top 30% of activated V1 voxels. Figure 5B, which plots the signal distribution of the top 30% of voxels, reveals that layers (I–III) contain the strongest BOLD responses, followed by moderate activation in the middle layers (IV), and the smallest signal changes in the deep layers (V and VI). BOLD signal changes in top layers were on average 33% and 81% greater than these in middle layers and deep layers, respectively; both differences were significant (both $p < 10^{-7}$). Thus, our results show, as expected, that the activations influenced by the large veins are the highest at the cortical surface.

As shown in our analysis above (Fig. 2F Slice #3 type), about 60% of the voxels are large vein-free among slices without principal veins. In other words, the bulk of influence from large veins will be eliminated by excluding the top 40% of activated voxels. In the plot in Fig. 5C, we excluded the top 40% and have plotted the activation distribution of the

remaining 60%. Accordingly, the removal of these voxels reduces the signal magnitude across all layers. However, this reduction is least in the middle layers, revealing an activation in middle layer that is significantly greater than top layers or deep layers (both $p < 10^{-7}$). On average, activations in middle layers were 30% and 32% greater than top layers and bottom layers, respectively.

In summary, we show that GE-BOLD signals were most contaminated by large veins at the cortical surface and in superficial layers. By excluding voxels potentially influenced by large veins, we are able to reveal the expected enhanced BOLD activation in layer IV in response to visual stimulation. Thus, the removal of contaminating influences from large pial veins leads to visualization of lamina-specific functional activation patterns in V1 of awake monkeys.

4. Discussion

In this study, we have shown that, by imaging at high magnetic field (4.7T) with a small (2 cm) surface coil, high spatial resolution BOLD imaging capable of revealing laminar specific functional activation can be achieved in awake monkeys. Below, we discuss the contributing factors, including optimizing voxel size while maintaining good *SNR* and *CNR*, reduction of animal motion, and removal of large vein contribution.

4.1. Optimizing voxel size

In functional MRI, voxel size is one of the most important variables to be considered. Voxel sizes which are too small will affect detection of activation, while those that are too large will reduce functional specificity. At the optimal voxel size, the highest temporal *SNR* can be achieved for the least loss in resolution. In human imaging, this optimal size occurs when the contribution of physiological noise equals thermal noise (Bodurka et al., 2007). However, due to differences in preparation, the values determined with human imaging data cannot be directly used to decide the optimal voxel size in behaving non-human primates. In awake monkeys, head immobilization (Gamlin et al., 2006; Logothetis et al., 1999; Pinsk et al., 2005; Vanduffel et al., 2001) and extensive training (Chen et al., 2012a) are required to minimize animal movement and decrease the contribution of physiological noise. A primary factor which differs from human studies is that fluid reward is used in almost all monkey training paradigms and causes additional noise due to mouth and body movements (Pfeuffer et al., 2007). These parameters must then be determined independently in the awake, behaving monkey.

In this study, in the awake monkey scanned at high field (4.7T) with a 2 cm surface coil, we examined the contributions from the physiological noise and thermal noise at voxel sizes ranging from 0.04 mm^3 to 4.5 mm^3 . We found that at a voxel size of 4.5 mm^3 , the *SNR* reaches values exceeding 1000, much higher than that previously reported in human subjects (Fig. 1A). The maximum attainable temporal *SNR* was around 90, close to that reported in human subjects (Kruger et al., 2001; Triantafyllou et al., 2005). These results are consistent with previous human fMRI studies showing that a linear increase in image *SNR* with voxel size is accompanied by a diminishing gain in temporal *SNR* (Kruger et al., 2001; Triantafyllou et al., 2005). The greatly improved anatomical *SNR* obtained here may be attributed to the higher magnetic field and smaller surface coils used in this study, and may also result from extensive behavioral training. As we show in Fig. 1B, the physiological noise is roughly four times larger than the thermal noise at a voxel size of 4.5 mm^3 . At a voxel size of 0.5 mm^3 , the physiological noise still exceeds thermal noise by a factor at least 2. Only when the voxel size is reduced to 0.04 mm^3 ($200 \times 200 \times 1000 \text{ } \mu\text{m}^3$) does the ratio of physiological and thermal noise approach 1. Therefore, using our 2-cm coil setup in awake monkeys in a 4.7T magnet, we find the optimal voxel size is 0.04 mm^3 . For comparison, the

optimal voxel size is 5.8 mm^3 with a 16-channel coil array in human subjects at 3T (Bodurka et al., 2007), and close to 0.5 mm^3 with a home-made coil array in anesthetized monkeys at 4.7T (Goense et al., 2010). Thus, the high magnetic field coupled with a small surface coil in well trained monkeys can result in high spatial resolution imaging with good *SNR*.

Due to hardware limitations, the thickness of slices used in this study was four times greater than the in-plane resolution ($1000 \text{ }\mu\text{m}$ vs. $200 \text{ }\mu\text{m}$). To minimize partial volume effects, we prescribed the functional slice from a relatively flat cortical area and oriented the slice orthogonal to the cortical surface. Two slices adjacent to the functional slice are shown in Supplementary Fig. 1. The mean displacements between cortical surface in the medial slice (Supplementary Fig. 1A) and the functional slice (Supplementary Fig. 1B) and between the lateral slice (Supplementary Fig. 1C) and the functional slice were $50 \text{ }\mu\text{m}$ and $45 \text{ }\mu\text{m}$, respectively. Both values were small compared to the in-plane resolution of $200 \text{ }\mu\text{m}$. Thus, with the slice thickness used in this study, the through-plane curvature does not significantly influence the location of the cortical surface (Supplementary Fig. 1D).

Selection of hardware also affects the activation contrast-to-noise ratio. Compared to our results (Chen et al., 2012a) measured with a volume coil alone, the *CNR* of the BOLD response reported here with voxel size of 0.04 mm^3 is about the same, which can be explained by combined effects from the greater sensitivity of the surface coil and the lower spatial resolution in previous study ($1500 \times 1500 \times 2000 \text{ }\mu\text{m}^3$). A study using the same 0.04 mm^3 voxel size reported a similar *CNR* in anesthetized and paralyzed monkeys (Smirnakis et al., 2007). This suggests that the motion related noise present in well trained monkeys contributes little to the overall noise, and, furthermore, that the small voxel size does not compromise our ability to detect changes in BOLD signals.

In the study, the ability to achieve high *SNR* and *CNR* permits us to achieve high spatial resolution and visualize laminar structure. In human high resolution fMRI studies (which are typically conducted at $700 \text{ }\mu\text{m}$ – 1 mm in plane resolution), there are less than 4 voxels representing the 2–3 mm depth of the gray matter (Koopmans et al., 2010; Polimeni et al., 2010; Ress et al., 2007). To infer detailed layer-specific information, interpolation techniques must be used to increase the nominal spatial resolution. However, such interpolation may introduce additional artifacts (Koopmans et al., 2010; Wald et al., 2006). In this study, we avoided such complications by collecting with higher resolution; all data analyses were based on raw data acquired without any spatial filtering or interpolation. With an in-plane voxel size of $200 \text{ }\mu\text{m}$, laminar activations were acquired with up to eight voxels across the 1.5 mm thickness of monkey V1. We show that this resolution was sufficient for revealing layer-specific activation without spatial interpolation.

4.2. Reduction of animal movement

Another major source of noise in high resolution fMRI in awake subjects is the motion artifact related to subtle head movement; this often causes blurring of layer-specific activation and a loss of data at edges. Motion correction may remove some of the large head movement related artifact. However, the potential problems related to spatial interpolation will be introduced due to re-sampling inherent in the image registration algorithm. To avoid these complications, we designed a customized head restraint system to control large head motion, and trained monkeys to keep their head still (Chen et al., 2012a). Results reported here were acquired after subjects received intensive training to adapt to MR environment and keep their head movement to a minimum during the MR scans. This resulted in displacements much smaller than the voxel size (Fig. 3B and Supplementary Fig. 2), rendering motion correction unnecessary. Therefore, compared to previous studies, the functional specificity of BOLD signal can be estimated more precisely.

4.3. Removal of large veins reveals high spatial specificity of BOLD

Prior to this study, it was unknown whether fMRI is sufficient to achieve submillimeter functional specificity in awake subjects. Previous studies have used hemodynamic point spread function (PSF), which represents the coupling between the neural activation and the GE-BOLD signal, as a measure of functional specificity. In two human studies, the PSF has been reported to be 2 mm (in-plane resolution of $1000 \times 1000 \mu\text{m}^2$ at 7T, (Shmuel et al., 2007)) and close to 4 mm (in-plane resolution of $2000 \times 2000 \mu\text{m}^2$ at 3T (Parkes et al., 2005)). Such estimates would make GE-BOLD incapable of detecting sub-millimeter functional structures. However, we believe these are likely to be overestimates of the true PSF.

As demonstrated by the pial vein pattern analysis in this study, we found that contamination related to pial veins becomes more serious with large voxels. For example, we found that large vein coverage is greater than 70% in almost all (over 90%) of slices collected with in-plane resolution of $1500 \times 1500 \mu\text{m}^2$. In comparison, with the voxel size used in our study, only 30% of slices collected have greater than 70% vein-coverage. Thus, in human PSF studies, even after spatially masking some voxels associated with large veins, PSF estimates are dominated by pial vein contribution, rather than functionally specific capillaries.

The voxel size used in this study ($200 \times 200 \times 1000 \mu\text{m}^3$) is, to our knowledge, the smallest size ever used in awake subject fMRI. Using an optimized slice orientation to minimize the influence from pial veins, the activations in the top layer were only slightly higher (~10%) than those in the middle layer (Fig. 4). To achieve further specificity, we used pial vein pattern analysis to divide the fMRI signals into two components and remove the component dominated by the draining veins, leaving the largely vein-influence free component. Consistent with previous studies (Goense et al., 2007; Smirnakis et al., 2007; Zhao et al., 2006), the vein dominated component (Fig. 5B) is strongest at the pial surface and decreases monotonically with cortical depth. In contrast, the vein-free component (Fig. 5C) exhibited clear laminar specificity, with the highest activation in layer IV.

Partial volume effects can also reduce the mean intensity value of voxels located close to the edge of the brain. As a consequence, the estimates of superficial activation can be artificially inflated. One method to compensate for this bias is to calculate the relative BOLD signal change with the mean intensity of the voxel taken into consideration (Koopmans et al., 2010). Supplementary Fig. 3 shows depth profiles calculated with this method. The results were z-normalized to account for inter-session differences in the cortical intensity. We found the activation from top layers were not significantly greater than middle layers (Supplementary Fig. 3A, $p = 0.30$). After the influence from the large veins were removed (Supplementary Fig. 3C), BOLD signal changes in middle layers were significantly greater than these in top layers ($p < 0.001$) and deep layers ($p = 0.005$). Interestingly, when only the top 30% of activated V1 voxels were used for analysis, the difference was not significant ($p = 0.78$) between BOLD responses in top layers and those in middle layers (Supplementary Fig. 3B). This indicates that some of these strong activations from superficial cortical regions may be artifacts related to the partial volume effect. Large voxel size may further exaggerate this type of artificial inflation.

These findings suggest that the failure of most previous studies to visualize functional laminar activation in V1 may be due to the combined partial volume effect related to large voxel size and effects from large draining veins on the cortical surface and within the gray matter. As our study is able to identify laminar specific activation, it suggests that the functional spatial specificity of GE-BOLD signal is better than 0.5 mm.

In summary, we have demonstrated that the spatial specificity of GE-BOLD has a spatial resolution of less than half a millimeter. This is significantly higher than that attained in previous studies and close to results acquired with contrast agents in anesthetized animals (Smirnakis et al., 2007; Zhao et al., 2006). This improves the potential for GE-BOLD based fMRI for detecting functional activation at the sub-millimeter scale in awake subjects, a capability that has useful application for studying *in vivo* laminar activation in normal subjects and in subjects with cortical pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

High resolution fMRI in awake monkeys with in-plane resolution of $200 \times 200 \mu\text{m}^2$
Contaminating vein influence minimized with pial vein analysis from optical imaging.
Sub-millimeter functional laminar specific activation is revealed by GE-BOLD signals.

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Figure 1. Optimal voxel size in awake monkeys

(A) Temporal *SNR* as a function of image *SNR*. Data collected at different voxel sizes in awake monkeys: 0.04 mm³ (red), 0.5 mm³ (green), and 4.5 mm³ (blue). Each point represents data from one significantly activated voxel in the log-log plot. As seen by the blue dots, the Image *SNR* improvement does not greatly benefit the temporal *SNR* at large voxel size. The dashed line shows the line of identity (temporal *SNR* = image *SNR*). (Inset) Image *SNR* increases linearly with the voxel size. (B) Ratio of physiological to thermal noise as a function of voxel size. Physiological noise is dominant in data collected with large voxel size. The optimal voxel size is the smallest volume size (200×200×1000 μm³) we tested. MR signals collected in awake monkeys at this spatial resolution have balanced contributions from the physiological and thermal noise (ratio close to 1).

Figure 2. Large pial vein coverage is slice orientation and thickness dependent

Cortical blood vessel maps from the primary visual cortex were taken under 570 nm (A) or 630 nm (B) illumination. (C) Cortical pial vessel pattern. Arteries are red, large veins are black (radius > 30 μ m), and small veins are gray. Most large pial veins course over V1 perpendicular to the lunate sulcus. (D) Location of three slices: two of them are perpendicular to the lunate sulcus (slice #3, pink rectangle, 1 mm thick slice) and (slice #2, gray rectangle, 2 mm thick slice), and the third one is parallel to the lunate sulcus (slice #1, blue rectangle, 2 mm thick slice). The surface vessel patterns within these three slices are shown enlarged in (E). At the bottom of each panel are shown voxels (200 μ m in-plane resolution) with (green) and without (white) large surface vein content. The percentages of pial vein containing voxels are 84%, 60%, and 36% for slice #1 ($n = 110$), #2 ($n = 104$), and #3 ($n = 135$), respectively. (F) In the three types of slices, the distribution of number of slices with different pial vein content. Slices perpendicular to the lunate sulcus with 1 mm in thickness (Slice #3 type) have the smallest percentage of venous voxel.

Figure 3. High spatial resolution fMRI images acquired in trained awake monkeys are high in contrast-to-noise ratio and low in motion artifacts

(A) The stimulus paradigm used in awake monkey fMRI experiments. Monkeys were trained to maintain fixation on a small square. Stimulations were divided into blocks with duration of 20 seconds each. The stimulation was either a flashing full field (16 deg) checkerboard or background with mean luminance. Animals received a drop of fluid reward at the end of each block (marked by arrows). (B) Effect of animal motion on image quality is small. Functional images were collected with 1-mm thick slices (200 μm in-plane resolution). The motion artifacts are estimated by in-plane rigid alignment. (C) Average time course over all activated voxels in a session ($p < 0.01$, clustered). Yellow shaded area: stimulus presentation period. Error bars: standard deviation. Red and green dots: time point of activations used in contrast-to-noise calculation as conditions of with and without stimulation, respectively.

Figure 4. Signal modulation as a function of cortical depth in awake monkeys revealed by high-resolution fMRI

(A) T_2^* weighted anatomical image. Green arrow: location of layer IV. Red arrows: mark extent of cortical area used for depth-specific signal analysis. (B) BOLD activation map collected in the same session in response to a flashing checkerboard obtained at $200 \times 200 \mu\text{m}^2$ in-plane resolution in an awake monkey. The slice is 1 mm in thickness and is oriented perpendicular both to the surface of the cortex and the lunate sulcus (roughly sagittal). Color scale: intensity of signal change. The large signal changes are located within the lunate sulcus and cortical surface associated with large veins (green arrows). The layer-specific activation of layer IV is indicated by a white arrow. (C) The average percentage change of

seven sessions from two monkeys as a function of cortical depth. The maximal activation is located on the cortical surface. Top layers are not significantly more activated than middle layers ($p = 0.07$). Vertical lines: approximate boundaries of superficial, middle, and deep cortical layers. Yellow shading: 95% confidence level. Scale bar: 5 mm.

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Figure 5. Layer IV is the most activated cortical layers after removed the influence from large veins

(A) Top (red, 0–300 μm in depth, layers I–III), middle (green, 500–900 μm in depth, layer IV), and deep (blue, 1100–1500 μm in depth, layers V–VI) cortical layers overlaid on anatomical image. The average percentage change (B) of the top 30% of voxels as a function of cortical depth. The top layers are significantly more activated than the middle layer and the bottom layers (both $p < 10^{-7}$). After the removal of venous voxels (top 40% of voxels), the average percentage change is plotted against the function of cortical depth in (C). The middle layers (layer IV) are significantly more activated than the top layer and the bottom layers (both $p < 10^{-7}$). Scale bar: 5 mm.