ONLINE METHODS

All surgical and experimental procedures were approved by the Vanderbilt Animal Care and Use Committees and conformed to the guidelines of the National Institutes of Health.

Animal preparation

The subjects in this experiment were two adult rhesus monkeys (*Macaca mulatta*, 5 and 8 kg, respectively). Prior to the imaging, under sterile surgical conditions, each monkey was anesthetized and implanted with a headpost and a chronic nylon imaging chamber overlying dorsal V4. Native dura in the chamber was replaced with a clear artificial dura (Tecoflex, Thermedics Polymer Products). The chamber was sealed with a nylon cap and opened under sterile conditions for image acquisition. The chamber was located on the right hemisphere of one monkey (cases 1 and 2) and on the left hemisphere of another (cases 3 and 4). Retinotopic mapping experiments were performed using a spot or bar stimulus beforehand to examine whether our imaging regions were surely located in V4. In all cases, stimulus closer to the vertical meridian activated regions closer to the lunate sulcus, consistent with known retinotopy in V4[15]. The procedures for surgery, anesthesia, and maintenance of chamber were previously described in detail[14,45].

Awake optical imaging

Monkeys were trained for juice reward to sit calmly with their heads fixed and to fixate a spot (0.15°) on a CRT monitor (fixation window radius, < 0.75°). Eye position was monitored with an infrared eye tracker (RK-801, ISCAN, or iView X, SensoMotoric Instruments). Monkeys were required to maintain fixation throughout each image acquisition

(4 s). The detailed imaging methods have been described previously[14,45]. Under 632-nm illumination, images of light reflectance from V4 cortex were captured (4 s/trial) through a CCD video camera (504 × 504 pixels, 8 × 8 mm; 1M60P, Dalsa) with a tandem lens system focused on the cortical surface, and digitised by Imager 3001 (12-bit resolution, 4 frames per second; Optical Imaging). Image acquisition included a 0.5 s prestimulus period, followed by 3.5 s stimulus presentation period. Each experiment contained 4–16 stimulus conditions and one blank condition (no stimulus except fixation point); each condition was repeated 40-100 times, pseudorandomly interleaved with at least a 1.5 s inter-trial interval. This means that the inter-stimulus interval was 2.0 s, including 0.5 s prestimulus period (see **Supplementary Note** online for the validity of this inter-trial interval). Only trials with successful fixation throughout were further analyzed. The success rate was 88.5 ± 3.7% (mean ± SD; n = 7 imaging sessions).

Visual stimuli

Visual stimuli were created using VSG 2/5 or ViSaGe (Cambridge Research Systems) and presented on the CRT monitor which was gamma corrected by using a photometer (Minolta Chroma Meter CS-100, Ramsey) and positioned 118 cm or 140 cm from the eyes. To examine color, luminance, and orientation preference in V4, we presented a circular patch filled with isoluminant red/green (RG) or luminance-contrast (100%) black/white (Lum) drifting sinusoidal gratings (1–2 cycles/degree, 0.5-1 degree/s drift rate, and two orthogonal orientations) on a uniform gray background. Drifting direction was randomized on every trial. Gratings had an average luminance of 26.8 cd/m^2, identical to the background luminance.

To study color preference, we used patches of color/black drifting square-wave gratings with 100% luminance contrast (2 cycles/degree spatial frequency, 1 degree/s drift rate, and two orthogonal orientations). We used six different hues and one white with CIE-*xy*

chromaticity coordinates (0.63, 0.34; red, R), (0.39, 0.53; yellow, Y), (0.28, 0.61; green, G), (0.22, 0.35; cyan, C), (0.15, 0.07; blue, B), (0.31, 0.16; magenta, M), and (0.30, 0.33; white, W) (**Fig. 6a**). All colors including white had the same luminance (11.5 cd/m^2) and the average luminance of gratings was the same as the background luminance (5.75 cd/m^2). In all cases, the size and location of patches were optimized to stimulate the imaged region. In Case 1, 2, 3, and 4, the size was 1°, 4°, 1.5°, and 4° in diameter and the center was located at 0.75°, 6°, 1.5°, and 4° eccentricity, respectively, unless otherwise specified.

Data analysis

Images in trials with successful fixation throughout were used. Image frames were analyzed offline using custom software (MATLAB, Mathworks). All images were first corrected for any brain movements (induced by animal movements), by aligning each frame to the first frame using blood vessels as landmarks. For each trial, frames between 1 s and 3.5 s after the stimulus onset were averaged, and then subtracted and divided by the frame 0.5 s before the onset on a pixel-by-pixel basis to obtain maps of reflectance change ($\Delta R/R$ map). To remove non-stimulus-specific global signal changes and reveal local modulation, each $\Delta R/R$ map was convolved with a 1.6 × 1.6 mm median filter and subtracted from the original maps (high-pass filtering, see **Supplementary Fig. 5**). Finally, all filtered $\Delta R/R$ maps obtained in one stimulus condition were averaged to form the single condition map. Difference maps between two stimulus conditions were obtained by calculating the average difference of filtered $\Delta R/R$ maps between the conditions.

We used a two-tailed *t*-test for a comparison of the response between two stimulus conditions and a one-way ANOVA to evaluate a response modulation caused by the difference in one feature dimension (orientation or hue). The *P* value was calculated at each

pixel in filtered $\Delta R/R$ maps by either *t*-test or ANOVA (*P*-value map), uncorrected for multiple comparisons. In the *P*-value maps, only regions which consisted of pixels with $P < 0.05$ and peak $P < 10^{-4}$ were regarded as regions of significant modulation and regions which did not meet this criteria were excluded. We did not apply any correction for multiple comparisons to our *P* values in the maps. When the number of statistical comparisons conducted increases, the chance of a false-positive result also increases[46]. To estimate the likelihood of false-positive modulation expected by chance in our imaging method, we compared two sets of filtered $\Delta R/R$ maps obtained during odd and even numbered trials in the blank condition by a two-tailed *t*-test. In all cases, no significant modulation was revealed by this comparison, indicating that it is unlikely the observed significant modulations occurred by chance. Color-coded angle and polar maps of orientation-preference and hue-preference were created by pixel-wise vectorial summation of single condition maps of 4 different orientations or 6 different hues, respectively[47]. To remove high-spatial-frequency noise from the *P*-value, angle, and polar maps, each $\Delta R/R$ map was smoothed prior to analysis by using a 240×240 μm mean filter.

Signals from pixels on and near large vessels were less reliable because of large trial-by-trial fluctuation[48], something that occurred even without visual stimulation. To exclude these regions from the analysis, we calculated pixel-wise SD of blank condition images across trials. Pixels with large SD (> the upper limit of 95% one-sided confidence interval based on the χ^2 distribution) were eliminated from further analysis (shaded in gray in the *P*-value and polar maps). To measure the size of orientation-selective and hue-selective activation regions, single condition maps were compared with the average "cocktail blank" response to all examined orientations and hues, respectively, by using two-tailed *t*-test. Regions which consisted of pixels with $P < 0.05$ and peak $P < 0.0001$ were regarded as

stimulus-specific local activations. The diameter of each activation region was measured by averaging its length (the diameter along the long axis) and width (the diameter along the short axis) with ImageJ (National Institutes of Health).

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