## SUPPLEMENTARY INFORMATION

### 1. Anatomical spacing of penetrating arterioles.

In rodent neocortex, the density of penetrating arterioles has been estimated to be anywhere between 4 and 13 per $mm^2$ (refs.[15,20,38]) and a similarly wide range has been reported in macaque neocortex as well[39,40]. In order to determine the spacing of arteries in our animals, we obtained large-scale z-stacks of the vasculature spanning 5.25 and 4.24 $mm^2$ per craniotomy in two cats. We used sequential *in vivo* two-photon imaging of 800 × 800 µm regions. These image stacks were then stitched together and the blood vessels in the large-scale stacks were traced using Neurolucida software (MBF Bioscience). We found that penetrating arteriole density was approximately 8–12 arterioles per $mm^2$, similar to what one report found in cats[41] (15.8 ± 1.2 arteries per $mm^2$). This density translates into an inter-arteriole spacing of approximately 300 µm and therefore a neural response window with 200 µm radius (400 µm diameter) around each vessel would provide adequate coverage, as we show in Figs. 2b,c and 3b. Our analysis also included other window sizes (100–600 µm diameter, see Figs. 2f and 3e).

### 2. Spatial scale of neurovascular coupling.

a. Techniques. Whether vascular signals correlate better with synaptic or spiking activity and the range of cortical tissue over which these signals are correlated are controversial[3-7,42-44]. Differences among past studies were in part likely due to the difficulty of measuring synaptic, spiking and hemodynamic signals over equivalent spatial scales. The spatial range over which neural activity may be pooled from extracellular electrophysiological recordings depends on a number of factors including the physical properties of the electrode as well as the type of neural activity being recorded. Well-isolated single-units (action potentials from different neurons) can typically be obtained from only a few cells per electrode and are heavily biased toward the largest and most active neurons near the electrode tip[45]. Multi-unit spiking activity can be obtained from up to a few thousand neurons over a range from tens to several hundred microns, and LFPs pool low-frequency electrical activity over a range from a few hundred microns to several millimeters[8,46,47]. Furthermore, coarse-resolution hemodynamic imaging techniques, like fMRI, typically pool over even larger regions, making direct correlations between spiking, synaptic, and vascular activity difficult. An additional complication in these studies is that, although LFPs are used as a surrogate measurement of the synaptic activity in a region, it is still

not known exactly how different features of neural activity contribute to the LFP[46,48]. By imaging all of the neurons over a field of view along with individual vessels in the same region and by having a direct measure of synaptic activity we overcome these limitations.

b. Results. Typically, studies using low-resolution hemodynamic imaging and extracellular electrophysiology have found that hemodynamic signals spread well beyond the region of neural activation with point spread functions (PSFs) generally > 1 mm[4,49-51], although some studies have found conditions in which the spatial scales more closely match[24,52,53]. These studies correlate neural and vascular signals over spatial scales much larger in size (i.e., 500 µm to a couple of millimeters) than the regions we integrated over. Although we do not directly measure the complete spatial extent of neural and vascular signals, our finding of vascular responses in tissue where there is little to no concomitant neural activity indicates that vascular signals spread beyond the region of neural activity. However, despite the widespread range of vascular activity, hemodynamic signals have been used to discriminate functional columns in cortex with spacing < 1 mm including orientation columns[27,28,54-59]. Our results are consistent with these earlier studies because the preferred orientation of neighboring arteries differs according to the preference of the neural tissue around each vessel. Thus, by simply looking at the preferred orientation of the dilation signal, orientation maps could be extracted from our data (see Figure 2b,c).

3. **Glutamate signaling driving vascular responses.**

We show that robust sensory-evoked vascular responses can often occur without a corresponding glutamate signal in the surrounding tissue, e.g., Extended Data Fig. 2. Previous studies using pharmacological manipulations and low-resolution hemodynamic imaging in the neocortex have typically found that blocking post-synaptic glutamate receptors attenuates vascular responses[60-62], leading to the conclusion that local synaptic activity drives vascular responses (but see ref. [[63]]). It is difficult to separate the effects of synaptic and spiking activity with a pharmacological approach because widespread application of glutamate receptor antagonists may also eliminate spiking activity across a large population of neurons. Note that because pharmacological agents are typically applied over very large areas of tissue, the remote activity generating the propagating dilation signal (as we propose) would be shut down. Therefore the localized uncoupling of neural and vascular activity that we observed may be

missed with a pharmacological approach. Indeed, an elegant two-photon imaging study in the olfactory bulb showed that a highly localized block of glutamate receptors did not affect blood flow but a widespread application of glutamate receptor antagonists reduced or eliminated it[64]. Note also that our finding of glutamate signals being less orientation selective than calcium signals is consistent with intracellular electrophysiological responses, e.g., excitatory postsynaptic inputs show less orientation selectivity than neuronal spiking[65,66].

**4. Origin of the selectivity of parenchymal vessel dilation to stimulus orientation.**

The main findings of our paper regarding the parenchymal vasculature relate to the selectivity of responses in individual blood vessels along with the spatial regions of neural activity where selectivity may be matched. We assay two aspects of response selectivity in vessels and neural regions—the preferred stimulus and the degree of tuning to a complete set of oriented stimuli. The matching of neural and vessel responses to the best stimulus reaffirms the basic principle of functional hyperemia—a blood flow increase is highest in the most active region of neural tissue. Finding a mismatch in the degree of orientation tuning between responses in vessels and the surrounding neural tissue was a complete surprise. This new information will be critical for the interpretation of data on response selectivity in all vascular-based imaging approaches such as intrinsic signal optical imaging, two-photon imaging and fMRI. Deciphering the exact origin of the less selective vascular signal could take many years to resolve and is beyond the scope of our study. However, below we present an analysis of our data that supports one possibility along with some alternatives that may be considered in future work.

a. Long-rage propagation of dilation from one cortical column into neighboring columns. We propose that dilation signals in the absence of local neural activity are generated through long range propagation through the vascular wall[23,25,51]. This propagation has been estimated to travel along the vasculature at a speed of 2.4 mm/second[25]. Given that individual iso-orientation domains have widths in the range of several hundred microns[2,67], the dilation could propagate over a region of cortex that represents the entire span of orientations (a hypercolumn) within one second. Even though our imaging rates were <1 Hz for the majority of the data, we were able to detect differences in dilation onset latencies that were consistent with this hypothesis of a long-range propagated signal. According to our prediction, the local neural activity should first drive dilation in nearby arterioles which then propagates up to the surface[23]. Indeed we found that the

onset latency was faster for parenchymal vessels (mean ± s.e.m. 1.63 ± 0.15 seconds; $n = 79$) than for pial vessels (2.12 ± 0.17 seconds; $n = 24$; $P < 0.05$, $t$-test; normal distributions confirmed with Lilliefors $P > 0.1$). Note that although we have a clear relative latency difference between the pial and the parenchymal vessels, because of the slow data acquisition rates used, the absolute numbers of the two groups may not reflect what would be obtained with faster sampling rates[12,23,25,68]. Our hypothesis also predicts that the broader vessel tuning relative to neural activity comes from the dilation signal initiated in vessels from different orientation columns into the vessel we are measuring. Thus, in parenchymal vessels, dilation to the preferred stimulus should appear earlier than dilation to the non-preferred stimulus. We computed the standardized mean difference (Hedge's *g*, see Methods) in dilation latency between the preferred and non-preferred orientations for each parenchymal vessel and found faster onset for the preferred orientation ($g = 0.15$; $P < 0.005$; Extended Data Fig. 5a). As a control, we randomized the assignment of the preferred and non-preferred orientations trial-by-trial across the population and found no latency difference ($g = 0.01$; $P = 0.78$; Extended Data Fig. 5b).

b. Long-range release of vasodilators. Another potential explanation for the vascular responses without corresponding neural activity is that blood vessels may be driven directly by neural activity from beyond the regions (300-μm-radius windows) we measured. However, it seems unlikely that vasodilating factors such as nitric oxide can diffuse over such long distances (> 300 μm) quickly enough to drive rapid stimulus-evoked dilation[69]. Additionally, because this distance substantially exceeds the inter-vessel spacing, it seems unlikely that a given penetrating arteriole would need to supply increased flow to such a large area of neural tissue and therefore should not be directly sensitive to distal neural activity. Long-range neuromodulatory inputs from extrastriate regions[6] and/or deep brain structures[5,70] have been proposed as the explanation of a large scale decoupling that has been found in awake behaving subjects. However, because our experiments were performed under anesthesia, the fine-scale spatial decoupling we observed here (e.g., Extended Data Fig. 2) is unlikely to originate from these neuromodulatory inputs.

c. Cortical laminar-dependent selectivity of neural responses. Compared to our primary measurements in cortical layer 2/3, it is possible that neural activity in other layers, e.g., layer 4, is broader (less tuned for stimulus orientation) and represents the main driver of vascular signals. These relatively weakly-tuned neural responses drive vessel dilation locally which might then propagate retrogradely over relatively short distances into layer 2/3, where we detect them (but

see below). This short-range retrograde propagation is distinct from the long-range propagation into neighboring columns that we propose in section 4a.

To examine the role of layer 1 activity in broadening the vascular response, we measured neuronal spiking and excitatory synaptic activity in layer 1 of cat visual cortex. Layer 1 in cat visual cortex has very few neuronal cell bodies but across three imaged regions in two cats, we obtained OSI values of spiking activity from 8 significantly responding neurons (Extended Data Fig. 10a) and found a mean value of 0.74. This layer 1 OSI of neuronal spiking activity was very similar to what we found in layer 2/3 neurons (see Fig. 2a). We also measured 400-µm-diameter windows of glutamate release in layer 1 of three cats and found a mean OSI of 0.38 (Extended Data Fig. 10b). This layer 1 OSI of synaptic activity was very similar to our layer 2/3 glutamate selectivity, but nearly twice as selective as the parenchymal blood vessel dilation (0.21). Thus layer 1 synaptic or spiking activity cannot be contributing the orientation selectivity of parenchymal dilation we measured in layer 2/3.

While existing techniques do not allow imaging the activity of the deeper layers (the layer 4 border is ~1 mm from the pial surface in cats), we think it is unlikely that neural activity in layers 4–6 contribute to the relatively broad orientation selectivity of hemodynamic responses in cat layer 2/3 blood vessels, for the following reasons:

*Spiking*. Almost all neurons in cat layer 4–6 have the same orientation selectivity as layer 2/3 neurons when measured with electrophysiological single-unit recording techniques[71-73].

*Synaptic*. Due to the uniformity of orientation selectivity of spiking activity across lamina[71-73] and the tendency of long-range connections to target similar orientation domains[74-76], the most likely cortical layer where synaptic activity could be untuned is where most of the thalamic inputs terminate in cat visual cortex, i.e., layer 4. Individual LGN neurons and their axonal inputs are not orientation selective[77,78]. However, even though these LGN inputs in cat visual cortex are strong with excitatory postsynaptic potentials (EPSPs) twice the size of unitary EPSPs evoked from other cortical neurons[79], these LGN inputs only account for 6% of the glutamate synapses in layer 4[80]. Silencing all recurrent intracortical connections while sparing thalamic input activity eliminates most of the visually-evoked synaptic response[81] and the global hemodynamic response[82,83]. Finally, there are theoretical models[84,85] that support a dominant role for intracortical connections in shaping the overall response and selectivity in cortical layer 4 neurons.

d. Inhibition. Another possible explanation for the mismatch between the selectivity in glutamate and vascular signals is that a specific subclass of neurons broadens the vascular response. In particular, certain types of inhibitory neurons have been shown to modulate arteriole diameter[86,87] and blood flow[60,88-90]. However, inhibitory neurons in layer 2/3 of the cat visual cortex are comparable to excitatory neurons in orientation selectivity[91]. Moreover, hemodynamic effects linked to inhibitory neurons are very slow, requiring up to dozens of seconds before arteriole diameter changes can be detected[86]. Ultimately, the development of fluorescent GABA sensors and the subsequent elucidation of spatial maps of GABAergic responses may resolve the specific role of GABA release in driving sensory-evoked hemodynamic responses. If combined with our iGluSnFR imaging of glutamate release, GABA imaging will provide more complete maps of synaptic transmission.

e. Astrocytes. Although astrocytes have been implicated in neurovascular coupling[16,92], we have not included them in our analysis. We examined the neural events that lead to vascular signals rather than the cellular and molecular mechanisms that transfer the neural signal to the vasculature. Thus whatever role astrocytes may play, it likely is downstream of the spiking and synaptic activity that initially generates the hemodynamic response. However, astrocytes have been shown to have sharp orientation selectivity[93] and so they might be artificially increasing the selectivity of the calcium windows. We rule out this possibility for the following reasons. Firstly, astrocytes respond very slowly with onsets greater than 5 seconds[93], which is near the end of our typical neural response window. Secondly, although astrocytes are labeled with OGB, the GCaMP6 AAV serotype and promoter we used exclusively labels neurons and the selectivity of OGB labeled regions (OSI mean ± s.e.m. = 0.5898 ± 0.032, $n$ = 12) was identical to that of the GCaMP6 labeled regions (0.5912 ± 0.034, $n$ = 7; $P$ = 0.95; Mann-Whitney test).


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