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Visual Cortex: Two-Photon Excitement

Current *in vivo* methods for imaging the visual cortex lack the ability to map response properties at the level of single cells. A new technique using two-photon imaging of calcium signals has now overcome this limitation.

Mark Hübener and Tobias Bonhoeffer

For many decades, by far the most important tool for visual neurophysiologists has been the microelectrode. When such a microelectrode was advanced tangentially through the visual cortex of a higher mammal, such as a cat or monkey, neighboring cells were often found to have very similar response properties. Visual field position, preferred stimulus orientation and direction, ocular dominance and other receptive field parameters were found to change slowly and gradually, indicating that the visual cortex contains smooth functional maps for these properties. Occasionally, however, sudden jumps for certain stimulus features were observed along the microelectrode penetrations [1], indicative of discontinuities in the feature maps. But microelectrode recordings alone are not well suited for elucidating the detailed structure of such maps, particularly in regions with borders or irregularities.

This situation changed when optical imaging techniques were first applied to the visual cortex some 20 years ago [2–4]. Intrinsic signal imaging — today the most popular of the cortical imaging techniques — exploits changes in light reflectance of activated cortical tissue that are mainly due to altered blood oxygenation levels. This technique has made it possible to image the detailed layout of cortical maps as well as the spatial relationships between different maps coexisting in the visual cortex [4–7]. And indeed, these studies showed that cortical feature maps are not always continuous: orientation preference varies smoothly across the cortical surface for the most part, but in some locations regions preferring all different orientations meet, forming a singularity now commonly referred to as a ‘pinwheel center’ [4]. Similarly, largely continuous maps for the preferred direction of stimulus motion contain ‘fractures’, with neurons located on either side of the fracture responding best to movement in exactly opposite directions [5,6] (Figure 1).

While these studies have considerably expanded our knowledge on the functional architecture of the visual cortex, they also have their substantial limitations. When it comes to unraveling the fine structure of singularities or borders in these maps, optical imaging of intrinsic signals fails: its spatial resolution is just not good enough to resolve the response properties of single neurons. The combination of imaging with electrode recordings from single cells has been employed to elucidate the fine structure of these regions in the visual cortex [5,8], but, the insights from these studies notwithstanding, this approach, too, could not reveal the precise anatomical location of the recorded neurons. What was needed was a technique that has been the dream of neurophysiologists all along: a technique that allows the simultaneous recording of the functional properties of many neurons together with their precise spatial localization in the visual cortex.

A major step in this direction has just been made: Clay Reid’s group [9] at Harvard Medical School has managed to record simultaneously the activity of hundreds of neurons in the visual cortex. For each of those neurons, Ohki *et al*. [9] could determine its preferred visual stimulus, and importantly, also its exact three-dimensional location in the visual cortex (Figure 2). They used a technique whose principle had been developed by Stosiek *et al.* [10]: a membrane-permeable calcium indicator dye, in this case Oregon Green 488 BAPTA-1 AM, is injected into the visual cortex, where it is taken up by the neurons. After crossing the membrane, the AM tail is cleaved by unspecific esterases, and thus the dye gets trapped inside cells. Two-photon microscopy can then be used to visualize the brightly

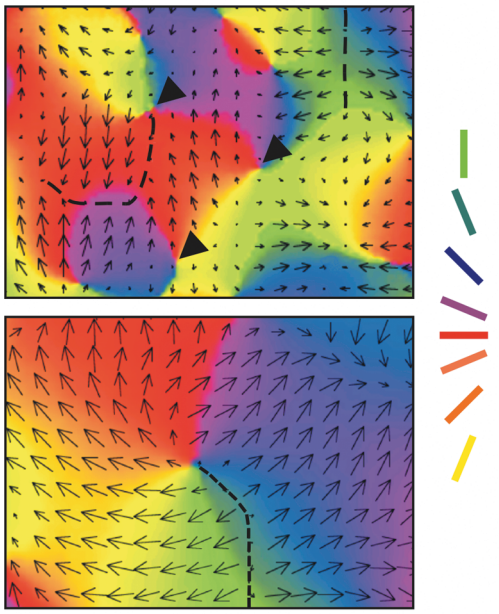

Figure 1. Orientation and direction maps from ferret visual cortex obtained by optical imaging of intrinsic signals.

The top panel shows an orientation preference map, with color coding for the preferred orientation of a visual stimulus, according to the legend at the right. Overlaid is a direction preference map, in which the arrows indicate the preferred direction of motion, and the length of an arrow codes for the magnitude of that preference. Note pinwheel centers in the orientation map (arrowheads), and fractures in the direction map (dashed lines). The bottom panel depicts a pinwheel center and a direction fracture (dashed line) at higher magnification. (Reproduced with permission from [6].)

labeled somata of individual neurons *in vivo*. This provides the locations of the neurons in the cortex, but not only that: upon activation by a visual stimulus, intracellular calcium levels rise, and the indicator dye fluoresces brighter. As the changes in calcium concentration are relatively slow, it is tricky to extract the number and timing of action potentials from these signals; however, the change in fluorescence serves as a good indicator of the cells' overall activity levels.

In initial experiments Ohki *et al.* [9] used this method to study orientation preference in rat visual cortex (Figure 2). While they found many cells with robust orientation tuning, there was no local order for this property, which is in agreement with previous rodent studies. Having demonstrated the principle feasibility of the new technique, Ohki *et al*. [9] turned their attention to direction maps in cat visual cortex. As expected, calcium imaging revealed large regions where the preferred direction of stimulus motion changed smoothly from neuron to neuron. They also found the sudden changes in direction preference that had already been reported in intrinsic imaging studies. The new method, however, provided much finer detail. The analysis of the response properties of individual neurons in these fracture zones revealed that the border between regions responding to opposite directions of motion is surprisingly sharp: in many instances preferred direction of motion was found to change by 180° over a distance of just 20–30 μm. At the same time, however, the degree of the neurons' preference for one movement direction over the other decreases towards the border. While a cell close to the border might, for example, respond best to upward movement, it is also — only more weakly — activated by downward movement. In contrast, a cell further away from the border responds strongly to upward, but not at all to downward motion.

The presence of sharp borders between neighboring regions with entirely different visual response characteristics indicates a high degree of specificity for the underlying cortical circuitry. While it is not completely clear how direction selectivity in the visual cortex is brought about, there is good evidence that intracortical excitatory and inhibitory connections contribute to its generation [11]. The fact that such abrupt changes of direction selectivity have now been observed suggests that intracortical connections have to be extremely precise. The degree of specificity required is even more astonishing when taking into account that the dendritic trees of most neurons in the visual cortex span several hundreds of micrometers in the horizontal plane.

So will we soon see the complete visual cortex charted at the level of individual cells? Probably not. Powerful as it is, this new technique also has its shortcomings, which need to be dealt with before making it a routine method. Dye loading is somewhat capricious, and for completely unknown reasons labeling of cells works quite reliably in young animals, but almost not at all in older ones. While there is good evidence from intrinsic imaging studies that functional maps in the visual cortex, once established, change only very little over time [12], this issue is exactly one of those, which we would like to see readdressed with single cell resolution. Similarly, studies on the plasticity of neurons in the visual cortex after modifying the visual input require that imaging can be carried on in older animals. Another obstacle is that the maximum depth at which signals from single cells can be picked up is about 400 μm. Thus, currently it is not possible to record from neurons in cortical layer 4, which is of particular interest, as it is the input layer of the cortex. Therefore while a tremendous advance, the approach described by Stosiek and Okhi and their co-workers [9,10] is also no magic bullet and it comes with a number of difficulties which still need to be solved.

For some of these problems solutions are on the horizon. In fact, what has been seen as the the major step forward, figuring out a way to load cells in the intact brain with a calcium sensitive dye, could be obsolete not before too long: genetically encoded calcium sensors finally seem to be working in mammals,

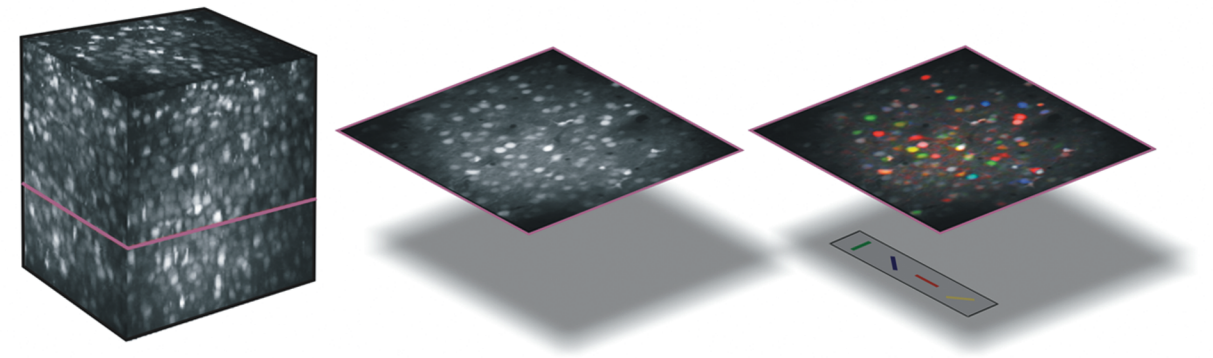

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Figure 2. Functional two-photon imaging with cellular resolution in the visual cortex.

The left panel shows an approximlately 300×300×300 μm^3 cube of tissue from a rat's visual cortex, containing more than 3000 neurons labeled with the calcium indicator dye. About 100 neurons can be identified in a single optical section (middle). The same section is shown again on the right, with the colors now indicating the neurons' preferred stimulus orientation. In rat visual cortex, neurons are randomly intermingled with regard to their orientation preference. (Reproduced with permission from [9].)

too [13]. And while these also still have their pitfalls, it seems safe to predict that important characteristics such as their dynamic range and quantum yield will improve in the future. In animals that are not directly amenable to transgenic techniques, like cats or monkeys, virus-mediated transfection could be an alternative [14]. Imaging of neurons deeper in the cortex will be aided by lasers with higher power, as well as by recently developed optical components, which allow for high resolution two-photon microendoscopy deep in the brain [15].

Whether or not some of these developments will eventually evolve into standard techniques in the lab, the new imaging method introduced by Ohki *et al.* [9] already allows questions to be addressed that we have not even dared ask until now. From the detailed cell by cell mapping of points of special interest in the visual cortex to longitudinal studies of a neuron's fate after manipulations of the sensory input, anything can be imagined. The latter question is of particular relevance, because *in vivo* two-photon imaging of neuronal morphology over extended periods has now been achieved by several groups [16,17]. Combining the latter approach with the new single cell calcium imaging technique will enable us to relate changes of a neuron's functional properties after alterations of the sensory environment to modifications in its morphology. To date, finding such correlations in the cerebral cortex has only been possible at the level of populations of cells, which made interpretation of the results highly ambiguous.

Finally, the ability to simultaneously record from large numbers of individual neurons might help to unravel one of the major mysteries of the visual cortex, namely the kind of computations that the cortex carries out when no external stimulus is present. Voltage sensitive dye recordings have shown that spontaneous activity in the visual cortex exhibits a high degree of spatio-temporal correlation [18], but it is not known whether this also holds on the single cell level. Calcium imaging in visual cortex slices [19], and electrical recordings from monkey prefrontal cortex [20] have revealed that spatially distributed neurons can fire action potentials in stereotyped sequences at surprising temporal precision. To understand the significance of such cortical songs [19] or synfire chains [20] it should be very enlightening to actually watch them play in the visual cortex *in vivo*.

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Plant Development: Auxin in Loops

Concentration gradients of the hormone auxin are associated with various patterning events in plants. Recent work has refined our picture of the complex and dynamic system of auxin transport underlying the formation of these gradients.

Stefan Kepinski[1,2] and Ottoline Leyser[1]

In the late 1960s, Wolpert [1] introduced the French flag hypothesis as a way of thinking about how cells can derive instructive positional information from the concentration gradient of a signalling molecule. Such signals, called morphogens, diffuse from a point source through a field of cells. Cells experiencing this morphogenetic gradient respond differently between different threshold concentrations and thus adopt fates according to their distance from the source [1,2]. There are many beautiful examples of morphogenetic gradient patterning in animals [2] but strictly speaking, none at all in plants.

Superficially, one of the most French flag-like patterning events in plants concerns the role of auxin in the root tip. Here, what appears to be a localised point of high auxin concentration patterns diverse cell types in the tissue [3]. The *Arabidopsis* root is a highly ordered affair, consisting of concentric cylinders of cells, with new cells being added at the distal root tip [4]. This radial pattern emanates, by a series of stereotyped divisions, from a small group of stem cells which surround four 'quiescent centre' cells at the heart of the root meristem (Figure 1). The quiescent centre has very little mitotic activity itself but functions to maintain the stem cell status of adjacent cells. Cells laid down in front of the advancing tip differentiate to form the root cap which protects the quiescent centre and stem cell niche as they push through the soil. In the cells left behind by the growing tip, division eventually gives way to a phase of rapid cell expansion without division which marks the end of the meristematic zone and the beginning of the elongation zone (Figure 1). Elongated cells then begin to differentiate, marked most clearly by the appearence of root hairs [4]. Thus, as well as its radial organisation, the root has a more general proximo-distal pattern of activities.

Previous work from the Scheres lab [3] showed a high level of induction of auxin responsive genes in a tightly focussed region just below the quiescent centre, suggesting a point source of auxin. Various experimental manipulations to increase, reduce or move this point were found to bring about concomitant changes in cell fate, consistent with a morphogen-like role for auxin in root tip patterning [3,5]. But any similarity to a French flag-type patterning mechanism turns out to be entirely illusory. Although stable gradients of auxin can be found here and throughout plant development, it is becoming increasingly clear that these are not generated by auxin movement away from a point source. Instead, the gradients are established, maintained, modified or even completely reversed by a complex interacting network of auxin transporters distributed throughout the region.

Evidence for the dynamic nature of auxin gradients has come from a series of seminal papers [5–8], the most recent of which [9] demonstrates the existence of a network of active auxin transport that recycles auxin around the root tip. The Blilou *et al.* [9] paper focuses on the PIN family of auxin efflux facilitator proteins. The PINs are transmembrane proteins and, in a mechanism which is not completely understood, they mediate the active pumping of auxin out of the cell [10]. Importantly, by means of asymmetric subcellular localisation patterns, their activity also confers a directionality to auxin transport [10]. At least five PIN proteins are expressed in specific and partially overlapping patterns in the *Arabidopsis* root [9]. Strangely, despite the clear importance of PIN-regulated auxin transport in root growth indicated by a whole raft of data [3,5,11,12], single *pin* mutants show only relatively minor defects in root patterning and growth. Blilou *et al*. [9] tackled this paradox by showing that, in certain single, double and triple *pin* mutant combinations, the remaining PIN proteins are often expressed ectopically in such a way as to mask *pin* mutant defects. This rather unusual example of gene family redundancy is most likely the result of altered auxin distributions in the *pin* mutant backgrounds leading to