

37. OTDK Biology Section



**Investigating mesoscale cortical maps
in the cat visual cortex using volumetric
functional ultrasound imaging**

Klaudia Csikós

Info-bionics Engineering MSc

Pázmány Péter Catholic University
Faculty of Information Technology and Bionics

2023

Supervisor
Dániel Hillier, PhD

TABLE OF CONTENTS

Abstract	3
Introduction	4
I. Literature review	6
Exploring functional maps and specialized domains in mammals.....	6
Essential techniques for the identification of cortical functional maps.....	7
Correspondence between cortical maps in animal models and humans.....	19
The effect of early visual experience on cortical map formation	20
II. Planning	21
Aim.....	21
Scientific motivation	21
Experimental apparatus	22
Visual stimuli.....	23
Anesthetized vs. awake measurement	24
III. Implementation.....	25
Modular interface for volumetric functional ultrasound imaging	25
Validating the stability of the interface.....	28
In vivo measurements.....	28
Analysis pipeline	29
IV. Results	31
Extracting activation maps	31
Orientation preference mapping.....	33
Consistency test.....	35
V. Conclusion	36
VI. Future plans.....	36
Acknowledgements	37
References	37
Attachments.....	40

Abstract

Understanding how the brain works poses a formidable challenge. Technology developments progressed hand in hand with new insights into how the brain processes sensory information. An overarching theme of the last 60 years has been the discovery of mesoscale functional maps that tile primary sensory cortical areas, like primary visual cortex in cats, monkeys and humans. In visual cortex, elementary visual features like stimulus orientation or motion direction are organized into functional maps. As brain activity recording technologies were invented, the existence of mesoscale organization and the interrelations of different functional maps within a cortical area have been uncovered. However, it remains unclear whether the layout and interrelations of cortical maps observed from surface layer activity extend into deeper layers of the cortex. Electrophysiology allows for precise temporal resolution, but it falls short in providing comprehensive spatial coverage, impeding a holistic comprehension of functional organization. On the other hand, optical imaging techniques have contributed valuable insights into cortical spatial organization, yet they are constrained by their limited penetration depth within the brain.

To bridge the gap between available technologies, I establish a novel measurement method for recording activity across the whole cortical depth using functional ultrasound technology. The new method contributes to a better understanding of cortical function by showing how the intertwined system of functional maps extend into the deeper layers of the primary visual cortex.

Introduction

The spatio-temporal dynamics of neural activity in the primary visual cortex (V1) blends external visual flow with an internal model of the visual environment. The cortical architecture implementing this computation was first probed with microelectrodes nearly 60 years ago by Hubel and Wiesel. They sketched a spatially organized, modular organization of the visual cortex (Hubel and Wiesel, 1962). Since their seminal work, many other groups extended and refined this framework by showing that neuronal selectivity for other visual stimulus features, such as position in visual space, orientation, spatial frequency, and motion direction are organized into functional maps that span V1 in carnivores and primates. The fundamental debate whether the cortical organization observed in functional recordings extends into deeper cortex in the form of radial “cortical columns” remains active (Szentágothai, 1983; Liedtke, 2017)

This debate about the relationship between cortical function and the underlying architecture of neuronal circuits has been fueled by the inconclusiveness of experiments emanating from the difficulty in connecting the functional and anatomical description of visual cortical organization. Probing neuronal function with electrophysiology has the advantage of finest possible temporal resolution across all layers of the cortex – at the expense of spotty coverage of spatial relationships. Electrophysiology therefore remains unfit for a comprehensive examination of the entire population of neurons within the visual cortex and can only yield a limited understanding of the functional organization across larger spatial scales.

Imaging is the most ancient technique to analyze brain organization: with simple microscopes, the retina as image sensor and the human brain as signal processor, anatomists have described brain organization across spatial scales. Imaging the activity of the brain became possible since about three decades, around the time when digital image recording technology was born (Grinvald, 1985). Significant progress has been made ever since in employing optical techniques to measure brain signals that reflect underlying neuronal selectivities and preferences. This development has put the spatial layout of functional maps across primary sensory cortices in the focus. In V1, several functional maps have been identified in imaging studies, thereby gradually refining the conceptual framework of functional cortical spatial organization. Optical imaging therefore has provided valuable support for the better understanding of the cortical functional architecture. However, using light to report brain

activity has an inherent limitation: light can be used to report activity at best from < 1 mm depth.

Technology development also opened the way for the analysis of anatomo-functional brain organization in 3D. Functional magnetic resonance imaging (fMRI) (Ogawa *et al.*, 1990) allows for the non-invasive recording of neural activity on the macroscopic scale. While there have been notable advancements to enhance the spatial and temporal capabilities of fMRI, the sensitivity and indirectness of the method to resolve smaller functional units likely cannot break the 0.5 mm limit. Therefore, our current understanding of how distinct 3D modules contribute to cortical function remains poorly understood.

To overcome these limitations, it is necessary to develop a methodology that:

1. enables the measurement of neural activity in the deeper layers of the cortex.
2. overcomes the spatiotemporal resolution constraints of current techniques (e.g. fMRI) and accurately detects the functional domains of the visual cortex on a mesoscopic scale.
3. facilitates seamless integration with other tools, allowing for the exploration of the relationship between the structure of cortical orientation maps and the functions of local cortical circuits at the microscopic scale.

In my work, I introduce a novel measurement method, along with an experimental setup and analysis, that holds the potential to map the functional organization of the visual cortex in-depth, in awake preclinical species with an accuracy unreachable before.

I. Literature review

The term “cortical map” refers to the existence of a nonrandom relationship between the location of a neuron and its associated physiological or anatomical descriptors. The first functional descriptor has been the receptive field of a neuron. In sensory cortex, e.g. in V1, this property delineates the region in visual coordinates that can elicit a response in the neuron. Other functional descriptors use more structured stimuli to describe what a neuron is “doing”, e.g. a neuron in V1 often responds vigorously to a specific orientation of a bar presented on a screen. Extensive research since the 1960s has focused on investigating these receptive field properties and has significantly contributed to our understanding of how the cortex segregates distinct stimulus features and how these segregated features are integrated to form a unified and coherent visual perception.

Exploring functional maps and specialized domains in mammals

During the exploration of the visual cortex in the mid-20th century, researchers made significant breakthroughs that revealed the intricate organization and processing of visual information in mammals. In the 1960s, Hubel and Wiesel delved into the visual cortex of cats, leading to groundbreaking discoveries. They uncovered the existence of ocular dominance columns, vertical arrangements of neurons in the primary visual cortex that exhibit a preference for inputs from one eye over the other. These columns form a striped pattern that spans V1. Each stripe clusters neurons that respond to activity transmitted from one eye. Additionally, Hubel and Wiesel identified orientation columns, which contain neurons selectively responsive to specific orientations of bars presented as stimulus. The orientation columns also form a functional map of orientation preferences that spans throughout the visual cortex (Grinvald *et al.*, 1986).

Other studies of the 1970s expanded our understanding of visual cortex organization by unraveling the retinotopic map, which describe the spatial arrangement of neurons based on their receptive fields. This map preserved the spatial relationships between visual stimuli, enabling the brain to maintain a coherent representation of the visual environment. Techniques employed in these studies included extracellular recording, optical imaging, anatomical tracing, and electrophysiological mapping. (Allman and Kaas, 1971; Tusa, Palmer and Rosenquist, 1978)

Advancing into the 1980s, Livingstone and Hubel (1984) could identify the so called “color blobs” within the visual cortex of macaque monkeys. These color blobs represent clusters of neurons that selectively responded to color stimuli. Concurrently, Tolhurst and Thompson

(1982) explored spatial frequency maps in cats during the same decade. Spatial frequency denotes the number of cycles of a periodic visual grating per unit of visual space. They revealed that neurons with similar spatial frequency preferences are tend to grouped together in distinct patches. In the following years, it was also discovered that neurons in the cortex were selective not only for the spatial frequency but also for the temporal frequency of the stimulus, although these clusters were much weaker compared to those observed in orientation or spatial frequency maps.(DeAngelis *et al.*, 1999)

Later on, researcher discovered that vast majority of neurons in the middle temporal visual area (MT) are direction selective, responding vigorously to movement in one direction within their receptive field and not responding to movements in the opposite direction. They have also described the columnar organization of cells sharing the same preferences (Albright, 1984).

Another significant discovery in the last century centered around binocular disparity. Binocular disparity refers to the differences in visual images received by the two eyes, enabling stereoscopic depth perception. Researchers focused on how neurons in the visual cortex responded to varying degrees of binocular disparity (Barlow, Blakemore and Pettigrew, 1967). Later it was also shown that these specialized neurons formed specific domains, providing the brain with crucial information about the depth and three-dimensional structure of the visual scene (Kara and Boyd, 2009).

Through the collective efforts of these pioneering researchers, utilizing high-acuity animal models such as cats and macaque monkeys, and employing techniques ranging from extracellular recording through optical imaging to anatomical tracing, the discovery of different functional maps within the visual cortex has significantly advanced our understanding of visual processing in mammals.

Essential techniques for the identification of cortical functional maps

Neuronal activity recording methods have played a pivotal role in expanding our understanding on the selectivity, spatial arrangement, and temporal dynamics of the neurons constituting these maps. By exploring each technological approach and its corresponding advancements, I will demonstrate how they have unlocked novel insights into the computations occurring in the primary visual cortex. Moreover, I discuss the limitations associated with each method, emphasizing the importance of utilizing multiple technologies to comprehensively investigate map organization.

Electrophysiology

Electrophysiology has played a crucial role in understanding the functional organization of the visual cortex in preclinical species. It involves the measurement of membrane potential changes from individual neurons or groups of neurons using techniques such as single-unit recording or multi-unit recording.

In the groundbreaking research conducted by Hubel and Wiesel, microelectrodes were utilized to record directly the electrical activity of individual neurons in the visual cortex while presenting various visual stimuli to animals. Apart from discovering that neurons in the primary visual cortex possess different receptive fields (simple and complex receptive fields), they also found that certain neurons exhibited distinct responses to visual stimuli based on its orientation and eye origin (Hubel and Wiesel, 1962).

By implanting several electrodes in adjacent areas, they could observe that neurons with similar orientation preferences forming columnar structures (Hubel and Wiesel, 1963). These results contributed to the formulation of the concept "functional architecture", which describes how different receptive fields are distributed and organized in the visual cortex.

Microelectrode measurements have also played a substantial role in the identification of spatial and temporal frequency clusters, describing the properties and organization of direction-selective neurons and revealing fundamental mechanism of binocular depth discrimination in various animal models.

However, this method has a significant limitation, namely its constrained capacity to examine a significant number of neurons within a limited spatial range. The limited sampling may not fully represent the diversity of neurons within the visual cortex, leading to potential biases in the observed results. In theory, the use of finely spaced microelectrode or tetrode recordings can potentially aid in addressing this issue, however, practically it is impossible to reconstruct the properties of even a small fraction of the neurons that contribute to a functional map or define the precise shape and arrangement of the columns. The pioneers of functional architecture in the visual cortex, recognizing the challenge of using a one-dimensional tool to tackle a three-dimensional problem, metaphorically compared it to attempting to “cut the back lawn with a pair of nail scissors,” emphasizing the difficulty and inefficiency of the task. Therefore, alternative techniques, such as advanced optical measurements or functional imaging methods, were necessary to provide more detailed and accurate insights into the exact structure and arrangement of these columns.

Optical imaging methods

In contrast to electrophysiology, optical imaging techniques provide enhanced spatial resolution and comprehensive coverage for functional mapping of the mammalian cortex. Moreover, they enable repeated imaging of the identical cortical area for extended periods, ranging from weeks to months, thus offering invaluable longitudinal observations into cortical development and the processing of sensory information. In the subsequent section, I will introduce several noteworthy optics-based technologies that have greatly contributed to our comprehension of the functional arrangement of the visual cortex.

Intrinsic optical imaging

Intrinsic signal optical imaging (ISOI) infers changes in neural activity by measuring metabolic changes by light microscopy. The term ‘intrinsic’ in the name refers to an important advantage of the technique: the activity measurement does not require any special staining procedure.

The source of the intrinsic signal is the early hemodynamic changes associated with neural activity, the local changes in the concentration of oxy-deoxy-hemoglobin and the increase in the local volume of cerebral blood flow, which causes a shift in the light absorption spectrum of the brain tissue around active neurons (Frostig *et al.*, 1990). ISOI allows for the high-resolution measurement of mesoscale cortical activity over relatively large areas, down to depths of 400-500 μm .

The technique was first implemented in 1986 by Grinvald and his colleagues to reveal the functional architecture of the visual cortex. Subsequently, it took an additional five years to demonstrate the precise layout of iso-orientation domains, which exhibited pinwheel-like arrangements within a cat’s secondary visual area (Bonhoeffer and Grinvald, 1991). They have observed that these domains are organized in a radial fashion, with multiple orientation preferences converging to a central point called pinwheel singularity. Surrounding each singularity point, there is a gradual transition of different orientation preferences, forming a swirling pattern, which can exhibit both clockwise and counterclockwise rotations as it is shown on Figure 1. Many studies have since confirmed the orientation pinwheel structure and added details that relate the map structure to receptive field properties. For instance, in the primary visual cortex (area 17) of the cat, the size of orientation domains increases with eccentricity

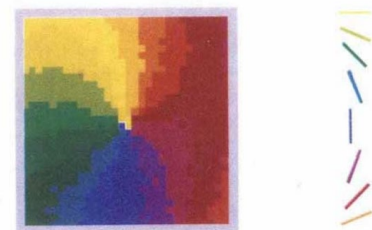


Figure 1. A close-up of a colour coded orientation map in the cat area 18 showing pinwheel-like structure around orientation centres. The preferred orientation coded according to the scheme shown on the right. (Grinvald et al. 1986)

(resulting in a decrease in pinwheel density), mirroring the increase in receptive field size with eccentricity (Xu *et al.*, 2007)

By investigating the direction preference of the neurons in the area 18 of the cat, researchers found a close relationship between the functional maps for direction and for orientation preference; typically, an iso-orientation patch was divided into regions that exhibited preference for opposite directions, orthogonal to the orientation (Shmuel and Grinvald, 1996).

Later on, researchers were also able to discern spatial connections among orientation-preference pinwheels, colour maps, and ocular-dominance columns in the primate cortex. This finding underscored a fundamental advantage of optical imaging: the capacity to visualize multiple maps within the same subject, thereby facilitating the exploration of geometric relationships between distinct functional architectures (Bartfeld and Grinvald, 1992).

Over the past 30 years, ISOI has become the prevailing method for mapping sensory cortical activity in large-animal species. Due to its applicability across different cortical regions and species, ISOI was employed to investigate the orientation preference and ocular dominance maps in the primary visual areas of species such as cats, ferrets, macaques, tree shrews and marmoset monkeys (Bonhoeffer and Grinvald, 1991; Bonhoeffer, 1995; Bosking *et al.*, 1997; Issa *et al.*, 1999; Schiessl and McLoughlin, 2003). Optical imaging has also revealed maps of motion direction preference (Shmuel and Grinvald, 1996), spatial frequency preference (Issa, Trepel and Stryker, 2000), form processing modules in macaque area V4 (Ghose and Ts'o, 1997), color-selective neuron clusters in V1 (Landisman and Ts'o, 2002), and hue-selective organization within the cytochrome oxidase stripes of V2 (Xiao and Felleman, 2004).

However, some inconsistencies arise in these studies, particularly regarding the organization of spatial frequency domains. Intrinsic imaging studies presented conflicting claims about the structure of spatial frequency (SF) maps, ranging from no detectable organization to clear relationships with other maps (Issa *et al.*, 2000; Xu *et al.*, 2007). Sirovich and Uglesich (Sirovich and Uglesich, 2004) claimed that the detected organization of the SF domain with intrinsic imaging could be primarily associated with vasculature contamination, and they found little apparent evidence for a columnar organization for spatial frequency in cats. To address this concern, researchers started to investigate the SF preference map by using autofluorescence imaging (Mallik *et al.*, 2008), which is far less susceptible to vascular artifacts.

Autofluorescence imaging

Autofluorescence imaging (AFI) is a technique that utilizes the natural fluorescence emitted by certain molecules within cells to visualize and study biological samples. In the context of cortical imaging, autofluorescence imaging involves the detection of fluorescence signals emitted by flavoproteins present in neuronal mitochondria. Flavoproteins, such as flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), are electron-carrying molecules involved in the cellular energy production process. When these flavoproteins are oxidized during increased metabolic activity in neurons, they emit fluorescence in a specific range of wavelengths when excited by light. By analyzing the intensity and spatial distribution of the autofluorescence signal, researchers can gain insights into the functional organization and activity patterns of the imaged tissue (Husson *et al.*, 2007).

Mallik and his colleagues (2008) compared the two different methods (ISOI and AFI) and utilized them to observe SF domains in the same animal. Their findings revealed that SF maps and tuning curves obtained through AFI and ISOI exhibited statistically similar structures. This similarity strongly suggests that the clustering of SF preference is not a result of vascular artifacts but rather a genuine characteristic of the organization in area 17. Furthermore, the researchers demonstrated several advantages of AFI over other imaging techniques. AFI enables a direct measurement of cellular metabolism and provides spatially restricted imaging at the level of individual cells, resulting in high-resolution data. Moreover, the autofluorescence signal is less prone to vascular artifacts compared to methods based on blood flow or oxygenation signals and has a faster time course. These attributes position autofluorescence imaging as a valuable tool for studying cortical activity and investigating functional organization.

Fluorescence Imaging

In contrast to intrinsic or autofluorescence imaging, widefield fluorescence microscopy requires the labeling of tissues with synthetic fluorescent dyes or genetically encoded fluorescent proteins. Synthetic fluorescent calcium-binding dyes and fluorescent voltage-sensitive dyes (VSDs) have been used since the 1970s to measure the activity of neuronal cell networks in response to changes in cell membrane voltage.

In the case of VSDI, a special dye is used to stain brain tissue. The dye molecules bind to the outer surface of neuronal membranes and emit a fluorescent signal that is proportional to changes in membrane potential. Depending on the experimental conditions, the VSD signal can be detected with sub-millimeter spatial accuracy over milliseconds of time (Grinvald and Hildesheim, 2004).

Leveraging these capabilities, Grinvald and Hildesheim (2004) addressed the question of how the interplay between feedforward processes and intracortical connections are contributing to the formation of cortical maps. To

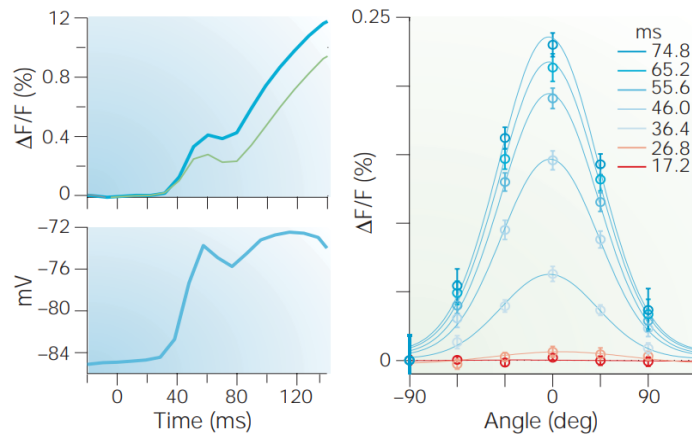


Figure 2. Left, top: The time-course of the evoked response to the preferred (blue) and orthogonal (green) orientations. Response to the preferred orientation is noticeably larger.

Left, bottom: An intriguing aspect of the evoked responses is the presence of the DA notch.

Right: Orientation tuning curves of single-condition responses at different times after stimulus onset: The curves maintain a consistent shape but differ in their amplitudes (Grinvald and Hildesheim, 2004).

investigate this question, single-condition responses were used to calculate orientation tuning, revealing tuning curves with a constant shape but changing amplitude (Figure 2., Right). They have observed that the time course of the evoked response to the preferred and orthogonal orientation have the same onset latency, but the response to the preferred orientation is larger from the beginning. During the investigation of the response dynamics, they also observed a unique pattern in the response, known as the “evoked DA notch,” which indicated a deceleration followed by acceleration in the neural activity (Figure 2., Left). This pattern suggested the involvement of specific inhibitory mechanisms. Interestingly, the evoked DA notch was more pronounced when the neurons were responding to orientations different from their preferred orientation. These findings suggest that the initial input from the thalamus influences the width of orientation preferences in cortical neurons, while subsequent processing within the cortex amplifies the selectivity. Furthermore, inhibitory processes help prevent responses to non-preferred orientations from increasing rapidly, ensuring a sharper tuning to preferred orientations.

Among optical techniques used to describe the temporal dynamics of visual responses, VSD has demonstrated its prominent position, however, it is important to bear in mind that VSDI is best suited for acute experimental conditions due to the necessity of repeating the staining procedure before each experiment and the quality of the staining can impact the reliability of

the measurements (O'Shea *et al.*, 2017). To overcome this limitation, one potential solution is the utilization of genetically encoded calcium indicators to achieve long-term fluorescent labeling, with the ability to target specific cell types, layers, and regions in the brain. Currently, the most frequently employed fluorescent protein family for optical imaging is GCaMP, which allows for the detection of rapid increases in calcium ion concentration accompanying neural activity.

Recently, Seidemann and colleagues (2016) successfully demonstrated this method, by virally targeting excitatory neurons in the V1 in macaques and could map orientation preference columns with a high signal-to-noise ratio (SNR) over several months. They have also developed a computational model that quantitatively links wide-field measurements with single-unit measurements and provides evidence that the widefield GCaMP signals are dominated by the summed spiking activity of local populations of neurons, while widefield VSD signals reflect the linearly summed local membrane potential. Consequently, GCaMP calcium imaging holds significant value for conducting further investigations into mesoscopic functional maps.

Two-photon imaging

Two-photon calcium imaging is a powerful technique that enables high-resolution, three-dimensional mapping of cortical areas. It uses nonlinear excitation technology to excite fluorescent molecules in tissue. The previously mentioned optical methods use linear, i.e. single-photon excitation, whose strong scattering in biological tissue can significantly reduce resolution, and targeting deeper cortical layers has severe limitations (Helmchen and Denk, 2005). Nonlinear excitation overcomes these limitations by simultaneously (within 0.5 fs) absorbing two photons in the fluorophore molecule, which together provide sufficient energy to excite the molecule. Because no fluorescence signal can enter the detectors from outside the focal point, it enables the visualization of single neurons within specific regions of the cortex, providing detailed insights into neural activity (Klioutchnikov *et al.*, 2020). This imaging method relies on the use of calcium-sensitive dyes or genetically encoded calcium indicators to measure activity-dependent fluorescent signals. By employing this technique, researchers have been able to sample large populations of neurons in macaque V1, revealing the organization of orientation, direction and spatial frequency preferences on the microscopic scale.

Okhi *et al.* (2005) labeled thousands of neurons in the visual cortex with a calcium-sensitive indicator and used two-photon microscopy to capture the activity of these neurons at single-cell resolution, up to a depth of 400 μm . They could observe that in area 18 of the cat visual cortex neurons with opposite preferences for stimulus direction were segregated with extraordinary

spatial precision in three dimensions, with columnar borders one to two cells wide. In a later study, they also reported that the pinwheel structure of orientation maps is also highly ordered: neurons selective to different orientations are clearly segregated even in the very center (Ohki *et al.*, 2006). This highly ordered arrangement at the level of single cells suggests great precision in the development of cortical circuits underlying direction and orientation selectivity.

Nauhaus and colleagues (2012) also employed two-photon imaging to examine the fine organization of spatial frequency maps in the visual cortex of macaque monkeys. One of their goals was to resolve a longstanding debate regarding the architectural organization of spatial frequency maps. By investigating the response properties on the cellular level and measuring the degree of clustering by looking at cell pairings at different distances within the cortex, they could demonstrate substantial structure in preferred spatial frequency - low or high spatial frequency preference is clustered with smooth transitions in between - and they also showed that this structure is consistent throughout the depth of layer II/III. Moreover, they discovered a systematic relationship between the orientation and spatial frequency maps: the smoothed contours of these maps ran perpendicular to each other, which highlights the joint representation and coordinated organization of orientation and spatial frequency preferences in the cortex (Figure 3.).

Recently, in vivo two-photon imaging of somatic calcium signals has been applied to investigate the connection between orientation and color-coding neurons in macaques. Initially, early studies proposed a strict segregation of color and orientation processing (Livingstone and Hubel, 1988), while later conflicting studies reported no correlation between these

features (Kiper, 2003). However, Garg and colleagues (2019) were able to address this issue by examining the response properties of individual neurons. Their findings revealed that a significant portion of V1 neurons exhibited a strong preference for equiluminant color over achromatic stimuli and were also orientation selective. These results indicates that orientation and color in V1 are mutually processed by overlapping circuits. Single neurons could precisely

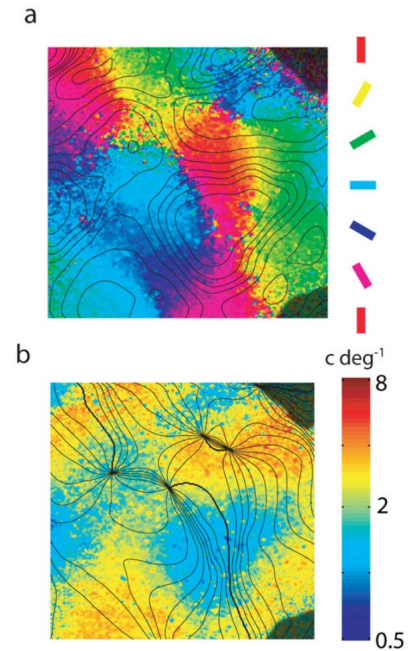


Figure 3. The correlation between orientation (ORI) and spatial frequency (SF) maps in the visual cortex of macaque monkeys obtained from large-scale imaging experiments. a) ORI maps are displayed with smoothed SF contours overlaid in black. b) Corresponding SF maps are shown with smoothed ORI contours overlaid in black. The preferred orientation and spatial frequency coded accordingly to the scheme shown on the right. (Nauhaus *et al.*, 2012)

and unambiguously code for both color and orientation, which implies that information in primate V1 is more sparsely coded than indicated by previous results. Additional analyses confirmed the systematic spatial relationships between color tuning and orientation selectivity, supporting the notion that form significantly influences color perception and vice versa.

Nevertheless, despite the higher wavelength which enables deeper penetration, two-photon imaging is still confined to the functional mapping of the superficial layers of the cortex (up to about the top of layer IV in large brains), while the activity of deeper layers remains hidden. Another significant drawback lies in the limited field of view, which restricts the simultaneous imaging of large cortical areas and may require stitching together multiple images to obtain a comprehensive map.

Functional optical coherence tomography

Functional optical coherence tomography (fOCT) is an advanced imaging technique that utilizes light interference to capture three-dimensional structures by detecting the light-scattering component of intrinsic signals. It is an extension of the optical coherence tomography (OCT) technique, which enables visualization of 3D structures using light interference. What sets OCT apart from traditional optical imaging methods is its ability to resolve light reflections from different depths. By employing an appropriate light source, OCT can achieve the necessary spatial resolution and measurement range to visualize mesoscopic cortical columns up to 1 mm in depth. The functional aspect of OCT comes into play by detecting changes in OCT signals, allowing it to function as a 3D imaging technique based on intrinsic signals.

Nakamichi and his colleagues (2018) used fOCT to investigate the three-dimensional structure of orientation coding in the visual cortex. The researchers could validate the vertically elongated columnar structure in iso-orientation domains, that had been predicted from electrophysiological recordings. Unexpectedly, the researchers have also made a novel discovery regarding the functional organization around pinwheel centers. Rather than observing straight solid bars connecting pinwheel centers and white matter, they discovered that these singularities followed various

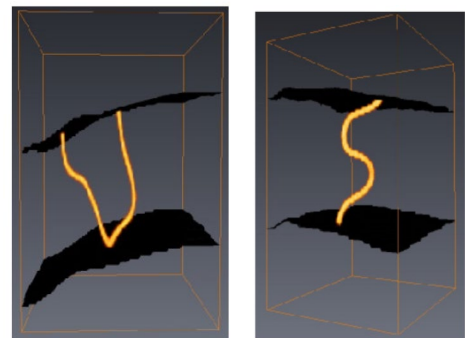


Figure 4. 3D intracortical structures of singularity strings observed in cats via fOCT (Nakamichi et al., 2018)

trajectories along the depth axis of the cortex. Their observations revealed that the columnar assemblies are distorted around pinwheel centers, forming twisted string-like structures that extended horizontally through the cortex. In some cases, the so-called “singularity strings”

formed a U-shape structure connecting the clockwise and counterclockwise orientation pinwheels on the surface, which could not be fully anticipated from two-dimensional surface measurements (Figure 4.). The findings highlight the importance of considering the depth axis in understanding the topology of orientation coding.

Functional magnetic resonance imaging

Functional magnetic resonance imaging (fMRI) is a widely used non-invasive imaging technique that measures the hemodynamic response associated with neural activity in the brain. When neurons are active, they require oxygen to support their metabolic demands. As a result, there is an increase in regional cerebral blood flow to supply oxygen-rich blood to the active brain regions. The increased blood flow leads to an increase in the delivery of oxygenated hemoglobin to the active areas, leading to an elevation in oxyhemoglobin concentration while simultaneously reducing the concentration of deoxygenated hemoglobin. This change in the relative levels of oxy- and deoxyhemoglobin is known as the hemodynamic response. Functional MRI takes advantage of these hemodynamic changes to indirectly measure neural activity non-invasively in the entire brain.

In contrast to previously introduced techniques, fMRI offers a significant advantage by enabling the observation of brain regions at different depths, encompassing both superficial and deep areas. However, its effectiveness has been hindered by its limited spatial (typically 1 mm³) and temporal resolution (1-3 sec). Therefore, certain patterns of brain activation, such as low-resolution retinotopy maps in humans could be resolved by this technique, but finer structures, such as orientation preference domains or ocular dominance bands in primates were too small to be resolved. The low spatial resolution causes signals to be blurred or averaged across multiple cortical columns, which can result in an imprecise or inaccurate depiction of cortical activation patterns (Kim, Duong and Kim, 2000).

Therefore, in recent years, there has been an increasing amount of research aimed at pushing the boundaries. By utilizing higher field strengths, it becomes possible to achieve higher spatial and temporal resolution in mapping, both in animals and humans (Kim, Duong and Kim, 2000). Besides, further investigations into imaging techniques such as the initial negative BOLD response (the "initial dip"), CBF signal, and CBV-based fMRI have shown promise in elucidating columnar and laminar organization in sensory cortices.

A significant example highlighting the progress in the field is the research conducted by Fukuda and his colleagues (2006), where they utilized CBV-weighted fMRI in conjunction with contrast

agents like monocrystalline iron oxide nanoparticles (MION) to investigate the cat's iso-orientation domains. This approach demonstrated a substantial improvement in resolution on the order of submillimeter. They could successfully map orientation-specific domains in the medial bank of cortical area 17 (suprasplenic and splenic gyri), which is inaccessible using optical imaging. Distinct activation patterns appeared as patches and bands on the recorded sagittal imaging slices, depending on the orientation of the imaging plane relative to the cortical surface and the arrangement of intracortical veins. However, there were limitations in accurately assigning stimulus orientation in regions perpendicular to the cortical surface due to neighboring column contributions and reduced signal sensitivity in anterior and posterior regions.

Other researchers have dedicated significant efforts to exploring the complexities of orientation selectivity variations across cortical layers through the utilization of fMRI methodologies. Cho et al. (2022) conducted a study using CBV-weighted fMRI to examine the orientation domains through all the cortical layers. While they successfully identified columnar structures, they noted inconsistencies in map precision, such as domains tilting away from the cortical surface and disruptions in map continuity in the deeper cortical layers (layer IV, V, VI). These irregularities have been also observed with functional optical coherence tomography imaging and single-unit recordings. However, the exact causes of these imperfections in orientation preference maps are still not clear.

It is plausible that hemodynamic signals in the cat visual cortex exhibit lower orientation selectivity compared to synaptic and spiking neural activity, as suggested by O'Herron et al. (2016). Additionally, it is conceivable that the deeper cortical layers demonstrate less orientation selectivity compared to other layers. However, the sensitivity of fMRI might not be adequate to resolve this matter.

Another constraint of fMRI recordings is the requirement for strict subject immobility due to the difficulties in correcting movement artifacts. As a result, fMRI is not suitable for studies involving freely moving subjects, despite the increasing interest in this research area. Furthermore, the high cost associated with fMRI presents a barrier to its widespread adoption, limiting its accessibility and utilization.

Functional ultrasound imaging

Functional ultrasound imaging, also known as fUSI, has emerged as a promising alternative to mesoscale techniques in the field of functional brain imaging. It offers high spatial and temporal

resolution and can be combined with other tools for comprehensive investigations. The technique indirectly captures spatial and temporal dynamics of neural activity by monitoring changes in blood flow, leveraging a mechanism called neurovascular coupling. This mechanism regulates local brain blood flow in response to activated neuron's increased oxygen and nutrient demand (Krishnamoorthy-Natarajan and Koide, 2016).

The physical principle behind functional ultrasound imaging is similar to conventional ultrasound imaging, where ultrasound waves are emitted into the tissue by a piezoelectric transducer, and the reflected echoes are converted back into electrical signals. Doppler ultrasound, a variant of ultrasound imaging, is used for functional measurements to determine the direction and flow rate of blood flow based on the Doppler effect (Moorthy, 2002). However, conventional Doppler ultrasound lacks the sensitivity required to map hemodynamic responses correlated with neural activity in the brain (Macé *et al.*, 2011).

Recent advancements have led to the development of ultrafast ultrasound imaging, which enables high-resolution neurovascular mapping at high frame rates. By emitting plane-wave ultrasound, this technique achieves high-sensitivity Doppler imaging with excellent spatial (approximately 100 μm) and temporal resolution (between 0.1 to 0.5 seconds) (Macé *et al.*, 2018). Functional ultrasound imaging relies on Doppler ultrasound measurements to record changes in cerebral blood volume (CBV) induced by neural activity. With an available imaging depth of up to 30 mm, functional ultrasound imaging can create comprehensive neural activity maps in rodents and small mammals, capturing the dynamics of both small cortical structures and large-scale networks (Demené *et al.*, 2017).

Recent studies have demonstrated the capability of fUS imaging for high-resolution mapping of 3D tonotopic organization in the auditory cortex and deeper structures such as the inferior colliculus in awake ferrets (Bimbard *et al.*, 2018). Researchers have also evaluated the reliability of fUS imaging for mapping the functional organization of the visual cortex in depth, such as retinotopic maps and ocular dominance columns, in awake primates with an accuracy unreachable before. Blaize *et al.* (2020) has demonstrated that fUS can reveal cortical layer selectivity of the visual cortex during ocular dominance mapping.

By applying fUS to the area 17 of anesthetized ferrets, the researchers were able to visualize retinotopic responses and orientation domains in 3D. The fUS activations revealed cone-shaped domains with larger areas near the cortical surface and narrowing towards the deeper layers. The spacing between neighboring iso-orientation domains was consistent with previous findings using optical imaging methods. Nonetheless, it is important to note that the current

study utilized only one orientation stimulation, thereby preventing the construction of a comprehensive 3D orientation preference map encompassing all possible orientations.

These findings demonstrate that fUS is a powerful tool for studying the 3D organization of functional domains in the primary visual cortex. It provides spatial resolution capable of revealing the functional architecture and can be applied to different cortical regions. It could expand our understanding of the visual cortex and its functional organization beyond the limitations of previous techniques.

With further development, functional ultrasound may provide a powerful mesoscopic-scale 3D functional brain imaging technology to fill the gap between two-photon microscopy and fMRI, and to map a broad array of functional cortical response properties and derive topological information.

Correspondence between cortical maps in animal models and humans

The cortical organization of the visual cortex exhibits notable differences between rodents and species with high-acuity vision, such as cats and monkeys. These dissimilarities hold crucial implications and must not be overlooked, especially when considering the relevance and translational value of research findings to humans.

In rodents, the visual cortex demonstrates a relatively simpler organization compared to high acuity species. While rodents do exhibit certain cortical maps to a limited extent, they are less intricate compared to those observed in cats and monkeys. For example, the retinotopic organization in the rodent visual cortex may be relatively coarse, but it lacks the same level of specificity and precision found in high-acuity species. Their visual system is predominantly focused on processing basic visual features, such as orientation and motion, necessary for survival and navigation in their environment. In visual cortex of mice (Dräger, 1975) and rats (Girman, Sauvé and Lund, 1999), neurons are sharply tuned to orientation and direction of motion, although no evidence has been found for orientation or direction maps. Two-photon calcium imaging confirmed that in both species, neurons with the different selectivity preference are mixed in a so-called “salt-and-pepper” organization, which starkly contrasts the pattern observed in cats or monkeys.

In contrast, high acuity species like cats and monkeys possess a more complex and specialized cortical organization that looks similar to what we know of human cortical organization. These animals rely heavily on fine spatial resolution and detailed object recognition, necessitating sophisticated cortical processing. As a result, their visual cortex exhibits well-defined

retinotopic, orientation selectivity, ocular dominance and motion selectivity maps, allowing for more sophisticated visual processing. The layout of these maps shows correspondence between the two species; however, functional and anatomical evidence highlights some differences between V1 in macaque monkeys and cat area 17, which should be considered.

The effect of early visual experience on cortical map formation

The influence of visual experience on cortical map formation has been a topic of great interest in the field of neuroscience. One of the first evidence illustrating the impact of visual experience on the maturation of the cortex emerged from studies investigating the development of orientation preference maps in cats (Hubel *et al.* 1977). Although the researchers found that the topological maps of visual space, orientation preference and ocular dominance are evident before birth in primates and before the postnatal separation of the eyelids in carnivores, patterns of retinal activity are necessary to achieve the finely tuned precision that is characteristic of the mature maps. Noisy or uncoordinated patterns of neural activity - such as sensory signals filtered through injured or diseased sensory organs - may weaken (or fail to strengthen) synaptic connections producing dysfunctional neural circuits and abrupted cortical maps.

Visual cortical plasticity is typically assessed by using the experimental paradigm of monocular deprivation, which will result in an unbalanced input from the two eyes. The consequences of this phenomenon include the atrophy of the lateral geniculate nucleus (LGN) layers that correspond to the deprived eye, as well as the reorganization of ocular dominance columns (Castaldi, Lunghi and Morrone, 2020). The deprived eye's dominance decreases, with only a small number of neurons responding to its stimulation (Hubel and Wiesel, 1970). In addition, the orientation map of the deprived eye vanishes (Figure 5.) (Crair *et al.*, 1997) and alternation can be

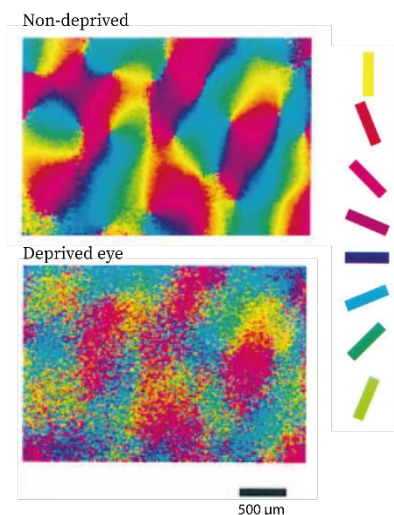


Figure 5. Orientation (ORI) preference maps from the visual cortex of a kitten, in which one eye was sutured closed for 1 week (P28–P35). Top: ORI map of the non-deprived eye showing structured columns. Bottom: ORI map of the deprived eye showing highly distorted orientation columns. The preferred orientations are indicated by the color bar inserts. (Crair *et al.*, 1997)

observed in the retinotopic organization (Smith and Trachtenberg, 2007). These changes manifests at the functional level as reduced visual acuity and reduced responsiveness to visual stimulation, a condition known as amblyopia (Levi, Knill and Bavelier, 2015).

In adults, once the critical period has closed, it is no longer possible to induce or treat amblyopia. Studies on adult cats have demonstrated minimal or no effects of monocular deprivation, indicating that the visual cortex exhibits very limited or no experience-dependent plasticity in adulthood (Wiesel and Hubel, 1963). Similarly, in humans, early visual deprivation or suboptimal visual experiences (such as untreated congenital cataracts, astigmatism, myopia, etc.) lead to plastic reorganization of the visual cortices and permanent visual impairments that are challenging to recover after the closure of critical period.

Considering that amblyopia is one of the most prevalent visual developmental disorders affecting approximately 4% of the global human population (Williams *et al.*, 2008), and its status as a primary cause of vision loss in children (Holmes and Clarke, 2006), emphasize the utmost importance of developing suitable therapeutic strategies. To achieve this, it is imperative to gain comprehensive insights into the underlying mechanisms of the disease and the regulatory principles governing neural circuits forming cortical maps.

II. Planning

Aim

My aim was to develop an experimental setup that enables the investigation of functional columns in all the six layers of the primary visual cortex of both anesthetized and awake cats by leveraging the innovative functional ultrasound technology. This required the design and implementation of a motorized head-implant system, the application of different visual stimulation protocols during in vivo measurement and a development of an analysis protocol to detect stimulus related changes in the visual cortex. Here, I will discuss the investigation of one of the extensively researched cortical organizations, namely the orientation preference columns.

Scientific motivation

Our motivation in the Visual Systems Neuroscience Research Group (Institute of Cognitive Neuroscience and Psychology at the Research Centre for Natural Science) is to uncover the causes of permanent loss of visual function resulting from amblyopia.

From a neuroscientific standpoint, experimentally induced amblyopia serves as one of the most extensively employed animal models for investigating the mechanisms underlying the development and plasticity of the visual cortex. Animal models of amblyopia provide a valuable platform to examine the influence of sensory experience on cortical development and to explore the potential for environmental and pharmacological interventions to induce neuroplasticity in

the adult brain. As neuroplasticity plays a central role in the recovery from various neurological and psychiatric disorders, studies utilizing amblyopia as a model hold promise in generating novel intervention strategies that have broad applicability across different disorders (e.g., stroke, addictive disorders, developmental disorders etc.).

Tracking changes in cortical maps (eye dominance, retinotopy, orientation, motion direction, spatial frequency) in long-term experiments, and comparing healthy, diseased and treated animals, we can gain valuable insight into those underlying mechanism which govern their formation (White and Fitzpatrick, 2007).

Our model animal is the cat, a large-animal preclinical species that - unlike rodents - faithfully models several features of the human visual system and has been used extensively to model binocular integration and its deficits in amblyopia due to the close correspondence between the visual deficits exhibited by visually deprived cats and those manifested by human amblyopes.

Experimental apparatus

Functional ultrasound imaging serves as a highly potent tool for investigating the three-dimensional organization of functional domains. However, certain limitations need to be addressed in order to maximize its utility.

Animal preparation Due to the substantial attenuation of the ultrasound signal by the skull, achieving high signal-to-noise ratio in fUS imaging necessitates the opening of a window in the animal's skull. This procedure requires the implementation of a chronic implant that not only facilitates the accessibility of tissue to ultrasound waves during measurements but also ensures the protection and preservation of the brain's normal state between measurements.

Extending imaging to the third dimension A practical limitation of fUS is its capability to capture only a single cross-section of the brain using a linear ultrasound probe, necessitating the motorization of the probe to scan the entire brain volume. A potential solution to this challenge was recently proposed by Brunner et al. (2021), who introduced a method involving the linear movement of the transducer across the target area using a motorized arm while fixing the mouse's head. My objective is to develop a head-mounted motorized interface that offers precise control over the positioning of the transducer above the exposed brain surface, thus enabling the mesoscopic and three-dimensional investigation of neural activity of the visual cortex.

Stability Stable imaging is crucial for accurate measurements in order to avoid complications arising from motion artifacts. Such artifacts can introduce inconsistencies in the extracted data, leading to erroneous conclusions. These motion artifacts can be caused by both voluntary and involuntary movements of the animal, including respiration and heartbeat. In case of fUS imaging, motion artifacts can not only result in signal blurring but also lead to inaccuracies in the activation patterns, since Doppler imaging relies on quantifying the relative displacement of red blood cells in relation to the position of the transducer enabling the tracking of blood volume changes. Therefore, the holder designed for fUS imaging should possess mechanical stability to effectively minimize artefacts caused by motion.

Visual stimuli

Traditionally, the assessment of orientation selectivity involves sweeping a bar or drifting gratings across the receptive field of neurons in various directions. Drifting grating stimuli involve the presentation of sinusoidal or square wave gratings (alternating light and dark bars) on a screen. These gratings have a specific orientation and exhibit motion in a direction perpendicular to their orientation. Some research group used a specific variation of drifting grating stimulation, which incorporating directional reversals during the stimulation period. Specifically, a static grating was initially presented, then it started drifting in a specific direction, and after a few seconds, the direction of the movement was reversed. The intention behind this alteration is to elicit responses from all neurons that are selective to the specific orientation, irrespective of their preferred motion direction (Husson *et al.*, 2007).

Another important consideration in stimulus design is that our measurement tool, the fUS captures indirectly the neural activity by detecting hemodynamic changes in the brain. To differentiate stimulus-related responses from background activity, we typically present a gray screen to the animal before and after stimulation, which help us to define the baseline activity of the brain. Besides, it also allows the visual system to adapt to a consistent brightness level, optimizing contrast sensitivity for detecting differences in neural responses. Additionally, the gray screen also facilitates experimental design by providing a clear separation between baseline and active conditions, enabling accurate analysis and interpretation of the fUS data within a defined temporal sequence. Alternatively, we can also use static grating pattern instead of gray screen.

In order to optimize our stimulation protocol and achieve precise detection of orientation domains, we tested multiple stimulation design during our in vivo measurements (Figure 6.):

- a) **Gray screen – grating moving in one direction.** In the first type of stimulation, the baseline period begins with a gray screen, which is then followed by the presentation of grids that appear in a specific orientation and drift during the stimulation period. Gray screen reappears after the stimulation.
- b) **Gray – grating moving in zig-zag.** The second type retains the gray baseline but introduces direction reversals during the stimulation period. After every 2 seconds of drifting, the grating changes its direction.
- c) **Static grating – grating moving in zig-zag.** In case of the third variation, the gratings change direction following the same pattern as in Type II., but instead of using a gray screen, static grating is displayed before and after stimulus.

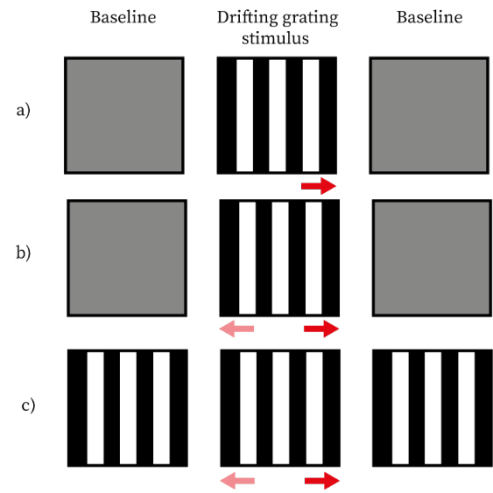


Figure 6. Schematic illustration of the different stimulus designs.

Anesthetized vs. awake measurement

Anesthetized Functional imaging studies are often performed under anesthesia to minimize motion artifacts or eliminate the need of behavior training. However, different type of anesthesia can have varying effect on neural activity or it may alter the coupling between neural and vascular responses (Franceschini *et al.*, 2010).

Decades of electrophysiological research in cats have provided valuable insights into the effects of different anesthetic agents on neural function. Typically, a common protocol involves continuous infusion of ketamine along with other agents like sodium pentobarbital, xylazine, or diazepam to induce and maintain anesthesia (Levine *et al.*, 2020). To minimize the amount of ketamine required for anesthesia and its suppressive effects, pre-medicating with ketamine and maintaining sedation with isoflurane gas has been employed for functional measurement. However, studies revealed that isoflurane can redirect cerebral blood flow and significantly reduce neural activity in various visual brain regions (Olman *et al.*, 2003).

As an alternative to the combined administration with ketamine, alfaxalone has emerged as a strong candidate as a primary anesthetic agent. Alfaxalone exhibits dose-dependent effects on cardiovascular, respiration, neuronal activity, and neuromusculature, which may yield more predictable changes in the neurovascular response (Warne *et al.*, 2015). Additionally, alfaxalone has been successfully administered intravenously for anesthesia in our cats during surgical

procedures with minimal physiological side effects. Therefore, we have chosen alfaxalone combined with ketamine for our anesthetized functional measurements.

Awake Performing awake functional measurements of cortical activity offers several advantages. Firstly, it allows for a more natural and physiologically relevant state, avoiding the confounding effects of anesthesia. Secondly, awake measurements facilitate the investigation of functional connectivity and network interactions, shedding light on the complex dynamics of the brain.

However, conducting experiments on awake and behaving cats poses certain challenges that need to be addressed. One critical aspect is reducing the noise caused by movement or by cardiac and respiratory pulsations. Typically, reducing movement artifacts during imaging requires head fixation, which can induce stress responses in the animal without prior habituation training. However, these habituation training sessions can be labor-intensive and time-consuming, lasting for days or even weeks, making them less efficient compared to anesthesia protocols.

Therefore, my objective was to develop an experimental setup that enables stable imaging during awake measurements without the need for head fixation.

III. Implementation

Modular interface for volumetric functional ultrasound imaging

To enable volumetric functional ultrasound imaging, I have developed a compact and customizable modular implant system that can be easily adjusted according to experimental needs. Combined with a unique motorized interface, this construct enables the acquisition of three-dimensional functional ultrasound images in both anesthetized and awake animals.

The guiding concept behind the design was modularity. The implant system can be divided into three main components:

- 1) Chronic chamber – shaped to conform to the contours of the skull on one side and provide flexible access to connect experiment-specific hardware on the other.
- 2) Inset chamber - stabilize the tissue and minimize motion artefacts
- 3) Motorized fUS holder – enables the attachment of the ultrasound transducer to the skull and offers precise control over the positioning of the transducer above the exposed brain surface during functional ultrasound imaging.

Chronic chamber

Due to the significant variations in size and geometry of the cats' skulls, an individualized implant was created for each cat based on their own structural MR images. Volumetric images of the cats' skulls were acquired using a 3 Tesla MRI machine (Siemens MAGNETOM Prisma) with a T1 VIBE sequence. After I have imported the MRI recordings in DICOM format into ITK-SNAP 3.8 software, and by utilizing a semi-automated segmentation method, the 3D model of their skull could be reconstructed. By aligning this model with the cortical volumetric reference atlas (Catlas, Stolzberg *et al.*, 2017), I could define the target region and optimize the shape and size of the implant accordingly. Subsequently, I fitted the chamber to the animal's skull to ensure easy implantation and stable fixation. (A comprehensive description of the specific methodology was presented at the Scientific Students' Association Conference in autumn 2021.) Figure 7 displays the final design fitted to a cat skull.

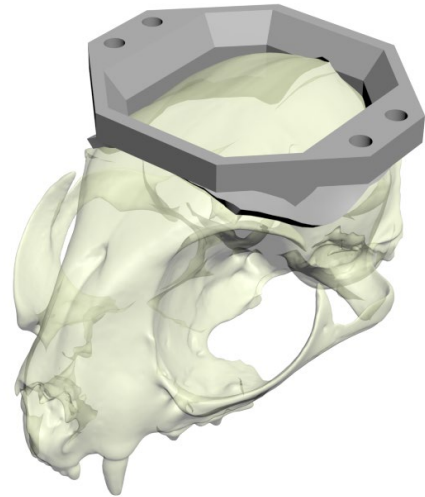


Figure 7. Customized chronic chamber design on a 3D cat skull model (Autodesk 3ds Max)

Inset chamber

Tissue stabilization is critical for stable imaging performance. For this reason, I designed an inset chamber to apply slight mechanical pressure to the tissue. With this approach we could effectively minimize the motion artefact caused by both voluntary and involuntary movements of the animal (respiration and heartbeat). A polymethyl pentene (PMP) film was glued to the lower surface of the inset chamber, as this lightweight, functional polymer has a tissue-like acoustic impedance that allows undistorted propagation of ultrasound waves. In addition, it provides excellent optical clarity and has good elasticity (Heo *et al.*, 2016).

Additionally, the designed inset element is flexible to be exchanged between measurements, thus providing accessibility for the delivery of different pharmacological agents or viruses into tissue.

Motorized fUS holder

To enable three-dimensional fUS imaging of freely moving subjects, I designed a module equipped with stepper motors that supports the 3-dimensional mapping of neural activity. The module was designed with key considerations in mind: maximizing the scannable area, minimizing size and weight, and ensuring stable imaging.

Nanotec's NEMA 8 motor (LA201S06-B-TDBA) proved to be an excellent choice for our purposes, as it is one of the smallest stepper motors available. With a step resolution of $5\mu\text{m}$, its compact size (20 mm x 20 mm x 33 mm), and a relatively light weight (54 g), it fulfilled the necessary criteria.

To achieve precise motor control, we utilized an Arduino Uno R3 microcontroller coupled with an Arduino Motor Shield Rev3 board. The AccelStepper library served as a programming tool, allowing us to tailor motor motions to our specific requirements.

The main component of the designed motorized system is depicted on the Figure 8.

By incorporating an intermediate interface, the motorized module can be easily connected to the chronic element. The axisymmetric design of the interface enables scanning of both sagittal and coronal axis, resulting in a scan area of 18.5 x 15.9 mm in both directions. Including the motors, the total weight of the construction is 150 g, which is less than 5% of the average cat's body weight.

Manufacturing

The designed elements were manufactured by using the Formlabs Form 2 desktop SLA printer. Rigid 10K resin was selected for its outstanding mechanical properties such as rigidity and tensile strength. Implants made from Rigid 10K resin maintained their structural integrity even under higher mechanical stress. To ensure biocompatibility, the sides of the chronic implant was

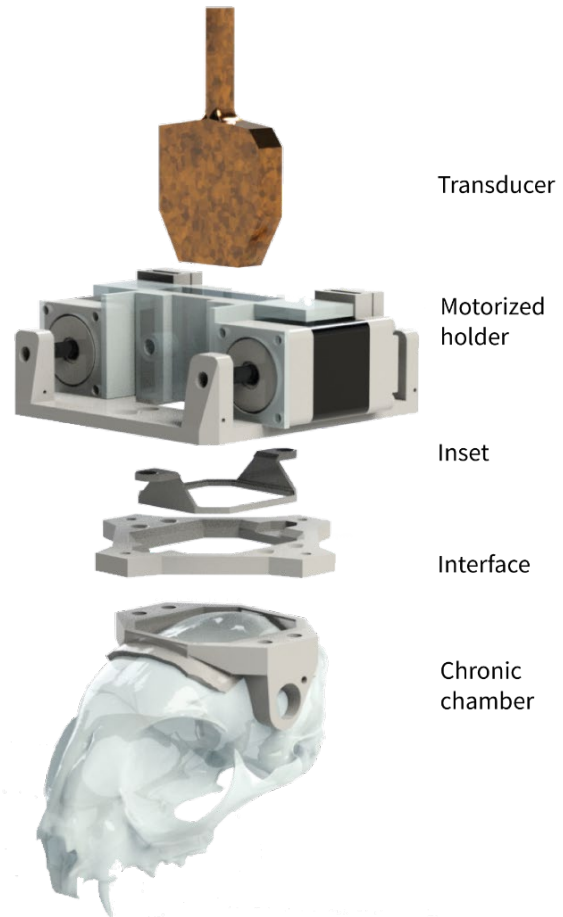


Figure 8. Exploded view of the motorized fUS implant system (SolidWorks)

coated with Palacos bone cement during surgery, promoting adherence of the skin and tissues to the chamber.

Validating the stability of the interface

The motorized implant system was validated by in vivo measurements. The images depicted in Figure 9 were acquired utilizing the Bmode and μ Doppler functions. The Bmode imaging is suitable for real-time visualization of brain morphology, thus aiding navigation in the brain prior to measurements, while the μ Doppler imaging technique is very sensitive to small tissue movements allowing us to check the stability of the transducer fixation before the stimulation protocol is initiated.

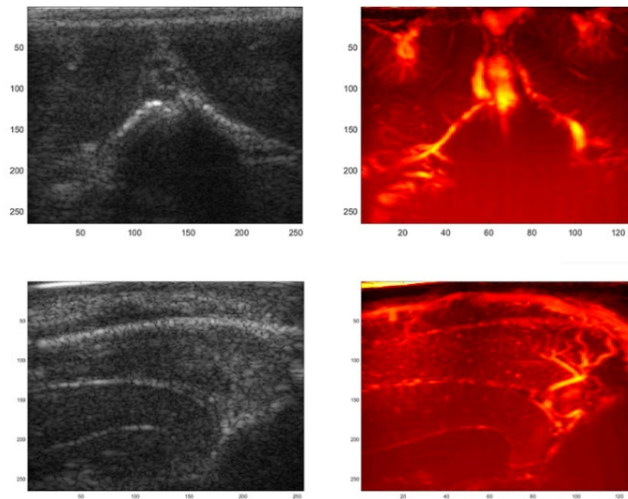


Figure 9. Bmode (left) and μ Doppler (right) images of the coronal (top row) and sagittal (bottom row) planes of the cat brain.

In vivo measurements

For in vivo ultrasound scanning, a 12 MHz transducer was used to capture images in multiple sagittal sections of the primary visual cortex. This transducer has the capability to detect activity at a depth of up to 15 mm within the brain, which allows us to monitor the complete cross-section of the cat's neocortex and even a fraction of the subcortical regions. The imaging frequency was 2 Hz. We utilized different stimulation protocols for measurements conducted during anesthesia and awake conditions.

During the anesthetized measurements (Alfaxalone: 5mg/kg/h, Ketamine: 0.72 mg/kg/h), drifting grating stimulation was projected on a monitor positioned 40 cm away from the animal's head. During one trial, a gray screen appeared in front of the animal for 10 seconds, then for

the next 6 seconds a grating pattern (spatial frequency: 0.05 cycles/second, temporal frequency: 1 cycle/second) drifted across the entire screen in a defined direction, followed by a steady gray screen for 10 seconds. Two different orientations of grating (0° , 270°) were applied during a complete protocol. Each protocol was repeated five times on a given slice to increase the signal-to-noise ratio. To obtain a three-dimensional map of brain activity, the stimulation protocol was replicated on 5 consecutive parasagittal planes over the marginal gyrus, with 200 μm step intervals.

For the awake measurements, we used zig-zag stimulation. We utilized grating stimulation with a spatial frequency of 0.5 cycles/degree and a temporal frequency of 2 cycles/second. The grating stimulation was projected in four different orientations, namely 0° , 225° , 270° , and 315° . In both types of stimulation, the protocol began with a 5 second baseline period (either gray screen or static grating pattern), which was followed by a 6 second drifting stimulation wherein the direction of movement changed in every 2 seconds. Finally, a 7 second baseline period concluded the trial. We repeated the measurement 5 times for a given orientation. Both measurements were performed in the same parasagittal slice over the marginal gyrus. Figure 10. illustrates the different orientations of the gratings utilized in the measurements.

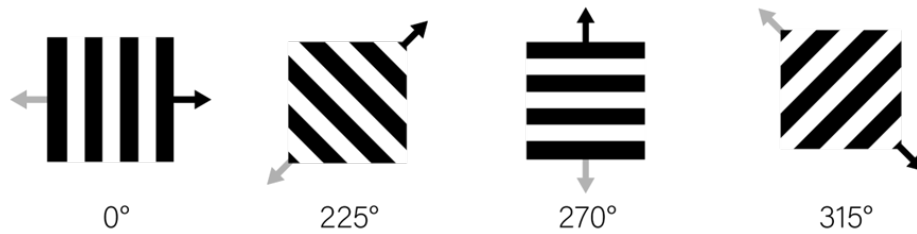


Figure 10. Schematic representation of different orientations of gratings, with arrows denoting the direction of movements. The gray arrows indicate the change in direction during zigzag stimulation.

Analysis pipeline

During functional ultrasound recordings, the CBV changes in the brain are captured with Doppler imaging during the baseline and the stimulation period. The acquired CBV signals are obtained for different grating orientations in multiple recording sessions. After preprocessing, the CBV signals are reconstructed into a 3D matrix format, where the third dimension represents the intensity changes of the captured image over time. The signals are averaged over the trials for each orientation, respectively.

To determine which region of the brain was active during each stimulus orientation I have implemented the following analysis steps in our analysis pipeline:

- 1) Generating predicted modulated stimulus pattern by convolving a hemodynamic response function with the stimulus pattern.

The hemodynamic response function (HRF) represents the characteristic temporal response of the brain's hemodynamic changes to neuronal activity. It is typically modeled as a mathematical function that approximates the observed physiological changes in blood flow, oxygenation, and volume. To generate the predicted modulated stimulus pattern, we can take the stimulus pattern, which represents experimental condition (e.g., duration of the visual stimulation) and convolve it with the HRF. This predicted pattern represents the expected changes in the fUS signal based

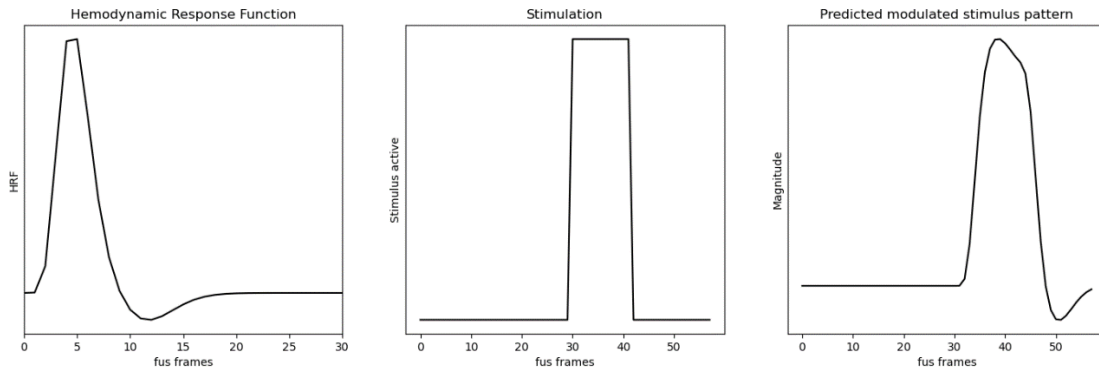


Figure 11. A predicted modulated stimulus pattern generated by convolving the hemodynamic response function with the stimulation pattern.

on the assumed relationship between neuronal activity and hemodynamic response (Figure 11.).

- 2) Producing correlation map by calculating the cross-correlation between the pixel signal over time and the predicted modulated stimulus pattern for each individual pixel.

By comparing the predicted and observed signals, we can determine the extent to which the measured fUS data aligns with the expected pattern of hemodynamic response. This approach allows us to detect spatial patterns of brain activity that are specifically associated with the stimuli.

- 3) Determine the statistical significance of the correlating pixels.

For each pixel in the correlation map, a t-score is calculated to measure the strength of the correlation while considering the sample size and variability. Using the t-score, one-tailed p-values are computed to assess the likelihood of observing a correlation as extreme or more extreme than what would be expected if there was no correlation. To determine statistical significance, a significance threshold (alpha) is chosen. In our case, a threshold of 0.05 is used, meaning that p-values below this threshold are considered statistically significant. Based on the

chosen significance threshold, a binary mask is created, designating pixels as significant if their associated p-values fall below the threshold.

Finally, the binary mask is displayed as an activity map over the Doppler image to identify active regions. This visualization enables us to identify and locate the active regions in the brain and provide a comprehensive and systematic approach to understand brain activity patterns during different stimulus orientations.

IV. Results

Extracting activation maps

To identify the active regions of the brain during stimulation, activity maps were generated for each orientation in each imaging plane using the analysis pipeline described in section III.

Figure 12b illustrates the time course of stimulation-induced hemodynamic responses (orange and blue curves) for two stimulus orientation (0° , 270°) originating from a manually chosen cortical region (Fig. 12a). The green curve representing the predicted modulated stimulus pattern closely approximates the actual CBV responses.

In order to evaluate the correlation between the predicted stimulus pattern and the actual response, cross-correlation was computed for each pixel. The correlation maps for one orientation (270°) are displayed in sub-figures c) and e), representing anesthetized and awake measurements, respectively. By employing a statistical significance test, the visually activated regions of the brain could be identified (Fig.12d,12f).

During the awake measurement, we could obtain a significantly improved signal-to-noise ratio in the correlated CBV responses compared to the measurements conducted under anesthesia. The activity maps demonstrate that a significantly larger region of the cortex was active during the awake measurements (Fig.12f), in comparison to anesthetized measurement (Fig.12d).

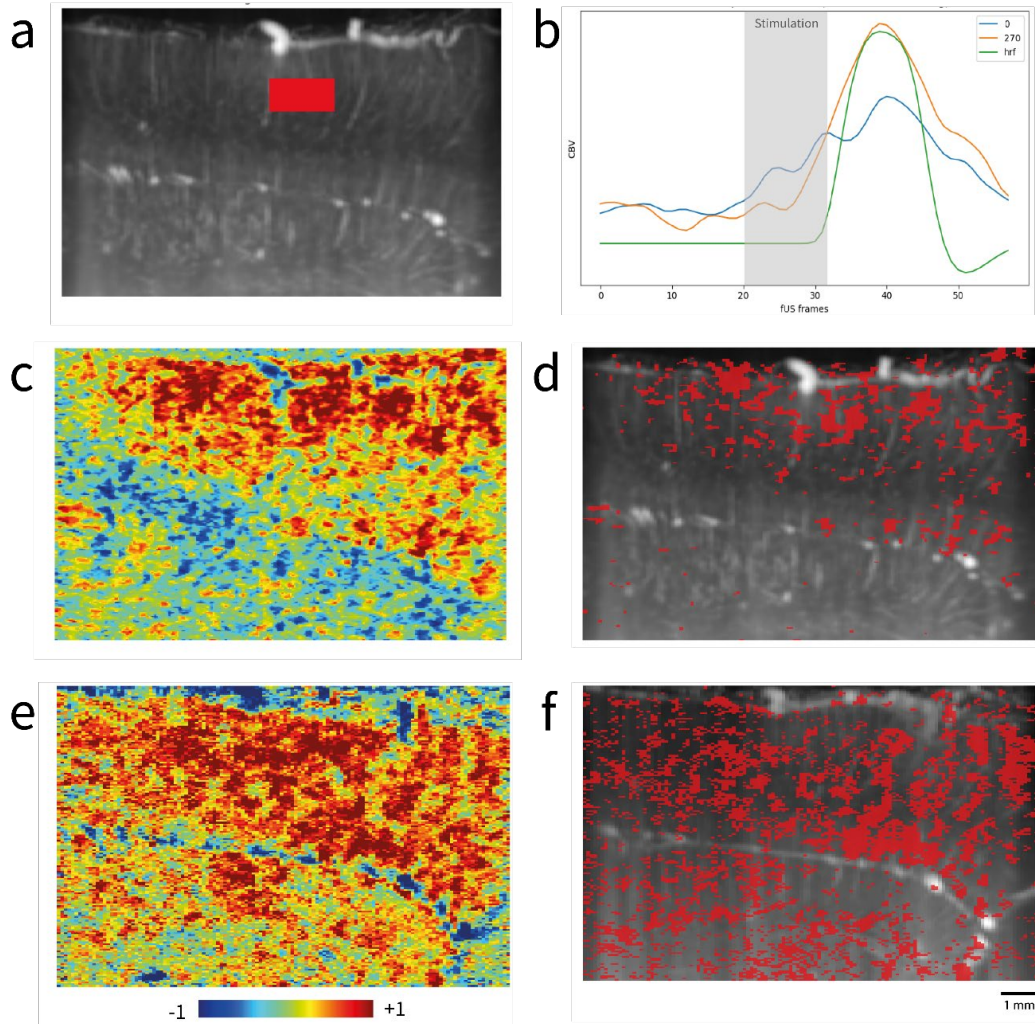


Figure 12. a) Time-averaged Doppler image in a sagittal cross-section of the cat brain. b) Visual stimulation induced CBV signal in anesthetized animals displayed with orange and blue curves for each stimulus orientation (0° , 270°) as a function of time, after applying Gaussian filter. The green curve represents the predicted modulated stimulus pattern labelled as hrf. c) Correlation map for the 270° stimulus orientation. Positive correlation values, depicted using warm colors, indicate a strong association between the predicted and actual response. Negative correlations, represented by cooler colors, indicating a dissimilar or opposing relationship between the predicted and actual responses. d) Activity map for the 270° stimulus orientation showing significance activation. e) Correlation map for the 270° stimulus orientation in awake animal. f) Activity map for the 270° stimulus orientation in awake animal.

Orientation preference mapping

The orientation preference maps were created by color-coding regions that exhibited significant activation to different stimulation orientations and projecting them onto the time-averaged Doppler image. In cases where regions exhibited significant activation for more than one stimulus orientations, their assignment to a specific orientation was determined based on the strength of correlation. Figure 13. shows the active regions detected during anesthesia for each orientation (red: 0° , blue: 270°) across five consecutive sagittal planes.

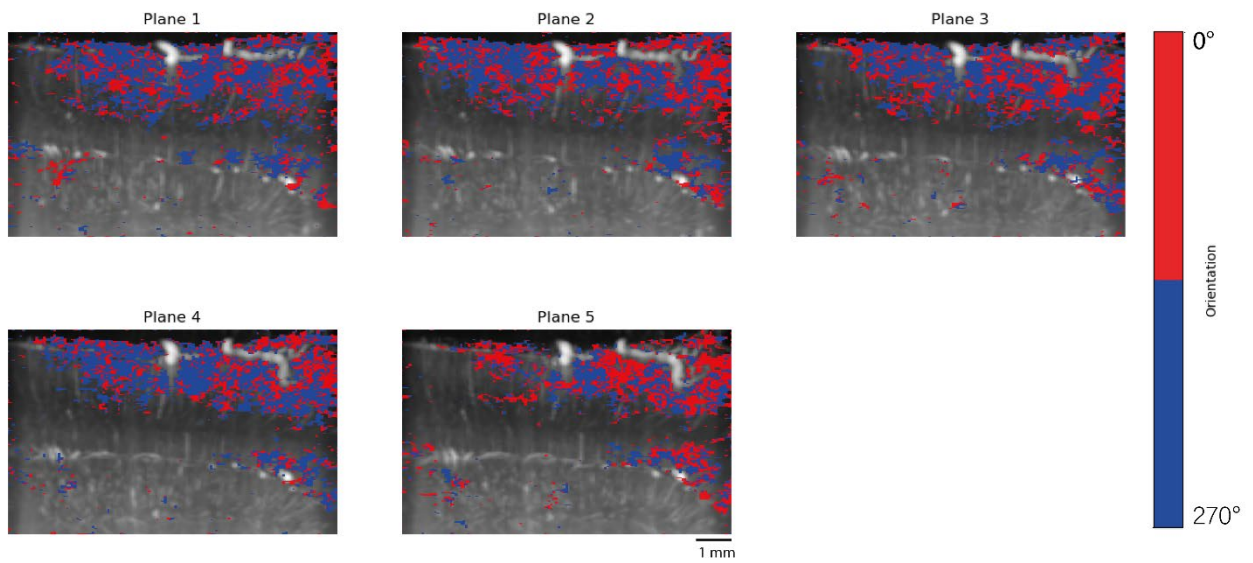


Figure 13. Orientation preference map in the cat visual cortex recorded in 5 consecutive parasagittal planes using fUSI. The orientation preferences of the activating pixels are represented by colors based on the color bar displayed on the right side.

Although the columnar structure described in the previous literature is not clearly visible, clusters of regions can be detected on each plane. These clusters appear to be considerably distorted, potentially due to several factors. One possible reason for distorted structure lies on that we indirectly measure neural activity, and the hemodynamic responses can propagate to the adjacent areas, therefore single orientation stimulus can activate multiple neighboring columns. Additionally, the use of only a subset of stimuli can significantly affect the map obtained (Issa et al., 2000).

Therefore, in the awake measurements we addressed this issue by covering the stimulation space more uniformly, by employing orientation 0° , 225° , 270° , and 315° .

The Figure 14 demonstrates the orientation preference maps generated for "static grating –zig-zag" type of stimulation (described in section III. Visual stimuli) in awake animal. Notably, we have observed a strong dependency of the orientation map structure on the chosen latency value of the predicted modulated stimulus pattern.

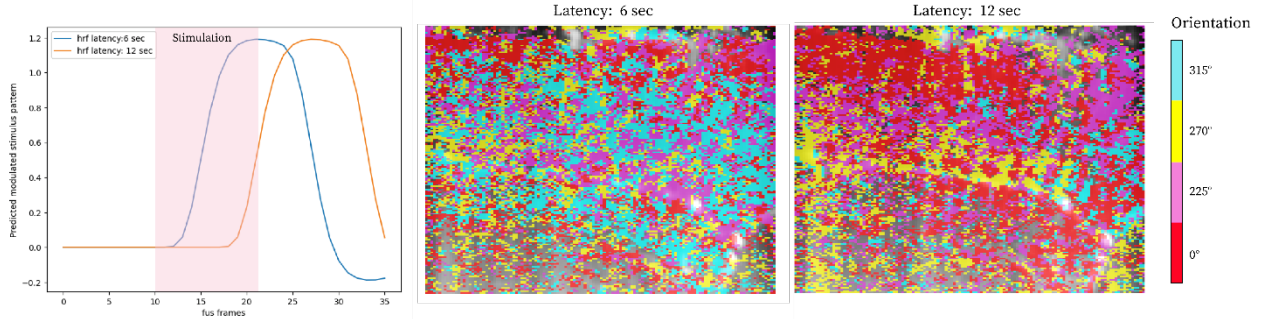


Figure 14. Left: Two predicted modulated stimulus patterns shifted with 6 seconds (12 fUS frames). Middle: Orientation preference map generated by the "low latency" (6 seconds) pattern in a sagittal imaging plane of a cat's brain. Right: Orientation preference map generated by the "high latency" (12 seconds) pattern. The orientation preferences of the activating pixels are color-coded according to the colour bar shown on the right.

These results are correlating with previous findings observed with fMRI and OIS imaging, which demonstrated that non-specific hemodynamic responses decreased with the stimulus duration, while the specific responses increased or remained constant (Moon, Fukuda and Kim, 2013; Fukuda, Poplawsky and Kim, 2016). Consequently, employing a longer stimulation time and selecting a longer latency value for generating the predicted modulated stimulus pattern can significantly enhance the spatial specificity of the orientation preference maps.

Figure 15 displays the orientation maps resulting from two distinct types of stimulation (gray vs. static grating baseline). Upon comparison, similarities can be observed, although there are notable differences in certain areas. The underlying cause of these differences is likely due to the eye movements during the awake measurement, which can significantly effect the response

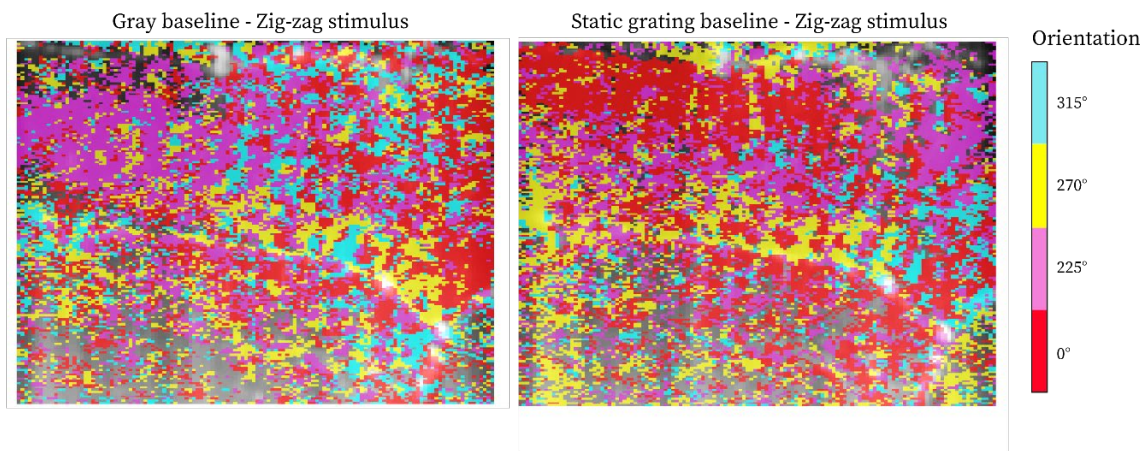


Figure 15. Orientation preference maps of two different stimulus designs measured in the same parasagittal section in the visual cortex of awake cats. The two stimulus designs differed in their baseline period. The orientation preferences of the activating pixels are represented by colors based on the color bar displayed on the right side.

dynamics. (The future direction of the research includes training the animals for gaze fixation and incorporating an eye-tracking apparatus to enhance the accuracy and consistency of our measurements.)

Nonetheless, I conducted measurements to determine the distance between adjacent iso-orientational bands present in both maps, which ranged between 1.2 and 1.6 mm. These values align closely with the reported distance between iso-orientation columns in optical imaging studies (1.07 ± 0.27 mm) (Chenchal Rao, Toth, & Sur, 1997). Although further investigation is needed to confirm these findings, these observations suggests that these bands are likely represent iso-orientation domains.

Consistency test

In order to assess the consistency of activating pixels in three dimensions, I calculated the total number of activated pixels along the medial-lateral axis separately for each orientation. These values were then plotted within a single parasagittal plane.

Based on the observations from Figure 16, it appears that pixels exhibiting the highest consistency (pixels showing activation in more than 80% of adjacent imaging planes along the medial-lateral dimension at the same dorsal-ventral/rostral-caudal position) were predominantly situated at the center of clusters comprising similarly consistent pixels.

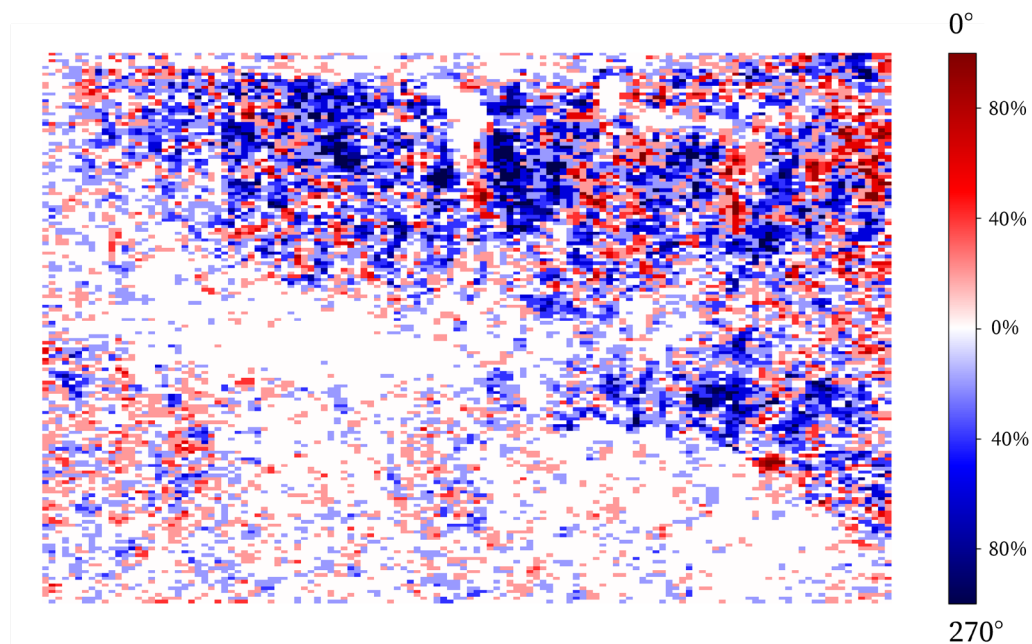


Figure 16. Consistency map of the anesthetized measurement. Significantly activated fUS pixels in response to the different orientational grating (0° , 270°) across five imaging planes, projected onto a single parasagittal plane. The color code of the image represents the percentage of planes in which pixels exhibit significant activation at the same location (e.g.: a value of 100% indicates that the pixel is activated for the same orientation in all five planes.)

Conversely, pixels with lower consistency (with 20-40% consistent pixel activations across imaging planes) were more likely found at the periphery of these clusters, while pixels with medium consistency were located between regions of high and low consistency. Collectively, these findings suggest that fUS activations can potentially discern spatially restricted, isotropic domains.

V. Conclusion

In this thesis, I present an extensive overview of the assessment of the functional cortical organization in large-bodied preclinical species. In the technological and scientific overview, I have provided a comprehensive description of functional measurement tools and their corresponding advancements, and demonstrated how these tools have contributed to the discovery of processes taking place in the primary visual cortex. By discussing the limitations associated with each method, I have emphasized the pressing need for the development of a new methodology capable of investigating the layout and interrelationships of cortical maps in the deeper layers of the cortex.

To address this issue, I have developed a novel modular implant system, which enables the investigation of deep cortical layers in three dimensions by leveraging functional ultrasound imaging. Furthermore, I presented the core components of the experimental setup, including the stimulation design, in vivo measurement protocols, and analysis pipeline. The established measurement method exhibits promising potential for mapping the functional organization of the visual cortex in-depth, both in anesthetized and in awake animals.

While further development and measurement are necessary to fully validate the system, preliminary data indicate a high probability of achieving high-resolution, three-dimensional functional maps of the visual cortex in the near future. This significant advancement would contribute to a deeper understanding of how the visual system organizes itself to process and represent visual information.

VI. Future plans

In the upcoming phase, I intend to refine the stimulation protocol and analysis procedures in order to create a comprehensive three-dimensional representation of orientation preference maps. Furthermore, I am committed to generating different types of functional maps, such as ocular dominance, spatial frequency, and temporal frequency maps, and exploring the interrelationships among these functional organizations within the deeper cortical layers.

To ensure the reliability of the obtained data, my aim is to cross-validate the acquired data by the functional ultrasound with our wide-field fluorescence imaging system. The modular implant system presented in this thesis allows for the asynchronous use of both modalities in the same animal. Through the comparative analysis of functional maps obtained in the superficial layers (layer I, II, III) via widefield fluorescence and functional ultrasound imaging, we can examine and contrast the responses arising from neural activity and vascular dynamics. This endeavor represents a significant methodological advancement as it establishes a correspondence between the two modalities, allowing for a deeper understanding of the relationship between neural and vascular processes.

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Dániel Hillier, for his tireless dedication, energy, and support, without which this thesis would not have been possible. I am also grateful to Zoltán Vidnyánszky and István Homolya for facilitating the creation of the MRI recordings, as well as to Alan Urban, Dries Kil, and Gabriel Montaldo for their indispensable assistance with the fUS experiments. Special thanks go to the members of the research group, particularly Domonkos Horváth, Ábel Petik, Kinga Gintner, and Fanni Somogyi, whose expertise and ideas significantly contributed to my work. Additionally, I wish to express my gratitude to Gergely Márton and István Ulbert for their mentorship. I am also thankful to the New National Excellence Program for their scholarship support, which enabled the completion of this work.

References

- Albright, T.D. (1984) 'Direction and orientation selectivity of neurons in visual area MT of the macaque', *Journal of Neurophysiology*, 52(6), pp. 1106–1130. Available at: <https://doi.org/10.1152/jn.1984.52.6.1106>.
- Allman, J.M. and Kaas, J.H. (1971) 'Representation of the visual field in striate and adjoining cortex of the owl monkey (*Aotus trivirgatus*)', *Brain Research*, 35(1), pp. 89–106. Available at: [https://doi.org/10.1016/0006-8993\(71\)90596-8](https://doi.org/10.1016/0006-8993(71)90596-8).
- Barlow, H.B., Blakemore, C. and Pettigrew, J.D. (1967) 'The neural mechanism of binocular depth discrimination', *The Journal of Physiology*, 193(2), pp. 327–342. Available at: <https://doi.org/10.1113/jphysiol.1967.sp008360>.
- Bartfeld, E. and Grinvald, A. (1992) 'Relationships between orientation-preference pinwheels, cytochrome oxidase blobs, and ocular-dominance columns in primate striate cortex.', *Proceedings of the National Academy of Sciences of the United States of America*, 89(24), pp. 11905–11909.
- Bimbard, C. *et al.* (2018) 'Multi-scale mapping along the auditory hierarchy using high-resolution functional UltraSound in the awake ferret', *eLife*. Edited by A.J. King, 7, p. e35028. Available at: <https://doi.org/10.7554/eLife.35028>.
- Blaize, K. *et al.* (2020) 'Functional ultrasound imaging of deep visual cortex in awake nonhuman primates', *Proceedings of the National Academy of Sciences*, 117(25), pp. 14453–14463. Available at: <https://doi.org/10.1073/pnas.1916787117>.
- Bonhoeffer, T. (1995) 'Optical imaging of intrinsic signals as a tool to visualize the functional architecture of adult and developing visual cortex', *Arzneimittel-Forschung*, 45(3A), pp. 351–356.

- Bonhoeffer, T. and Grinvald, A. (1991) 'Iso-orientation domains in cat visual cortex are arranged in pinwheel-like patterns', *Nature*, 353(6343), pp. 429–431. Available at: <https://doi.org/10.1038/353429a0>.
- Bosking, W.H. *et al.* (1997) 'Orientation Selectivity and the Arrangement of Horizontal Connections in Tree Shrew Striate Cortex', *Journal of Neuroscience*, 17(6), pp. 2112–2127. Available at: <https://doi.org/10.1523/JNEUROSCI.17-06-02112.1997>.
- Brunner, C. *et al.* (2021) 'Whole-brain functional ultrasound imaging in awake head-fixed mice', *Nature Protocols*, 16(7), pp. 3547–3571. Available at: <https://doi.org/10.1038/s41596-021-00548-8>.
- Castaldi, E., Lunghi, C. and Morrone, M.C. (2020) 'Neuroplasticity in adult human visual cortex', *Neuroscience & Biobehavioral Reviews*, 112, pp. 542–552. Available at: <https://doi.org/10.1016/j.neubiorev.2020.02.028>.
- Cho, S. *et al.* (2022) 'Cortical layer-specific differences in stimulus selectivity revealed with high-field fMRI and single-vessel resolution optical imaging of the primary visual cortex', *NeuroImage*, 251, p. 118978. Available at: <https://doi.org/10.1016/j.neuroimage.2022.118978>.
- Crair, M.C. *et al.* (1997) 'Ocular Dominance Peaks at Pinwheel Center Singularities of the Orientation Map in Cat Visual Cortex', *Journal of Neurophysiology*, 77(6), pp. 3381–3385. Available at: <https://doi.org/10.1152/jn.1997.77.6.3381>.
- DeAngelis, G.C. *et al.* (1999) 'Functional Micro-Organization of Primary Visual Cortex: Receptive Field Analysis of Nearby Neurons', *The Journal of Neuroscience*, 19(10), pp. 4046–4064. Available at: <https://doi.org/10.1523/JNEUROSCI.19-10-04046.1999>.
- Demené, C. *et al.* (2017) 'Functional ultrasound imaging of brain activity in human newborns', *Science Translational Medicine*, 9, p. eaah6756. Available at: <https://doi.org/10.1126/scitranslmed.aah6756>.
- Dräger, U.C. (1975) 'Receptive fields of single cells and topography in mouse visual cortex', *The Journal of Comparative Neurology*, 160(3), pp. 269–290. Available at: <https://doi.org/10.1002/cne.901600302>.
- Franceschini, M.A. *et al.* (2010) 'The effect of different anesthetics on neurovascular coupling', *NeuroImage*, 51(4), pp. 1367–1377. Available at: <https://doi.org/10.1016/j.neuroimage.2010.03.060>.
- Frostig, R.D. *et al.* (1990) 'Cortical functional architecture and local coupling between neuronal activity and the microcirculation revealed by in vivo high-resolution optical imaging of intrinsic signals.', *Proceedings of the National Academy of Sciences of the United States of America*, 87(16), pp. 6082–6086.
- Fukuda, M. *et al.* (2006) 'Mapping Iso-Orientation Columns by Contrast Agent-Enhanced Functional Magnetic Resonance Imaging: Reproducibility, Specificity, and Evaluation by Optical Imaging of Intrinsic Signal', *The Journal of Neuroscience*, 26(46), pp. 11821–11832. Available at: <https://doi.org/10.1523/JNEUROSCI.3098-06.2006>.
- Fukuda, M., Poplawsky, A.J. and Kim, S.-G. (2016) 'Chapter 6 - Submillimeter-resolution fMRI: Toward understanding local neural processing', in K. Masamoto, H. Hirase, and K. Yamada (eds) *Progress in Brain Research*. Elsevier (New Horizons in Neurovascular Coupling: A Bridge Between Brain Circulation and Neural Plasticity), pp. 123–152. Available at: <https://doi.org/10.1016/bs.pbr.2016.03.003>.
- Garg, A.K. *et al.* (2019) 'Color and orientation are jointly coded and spatially organized in primate primary visual cortex', *Science*, 364(6447), pp. 1275–1279. Available at: <https://doi.org/10.1126/science.aaw5868>.
- Ghose, G.M. and Ts'O, D.Y. (1997) 'Form Processing Modules in Primate Area V4', *Journal of Neurophysiology*, 77(4), pp. 2191–2196. Available at: <https://doi.org/10.1152/jn.1997.77.4.2191>.
- Girman, S.V., Sauvé, Y. and Lund, R.D. (1999) 'Receptive field properties of single neurons in rat primary visual cortex', *Journal of Neurophysiology*, 82(1), pp. 301–311. Available at: <https://doi.org/10.1152/jn.1999.82.1.301>.
- Grinvald, A. (1985) 'Real-time optical mapping of neuronal activity: from single growth cones to the intact mammalian brain', *Annual Review of Neuroscience*, 8, pp. 263–305. Available at: <https://doi.org/10.1146/annurev.ne.08.030185.001403>.
- Grinvald, A. *et al.* (1986) 'Functional architecture of cortex revealed by optical imaging of intrinsic signals', *Nature*, 324(6095), pp. 361–364. Available at: <https://doi.org/10.1038/324361a0>.
- Grinvald, A. and Hildesheim, R. (2004) 'VSFI: a new era in functional imaging of cortical dynamics', *Nature Reviews Neuroscience*, 5(11), pp. 874–885. Available at: <https://doi.org/10.1038/nrn1536>.
- Helmchen, F. and Denk, W. (2005) 'Deep tissue two-photon microscopy', *Nature Methods*, 2(12), pp. 932–940. Available at: <https://doi.org/10.1038/nmeth818>.
- Heo, C. *et al.* (2016) 'A soft, transparent, freely accessible cranial window for chronic imaging and electrophysiology', *Scientific Reports*, 6(1), p. 27818. Available at: <https://doi.org/10.1038/srep27818>.
- Holmes, J.M. and Clarke, M.P. (2006) 'Amblyopia', *The Lancet*, 367(9519), pp. 1343–1351. Available at: [https://doi.org/10.1016/S0140-6736\(06\)68581-4](https://doi.org/10.1016/S0140-6736(06)68581-4).

- Hubel, D.H. and Wiesel, T.N. (1962) 'Receptive fields, binocular interaction and functional architecture in the cat's visual cortex', *The Journal of Physiology*, 160(1), pp. 106-154.2.
- Husson, T.R. *et al.* (2007) 'Functional Imaging of Primary Visual Cortex Using Flavoprotein Autofluorescence', *The Journal of Neuroscience*, 27(32), pp. 8665–8675. Available at: <https://doi.org/10.1523/JNEUROSCI.2156-07.2007>.
- Issa, N.P. *et al.* (1999) 'The Critical Period for Ocular Dominance Plasticity in the Ferret's Visual Cortex', *Journal of Neuroscience*, 19(16), pp. 6965–6978. Available at: <https://doi.org/10.1523/JNEUROSCI.19-16-06965.1999>.
- Issa, N.P., Trepel, C. and Stryker, M.P. (2000) 'Spatial Frequency Maps in Cat Visual Cortex', *Journal of Neuroscience*, 20(22), pp. 8504–8514. Available at: <https://doi.org/10.1523/JNEUROSCI.20-22-08504.2000>.
- Kara, P. and Boyd, J.D. (2009) 'A micro-architecture for binocular disparity and ocular dominance in visual cortex', *Nature*, 458(7238), pp. 627–631. Available at: <https://doi.org/10.1038/nature07721>.
- Kim, D.S. and Bonhoeffer, T. (1994) 'Reverse occlusion leads to a precise restoration of orientation preference maps in visual cortex', *Nature*, 370(6488), pp. 370–372. Available at: <https://doi.org/10.1038/370370a0>.
- Kim, D.-S., Duong, T.Q. and Kim, S.-G. (2000) 'High-resolution mapping of iso-orientation columns by fMRI', *Nature Neuroscience*, 3(2), pp. 164–169. Available at: <https://doi.org/10.1038/72109>.
- Kiper, D.C. (2003) 'Colour and form in the early stages of cortical processing', *The Journal of Physiology*, 548(Pt 2), p. 335. Available at: <https://doi.org/10.1113/jphysiol.2003.039974>.
- Klioutchnikov, A. *et al.* (2020) 'Three-photon head-mounted microscope for imaging deep cortical layers in freely moving rats', *Nature Methods*, 17(5), pp. 509–513. Available at: <https://doi.org/10.1038/s41592-020-0817-9>.
- Krishnamoorthy-Natarajan, G. and Koide, M. (2016) 'Chapter Eleven - BK Channels in the Vascular System', in C. Contet (ed.) *International Review of Neurobiology*. Academic Press (Big on Bk), pp. 401–438. Available at: <https://doi.org/10.1016/bs.irm.2016.03.017>.
- Landisman, C.E. and Ts'o, D.Y. (2002) 'Color Processing in Macaque Striate Cortex: Relationships to Ocular Dominance, Cytochrome Oxidase, and Orientation', *Journal of Neurophysiology*, 87(6), pp. 3126–3137. Available at: <https://doi.org/10.1152/jn.2002.87.6.3126>.
- Levi, D.M., Knill, D.C. and Bavelier, D. (2015) 'Stereopsis and amblyopia: A mini-review', *Vision research*, 114, pp. 17–30. Available at: <https://doi.org/10.1016/j.visres.2015.01.002>.
- Levine, A.T. *et al.* (2020) 'Assessment of anesthesia on physiological stability and BOLD signal reliability during visual or acoustic stimulation in the cat', *Journal of Neuroscience Methods*, 334, p. 108603. Available at: <https://doi.org/10.1016/j.jneumeth.2020.108603>.
- Liedtke, J. (2017) *Genetic determination and layout rules of visual cortical architecture*. Georg-August-Universität Göttingen. Available at: https://ediss.uni-goettingen.de/bitstream/handle/11858/00-1735-0000-0023-3EE8-5/Dissertation_J_Liedtke.pdf?sequence=1 (Accessed: 9 June 2023).
- Livingstone, M. and Hubel, D. (1984) 'Anatomy and physiology of a color system in the primate visual cortex', *The Journal of Neuroscience*, 4(1), pp. 309–356. Available at: <https://doi.org/10.1523/JNEUROSCI.04-01-00309.1984>.
- Livingstone, M. and Hubel, D. (1988) 'Segregation of Form, Color, Movement, and Depth: Anatomy, Physiology, and Perception', *Science*, 240(4853), pp. 740–749. Available at: <https://doi.org/10.1126/science.3283936>.
- Macé, E. *et al.* (2011) 'Functional ultrasound imaging of the brain', *Nature Methods*, 8(8), pp. 662–664. Available at: <https://doi.org/10.1038/nmeth.1641>.
- Macé, É. *et al.* (2018) 'Whole-Brain Functional Ultrasound Imaging Reveals Brain Modules for Visuomotor Integration', *Neuron*, 100(5), pp. 1241-1251.e7. Available at: <https://doi.org/10.1016/j.neuron.2018.11.031>.
- Mallik, A.K. *et al.* (2008) 'The organization of spatial frequency maps measured by cortical flavoprotein autofluorescence', *Vision Research*, 48(14), pp. 1545–1553. Available at: <https://doi.org/10.1016/j.visres.2008.04.014>.
- Moon, C.H., Fukuda, M. and Kim, S.-G. (2013) 'Spatiotemporal characteristics and vascular sources of neural-specific and -nonspecific fMRI signals at submillimeter columnar resolution', *NeuroImage*, 64, pp. 91–103. Available at: <https://doi.org/10.1016/j.neuroimage.2012.08.064>.
- Moorthy, R. (2002) 'DOPPLER ULTRASOUND', *Medical Journal, Armed Forces India*, 58(1), pp. 1–2. Available at: [https://doi.org/10.1016/S0377-1237\(02\)80001-6](https://doi.org/10.1016/S0377-1237(02)80001-6).
- Nakamichi, Y. *et al.* (2018) '3D topology of orientation columns in visual cortex revealed by functional optical coherence tomography', *Journal of Neurophysiology*, 119(4), pp. 1562–1575. Available at: <https://doi.org/10.1152/jn.00921.2017>.

- Ogawa, S. *et al.* (1990) 'Brain magnetic resonance imaging with contrast dependent on blood oxygenation.', *Proceedings of the National Academy of Sciences of the United States of America*, 87(24), pp. 9868–9872.
- O'Herron, P. *et al.* (2016) 'Neural correlates of single-vessel haemodynamic responses in vivo', *Nature*, 534(7607), pp. 378–382. Available at: <https://doi.org/10.1038/nature17965>.
- Ohki, K. *et al.* (2006) 'Highly ordered arrangement of single neurons in orientation pinwheels', *Nature*, 442(7105), pp. 925–928. Available at: <https://doi.org/10.1038/nature05019>.
- Olman, C. *et al.* (2003) 'Retinotopic mapping in cat visual cortex using high-field functional magnetic resonance imaging', *Journal of Neuroscience Methods*, 131(1), pp. 161–170. Available at: <https://doi.org/10.1016/j.jneumeth.2003.08.009>.
- O'Shea, D.J. *et al.* (2017) 'The need for calcium imaging in nonhuman primates: New motor neuroscience and brain-machine interfaces', *Experimental Neurology*, 287, pp. 437–451. Available at: <https://doi.org/10.1016/j.expneurol.2016.08.003>.
- Schiessl, I. and McLoughlin, N. (2003) 'Optical imaging of the retinotopic organization of V1 in the common marmoset', *NeuroImage*, 20(3), pp. 1857–1864. Available at: <https://doi.org/10.1016/j.neuroimage.2003.07.023>.
- Shmuel, A. and Grinvald, A. (1996) 'Functional organization for direction of motion and its relationship to orientation maps in cat area 18', *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 16(21), pp. 6945–6964. Available at: <https://doi.org/10.1523/JNEUROSCI.16-21-06945.1996>.
- Sirovich, L. and Uglesich, R. (2004) 'The organization of orientation and spatial frequency in primary visual cortex', *Proceedings of the National Academy of Sciences of the United States of America*, 101(48), pp. 16941–16946. Available at: <https://doi.org/10.1073/pnas.0407450101>.
- Smith, S.L. and Trachtenberg, J.T. (2007) 'Experience-dependent binocular competition in the visual cortex begins at eye opening', *Nature Neuroscience*, 10(3), pp. 370–375. Available at: <https://doi.org/10.1038/nn1844>.
- Stolzberg, D. *et al.* (2017) 'Atlas: An magnetic resonance imaging-based three-dimensional cortical atlas and tissue probability maps for the domestic cat (*Felis catus*)', *The Journal of Comparative Neurology*, 525(15), pp. 3190–3206. Available at: <https://doi.org/10.1002/cne.24271>.
- Szentágothai, J. (1983) 'Cortical organization: A plea for better understanding, clearer definition, and more correct use of the term "column"', *Behavioral and Brain Sciences*, 6(1), pp. 169–171. Available at: <https://doi.org/10.1017/S0140525X00015326>.
- Tolhurst, D.J. and Thompson, I.D. (1982) 'Organization of neurones preferring similar spatial frequencies in cat striate cortex', *Experimental Brain Research*, 48(2), pp. 217–227. Available at: <https://doi.org/10.1007/BF00237217>.
- Tusa, R.J., Palmer, L.A. and Rosenquist, A.C. (1978) 'The retinotopic organization of area 17 (striate cortex) in the cat', *Journal of Comparative Neurology*, 177(2), pp. 213–235. Available at: <https://doi.org/10.1002/cne.901770204>.
- Warne, L.N. *et al.* (2015) 'A review of the pharmacology and clinical application of alfaxalone in cats', *The Veterinary Journal*, 203(2), pp. 141–148. Available at: <https://doi.org/10.1016/j.tvjl.2014.12.011>.
- White, L.E. and Fitzpatrick, D. (2007) 'Vision and Cortical Map Development', *Neuron*, 56(2), pp. 327–338. Available at: <https://doi.org/10.1016/j.neuron.2007.10.011>.
- Wiesel, T.N. and Hubel, D.H. (1963) 'SINGLE-CELL RESPONSES IN STRIATE CORTEX OF KITTENS DEPRIVED OF VISION IN ONE EYE', *Journal of Neurophysiology*, 26, pp. 1003–1017. Available at: <https://doi.org/10.1152/jn.1963.26.6.1003>.
- Williams, C. *et al.* (2008) 'Prevalence and risk factors for common vision problems in children: data from the ALSPAC study', *The British Journal of Ophthalmology*, 92(7), pp. 959–964. Available at: <https://doi.org/10.1136/bjo.2007.134700>.
- Xiao, Y. and Felleman, D.J. (2004) 'Projections from primary visual cortex to cytochrome oxidase thin stripes and interstripes of macaque visual area 2', *Proceedings of the National Academy of Sciences*, 101(18), pp. 7147–7151. Available at: <https://doi.org/10.1073/pnas.0402052101>.
- Xu, T. *et al.* (2015) 'Orientation selectivity in cat primary visual cortex: local and global measurement', *Neuroscience Bulletin*, 31(5), pp. 561–571. Available at: <https://doi.org/10.1007/s12264-014-1535-7>.

Attachments

The code for the analysis can be found on the following public links: <http://bit.ly/3CrEFbf>