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# **Rapidly mapping the brain of large animals using functional imaging**

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# Abstract

Amblyopia, a prevalent condition affecting a substantial portion of the global population, serves as an invaluable model for understanding neurodevelopmental disorders. This thesis provides an overview of amblyopia and highlights its potential as a gateway to unravelling the complexities of various related conditions. Building upon this foundation, a long-term research plan is outlined, aiming to map the functional organisation of both healthy and perturbed brains and subsequently compare them. The overarching objective is to enhance our understanding of developmental disorders and translate this knowledge into more effective diagnostic and treatment methods.

To accomplish these goals, the thesis discusses the prominent functional mapping techniques employed in neuroscience and proposes the integration of two specific methods to gain a comprehensive understanding of brain function. Notably, the experiments are conducted in large animals – cats –, a species much closer to the human in the organisation of its visual system, than the much more widely used rodent models.

The thesis then continues to describe the planning and development of the software environments required for conducting functional mapping experiments and analysing the acquired data. Detailed descriptions of the functional ultrasound imaging and wide field optical imaging software environments are provided, encompassing their feature sets, visual stimuli, and analysis protocols.

Lastly, the thesis presents the results obtained using the developed software, serving as validation for the methodological advancements achieved. The focus lies on the generation of orientation preference, ocular dominance, and retinotopy maps. Moreover, significant progress has been made in optimising the analysis program to provide real-time feedback to experimenters, facilitating its practical implementation.

In conclusion, this thesis implements neuroimaging techniques and methodologies in the quest to unravel the functional organisation of the healthy and perturbed brain. By leveraging the potential of amblyopia as a model, the work contributes to a better understanding of developmental disorders and paves the way for improved diagnostic and therapeutic strategies. The integration of functional ultrasound imaging and wide field optical imaging, combined with the developed software environments, demonstrates promising results, and sets the stage for further investigations in this field.

# I. Introduction

Vision is arguably the most important sense in our everyday lives. It enables us to perceive the world around us, navigate our environment, and interact with other individuals. Our ability to see provides us with information about the shape, colour, size, and texture of objects, which allows us to recognise them and make informed decisions. Vision also helps us to detect potential hazards and avoid danger, such as avoiding obstacles while walking or driving. In addition, our eyes contribute significantly to our social interactions by providing us with visual cues about the emotions and intentions of others. Any loss or impairment of vision has significant negative effects on our physical, emotional, and social well-being.

Both genetic and environmental factors can disrupt visual function. Monogenic causative factors can underlie retina degeneration that leads to blindness. Myopia has a dominant environmental origin that occurs during childhood if children do not have enough opportunity to play outside. Amblyopia is a developmental disease that has primary origin in the eyes with a clear – but not well-understood – genetic component. Left untreated, amblyopia develops into a significant loss of visual acuity in the part of the visual field viewed by the affected eye and can lead to unilateral blindness.

Identifying the origins of amblyopia remains important for two reasons. First, more than 100 million people live with the burden of amblyopia<sup>1</sup>, motivating the development of better diagnosis and treatment for affected patients. Second, amblyopia remains one of the cleanest models of developmental diseases. Brain development proceeds in posterior-to-anterior progressing critical periods of maturation<sup>2</sup>. The critical period of visual development is characterised by a highly plastic state of neuronal connectivity in subcortical and visual cortical brain regions. Altered visual input modifies the maturation of neuronal circuits and the incorrectly wired circuits cannot be modified once the critical period of visual development finishes at the end of the first decade of life. Animal models that largely recapitulate human amblyopia phenotypes are established allowing the understanding of how developmental diseases occur, what physiological and molecular pathways get affected. Gaining insights into the development and origins of amblyopia, as well as the co-occurring plasticity mechanisms, can provide a framework for understanding other developmental conditions across various sensory and psychiatric domains.

Hubel and Wiesel made pioneering findings on the architecture of the visual cortex in cats in 1962, which were subsequently extended to macaque monkeys<sup>3,4</sup>. In the 1980s, Horton

demonstrated that the human visual cortex shares similar cortical architecture with cats and monkeys<sup>5</sup>. One could assume that lower mammalian species also share at least some of the architectural traits of human vision. Mice are the most widely used mammalian model species, yet strong evidence pointing at fundamental differences between mice and large-animal models were provided only in the 21<sup>st</sup> century<sup>6</sup>. Conversely, extensive research has focused on investigating functional domains such as ocular dominance, orientation preference, and spatial frequency in large animal models. This is why we have chosen the cat as our model animal for studying the visual system.

Our research paradigm relies on state-of-the-art functional imaging methods to map the healthy brain and its functionality, creating a baseline model from these recordings. We then introduce a perturbation into the system to model the disease of interest, specifically amblyopia. After the perturbation, we redo the mappings to create a model of the disease and to observe how it changes the functional structure of the affected visual areas. By comparing the two different models, we can gain insights into how the disease affects the functional structure of the visual system.

Currently, we are in the initial stage of this process, which involves developing our imaging methods and mapping the functional structure of the healthy brain. My main contribution to the project is the development of software environments for performing and analysing the great number of imaging experiments, that are needed for such a comparative study. My responsibilities also include experiment planning, execution, and analysis, with hardware development as needed, which includes hardware assembly and testing.

## II. Amblyopia and research – an overview

### 1. Amblyopia

The first known usage of the term amblyopia was by Hippocrates in the fifth century BC. The word could be translated as dull vision, and it was used to describe reduced visual acuity corresponding to eyes that appear to be healthy<sup>7</sup>. Therefore, it was used as a more general term than today. Despite its early recognition, some of the most basic constituents of its aetiology were only explained following the neurophysiological experiments of Hubel and Wiesel in the early 1960s<sup>8,9</sup>.

The global prevalence of amblyopia is 1.44%<sup>1</sup>, and according to different estimates, it affects 1.3% to 3.6% of children below the age of 9 years, making it the most frequent cause of monocular visual loss in minors<sup>10</sup>. Most affected individuals suffer long-term effects of the condition, even if therapy is applied to improve visual acuity following generally accepted protocols<sup>11</sup>. Without such treatment, the long-term effects are expected to be worse. The consequences of amblyopia include permanent disruption of binocular vision, diminished fine motor skills, lower school performance and can even disrupt social interactions<sup>12–14</sup>. Based on these, a better understanding of amblyopia is clearly something worth pursuing.

#### a. Amblyopia as a model for developmental disruption of brain function

The primary factors underlying the development of amblyopia have been extensively studied, with some showing a strong dependence on genetic factors, including strabismus<sup>15</sup>, myopia, hyperopia and astigmatism<sup>16</sup>. These factors disrupt normal brain development, a phenomenon observed in various neurological conditions. By inducing amblyopia in animal models, we create a controlled experimental model not only for amblyopia but also for other conditions resulting from disrupted brain development. Consequently, if we can modify or reverse the progression of this well-established brain disorder, we may uncover insights and develop effective interventions applicable to a wide range of sensory and neurological conditions.

Therefore, our research has implications beyond amblyopia, extending to numerous individuals affected by various neural developmental disorders. By unravelling the mechanisms and discovering appropriate therapeutic approaches, we have the potential to benefit individuals impacted by diverse neurological conditions.

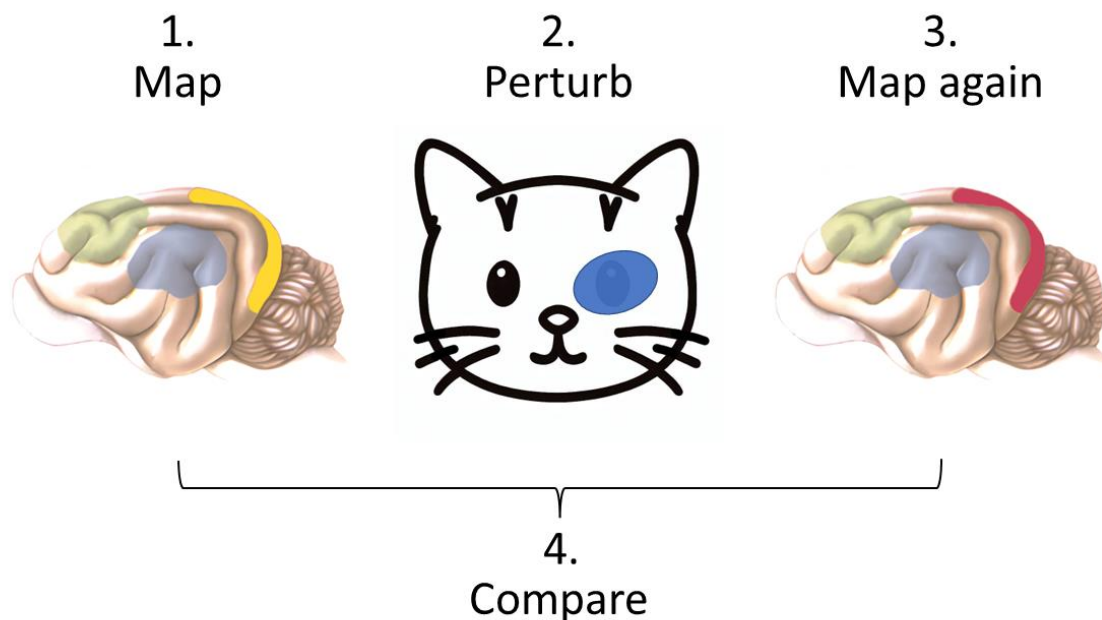
## 2. Research paradigm

As it was mentioned in the previous section, amblyopia causes functional and structural changes in the visual cortex. I believe that the identification and understanding of these changes are crucial for the development of more effective treatment for patients. Most methods granting access to the brain at the meso- to microscale are too invasive to be used in humans. We instead perform mapping experiments in model animals to gain a better understanding of the neural mechanisms underlying amblyopia.

The cortical neurons of higher-order mammals such as cats, primates and humans exhibit a spatial clustering based on their functional properties. This spatial organisation can depend on e.g., which eye innervates the given neuron. Mapping this organisation experimentally in a region results in a functional map called the ocular dominance map. Such functional maps can give us insight into how the given brain area operates<sup>17</sup>.

I propose the following paradigm for my research (Figure 1).

First, I aim to create functional mappings of the healthy brain, focusing on the brain regions involved in vision, such as the lateral geniculate nucleus, the primary visual cortex, and higher-order visual areas. It is well established that functional maps can vary significantly among



**Figure 1 Illustration of the research paradigm**

The first step is the creation of functional maps from the healthy brain. Second is the introduction of perturbation, monocular deprivation, to the animal, to have a model of the condition. The third step is the creation of the functional maps from the disease model animal. Lastly, the comparison of the healthy and diseased brain maps. Please note, that generally it is not possible to acquire the healthy and diseased maps from the same animal, as monocular deprivation for modelling amblyopia is only effective in the critical period of development, therefore the healthy maps would have to be recorded before the visual system is completely matured, which would result in erroneous data.

individuals<sup>18</sup>. Consequently, in order to account for these variations, it is crucial to conduct mapping studies with a large number of subjects. This highlights the need for a highly optimised mapping protocol. By generating these functional maps in multiple animals, I will establish a baseline model to serve as a reference for comparing with the diseased brain. Methods for producing functional maps and the most relevant map types for my research are described in subsections 3 and 4 respectively.

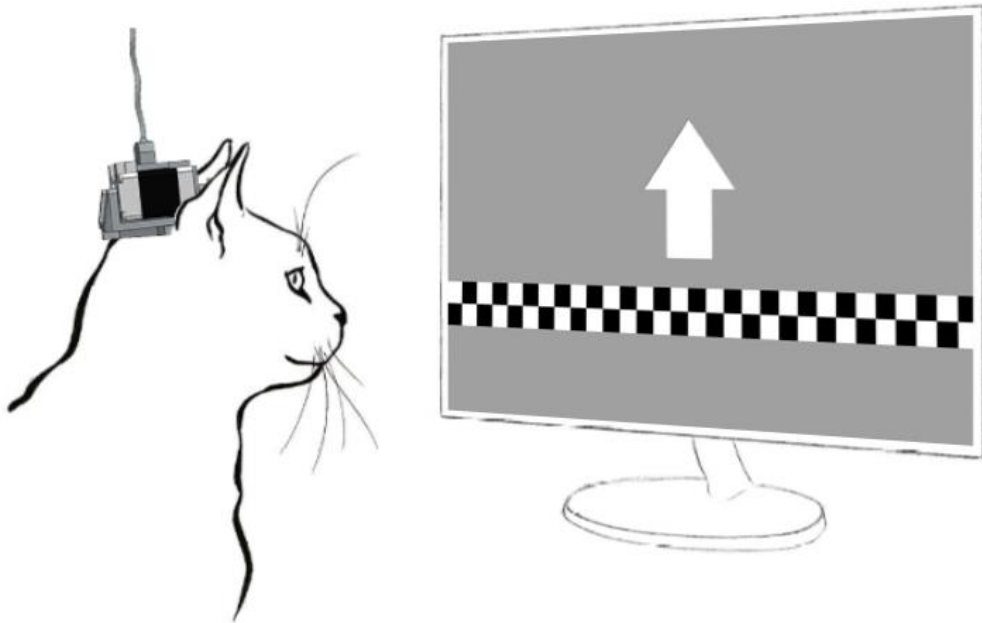
In the second step, I will introduce a perturbation into the system, which, in the case of amblyopia, is typically monocular deprivation, i.e., covering one eye during the critical period of development, or some other form of eye inhibition. Monocular deprivation has been the most widely adopted method for creating a disease model for amblyopia. It is important to note however, that different types of amblyopia based on their cause exhibit diverse effects, for example in the eye preference characteristics of neurons in the striate cortex of monocularly deprived opposed to strabismic kittens<sup>19</sup>. For this reason, we are also exploring newer techniques to induce perturbations that are more precisely controlled. Through these perturbations, I will create a disease model in animals.

As the third step, I will repeat the functional mappings in the diseased animals. From the recorded signals, I will generate a model of the diseased brain. Creating models by fitting them to all our experimental data is crucial to account for individual differences between animals.

As the final step, I will define both qualitative and quantitative measures to compare the baseline model to the disease model. By identifying differences between these models, I will investigate the development of the observed changes and establish cause-and-effect relationships. Through this understanding, I hope to develop therapies that target the root cause of the putative suppression imbalance in the diseased brain.

### 3. Functional mapping techniques

The experimental setup for an *in vivo* functional mapping experiment is a relatively simple one, as it is depicted in Figure 2. The first component of the setup is a large screen displaying carefully crafted stimuli to evoke responses in the visual system of the experimental animal. The stimuli are generally geometric shapes flashing or drifting across the screen designed to probe the visual system of the animal based on certain properties, such as contrast, the orientation of edges or movement direction. The exact types of stimuli used in my experiments are detailed in section IV.



**Figure 2 Main components of a functional mapping experiment of the visual system**

The three main constituents depicted are the stimulation screen displaying a drifting flickering checkerboard bar, the experimental animal, and the neural activity recording device of choice. In the figure an ultrasound transducer is shown, mounted to the skull of the animal using a chronic modular neural interface designed in-house. Note that the transducer is connected to an ultrasound system through a cable, and the ultrasound and the visual stimuli are controlled by computers, that are not depicted here.

The second constituent element is the already mentioned experimental animal, which serves as an essential component in our research. While our focus lies on understanding a disease that affects the human visual system, conducting experiments directly on human subjects is neither practical nor ethical due to the invasive nature of most functional mapping techniques. As a result, neuroscientists have turned to animal models as a substitute for human subjects to further our understanding of the disease in question.

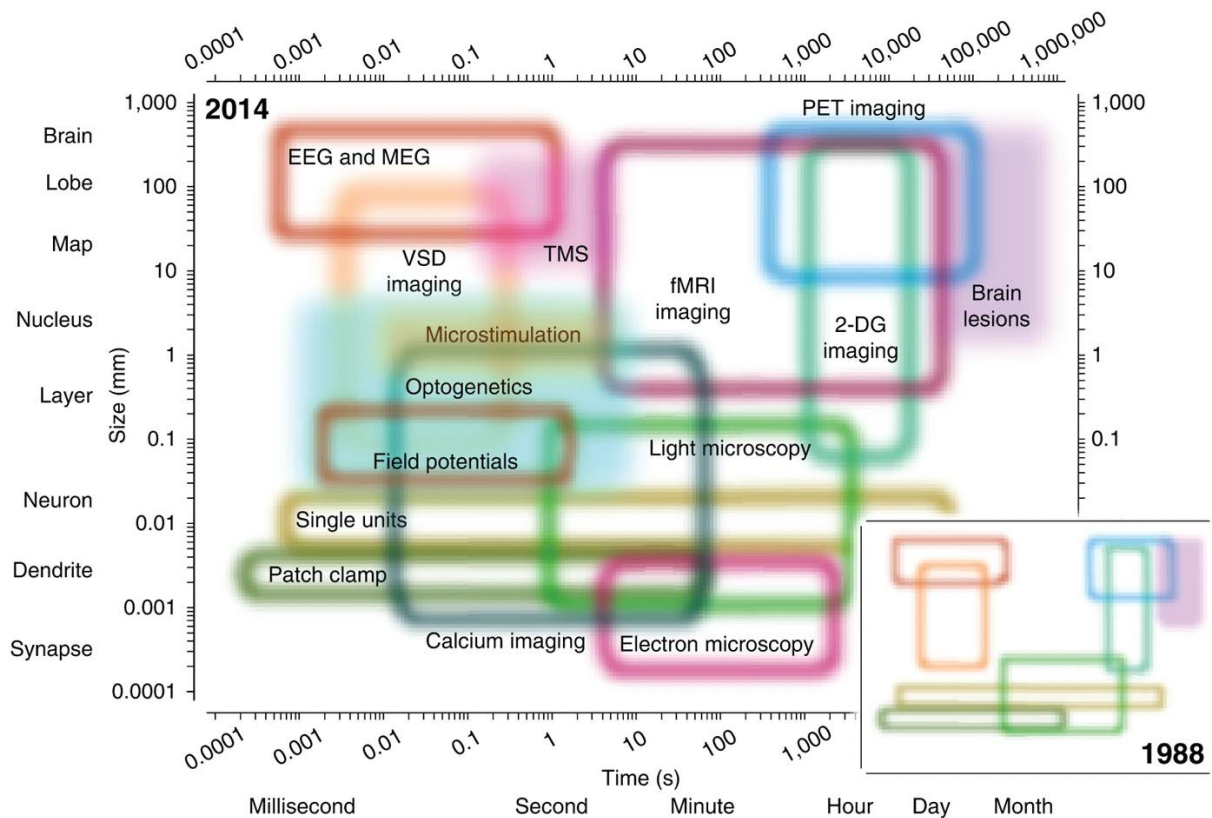
Our primary model animal is the cat. This species has been used since the dawn of modern visual cortex research in the 1960s by Hubel and Wiesel, who made the first discoveries about the functional architecture of this brain area<sup>3</sup>. Later, a similar cortical organisation was observed in macaque monkeys and even in the human visual cortex<sup>4,5</sup>. In contrast, in the past decades rodents, such as mice, have become the most widely adopted mammals for neuroscientific research due to their lower upkeep cost, higher reproductive rates, and ease of handling as small animals. Nevertheless, when it comes to comprehending the functioning of the human brain in both health and disease, the limitations of the mouse model become increasingly evident. The most significant disparity between mice and humans lies in the absence of high acuity vision, lack of forward-looking eyes and the fundamental differences in the mesoscale functional organisation of the visual system. Even though neurons in the mouse visual cortex have similar stimulus preferences as higher species, their emergence seems to be different compared to cats

or humans<sup>20</sup>, and they lack the spatial organisation of these neurons present in cats and humans<sup>6,21</sup>. Despite these limitations, rodents are integral elements in neuroscientific research. Generally, animals are anaesthetised and head-fixed during functional mapping experiments. The majority of neural activity recording methods encounter challenges when attempting to record through the skull, due to its high light scattering and sound attenuation properties<sup>22,23</sup>. Consequently, they require either the creation of holes in the animal's skull for inserting electrophysiological probes or the establishment of a cranial window, known as a craniotomy, for optical or ultrasound-based methods<sup>24</sup>. In certain cases, particularly with the usage of optical methods in large animals, a durotomy, the removal of the dura mater, is also necessary to obtain a clearer image of the brain surface. For long-term experiments, which align with our research interests, a surgically implanted chronic chamber is utilised in place of the removed bone. By employing such a chamber, experimental animals can live for extended periods, ranging from months to even years, facilitating invaluable insights by enabling the accumulation of larger amounts of data, and particularly in studying long-term brain plasticity<sup>25,26</sup>.

The last essential component of a functional mapping experiment is the neural activity recording device. Depending on the chosen recording technique, the device is either implanted into the brain, mounted on the head of the animal, or positioned above the cranial window. The most widely used measurement modalities are described in the following subsections.

Mapping the functional organisation of the brain requires choosing from a variety of techniques with different temporal and spatial resolutions, as seen in Figure 3. These methods also provide access to different organisational levels of the brain, ranging from the single-cell to the whole-brain level<sup>27</sup>. Additionally, the methods differ in invasiveness, with some requiring the introduction of artificial reporters or devices into the brain and possibly damaging it, while others can be used without any such tools.

Given these different aspects, in this section, I will provide an overview of the most popular methods used for functional mapping, along with their advantages and disadvantages.



**Figure 3 The available spatiotemporal domain accessible through different methods (Sejnowski et al., 2014)**

Each depicted region shows the practical spatial and temporal domain of a given method for the study of the nervous system. Clear regions show measurement techniques, while filled regions represent perturbation techniques. The inset shows the same for available methods in 1988. The regions shown are estimates by the authors of the figure. EEG, electroencephalography; MEG, magnetoencephalography; PET, positron emission tomography; TMS, transcranial magnetic stimulation; VSD, voltage-sensitive dye; 2-DG, 2-deoxyglucose

#### a. Electrophysiology

Electrophysiology was historically the first method used to investigate neural activity, including the discovery and description of action potentials and the already mentioned seminal works of Hubel and Wiesel on the functional architecture of the visual cortex<sup>3,28</sup>. The greatest advantage of using electrophysiology over other neural activity recording methods is its practically “unlimited” temporal resolution, meaning that the recording hardware can operate at a higher sampling frequency than any bioelectrical signal it records<sup>29</sup>.

The spatial resolution of electrophysiological methods is challenging to define. Implantable multi-electrode arrays have constantly increased their number of recording sites and concurrently usable channels, allowing for the monitoring of hundreds of neurons on the action potential level<sup>30,31</sup>. This can be seen as very high spatial resolution, as the activity of single cells can be distinguished. However, these few hundred neurons are confined to a small volume of neural tissue, therefore, despite the access to spiking activity of single neurons, using electrode

arrays can only provide insight into the operation of a small portion of the brain, compared to the 86 billion neurons of the human brain<sup>32</sup>.

Implanted electrodes and microelectrode arrays are among the most invasive devices used today to measure neural activity. Despite great efforts to make them more biocompatible, stable over time and less damaging during implantation, signal degradation due to the foreign body response of the brain and degradation of the hardware in the hostile biochemical environment is unsolved yet<sup>33</sup>. These challenges hinder their clinical adoption<sup>34</sup>.

#### b. Functional magnetic resonance imaging

Functional magnetic resonance imaging (fMRI) represents the other end of the spectrum of neural activity measurement techniques compared to single-cell resolving electrophysiology. It is a widely used technique for functional brain imaging, and its popularity is due to its predominantly noninvasive nature while providing an image of the whole brain, even in large animals and humans.

The signal most commonly recorded by fMRI, called the blood oxygen level-dependent (BOLD) signal, is based on the haemodynamic response of the brain<sup>35</sup>. In simple words, when there is increased neuronal activity in the brain, there is increased metabolic activity which results in an increased blood flow to the area. This phenomenon is referred to as neurovascular coupling. The change in blood flow is two-fold. There is an increase in blood volume and also a change in the relative concentration of oxygenated versus deoxygenated haemoglobin in the blood. These changes can be detected by appropriate fMRI imaging sequences; therefore, this readout measures a surrogate signal from which we can infer brain activity<sup>36</sup>. It is important to keep this limitation in mind when designing experiments, analyses, and interpreting results.

One of the main drawbacks of fMRI is its limited spatial and temporal resolution, especially when imaging the whole brain, with the best achievable spatial resolution being above 1 millimetre even in a high field 7T scanner<sup>37</sup>, and its temporal resolution being in the seconds domain. The use of magnetic coils implanted into the skull allows for fMRI studies with higher spatial resolution, however, this comes at the price of a great loss in the field of view and the loss of non-invasiveness<sup>38</sup>.

The ability of fMRI to give an image of the whole brain simultaneously, even with a limited spatial and temporal resolution, makes it a great tool for understanding the functional connectivity of different brain areas, such as can be done in resting-state fMRI studies<sup>39</sup>, but it is not well suited for understanding the functional connectivity inside brain areas and in local

neuronal networks, let alone single cells. Additionally, fMRI recordings require complete subject immobility since movement artefacts are hard to correct<sup>40</sup>. This makes it unsuitable for recordings in freely moving subjects, an area of great interest lately. Lastly, fMRI is quite costly, which stands in the way of the technique having a wider adoption.

### c. Optical imaging

Optical imaging methods have become the most widely used imaging techniques for systems neuroscience for measuring brain activity across the meso- to subcellular scale. As such, they are positioned between fMRI and single-cell electrophysiological methods. Their widespread adoption is primarily thanks to their versatility. They can be used on the mesoscale without the need for introducing any foreign reporters into the examined tissue taking advantage of intrinsic signals<sup>41</sup>, or with genetically encoded fluorescent indicators that can reach a subcellular resolution when used in combination with two- or three-photon microscopy<sup>42</sup>.

Despite their widespread usage, they have a very severe limitation concerning the imaging of deep brain areas or even the deeper layers of the cortex. Since nervous tissue has a substantial light scattering effect, classical single-photon excitation methods can only reach a maximum depth of approximately 500  $\mu\text{m}$ <sup>43</sup>. Newer multi-photon excitation microscopy methods exploit the fact that longer wavelength light penetrates neural tissue better, but even the most advanced three-photon microscopy methods are limited to approximately the superficial 1 mm of the cortex<sup>44,45</sup>. Reaching deeper layers is only possible through the employment of undesirable methods, such as imaging through invasive GRIN lenses<sup>46</sup>. Despite this limitation, they are still very effective for studying the visual cortex, one of our primary objectives.

Widefield optical imaging (WFOI) refers to several optical imaging techniques, where the entire examined sample is exposed to light and that utilise a macroscope, in essence, a microscope with a small magnification value, in the range of 0.5 to 10 times. Consequently, the field of view of these macrosopes is large, often covering centimetres of the surface of the primary visual cortex<sup>26</sup>. However – due mostly to the small magnification – single cells are generally not discernible in WFOI recordings. This is in contrast to multi-photon laser scanning microscopes, which have a limited field of view in the few hundred  $\mu\text{m}$  range, but make the identification of single neurons and their activity possible<sup>44</sup>. Therefore, optical imaging on the mesoscale generally refers to WFOI, while on the micro-to-cellular scale, it refers to multi-photon microscopy methods.

In conclusion, optical imaging methods are very powerful for studying neural activity, with high spatiotemporal resolution. Their drawbacks are the need for opening the skull and dura mater, and most importantly, the very limited imaging depth.

#### d. Functional ultrasound imaging

Functional ultrasound imaging (fUSI) is a relatively new technique that aims to bridge the gap between full brain imaging methods like fMRI with low spatiotemporal resolution and techniques with a limited field of view but higher resolution such as optical imaging or electrophysiological recordings. fUSI owes its invention to the technical advancement in the development of fast ultrasound scanners, and the usage of plane-wave illumination, making imaging at 1 kHz frame rate possible. This improvement enabled the imaging and measurement of the movement of red blood cells in the microvasculature. This type of ultrasound imaging is called micro-Doppler imaging. From the movement of the red blood cells, the “power Doppler” image can be calculated, which is proportional to local blood volume<sup>47</sup>. Due to the earlier discussed neurovascular coupling, we can infer neural activity in a given area from the changes in local blood volume. Thus, fUSI is dependent on the haemodynamic signal, which makes it similar to fMRI and intrinsic signal optical imaging.

I have already described the limitations that haemodynamic signal based methods face, namely limited temporal resolution and the indirect nature of the activity measurement. These are true for fUSI as well, limiting its temporal resolution to  $\sim 100$  ms<sup>48</sup>. However, the advantage of measuring the haemodynamic signal applies too, thus no reporter or contrast agents need to be introduced into the experimental subject. This benefit combined with the  $\sim 100$   $\mu$ m spatial resolution makes it stand out among whole-brain imaging methods.

fUSI images are created perpendicularly to the cortical surface, resulting in “slices” of the brain. This means that the recordings provide information about the brain in depth rather than from the surface as in optical imaging techniques. The maximal achievable depth is in the few centimetres range, which makes fUSI a whole-brain imaging method in rodents<sup>49</sup>, and enables the observation of anatomical features and functional signals deep below the surface even in large animal models<sup>50</sup>.

Linear transducers used today only image a single plane of the brain at a time. Our group has designed and created a motorised transducer holder, that can step the transducer, making volumetric scans of the brain possible. This in itself is enough for creating 3D anatomical scans. In addition, by repeating the same stimulation protocol for each recorded position, we can also

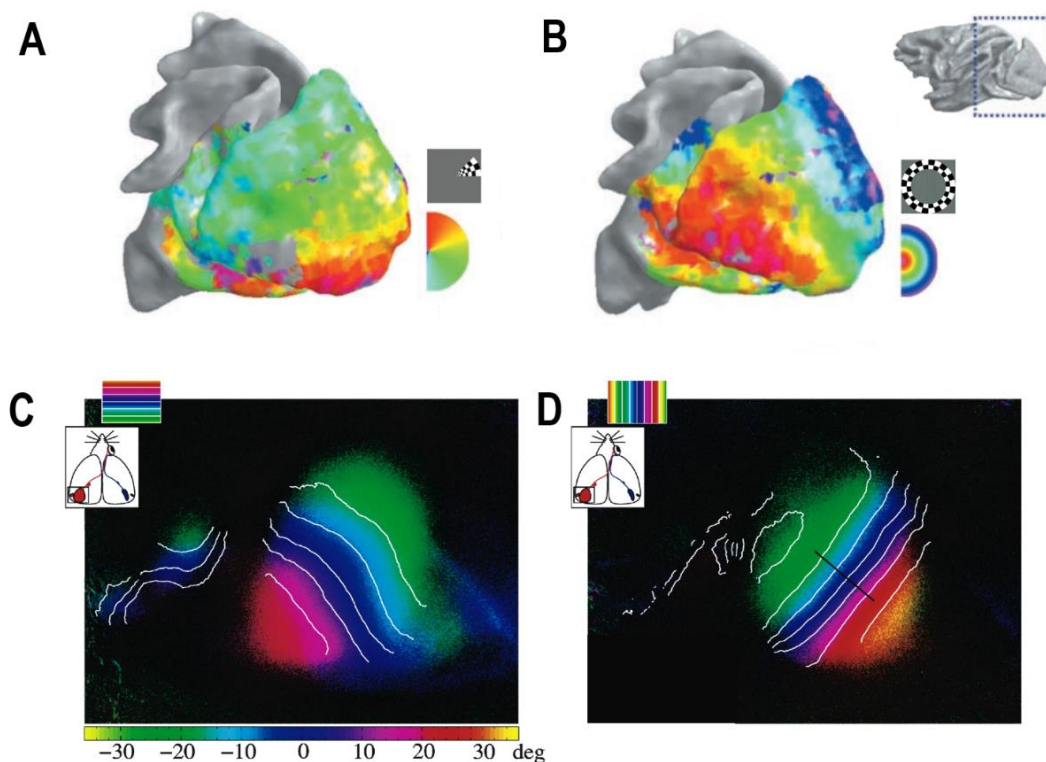
construct 3D activation maps of the brain. It is also worth mentioning that the usage of matrix array transducers for fUSI has been demonstrated, which can image a volume simultaneously<sup>51</sup>. The whole-brain images created by fUSI are even more appealing if we consider the spatial resolution of the technique. It is approximately 100  $\mu\text{m}$ , although voxel sizes differ depending on imaging depth<sup>49</sup>. This places fUSI above fMRI based on resolution, although we must keep in mind, that fUSI requires a craniotomy, which makes it the more invasive method of the two. fUSI is highly sensitive to movement artefacts, as the recorded signal itself is based on the motion of red blood cells. This sensitivity is increased by the need to capture numerous images at high frame rates (1 kHz) and subsequently generate a compound image (2 Hz). Consequently, applying traditional motion correction methods becomes challenging, as they would need to be applied to each of the 500 raw images, which is impractical due to contemporary computational limitations. However, in our experience, minor head movements in awake animal recordings do not significantly impact the results. In cases where head shaking occurs during a recording, we can exclude that specific repetition from our analysis, ensuring it does not affect the final outcomes. In summary, functional ultrasound imaging exhibits greater tolerance to movement compared to fMRI, allowing for recordings in awake animals without the need for stringent head fixation required in fMRI experiments.

In this section, I have presented an overview of four fundamentally different neuroimaging techniques. They address different aspects of brain function and activity, encompassing various spatial and temporal scales. To achieve a comprehensive understanding of how the brain functions and changes in disease, it is necessary to combine multiple methods, since no single method encompasses all aspects of brain activity across all levels.

#### 4. Functional maps of the visual cortex

The neurons in the visual cortex display a distinctive spatial distribution based on their functional properties. For instance, certain neurons may be more strongly driven by input from one eye compared to the other, while others may exhibit selectivity for specific stimulus orientations, and similar neurons with respect to these properties tend to be clustered together<sup>52</sup>. By systematically measuring these properties across a large population of neurons or within a specific cortical area, it is possible to create a functional map that illustrates the spatial arrangement of these response preferences. This map provides valuable insights into the specialised functional organisation of the visual cortex and helps us understand how different stimuli are represented and processed within the visual system.

The retinotopic map, which describes the mapping between the visual field and the early visual pathway, was the first functional map to be discovered<sup>53</sup>. Retinotopy, in simple terms, refers to the fact that adjacent points on the retina are also represented as adjacent points in the primary visual cortex<sup>54</sup>. Over time, more sophisticated techniques have been used to measure retinotopy, including electrophysiological studies<sup>3</sup>, optical imaging<sup>55</sup>, and functional magnetic resonance imaging<sup>56</sup>. The key to successful mapping of retinotopy is the utilisation of a stimulus that is relatively small, and its position in the visual field of the experimental animal is known. By slowly moving this stimulus on the stimulation screen and correlating the activity of different regions with the position of the stimulation, it is possible to create this mapping between the visual field and the primary visual cortex. There are two distinctive stimulation approaches for retinotopic mapping. The first uses an annulus for mapping eccentricity, i.e., distance from the centre of the visual field, and a wedge for mapping visual angle (Figure 4, A, B). The second approach utilises a horizontal bar for mapping altitude, i.e., vertical position on the screen and a vertical bar for mapping azimuth, horizontal position on the screen (Figure 4, C, D).

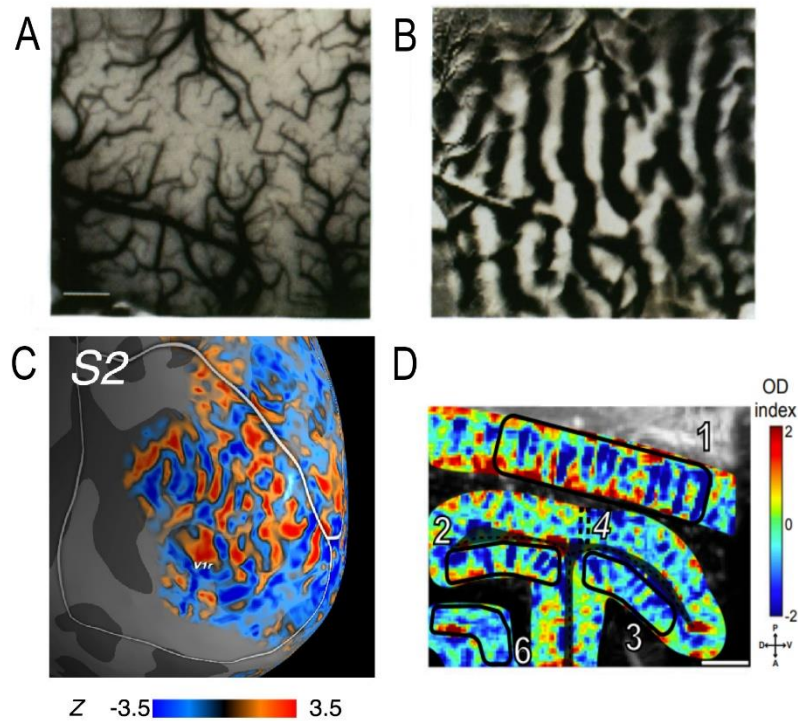


**Figure 4 Retinotopy maps (Brewer et al., 2002; Kalatsky and Stryker, 2003)**

A and B, Angular and Eccentricity maps of macaque brain recorded using fMRI. (See text for explanation.) Inset on the top right shows the imaged region of the brain. Coloured semicircles show what colour corresponds to what angle and eccentricity in the visual field of the animal respectively. Above the colourmaps the visual stimuli used for each of the two mapping types are represented.

C and D, Altitude and azimuth maps of mouse brain recorded using intrinsic signal optical imaging. Inset shows the stimulated eye and the imaged region of the brain. Colourful rectangles show which colour corresponds to what vertical and horizontal positions in the visual field of the animal.

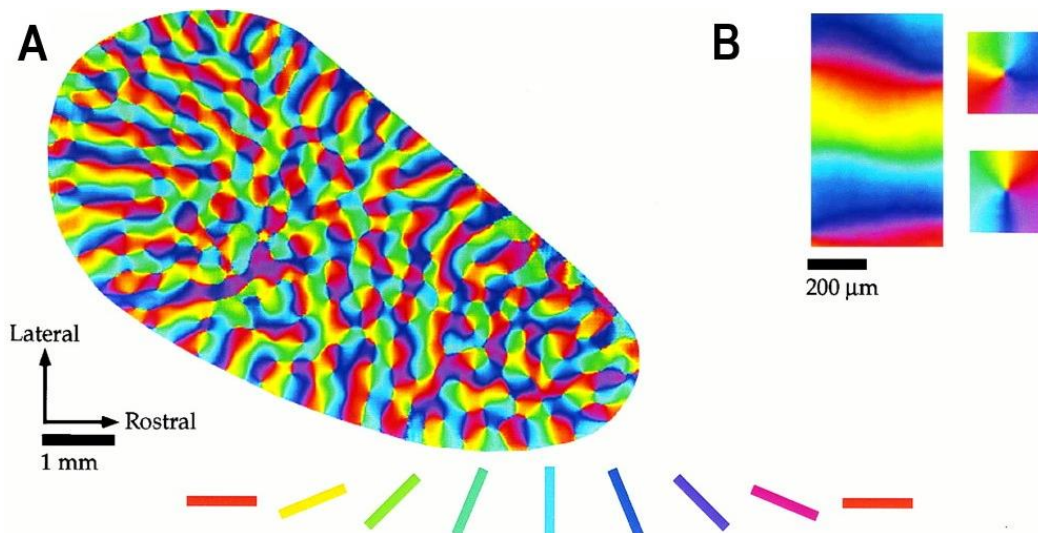
One of the most important functional maps in the context of amblyopia is the ocular dominance map, which reveals the regions of the visual cortex that are predominantly innervated by one eye over the other<sup>57</sup>. Example maps are shown in Figure 5. This map is based on the columnar organisation of the primary visual cortex, where individual columns receive input predominantly from the left or right eye. To create this map, arbitrary stimuli can be used, with the condition that one eye is covered while the other eye is stimulated. By subtracting the activity maps obtained from stimulating each eye separately, a contrast map of the visual cortex, known as the ocular dominance map, can be generated. This map provides valuable insights into the functional connectivity between the eyes and the visual cortex, highlighting any imbalances or deviations in neural processing associated with amblyopia<sup>58</sup>.



**Figure 5 Ocular dominance maps (Frostig et al., 1990; Hollander et al., 2021; Blaize et al., 2020)**

A and B, Image of cortical surface and corresponding ocular dominance map created using intrinsic signal optical imaging. Dark and bright stripes in B correspond to areas dominantly innervated by the right or the left eye. C, Ocular dominance map of human visual cortex using fMRI, with restricted field-of-view to the occipital lobe, optimised to the calcarine sulcus. Colourmap shows eye dominance through corresponding Z-score. D, Sagittal section ocular dominance map of macaque deep visual cortex. Colourmap to the right represents ocular dominance index.

Orientation preference maps convey information about how different patches of the examined area respond with a larger intensity to stimuli with edges of a certain orientation. These patches that show a preference for a certain orientation form so-called pinwheels on the surface of the cortex<sup>21</sup>. Commonly used visual stimuli for creating orientation preference maps are sinusoidal



**Figure 6 Orientation preference map (Bosking et al., 1997)**

A, Orientation preference map in tree shrew visual cortex created using intrinsic signal optical imaging. Orientation preference of each location is colour-coded according to the key shown below. B, Magnified parts of the orientation map shown in A, to demonstrate linear and pinwheel arrangements in the map.

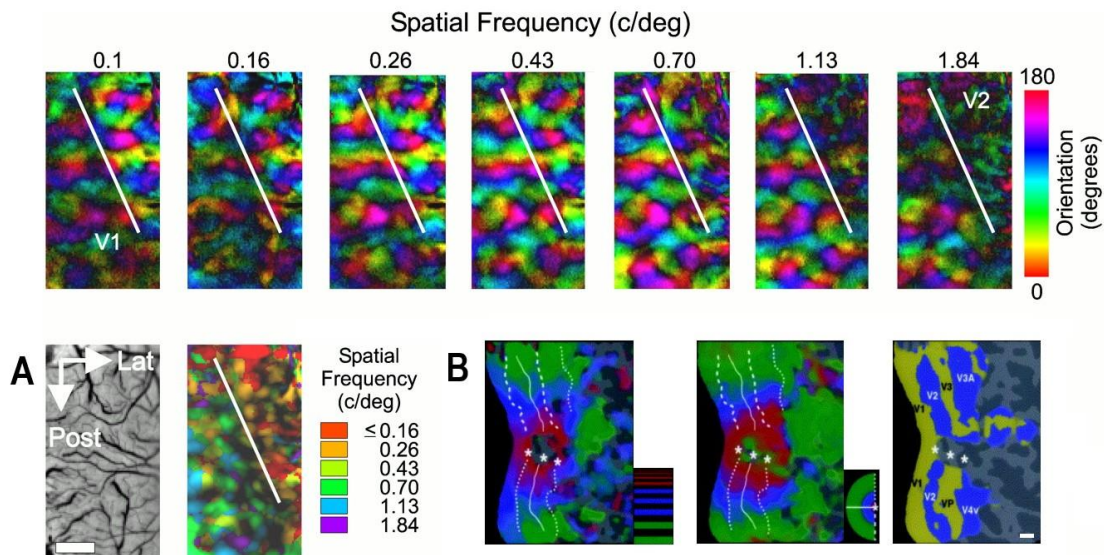
or square wave grating patterns consisting of iso-oriented bars<sup>59</sup>. An example orientation preference map is depicted in Figure 6.

Spatial frequency maps are closely related to orientation preference since they can be mapped using the same stimuli. But instead of varying stimulus orientation, the spatial frequency of the displayed grating pattern is varied<sup>60</sup>. The spatial frequency of grating patterns is measured in cycles over visual degrees (c/deg), i.e., the ratio of a single black-and-white cycle covered by one degree of the visual field.

It is visible in Figure 7, Bottom, B, that the areas closer to the fovea prefer higher spatial frequencies, i.e., more fine details in the stimuli, than the parts corresponding to peripheral vision. This is in accordance with our knowledge about foveal and peripheral visual acuity<sup>61</sup>.

For this mapping to be successful, one has to first measure the preferred orientation of the smallest measured unit – neuron or pixel – and perform the measurement of the preferred spatial frequency using the found orientation<sup>62</sup>. This mapping can be done by simultaneously varying the orientation and the spatial frequency of the grating too, however, this substantially increases the number of distinct stimulus conditions, which leads to long experiments.

In this section, I have presented known functional maps of the visual cortex. As Figures 4-7 show, these have all been successfully mapped using optical imaging methods. The maps created with optical techniques provide valuable insights into the spatial and functional organisation of neurons on the brain surface. However, mapping these functional domains in depth poses a significant challenge. Although I have included a map illustrating ocular



**Figure 7 Spatial frequency maps (Issa et al., 2000; Tootell et al., 1998)**

Top, Orientation maps recorded using different stimulus spatial frequencies in the cat visual cortex. Hue represents preferred orientation as shown by colourmap on the right. Colour lightness represents degree of tuning, i.e., a light colour shows that the given position gave a large response to the used spatial frequency. Border of primary and secondary visual cortices is marked by white line.

Bottom, A, Left panel shows vasculature in the imaged region of the cortex. Scale bar represents 1 mm (applies to top row as well). Right panel shows spatial frequency map calculated from images in the top row. Brightness represents response intensity.

B, Functional maps created using fMRI in human visual cortex. Cortical surface is flattened for visualisation. Left, Spatial frequency map from  $\sim 3$  c/deg (red) to  $\sim 0.75$  c/deg (green). Representation of the fovea is marked with white asterisks. Middle, Retinotopy eccentricity map in the same area. The systematic organisation of spatial frequency preference is remarkably similar to that of retinotopic eccentricity. Right, Identified visual areas.

dominance structures in deeper layers of the cortex in Figure 5, not all the shown mappings have been demonstrated in depth. It is of great interest to obtain a comprehensive understanding of these functional architectures in three dimensions with high spatial resolution.

To address this, I have chosen to combine optical imaging methods and fUSI for my research paradigm described in section 2, based on their properties described in the previous section – namely, access on the surface and in depth with high spatiotemporal resolution and relatively low invasiveness – and the example functional maps created with each method. I aim to integrate the functional maps generated using optical methods with those of the deep visual cortex using fUSI. To the best of my knowledge, the integration of these two methods in the same animal has not been demonstrated yet. This novel approach will provide a more comprehensive understanding of the functional organisation of the visual cortex.

This concludes my review on amblyopia, with research methods applicable to it and other neurodevelopmental conditions. The subsequent sections delve into the detailed description of my primary contributions to this project, shedding light on their significance and implications.

### III. Planning

My primary responsibility in this project is to develop the software environment for experimental and analysis purposes in the chosen neuroimaging methods, functional ultrasound and widefield optical imaging (WFOI). This includes stimulus generation, control, and synchronisation of hardware devices for experiments, and analysis of the recordings.

As mentioned previously, the functional maps of interest vary between individuals. However, the main focus of my research are the specific alterations in functional maps caused by amblyopia independent of individual differences. This makes it necessary to perform the mappings in each experimental animal. Given the substantial workload involved in conducting these mappings across numerous subjects, it becomes imperative to streamline the experimental procedures and develop highly optimised software for conducting experiments and analysing data. The optimisation of such software is not merely a convenience for the experimenter but a critical factor that directly impacts the success and viability of the entire research project.

In this section, I will first describe the motivation behind the development of these software environments. Then, I will outline my objectives for the resulting software and discuss some additional factors that were considered during the development process.

As the two software environments developed for fUSI and WFOI have a lot in common, I will describe the process of their development in parallel.

#### 1. Motivation

##### a. Functional ultrasound imaging

The fUSI system initially had three software components for conducting experiments and analyses, which were created by the developers of the imaging system. We originally utilised these components in our research. The first software, written in MATLAB, controlled the ultrasound hardware and processed the raw data during recordings. It included a graphical user interface (GUI) for managing the recordings and functional ultrasound hardware parameters. The second software was responsible for stimulus presentation during functional imaging experiments, and consisted of many separate Python scripts that utilised the PsychoPy package. Although this software was functional, it consisted of separate scripts rather than coherent modules with many repeated code sequences and was therefore very tedious to modify. The third component was the fUSIAnalyzer software, also written in MATLAB, which offered a GUI for analysing the recordings. Despite the graphical interface making it seem user-friendly

at first glance, it became apparent that using this software for analysing large numbers of recordings was impractical due to the constant text and mouse inputs required. Analysing a single recording took around 10 minutes using this software.

Since the first software worked reliably and it was complex, we did not wish to modify it. However, we felt that the other two components were limiting us, and thus decided to replace them. I considered using freely accessible or open-source software however, due to the relative novelty of fUSI, there are no well-established open solutions that cover all the aspects we would like to use it for<sup>63</sup>. Therefore, I have decided to develop my own software solution.

#### b. Widefield optical imaging

In our research group, we work on projects that span multiple years and require software that is both stable and flexible, since occasionally the initially laid out plan goes through considerable changes based on the intermediate results. This necessitates that the software is maintainable, meaning it should be easy to modify, debug, and extend over time. To achieve this, I follow several best practices in software development.

Firstly, I use a version control system – Git – to keep track of changes to the project<sup>64</sup>. This allows me to collaborate more efficiently with others, maintain a history of development through commit messages, and roll back changes in case of errors.

A consistent coding style is crucial for creating easily readable and understandable projects. I use formatting, naming conventions, and indentation to ensure a consistent coding style. In addition, I use docstrings and comments to improve the clarity of the code, and I provide separate written guides to help people with less technical knowledge.

I also employ a modular design where every functional piece of code is its own well-defined unit. This makes it easier to modify a certain part of the project without affecting the rest. Automated testing is also crucial for building maintainable software. With tests for each module, I can make changes to the codebase with confidence, knowing that I can easily test whether the changes have broken existing functionality.

Finally, I regularly check and refactor the codebase to remove unused parts and restructure modules more logically.

The original software project I created for WFOI was missing many of these features due to my lack of programming experience at the time. The coding style was inconsistent, the documentation was incomplete, and there were almost no unit tests. The project also depended on multiple old, poorly maintained code repositories, which made it difficult to keep

functioning with newer versions of Python and its packages due to regular version conflicts and incompatibilities. As a result, the project became hard to maintain, and it was particularly challenging to modify or extend.

Another issue that arose when we started using fUSI was the use of different software packages for generating visual stimuli in functional ultrasound and widefield imaging experiments, resulting in slightly different stimuli and protocols, which could compromise the comparability of results across modalities in future studies. To address this issue, the decision was made to adopt the same visual stimuli for WFOI that we used for functional ultrasound experiments.

Lastly, I have discovered a design flaw in the time synchronisation between the recorded camera frames and the displayed visual stimuli, that would require a significant effort to fix. Therefore, developing a new widefield imaging software environment from scratch, using the functional ultrasound repository as a model, was deemed necessary.

Just like in the case of fUSI, I initially explored the possibility of utilising existing comprehensive open-source software solutions<sup>26</sup>. However, I found that these solutions were not well suited for our specific hardware setup, e.g., not compatible with our camera. Furthermore, most of them were either too specialised, limiting our freedom in designing experiments<sup>65</sup>, or were overly comprehensive tools that proved challenging for regular users. For instance, the PsychoPy package<sup>66</sup> – which I later utilised to create visual stimuli – is highly complex with an abundance of features and requires a more user-friendly interface or wrapper for practical experimental use.

The parallel development of the two software environments – that is fUSI and WFOI – enables me to bring both their experimental and analysis parts closer together, which can increase maintainability and development speed, and therefore repay the effort put into their original development.

## 2. Aims

In this section, I will describe the aims that guided the development of the two software environments, which apply to both modalities. Firstly, a single code repository should contain all the necessary codes for performing and analysing experiments for a given modality. This approach ensures maintainability as described earlier. Secondly, the interface should be user-friendly, even for those without programming expertise. This does not necessarily require a graphical user interface but rather a logical layout and minimal user input. Ideally, everything related to a single experiment type should be controllable from a single script.

Thirdly, the software should be efficient, responding quickly to user input and generating and displaying stimuli while controlling recording hardware. For the widefield imaging program, this involves processing and saving a large data stream continuously and in parallel with stimulus display. The analysis must also be fast to provide timely feedback to experimenters on progress. This feedback enables the experimenter to decide whether to continue recording from a specific position or move to a different region. Rapid analysis is also valuable for optimising stimulus parameters, as experimenters can obtain results in a few minutes and adjust parameters accordingly. The ease of use of the software can play a part in fast operation too, as waiting for user input is in some cases the biggest time loss during runtime. Therefore, this aim further strengthens the need for minimising required user input.

Finally, the software must be easy to modify and extend to accommodate new types of visual stimuli and corresponding analyses required to create new functional maps. These new stimuli might also include various types of new stimulating hardware, such as optogenetic or somatosensory stimulator devices, as we are not solely interested in using visual stimuli. The need for new types of stimuli can arise at any time during a project, therefore implementing a new type of stimulus and its analysis should take a maximum of a few working days.

### 3. Considerations

I have taken several considerations into account while developing the two software.

**Reproducibility** is a crucial aspect of experimental science. We aim to save all parameters related to a single experiment, including stimulation, and recording settings, into a single file to facilitate data management. This approach allows us to move the raw data freely e.g., for analysis on a different computer, without any additional effort or complexity. To support reproducibility, we have chosen the Hierarchical Data Format 5 (HDF5) for storing our data. This file format can hold heterogeneous data in a logically structured format; therefore, it provides us with the necessary properties and freedom required for our experiments. Furthermore, we perform all analysis steps using deterministic operations, which means that if the same parameters are used, the same results are obtained, regardless of when and where, on what computer, the analysis is performed.

**Efficiency** in software can be interpreted in different ways, including resource versus time efficiency. For example, in the analysis of large recordings, an efficient program would load parts of the recording sequentially and analyse them without requiring large amounts of computer memory. On the other hand, if we want to be efficient with time, we can load the

whole recording into memory, even in the range of tens of gigabytes, and run the analysis entirely in memory, saving precious time. Thus, efficiency is not a simple principle to follow during development, it always requires the assessment of a certain situation and the application of the principle accordingly. Another reason for choosing the HDF5 file format – besides holding an arbitrary amount of heterogeneous data – is that it supports advanced compression algorithms, which can reduce file sizes by up to 50% in the case of WFOI recordings.

**Performance** is another critical consideration in our experiments. By performance, I mean that our programs should utilise the hardware as much as possible. Although Python is not known for having outstanding performance, this shortcoming can be greatly improved by implementing parallel computing. This consideration is especially important for WFOI where a single consumer-level notebook runs the stimulation and recording in parallel.

**Maintainability** is also an essential consideration, and we plan to build our software on as few Python packages as possible. We essentially build only on a handful of Python packages that are not part of the Python Standard Library.

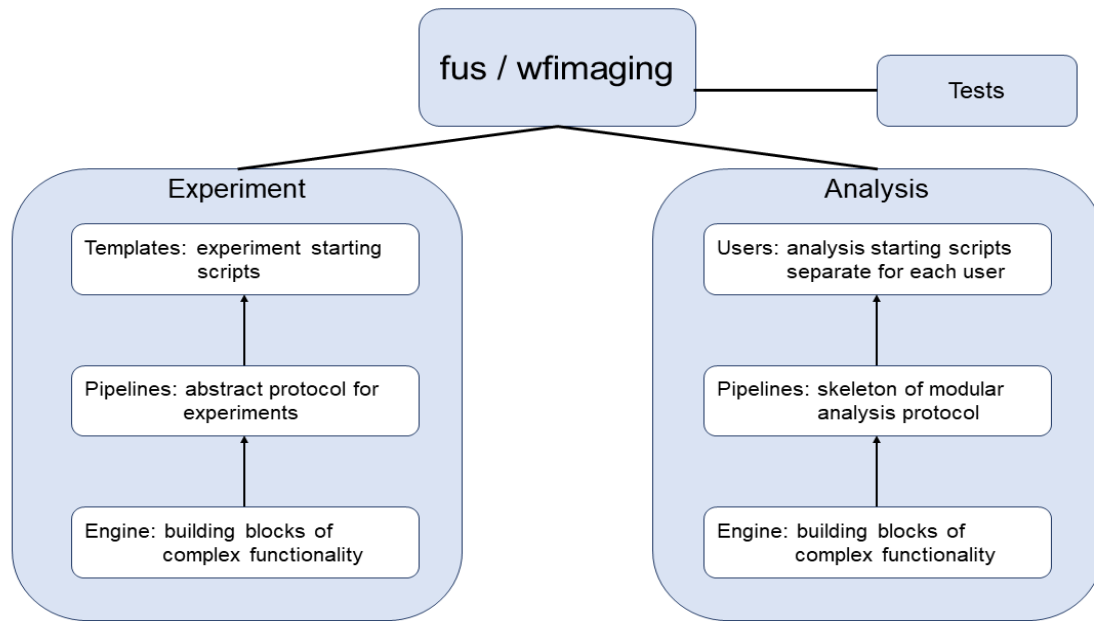
**Cross-platform** compatibility is not our main priority, but we aim to ensure that our system runs without errors on both Linux and Windows since Python allows for this, and it is very practical in the real world. Our experimental machines are slowly being transferred to Linux due to its superior stability and timing properties<sup>67</sup>. However, many users want to run the analysis on their personal machines running Windows. Therefore, we can only use packages and functions that have the same behaviour on different operating systems. A great tool for achieving cross-platform compatibility is the Poetry Python package manager software, which ensures that the exact same package versions are installed on every system where we deploy our analysis software<sup>68</sup>.

**Simplicity** versus overengineering is a crucial consideration since development time is finite. For example, we identify recording file names using their creation time given with one-second resolution. Since there is only one fUSI and one WFOI setup in the lab, there is no need for a more complicated naming scheme, because no two recordings can be started simultaneously. Such focus on well-thought-out yet simple design is essential to finish the development of the usable software within a reasonable time frame.

## IV. Development

### 1. Project structure

In this section, I provide a description of the software architecture of both software environments, which is illustrated in Figure 8. There are two main modules in both projects, along with a separate tests module. The first main module is the experiment module, which contains all the necessary submodules for performing functional mapping experiments.



**Figure 8 Project architecture of the imaging software environments**

This figure represents the architecture of both the fus and the wfimaging projects. Arrows represent dependence between modules. The experiment and analysis main modules are not dependent on each other. This makes it possible to use e.g., the analysis module without installing the dependencies of the experiment module.

Inside the experiment module, we find the engine submodule. Engine holds the building blocks of complex protocols, such as data types, file types, file handling, importers, exporters, and other related components. Additionally, the base classes for visual stimuli are defined in this module.

The next module is called pipelines, which contains abstract protocols built using the building blocks defined in the engine module. Abstract protocol means that it does not utilise a specific stimulation type or any predefined parameters. Instead, it forms a scaffold which can be easily parametrised to conduct experiments. Keeping these protocols separate from the original building blocks makes it easier to implement new experimental paradigms, e.g., a new method to determine the baseline signal, without modifying the existing components.

The last submodule is called templates, which stores the experimental starting scripts. These scripts include all the necessary parameters for the user to define and customise an experiment. Essentially these scripts simply parametrise and call one of the protocols defined in the pipelines module. It was crucial to design these scripts to be as easy-to-use as possible while providing the user with sufficient control over the experiment. Consequently, all the parameters in the scripts are described in concise comments, and during an experimental session, users typically only need to modify a few settings in these files, e.g., which eye is stimulated or what recording position the imaging device is in. Once all the settings are adjusted, the user can run a single script that initiates the stimulation and the recording, besides it ensures the proper saving of data. In case of errors, the user is promptly notified, allowing for timely adjustments without significant data loss.

By organising the software structure in this manner, I aim to provide researchers with a modular and flexible framework for conducting experiments. The separation of modules, building blocks, protocols, and templates allows for easier customisation, scalability, and the incorporation of new experimental paradigms.

The second half of the project is the analysis module. It is important to highlight here the rationale behind separating the analysis module from the experiment one, even though they are two closely related parts of the same project.

The reason for this separation lies in the practicality of installing the project on a new computer. By dividing the project into separate modules, it becomes easier to install only the necessary components on a specific computer. For instance, if a user intends to perform analysis on their own computer using recorded data, they can install only the dependencies of the analysis module, making the installation simpler. This approach is particularly beneficial considering that certain Python packages have many dependencies, some of which may not always be reliably installed, even when using a sophisticated package manager such as Poetry.

The analysis module, like the experiment module, also consists of submodules. One of these submodules is once again called engine, which contains data type definitions and atomic functions that are commonly used throughout the analysis process. Due to the diversity of experiment types and their corresponding analysis requirements, the engine submodule is the largest submodule within the analysis module.

The next submodule is pipelines, which defines the skeleton of analysis protocols. Similar to the experiment module, the analysis pipelines are separated from the atomic functions to make the creation of new pipelines easier without overwriting existing functionalities. They are also

abstract, meaning that the actual steps to be performed during the analysis along with their running parameters are all defined elsewhere.

Lastly, there is the users submodule. This submodule serves a similar purpose to the experiment templates submodule. However, for analysis, I considered it more appropriate to separate the analysis starting scripts for each user. This decision was made to accommodate the fact that different users work on different experiments and may require customised analysis pipelines and default settings. By providing each user with their own space and version of the analysis scripts, it becomes easier for them to make changes to the pipelines and adjust default settings according to their specific needs. Due to the small number of users and the small size of the analysis starting scripts, this decision does not lead to an excessive amount of repeated code segments.

In summary, the analysis module includes the engine submodule for data type definitions and atomic functions, the pipelines submodule for housing analysis pipelines for different analysis protocols, and the users submodule for separating analysis starting scripts for individual users. This modular structure ensures flexibility, customisation, and ease of maintenance for the analysis component of the software environment.

## 2. Visual stimuli

In this section, I provide an overview of the visual stimuli used in our functional mapping experiments. Please note that not all the stimuli I have created are described here, as I have selected those that work best based on our experience.

All visual stimuli were generated using the PsychoPy Python package<sup>66</sup>, which offers a convenient framework for creating geometric, moving, and flashing stimuli commonly used in such experiments.

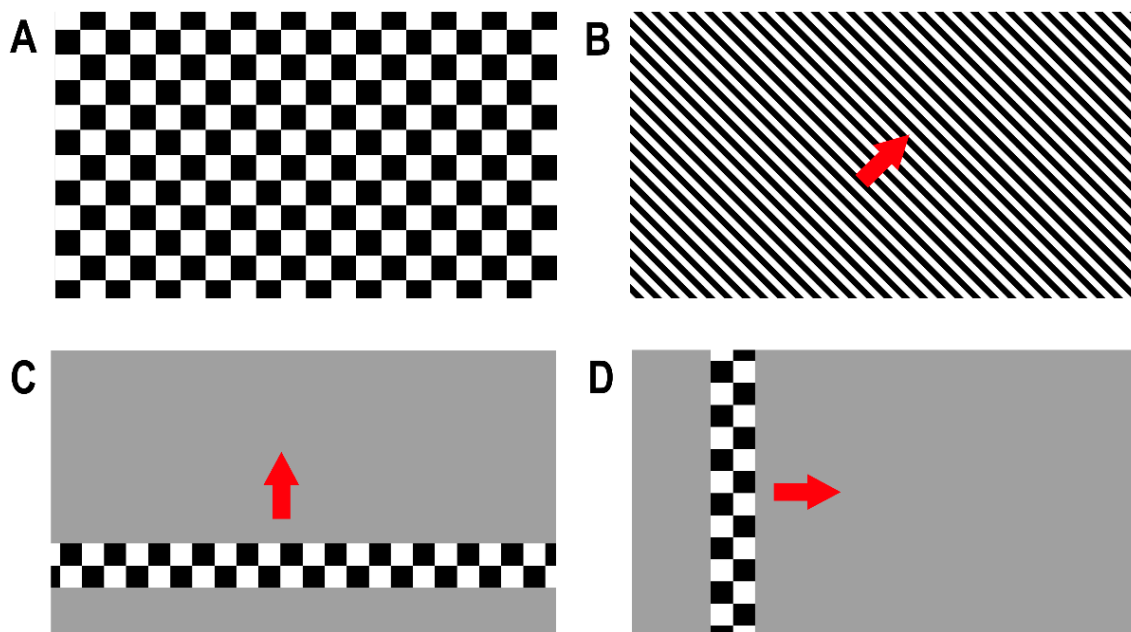
The first stimulation we utilise for rapid assessment of brain activity is the full-screen checkerboard stimulation (Figure 9, A). During this stimulus, the screen is grey in the baseline period and displays a checkerboard pattern that reverses its colours at a given temporal frequency during the stimulation period. The experimenter has the flexibility to adjust the spatial frequency of the checkerboard pattern and the temporal frequency of colour inversion.

For retinotopic mapping, we employ a drifting checkerboard bar stimulus (Figure 9, C, D). This stimulus consists of a bar that is narrow in one orientation but spans the entire screen in the other orientation. The bar drifts slowly across the screen to evoke activity in the animal's visual cortex sequentially. We perform this stimulation twice, once with a vertically drifting bar and

once with a horizontally drifting bar, allowing us to extract altitude and azimuth maps, respectively. Surprisingly, in some of our experiments, this stimulation evoked larger activation in the visual cortex of the animal compared to the full-screen checkerboard stimulation, which is counterintuitive, since that uses the same stimulation pattern, but covers a much larger part of the visual field. My hypothesis is that the combination of flashing and drifting movement may contribute to the enhanced response, although this hypothesis is yet to be confirmed.

Another important category of stimulation is the family of drifting grating stimuli. These stimuli consist of sinusoidal or square wave gratings displayed on the screen with a specific orientation, and they generally drift in a direction perpendicular to their orientation (Figure 9, B). Drifting gratings are the most widely used stimuli for mapping orientation preference in the visual cortex, and there are several variants available. I have developed three different types of drifting grating stimuli to be used in our experiments.

The first type is the simplest drifting grating stimulus, where the screen is grey during the baseline period, and the gratings appear and drift in a single direction perpendicular to their orientation during the stimulation period.



**Figure 9 Visual stimuli for functional mapping experiments**

The images presented here are example screenshots of the visual stimuli I have created in the functional imaging software environments. A, Full screen checkerboard stimulation. This pattern is inverted with a user specified temporal frequency. B, Drifting grating stimulation used for orientation preference mapping. C, Drifting checkerboard bar stimulation used for mapping altitude in retinotopy mapping experiments. D, Same as in C but for azimuth mapping. The red arrows in B, C and D denote the direction in which the stimulation pattern is moving.

The second type maintains the same grey baseline but introduces reversals in the direction of movement during the stimulation period. Specifically, after the grating starts drifting for 2 seconds, it reverses its direction, and this reversal is repeated again after another 2 seconds. The purpose of this change in direction is to stimulate all neurons driven by the specific orientation, regardless of their preferred direction of motion.

The third variant of drifting grating stimulation involves displaying a static grating on the screen during the baseline period, which only starts to move during the stimulation period.

These different variants of drifting grating stimuli provide us with valuable information for mapping orientation preference in the visual cortex and allow us to capture the responses of neurons driven by specific orientations and directions of motion.

### 3. Standard analysis protocol

In this section, I will provide an overview of the main analysis steps involved in processing a single recording, illustrating the steps with relevant figures from the analysis of fUSI recordings.

The first step of the analysis is importing the data to be analysed. This involves locating the recording specified by the user in the analysis starting script. For functional ultrasound recordings, the importing process begins with merging the file containing stimulation parameters and logs with the actual imaging data. Then the recording is sliced into shorter pieces corresponding to a single repetition of a given stimulus presentation. An optional step during import is the manual curation of individual repetitions of the stimulation. This step can be useful for filtering out repetitions with movement artefacts that could potentially affect the final analysis results.

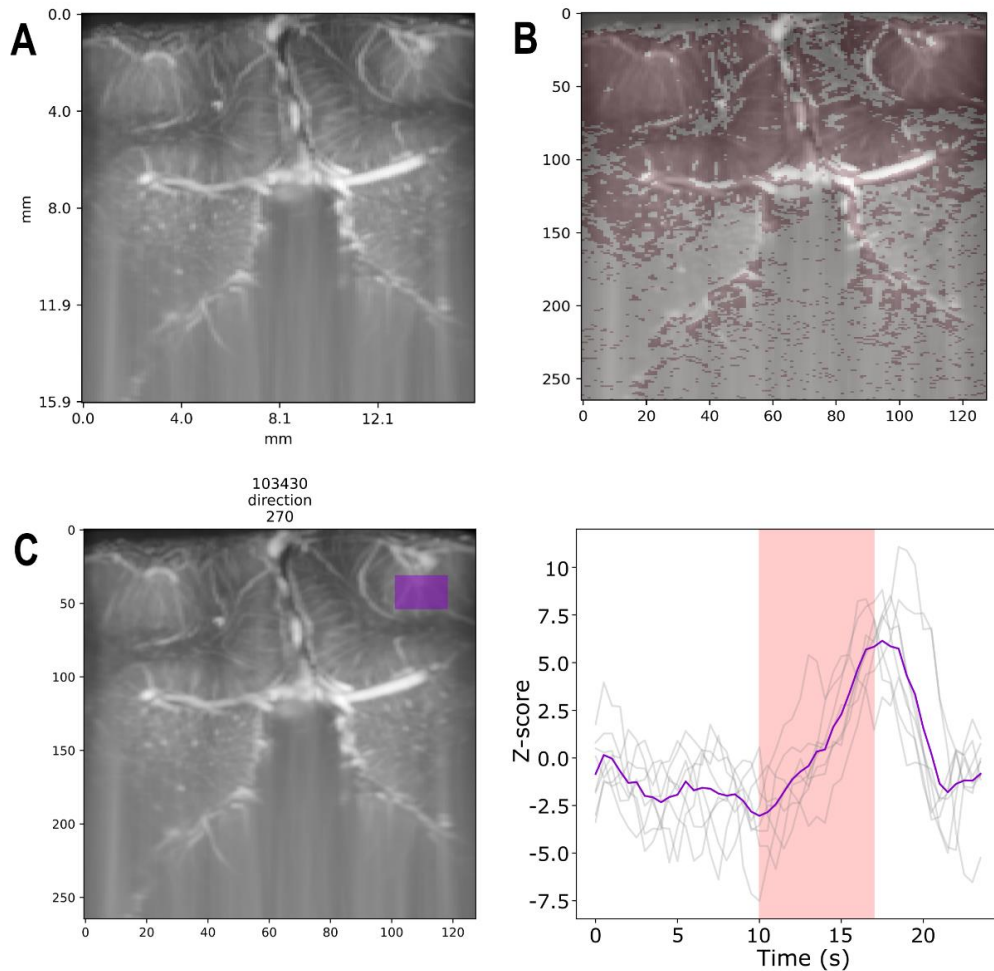
The next step in the standard fUSI analysis protocol is generating an anatomy map from the micro-Doppler recording (Figure 10, A). This map is an average of the entire recording over the time axis. This image is used for creating figures later during the analysis. It also provides a visual quality check for the user, because due to the time averaging, movements in the recording will render the anatomical image blurry.

Following the anatomy map generation, the repetitions of a specific stimulus condition or all repetitions of all conditions are averaged, and the responses to the stimulation are visualised. This step involves the use of a graphical user interface element called the "region of interest picker," as shown in the figure. The user can select a region of interest on the anatomical image, and the mean activity of that region is displayed on the right (Figure 10, C). This step can be

time-consuming as it requires user inputs, and finding the best region of interest manually can sometimes be challenging.

The subsequent step involves a preliminary calculation of response significance using the Mann-Whitney U test on a pixel-by-pixel basis (Figure 10, B). This test determines if a given pixel's response exhibits a significant increase compared to the baseline. It's important to note that this test, at this stage, is not corrected for multiple tests conducted simultaneously. Therefore, its purpose is to guide the user toward areas of potential activation rather than providing conclusive statistical analysis.

Stimulation-specific analysis steps are usually included in the analysis protocol at this point. For example, if retinotopy mapping or orientation map calculation is required, it would be



**Figure 10 Analysis steps of an awake fUSI recording in cat.**

All the figures shown are from the same recording, in an awake non-head-fixed cat. The ultrasound transducer was placed in coronal orientation above the visual cortex of the animal. The stimulation used was a drifting grating displayed for 7 seconds in a single orientation. A, Anatomy map extracted from the recording. X and Y scales show relative lateral position and depth respectively. B, Pixel-wise response significance map. See text for more details. C, User interface of region of interest picker module. The user can select region on the left, the extracted single repetition and mean activity of which is plotted on the right.

incorporated in this section. If no specific steps are needed, the analysis proceeds to the final step.

The last step of the analysis involves generating a PDF report that encompasses the basic experiment description, such as the animal's name and the type of stimulation, along with all the analysis steps and their corresponding running parameters. Additionally, all the figures generated during the analysis are automatically saved and included in the PDF report. This report reflects our philosophy of centralising all relevant output and storing it in one easily understandable file that can be shared and understood by everyone involved.

#### 4. Special analysis methods

The standard analysis protocol described in the previous section is useful for identifying valuable signals in recordings and confirming whether the desired data was successfully captured. However, to construct more specialised functional maps, such as retinotopy, orientation preference, and ocular dominance maps, additional analysis methods are required. I will provide a brief description of each. The figures created using the methods described here are presented in the next – Results – section.

Retinotopy mapping involves the slow drift of a stimulating checkerboard bar across the screen. As the bar sweeps across the visual field of the subject, it activates the part of the visual cortex that corresponds to that position sequentially. Therefore, by determining the time of maximal activation for each pixel in the cortex, we can observe how the horizontal and vertical positions of the bar are mapped onto the visual cortex of the experimental animal.

For the orientation preference mapping, we have created a new calculation of per-pixel significant activation following the protocol described by Brunner et al.<sup>63</sup>. Using this significance analysis, we find the pixels that give a significant response to a grating stimulus of a certain orientation. To create the combined map showing all the orientations, for each pixel we show the orientation to which the given pixel gave the largest response. Resulting map created from preliminary data is shown in Figure 12. The same method can be applied to create an ocular dominance map, the only difference being that the comparison is between the responses given to a certain eye, not a certain stimulus orientation.

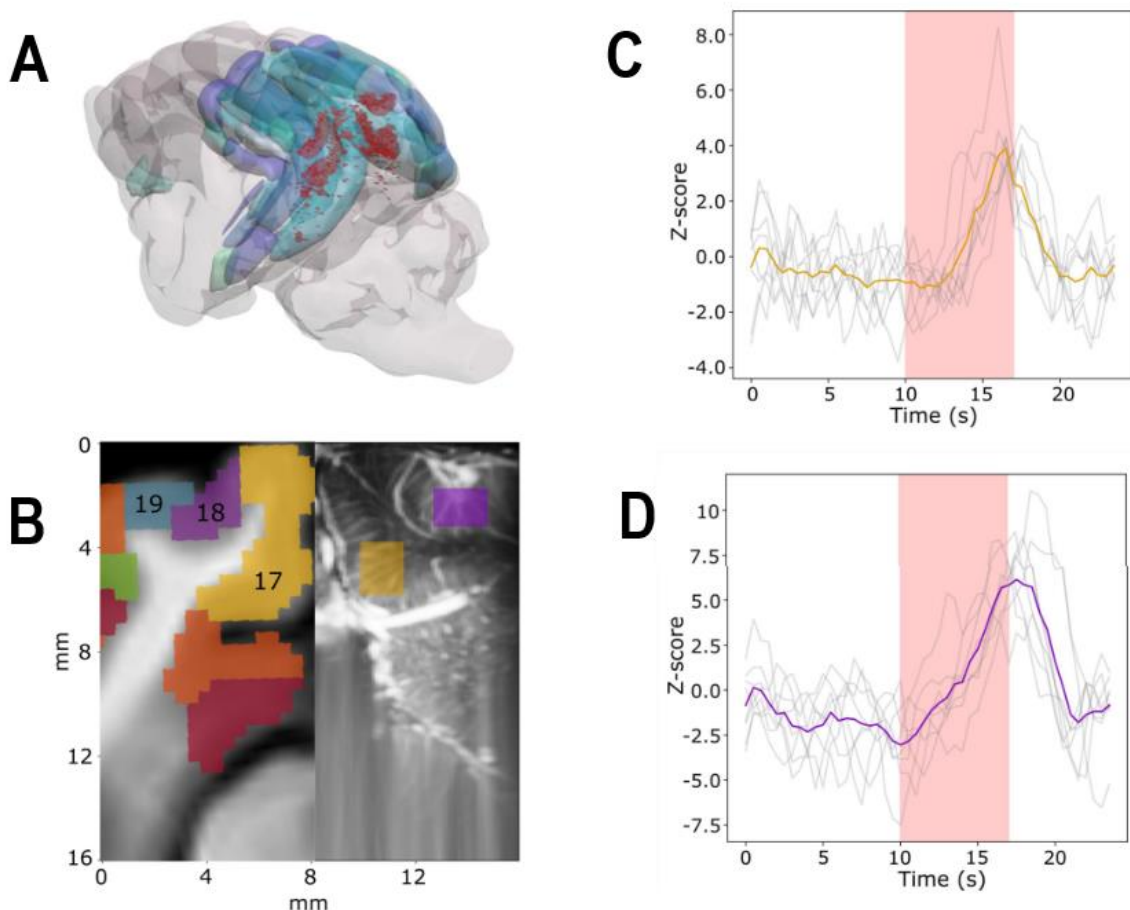
## V. Results

Throughout my work, I have developed methods for performing and analysing experiments. Therefore, my main results are the software packages themselves. In this section, I would like to show the preliminary experimental results I have reached using the software, as a validation of their usability.

The fus software has been in use in the lab for a substantial amount of time, while still being actively developed. Therefore, the data presented here has been gathered from multiple cats across multiple experimental sessions.

### 1. Reliable detection of single-trial responses

I have recorded visually evoked responses in Brodmann areas 17, 18 and 19 – all of which are early visual areas – in cats, in deep layers of the cortex, achieving sufficient signal-to-noise



**Figure 11 Visually evoked response in cat visual cortex recorded using fUSI.**

A, Cat brain model with visual areas highlighted and activity heatmap overlain. B, Left, Magnetic resonance image of cat brain, with anatomical area segmentation overlay, showing Brodmann areas 17, 18 and 19. Right, fUSI of the same coronal plane, with two regions marked for activity analysis in C and D. C, D, Single-trial (grey) and mean response (8 trials) to drifting grating stimulation shown in the pink shaded period.

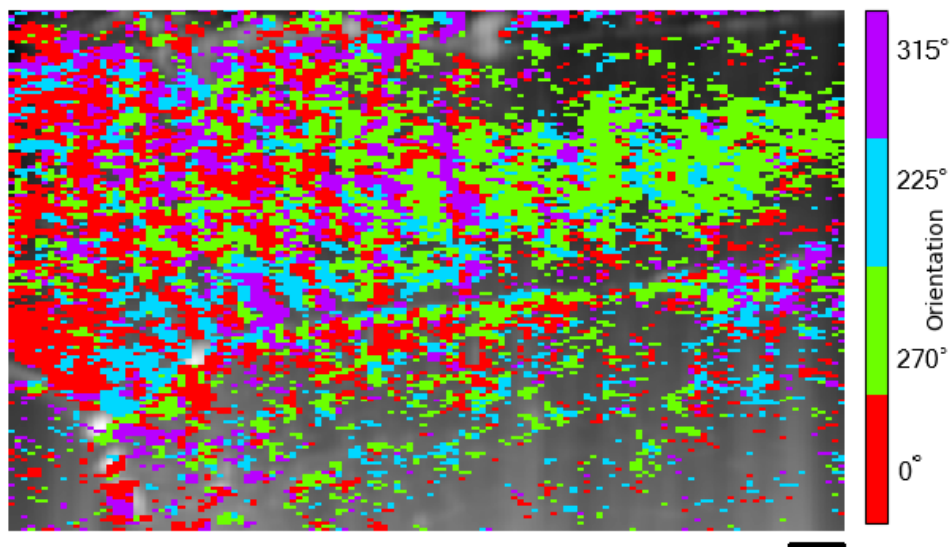
ratio for the reliable detection of single-trial responses, as shown in Figure 11. For this experiment the animal was awake and not in head-fixation. Both eyes were stimulated concurrently with a single type of drifting grating stimulation (horizontal bars drifting upwards) repeated 8 times.

High SNR in a recording is especially beneficial in awake experiments, as the length of the experiment session is quite limited – due to the animal getting tired and losing focus – but thanks to the reliable detection of responses I can reduce the number of repetitions recorded for each functional mapping procedure.

To the best of my knowledge, the reliable detection of visually evoked activity using fUSI in awake, non-head-fixed animals has not been demonstrated yet.

## 2. Orientation preference map

We have recorded the responses of cat visual cortex to drifting grating stimuli in four different orientations. Using the analysis method described in the previous section, we have created a mapping of orientation preference. The result of this mapping is shown in Figure 12. We have not yet confirmed that the pattern of orientation preference we have recorded is stable in time, nor have we cross-validated this map using other techniques. Therefore, the figure shown here is for displaying the capability of the imaging and analysis system, to construct such a map from experimental data. Nevertheless, there may be some column-like structures formed by the



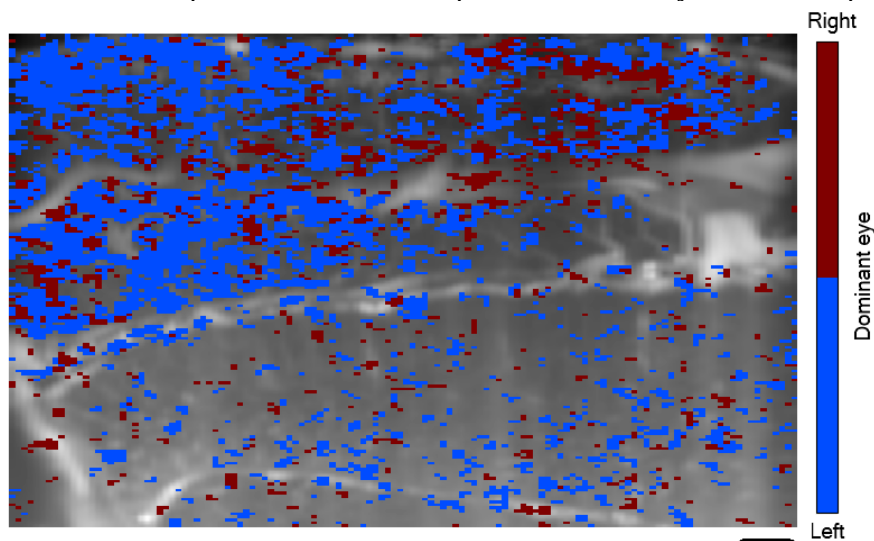
**Figure 12 Orientation preference map in cat visual cortex recorded using fUSI.**

Every pixel is coloured according to which stimulus grating orientation elicited the biggest response in its position. Colourmap of orientations is shown on the right. Pixels that did not show a significant response to any orientation are left without colouring. Note that there is no spatial filtering applied to this figure. It is possible that with an appropriate spatial filter, continuous structures would be easier to detect. Scale bar in bottom right corresponds to 1 mm.

colours visible – although very faintly – in the upper half of the figure. These might be the cross-sections of the orientation columns that we get if we extend the orientation preference map on the surface of the cortex – as shown in Figure 6 – however, this is difficult to state, as such a mapping of orientation preference in depth with such high resolution has not been performed before<sup>69</sup>.

### 3. Ocular dominance map

I have recorded evoked responses to drifting grating stimuli in cat visual cortex, first in the left eye with the right eye covered, and then vice versa. I applied the same analysis to these recordings as for the orientation preference mapping. The resulting map is shown in Figure 13. It is apparent, that the left eye stimulated a large part of the cortex more effectively than the right eye. This was the expected outcome of the measurement, as the right eye of the experimental animal was deprived in the critical period of visual system development.



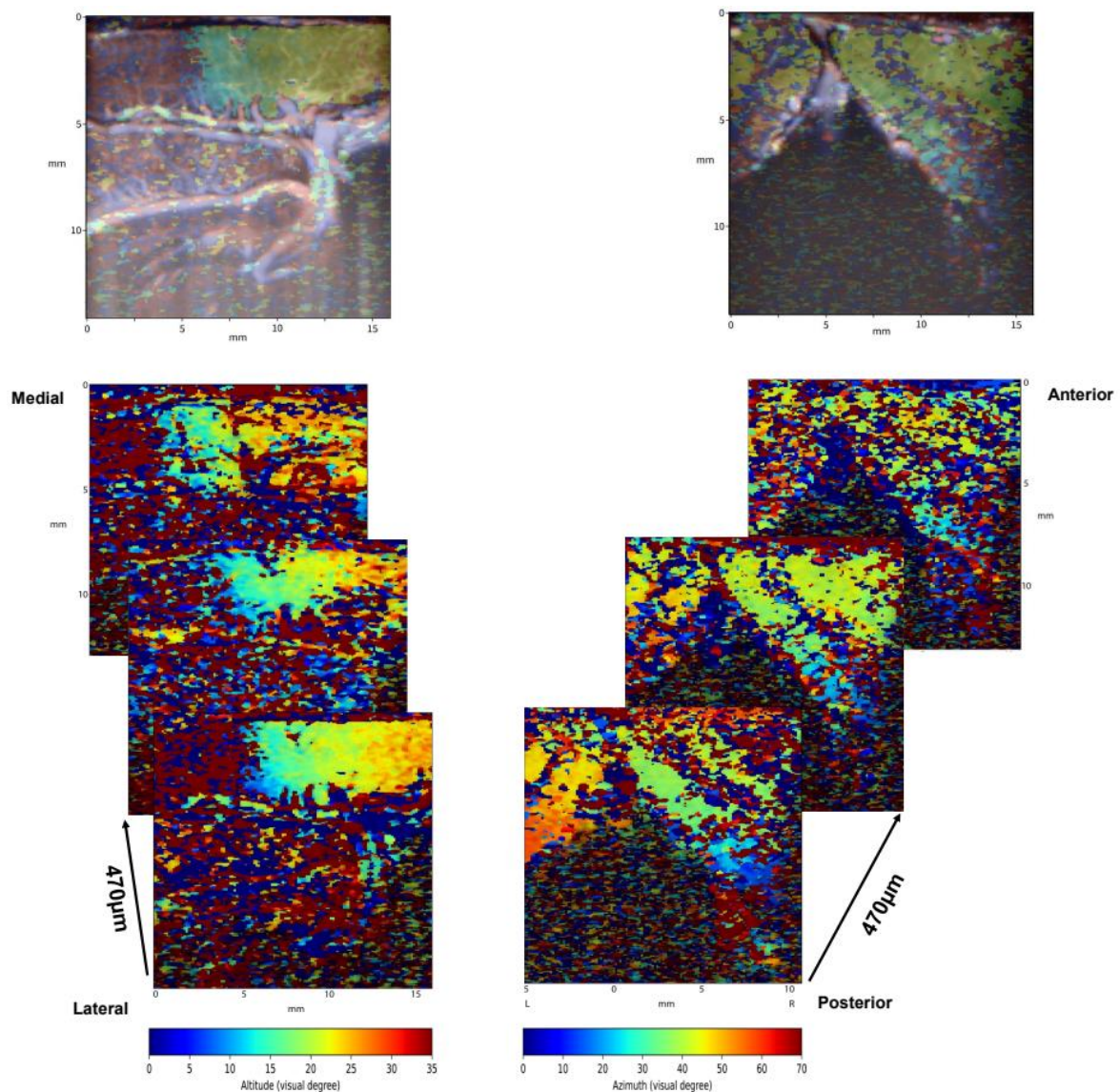
**Figure 13 Ocular dominance map in cat visual cortex recorded using fUSI.**

Every pixel is coloured according to stimulation through which eye elicited the biggest response in its position. Colour coding of eyes is shown on the right. Pixels that did not show a significant response to any orientation are left without colouring. Scale bar in bottom right corresponds to 1 mm.

### 4. Retinotopy map

I have constructed retinotopy maps of deep visual cortex in anaesthetised, head-fixed cats. Before the measurement, we covered one eye of the animal and mapped the area centralis of the other eye to the centre of the stimulation screen using an ophthalmoscope. This was to ensure that the stimulating screen covers as much of the visual field of the animal as possible. I used horizontally and vertically oriented flickering checkerboard bar stimuli drifting through the screen perpendicular to their orientation, as it was shown in Figure 9. Utilising our novel

motorised imaging chamber design, I can record functional maps in adjacent positions. By combining such recordings, I can reconstruct large-volume 3D functional maps. The recording of such a map in three sagittal and coronal sections is shown in Figure 14. On the shown maps a continuous colour gradient represents that the areas covered by that gradient were activated sequentially as the stimulating bar swept across the screen. The recorded maps correspond well to the known organisation of retinotopy in cat visual cortex<sup>70,71</sup>.



**Figure 14 3D retinotopy map in cat visual cortex recorded using fUSI.**

Left, Sagittal plane retinotopy maps. Top, Retinotopy map overlain on anatomy image to show how the map relates to large vasculature. Bottom, Altitude maps in three adjacent sections of deep visual cortex. Colour represents “vertical” position in the visual field, while brightness shows response intensity. Right, Same as on the left but for azimuth mapping in coronal orientation.

## 5. Additional results

In the originally used fUSI software each repetition of a certain stimulation was recorded separately. The stopping, restarting and signal stabilisation of the fUSI recording resulted in a loss of approximately 8 s each repetition. By reworking the stimulation and recording protocol to consist of a single continuous recording, I could avoid this loss. For some of the functional mapping experiments this resulted in a 30% reduction in time requirement. This reduction directly translates to more mappings being possible in a single experimental session. Such an increase in efficiency eases the burden of the large number of experiments and may also reduce the number of experimental animals needed for my research, by being more effective at extracting data from fewer animals.

All the shown functional maps take less than 10 minutes to record in a single position, making my method a rapid technique of mapping the functional organisation of the brain of large animals compared to the state of the art<sup>69,50</sup>.

As a test for the speed of the analysis, I have measured the amount of time it takes to analyse all the recordings made in a single experimental session. The complete analysis of 30 recordings – including the manual selection of regions for finding the most responsive areas – took ~50 minutes. Therefore, the average time needed for analysing a single recording is below 2 minutes. This means, that the experimenter can see the results of a recording in close to real-time, which is crucial for making informed decisions about how the experiment should be continued.

Due to the well-organised nature of the created data, it is easy to move the recordings to another computer for analysis. By doing this, the next experiment can be started while performing the analysis. Consequently, the downtime between experiments can be reduced to ~1 minute, at which point it is practically no longer a limiting factor of experimental progress.

I have created prototype experimental scripts that run multiple stimulation protocols sequentially without the need for user input, however, due to the value of virtually instant feedback about the success of a single experiment we do not use these in practice yet.

The lack of results shown using the wfanalysis software is because it is relatively new and has not been tested in animals yet. Nevertheless, since it shares many similarities with the fus experimental and analysis environment, it is certainly capable of producing the same type of functional maps, but from a different point of view.

## VI. Conclusion

In this thesis, I have provided an overview of amblyopia as a prevalent condition affecting a significant portion of the global population. Furthermore, I have emphasised its potential as a model for understanding various neurodevelopmental disorders. Based on this background, I have outlined my long-term research plan, which aims to map the functional organisation of both healthy and perturbed brains, then compare them. The ultimate goal of this research is to enhance our comprehension of developmental disorders and apply this knowledge to develop more effective diagnostic and therapeutic approaches.

To achieve these objectives, I have discussed the currently most utilised functional mapping techniques in neuroscience and proposed the integration of two specific methods to gain a more comprehensive understanding of brain function. My primary responsibility in this project is the creation and maintenance of software necessary for conducting functional mapping experiments and analysing the acquired data. I have provided detailed descriptions of the functional ultrasound imaging and widefield optical imaging software environments, including their feature sets, visual stimuli, and analysis protocols.

Furthermore, I have presented the results obtained using the developed software, which serve to validate the methodological advancements made in this study. Specifically, I have focused on the production of orientation preference, ocular dominance, and retinotopy maps. Additionally, I have made significant progress in optimising the analysis program to provide real-time feedback for experimenters, contributing to its practical implementation. Throughout the project, I have been actively engaged in continuous testing and participated in experiments, data collection, and analysis.

Overall, the accomplishments described align with the goals outlined in the research paradigm, highlighting the successful development of visual stimuli and analysis protocols, as well as the continuous refinement of the software environments. These achievements demonstrate the potential and effectiveness of the methodology employed in this study.

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## VIII. Attachments

The code repositories for the two software environments described in the thesis can be found on the following public links:

Software environment for fUSI experiments and analysis:

<https://gitfront.io/r/abelpetik/jNXsxgvmqhAx/fus/>

Software environment for WFOI experiments and analysis:

<https://gitfront.io/r/abelpetik/Yb8drcHp6kVs/wfimaging/>