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Functional Imaging

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Definition

A technique that records neural activity in many places in parallel, generally using light. Activity patterns can be visualized as images that reflect both anatomy in space and activity in time. Subtechniques include optical imaging (e.g. using intrinsic signals or voltage sensitive dyes), calcium imaging (using fluorescent dyes, ▶ ratiometric dyes, measure of ▶ deltaF/F), and functional magnetic resonance tomography (fMRT), among others.

Characteristics

Imaging Techniques

Brains consist of a high number of neurons that are interconnected and work as an ensemble. For example, the human brain has 10 billion neurons, and even the brain of a humble honeybee has 1 million neurons. Understanding their function requires recording from these neurons under physiologically controlled conditions. Single-cell electrophysiology gives information about neural activity in individual cells as a whole. Functional imaging techniques have been developed in order to record neural activity both in space and in time simultaneously. Space can include the analysis of activity along the branching pattern of a single neuron, or across large neuron populations. As a consequence, functional imaging has gained an important role in understanding the relationship between the morphology of neurons and their function, and in understanding combinatorial information processing in the brain.

In the following, I will review the major techniques that have been used in recent years, first from the point of view of the signal that is measured (intrinsic signals, dyes, reporter proteins), next from the point of view of the neurons measured (from single neurons to entire brains), including examples from the analysis of olfactory systems.

Optical Imaging: Intrinsic Signals

Neurons change their light properties when they are active, though the exact physics of this phenomenon is as yet unknown. While these changes were used in the very early days of optical imaging, today intrinsic signals are used mostly for *in vivo* studies of mammalian brains. Here, the main signal derives from blood, which changes its color in its transition from the oxygenated to the deoxygenated state. Therefore, the brain is illuminated with a red light, and the reflectance is measured. While the skull has to be removed in order to gain optical access to the brain in most cases, in small mammals, notably rodents, successful measurements have been done through the thinned skull bone layer. The advantage of this technique is its independence from dyes, and therefore the brain is measured with very little interference. However, the signal is only an indirect measure of neural activity, since it consists of a blood oxygenation response, and therefore it is relatively slow and has a limited spatial resolution. Hence the strength of this technique to map spatial activity patterns.

Optical Imaging: Voltage Sensitive Dyes

Much faster measurements are possible with voltage sensitive dyes (VSD), and when only neurons are stained with these dyes, the signal is exquisitely neuronal. VSDs are generally fluorescent dyes, with a charged chromophore attached to a lipophilic moiety that integrates into the cell membrane. As the voltage across the membrane changes, the charged chromophore moves with respect to the membrane, and the fluorescent spectrum shifts. When using a ▶ fluorescence excitation and emission setting that hits the dye's maximum, a shift in fluorescent spectrum of the dye results in a fluorescence decrease. This decrease is proportional to the membrane's voltage change. VSDs are generally fast, which is their greatest advantage. In suitable preparations it is possible to resolve single spike events. However, changes in fluorescence are small, often resulting in a poor ▶ signal to noise ratio. Dyes can be applied by soaking the brain in it, or by selective staining (see below). Because optical access to the brain is necessary, these techniques are always invasive.

Optical Imaging: Calcium Signals

Most studies done with animals as opposed to humans have used calcium as an indicator for cellular activity. This has specific reasons: calcium is the ion that has the greatest concentration change when a neuron is active. Within cells, calcium concentration is in the range of 200 nm, while outside concentration can easily reach millimolar concentrations, giving a concentration difference of up to 10,000-fold. This enormous gradient is actively created by the cells with calcium pumps that keep intracellular concentration low, and is used for intracellular messaging: calcium plays an important role in many intracellular information cascades, ranging from neurotransmitter release to second messenger cascades, including sensory transduction cascades. Most cells express voltage sensitive calcium channels, so that intracellular calcium concentration increases when the neuron depolarizes. However, although in most cases the correlation between calcium concentration and depolarization and/or spiking activity is very good, this relationship is not one-to-one, given that calcium fulfils many more tasks within the cell.

Calcium dyes are generally modified ▶calcium chelators with a fluorescent chromophore attached to it. The calcium chelator has a predominance of negative charges that are compensated when the positive calcium ion is trapped in it. Thus, the fluorescence chromophore is modified, with a resulting change in fluorescent properties. In some dyes, such as *calcium green* or *fluo-4*, fluorescent intensity increases with increasing calcium concentration. In other dyes, such as *fura*, increase or decrease depend on the excitation wavelength. Specifically, in *fura* measurements, fluorescence with excitation light of 340 nm increases, and with excitation light of 380 nm it decreases (at approx. 360 nm there is an isosbestic point, i.e. a point where fluorescence is calcium-concentration independent).

Optical Imaging: Other Dyes

There are a variety of other dyes to monitor cell activity, including dyes for ions such as chloride and potassium, pH-sensitive dyes, or more sophisticated variants that monitor transmitter vesicle release. It is beyond the scope of this chapter to list them all.

Optical Imaging: Genetically Engineered Dyes

An area of fast and important growth is the development of genetically engineered reporter proteins. The basic concept is easy: a fluorescent protein is linked to another protein that is sensitive to the metabolic status of a cell in such a way that changes in metabolism result in fluorescent changes. Then, the gene for this artificial protein is inserted into an animal under the control of a cell-specific promotor creating a transgenic organism. As a result, a genetically defined population of cells will express the reporter gene, and measurements of these cells can be done with optical methods.

Take, as an example, the calcium sensitive G-CaMP. One component of this protein is a modified GFP-protein (GFP – green fluorescent protein – was the first of what is now a large family of proteins that are fluorescent. Several animals naturally express fluorescent proteins. GFP was isolated from the jellyfish *Aequorea victoria* that uses it together with a bioluminescent protein to produce light of controlled wavelength). The other component of G-CaMP is derived from the calcium-sensitive natural protein calmodulin. Thus, borrowing from natural proteins as if it were building blocks in a child's game, a new calcium-sensitive fluorescent protein was created (of course, the technical details make the process much more complex). Finally, in order to use these proteins for functional imaging, their genes have to be inserted into the genome under the control of a promotor. It is the choice of the promotor that will lead to expression in neurons rather than in other cells, or even in genetically defined specific populations of neurons, e.g. only receptor cells, or only inhibitory cells – in fact, any cell populations for which a specific promotor is known. Alternative approaches include a random gene expression using a gene-gun, where gold particles are coated with the genetic material, shot into the tissue, taken up by some but not all cells, and then expressed, resulting in a situation where some but not all cells are labeled with the reporter protein.

Once the cells of interest have been labeled, recording follows the same procedures as for synthetic dyes. Being fluorescent dyes, it is necessary to gain optical access to these neurons (e.g. by removing the skull in the case of in-vivo measurements), then an excitation light is shone on the neurons, and the fluorescent light is measured using a light sensitive device, e.g. a photomultiplier or a CCD-Camera.

Just as calcium sensitive proteins have been created from calcium-sensitive cell constituents, proteins sensitive to other cell properties have been and/or are being developed. These include probes for membrane potential, for pH value, for synaptic transmission, and for second messengers such as cAMP. A further development is to create probes that interfere with cellular events: here we leave functional imaging, and enter the realm of targeted functional manipulation.

fMRT: BOLD Signal

An increasingly important technique in biomedical imaging measures the BOLD signal (Blood Oxygen Level Dependency) with fMRT (functional Magnetic Resonance Tomography). This is a technique that uses intrinsic signals (see above), but does not involve light. Rather, a strong magnetic field is applied as a pulse, and the magnetic relaxation is measured. The BOLD-signal

is related to blood flow, and therefore also to neuronal activity. Just as for other intrinsic signals, this is an indirect measure of neuronal activity, and therefore intrinsically slow. Furthermore, the spatial resolution is limited by the strength of the magnetic fields used, and by the volume of blood affected by neural activity. Therefore, single cell analysis will never be possible with this technique. However, brain areas can be investigated. For example, in the olfactory system, the analysis of individual olfactory glomeruli is already possible. The greatest advantage of this technique is that the animals remain intact: no chirurgical manipulation is necessary, and no dye or contrast agent needs to be administered. As a consequence, fMRT gains increasing importance in studying brain activity in humans. Most importantly, results from invasive experiments on animals can in many cases be verified for their relevance to the human system by using fMRT.

Imaged Structures

Functional imaging studies play an important role in neuroscience research, because they allow measuring neuronal activity over time and space. However, the kind of results that can be obtained depend strongly on the technique used, and on the structures that are imaged. It is the combination of different staining techniques that is allowing scientist to study brain function just as putting together a jigsaw-puzzle.

Single Neurons: Selective Loadings

The unit of brain activity is the individual neuron. After loading a single neuron with dye using a microelectrode (effectively a hollow glass tube with a microscopic tip that penetrates into the cell), functional imaging allows to analyze how the neuron itself is substructured into functional domains. A textbook neuron consists of an input branch (the dendrites) and an output site (the axon), joined by the cell body. Using functional imaging, these compartments can be characterized: their electrical and biochemical properties can be analyzed separately. For example, many cells have spines on their dendrites, small protuberances that are the sites of synaptic input. Imaging studies showed that each spine constitutes a compartment on its own, acting in concert with the rest of the cell, but independently to a great extent. In more complex neurons, where the input and the output regions are not separated so clearly, functional imaging is used to map input and output sites, and characterize their properties. For example, local neurons within the olfactory bulb create microcircuits mediating activity of neighboring mitral cell dendrites. Thus, these neurons do not really act as a unit, but themselves as a complex of many, interconnected units.

Neuron Populations: Selective Staining

Neural processing occurs in neural networks of interconnected units and networks. By selectively staining particular populations of neurons it is possible to follow neural processing along its steps. For example, in the olfactory system odor coding can be followed from receptor neurons, to populations of local neurons in the first olfactory epithelium (the mammalian olfactory bulb, or the insect antennal lobe), to higher order brain centers, including the mammalian cortex. Such studies are only possible by selectively staining specific populations of neurons, because in any one brain area there are many neurons that contribute in different ways to processing in that area. Techniques for selective staining include filling many neurons by injecting a bolus of highly concentrated dye or dye crystals into a brain region, with the result that many neurons in that region will pick up the dye. As a result, in other areas of the brain neurons will not be stained, unless they have a projection (e.g. an axon) into the treated area – with other words, here the staining is selective for those neurons that have specific connections to the injected area.

Neuron Populations: Genetic Labeling

Many neurons that work in groups are not easy to label by dye injection. For example, in all brain regions there are local neurons that do not project to other areas. These neurons cannot be stained by dye injection without incurring into unspecific staining of other neuron populations. In many cases, however, it has been possible to find genetic promotors that are specific to local neurons, and to use these for expressing reporter genes in these neurons. The fruit fly *Drosophila melanogaster*, for example, is among the animals with the best genetic tractability. Here, it was possible to characterize at least three distinct populations of local neurons in the first olfactory processing area, the antennal lobe, and to show that they all have a distinct role in odor processing, and different functional properties.

As another example, many olfactory sensory cells express the same olfactory receptors, and consequently respond to the same odors. This is a property that derives from their genetic instruction, and has been used to create animals were only neurons expressing a particular receptor type express a reporter gene. Such preparations can be used to characterize the molecular response profiles of olfactory receptors, i.e. the entire description of the odors that bind to a particular receptor.

Neuron Populations: Non-Selective Staining

Non-selective stainings are those that make no distinction of different cell types. One technique is to bath the brain tissue in dissolved dye, and to allow all neurons to

incorporate the dye. These studies do not allow dissecting the cellular steps of neural processing. However, they allow investigating the spatial arrangement of information processing in the brain. For example, the olfactory system creates a functional map of activated glomeruli in the antennal lobe (insects) or the olfactory bulb (mammals). These maps can be directly measured using non-selective staining techniques. Such measurements have revealed the basic principles of olfactory coding: a combinatorial scheme, where each odor evokes activity not in one, but in many glomeruli, and the information about the odor resides not in any single glomerulus, but in its combinatorial arrangement. Thus, a single glomerulus will contribute to the code of very different odors, and the task of the brain is to extract the olfactory significance in each combinatorial pattern.