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Review

Calcium imaging in retinal research: challenges and prospects for its application

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Abstract: Calcium ions are universal signaling particles used alongside other signaling systems in animal cells. Changes in intracellular calcium concentration trigger vital reactions in different types of eukaryotic cells by acting through molecular calcium sensors. Over several decades, calcium imaging has been developed to study calcium-based signaling pathways. This method visualizes changes in intracellular free calcium using special fluorescent indicators. The retina, a type of nervous tissue in the eye, is responsible for perception, primary processing, and transmission of visual information to the brain. Signal cascades within retinal cells and synaptic transmission between cells play a crucial role in implementing these functions. Calcium plays a significant and diverse role in the functioning of the retina, in both normal and pathological conditions. Studying the fundamental processes of visual perception at the retinal level requires the visualization of changes in intracellular calcium concentration at different time scales, including very rapid changes. Consequently, the calcium imaging method, which was originally developed and used for studying other tissues, has now entered the field of visual neuroscience. While there are currently many examples of calcium imaging being used to study the functioning principles of all major types of retinal cells, adapting this method to the study of this tissue presents a number of difficulties. This review discussed these problems and how to solve them.

Keywords: calcium imaging; retina; vision; calcium intracellular signaling

1. Introduction

Calcium ions (Ca^{2+}) are universal messengers used alongside other signaling systems in animal cells. Changes in the intracellular concentration of this ion trigger a variety of reactions in different types of eukaryotic cells by acting through molecular calcium sensors. In activated cells, the concentration of Ca^{2+} increases, which serves as a trigger for the initiation of signaling pathways. The process of calcium signaling is involved in the regulation of a number of processes, including cell death (including apoptosis), proliferation, differentiation, and autophagy [1–5]. Other processes in which calcium signaling is involved include muscle contraction in skeletal muscles and myocardium [6–8], other types of cell motility, and secretion in endocrine glands [9,10]. Perhaps the most complex system based on Ca^{2+} signaling is the synaptic transmission in the nervous system [11,12]; this calcium function (among others) is associated with its important role in sensory system function [13–15].

The role of Ca^{2+} as a universal secondary messenger depends upon the ability to rapidly and significantly fluctuate its cytoplasmic concentration. This is due to the high Ca^{2+} gradient across the membrane, with a concentration maintained at submicromolar levels in the intracellular environment and at 1–10 mM in the extracellular environment [16]. The balance of free Ca^{2+} in the cell cytosol is determined by its entry through the membrane via ion channels, its removal from the cell by transporters or exchangers, and its binding by intracellular buffers (or chelators). Short-term increases in cytosolic calcium concentration occur due to influx from the extracellular space or release from intracellular stores [17]. Ion channels that allow Ca^{2+} to enter the cell include voltage-gated Ca^{2+} channels (VGCCs), Ca^{2+}-permeable mechanosensitive channels such as PIEZO, ionotropic glutamate receptors (iGluRs), cyclic nucleotide-gated channels (CNGCs), and hyperosmolality-gated calcium-permeable channels (OSCAs) [18]. Small molecules and macromolecules can act as calcium buffers, having affinity for Ca^{2+}. In a resting cell, calcium-binding proteins play the most important role. These proteins have an affinity for Ca^{2+} at the micromolar level and sufficient selectivity, despite competing with Mg^{2+} at the millimolar level. Examples of such calcium-binding proteins include troponin C in muscle cells and calmodulin, which is found in all tissues [19]. Proteins with lower affinity for calcium, such as calsequestrin and calreticulin, play an important role within cell compartments where the physiological level of calcium is in the order of hundreds of micromoles, such as in the sarcoplasmic reticulum, the endoplasmic reticulum of non-muscle cells, and mitochondria. Calcium pumps and exchangers alter the concentration of Ca^{2+} in the cytosol or in intracellular calcium stores by pumping it out. Regardless of their location in the cell, all calcium pumps are P-type ATPases. They have a high affinity for calcium and belong to active transport systems, using ATP energy to undergo conformational changes and transport Ca^{2+} ions against their electrochemical gradient [20]. Calcium pumps can interact with calmodulin, making up a Ca^{2+}-dependent pump. Another way in which calcium is removed from the cell is through cation exchangers, such as Ca^{2+}/Na^{+} exchangers (NCX), which exchange one calcium ion for three sodium ions [21], and H^{+}/Ca^{2+} exchangers (HCX), which are found in the plasma membrane of some cell types and play a greater role in mitochondrial Ca^{2+} uptake [22].

Intracellular depots play an important role in maintaining balance and facilitating rapid changes in intracellular calcium concentration. These include certain cell organelles that can efficiently capture and release Ca^{2+}, the most important of which are the endoplasmic reticulum/sarcoplasmic

reticulum (ER/SR) and mitochondria. In animal cells, calcium is actively pumped from the cytosol into the ER/SR lumen by a Ca^{2+}-ATPase pump. Two receptors—the ryanodine receptor and the IP_3 receptor—are responsible for rapidly releasing calcium from the ER/SR in response to a primary stimulus, such as an action potential or a hormone [23,24]. Mitochondria are the second most important Ca^{2+} depot, regulating the amplitude and type of Ca^{2+} signal (rapid increase or oscillations). They capture Ca^{2+} from the cytosol using the mitochondrial Ca^{2+} uniporter (MCU) [25]. The MCU is sensitive to cytosolic calcium levels due to MICU regulatory factors containing EF-hand motifs.

To interpret changes in calcium levels within a cell in response to a particular stimulus, calcium-binding proteins are required. It is believed that, in the early stages of evolution, these proteins were responsible for detoxifying high concentrations of Ca^{2+}, subsequently taking on the function of decoding the Ca^{2+} signal [16]. These proteins bind to Ca^{2+} and undergo conformational changes, which leads to a change in activity or the implementation of a specific function. The most extensively studied Ca^{2+}-binding proteins are calmodulins [26]. These are Ca^{2+} sensors with an EF-hand motif, along with several other Ca^{2+}-binding proteins. These proteins have a structure consisting of six helices named A, B, C, D, E, and F. Two of these helices, E and F, form a "hand", with sites on this hand that can selectively bind calcium at micromolar levels. High selectivity is achieved by the structural features of the binding loop. Other proteins with the EF-hands motif include parvalbumin, recoverin, calretinin, and GCAP [19]. Interestingly, the EF-hands motif is highly conserved and widespread among calcium-binding proteins, which are found in the cells of all eukaryotic organisms. In addition to EF-hand proteins, other types of Ca^{2+}-binding proteins have been discovered, such as those with a C2 motif [including protein kinase C (PKC), phospholipase C (PLC), synaptotagmin, and several others] [27,28]. After binding to calcium, calmodulin and other calcium-binding proteins interact with various targets, including kinases, phosphatases, transporters, cytoskeletal components, transcription factors, and ion channels. This wide range of targets enables calcium-binding proteins to trigger a broad spectrum of cellular functions, as mentioned above.

The retina is a type of nervous tissue and a neural network that is responsible for the perception and primary (and highly complex) processing of visual information, as well as transmitting this information to the brain. Signaling cascades in different types of cells and synaptic transmission between cells are clearly significant in the implementation of these functions. Consequently, calcium plays significant and diverse roles in the functioning of the retina in both normal and pathological conditions (see [29] for an overview). Visualization of changes in intracellular calcium concentration at different timescales, including very rapid changes, is required to study the fundamental processes of visual perception at the retinal level. Consequently, the calcium imaging method, which was originally developed to study other tissues, is being established in the field of visual neuroscience. While there are currently many examples of calcium imaging being used to study all major types of retinal cell, adapting this method to the study of this tissue presents a number of difficulties. This review will discuss these problems and ways to solve them.

2. General principles of calcium imaging of animal cells

2.1. The fundamentals of the calcium imaging method

As previously mentioned, the Ca^{2+} ion acts as a universal secondary messenger; fluctuations in its cytosolic concentration are closely correlated with key cellular processes and functional states.

Calcium signaling is accompanied by changes in intracellular calcium concentration by several orders of magnitude, from submicromolar to tens of micromoles [19,30]. In order to study such processes, the scientific community required a tool with which to measure free intracellular calcium with adequate temporal and spatial resolution. Some of the earliest methods used ion-selective electrodes and absorbing dyes. Ion-selective electrodes for measuring calcium levels have been in use for over half a century [31,32] and are useful for measuring extracellular Ca^{2+} concentrations. However, the approach using intracellular fluorescent dyes has proven more effective for studying intracellular calcium events. Absorbing dyes (e.g., arsenazo III) played an important role in the study of Ca^{2+} signaling [33] but were also later replaced by fluorescent dyes. The development of calcium imaging has historically been influenced by two parallel processes: the development of instrumentation (fluorescent microscopes) and the continuous improvement of calcium indicators. As for the instrumentation, scientists initially used simple fluorescence microscopes to visualize fluorescent dyes, followed by confocal microscopes [34], which produced significantly less noisy images. Later, confocal laser scanning microscopy was used [35] and, particularly important for studying the retina, two-photon microscopy [36]. Calcium sensors for imaging have also evolved significantly. One of the first bioluminescent calcium sensor proteins discovered in nature was aquarin [37], which emits light in the blue part of the spectrum when bound to calcium. While the advantage of such bioluminescent sensors is that no external light source is required for measurement, there are also disadvantages. Since then, several new generations of compounds have been developed for this purpose, with improvements in delivery methods, selectivity, efficiency, and other characteristics.

Calcium imaging is a method for quantifying the concentration of intracellular Ca^{2+}, and it is also used to visualize its spatiotemporal dynamics. This method is based on the use of fluorescent calcium sensors (indicators) that alter their fluorescence properties when bound to Ca^{2+} ions [30]. Depending on the type of indicator, this change can manifest as either a change in the ratio of fluorescence intensities at the maximum excitation and emission wavelengths (ratiometric indicators, e.g., Fura-2 and Indo-1) or a change in fluorescence intensity at single wavelengths (e.g., Fluo-3 and Fluo-4).

This method has several advantages:

- Relatively high temporal resolution: Changes in Ca^{2+} concentration can be recorded with a resolution of tens or hundreds of milliseconds. The limiting factor is usually the speed of the imaging system (scanner or camera) or the light stimulation system.
- Wide sensitivity range: Modern indicators can detect changes in Ca^{2+} across a wide concentration range [18]. However, the sensitivity range of each specific indicator is limited (i.e., the range of calcium concentrations at which the indicator detects the ion and does not saturate), so careful selection of the indicator is required for a specific experimental task [38].
- Multicellular recording (wide-field imaging) is possible: Many indicators can be loaded effectively into cell populations, enabling the parallel recording of calcium signals in multiple cells. This is particularly valuable for studying patterns of neural activity and lateral interactions in tissues such as the retina, where complex convergence and integration of signals occur.
- High spatial resolution: Modern indicators enable the visualization of Ca^{2+} dynamics at the level of individual cells or even subcellular compartments, providing accurate signal localization [18].

These last two points distinguish imaging from electrophysiological methods, which are alternative approaches to studying neural circuits.

2.2. Calcium indicators used in calcium imaging

Depending on their chemical nature, calcium indicators can be divided into two large subgroups: synthetic and genetically encoded (protein) (see [18] for an overview). It is also worth considering the division of indicators into ratiometric and non-ratiometric types [30]. Regardless of type, the key characteristics of calcium indicators are their affinity for calcium, indicated by their dissociation constant (Kd), which ranges from tens of nanomoles (basal level) to tens of micromoles (peak concentrations in synapses and organelles); their spectral properties, such as the wavelengths of excitation and fluorescence emission; and quantum yield of photon emission in the presence and absence of Ca^{2+}. Additional characteristics include bleaching rate, buffer capacity, and Ca^{2+} binding kinetics [39]. The properties and quantitative characteristics of calcium indicators are discussed in detail in the reviews [18] and [30].

Synthetic calcium indicators are small molecules, typically consisting of two parts: a calcium chelator and a fluorophore. The fluorophore is excited by a photon of one wavelength and emits a photon of another wavelength. Binding calcium ions to the indicator leads to conformational changes in the molecule, causing a change in fluorescence parameters (see Figure 1A). These changes can be used to estimate calcium concentrations and dynamics. Synthetic indicators are characterized by their low molecular weight, which could ensure rapid diffusion into cells. However, the polar nature of the molecule (which is necessary for binding to Ca^{2+}) can prevent effective penetration through the cell membrane. To address this issue, AM-form indicators were developed [32], where the polar group is chemically bound to an acetoxymethyl (AM) group, rendering the compound hydrophobic. With this configuration, the indicator has a minimal chance of binding Ca^{2+} ions (provided there are no extracellular esterases). AM-modified indicators penetrate the membrane and undergo intracellular hydrolysis by esterases, releasing the chelator. This approach improves the signal-to-noise ratio by reducing the level of extracellular fluorescence (non-esterified forms of indicators are characterized by relatively high extracellular fluorescence intensity). Once inside the cell, most synthetic indicators are removed by active transport mediated by anion transporters within a matter of minutes, hours, or days, depending on the cell type and its condition. The use of, for example, probenecid, an inhibitor of anion transporters, increases the retention time of the indicator inside the cell, but this can have an adverse effect on the physiological state of the test object [40]. Due to their hydrophobicity, AM forms can penetrate organelles, potentially leading to high basal fluorescence levels [30]. The latest generation of indicators (Calibryte) demonstrates improved retention in cells without the need for inhibitors. Representatives of the synthetic indicator group differ from each other in a number of characteristics. For instance, fura-2 with single emission exhibits decreased fluorescence when calcium levels increase, whereas indicators such as Oregon Green BAPTA and fluo exhibit increased fluorescence. Different synthetic calcium indicators also differ in their K_d value for Ca^{2+} and in the wavelength of fluorescence excitation/emission. Synthetic indicators are used in ex vivo experiments, and there are various ways of treating isolated tissue, such as brain slices, as part of the delivery methods. These delivery methods include incubation with the AM form, loading in a dextran-conjugated form, pumping into single cells using microelectrodes, and electroporation of the entire tissue preparation [41].

Genetically encoded calcium indicators are proteins expressed based on an introduced genetic construct. These include single-fluorophore (intensiometric) and two-fluorophore indicators, which are based on the phenomenon of FRET (Förster resonance energy transfer). Figure 1B illustrates the

principle of action of single-fluorophore GECIs using the example of perhaps the most popular representatives of this group—GCaMP proteins. These proteins have a structure that includes a calcium-binding CaM domain, circularly permuted GFP (cpGFP), and an M13 peptide motif. The CaM domain binds calcium ions, undergoes conformational changes, and enables the M13 peptide motif to bind to it. Due to the deprotonation state of GFP, its emission intensity increases [42]; however, the degree of deprotonation also depends on the pH level, which should be taken into account when conducting certain experiments where the pH may change [43].

In addition to GCaMP, various other genetically encoded indicators have been developed, including Camgaroo 1 and 2, Yellow Cameleon, TN, and Inverse Pericam (see [41] for an overview). There are two main approaches to delivering genetic constructs encoding indicators to the cells of a living organism: direct delivery to target tissues of an adult organism (e.g., by viral transduction or electroporation in utero) or creating transgenic animals. Both approaches present technological challenges and incur costs. The second approach is particularly expensive, especially in higher vertebrates. Therefore, they have only been implemented for a limited number of species [44–48]. However, this group of indicators also has undeniable advantages. This method enables in vivo experiments to be conducted with minimal impact and multiple repetitions over a long period of time [44]. Genetic coding can also ensure the selective delivery of indicators to specific cell populations or compartments [18,29].

Additionally, calcium indicators fall into two subgroups: ratiometric and non-ratiometric [19,30]. Ratiometric indicators have more than one wavelength of absorption or emission, or both. The ratio of fluorescence signal intensities at different absorption or emission wavelengths enables accurate Ca^{2+} concentration determination while minimizing the effects of uneven dye distribution, leakage, photobleaching, and differences in cell thickness, and subsequent computational processing simplifies the correction of artifacts caused by specimen movement along the X, Y, and Z axes. Non-ratiometric indicators are characterized by single absorption and emission wavelengths. This simplifies the technical setup and reduces the baseline fluorescence level at low Ca^{2+} concentrations.

2.3. Stages of the calcium imaging experiment and configuration of the experimental setups

Two fundamentally different approaches can be used for intracellular Ca^{2+} visualization studies in animal cells: ex vivo calcium imaging (performed on tissue samples or in cell cultures) and in vivo imaging (performed on the tissues of a living animal when access to the tissues is organized and the life and functional state of the organism can be preserved). In vivo calcium imaging uses miniature signal imaging devices such as micro-optical probes connected to an endoscope or a wearable miniscope, which is often attached to the head of the animal being studied. This allows the dynamics of calcium in neurons in specific parts of the brain to be studied [49–52].

This review focuses on calcium imaging of retinal cells. For this tissue, it is more relevant to work with isolated retinal preparations. We will therefore discuss the experimental protocol and necessary equipment for the ex vivo calcium imaging approach in more detail. The main components of a typical experimental procedure are shown in Figure 1B. In order to visualize intracellular Ca^{2+} in surviving tissue preparations or cell cultures, the following are required: a light source to excite fluorescence in the indicator; a means of capturing the fluorescent image; and a device to receive, store, and undergo primary processing of the data, as well as control signal capture (computer). The excitation light source can be a mercury or xenon lamp with a light filter system, LEDs with a

specified emission spectrum, or a laser, which is an integral part of a confocal or two-photon microscope (see [41] for an overview). The image is captured using photodiode arrays (a rare option), CCD-based cameras, or CMOS-based cameras, which are connected to a wide-field or fluorescence microscope on whose stage the specimen is placed. A more modern and productive option is to use confocal microscopes for the single-photon excitation of Ca^{2+} indicator fluorescence. In this technique, an image is created by scanning a laser beam across a tissue sample and decoding the resulting signal. A significant breakthrough in calcium imaging was achieved with the introduction of two-photon microscopy [36,53]. In two-photon microscopy, two low-energy photons in the near-infrared region of the spectrum are used to excite the indicator molecule instead of one high-energy photon. Two-photon microscopy offers several clear advantages for fluorescent tissue imaging: reduced chances of excitation outside the optical focus, less bleaching and photodamage to tissues, improved penetration of excitation light into tissue depths, and high resolution. When studying the retina, two-photon microscopes offer additional advantages and overcome previously existing problems, which we will discuss in more detail in Section 3.1.

A typical calcium imaging experiment with isolated living tissue (such as a brain slice or retina) involves the following steps: First, the tissue is isolated from the animal in a way that preserves maximum viability and functionality of the preparation, according to recommended protocols. Next, the tissue explant is loaded with the selected calcium indicator and placed on the stage of a fluorescence, confocal, or two-photon microscope. For particularly vulnerable preparations, such as warm-blooded animal brain sections and retinal preparations, the tissue is usually placed on the microscope stage in a special perfusion bath. A life-supporting solution at the required temperature, enriched with O_2, constantly flows through this bath. The loading and viability of the specimen are then assessed, after which further stimuli are applied depending on the research task at hand. Fluorescent images of the tissue are captured at the required sampling rate and transferred to a computer for storage and subsequent analysis (see Figure 1B).

2.4. Processing and interpretation of data recorded during calcium imaging

Modern calcium imaging techniques produce large volumes of data requiring complex calculations.

Pre-processing the obtained video recordings involves several key stages: correcting motion artefacts, accurately isolating the signal sources [defining the regions of interest (ROI)], and finally obtaining the primary time series, which reflects calcium oscillations and is free from artefacts associated with the dynamics of the fluorescent indicator itself [54].

Modern algorithms aim to automate these procedures with varying degrees of human involvement [55−57]. In recent years, the active development of AI methods has led to their widespread implementation in these processes [58]. These approaches effectively solve problems such as cell segmentation and neuron detection and are employed for signal cleaning and spike activity extraction [55,59−63]. At the same time, machine learning methods demonstrate efficiency comparable to that of traditional deterministic algorithms and expert assessment [55,56].

After obtaining time series that characterize calcium dynamics in individual cells, subsequent analyses use correlation analysis to identify functional connections between neurons and clustering to identify populations of neurons with similar activity patterns. More complex methods are also employed, including AI-based approaches, to classify cell types, analyze population neural dynamics,

and identify functional states [54,56,58,59,64].

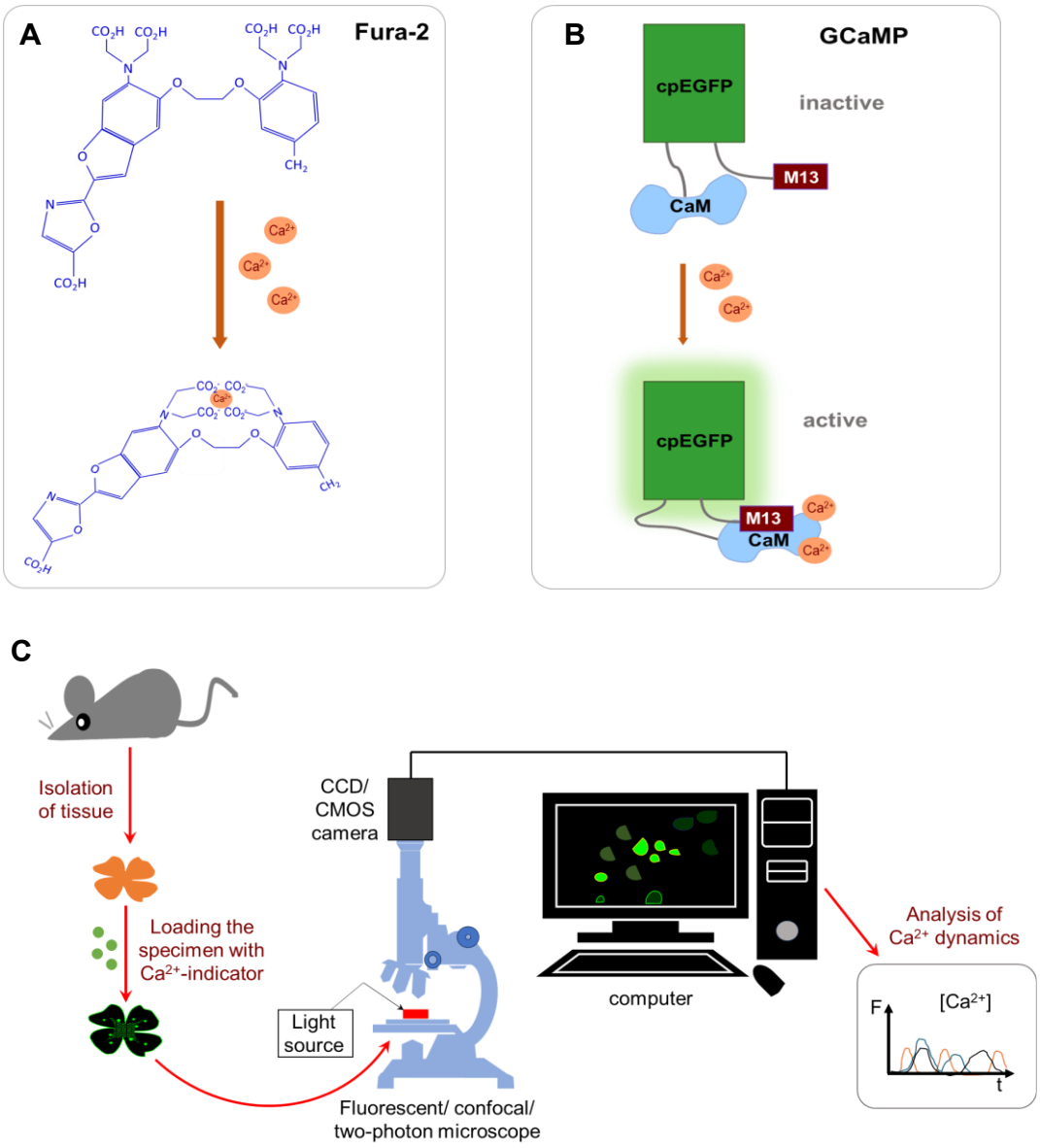

Figure 1. Types of calcium indicators and their principle of action. (A) The principle of action of a synthetic indicator, illustrated using fura-2. The binding of calcium by the indicator's chelating group leads to conformational changes and increased indicator fluorescence. (B) The principle of operation of a genetically encoded indicator, using GCaMP as an example. GCaMP's structure includes a Ca^{2+}-binding CaM domain, circularly permuted GFP (cpGFP), and an M13 peptide motif. The CaM domain binds calcium ions, undergoes conformational changes, and enables the M13 peptide motif to bind to it. Due to the deprotonation state of GFP, its fluorescence intensity increases. (C) The stages of an experiment on ex vivo calcium imaging for isolated tissue (using the retina as an example), and the layout of a typical experimental setup.

3. Calcium imaging of retinal cells

3.1. The structure of the vertebrate retina

In order to understand the value of calcium imaging in studying the retina, as well as the specific problems that arise when applying this method to such a complex biological system, it is necessary to provide a brief overview of the retina's structure and how it functions. The vertebrate eye consists of two parts: the optical system and the neurosensory system. The optical system comprises the cornea, lens, iris, and vitreous body, which focus light onto the light-sensitive retinal tissue. The structure of the retina is illustrated in Figure 2.

The retina ranges in thickness from 0.1 to 0.5 mm. Its thickness varies not only between species, but also within the same organism. Areas with high visual acuity have a higher cell density and differ in thickness compared to other areas of the retina [65]. The retina consists of several morphologically distinct layers, each performing a specific function. These layers are the outer nuclear layer, the inner nuclear layer, and the ganglion cell layer. The inner and outer plexiform layers separate these layers and are areas where synaptic connections between retinal neurons are formed.

The outer nuclear layer comprises the bodies of photoreceptor cells. Photoreceptors consist of two main segments: the outer segment and the inner segment. The outer segment is a specialized structure containing membrane discs in which visual pigments are localized. It is here that phototransduction occurs, i.e., the conversion of photon energy into a change in membrane potential. The inner segment contains most of the organelles, excluding the nucleus, which is located in the cell body. The inner segment supports ion gradients, energy metabolism, and protein synthesis [66]. The inner and outer segments are connected by a cilium through which substances and membrane potential are transported [67]. In the dark, photoreceptors become depolarized and release the neurotransmitter glutamate into the synaptic cleft, which is then taken up by bipolar and horizontal cells. Glutamate release decreases as light intensity increases, reaching a minimum at a certain brightness. It is important to note that the signal generated by photoreceptors is gradual in nature.

The inner nuclear layer comprises the cell bodies of bipolar, horizontal, and amacrine cells. Bipolar cells are the second link in the chain of direct visual signal transmission and extend their dendrites toward photoreceptors [68]. Changes in the level of glutamate release indicate variations in illumination. Depending on their response to these changes, bipolar cells are classified as ON cells (which depolarize in response to light), OFF cells (which hyperpolarize in response to light), and ON-OFF cells (which respond to the onset and end of a light pulse). Like photoreceptors, the responses of bipolar cells are often gradual, but certain bipolar cells are capable of generating spikes under specific conditions [69–71]. Horizontal cells maintain lateral connections between photoreceptors and bipolar cells, forming complex networks of synaptic contacts. They play an active role in ensuring the transition between scotopic and photopic vision and in the formation of color vision [72−74]. Amacrine cells also provide lateral connections, but between ganglion cells. Amacrine cells represent a large, heterogeneous population with diverse functions [65] and are still the subject of active study and the discovery of new cell subtypes.

The third functional layer consists of ganglion cells that collect and process signals from the underlying layers of cells and send an integrated signal to the brain. These cells are also categorized as ON, OFF, or ON-OFF [75]. Unlike bipolar cells, however, all signals originating from ganglion cells are spike sequences. The frequency of these spikes is modulated by the activity of bipolar and

amacrine cells within the receptive field of a given ganglion cell. Ganglion cells also differ in the size of their soma, stratum, and receptive field. They are also often classified according to their morphofunctional characteristics. In some vertebrates, photosensitive melanopsin-containing ganglion cells are distinguished separately as intrinsically photosensitive retinal ganglion cells (ipRGCs) [76,77]. These cells play a role in the formation and maintenance of circadian rhythms, as well as in the formation of the pupillary reflex. However, the classification of ganglion cells is not limited to these cells alone. They also form groups based on the colors of visual stimuli they prefer, their ON-OFF responses, the direction of movement of the light source in the receptive field, and contrast [75].

Glial cells, particularly Müller cells, spread throughout the entire thickness of the retina and perform several vital functions. These include maintaining ion and water homeostasis, removing neurotransmitters from the synaptic cleft, providing metabolic support to retinal neurons, and participating in the formation of the blood–retinal barrier [65,78].

The retina also contains two barrier structures: the inner and outer limiting membranes. The outer limiting membrane is located at the level of the inner segments of the photoreceptors and their bodies, and it is formed by the feet of Müller cells with tight adhesion contacts. The inner limiting membrane is a basement membrane, which, according to the current understanding, is most likely formed by Müller cell processes. This membrane acts as a barrier that separates the retina from the vitreous body. It also partially maintains the retina's shape and represents a fairly strong, impermeable, 500-nm-thick mechanical barrier [79−81]. The inner limiting membrane is composed of collagen IV and laminin [80]. Different ratios of these compounds on either side of the membrane determine its polarity, a factor that must be considered when developing methods for delivering various substances, including calcium indicators.

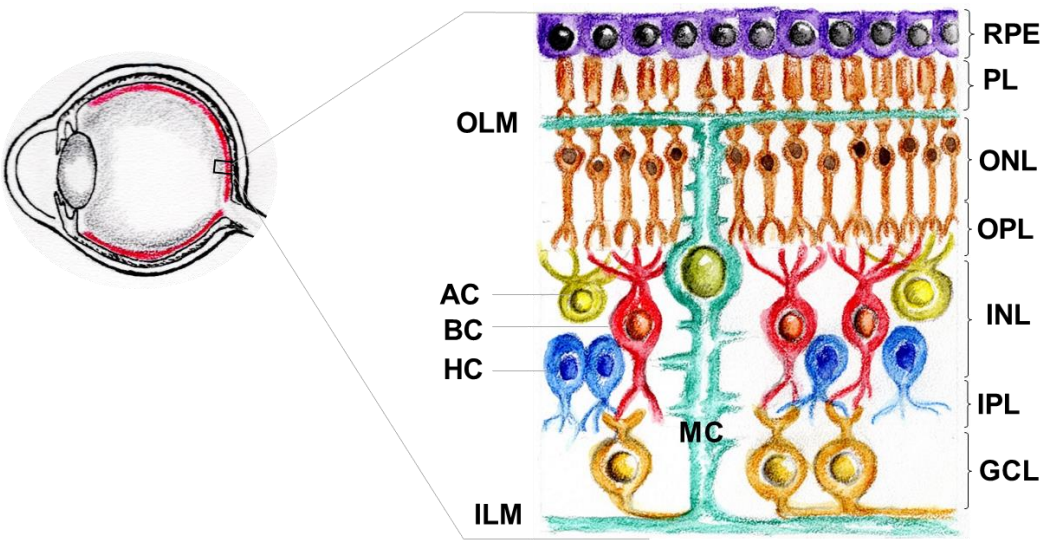

Figure 2. Diagram of the structure of the vertebrate retina. Abbreviations: RPE, pigment epithelium; PL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; OLM, outer limiting membrane; ILM, outer limiting membrane; AC, amacrine cell; BC, bipolar cell; HC, horizontal cell; MC, Müller cell.

3.2. Intracellular calcium dynamics and signaling in vertebrate retinal cells

3.2.1. Photoreceptor cells

In photoreceptor cells (rods and cones), calcium ions do not act as secondary messengers in the phototransduction cascade. However, they play a crucial role in shutting down the cascade after activation by light, as well as in light adaptation [15,82]. Unlike most cell types in the animal body, in photoreceptors, the trigger for the calcium response (i.e., the activation of calcium feedback) is a decrease, not an increase, in its intracellular concentration. Measurements of intracellular calcium in the outer segments of photoreceptors in toads, frogs, and geckos have shown that Ca^{2+} decreases from a dark level of approximately 500–700 nM to approximately 30–50 nM under saturating light [83−85] (see [86] for a review), which corresponds to a change in concentration of approximately one order of magnitude. This decrease in Ca^{2+} levels in the outer segment is due to the closure of cyclic nucleotide-gated ion channels, which allow Ca^{2+} to enter the cell in the dark (Ca^{2+} current accounts for around 15% of the membrane current conducted through these channels), and to the continued operation of the Na^{+}/Ca^{2+}-K^{+} exchanger, which is independent of light [15,87].

In the outer segment of photoreceptors, changes in intracellular Ca^{2+} play a special and important role in the phototransduction cascade. They modulate the shutdown of the photoresponse and are perhaps the main factor in light adaptation [85,86]. Phototransduction cascade consists of a series of biochemical reactions: the visual pigment enters an excited state when exposed to light and interacts with the G protein transducin, activating it. The G protein then interacts with type-6 phosphodiesterase (PDE6), which increases the rate of cGMP (cyclic guanosine monophosphate) hydrolysis. This sharply reduces the concentration of cGMP in the outer segment and closes cyclic nucleotide-gated (CNG) channels. This constitutes a response to light, which is then transmitted to the next links in the retina via the synaptic terminal. The mechanisms of light adaptation are triggered by calcium feedbacks when the CNG channels remain closed due to continued exposure to light. Calcium does not enter the outer segment but is still pumped out by the Na+/Ca^{2+}-K^{+} exchanger, resulting in a decrease in its concentration. This leads to an increase in the activity of G-protein-coupled receptor kinases (GRKs) via the calcium sensor recoverin and an increase in the activity of guanylate cyclase (GCs) via the calcium sensor guanylate cyclase-activating protein (GCAPs) [88−90], as well as an increase in the affinity of CNG channels for cGMP via the calcium sensor calmodulin [29]. Interestingly, the trigger for calcium feedback during photoreceptor light adaptation is a decrease, rather than an increase, in intracellular Ca^{2+} concentration.

Although changes in potential in the outer segment of photoreceptor cells play a major role in transducing visual signals, Ca^{2+}-activated channels in the inner segment and synaptic terminals modulate signals transmitted through the synapse to the second link in the retinal neural circuit [29]. These include L-type channels, which are located near the synaptic ribbon. Additionally, cGMP-gated Ca^{2+} currents, regulated by nitric oxide, appear to be present in the synaptic terminals of cones [91]. Thus, calcium currents in the inner segment and synaptic terminal of photoreceptors are modulated by various factors and neurotransmitters. The resulting change in Ca^{2+} levels regulates the exocytosis of glutamate vesicles in the terminals, affecting the final signal transmitted to bipolar cells [92]. A schematic diagram illustrating calcium exchange and its functions in photoreceptor cells is presented in Figure 3A.

3.2.2. Bipolar cells

The current understanding is that Ca^{2+} does not play a significant modulatory role in the transduction cascade of bipolar cells. However, its participation in synaptic transmission from bipolar cells to underlying retinal neurons (amacrine and ganglion cells) is significant and is the subject of active research. In these cells, and more specifically in ribbon-type synapses, L-, T-, P/Q-, and N-type Ca^{2+} channels have been discovered [93]. These channels can conduct both gradual and transient Ca^{2+} currents [69,92,94]. However, the distribution of calcium channels in bipolar cells is heterogeneous, and the expression of certain types of these channels forms the basis for their classification [70,92]. Figure 3B shows a schematic diagram of calcium exchange in a bipolar cell.

3.2.3. Ganglion cells

Ganglion cells are the final link in the transmission of signals across the retina. They collect excitatory signals from bipolar cells and inhibitory signals from amacrine cells. They then send this information along their axons to the brain. Unlike other types of retinal cell, ganglion cells generate spikes. This is explained by the presence of Na_v channels [92]. At the same time, they also express various types of voltage-activated Ca_v channels of L-, T-, N-, and P/Q-type. However, the distribution of these channels among ganglion cells is heterogeneous [92].

When an action potential occurs in the axons of ganglion cells, it is accompanied by a massive influx of Ca^{2+} into the axon. Pharmacological analysis has shown that this influx is mainly mediated by N-type Ca^{2+} channels in myelinated axons and by L-type channels in unmyelinated axons [95]. It has been suggested that L-, T-, N-, and P/Q-type Ca^{2+} channels play a significant role in regulating non-electrogenic functions of ganglion cells, such as secretion, gene expression, and enzymatic activity, rather than in membrane potential changes and spike generation [92]. Additionally, one might expect changes in calcium levels in the axon to modulate the synapses formed with neurons in the visual nucleus. However, there is no reliable information on this.

The transient influx of Ca^{2+} into ganglion cells during action potential generation allows calcium imaging to be used to study their neuronal activity. This provides a wide range of opportunities to study the functional properties of ganglion cells and how the retinal neural network responds to various stimuli, since ganglion cells are the final integrating link in this network. Figure 3C shows a schematic diagram of calcium exchange in a ganglion cell.

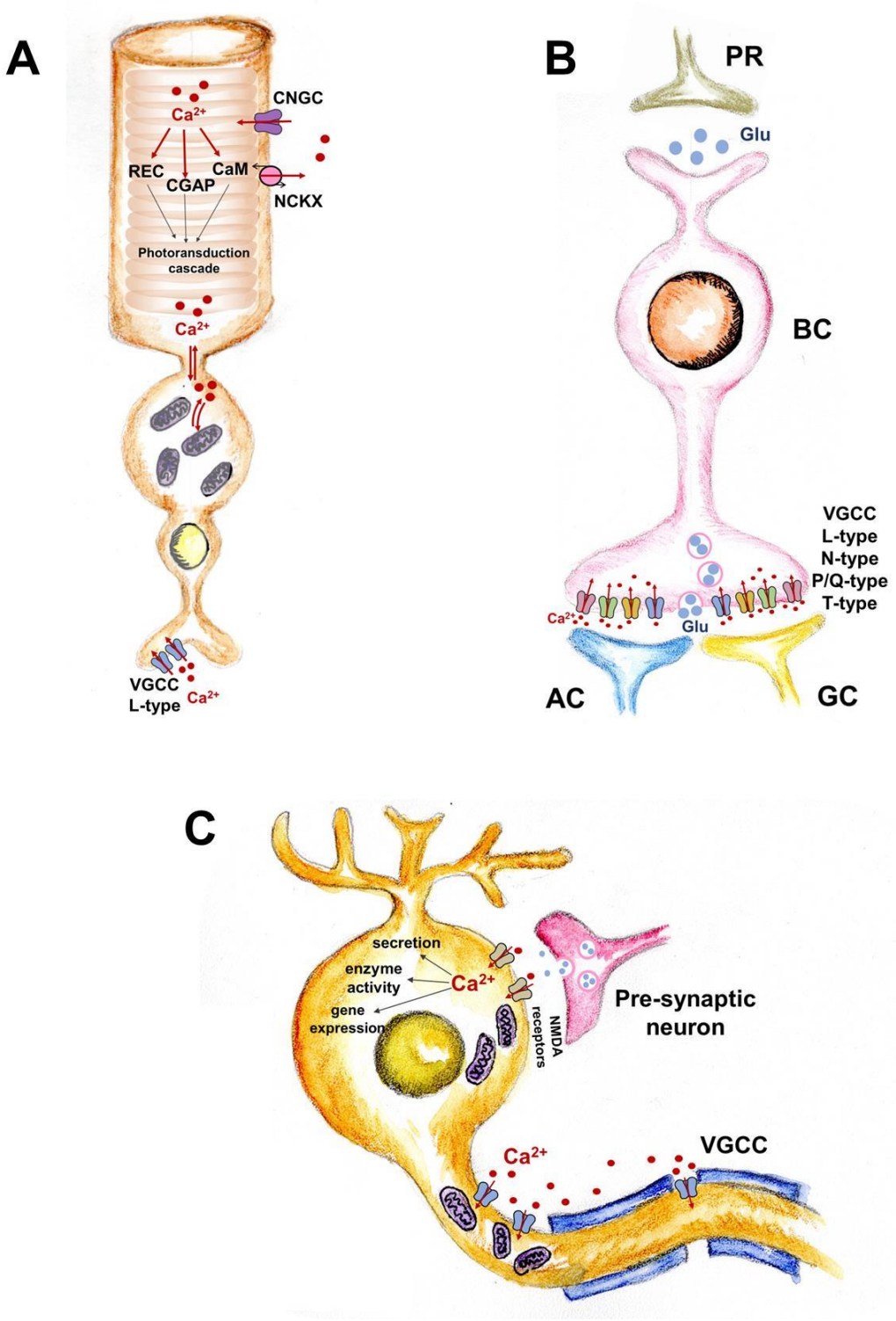

Figure 3. The role and maintenance of Ca^{2+} ion balance in some of the main types of retinal cells. (A) Photoreceptor cell (rod). (B) Bipolar cell. (C) Ganglion cell. Abbreviations: CNGC, cyclic nucleotide-gated channels; NCKX, Na^{+}/Ca^{2+}, K^{+}-exchanger; REC, recoverin; CaM, calmodulin; GCAP, guanylyl cyclase activating protein; VGCC, voltage-gated calcium channels; Glu, glutamate; AC, amacrine cell; BC, bipolar cell; GC, ganglion cell; PR, photoreceptor.

3.2.4. Intrinsically photosensitive retinal ganglion cells

ipRGC are a distinct type of ganglion cell that, as mentioned above, responds to light, which is used to regulate non-visual functions (e.g., circadian rhythms and the pupillary reflex). The phototransduction signaling cascade in ipRGCs differs greatly from that in photoreceptors. When excited by light, melanopsin interacts with G proteins of the q/11 family (Gα_q, $G\alpha_{11}$, and $G\alpha_{14}$), which activate phospholipase $C\beta_4$. This leads to the opening of $TrpC_6$/$TrpC_7$ non-selective cation channels [96]. In at least one type of ipRGC (M1), increasing Ca^{2+} concentration during the development of the depolarizing response has been shown to play an important role in switching off the photoresponse. L-type Ca^{2+} channels are responsible for this Ca^{2+} input [97].

3.2.5. Horizontal cells

As horizontal cells in the retina modulate the transmitted signal through synapses with photoreceptors, calcium plays an important role in synaptic transmission. In these synapses, Ca^{2+} regulates the exocytosis of GABA and glycine [92]. At the same time, horizontal cells modulate the Ca^{2+} current in the synaptic terminals of photoreceptors [72]. Horizontal cells have both L- and T-type calcium channels [92,94]. Some studies suggest that horizontal cells in certain species exhibit a transient Ca^{2+} current that could contribute to membrane repolarization [92].

3.2.6. Amacrine cells

In the vertebrate retina, the amacrine cells are inhibitory cells for the transmission of visual signals, and the electrical synapses that connect them to other cell types play an important role in this function. Numerous studies have demonstrated the important role of calcium in regulating the function of electrical synapses, suggesting that calcium plays a crucial role in the functioning of these cells. In a recent study [98], a line of transgenic mice was developed that expressed the calcium biosensor GCaMP linked to connexin 36 in electrical synapses. This allowed the calcium dynamics near electrical synapses to be traced, revealing that, when the retina is activated by light, the connexins couple to connect AII cells into a network. This process is based on Ca^{2+} concentration waves arising from excess glutamate and the opening of non-synaptic NMDA receptor channels [99]. Therefore, the regulation of calcium in AII amacrine cells may be the basis for the plasticity of electrical synapses.

3.2.7. Retinal glial cells

Müller cells are a type of retinal glial cell that also experiences fluctuations in Ca^{2+} concentration associated with signal transmission. When visual stimuli reach the retina, neurotransmitters such as glutamate and ATP are released, which can lead to temporary fluctuations in Ca^{2+} concentration in glial cells [100]. In Müller cells, calcium waves caused by light stimuli can propagate in different directions. In rats, for example, calcium waves have been recorded propagating from the inner plexiform layer to the endfeet of the Müller cell located in the ganglion cell layer [100]. In other animal species, such as salamanders, however, waves have been observed to originate at the endfeet of the cell and propagate toward the cell body and outer stem processes [101].

Additionally, the differentiation of Müller cells in the rat retina has been shown to be accompanied by a change in these cells' calcium response pattern, with the calcium response in the cell endfeet persisting while gradually disappearing in the cell body [102]. Evidence also suggests that electrical and mechanical stimulation can lead to the formation of calcium signal waves that propagate laterally throughout the retinal glial network formed by astrocytes and Müller cells [103,104]. Such calcium waves in the glial network may contribute to the spatial regulation of neuronal activity by releasing glial transmitters. They may also regulate the volume of extracellular fluid and blood flow to the retina. Furthermore, they may play a role in the pathogenesis of gliosis and retinal neurodegeneration [105].

3.3. Problems associated with calcium imaging of the retina and ways to solve them

3.3.1. Excitation of the retina with a light source exciting the fluorescence of indicators

Unlike other tissues, the retina responds to light stimuli and, in this respect, is a highly sensitive sensor. The photoreceptors responsible for night vision, the rods, can generate and transmit responses to a single photon across the retina. At the same time, calcium imaging is based on exciting fluorescence in a Ca^{2+} indicator using a light source. Since single-photon fluorescence and confocal microscopy use light with wavelengths in the visible range to excite fluorescence, such illumination causes a physiological response to light in the photoreceptors and subsequent cells in the signal transmission pathway, elucidating the responses to both the experimental stimulus and the light used to excite fluorescence [106]. Furthermore, the intensities of the light required to cause fluorescence are so high that they lead to the saturation or even hypersaturation of photoreceptor cells. Consequently, the presentation of the light stimuli under study becomes meaningless (except in the specific case where calcium imaging is used in animal models with degenerated photoreceptors to study signal transmission in the diseased retina). This situation would have been hopeless if two-photon microscopy had not been invented and implemented [36]. As mentioned above, two-photon microscopy uses two low-energy photons instead of one high-energy photon from the visible part of the spectrum. The typical wavelengths of the lasers used as excitation light sources in two-photon microscopes are in the range of 850–1050 nm. However, all known opsins, including long-wave ones, have absorption spectra that hardly overlap with this region [106]. Consequently, two-photon imaging significantly reduces the problem of photoreceptor and other retinal cell excitation and saturation by light for all types of retinal fluorescence imaging, including calcium imaging [107,108]. Despite two-photon microscopy being widely used for retinal imaging and yielding valuable data, it should be noted that there is still a possibility of photoreceptor excitation from light re-emitted by the fluorophore or from two-photon excitation of long-wavelength light itself [109,110], which could affect interpretation of results.

It should also be kept in mind that two-photon imaging systems are expensive, primarily due to the high cost of lasers capable of generating sufficient far-infrared photon flux, as highlighted by several authors [111]. As an alternative, Roy et al. [111] proposed a calcium imaging platform based on single-photon fluorescence excitation. In this case, the beam from the excitation laser passes through the plane of the retinal preparation; being sufficiently narrow, it affects only the layer of cells under study (e.g., ganglion cells) without affecting the photoreceptors. Another alternative to two-photon microscopy is the use of a calcium indicator excited by near-infrared light (e.g., as

proposed by Shemetov et al. [112]), in combination with single-photon microscopy. However, we are not yet aware of any work on calcium imaging of the retina using such IR-excited indicators.

3.3.2. Separation of detected fluorescence and light stimulus for testing visual functions

When examining the retina to elicit visual responses, it makes sense to use light stimuli that correspond to the absorption spectra of the photoreceptor visual pigments of the species under study. However, this can lead to overlap between the light stimulation spectra and the fluorescence detected during imaging, resulting in imaging artefacts. This occurs when the light stimulus passing through the retina and captured by the imaging system is added to the fluorescence signal, creating an apparent increase in intensity. Therefore, special attention should be paid to this problem when designing an experiment. To avoid fluorescence artefacts during light stimulation of the preparation, it has been proposed that light stimuli and indicator fluorescence should be separated by emission spectra, or that they should be spread out in time [113,114].

3.3.3. Loading and retention of calcium indicators in retinal cells

One problem that arose when using first-generation calcium indicators for retinal imaging was their low ability to remain in the cell body. This made it impossible to conduct long-term experimental protocols. To address this issue, manufacturers have developed subsequent generations of dyes. Alternative options include using anion transporter inhibitors or increasing the molecular weight of the indicator by adding dextran.

The issue of loading dyes into retinal cells merits separate discussion. In a living eye or eye cup, the retina is in close contact with the opaque pigment epithelium, which greatly complicates imaging. Therefore, explants, or "flat-mounts", in which the connections between layers and lateral connections are preserved and all major layers can be accessed, have become the most popular option for retinal imaging. Less commonly, cross-sections of the retina that are 200–300 μm thick are used for imaging. Although these have some advantages for visualization, a major disadvantage is the loss of lateral connections present in vital retinas [106]. In this subsection, we will therefore discuss the challenges of delivering synthetic indicators to retinal preparations for ex vivo experiments in a flat-mount configuration.

Regardless of the configuration of the preparation, the issue of polar indicator penetration into the cell through the membrane is solved by using a special AM form of the indicator. This increases the probability of the dye entering the cell and promotes preferential binding of calcium inside the cell after enzymatic hydrolysis. This provides a reduced extracellular background signal, which is characteristic of polar forms of synthetic indicators, and a relatively high intracellular signal. However, overcoming the barrier of the inner limiting membrane is another difficult task that different researchers solve in different ways. The calcium indicator used to load cells in the inner layers of the retina is applied from the ganglion cell side. This means that the first barrier it encounters is the inner limiting membrane (see Figure 2).

As previously mentioned, this barrier is both mechanical and electrostatic [115,116]. While some studies indicate that this membrane filters out only molecules with a molecular weight of several tens of kilodaltons [115,117], most studies using calcium imaging of the retina claim that molecules with significantly lower masses cannot pass through it. Several basic approaches to

overcoming the inner limiting membrane with synthetic indicators are currently known and have been tested, with some suggested by calcium indicator manufacturers in their technical documentation.

According to the results of several studies [118−120], indicators can be loaded retrograde into retinal ganglion cells via the optic nerve (or its stump), bypassing the inner limiting membrane. While this method does not have a serious adverse effect on the retina itself, it does require prolonged incubation (approximately 4–6 hours). This not only changes the functional status of the retina, but depending on the indicator used, it can also reduce the efficiency of loading due to active removal during transport along the axon. However, as the authors of the study [118] pointed out, it is logical to assume that an inhibitor of anion transporters would reduce the rate of removal. Indicators with bound dextran can also be used for this purpose and help increase the time that the calcium indicator remains in the cell.

There are studies in which indicators were delivered to the cells of the inner layers of the retina using microinjections [121,122]; this method is also currently recommended by some indicator manufacturers. Microinjections are performed on a prepared flat-mount retinal specimen using a micropipette prepared in the same way as for patch clamping, with the aid of a micromanipulator. The pipette is inserted from the side of the inner limiting membrane, mechanically piercing it. Then, the indicator is injected under pressure from the pipette into the thickness of the retina. This approach bypasses the inner limiting membrane or cell membrane. Additionally, unlike retrograde loading, the indicator is placed in a location near the cell bodies, reducing the loading time and increasing the total experiment time. However, this approach has one clear disadvantage: the indicator diffuses only a short distance in the retinal plane. Consequently, conducting an experiment over a large area requires multiple injections.

Since the main problem when loading indicators from inside the retina is the inner limiting membrane, this can be removed or its integrity can be disrupted. This can be achieved using enzymes that break down such structures. In their work [123], Cameron and coauthors described using the enzyme papain as one possible approach. However, this approach requires consideration of many nuances and has a strong, nonspecific impact. In addition to breaking down the inner limiting membrane, it can also disrupt cell integrity if the correct concentration and incubation conditions are not selected. Papain requires specific temperature and pH conditions; otherwise, its effect will be minimal. Although the enzyme's optimal conditions are similar to many physiological conditions, they are not suitable for all organisms. Exposure to enzymes can have both short-term and long-term negative effects and can lead to the rapid death of cells (including ganglion cells) [81]. It should also be noted that exposure can destroy the outer segments of photoreceptors if the pigment epithelium is removed.

One of the main approaches recommended by indicator manufacturers is electroporation. A study by Briggman et al. [124] describes using electroporation to deliver the indicator to ganglion cells when loading it from the inner surface of the retina. This approach enables the indicator to be loaded in a very short time compared to other loading techniques. However, the retinal electroporation protocol requires careful consideration of various factors, including the shape of the electrodes, the intensity of the electric field (which should not exceed several kV/m), the number and duration of pulses, and the composition of the solution (e.g., ensuring it does not contain Ca^{2+}) [125−127]. After electroporation, the retina also needs a rest period of several tens of minutes to return to its original functional state. Additionally, an important consideration is how electroporation

affects elongated cells aligned with the electric field, as there is an increased likelihood of membrane pore formation compared to other cell types [128]. This is particularly pertinent for photoreceptors.

There are a number of procedures that are designed to detach the inner limiting membrane. Halfter described a method of mechanically separating the membrane from the retina using poly-L-lysine [79]. The authors were primarily interested in the inner limiting membrane itself rather than in the integrity and functionality of the retina. However, this approach could be adopted to load Ca^{2+} indicator into the inner layers of the retina for subsequent imaging. It is expected that removing the membrane will dramatically increase the efficiency of indicator loading; however, it remains to be seen whether this procedure will compromise the viability of the retinal preparation.

Another interesting procedure is the ophthalmological technique of detaching the internal limiting membrane for certain disease groups [80]. With this approach, the ILM is surgically excised locally in patients under anesthesia, a procedure that could be extended to some types of experimental animals. This would reduce the entire protocol to calcium imaging of nerve tissue, eliminating the need for additional measures to overcome barriers.

The latter two approaches are not documented in the literature as techniques for enhancing the loading of synthetic indicators into the inner layers of retinal explants. These two methods also involve removing large areas of the inner limiting membrane, which can have negative effects. However, ophthalmologists believe that the surgical removal of a small portion of the inner limiting membrane is not seriously detrimental [80]. Figure 4 illustrates the main techniques for delivering synthetic calcium indicators to the inner layers of the retina for subsequent ex vivo calcium imaging.

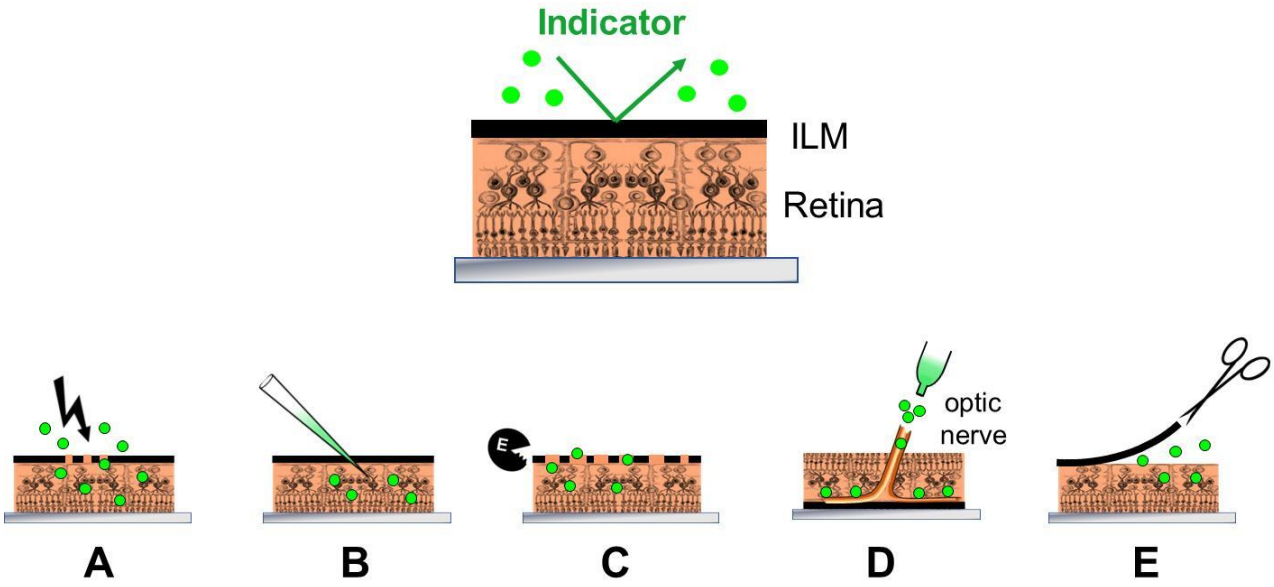

Figure 4. The problem of loading synthetic Ca^{2+} indicators into ex vivo retinal explants from the inner side, and solutions to this problem. (A) Loading by bulk electroporation. (B) Injection of the indicator under the ILM. (C) ILM enzymatic digestion (most commonly using papain), followed by indicator incubation. (D) Loading through cutting the optic nerve. (E) Surgical peeling of the ILM, followed by incubation with the indicator. ILM, inner limiting membrane of the retina.

4. Application of calcium imaging of retinal cells in fundamental and applied research

Calcium imaging is clearly a powerful tool for fundamental studies of the retina. It can shed light on many aspects of how individual cell types and the entire retinal neural network function. Despite some limitations and problems, which we have discussed in this review, this method is becoming increasingly popular within the visual neuroscience community. To date, a vast amount of data has been obtained for the retina using this method, either on its own or alongside electrophysiological and/or molecular biological approaches. Perhaps the most widespread application of calcium imaging has been in investigating the operation of ganglion cells. Aspects of their functioning that have been studied using this method include the role of tight junctions in fine-tuning visual signal encoding by ganglion cells [129] and the selective modulation of nitric oxide in one subset of ganglion cells related to contrast detection [130]. Furthermore, calcium imaging of ganglion cells enables the characterization of their functional properties and the investigation of information transmission channels across the retina as a whole. One example of this type of research is the extensive study by Baden et al. [131], which combined two-photon calcium imaging of the mouse retina with the clustering of over 11,000 cells. This revealed the presence of over 30 functional output channels and several new types of ganglion cells.

Progress has also been made in studying the functions of bipolar cells in the retina thanks to calcium imaging. For instance, Baden et al. used two-photon calcium imaging combined with reconstruction of the morphology of mouse bipolar cell axon terminals to study these cells in detail [70]. This allowed them to divide the cells into eight types that differ in their physiological properties and identify groups that generate calcium spikes in response to light.

Calcium imaging has also been successfully used to study the function of horizontal cells. Chapot et al. used two-photon calcium imaging with the genetic indicator GCaMP3 to obtain data indicating that horizontal cells locally modulate the input signal to the cones, and that this previously unknown feedback modulates the temporal properties of the cones' output signal [132].

Calcium imaging has also been applied to studying amacrine cells, yielding interesting results. For instance, the pivotal role of starburst amacrine cells in forming directional selectivity in the perception of visual motion was demonstrated [133], as was the involvement of an autonomous dendritic mechanism in this process [134].

As for the application of calcium imaging to the retina in applied research, there are currently very few examples of such work. One such example is the use of ex vivo calcium imaging to study the changes in function of different types of ganglion cells as retinal degeneration progresses in rd10 mice (an animal model of slow degeneration) [135]. The authors of this study demonstrated that OFF and ON–OFF ganglion cells were the most vulnerable, and that direction-selective ganglion cells were more vulnerable than orientation-selective ones. Additionally, the first studies using ex vivo calcium imaging to test electrical stimulation of the retina have appeared. Electronic retinal prostheses consisting of an implanted epiretinal or subretinal electrode array are considered one of the most promising approaches to restoring lost vision [136]. Such prostheses electrically stimulate remaining viable retinal cells. In the study by Haq et al. [137], calcium imaging combined with multi-electrode arrays was employed to determine the feasibility of modulating retinal network activity via electrical stimulation in a mouse model of retinal degeneration (rd1). In a study by Azrad Leibovitch et al. [138], the authors took things a step further and developed a new rat model to evaluate the effects of electrical stimulation. This model combines retinal degeneration with high

levels of GCaMP6f indicator expression in ganglion cells. Using this model, the authors demonstrated that the sensitivity threshold to electrical stimulation increases in cases of retinal degeneration, and increasing the stimulation current expands the stimulated area.

An important step toward applying retinal calcium imaging to preclinical studies of therapeutic options aimed at restoring or replacing retinal cell functions under pathological conditions is developing in vivo imaging protocols. The possibility of visualizing calcium dynamics in vivo in retinal neurons was first demonstrated in mice using viral transduction of genetically encoded calcium indicators in the retina and adaptive optics [139,140]. More recently, this has been achieved for the functional assessment of ganglion cells in the living eye of non-human primates, initially in the fovea [141] and subsequently in other areas of the retina [142]. The study by Baez et al. achieved significant progress by combining an ILM peel with subsequent viral transduction of the calcium indicator GCaMP8 into the retina and using adaptive optics scanning light ophthalmoscopy to visualize the signal. These in vivo calcium imaging protocols pave the way for assessing retinal function, detecting vision loss, and testing new therapeutic approaches. Methodological details of some of the studies discussed in Sections 3 and 4 are summarized in Table 1.

Table 1. Selected examples of studies using calcium imaging in retinal research and methodological details of the experiments.

Authors and references	Purpose of the study	Calcium indicator used	Indicator properties	Ex vivo/in vivo imaging	Tissue preparation and indicator loading
Blankenship et al., 2009 [122]	To provide key insights into the mechanisms underlying glutamatergic stage III waves in the retina's glutamatergic circuits	Oregon Green BAPTA-1-AM	Synthetic calcium indicator; non-ratiometric; K_d 170 nM; $\lambda_{excitation}$ 494 nm; $\lambda_{emission}$ 523 nm	Ex vivo	Retinas of C57Bl/6 mice were bulk loaded with the calcium indicator using the bolus loading technique: the solution was pressure-ejected from a glass micropipette just under the inner limiting membrane, and the dye was injected in 3−5 locations per retina.
Briggman and Euler, 2011 [124]	To develop an electroporation method to label the complete ganglion cell layer of the adult mammalian retina	Oregon Green BAPTA-1 Oregon Green BAPTA-2 Oregon Green BAPTA-6F	Synthetic calcium indicators; non-ratiometric; K_d 170, 580 and 3000 nM; $\lambda_{excitation}$ 494 nm; $\lambda_{emission}$ 523 nm	Ex vivo	For indicator loading, whole-mounted retinas of C57Bl/6 mice were bulk-electroporated with the following parameters: specialized electroporator (CUY21; Nepagene/BioVendor), +13-V (top electrode, on ganglion cell side), 10 square wave pulses 10 ms each with 1 Hz frequency.

Continued on next page

Authors and references	Purpose of the study	Calcium indicator used	Indicator properties	Ex vivo/in vivo imaging	Tissue preparation and indicator loading
Cameron et al., 2017 [123]	To develop a procedure for maintaining viable neuronal and retinal tissue for prolonged periods, including prolonged incubation with a calcium indicator	Fura-2AM Fluo-8AM Fluo-4AM	Synthetic calcium indicators; K_d 135–258, 100 and > 200 nM; $\lambda_{excitation}$ 363/335, 491/494, and 490 nm; $\lambda_{emission}$ 512/505, 516, and 514 nm	Ex vivo	For indicator loading, whole-mounted retinas of C57BL/6 and C3H/He mice were papain-treated (30 U/mL papain at 37 °C for 20 min) and then incubated with the selected indicator for 45 min at 37 °C (Fura-2 AM) or at RT (Fluo-4 AM; Fluo-8 AM).
Szarka et al., 2024 [129]	To study how gap junctions of retinal ganglion cells fine-tune the light response kinetics prior to conveying information toward visual brain centers	Thy1-GCaMP3	GECI; non-ratiometric; $\lambda_{excitation}$ 480 nm; $\lambda_{emission}$ 510 nm	Ex vivo	For calcium imaging, whole-mounted retinas of Thy1-GCaMP3-transgenic mice were used.
Baden et al., 2016 [131]	To investigate how many types of retinal ganglion cells exist in mammalian retina and how they should be classified	Oregon-Green BAPTA-1	Synthetic calcium indicator; non-ratiometric; K_d 170 nM; $\lambda_{excitation}$ 494 nm; $\lambda_{emission}$ 523 nm	Ex vivo	For indicator loading, whole-mounted retinas of C57Bl/6 mice were bulk-electroporated with parameters as in [124].
Dyszkant et al., 2025 [135]	To investigate how the response diversity of functional ganglion cell types changes with retinal degeneration progression	Oregon-Green BAPTA-1	Synthetic calcium indicator; non-ratiometric; K_d 170 nM; $\lambda_{excitation}$ 494 nm; $\lambda_{emission}$ 523 nm	Ex vivo	For indicator loading, whole-mounted retinas of C57Bl/6 (wild-type) and rd1 (retinal degeneration) mice were bulk-electroporated with parameters as in [124].

Continued on next page

Authors and references	Purpose of the study	Calcium indicator used	Indicator properties	Ex vivo/in vivo imaging	Tissue preparation and indicator loading
Haq et al., 2018 [137]	To investigate whether electrical stimulation can evoke responses in the degenerated mammalian retina	Fura-2	Synthetic indicator; ratiometric; K_d 135–258 nM; $\lambda_{excitation}$ 363/335 nm; $\lambda_{emission}$ 512/505 nm	Ex vivo	For indicator loading, whole-mounted retinas of rd1 (retinal degeneration) mice were incubated in an extracellular solution containing 0.27 μM Fura-2-AM and 0.1% pluronic acid for 35 min at room temperature
Azrad Leibovitch et al., 2024 [138]	To develop and test a novel rat model for optical recording of ganglion cell activity under retinal degeneration	GCaMP6f	GECI; ratiometric; $\lambda_{excitation}$ ~ 488 nm; $\lambda_{emission}$ ~ 512 nm	Ex vivo	Cross-breeding a retinal degenerated rat with a transgenic line expressing the genetic calcium indicator. Recordings of ganglion cell function in response to ex vivo subretinal electrical stimulations.
Yin et al., 2013 [139]	To develop a novel method for high-resolution in vivo imaging of the function of individual mouse retinal ganglion cells	GCaMP3	GECI; non-ratiometric; $\lambda_{excitation}$ 497 nm; $\lambda_{emission}$ 514 nm	In vivo	Viral transduction of GCaMP3 expression in retinal cells by intracranial injection of rabies vector.
Baez et al., 2014 [142]	To develop a novel method for high-resolution in vivo imaging of the function of individual primate retinal ganglion cells using adaptive optics scanning light ophthalmoscopy	GCaMP8	GECI; non-ratiometric $\lambda_{excitation}$ 480–490 nm; $\lambda_{emission}$ 500–600 nm	In vivo	Eyes from three *Macaca fascicularis* underwent vitrectomy and ILM peeling centered on the fovea prior to intravitreal delivery of 7m8: SNCG: GCaMP8.

5. Conclusions

5.1. Limitations of the method

Calcium imaging is a powerful tool for studying neuronal tissues, particularly the retina. It allows changes in calcium levels in the cytosol or other cell compartments to be visualized in response to incoming signals and physiological events. This reflects the occurrence of action potentials and other changes associated with signal generation and transmission within cells or across neural networks. However, this methodology has its limitations, which must be taken into account and, in some cases, overcome. The general limitations of calcium imaging when applied to nervous tissue include the following: 1) a relatively low signal-to-noise ratio, which makes it difficult to detect dynamic signals and identify small deviations in calcium concentrations that may be physiologically significant in individual cells; 2) the fluorescent signal of calcium indicators does not fully correspond to the kinetics of action potentials, as the calcium signal is slower; 3) calcium indicators have calcium buffering properties, meaning that when they enter cells they compete with natural buffers; this affects intracellular calcium dynamics in intact cells, leading to the recording of calcium concentrations that are not entirely viable, both at rest and when cells respond to stimuli; and 4) the use of genetically encoded calcium indicators, although significantly expanding the capabilities of calcium imaging, at the same time requires additional time-consuming and often complicated procedures, such as direct delivery of genetic constructs to target tissues or the creation of transgenic animals.

In addition to the general limitations listed above, calcium imaging for studying the retina faces specific difficulties. These include: 1) excitation of the retina using a light source that activates the fluorescence of the indicators. This limitation is partially resolved by the use of two-photon microscopes, but not completely. 2) The need to separate detected fluorescence and light stimulus in order to test visual functions. 3) A serious mechanical and electrostatic barrier—the ILM—must be overcome for a calcium indicator (synthetic in ex vivo conditions or genetically encoded in intravitreal injections in ex vivo conditions). Several approaches have been suggested to overcome this limitation, but these may also adversely affect the function and viability of the retina.

5.2. Future directions

Despite the abundance of successful applications of calcium imaging in retinal research, there are still areas in which this methodological approach could be utilized. One of the most pressing tasks, for example, is adapting the method to the study of the avian retina using synthetic and/or genetically encoded indicators. First, birds have an exceptionally complex retina and visual information processing mechanisms (due to their highly developed color vision and the need for high-speed information processing in connection with their lifestyle). Second, there is growing interest in identifying the mechanism of the magnetic compass in birds, which, according to the prevailing hypothesis, is located in the retina [143−145].

Additionally, calcium imaging could be introduced to study photoreceptor cell function in more detail, as the precise mechanisms of calcium modulation in phototransduction (e.g., its effect on light adaptation and the nature of slow calcium buffers) remain unclear. We believe that direct

measurements of free calcium are required to clarify these mechanisms. However, solving this problem with the current level of methodology development still seems difficult. As mentioned above, the introduction of two-photon microscopy has made it possible to shift the wavelengths of fluorescence-exciting light further away from the absorption maxima of visual pigments that trigger phototransduction; however, the lasers used still activate the cascade processes to some extent. This means that, currently, we lack a tool for studying calcium dynamics in photoreceptors in their dark-adapted state. Perhaps future methodological breakthroughs will bring the scientific community closer to solving this problem.

In addition, we predict that calcium imaging will play an increasingly important role in applied research in the future. It will become more widely used for assessing pathological changes in the different layers of the retina in various diseases, including degenerative conditions, as well as for testing different therapeutic approaches, such as cell replacement therapy, optogenetic and optopharmacological prosthetics, and electronic prostheses. However, calcium imaging's role in this field will likely be confined to preclinical models, given the highly invasive nature of this methodology and the current difficulty in envisaging applications for calcium imaging of the retina in human patients.

Use of generative-AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article, except for grammar checking.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Y.V.: conceptualization, original draft preparation, review, editing and approval of the final version of the manuscript; L.A.: conceptualization, curation, original draft preparation, review, editing and approval of the final version of the manuscript.

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