Ionic Homeostasis in Glia: A fluorescence microscopy approach

by

Jean-Yves Chatton, PhD

Department of Fundamental Neurosciences

University of Lausanne, Switzerland

Contact details:

Jean-Yves Chatton, PhD
Department of Fundamental Neurosciences
University of Lausanne
Rue du Bugnon 9
CH-1005 Lausanne
Switzerland
Tel.: +41-21-692-5106
Fax: +41-21-692-5105
e-mail: jean-yves.chatton@unil.ch

Keywords

Astrocytes

Calcium imaging

Confocal microscopy

Flash photolysis

Fluorescent indicators

Genetically-encoded indicators

GFP

Glial cells

Ion sensitive probes

Microscopy

pH

Sodium imaging

Total internal reflection microscopy

Two-photon microscopy

Abstract

The article introduces recent progress in cellular imaging technology and reviews important aspects of the current understanding of ion homeostasis in glial cells and their relationship with integrated cellular and network functions. An evaluation of the respective benefits of electrophysiology and imaging approaches is proposed. Choices of optical sensor molecules (*i.e.* organic fluorescent indicators and genetically encoded biosensors), and microscope configurations (*i.e.* widefield, confocal, and two-photon microscopy) for quantitative intracellular ion measurement are discussed. A brief review of membrane transporters and ion conductances expressed in glial cells introduces how imaging technology contributes to investigations of Ca^{2+}, pH and Na^{+} regulation.

Introduction

Glial cells and in particular astrocytes have been assigned the main role of supporting neurons for their function in the brain. Among the most recognized roles are those of buffering the extracellular potassium and providing energetic support. Recent advances in glial research have uncovered a more active behavior of astrocytes being able to perform functions traditionally held by neurons such as intercellular communication or neurotransmitter release, some of them directly or indirectly modulating neuronal network activity. To a large extent, what critically contributes to the new understanding of glial functions is the development of methodological approaches centered around fluorescence imaging. We will first introduce recent progress in imaging technology and then review some aspects of the current understanding of ion homeostasis in glial cells and their relationship with integrated cellular and network functions.

Technological approaches

Fluorescence imaging and electrophysiology

Glial cells being unable to generate action potentials and having a membrane potential generally maintained stable by an abundance of K^+ channels at their membrane, they have not attracted the attention of electrophysiologists as much as neurons. Moreover, when studied in primary culture, astrocytes generally adopt an extremely flat shape with a thickness of a few micrometers rendering them difficult to impale with microelectrodes. On the other hand, the last decade has brought a number of innovative imaging approaches of both high sensitivity and resolution—both temporal and spatial—that perfectly complement electrophysiological signal measurements.

The temporal resolution brought about by electrophysiological techniques along with their exquisite sensitivity making them able to monitor single channel kinetics, are currently not matched by imaging techniques, even though the gap becomes increasingly small. However, imaging approaches bring several decisive advantages over electrophysiological techniques, the most obvious one being the ability to monitor parameters with high spatial resolution. It is thus possible to simultaneously monitor responses from many cells or many sites within one cell. This approach enabled several discoveries that encompass the long range intercellular mode of communication called Ca^{2+} waves in glial cells.

The resolution of the optical microscope (~0.3 μm laterally and 0.5-1.0 μm axially) enables it to resolve sub-cellular structures such as organelles or large secretary vesicles. It can also detect, without spatially resolving them, a signal originating from smaller structures or even single molecules. Combining these two features has enabled the measurement of ion changes in microdomains hardly accessible to microelectrodes, such as the mitochondrial matrix. Another advantage is the possibility of placing more than one fluorescent probe in a cell which allows the monitoring of more than one parameter simultaneously, for example intracellular Ca^{2+} and Na^+ concentrations, or Ca^{2+} concentration changes in the cytosol and in organelles such as mitochondria. This feature provides a decisive advantage for a more complete and integrated understanding of cellular functions.

Organic fluorescent indicators

Since their first appearance in the late 1980s, a wide selection of ion sensitive fluorescence molecules have been synthesized and made commercially available. A major step associated with the development of these probes was the idea of producing membrane-permeant analogues that render them capable of accumulating in cells. Cation sensitive dyes are usually weak acids containing carboxyl groups and normally negatively charged at physiological pH. Esterification of these molecules (using ethyl esters or acetoxymethyl ester) allow them to undergo passive diffusion across the cell membrane, and once inside the cell, esterases with broad selectivity hydrolyze the esters releasing the free cation-sensitive and electrically negative molecule which remains trapped inside the cell. Three major advantages of this loading method can be cited: (1) it is strictly non-invasive; (2) a large number of cells can be simultaneously loaded at once; (3) because the ester analogue is continuously hydrolyzed inside the cell, there is an actual accumulation of the dye well above the concentration of the compound in the bath. It is noteworthy to mention that acetoxymethyl ester probes preferentially load into astrocytes in brain slices rather than neurons, which is a significant advantage for glial research. However, when it is required to load both neurons and astrocytes, the technique of bolus loading may be applied. It consists in locally applying the acetoxymethyl ester dye dissolved at relatively high concentration (10mM) in 20% Pluronic F-127, a dispersing agent. A pipette is carefully inserted into the tissue using a micromanipulator and the dye is pressure ejected into the tissue by application of a positive pressure. This technique provides relatively non-invasive staining of both neurons and glia with small molecule Ca^{2+} indicators thus allowing real-time imaging of brain activity. However, the concurrent staining of astrocytes using Sulforhodamine 101 may be required to distinguish astrocytes from neurons in the brain tissue.

Most of the indicators discussed below can be purchased as membrane-permeant analogues from several sources. However, for fluorescent probes that do not exist as membrane permeant precursors other more invasive approaches have to be used such as microinjection, incorporation of the dye through the pipette in whole-cell patch-clamp studies, or pinocytosis.

1) pH

Intracellular pH, i.e. H^+ concentration, has been one of the first parameters that could be successfully monitored by fluorescence imaging, as the fluorescein molecule and several of its derivatives display intrinsic changes in their fluorescence characteristics (intensity and spectral shifts) depending on ambient pH. This has opened the possibility of studying important membrane transporter activity involved in intracellular pH homeostasis. The most widely used pH dye is 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, which possesses a pKa of ~7.00. This is close to the cellular pH and enables the monitoring of pH in an optimal dynamic range. This dye has its excitation maximum at a wavelength of ~490 nm, which shows pH sensitivity. When excited at ~440 nm, the fluorescence is no longer influenced by pH. This particular point in the spectrum is called the isosbestic point and is used as an internal reference point allowing correction for dye concentration changes or path length/thickness differences. This dye is called an excitation ratio probe. Other pH dyes, such as

SNARF-1, display a similar pH-dependency within their emission spectra and can be similarly used in the so-called emission ratio mode, which is particularly useful with confocal microscopy.

2) Ca^{2+}

Intracellular Ca^{2+} has been recognized as a second messenger of prime importance critically influencing a number of normal cellular functions and playing a central role in pathological conditions, such as ischemia. The resting cytosolic Ca^{2+} concentration in glia is in the range of 100-200 nM and can reach the low micromolar during transients. Its detection *in situ* by fluorescent probes is probably one of the elements at the origin of the tremendous success that fluorescence imaging techniques has been receiving. Ca^{2+} has thus become the most studied cation in cell biology which is reflected by the wide array of probes made commercially available.

One can classify Ca^{2+} probes into low and high Ca^{2+} affinity probes, and according to their spectral properties (excitation in the UV or visible spectrum). The most widely used Ca^{2+} probes are Fura-2 (high affinity, UV) and Fluo-3, Fluo-4, or Oregon Green BAPTA-1 (high affinity, visible). Low affinity probes enable a lower tendency to buffer intracellular Ca^{2+} changes and are more suited to resolve large amplitude Ca^{2+} transients. Examples of low affinity probes are Fluo-5F (low affinity, visible) and Mag-Fura-2 (low affinity, UV). As a non-excitable cell, the astrocyte does not usually generate large Ca^{2+} spikes and high affinity dyes are more often used.

In addition, changes in Ca^{2+} concentration in the microdomains facing the cell membrane, *i.e.* close to the channel mouth, have different amplitudes and kinetics than bulk cytosolic Ca^{2+} changes. Lipophilic derivatives of Ca^{2+} probes (e.g. fura-C18, FFP-18) that bind to the membrane surface can be used to selectively respond to Ca^{2+} changes only in the immediate vicinity of membrane channels and transporters. Finally, a class of Ca^{2+} probes can report changes in mitochondrial Ca^{2+}. These molecules (Rhod-2 AM and derivatives) harbor an intrinsic positive charge and preferentially localize in mitochondria of living cells, including in glia and neurons.

3) Mg^{2+}

Intracellular Mg^{2+} plays an important role as a mediator of enzymatic reactions, DNA synthesis, hormonal secretion and muscular contraction. Cytosolic Mg^{2+} concentrations found in cells typically range from about 0.1 mM to 2 mM. Several probes exist with relatively good Mg^{2+} selectivity. As for Ca^{2+}, one can distinguish probes used with visible or UV excitation. The most used Mg^{2+} probes are Mag-fura-2 (UV) and Magnesium Green (visible). However, it should be highlighted that most Mg^{2+} probes exhibit a substantial Ca^{2+} sensitivity, which make them usually good low affinity Ca^{2+} dyes but limit their application as Mg^{2+} probes in situations where important intracellular Ca^{2+} changes are expected.

Monitoring of intracellular free Mg^{2+} has been used as an indirect but real-time in situ measurement of ATP hydrolysis. At the origin of this strategy is the fact that ATP displays a ~10-fold higher affinity for Mg^{2+} than ADP and binds a large proportion of cellular Mg^{2+}. As a consequence, upon hydrolysis of ATP into ADP, one Mg^{2+} ion is released and leads to a detectable increase in the free Mg^{2+} concentration.

4) Na^+

The development of fluorescent probes for measuring Na^+ has been more challenging than that of Ca^{2+} probes. The most widely used probe, sodium-binding benzofuran isophthalate (SBFI), is an ultraviolet excitable dye with similar spectral characteristics as the Ca^{2+} probe Fura-2. Its dissociation constant for Na^+ in the presence of physiological concentrations of K^+ is ~11mM whereas its selectivity for Na^+ is about 18-fold higher than for K^+. Na^+ probes with visible excitation currently available, *i.e.* Sodium Green and CoroNa Green have given mixed results depending on the preparation studied. Asante Natrium Green, a sodium indicator excited around 500nm and emitting in the green/yellow region of the spectrum has recently been released and shown to very successfully monitor Na^+ in astrocytes. A red fluorescence emitting Na^+-sensitive dye, named CoroNa Red, has been released and has been shown to localize in mitochondria and therefore could be used to monitor Na^+ concentration changes occurring in the mitochondrial matrix. This indicator has however been discontinued from the supplier.

5) Cl^-

Chloride has traditionally been difficult to monitor by fluorescence microscopy. Available dyes include MEQ, SPQ, MQAE, and have in common that their fluorescence decreases by collisional quenching, when the intracellular Cl^- concentration increases. In contrast to cation-sensitive dyes, Cl^- dyes are not available as membrane permeant form (i.e. acetoxymethyl esters). However, MEQ can be loaded in cell populations in a non-invasive manner when administered as its chemically reduced form dihydroMEQ that readily crosses cell membranes. Once inside the cell, it undergoes oxidation into a polar form that remains trapped inside the cell. However, because the reduced form is extremely labile, MEQ is not commercially available in this membrane permeant form, making it necessary to perform the chemical reduction reaction immediately before loading.

Genetically engineered sensors

One of the elements that contributed to today's success of live-cell imaging was the development of the green fluorescent protein (GFP) of the jellyfish *Aequora victoria* as a fluorescent label that can be incorporated into proteins by genetic fusion. Since the original studies describing the use of GFP, several improved variants have been produced, having higher fluorescence quantum yield or improved stability. A powerful feature of these reporter proteins is the ability to target their expression to given cellular compartments like mitochondria or endoplasmic reticulum, as well as to given cell types in the living organisms. In particular, constructs bearing the glial fibrillary acidic protein (GFAP) promoter have been produced to specifically direct the expression of the fluorescent protein constructs to astrocytes in the brain, and, as an extension, transgenic animals with such type of mutations have been produced. In parallel to the development of these reporters used as morphological markers, fluorescent protein constructs have also been modified to reflect the chemical environment. For instance, GFP mutants have been produced to report pH changes within acidic compartments like secretory vesicles.

Another strategy has been employed to build biosensors, namely to rely on sensing elements (*e.g.* protein sequences) that are the result of the evolution of living organisms which use them to selectively detect changes in the environment. These sensing elements can be coupled to fluorescent proteins by genetic engineering to produce an optical biosensor. The optical detection relies mainly two alternative approaches: (1) One is based on Förster resonance energy transfer (FRET), in which two fluorescent proteins with overlapping fluorescence spectra may transfer the absorbed energy from one (donor) to the other (acceptor) in a radiationless process. This occurs when the two molecules are situated within a distance of 1-10 nanometers. Based on this general scheme, an increasing list of optical sensors have been developed to detect ions (Ca^{2+}, H^{+}, Cl^{-}), membrane potential, as well as compounds such as inositol-1,4,5-trisphosphate, cyclic nucleotides or sugars. As an example, the first generation of genetically encoded Ca^{2+} indicators made use of conformational changes in calmodulin induced by Ca^{2+} binding in the presence of a tethered calmodulin binding peptide M13. The readout was achieved by detecting changes in the FRET efficiency between two spectral variants of fluorescent proteins, namely cyan and yellow fluorescent proteins, linked to the calmodulin and the M13 part of the construct, respectively. (2) The other approach is based on circularly permutated GFP variants in which the original N- and C-termini are fused, creating a new terminus in the middle of the protein. The principle described above for sensing Ca^{2+} (i.e. calmodulin and the tethered M13 peptide) is used in a similar way, however by inserting this sensing domain in the newly created termini. The circular permutation in the absence of Ca^{2+} binding causes solvent exposure of the inner beta-barrel, causing protonation of key chromophore residues and resulting in a low-florescence state. Upon calmodulin binding of Ca^{2+} solvent access to the barrel is prevented and chromophore undergoes deprotonation, leading to a highly fluorescent state. This approach led to a generation of the genetically encoded calcium indicator GCAMP, followed by several improved versions, brighter fluorescence, improved signal-to-noise, and faster response time.

This approach for detecting ion changes has many advantages, but suffers from several drawbacks in comparison with classical organic probes. For instance, the dynamic range and amplitude of the response is generally much smaller in FRET Ca^{2+} sensors than with dyes, and transfecting cells can be more invasive than dye loading using membrane-permeant indicators. It is noteworthy to mention that the newer versions of GCAMPs (e.g. GCAMP6) tend to equal the dynamic range for Ca^{2+} detection offered by organic indicators. In addition, protein sensors have obviously a much larger mass than conventional non-protein indicators and steric interactions can potentially perturb the normal physiology of the living cell. Nevertheless, the prospect of having transgenic animals expressing such biosensors in given cell types of the brain, or attached to given subcellular structures, will push the development of these approaches.

Fluorescence imaging and detection methods

Fluorescence microscopy approaches are generally divided into widefield (or conventional) imaging and confocal imaging. In both cases however, the basic design is based on the epifluorescence principle where the strong excitation light is separated from the much weaker emitted fluorescence light by means of a dichroic mirror placed at 45° into the optical path of the microscope. Today's

research microscopes make extensive use of photonics technologies and routinely use lasers, acousto-optical devices for wavelength selection, and are equipped with high-performance interference filters.

Widefield imaging

In widefield epifluorescence, the entire field of view is illuminated by the excitation light and fluorescence is visually observed through the oculars or detected using a camera. The main advantages of this approach is that it allows a fast detection and requires lower levels of light, which are both crucial parameters for fluorescence imaging of living specimens. Until recently, low-light imaging devices used for fluorescence microscopy belonged to the class of the extremely sensitive and fast intensified cameras, however with limited resolution, or to the digital charged-coupled device (CCD) cameras having excellent resolution but lower sensitivity and reduced speed of acquisition at low light levels. Whereas digital CCD cameras have lately dominated the field of fluorescence imaging, a new development, the so-called electron multiplying CCD, which pushes the sensitivity and speed of digital CCD cameras closer to the intensified cameras, is becoming the solution of choice.

Confocal imaging

<Figure 1 near here>

In confocal microscopy, the excitation light originating from a laser beam is focused by the objective lens onto a diffraction-limited spot in the specimen plane, with the consequence that fluorescence is excited only in this specific point of the specimen (**Figure 1**). To obtain an image of the specimen, the whole field has to be visited by the spot of excitation light generally using a scanning device. Several variations of this basic principle are currently implemented in confocal microscopes, using either a single point scanner or multiple point parallel illumination (*e.g. using Nipkow disks, line and slit scanning, array scanner).* The second main characteristic of confocal imaging is that, to be detected, the fluorescent light has to pass through a so-called pinhole, *i.e.* a small aperture diaphragm, placed in the conjugate plane before the detector. Because light emanating from above or below the focal plane in the specimen will be rejected by the pinhole, this arrangement enables obtaining sharp optical sections of the specimen, that otherwise appears blurred in widefield fluorescence imaging. In thick specimens, like the brain slice preparation, confocal microscopes enable obtaining sharp images of fluorescent cells or cells loaded with indicators relatively deep into the tissue. This major advantage of confocal microscopy has to be balanced with the disadvantage of being slower and requiring much more intense illumination than its widefield counterpart. It is beyond the scope of this chapter to describe the wide array of technological approaches used by confocal microscopes available today.

Advanced imaging and optical approaches

In conjunction with or as extensions of widefield or confocal imaging, several specialized optical approaches have become invaluable tools for neuroscience research.

Two-photon microscopy

This modification of laser scanning confocal microscopy considerably improves imaging in thick living tissue, such as brain slices or *in vivo* brains. The basic principle used is that instead of exciting fluorophores at its maximum of absorption (e.g. 480 nm for GFP), a wavelength of approximately twice this wavelength is used (e.g. ~930 nm for GFP), which corresponds to infrared light. Photons of this wavelength having about half the required energy would not be able to excite the fluorophore under normal conditions. However, when two such photons are delivered simultaneously (*i.e.* within 10^{-15} second) on the fluorophore, they can interact with it in a non-linear manner to excite electrons of the molecule to an excited-state molecular orbital. The relaxation from the excited state to the ground state occurs with the emission of a photon, as for conventional fluorescence. An extremely high density of photons needs to be produced by a pulsed light infrared source and when focused by the microscope objective lens, multi-photon absorption can occur only at the focal point where light is both spatially and temporally compressed to have two infrared photons reaching the same molecule simultaneously. Two major advantages can be seen: fluorescence will be emitted only from the focal point, eliminating the problem of out-of-focus background signal (**Figure 2**), and infrared light allows better penetration as compared with visible or ultraviolet light. Two-photon microscopy therefore enables imaging fluorescent reporter molecules or ion-sensitive probes deeper into the tissue. Even though this technology has recently been made somewhat easier to implement, the high cost of the laser source (generally a femtosecond Ti:Sapphire laser) remain a significant hurdle for its widespread use. Moreover, several fluorophores suffer from poor two-photon absorption capability. **<Figure 2 near here>**

Flash photolysis (UV uncaging)

Uncaging techniques allow the fast and spatially defined application of bioactive molecules from photoactivatable inert precursors and is therefore a powerful tool for the dynamic study of the molecular mechanisms underlying physiological processes at the cellular level. The photoactivatable molecules, called caged compounds, are composed of active molecules that are bound to a photoabsorbing group resulting in a photolabile, biologically inert molecule. Upon brief and intense ultraviolet illumination, the photolabile caged compound releases the free, biologically active molecule along with the free caging group (**Figure 3**). The sources of UV light able to generate sufficient power in the 340-380nm region of the spectrum that have been recently used are UV lasers (355nm diode-pumped pulsed Nd:YAG lasers) and 365nm high power light emitting diodes. Two-photon uncaging is also used by tuning the infrared Ti:Sapphire laser to ~730nm, with the advantage for thick tissue of restricting the photochemical to the focal volume only. Photoactivation approaches can be combined with electrophysiological techniques (*e.g.* patch-clamp) or cellular imaging techniques (*e.g.* fluorescence microscopy) to monitor cellular responses to photoactivation of the caged molecules. Several classes of compounds such as neurotransmitters, nucleotides, Ca^{2+} and Ca^{2+} chelators, fluorescent dyes, or second messengers are commercially available as caged compounds and can be exploited for a wide panel of biological applications in neuroscience research. **<Figure 3 near here>**

Total internal reflection microscopy (TIRF)

TIRF uses the property that a light beam reaching the interface between two media with different refractive indexes with an incident angle exceeding a critical value is totally reflected. Only a small portion of the radiation, called the evanescent wave, propagates into the distal medium. The evanescent wave will continue to travel into the medium of higher refractive index, but its strength will decay exponentially. This light escaping in this restricted region of the specimen immediately adjacent to the glass-water interface has a thickness usually lower than 200 nanometers and can serve to selectively illuminate and excite fluorophores. This technique is particularly useful with cultured cells to visualize processes such as vesicle exocytosis and trafficking happening in the close vicinity of the cell membrane facing the coverslip. It was for instance used to demonstrate that astrocytes possess a vesicular pool involved in glutamate release.

Glial transporters and channels

Glial cells express a large array of transporters and channels at their membrane that allow them to play a central role in regulating the extracellular milieu in the brain. The main primary element energizing these transmembrane ion fluxes comes from the Na^+/K^+-ATPase, which is abundantly expressed by glial cells. This pump utilizes the energy liberated by the hydrolysis of ATP into ADP to export three Na^+ ions and takes up two K^+ ions per cycle, thereby enabling the buildup of Na^+ and K^+ gradients in opposite directions. As glial cells are not excitable, the Na^+ gradient has the main purpose of serving as the driving force of secondary active transport systems, i.e. cotransporters and antiporters (see below). It is estimated that the Na^+/K^+-ATPase accounts for about half of the total cellular ATP consumption.

Transporters

Glial cells, and astrocytes in particular, express several Na^+-coupled transport systems such as those involved in pH regulation (Na^+/H^+ exchanger, Na^+/HCO_3^- cotransporter), in Ca^{2+} homeostasis (Na^+/Ca^{2+} exchanger), in neurotransmitter uptake (glutamate, GABA, serotonin, glycine), in amino acid uptake, as well as the $Na^+/K/2Cl^-$ cotransporter. They also express anion exchangers (Cl^-/HCO_3^-, Cl^-/OH^-) with a role in pH regulation.

Ion channels

Several ion conductances have been described in glial cells. The most studied ones are K^+ channels which represent the most abundant conductance detected in glia and dominate all other ion conductances. They have been assigned the main role of buffering increases in interstitial K^+ concentration resulting from neuronal excitation. In addition, voltage-gated Ca^{2+} channels or voltage-gated Na^+ channels (TTX sensitive) were reported in several studies, although it is unclear what role this latter channel could play in non-excitable glial cells. Finally, anion channels are also expressed in glial cells and probably involved in volume regulation.

Ionotropic receptors

Initially thought to be a genuinely neuronal attribute, ionotropic neurotransmitter receptors have been found to be functionally expressed in glia, such as non-NMDA glutamate receptors (AMPA and kainate receptors), and acetylcholine nicotinic receptors. NMDA receptors appear to be primarily associated with oligodendrocytes rather than astrocytes. No clear consensus exists on the purpose of their presence in glial cells. $GABA_A$ receptors as well as nicotinic acetylcholine receptor currents have also been observed.

Gap junctions and connexins

Connexins are proteins that form hexameric structures in the plasma membrane, called hemichannels, which harbor a central pore. When hemichannels of two adjacent cells dock to each other they form a gap junction channel that enables the intercellular diffusion of ions and molecules of up to 1kDa mass. It is generally assumed that gap junctions contribute to long-range intercellular signaling as well as tissue homeostasis, including the transport of nutrients across the glial network and the spatial K^+ buffering. The gap junction channels can be gated by several stimuli such as low pH and various pharmacological agents, and hemichannels not apposed to another cell are normally closed. However, under certain conditions, like low extracellular Ca^{2+} levels, they can open and mediate the release of molecules such as ATP or glutamate from the cell as well as equilibrate ion gradients. Whether such hemichannel openings have significance in non-pathological conditions is currently debated.

Investigating ion homeostasis in relationship with cellular functions

The availability of fluorescent indicators coupled to sensitive detection devices has allowed the exploration of detailed mechanisms underlying the regulation of H^+, Na^+, and Ca^{2+}, and to a lesser extent of Mg^{2+}. In several cases, a link could be drawn to their role in certain functions at the level of the cell or the tissue. We will select a few examples

Ca^{2+} homeostasis and signaling

Researchers started recognizing the decisive advantage of fluorescence imaging technology with the ability of monitoring intracellular Ca^{2+} with spatial resolution using indicators that reliably report changes in free Ca^{2+} concentration in the nanomolar to sub-milimolar range. Imaging techniques have allowed the demonstration that glial cells, although not electrically excitable, show a complex repertoire of Ca^{2+} responses to various electrical, mechanical and chemical stimuli. Astrocytes are for instance able to generate Ca^{2+} signals by Ca^{2+} entry via voltage-gated Ca^{2+} channels, via AMPA receptor activation, and by IP_3-induced Ca^{2+} release following metabotropic receptor stimulation. Ca^{2+} transients in astrocytes have been shown to trigger the release of various transmitters such as glutamate or ATP, which in turn can drive neuronal excitation. **<Figure 4 near here>** In addition, astrocytes are also involved in long range signaling as they can initiate and propagate the intercellular spreading of Ca^{2+} signals, known as Ca^{2+} waves (**Figure 4**, **Movie 1**). Until recently, this type of communication was thought to be an exclusive property of neurons. This form of excitability, also called calcium excitability, needed optical approaches to be uncovered.

pH regulation

Glial cells play a prominent role in the overall H^+ homeostasis in the nervous system. Fluorescent indicators are a considerable improvement over ion-sensitive microelectrodes since they are not as invasive as cell impalements, and they provide spatial information on intracellular pH distribution. Studies using fluorescent indicators have provided crucial details on the cytosolic H^+ buffering, transmembrane movements of acid/base equivalents, metabolic production of H^+ or sequestration of H^+ into intracellular organelles. Intracellular pH is regulated passively by the buffering capacity of cell, and most importantly actively by mechanisms that glial cells share with other cells types, such as exchangers (Na^+/H^+ or Cl^-/ HCO_3^-) or cotransporters (Na^+/HCO_3^-). Changes in glial pH following various physiological stimuli can range from seconds to minutes or longer. They may also occur in pathological situations such as ischemia or epilepsy.

Activation of neurotransmitter receptors and transporters expressed by glial cells can induce pH transients. For instance, stimulation of $GABA_A$ receptor-coupled chloride channels, which present a significant permeability to bicarbonate and are expressed in astrocytes, lead to cellular alkalinization because the HCO_3^- gradient is outwardly directed. Inversely, glutamate causes an intracellular pH to drop by activating AMPA and kainate receptors, as well as by activation of Na^+-glutamate transporters which co-transport with one proton (or exchange a hydroxyl ion). pH changes in glial cells are thought to be important regulatory signals for function and metabolism, such as the glutamate-glutamine conversion.

Na^+ homeostasis and energy metabolism

Studying Na^+ homeostasis with high spatial and temporal resolution, which cannot be achieved using radioactively labeled tracers or ion-sensitive microelectrodes, can be performed using fluorescent indicators. The transmembrane Na^+ gradient generated by the Na^+/K^+-ATPase plays a central role in providing the driving force for secondary transport systems, such as the Na^+/H^+ or Na^+/Ca^{2+} exchangers, which are important regulators of intracellular pH and Ca^{2+}, respectively. One of the most specific functions of glial cells is the recapture of extracellular glutamate by Na^+-glutamate cotransporters, thereby preventing its buildup to excitotoxic levels during neuronal activity. Because of the prominent activity and density of Na^+-dependent glutamate transporters in glia, glutamate transport is accompanied with a substantial Na^+ influx that causes a three to four-fold increase in the intracellular Na^+ concentration (Na^+_i) which is detectable with good accuracy using dyes, in particular SBFI or Asante Natrium Green-1. Such measurements are therefore an indirect means of detecting the spatial distribution of glutamate transport activity in real time. Studies have shown that Na^+_i is the result of the balance between the rate of Na^+ influx and extrusion by the Na^+/K^+ ATPase. Fluorescence imaging studies have demonstrated that glutamate transport activity causes a substantial energy burden to the cell by increasing the overall activity of the Na^+/K^+ ATPase by a factor of two to three, increasing in turn its associated ATP consumption by a corresponding factor.

Change history

……

Further reading

Bennett M.V., Contreras J.E., Bukauskas F.F., Saez J.C. (2003). New roles for astrocytes: gap junction hemichannels have something to communicate. *TRENDS in Neurosciences* **26**:610-7

Brookes N. (1997) Intracellular pH as a regulatory signal in astrocyte metabolism. *Glia* **21**:64-73.

Inoué S., Spring K.R. (1997). *Video Microscopy: The fundamentals*. New York and London: Plenum Press.

Knopfel T., Diez-Garcia J., Akemann W. (2006). Optical probing of neuronal circuit dynamics: genetically encoded versus classical fluorescent sensors. *TRENDS in Neurosciences* **29**:160-6

Pawley J.B. (ed.) (2006). Handbook of biological confocal microscopy. New York and London: Plenum Press.

Verkhratsky A., Orkand R.K., Kettenmann H. (1998). Glial calcium: Homeostasis and signaling function. *Physiological Reviews* **78**:99-141.

Verkhratsky A., Steinhauser C. (2000). Ion channels in glial cells. *Brain Research Reviews* **32**, 380-412.

Volterra A., Magistretti P.J., Haydon P.G., eds. (2002). *The Tripartite Synapse*. Oxford: Oxford University Press.

Yuste, R., Lanni F. and Konnerth, A (eds.) (2000). *Imaging neurons: A laboratory manual*. New York: Cold Spring Harbor Press.

Araque A., Carmignoto G., Haydon P. G., Oliet S. H., Robitaille R., Volterra A. (2014) Gliotransmitters Travel in Time and Space. *Neuron* **81**,728-739.

Kirischuk S., Parpura V., Verkhratsky A. (2012) Sodium dynamics: another key to astroglial excitability? *Trends Neurosci* **35**,497-506.

Tian L., Hires S. A., Looger L. L. (2012) Imaging neuronal activity with genetically encoded calcium indicators. *Cold Spring Harbor protocols* **2012**,647-656.

2.

2691*

2692*

2693*

Related citations

See also:

→ Links to other chapters that should be updated once all chapters and titles are definitive

Neuron-glia pH regulation (Deitmer)

Ionic fluxes in glia (Brookes)

Potassium homeostasis in glia (Newman)

Calcium homeostasis in glia (Pozzan)

Calcium waves (Kettenmann)

Communication through gap junctions (Giaume)

Na^+/K^+-ATPase (Sweadner)

Figure legends

Figure 1. Comparison of fluorescence illumination in widefield versus confocal microscopy. Whereas in widefield fluorescence microscopes, the whole field of view is homogenously illuminated, point scanning confocal microscopes illuminate only a diffraction-limited spot focused by the objective lens. In this configuration the intensity of excitation is maximal at the focal plane.

Figure 2. Comparison of fluorescence illumination in one-photon versus two-photon confocal microscopy. In two-photon microscopy, fluorescence excitation only occurs in a small focal volume where the light density is sufficient for simultaneous absorption of two infrared photons. No fluorescence is excited above or below this focal point, considerably reducing the background signal and allowing direct optical sectioning. Two-photon imaging is particularly useful for imaging in scattering media and thick specimens, such as the brain slice preparation or the whole living brain.

Figure 3. Intracellular Ca^{2+} photorelease by ultraviolet flash photolysis. (**A**) Upon intense illumination by ultraviolet light, the caged Ca^{2+} molecule—nitrophenyl EGTA—with a bound Ca^{2+} ion undergoes a photochemical reaction leading to the breakdown of the molecule into two parts liberating the free Ca^{2+} ion. (**B**) Ca^{2+} uncaging performed in astrocytes loaded with the caged Ca^{2+} compound NP-EGTA AM and the Ca^{2+} probe Fluo-4 AM (top image). The UV beam, produced by a powerful light emitting diode, was aimed at cell 1 in the center. The false color overlay in the bottom image shows that only the targeted cell 1 exhibited a significant elevation of cytosolic Ca^{2+} shown as Fluo-4 fluorescence intensity. (**C**) Fluo-4 fluorescence intensities plotted against time, showing that repetitive Ca^{2+}_i responses can be triggered by NP-EGTA uncaging in the targeted cell. Scale bar, 100µm. (Panels B&C are adapted from Figure 2 in Bernardinelli Y, Haeberli C, and Chatton JY (2005) Flash photolysis using a light emitting diode: an efficient, compact, and affordable solution. *Cell Calcium*. 37:565-572).

Figure 4. Intercellular Ca^{2+} wave in cultured astrocytes. Sequence of images of mouse astrocytes in primary culture loaded with the Ca^{2+} probe Fluo-4 AM. A Ca^{2+} response was triggered using a stimulation microelectrode placed 20 µm above the surface of one cell in the center of the field. The timecourse of the Ca^{2+} wave spreading from the center can be seen. Scale bar: 100 µm.

Movie 1. Intercellular Ca^{2+} wave in cultured astrocytes. Movie showing mouse astrocytes in primary culture loaded with the Ca^{2+} probe Fluo-4 AM. A Ca^{2+} response was triggered using a microelectrode placed 20 µm above one cell in the center of the field about 4 seconds after the beginning of the movie. The timecourse of the Ca^{2+} wave spreading from the center can be seen. The movie was accelerated by a factor of 3 compared with real time. Scale bar: 100 µm.

Araque A., Carmignoto G., Haydon P. G., Oliet S. H., Robitaille R., Volterra A. (2014) Gliotransmitters Travel in Time and Space. *Neuron* **81**,728-739.

Kirischuk S., Parpura V., Verkhratsky A. (2012) Sodium dynamics: another key to astroglial excitability? *Trends Neurosci* **35**,497-506.

Tian L., Hires S. A., Looger L. L. (2012) Imaging neuronal activity with genetically encoded calcium indicators. *Cold Spring Harbor protocols* **2012**,647-656.

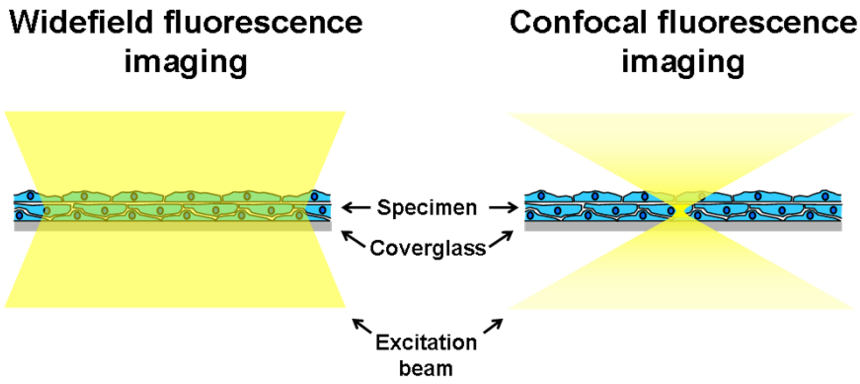
Widefield fluorescence imaging
Confocal fluorescence imaging
Specimen
Coverglass
Excitation beam

Figure 1

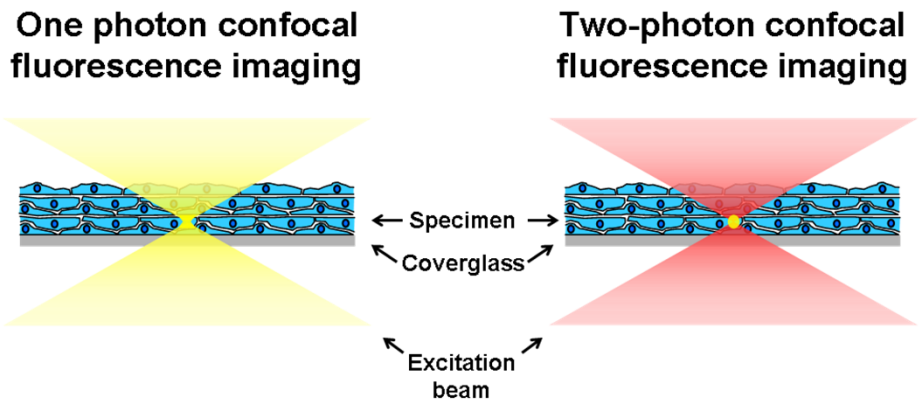
One photon confocal fluorescence imaging
Two-photon confocal fluorescence imaging
Specimen
Coverglass
Excitation beam

Figure 2

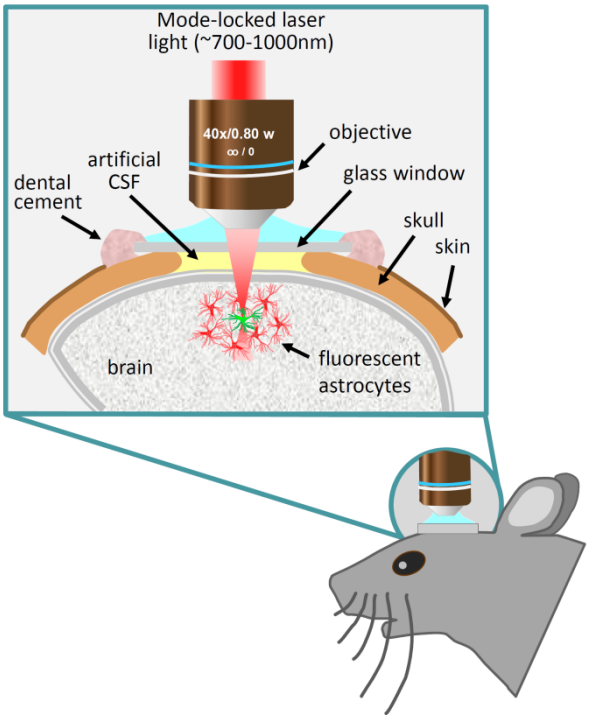
Mode-locked laser
light (~700-1000nm)
40x/0.80 w
∞ / 0
objective
artificial
CSF
glass window
dental
cement
skull
skin
brain
fluorescent
astrocytes

Figure 3

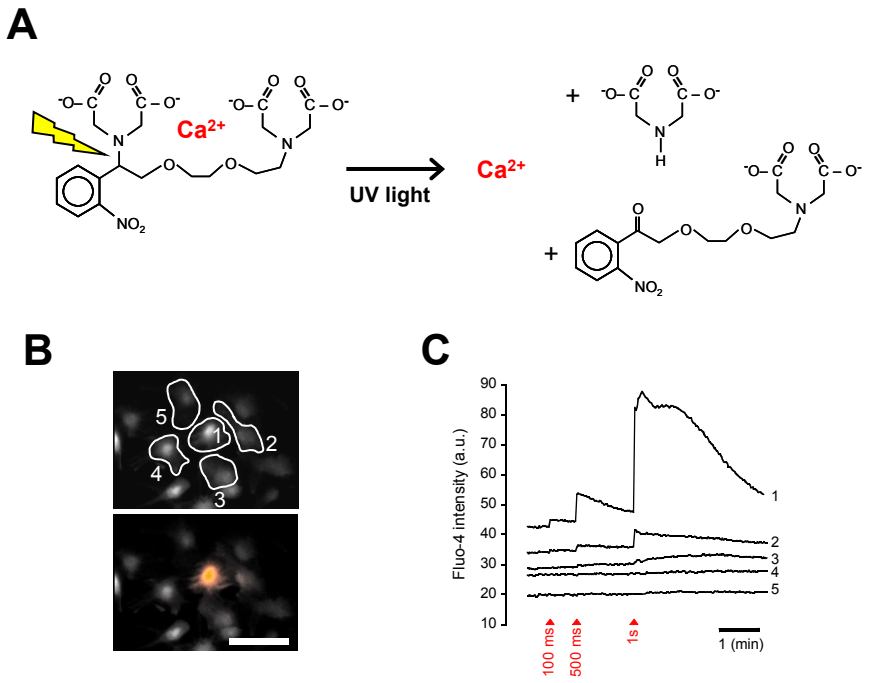
A
Ca2+
UV light
Ca2+
NO2
NO2
B
1
2
3
4
5
C
Fluo-4 intensity (a.u.)
90
80
70
60
50
40
30
20
10
100 ms
500 ms
1s
1 (min)
1
2
3
4
5

Figure 4

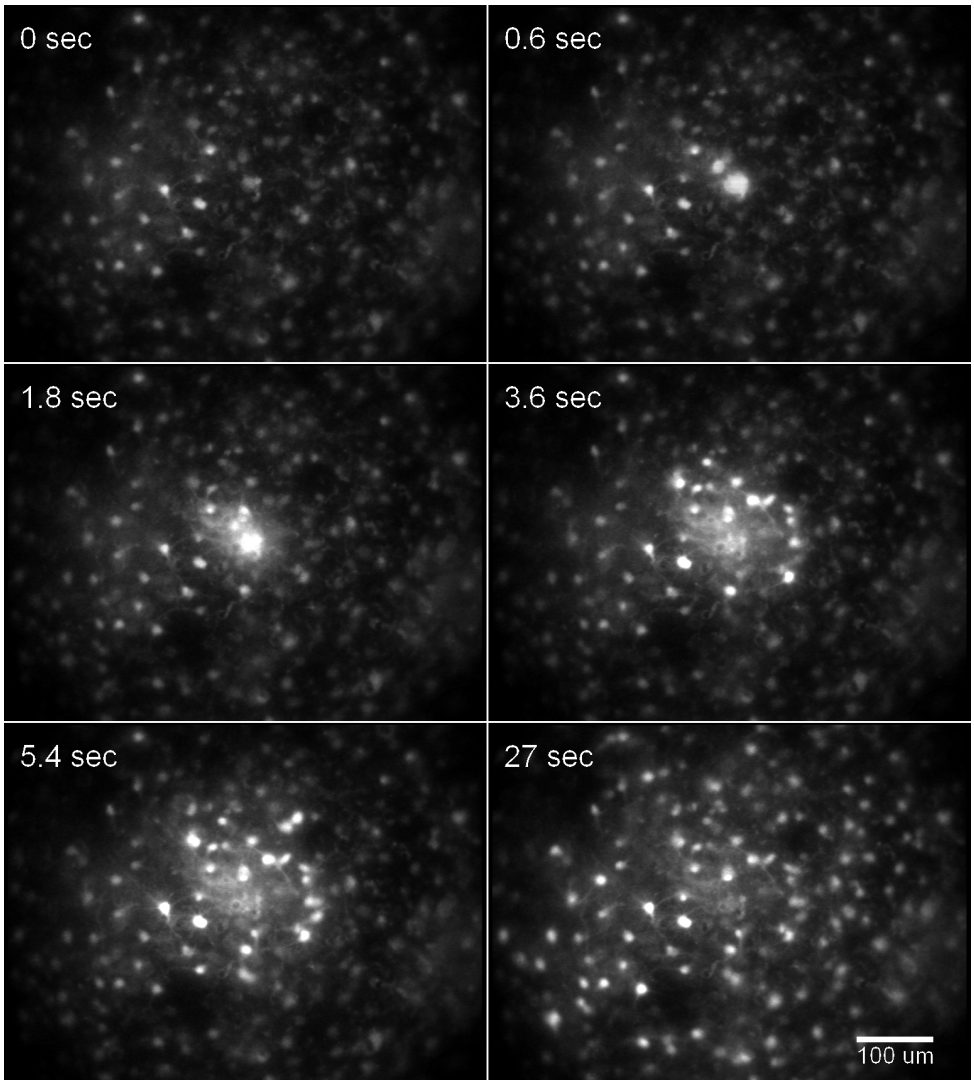

Figure 5