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Synaptic plasticity and Ca^{2+} signalling in astrocytes

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There is a growing body of evidence suggesting a functional relationship between Ca^{2+} signals generated in astroglia and the functioning of nearby excitatory synapses. Interference with endogenous Ca^{2+} homeostasis inside individual astrocytes has been shown to affect synaptic transmission and its use-dependent changes. However, establishing the causal link between source-specific, physiologically relevant intracellular Ca^{2+} signals, the astrocytic release machinery and the consequent effects on synaptic transmission has proved difficult. Improved methods of Ca^{2+} monitoring in situ will be essential for resolving the ambiguity in understanding the underlying Ca^{2+} signalling cascades.

Keywords: Astrocyte, plasticity, glia, synapse

INTRODUCTION

The active role of astroglia in neural information processing and storage, as opposed to providing purely metabolic support to neurons, is a relatively novel concept in our understanding of the brain machinery. Rapidly emerging experimental evidence appears to implicate Ca^{2+}-dependent activity of astrocytes in regulation of neural network activity and synaptic transmission including its use-dependent modifications (see Fiacco *et al.*, 2009; Hamilton and Attwell, 2010; Perea and Araque, 2010 for recent reviews). Because the latter phenomenon is thought to underlie learning and memory formation in the brain, the potential involvement of astroglia attracts intense attention across neuroscience areas. A widely accepted experimental model to explore mechanisms of synaptic memory formation in the brain is long-term potentiation (LTP) of excitatory transmission, in particular its classical forms documented in the hippocampal synaptic circuitry (Bliss and Lomo, 1973; Bliss and Collingridge, 1993). Although the basic neuronal mechanisms of LTP induction and expression have largely been established, the picture is far from complete. The most common form of LTP in the hippocampus relies on postsynaptic Ca^{2+} entry through *N*-methyl-D-aspartic acid (NMDA) receptors (NMDARs). In addition to glutamate, however, activation of NMDARs depends on the presence of a receptor co-agonist, either glycine or D-serine (Johnson and Ascher, 1987), and LTP does require the activation of the NMDAR co-agonist site (Bashir *et al.*, 1990). Astroglia can synthesise D-serine and release it in a Ca^{2+}-dependent manner (Mothet *et al.*, 2005). At the same time, excitatory synaptic activity could evoke a variety of Ca^{2+} responses in astrocytes, most notably through activation of metabotropic glutamate receptors (mGluRs) involving intracellular molecular cascades associated with G-protein coupled receptors (GPCRs) (reviewed in Porter and McCarthy, 1997; Haydon, 2001; Volterra and Meldolesi, 2005). In turn, Ca^{2+} waves induced inside astrocytes by experimental activation of such cascades, or by direct triggering of the inositol tris-phosphate (IP_3)-dependent signalling using photolytic uncaging, have been shown to affect synaptic transmission, by either depressing or enhancing it, in the neighbouring neuropil (Pasti *et al.*, 1997; Fiacco and McCarthy, 2004; Perea and Araque, 2007; Navarrete and Araque, 2008). While the precise physiological circumstances in which such induction may occur *in vivo* remain to be further investigated, experiments that explored the classical LTP paradigm found a link between astrocytic release of D-serine and use-dependent plasticity of glutamatergic transmission (Yang *et al.*, 2003; Panatier *et al.*, 2006). Indeed, combining two-photon excitation microscopy with patch-clamp electrophysiology in acute hippocampal slices has provided evidence that interfering with intracellular Ca^{2+} homeostasis in area CA1 astrocytes blocks induction of the classical NMDAR-dependent LTP at nearby excitatory synapses and that the underlying mechanism involves Ca^{2+}-dependent release of D-serine from astrocytes (Henneberger *et al.*, 2010). However, the signalling cascade controlling D-serine release remains to be determined: although increased synaptic activity does seem to enhance supply of D-serine by astrocytes, the receptor mechanism which triggers such increases is not known. In addition, the causal connection between mGluR activation and LTP in area CA1 has been a subject of debate in the synaptic literature, with various forms of potentiation involving these receptors depending on the induction protocol used (Bashir *et al.*, 1993; Selig *et al.*, 1995; Lu *et al.*, 1997; Manahan-Vaughan, 1997).

GENETIC PROBING OF IP3-DEPENDENT SIGNALLING AND OTHER APPROACHES TO PHYSIOLOGICAL PLAUSIBILITY

Although a major role of mGluRs in generating astrocyte Ca^{2+} responses in situ has long been demonstrated (Porter and McCarthy, 1996), the physiological consequences of astrocyte

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Ca^{2+} signalling have been difficult to establish, mainly because the pharmacological action aimed at mGluRs is not normally restricted to astrocytes. However, advances in genetics have enabled researchers to generate genotypes in which astrocytes could be targeted with high specificity (Fiacco *et al.*, 2007, 2009). A recent study, which used genetic deletion of the IP_3R2 receptor and genetic insertion of MrgA1 receptors in young mice, was unable to associate the astrocyte-specific, G_q GPCR-dependent Ca^{2+} signalling with excitatory synaptic transmission or its plasticity (Agulhon *et al.*, 2010). Based on these results, the authors dispute the involvement of astrocytic Ca^{2+} signalling, in particular G_q/IP3-dependent Ca^{2+} signalling, in neural function (Agulhon *et al.*, 2008, 2010). They argue that the widely reported effects of Ca^{2+}-dependent release of signalling molecules from astroglia on synaptic circuitry are likely to involve either non-physiological manipulations (such as mechanical disturbance of glia or the photolytic uncaging of IP3) or direct physiological action outside astroglia.

Indeed, achieving conditions compatible with brain physiology is an issue of paramount importance in experimental studies in vitro. Ideally, one would aim to identify and activate selectively the molecular trigger(s) of Ca^{2+}-dependent release in astrocytes. One conceptual difficulty is that the current experimental techniques employed to evoke intra-astrocytic Ca^{2+} signalling (such as uncaging of IP3 or activation of MrgA1) are controlled by detecting Ca^{2+} elevations throughout the cell (Fiacco and McCarthy, 2004; Fiacco *et al.*, 2007). Whether such elevations reproduce the spatiotemporal aspects of physiological Ca^{2+} signalling is not known. The uncertainty is highlighted by the reports that uncaging of IP3 in astrocytes does affect spontaneous synaptic transmission in the hippocampus (Fiacco and McCarthy, 2004) whereas activation of MrgA1 does not (Fiacco *et al.*, 2007; Agulhon *et al.*, 2010) even though the evoked astrocytic Ca^{2+} signals appear similar. Furthermore, Ca^{2+}-dependent glutamate release from astrocytes has been associated with an action of *endogenous* endocannabinoids released by neurons (Navarrete and Araque, 2008). This disparity suggests that the origin and propagation of a physiological Ca^{2+} signal could depend critically on GPCR localisation or, more generally, on the spatial relationships among the intracellular players involved in Ca^{2+} signalling in astrocytes: GPCRs, IP_3Rs, Ca^{2+} stores and the Ca^{2+}-sensing molecular targets such as the trigger of release. Little is known about the intracellular distribution of such players, either on the scale of the entire astrocytic arbour or within the fine astrocyte processes that approach synaptic structures.

By the same token, a selective alteration of a genome that would boost the expression of a particular receptor, or otherwise would suppress the expression of another receptor, is not expected to habitually occur in physiological circumstances. Although such experimental manipulations provide a powerful tool to address molecular specificity of a particular signalling mechanism, a careful consideration should be given to the potential compensatory or concomitant changes in the function, expression and distribution of signalling molecules that might play part in the effect under study (Hamilton and Attwell, 2010).

Indeed, several recent reports urge caution against the wide extrapolation of the negative results based on the above genetic approach. A study in which the aforementioned genetic model was used has shown that MrgA1 receptor-evoked Ca^{2+} signals in astrocytes do lead to the enhanced NMDAR activity in nearby neuroblasts migrating to the olfactory bulb (Platel *et al.*, 2010). Although these experiments have been carried in a different brain area and focused on a different neural function, they do demonstrate that the Ca^{2+} activity restricted to astrocytes through a genetic tool could affect activation of NMDARs on other cells. Earlier, conditional astrocyte-selective expression of the SNARE domain of the protein synaptobrevin II (dnSNARE), which suppresses exocytosis in the host cells, was used to associate activity of astrocytes *in vivo* with modulation of sleep homeostasis, in an ATP-dependent (Halassa *et al.*, 2009) and NMDAR activation-dependent (Fellin *et al.*, 2009) manner. Most recently, Ca^{2+} signalling in pH-sensitive astrocytes, evoked either endogenously or exogenously, has been found to cause activation of ATP-sensitive neurons in the brainstem *in vivo* which in turn induced adaptive changes in breathing (Gourine *et al.*, 2010). These studies have therefore used genetic approaches that are not conceptually dissimilar to that used by Agulhon and co-authors (Agulhon *et al.*, 2008, 2010) and yet found what appears to be a causal connection between astrocytic Ca^{2+}-dependent activity and neural function.

An alternative to the genetic targeting of astrocytes, which is designed to facilitate a physiologically plausible *exogenous* stimulus, is the interference with *endogenous* signalling from within an individual astrocyte held in whole-cell mode. One common target is Ca^{2+} signalling cascades which can be modulated by adding combinations of Ca^{2+} buffers or by the blockade of the Ca^{2+}-sensing effector. Indeed, this type of experimental interference in astroglia has been shown to affect neural function in situ (Jourdain *et al.*, 2007; Perea and Araque, 2007; Andersson and Hanse, 2010; Gomez-Gonzalo *et al.*, 2010; Henneberger *et al.*, 2010; Todd *et al.*, 2010). One important advantage of these methods is that they allow direct electrophysiological control of an identified astrocyte, thus enabling the monitoring of astrocytic and neuronal responses in real time. Because perturbation of the intracellular medium is inherent to such approaches, tests with control intracellular solutions are normally required to confirm that Ca^{2+} mechanisms in question are preserved in whole-cell configuration *per se*.

A common issue in comparative studies of astroglia-neuron signalling is that the effects may depend not only on the maturity of the astrocytes but also on the developmental stage of the neuronal network. Historically, most Ca^{2+} imaging studies in astrocytes have been performed in tissue obtained from early postnatal to 2–3-week-old rodents, mainly for technical reasons. However, this postnatal period is characterised by the rapid development of the neuronal circuitry: the scope of neuronal plasticity modes and the requirements for their induction vary greatly (Dudek and Bear, 1993; Bolshakov and Siegelbaum, 1995; Buchanan and Mellor, 2007), and the astrocyte GPCR signalling itself continues to develop (Cai *et al.*, 2000). This implies that careful age matching is required to make physiologically relevant comparisons among astrocytic mechanisms under study.

DISTINGUISHING BETWEEN CAUSAL AND CONSEQUENTIAL

More generally, the observation that the GPCR-dependent Ca^{2+} rises have no detectable effect on synapses (Fiacco

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et al., 2007; Agulhon *et al.*, 2010) leaves open the possibility that in physiological circumstances such Ca^{2+} rises could have a common upstream trigger, but not necessarily a causal link, to the Ca^{2+}-dependent astrocytic release machinery. For example, nicotinic acid adenine dinucleotide phosphate (NAADP) elicits relatively small and localised Ca^{2+} signals that are later amplified many-fold, in a second step prompted by an IP3R-dependent mechanism in mammalian cells (Ruas *et al.*, 2010). An initial small Ca^{2+} transient could easily occur undetected under the current imaging protocols while still providing an efficient molecular signal. Indeed, not only Ca^{2+} signals in astrocytes seem to have an inherently oscillatory nature and are orders of magnitude slower than evoked Ca^{2+} entry in neuronal axons or dendrites, such signals often become detectable only seconds after the stimulus (Verkhratsky and Kettenmann, 1996; Pasti *et al.*, 1997; Haydon and Carmignoto, 2006; Henneberger *et al.*, 2010) (Fig. 1). What happens to small Ca^{2+} fluctuations during this 'dormant' period and whether GPCRs are involved in the underlying machinery is beyond resolution or sensitivity of the currently available imaging approaches.

Indeed, non-GPCR sources of Ca^{2+} signalling provide an additional dimension to the quest for the mechanisms involved in generation of astroglial communication signals. Before GPCRs had taken centre stage in our understanding of how external signalling is translated into intracellular Ca^{2+} regulation, studies of astroglia documented a variety of other Ca^{2+} signal transduction mechanisms, including voltage-gated Ca^{2+} channels, ionotropic receptors or ion exchangers acting in situ (see Porter and McCarthy, 1997; Verkhratsky *et al.*, 1998 for a review). In their pioneering study, Porter and McCarthy reported as early as in 1996 that synaptic activity could evoke astrocytic Ca^{2+} rises when both metabotropic and ionotropic glutamate receptors are effectively blocked (Porter and McCarthy, 1996). These Ca^{2+} mechanisms might well be spatially separated from the GPCR-dependent Ca^{2+} signalling domains because free Ca^{2+} inside cells is likely to be highly compartmentalised due to its rapid diffusion and therefore steep concentration gradients arising near local Ca^{2+} sources and sinks (Fig. 2A). On the other hand, GPCR-independent Ca^{2+} sources could interact and interfere with store-dependent Ca^{2+} signalling because the gating of the IP3R itself not only depends on the concentration of IP3 but is also highly sensitive to intracellular Ca^{2+} levels (see Foskett *et al.*, 2007 for a recent review).

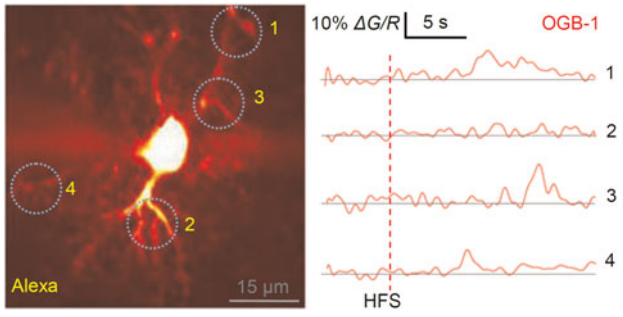


Fig. 1. Processes of a stratum radiatum astrocyte often show delayed Ca^{2+} responses to a synaptic stimulus, much unlike Ca^{2+} responses evoked in neuronal axons or dendrites. Left: a CA1 astrocyte held in whole cell mode (a single 2P excitation plane, 40 µM Alexa Fluor 594 'red' channel, λ_x^{2p} = 800 nm) depicting four regions of interests (ROIs, 1–4). Right: the corresponding Ca^{2+} responses ($\Delta G/R$, the OGB-1 'green' channel signal increment related to 'red' Alexa channel signal to cancel out focus drift (Oertner *et al.*, 2002), 200 µM OGB-1); HFS, 100 pulses at 100 Hz applied to Schaffer collateral fibres (Henneberger *et al.*, unpublished observations).

CA^{2+} NANODOMAINS

An uneven landscape of free intracellular Ca^{2+} (Fig. 2A) could potentially be an important factor in considering how Ca^{2+}-dependent signalling is generated inside astrocytes and how the exogenous manipulations might affect it. Indeed, mechanisms that shape Ca^{2+} activity of astroglia are still poorly understood even at the conceptual level, although important initial data have been obtained in cultured astrocytes addressing the occurrence and basic properties of microscopic Ca^{2+} domains near vesicle release sites (Marchaland *et al.*, 2008) or in thin astrocytic processes (Shigetomi *et al.*, 2010). One technical limitation is that individual astrocyte processes in situ (organised brain tissue) could be as thin as 20–30 nm (Ventura and Harris, 1999; Rollenhagen *et al.*, 2007; Witcher *et al.*, 2007) whereas signals recorded with two-photon excitation in organised brain tissue represent

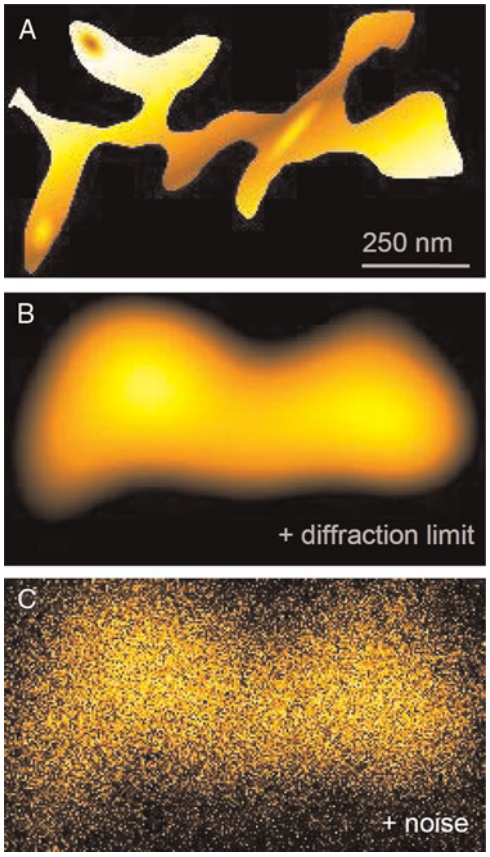


Fig. 2. Ca^{2+} imaging does not reveal the nanoscopic landscape of intracellular Ca^{2+}. (A) A hypothetical distribution of nanoscopic Ca^{2+} sources and sinks in a planar section of an astrocyte process. False colour scale (from dark to bright orange) depicts Ca^{2+} concentration. (B) Diffraction-limited resolution (~250 nm, Gaussian point-spread function) is imposed on image A. (C) Optics registration noise (~25% of the maximal fluorescence) is added to image B. These transformations represent some typical experimental distortions in two-photon excitation Ca^{2+} imaging experiments in organised brain tissue. The distortion is likely to be higher with non-confocal registration.

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fluorescence volume averaged on an at least an order magnitude coarser scale (see an example in Scott and Rusakov, 2006). This averaging will potentially mask a complex distribution of active nanoscopic sources and sinks that might contribute to local Ca^{2+} homeostasis (Fig. 2B,C), the scenario similar to Ca^{2+} imaging of thin axons and their synaptic terminals (Koester and Sakmann, 2000; Scott *et al.*, 2008).

A particularly important aspect of the uncertainty surrounding the interpretation of recorded Ca^{2+} activity in astrocytes is that, similar to presynaptic terminals, critical release events in these cells could be controlled by hotspots of Ca^{2+} generated by Ca^{2+} entry on a 10 nm scale, or Ca^{2+} nanodomains. If the molecular trigger of release is located strategically in the immediate vicinity of a Ca^{2+} source, the local concentration of Ca^{2+} ions entering the cytoplasm is likely to dwarf the numbers of Ca^{2+} buffer molecules (endogenous or exogenous) stationed in the same locality. This scenario might explain, for instance, why loading astrocytes with EGTA, a high-affinity but relatively slow-binding Ca^{2+} buffer in whole-cell mode has had little effect on synaptic plasticity at nearby excitatory synapses in the hippocampus (Diamond *et al.*, 1998; Luscher *et al.*, 1998; Ge and Duan, 2007). However, high concentrations of the fast-binding, high-affinity Ca^{2+} buffer BAPTA could convincingly block Ca^{2+} and astrocyte-dependent effects on some forms of synaptic activity (see Andersson and Hanse, 2010; Todd *et al.*, 2010 for most recent examples), suggesting that Ca^{2+} nanodomains occurring near the release trigger machinery could be a preferred mechanism in such cases (Bucurenciu *et al.*, 2008).

Perhaps surprisingly, high concentrations of intracellular BAPTA did not block LTP to the same extent as Ca^{2+} clamp, nor did they completely suppress slow changes of intracellular Ca^{2+} in response to stimulation of afferent fibres in the hippocampal area CA1 (Henneberger *et al.*, unpublished observations). In contrast, an intracellular solution that contains an equilibrated mixture of Ca^{2+} buffers and Ca^{2+} ions, a so-called 'Ca^{2+} clamp' approach (Baker *et al.*, 1985; Belan *et al.*, 1993), appears to interfere with this dynamic equilibrium suppressing astrocytic release of the NMDAR co-agonist D-serine and thus inhibiting synaptic potentiation (Henneberger *et al.*, 2010). One principal difference between the Ca^{2+} clamp solution and Ca^{2+} buffers alone is that the unlimited supply of Ca^{2+} ions in the former case is likely to prevent free Ca^{2+} from dropping *below* the clamped level whereas Ca^{2+} buffers on their own prevent Ca^{2+} from going *above* its equilibrated concentration. This may suggest that the signalling mechanism that eventually leads to D-serine release requires, at some stage, *fluctuations* of the local Ca^{2+} concentration rather than monotonic Ca^{2+} rises. Again, this ambiguity highlights the need for understanding spatial relationships among those cellular systems that (i) trigger the Ca^{2+} signal, (ii) propagate it on a local scale and (iii) host the molecular target(s) of this signal. An additional complication may arise when individual target systems, such as the Ca^{2+}-dependent release machinery, Ca^{2+}-dependent potassium channels or the sodium–calcium exchanger display different sensitivities to the same local Ca^{2+} signalling event.

FUTURE DIRECTIONS

It would seem reasonable to conclude that our understanding of Ca^{2+} signalling in astrocytes is in its infancy. Do the signalling cascades relaying neural code to glial activity occur homogenously across the astrocytic arbour, near synapses or in some specialised areas? What are the origin, spatiotemporal properties and the adaptive purpose of 'spontaneous' Ca^{2+} signals in astrocytes, i.e. cellular Ca^{2+} elevations that cannot be directly associated with synchronous nerve cell firing or synaptic discharges? How, where and on what time scale do these mechanisms respond to changing patterns of neural activity or its pathological changes? To approach these questions, it would appear critical to improve the sensitivity of Ca^{2+} measurements and its spatial resolution. The spatial resolution of optical microscopy (including two-photon excitation microscopy) is by definition diffraction limited to 0.3–0.5 μm, which is an order of a magnitude greater than small astrocyte branches (Fig. 2). However, several recently developed super-resolution methodologies may help to overcome this limitation (reviewed in Hell, 2007; Wilt *et al.*, 2009). Stimulated emission depletion (STED) microscopy is based on suppressing emission from the outer, doughnut-shaped part of the regular diffraction-limited volume using intense de-excitation light generated by a second laser source. This could potentially enhance resolution by an order of magnitude, and it has successfully been applied to monitor nanoscopic compartments of neuronal dendrites (Nagerl *et al.*, 2008; Ding *et al.*, 2009). The increased resolution of STED microscopy, however, will require increased fluorescence yield and/or excitation light exposure to maintain the acceptable signal-to-noise ratio in turbid media such as organised brain tissue. The latter implies trade-off between achievable resolution, time scale of measurements and potential phototoxic effects. Currently, the light intensity required to obtain STED images seems to be compatible with only a few images per experiment, and using STED in organised brain tissue, may impose additional limitations related to the wavelength- and depth-dependent light scattering. Other advancing imaging methodologies do not rely on intensity measurements but use instead fluorescence parameters independent of fluorophore concentration. Fluorescent lifetime imaging measures the average decay in fluorescence intensity post-excitation on a nanosecond scale. This methodology involves therefore registering photons emitted by fluorophore molecules at different time intervals (1–10 ns) following a very brief (50–200 fs) laser pulse. Because individual signals, or photon counts, recorded during each such duty cycle are very small, thousands of duty cycles are often required to produce the average fluorescence lifetime curve. Fortunately, some Ca^{2+} indicators, such as the Oregon Green BAPTA series, do have fluorescence lifetimes sensitive to Ca^{2+} binding (Wilms *et al.*, 2006; Gersbach *et al.*, 2009). This should enable direct readout of the free-Ca^{2+} concentration, albeit on a time scale which has to be sufficiently slow to accommodate multiple duty cycles required to obtain an acceptable signal-to-noise ratio. As for localisation of individual identified molecular targets, much promise is held by photo-activated localisation microscopy (PALM) and stochastic optical reconstruction microscopy (STORM). These two techniques are based on repeated imaging of a small subset of fluorescent molecules (such as protein tags) excited at a low density to avoid overlap between individual point sources of emission. Because each such source could be de-convolved to a relatively precise pair of coordinates, accumulation of data on individual sources in the same preparation generates a combined image with an effective resolution of ~20 nm (Wilt *et al.*, 2009). Again, successful

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implementation of these two techniques in real-time imaging will depend on the experimental requirements in the time domain: in general, cellular mechanisms under study have to be quasi-stationary during the multiple duty cycles required to construct a complete PALM/STORM image. Accordingly, this imposes a set of constraints on the fluorescent probes, optical system and the acquisition techniques involved, and the entire experiment might thus require a relatively unique combination of all such factors to achieve an optimal outcome. Notwithstanding these potential technical obstacles, such techniques should undoubtedly help to provide important advances in our ability to understand Ca^{2+}-dependent mechanisms underlying functional relationship between neurons and astroglia.

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