Calcium imaging at kHz frame rates resolves millisecond timing in neuronal circuits and varicosities

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Abstract: We have configured a widefield fast imaging system that allows imaging at 1000 frames per second (512x512 pixels). The system was extended with custom processing tools including a time correlation method to facilitate the analysis of static subcellular compartments (e.g. neuronal varicosities) with enhanced contrast, as well as a dynamic intensity processing (DIP) algorithm that aids in data size reduction and fast visualization and interpretation of timing and directionality in neuronal circuits. This system, together with our custom developed processing tools enables efficient detection of fast physiological events, such as action potential dependent calcium steps. We show, using a specific blocker of nerve communication, that with this setup it is possible to discriminate between a pre and post synaptic event in an all optical way.

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To capture all facets of cellular communication in an optical way, it is of utmost importance to record with high temporal resolution as many biological interactions occur faster than the human eye can see or a classic camera can capture. Action potential firing and the function it is important to resolve timing at this scale because it allows assessing the chronology of events taking place in multicellular neuronal networks. Electrophysiological techniques such as patch clamping are so far the only approaches that deliver sufficient temporal resolution [1]. However, they are quite limited in terms of spatial resolution as only one or a few [2–4] cells can be monitored simultaneously. Furthermore, these recordings can compromise the physiological environment because of their invasive nature. Optical readouts have a clear advantage as they are minimally-invasive and allow recordings from many different cells in a network at the same time.

To overcome these spatiotemporal limitations, different types of ‘fast imaging’ have been implemented. Confocal approaches have been optimized for speed based on resonant scanning technology, however they still make a tradeoff between temporal and spatial resolution [5]. A point scanning confocal microscope remains relatively slow because the sample is scanned point by point, which classically takes seconds to minutes to acquire a full field of view. Spinning disk confocal microscopy, on the other hand, is able to acquire whole frames at once, resulting in an increased recording speed up to a few hundreds of Hz [6], again partially trading spatial (mainly axial) resolution for speed [7].

However, when maximum temporal resolution is required widefield fluorescence microscopy is still the optical method of choice [8–11]. Using high speed cameras that are optimally designed for fast acquisition, enough photons can be caught in very short time intervals. Especially EMCCD’s and sCMOS now allow full frame recordings at a few hundreds of Hz [12]. Some setups even reach up to 10 kHz, which is at the expense of the
number of pixels on the sensor chip (e.g. 80x80) [8, 9], and therefore spatial resolution. Also, signal to noise ratio issues arise at these kHz frame rates as only a limited number of photons – if any – can be collected per time point. As an alternative to these camera approaches, photodiode arrays can be used but they are also limited in the number of spatial data points [13–15]. Because of the restriction in spatial resolution of the currently available systems, it remains extremely challenging to accurately record fast events in smaller cellular components such as neuronal processes.

In this study we report the successful development of a microscopy configuration based on a widefield approach with a dual camera system that allows recording images both at fast (up to 2000 Hz) and standard (2-10 Hz) frame rates with sufficient spatial resolution. With this setup it is possible to resolve individual neurotransmitter release sites and record their activity as they operate in a neuronal network.

In combination with our custom image processing tools we are able to detect the chronology of firing using the calcium indicator Fluo4 AM in neuronal circuits both in cell culture and tissue preparations. Furthermore, the approach yields high resolution feedback from sub-millisecond stimuli and allows calculating the propagation speed of the calcium wave front.

2. Experimental setup

2.1 Microscopy setup

A Zeiss Examiner Z1 upright microscope was used as the core of our experimental physiology setup. A cooled CCD camera (PCO; Kelheim, Germany) mounted on the top port was used to record up to 2 Hz and a CMOS camera (Focuscope SV200-i, Photron; Tokyo, Japan) was attached to the side port [Fig. 1(a)] and allowed us to record images at a maximum speed of 2 kHz. This camera has a CMOS sensor of 512 by 512 pixels equipped with a GaAsP photocathode surface. It incorporates a manually adjustable image intensifier allowing full frame high speed recordings up to 2000 Hz, even for low light fluorescence. A custom-made automated mirror switches mechanically between both cameras in about 220 ms. Images were acquired through a water dipping lens (20x, 1.0 NA) and excitation light for fluorescence imaging was generated by a Poly V monochromator (Till Vision; Gräfelfing, Germany). Furthermore, an electric Grass S88 stimulator (Grass Technologies; Warwick, USA), a picospritzer (General Valve Corporation; Fairfield, USA) and a gravity-fed perfusion system (1 ml/min) were used to provide physiological stimuli and buffered solutions to the studied cells and tissues. All components (switching mirror included) were automatically controlled via a control unit and protocol editor available in the Till Vision software (Gräfelfing, Germany). The electrical stimuli (300 µs) were delivered via a local Pt/Ir electrode (Ø 50 µm) to the interconnectives of the nerve network either as single or train (20 Hz) pulses.

2.2 kHz camera performance

To test the performance of the Photron CMOS camera, 50 bias frames were recorded with a minimal exposure time of 3.7 µs for each intensifier voltage, increasing the image intensifier up to 850 (Δ = 50). All these frames had zero counts as a consequence of the preset threshold of the camera. It also shows that read noise, at least up to this threshold, is not influenced by the image intensifier. Secondly, 50 dark frames were recorded with an exposure time of about 1 ms for each intensifier voltage, increasing the image intensifier up to 850 (Δ = 50). For most pixels the counts were again zero, except for some noise that is only present at higher intensifier voltages [Fig. 1(b)]. This noise is single pixel limited as 98% of the spot noise population over all 850 dark frames appears in one single pixel only [Fig. 1(c)]. Possibly this is caused by cosmic radiation that is able to penetrate the camera shielding during dark frame acquisition and is then enhanced by the image intensifier. In order to have a good estimate of
the overall noise levels we set out the standard deviation versus size of region of interest (spatial signal integration) and intensifier voltage measured on a fixed sample of comparable fluorescence intensity to our live tissue samples [Fig. 1(d)].

We recorded at 1000 Hz for all experiments, except for a specific set of calcium wave speed measurements that were recorded at the maximum of 2000 Hz. Thus, we benefit from the increased exposure time and reduced (shot) noise due to a lower intensifier voltage, while still retaining 2 extra orders of magnitude compared to standard (~10 Hz) acquisition rates. The A/D converter’s 10 bit stacks were sampled down to 8 bit to reduce file size and to facilitate software handling. Recordings in live tissues proved that our system has sufficient resolution and sensitivity to resolve individual varicosities and their responses with high signal-to-noise ratio [Fig. 1(e)] at kHz recording rates.

2.3 Tissue and cell culture preparation

For live experiments we used enteric neurons, which reside in two specific layers within the intestinal wall: the submucosal and myenteric plexus [16]. These plexus consist of a complex network of neurons and glia and especially the myenteric plexus is organized in a cellular layer, which is ideally suited for widefield imaging [17]. These nerves communicate via classic synaptic contacts but many processes also have en route varicose release sites, which are referred to as boutons or varicosities in the remainder of the manuscript. For our recordings, we used both enteric nerve cell cultures (small intestine) and whole mount myenteric plexus preparations, taken from the mouse large intestine.
Mice (C57/Bl6) were killed by cervical dislocation and the large intestine was removed and placed in Krebs solution (in mM: 120.9 NaCl, 5.9 KCl, 1.2 MgCl2, 2.5 CaCl2, 1.2 NaH2PO4, 14.4 NaHCO3, 11.5 Glucose). All animal procedures were approved by the ethical committee of the University of Leuven (Leuven, Belgium). The myenteric plexus from the large intestine was loaded for 20 minutes with the fluorescent calcium indicator Fluo4 AM (1E-6 M). Cremophor (0.01% v/v) was added to enhance uniform loading. After washing (10 minutes in Krebs solution) the tissue was mounted on the microscope stage while continuously perfused with carbogenated (95% O2 –5% CO2) Krebs (22°C). Apart from tissue primary neuronal cell cultures were also used for some tests. These were made from mouse (C57/Bl6) intestine and grown for 3 days at 37°C and 5% CO2. They were then loaded with Fluo4 AM (5E-6 M), washed for 10 minutes and kept under constant perfusion with HEPES buffered solution during the experiment (22°C) [17,18] [Fig. 2(a) and 2(b)].

After live experiments, some tissues were fixed at room temperature using 4% PFA (30 min) and processed for immunohistochemistry to identify the cellular components that were recorded from: rabbit anti-synaptotagmin (gift from Dr. R. Jahn, Göttingen, Germany) was used to identify varicosities, mouse anti-Hu C/D (Invitrogen, Merelbeke, Belgium) for neuronal cell soma and goat anti-peripherin (Santa Cruz, Heidelberg, Germany) to label neuronal fibers. Secondary antibodies were coupled to AMCA (Jackson ImmunoResearch, West Grove, USA), alexa594 and alexa488 (Invitrogen, Merelbeke, Belgium) [Fig. 2(c)]. These tissues were then imaged with a scanning 2-photon/confocal microscope (LSM510 META, Zeiss, Germany) in the Cell Imaging Core (CIC, University of Leuven).

3. Millisecond resolved imaging of neuronal circuit activity

3.1 Autocorrelation of high frame rate images allows accurate localization and visualization of individual varicosities

Even though spatial resolution in our setup is significantly higher (512x512) than fast CCD (80x80) based systems, not all varicosities can easily be discerned from a single (1 ms exposure) frame. Therefore we designed a software tool based on an autocorrelation approach, which due to the high temporal resolution performs well to assist and facilitate varicosity identification. The algorithm calculates the arithmetic mean (V) of the autocorrelation [Eq. (1)] over a user-defined time window to construct a pixel by pixel autocorrelation image. V is calculated for each pixel with f(t) the fluorescence over time in this pixel, N the user-defined time window and M the total number of points in the autocorrelation function. By subtracting autocorrelation images produced before, from images at the time of stimulation [Fig. 2(d) and 2(e)], high-contrast images are generated that highlight the responding varicosities with a strongly reduced background [Fig. 2(f)].

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V = \frac{\sum_{T=0}^{M-1} (f(t) \cdot f(t+T))}{M}
\]  

(1)
Fig. 2. Localization and identification of varicose release sites in a myenteric ganglion dissected from the mouse large intestine. Dashed red lines indicate the region shown in (c) (63x objective). (a) Snapshot images taken with a standard CCD camera (20x objective) yield highly detailed information. Inset Image recorded (standard CCD) with 5x objective shows the ganglionic network and the position of the stimulation electrode on the tissue. (b) Single original frame (20x objective) acquired with 1 ms exposure has sufficient signal-to-noise to resolve individual varicosities of neuronal fibers in live imaging experiments. Arrows indicate active varicosities. (c) Immunohistochemical staining (63x objective) to identify varicosities – synaptotagmin (red arrows), neuronal processes-peripherin (green arrowheads) and neuronal soma-HuC/D (white asterisks). (d-f) Images resulting from our autocorrelation software tool. Output of the autocorrelation tool in a time window of 500 ms before (d) and after (e) the stimulation. (f) Image that results from the subtraction of (d) from (e), which selectively shows those parts of the network that respond within 500 ms after stimulation, clearly highlighting responding varicosities.

3.2 Detection of fast calcium events

Calcium responses could be measured in individual varicosities from Fluo4 loaded tissue using 3 different types of stimuli. The response to a single electric pulse was typically characterized by a sharp upstroke reaching its maximum in less than 10 ms (3 mice, 4 ganglia, max F/F₀ = 2.2 ± 0.45) [Fig. 3(b)]. Using a 20 Hz pulse train stimulus, we could show that our detection is fast enough to resolve individual calcium steps at a frequency that matches the stimulation frequency (5 mice, 8 ganglia, max F/F₀ = 3.2 ± 0.59) [Fig. 3(c)]. This response frequency was confirmed using fast Fourier transformations that reveal a ~20Hz dominant peak in the spectra [Fig. 3(e) and 3(f)]. A stepwise calcium response was also observed when the neurotransmitter serotonin (5-HT) was locally applied to the tissue using a pressure spritzer (5 mice, 5 ganglia, max F/F₀ = 1.7 ± 0.19) [Fig. 3(a)]. To improve signal to noise, a low pass filter can be applied either directly or after taking the derivative of the signal [Fig. 3(d)], which facilitates automatic step detection as the derivative signal clearly divulges the individual calcium step location.
Next, we tested whether the improved temporal and spatial resolution could be used to assess calcium signal propagation in neuronal fibers. To address this question, interganglionic fiber tracts were stimulated with a single electrical pulse, classically used to elicit fast excitatory postsynaptic potentials in the enteric nervous system [8]. By recording at 2 kHz, we were able to continuously monitor the calcium signal as it travelled through ganglia and connecting fibers at a calculated propagation speed of about 80 mm/s (7 ganglia, 4 mice). Remarkably this speed is in the same order of magnitude as the one extrapolated from recordings of calcium waves in the intestinal muscles layers [19].

3.3 Computation and visualization of neuronal activity spread using dynamic intensity processing (DIP)

To highlight the importance of timing in the sequence of biological events such as communication in neuronal networks, we sought to further improve the temporal contrast of our recordings by designing an algorithm that comprises image thresholding, peak detection and color mapping as schematized in Fig. 4.
Fig. 4. Dynamic intensity processing (DIP) visualized by input (a,b,c), processing (d,e,f) and output (g,h,i). The input (a) time lapse stack of Fluo4 loaded neuronal cell culture. (b) Time lapse stack of Fluo4 loaded live neuronal tissue. (c) Calcium peaks in these recordings differ only by a few ms, while they are quite distant in space. Therefore, while interpreting the raw data, it is impossible to distinguish when these events occur compared to others. (d) Creating a threshold mask (red) with user defined threshold value. (e) For each non-thresholded pixel: Starting from the time trace of each pixel (upper trace), differentiate (middle trace) and low-pass filter (lower trace) the differential trace. (f) The square of this trace is ideally suited for Igor Pro’s peak detection algorithm (Maximum peaks = 1; Minimum peak percent = 20) that finds the time coordinate of the peak’s maximum. This value is then color coded (using a rainbow color map spread over a user defined time window: from stimulus start until the moment the final responses occur). Pixels with values outside the user defined window are color mapped in black. (g,h) Output of the resulting color coded peak position time value in a new image using the pixel coordinates of the original pixel for either cell culture (g) (Media 1) or nerve tissue (h). Arrows indicate varicosities. Thresholded pixels are not processed and receive a value of 0. (i) The color coded result resolves the spatiotemporal interpretation difficulties of the raw data.
The initial thresholding [Fig. 4(d)] is introduced to discard background pixels and reduce computation time. In a following step (b) the signal is differentiated to transform responses into peaks. An increase in signal-to-noise results from low-pass filtering [Fig. 3(d)] (blue) and Fig. 4(e) (lower trace) and squaring [Fig. 4(f)]. Next, Igor Pro’s peak detection algorithm based on smoothed derivatives is called to define the peak location. Once the new image is calculated the rainbow color scale can be adjusted at any time to color code a specific time window [Fig. 4(f)]. This allows discriminating both very fast calcium rises appearing within ms after the stimulus as well as cells or fibers that respond within a few tens of ms after the stimulus. By using this millisecond time gating, specific structures and responses can be isolated, even for samples with ample background fluorescence as is the case in Fluo4 loaded myenteric plexus tissues. This image processing tool, optimized for fast recordings, is similar in readout to the time-to-maximum images earlier described by our group for standard recordings [17]. DIP generates MB size images from GB size time lapse stacks clearly summarizing the temporal aspects of activity spread in individual varicosities and neuronal networks in both cell culture (g) and tissue (h). This reveals that fibers, even though they are physically close to the stimulated cell are not necessarily the first responders. Figure 4(g) shows that fibers are responding in the direction towards the electrode (bottom right), rather than away from it (Media 1). This suggests that these processes belong to neurons that are among the last to respond in a cascade of responders that starts at the electrode, with the connecting circuitry mainly present outside the field of view. Alternatively we are not sure whether some signals remain under the detection limit. These color maps allow fast interpretation of large data volumes and introduce time with ms resolution as a practical scientific research parameter.

Specific color map selections [Fig. 5] will give a better contrast to the human eye and can be used to highlight different aspects (varicosities [Fig. 5(c)]; overall flow [Fig. 5(d)]) of the information flow in a neuronal network. In order to cover the full time window of interest and to increase contrast for local and consecutive physiological events a follow-up procedure was created that produces either a movie that expands (Media 1 and Media 2) or one that shifts the color scale over the values from the DIP image [Fig. 5(a) and 5(d)-5(f)]. Both movies clearly show directionality of intracellular calcium rises in the millisecond range.
3.4 Pre- and postsynaptic intracellular calcium responses can be temporally resolved

To further address the synchronicity in fiber and varicosity responses in a network, we used a cell culture model of enteric neurons and took advantage of our newly developed DIP to distinguish between timing of responsive elements. Even though some network elements are spatially close together, it is still possible to detect a ~20 ms delay between some of their responses [Fig. 6]. We used the nicotinic receptor blocker hexamethonium to silence cholinergic transmission, the dominant component of fast neurotransmission in the ENS, and found that the slower fibers [Fig. 6(a)] were delayed even further [Fig. 6(b)]. Upon washout of the drug, this effect was reversed [Fig. 6(c)]. This indicates that cholinergic transmission is implicated in the complex wiring most likely via autaptic communication (as present in the artificial conditions of the cell culture system). We found that the effect of hexamethonium can be predicted from the maximum response delay in the first recording, as only in those recordings with substantial delays (17 ± 1.4 ms) blocking of nicotinic receptors was able to amplify the delay (27.5 ± 3.5 ms), while in other recordings with synchronous responses (10.2 ± 2.5 ms) hexamethonium did not have any effect (10.6 ± 2.4 ms). Therefore, we expect that the observed distinct delays reflect the length of the network element before it reappears in the field of view.
Instead of focusing on different response elements in a network, we also analyzed individual varicosities to examine whether circuitry information could be deduced from a single spot [Fig. 7]. For this set of experiments we used isolated tissues, which have an advantage in that they contain the native wiring of the network. The responses evoked by a single electrical stimulus were highly repeatable over sequential trials (3 repeats, 258 varicosities from 2 mice). The majority of varicosities displayed one single sharp upstroke in response to the single stimulus [Fig. 3(b)], however in a select number of cases (~10%) a biphasic response could be detected [Fig. 7(a)] suggestive of a recurrent or delayed secondary activation. These secondary responses seemed to be hard wired as they were also consistent in repeated recordings. To investigate the nature of this second peak, we again used hexamethonium and imaged the varicosities (330 varicosities from 6 mice) first in control and secondly in the presence of hexamethonium (1E-4 M, 2.5 min). In over 40 varicosities, secondary responses were observed. Hexamethonium completely abolished the secondary response in 14 varicosities [Fig. 7(b)] while the double response in 26 varicosities was not affected by hexamethonium. After washout (2.5 min) all secondary responses reappeared [Fig. 7(c)], clearly proving the involvement of an intermediate cholinergic connection.

We conclude that with our microscopy setup it is possible to simultaneously record and discriminate between pre- (direct/antidromic) and postsynaptic activity in an all optical way.
4. Discussion

Live fluorescence microscopy is a powerful and heavily used tool in biomedical research mainly because it yields an optical readout from many cells simultaneously, which creates the possibility to visualize cellular interactions and circuits. The main disadvantage of these techniques is the lack of speed at which recordings can be made in comparison to electrophysiological approaches [20]. To overcome this, strategies have been designed to improve camera capture speed or – in case of scanning microscopy – enhance the (resonating) speed of the scanner.

Confocal measurements on the millisecond time scale are an option but only by limiting the recorded area to single lines or single points. In this case, the increase in temporal resolution is at the expense of a general overview. As an alternative approach to increasing temporal resolution resonant scanners have been implemented [21,22], which sweep the excitation beam over the field of view much faster than classic linear galvanometric scanning mirrors. Here the disadvantage is that pixel dwell times are often too short to collect a sufficient amount of photons. Alternatively acousto-optic deflectors can be used to change the beam direction in a non-mechanical way [23].

Unlike point or line scanning systems, also fast (kHz) cameras have been elegantly used in cardiac [24], peripheral [8,9] and central nervous system [10] research to improve recording speed. However, to maximize readout speed, sensitivity has to be raised, very often accomplished by increasing pixel size and therefore lowering spatial resolution. Here we report a microscopy configuration with an additional high spatial resolution fast camera (512 by 512 readout), which makes it possible to record both fast and slow events in small (close to diffraction limited) objects, such as varicosities in a neuronal network. A custom-made mechanical sliding mirror allows switching (220 ms delay) back and forth from fast to standard recordings automatically. Our hardware setup, combined with custom made analysis tools, close an important gap in the available microscopy techniques because of the obvious
speed advantage while still having the possibility to simultaneously record from different components in an integrated network. Another advantage relates to the flexibility of the technique as it can easily be integrated in existing microscopy setups.

Our system (hard- and software) is unique in terms of temporal and spatial resolution as it reveals events that could not be detected before in an optical way. The distinct intracellular calcium steps that we recorded in individual varicosities during serotonin application are a good example. Most likely each step is the consequence of a unitary event like a single action potential or the opening of a 5HT3 receptor (cluster). This is in line with the stepwise intracellular calcium rise that was measured for nicotinic receptor stimulation [8] of enteric neurons, which was indeed associated with action potential firing. We further show that we can resolve distinct intracellular calcium steps in varicosities using action potential pulse trains (20 Hz), thus yielding much more detail about the response upstroke compared to classic CCD cameras.

Our image capture speed proved fast enough to deduce calcium propagation speed (~80 mm/s) in unmyelinated neuronal fibers of the enteric nervous system, which is remarkably similar to what was extrapolated from muscle recordings [19]. It remains to be elucidated why these high speeds are necessary to control physiological behavior (intestinal motility, mixing) that is far slower. It could be argued that this conduction speed is that high to allow signal integration over larger areas generating concerted actions of smooth muscle units in the gut wall. Muscle twitch indeed happens simultaneously for groups of cells spread over 100-200 µm, a distance that is bridged in ~2 ms at the measured calcium propagation speed. Compared to action potential propagation in unmyelinated fibers the calcium propagation speed is still low. This may be due to the fact that the experiments were performed at room temperature or caused by a true mismatch between Ca²⁺ signal and action potential propagation. The latter could be explained if Na⁺ and Ca²⁺ channels are not evenly distributed along the fiber or if the volume of the process is not constant. We do not expect that delays are due to the properties of the indicator, as Fluo4 has a sufficiently high affinity (Kd = 345 nM), S/N ratio and Ca²⁺ binding kinetics [25,26] to generate enough photons during the short (ms) integration times. The temperature dependency of Fluo4 [27] is most likely negligible in light of the effect temperature will have on the physiology of the network. Higher conduction velocities as present in myelinated (motor) neurons cannot be measured at the moment, since this would require imaging larger fields of view with lower magnification lenses and therefore less detail.

The first of our processing tools allows summarizing a GB time lapse stack into MB images that are easy and fast to interpret. They either enhance spatial contrast (time correlation) or temporal contrast (DIP). Despite these obvious advantages, it is important to realize that this type of computation takes time, for instance a time lapse stack reduced to 350 frames around the event of interest requires typically around 10 min to be processed because of the pixel by pixel multi-step analysis. This process can be sped up by adjusting the threshold value, which is especially useful in recordings from cell cultures as more background pixels can be discarded from the calculations.

A second tool, DIP, also allows time gating at ms resolution, which can be used to identify varicosities that fire in synchrony. This technique can be employed to separate single active fibers within complex neuronal circuits from background information, even using widefield microscopy. Furthermore, it allows determining signaling order within neuronal networks as well as distinguishing between direct and synaptic or long-distance communication. Indeed, using hexamethonium, a drug that blocks the most important fast component of neurotransmission in the enteric nervous system [28], we were able to delay responses by forcing the response propagation over longer (non-cholinergic) connections.

In tissue experiments, where neuronal wiring is still intact, single fast responses to fiber tract stimulation dominate the response spectrum of varicosities in the entire field of view. Recurring post-synaptic activity within the circuitry is limited to a stochastic minimum.
Although altered intracellular calcium buffering, summation of inputs or paracrine factors may influence the response in a single varicosity at any given moment, most varicosities in which a secondary intracellular calcium step occurred, were shown to faithfully repeat that response in up to 3 consecutive recordings. Only in the presence of hexamethonium, the secondary response was completely abolished in a subgroup of varicosities. These data indicate that at least one cholinergic release site is involved in the generation of the secondary response, which proves the potential of fast imaging to distinguish between direct and circuit related activation. Synaptic contact redundancy or involvement of a neurotransmitter other than acetylcholine may explain the presence of hexamethonium resistant responses.

In summary, we show that high resolution full frame kHz imaging further expands the application spectrum of calcium imaging, since calcium propagation speed and stepwise calcium increases associated with single action potentials in individual varicosities can be detected. Our image processing tools assist in the discrimination of neuronal network activity on the millisecond timescale.

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