In vivo two-photon laser-scanning microscopy of Ca^{2+} dynamics in visual motion-sensitive neurons

Julia Kalb,[a] Tim Nielsen,[b,1] Matthias Fricke,[b] Martin Egelhaaf,[a] and Rafael Kurtz[a,*]

[a] *Lehrstuhl für Neurobiologie, Fakultät für Biologie, Universität Bielefeld, Postfach 100131, D-33501 Bielefeld, Germany*
[b] *Lehrstuhl für angewandte Laserphysik, Fakultät für Physik, Universität Bielefeld, Postfach 100131, D-33501 Bielefeld, Germany*

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Abstract

We applied two-photon laser-scanning microscopy (TPLSM) to motion-sensitive visual interneurons of the fly to study Ca^{2+} dynamics in vivo at a higher spatial and temporal resolution than possible with conventional fluorescence microscopy. Based on a custom-built two-photon microscope, we performed line scans to measure changes in presynaptic Ca^{2+} concentrations elicited by visual stimulation. We used a fast avalanche photodiode (APD) with a high quantum efficiency to detect even low levels of emitted fluorescence. Our experiments show that our in vivo preparation is amenable to TPLSM: with excitation intensities low enough not to cause photodamage, activity-dependent fluorescence changes of Ca^{2+}-sensitive dyes can be detected in small neuronal branches. The performance of two-photon and conventional Ca^{2+} imaging carried out consecutively at the same neuron is compared and it is demonstrated that two-photon imaging allows us to detect differences in Ca^{2+} dynamics between individual neurites.

Keywords: Calcium; fluo-4; Fluorescence imaging; Fly; Motion vision; Neuronal processing; Synapse; Tangential cell; Two-photon microscopy

Cytosolic changes in Ca^{2+} concentration control the computational properties of a neuron by regulating a variety of neuronal processes (reviews: [1–3]). Ca^{2+} molecules act as charge carriers for integrating synaptic inputs and can induce changes in membrane excitability. As a second messenger, Ca^{2+} molecules trigger biochemical pathways mediating modifications of synaptic strength as well as genetic signalling pathways. A prominent function of Ca^{2+} is to regulate the output signal of a neuron at chemical synapses (reviews: [4,5]).

Optical imaging techniques are commonly used to study the role of neuronal Ca^{2+} signals by visualizing the intracellular Ca^{2+} concentration (review: [6]). To allow for conclusions about the functional relevance of measured Ca^{2+} signals, a high temporal and spatial resolution of Ca^{2+} imaging is necessary in order to visualize Ca^{2+} close to the sites of transmitter release. TPLSM provides an important tool for high-resolution functional brain imaging ([7]; reviews: [8–10]). Due to the spatial restriction of excitation to the focal region, photobleaching and photodamage are confined to this small volume. By use of a near-infrared laser, less scattering and a better penetration depth than in conventional and confocal microscopy are achieved. Moreover, the poor absorption of infrared laser light by photoreceptors allows functional imaging in the visual system without unwanted visual stimulation by the excitation light, which may cause great problems during conventional imaging. All these aspects make TPLSM especially well suited for use in dense, compact brain tissues of intact animals (review: [11]).

In visual interneurons of the fly, the so-called tangential cells (TCs), the functional significance of activity-dependent Ca^{2+} signals has been analysed [12–17]. TCs are located in the third visual neuropile of the fly's brain and spatially pool the outputs of numerous retinotopically arranged local motion-sensitive elements. Hence, they respond to optic flow as generated by self-motion of the animal in a directionally selective way. Many TCs which are thought to be involved in optomotor course control are individually identifiable due to their

* Corresponding author. Fax: +49-521-106-6038.
E-mail address: rafael.kurtz@uni-bielefeld.de (R. Kurtz).
[1] Present address: Philips Research, Research Sector Technical Systems, Röntgenstrasse 24–26, D-22335 Hamburg, Germany.

anatomical characteristics and response properties (reviews: [18–23]).

Until present, intracellular Ca^{2+} concentration changes in TCs have been visualized with conventional wide-field fluorescence microscopy. To exert its functions as a second messenger Ca^{2+} is regulated in a highly localized and dynamical way (review: [24]). Conventional imaging techniques, however, provide only raw estimates of functionally relevant Ca^{2+} dynamics, because their spatial and temporal resolution is limited. Thus, conclusions drawn about the relationship between presynaptic Ca^{2+} and transmitter release remain fairly indirect [14]. Moreover, a reliable determination of Ca^{2+} concentration changes is not possible during presentation of stimuli which continually fluctuate in their velocity, as is the case under natural conditions.

In this paper we demonstrate for the first time the application of TPLSM to the in vivo preparation of the fly visual system. Line-scan measurements of presynaptic Ca^{2+} accumulation in a TC are performed and these measurements are compared to conventional fluorescence measurements carried out in one and the same neuron. Thus, two-photon microscopy can be critically evaluated with respect to its suitability for functional in vivo measurements in neurons of the fly visual system.

Materials and methods

Line-scan measurements under two-photon excitation were performed in vivo on a VS-neuron in a 1- to 2-day-old female blowfly (*Calliphora vicina*). VS-cells can be identified by the localization of their receptive fields and their anatomy using fluorescence dye staining [25,26]. These motion-sensitive neurons belong to a group of TCs being predominantly sensitive to vertical motion of optic flow. VS-cells respond to ipsilateral downward motion by graded depolarizations which may be superimposed by spike-like depolarizations of variable amplitude. During ipsilateral upward motion their membrane potential hyperpolarizes. VS-cells are presynaptic to a spiking neuron, the V1-cell, which relays the motion information to the contralateral brain hemisphere [14,27].

Animal preparation followed [13] with the modification that the upper part of the thorax was removed such that a 40× water immersion objective could be positioned sufficiently close to the axon terminal of the VS-cell.

Electrophysiology. Followed [14] except that the tip of the intracellular recording electrode was filled with a solution containing the Ca^{2+}-sensitive fluorescent dye fluo-4 [chemical composition in mM: KCl: 33.3 (Sigma); KOH: 1.7 (Merck); Hepes 33.3 (Sigma), pH 7.3; and fluo-4 pentapotasium salt: 17 (Molecular probes)]. Electrophysiological procedures were performed in a conventional fluorescence imaging set-up. For subsequent two-photon fluorescence imaging the fly was mounted in the TPLSM.

Conventional imaging set-up. The epifluorescence measurements of presynaptic Ca^{2+} concentration changes followed [14] except for the CCD camera. Here we used a Quantix 57 (Photometrix, chip size $512 * 512$, pixel size $13 \times 13\,\mu m^2$) to acquire $128 * 128$ pixel images (i.e., binning factor 4) controlled by PMIS Software (GKR Computer Consulting) at a frame rate of 14 Hz.

Two-photon imaging set-up. Line-scan measurements were carried out with a custom-made TPLSM based on an inverted fluorescence microscope (Olympus IX 70) (Fig. 1). A mode-locked Ti:Sapphire laser (Coherent Vitesse) produces laser pulses at a fixed wavelength of 800 nm with an average power of 600–950 mW and a repetition rate of 80 MHz. A rotatable half-wave plate followed by a polarizer was used to attenuate the laser intensity, such that incident laser power at the specimen did not exceed 10 mW, reported to be a critical level for the onset of photodamage [28,29]. The laser beam passes a pair of prisms (SF10) for compensation of the dispersion of the system to achieve a pulse length inside the sample of 42 fs. Dispersion compensation is important to ensure maximum fluorescence signal for a fixed pulse energy.

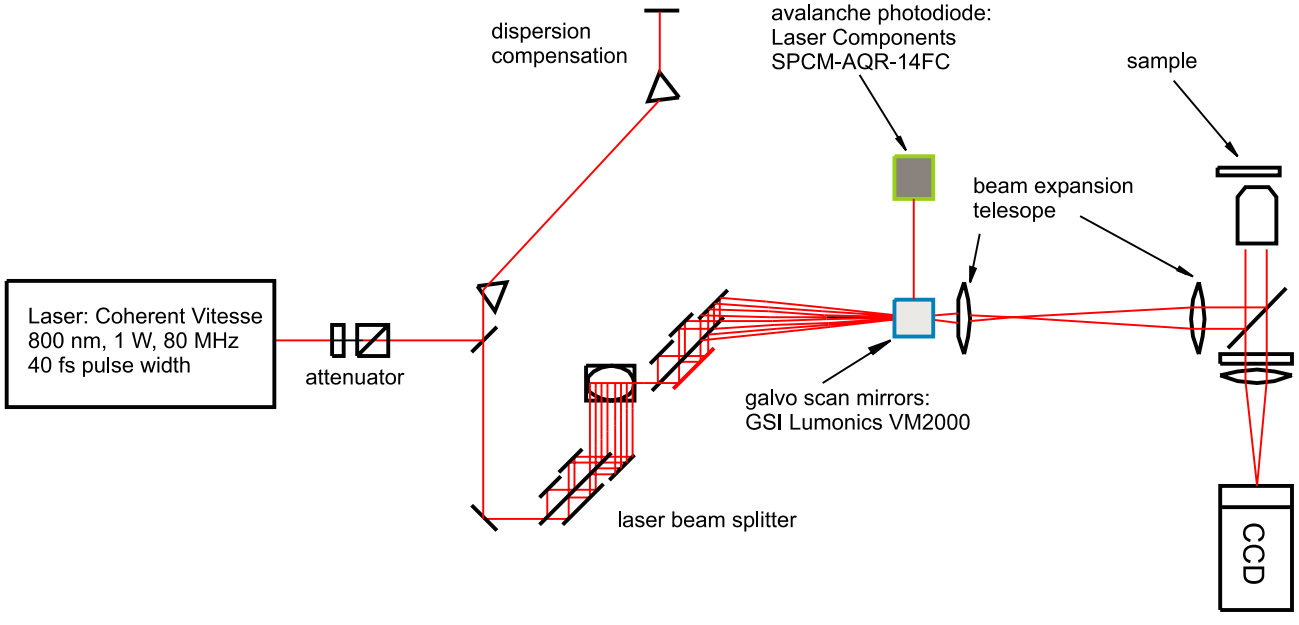

Fig. 1. Two-photon laser-scanning set-up for fast imaging with optional multifocal excitation. Schematic view of the two-photon imaging set-up. For technical details see Materials and methods.

Our experimental set-up is equipped with a beam-splitter which allows one to generate multiple laser beams to perform multifocal two-photon microscopy [30]. This mode of operation differs from conventional two-photon microscopy in that multiple laser foci are generated in the imaging plane. Scanning along several lines then allows fast simultaneous imaging of structures located apart from each other, thus bridging the gap between fast line scanning and slow acquisition of two-dimensional images. The principle of the beam-splitter is to combine a central 50% mirror with high-reflectivity mirrors to both of its sides. The 50% mirror provides doubling of incoming beams and the high-reflectivity mirrors send the beams repeatedly back to the central 50% mirror. All beams leave the device in a plane. The measurements shown in this paper were, however, performed with only a single laser beam, since the aim was to test with experimental complexity and expense kept as low as possible, if two-photon microscopy is generally suitable for Ca^{2+} measurements in the intact fly brain and to compare its performance with that of conventional fluorescence microscopy. In order to perform imaging along multiple scan-lines in future experiments, the set-up has to be modified extensively, since the fluorescence light emitted from individual laser foci has to be detected separately, e.g., by installing additional photodiodes.

The scanbox-device consisted of two non-resonant galvo scan-mirrors (VM2000, GSI Lumonics; controlled by SC2000-microprocessors) which facilitate two-dimensional scanning inside the focal plane. A telescope magnifies the beam diameter so that it overfills the aperture of the objective lens (water immersion objective, LUMPLFK40×, NA 0.8 W, Olympus).

The fluorescence emitted from the sample was collected by the same objective and directed to an APD (silicon avalanche photodiode, SPCM-AQR-14FC photon counting module, LaserComponents GmbH). Due to the small detection area of this detector it was necessary to descan the emitted fluorescence. This was realized by coupling the laser beam by a broad band high-reflectivity mirror into the microscope. This mirror reflects the emitted fluorescence back through the galvo-scanner system. Behind this system the light entered a dichroic mirror, separating excitation and emission wavelengths. The fluorescence was then focussed by a lens onto an optical fibre connected to the APD. In our set-up, fluorescence detection in the non-descanned mode with a large-area photomultiplier tube was not an option, because this detection mode would not allow us to modify the set-up for multifocal two-photon microscopy, as it is planned for the future (see above). The APD signals were recorded by a PC with a pulse counting card (PMM 328, Becker & Hickl GmbH).

In order to control the location of scan-lines in relation to the entire neuronal structures, fluorescence from two-photon excitation and from epifluorescence excitation with a mercury bulb could both be imaged by a CCD camera (Imager 3, LaVision, chip-size 1280 (horizontal) × 1024 (vertical), pixel size $6.7 \times 6.7\,\mu m^2$). This was done by replacing the high-reflectivity mirror in the optical path with a dichroic mirror which separated the fluorescence from the laser beam. Both in front of the CCD camera and in front of the lenses focussing the fluorescence onto the optical fiber, edge filters (BG39) suppressed remaining excitation light.

Data acquisition and analysis. For electrophysiology and conventional fluorescence microscopy of Ca^{2+} signals data acquisition and analysis followed [14]. TPLSM line-scan measurements and their analysis were performed with laboratory written software based on the programming software C++ (Borland compiler 5.01, Borland International) and the data acquisition software DaVis (6.2.0, LaVision). For the measurements presented in this paper only the original laser beam had been used while being moved in one dimension by a galvanometric scan mirror. The deflection of the scan mirror is sinusoidal. In order to get approximately equal pixel dwell-times, the descanned fluorescence was collected by the APD only during those time intervals where the mirror produced nearly linear deflections. The extent of linear mirror-deflection corresponded to approximately 80% of the maximum amplitude of deflection. Here, both directions of mirror movement were used for data sampling. Additionally, phase shifts due to inertia of the mirror system were compensated during the line-scan measurements by our data acquisition software.

Ca^{2+} transients were acquired using line scans at a rate of 2.5 ms per line. For data analysis the line-scan images from particular differently sized neurites were reconstructed from raw-fluorescence data. To calculate the corresponding Ca^{2+} dynamics, values were extracted from the line-scan images for each time point by averaging across the spatial axis along areas of interest. After correcting for the dark signal by subtraction of a line-scan image acquired without two-photon excitation, intracellular Ca^{2+} changes were evaluated as relative fluorescence changes ($\Delta F/F_0$). Baseline fluorescence (F_0) was determined in a time window before visual stimulation (0–100 ms). To estimate photobleaching of the Ca^{2+} indicator line-scan measurements were performed without sensory stimulation and single exponential decay functions were fitted to the fluorescence time courses. These decay functions were used to apply a bleach correction to the fluorescence time courses measured during visual stimulation.

Visual stimulus. Stimulus procedures followed [14]. In brief, a board with $48 * 30$ LEDs was used to produce motion stimuli. Switching on and off neighbouring LED rows with an appropriate time shift resulted in apparent vertical motion in the visual field of the fly. The spatial wavelength of the square-wave grating was 32° and it moved with a temporal frequency of 8 Hz.

Results and discussion

Line-scan measurements of visually induced Ca^{2+} transients in a VS-cell terminal under two-photon excitation

The cytosolic Ca^{2+} concentration increases in the synaptic output areas of motion-sensitive VS-neurons in the fly's brain when a visual stimulus in the receptive field moves in the preferred direction. So far, presynaptic Ca^{2+} signals have been analysed by the use of conventional wide-field fluorescence microscopy [14,31]. As a consequence of limited spatial and temporal resolution associated with this imaging technique, the measured Ca^{2+} signals allow only for indirect conclusions about the role of Ca^{2+} for synaptic transmission. These Ca^{2+} signals reflect bulk cytosolic Ca^{2+} concentrations rather than changes in Ca^{2+} concentration near the membrane surface which are directly relevant for transmitter release. Particularly the analysis of Ca^{2+} concentration changes in fine arborizations is critical. Due to light scattering, Ca^{2+} measurements in arborizations with small diameters are distorted by fluorescence from outside the region of interest. As a first step to assess changes in Ca^{2+} concentration at a better temporal and spatial resolution, we applied TPLSM to the output region of a VS-neuron, the VS2-cell, in our in vivo preparation. Here, we adapted a custom-built two-photon scanning system with an APD which—due to its high quantum efficiency of more than 50% at the emission maximum of fluo-4 (520 nm)—allows fast imaging even at dim light levels inherent to TPLSM and the small excitation volumes associated with high spatial resolution. In order to critically check Ca^{2+} measurements by TPLSM for artefacts and to compare with

established methods, we applied Ca^{2+} imaging with a CCD camera at a conventional epifluorescence microscope to the same preparation.

In an axonal recording close to its output region VS2 responds to downward vertical motion with a graded depolarization of its membrane potential which was superimposed by spike-like depolarizations (Fig. 2B). With two-photon excitation of the Ca^{2+}-sensitive fluorescent dye fluo-4 we performed line-scan measurements in the output area to measure presynaptic Ca^{2+} dynamics. During visual motion in the preferred direction, an increase in fluorescence was detected along lines crossing arborizations in the terminal region. The fluorescence elicited by less than 10 mW incident laser power at the specimen was sufficient to detect activity-dependent fluorescence changes even though fractions of fluorescence were lost in the optical path because it was necessary to descan the emitted light during the line-scan measurements with the APD (see Materials and methods). In TPLSM the fluorescence signal depends quadratically on the laser intensity. Hence, the signal can in principle be improved considerably by using higher intensities in the focus. However, the use of high laser intensities is limited by the onset of cell-damage producing effects. So far, only little is known about the dependence of photodamage on laser power in TPLSM (but see e.g.: [28,29,32]). The influence of laser power was investigated in earlier experiments. At average powers above 10 mW we observed membrane blebbing and bright fluorescent bursts upon prolonged scanning (20 min) which suggests cell damage (data not shown).

During motion stimulation Ca^{2+} signals could be acquired in single neurites at a scanning frequency of 200 Hz along a line-length of 10 μm (Fig. 2C). The spatial resolution was limited by the diameter of the excitation volume (i.e., the laser focus), which we determined to be 0.3 μm in the *xy*-plane and 0.7 μm in the *z*-dimension. Since these values were determined in water, the actual spatial resolution in the tissue might be slightly lower. By conventional fluorescence imaging with a CCD camera, image sequences with $128 * 128$ pixels could be acquired at a temporal resolution of 14 Hz and a pixel resolution of 1.3 μm (Fig. 2E). Spatial resolution during wide-field fluorescence might, however, be limited by light scattering in the tissue rather than by the pixel resolution of the CCD. Temporally low-pass filtered versions of the Ca^{2+} concentration time courses acquired by TPLSM look very similar to those calculated for small regions of interest from conventional fluorescence image sequences (cf. Figs. 2D and E): the Ca^{2+} concentration increases during the whole 1-s period of visual stimulation by 40–80% of its baseline value and recovers within several seconds after cessation of stimulus motion. In the unfiltered two-photon signal, additional fast fluctuations are visible (Fig. 2D). At the moment it is not possible to say whether these fast fluctuations represent noise or actual changes in the Ca^{2+} concentration. To discern between these possibilities, membrane potential recordings and two-photon Ca^{2+} imaging would have to be performed simultaneously. For technical reasons, this has not been done yet.

Decay kinetics of Ca^{2+} after cessation of visual motion in differently sized single presynaptic neurites

At higher magnifications, the output area of VS2 shows a branched structure instead of a single ending (Figs. 2C and E). We used two-photon line-scan measurements to compare the motion-induced changes in Ca^{2+} concentration in differently sized branches within the presynaptic output area of the VS2-cell. As shown in Fig. 2C, the scan line was placed on different regions in consecutive measurements. In order to compare the kinetics of Ca^{2+} decay after the cessation of visual motion, data were normalized to peak values of relative changes in fluorescence (peak value of $\Delta F/F_0$ set to 1, see Fig. 2D). The Ca^{2+} decay appeared faster in the line scan covering the thin side branch than in that covering the thicker main branch in the output area. A parsimonious explanation for the dependency of Ca^{2+} kinetics on branch diameter is that Ca^{2+} concentration changes are mainly due to transport via the outer membrane. This would lead to faster Ca^{2+} regulation in regions with large surface area to volume ratio. Such an explanation is corroborated by the finding that Ca^{2+} clearance is also faster in thin than in thick TC neurites when Ca^{2+} is homogeneously raised by flash photolysis of caged Ca^{2+} (Kurtz, in revision).

Comparison of Ca^{2+} imaging with TPLSM and with conventional fluorescence microscopy

Both with conventional wide-field fluorescence microscopy and with TPLSM measurements of Ca^{2+} concentration changes in TCs can be performed in vivo during visual stimulation. Whereas conventional imaging provides two-dimensional images, TPLSM allows measuring along single lines with much faster acquisition rate (200 vs. 14 Hz). The overall similarity of the measured signals indicates that with TPLSM at 10 mW or less incident laser power the neurons remain as viable as with conventional fluorescence microscopy, and that Ca^{2+} regulation remains intact. Bleaching of the dye, however, was stronger in TPLSM than in conventional imaging. This is due to the fact that, in order to achieve a high temporal resolution, one line was continually scanned by the laser for several seconds. The exponential decay time constants of bleaching, which were determined by scanning without visual stimulation and used for correcting the time courses shown in Fig. 2D, were 15.2 s in the thick neurite and 6.8 s in the fine arborization, respectively. In contrast, during conven-

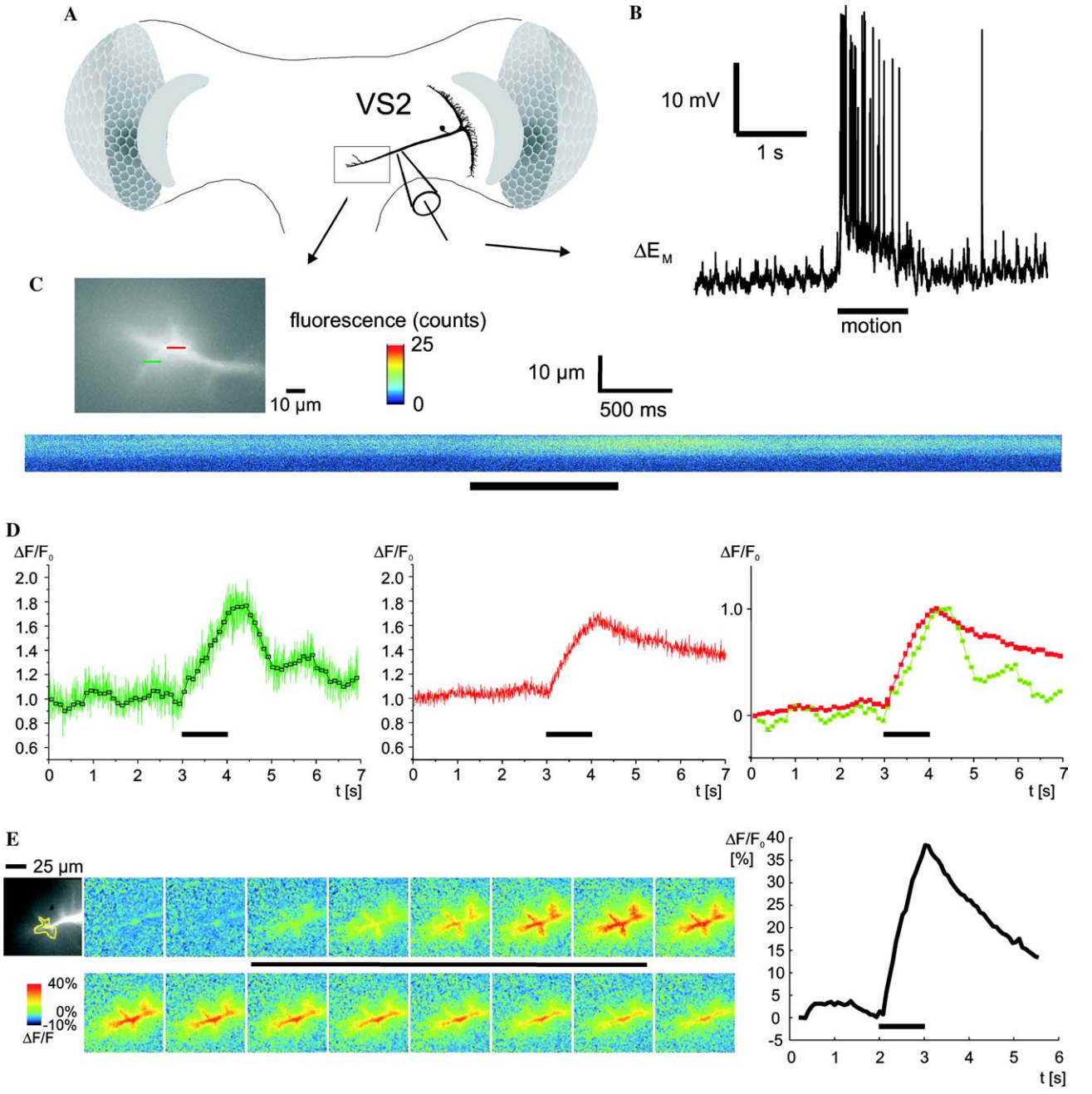

Fig. 2. Two-photon laser scanning and conventional Ca^{2+} imaging in the presynaptic output region of a motion-sensitive neuron. (A) Schematic of a fly's head (caudal view) with the visual motion-sensitive VS2-neuron. (B) Electrical recording of the membrane potential of VS2 during presentation of a motion-stimulus in the neuron's preferred direction. The black bar indicates stimulus motion. The neuron responds to visual motion with a graded depolarization that is superimposed by spike-like depolarizations. (C) Upper left: epifluorescence image taken with a CCD camera of the synaptic terminal region of a VS2-neuron filled with the Ca^{2+}-sensitive fluorescent dye fluo-4. An image taken during two-photon line scanning is superimposed on the epifluorescence image. The location of this scan line is additionally marked by a red bar. The green bar indicates the location where the scan line was located in a further experiment (see below). Below: two-photon laser-scanning measurement of visual motion-induced Ca^{2+} concentration changes in the presynaptic arborization along a line of 10 µm width indicated by the green bar in the epifluorescence image. A Ca^{2+} concentration increase during stimulus motion (black bar) is indicated by warm colours in the false-colour coded image. Scanning frequency is 200 Hz. (D) Left: time course of photobleach corrected $\Delta F/F_0$ of the line-scan measurement shown above. A smoothed version of the time-course, generated by averaging periods of 20 datapoints, is indicated by the boxes. Middle: time-course of line-scan along the red bar shown in (C). Right: comparison of the line-scans along the green and red bar in (C), respectively, smoothed to 10 Hz and normalized to their peak values. (E) The same VS2-neuron during conventional wide-field Ca^{2+} fluorimetry with a CCD camera. Ca^{2+} accumulation during presentation of visual motion (black bar) is shown in a sequence of false-colour coded images (left) and in a $\Delta F/F_0$ time course (right). Only every third image of the entire series acquired with 14 Hz temporal resolution is shown. $\Delta F/F_0$ was calculated in the region of interest indicated by the yellow line in the raw-fluorescence image (upper left). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

tional imaging the excitation intensity was attenuated with neutral density filters until virtually no bleaching was detectable. However, in several respects TPLSM seems to be superior to conventional imaging:

First, during TPLSM there is no unwanted visual stimulation because fly photoreceptors are virtually insensitive to wavelengths above 700 nm. This differs in conventional fluorescence imaging with excitation light in the visible or UV range, which excites fly photoreceptors strongly: although being fairly specific to motion, VS-neurons also respond to contrast modulations and are thus stimulated by the excitation light. This is reflected in a Ca^{2+} concentration increase at the beginning of image acquisition (see Fig. 2E).

Second, spatial resolution of TPLSM is superior to conventional fluorescence imaging. This higher resolution is only insufficiently described by the difference between focal volume and pixel resolution of TPLSM and conventional imaging, respectively (0.3 vs 1.3 μm in the *xy*-plane). By far more important, especially for comparisons of Ca^{2+} signals between different regions, is the fact that there is considerable crosstalk between neighbouring cellular regions during wide-field fluorescence excitation. Therefore, signals collected at thin neurites can be expected to include to a large extent fluorescence scattered from adjacent larger, more brightly stained, neurites. In contrast, when imaging thin neurites with TPLSM, the detected signal represents exclusively fluorescence emanating from the imaged structure, because the excitation volume itself is limited. Thus, Ca^{2+} signals from adjacent cellular structures, which might be smeared during wide-field excitation, are clearly separable with TPLSM.

Conclusions and outlook

In this paper we have shown that fast imaging of neuronal Ca^{2+} concentrations in the intact fly brain with our custom-built TPLSM is feasible and in several respects superior to conventional wide-field fluorescence imaging. One drawback of TPLSM and confocal one-photon microscopy is that fast imaging rates can only be achieved during line scanning. Two-dimensional laser scanning, in contrast, requires acquisition times comparable to conventional CCD camera based imaging. In order to fully exploit the benefits of two-photon imaging we intend to bridge the gap between fast line scanning and slow two-dimensional scanning. Therefore, the next step will be to make use of the laser beam splitter which is already installed in our set-up. By detecting the fluorescence emitted along multiple lines with separate APDs, Ca^{2+} concentration changes can then be measured on a fast timescale simultaneously for neuronal structures at different locations in the imaging plane.

References

[1] M.J. Berridge, Neuronal calcium signaling, Neuron 21 (1998) 13–26.
[2] R.S. Zucker, Calcium- and activity-dependent synaptic plasticity, Curr. Opin. Neurobiol. 9 (1999) 305–313.
[3] M.D. Bootman, T.J. Collins, C.M. Peppiatt, L.S. Prothero, L. MacKenzie, P. De Smet, M. Travers, S.C. Tovey, J.T. Seo, M.J. Berridge, F. Ciccolini, P. Lipp, Calcium signalling—an overview, Semin. Cell Dev. Biol. 12 (2001) 3–10.
[4] J.D. Spafford, G.W. Zamponi, Functional interactions between presynaptic calcium channels and the neurotransmitter release machinery, Curr. Opin. Neurobiol. 13 (2003) 308–314.
[5] E.F. Stanley, The calcium channel and the organization of the presynaptic transmitter release face, Trends Neurosci. 20 (1997) 404–409.
[6] A. Takahashi, P. Camacho, J.D. Lechleiter, B. Herman, Measurement of intracellular calcium, Physiol. Rev. 79 (1999) 1089–1125.
[7] W. Denk, J.H. Strickler, W.W. Webb, Two-photon laser scanning fluorescence microscopy, Science 248 (1990) 73–76.
[8] Z.F. Mainen, M. Maletic-Savatic, S.H. Shi, Y. Hayashi, R. Malinow, K. Svoboda, Two-photon imaging in living brain slices, Methods 18 (1999) 231–239.
[9] D.W. Piston, Imaging living cells and tissues by two-photon excitation microscopy, Trends Cell Biol. 9 (1999) 66–69.
[10] F. Helmchen, W. Denk, New developments in multiphoton microscopy, Curr. Opin. Neurobiol. 12 (2002) 593–601.
[11] F. Helmchen, J. Waters, Ca^{2+} imaging in the mammalian brain in vivo, Eur. J. Pharmacol. 447 (2002) 119–129.
[12] A. Borst, M. Egelhaaf, In vivo imaging of calcium accumulation in fly interneurons as elicited by visual motion stimulation, Proc. Natl. Acad. Sci. USA 89 (1992) 4139–4143.
[13] V. Dürr, M. Egelhaaf, In vivo calcium accumulation in presynaptic and postsynaptic dendrites of visual interneurons, J. Neurophysiol. 82 (1999) 3327–3338.
[14] R. Kurtz, A.K. Warzecha, M. Egelhaaf, Transfer of visual motion information via graded synapses operates linearly in the natural activity range, J. Neurosci. 21 (2001) 6957–6966.
[15] S. Single, A. Borst, Dendritic integration and its role in computing image velocity, Science 281 (1998) 1848–1850.
[16] M. Egelhaaf, A. Borst, Calcium accumulation in visual interneurons of the fly: stimulus dependence and relationship to membrane potential, J. Neurophysiol. 73 (1995) 2540–2552.
[17] R. Kurtz, V. Dürr, M. Egelhaaf, Dendritic calcium accumulation associated with direction-selective adaptation in visual motion-sensitive neurons in vivo, J. Neurophysiol. 84 (2000) 1914–1923.
[18] M. Egelhaaf, A. Borst, Movement detection in arthropods, Rev. Oculomot. Res. 5 (1993) 53–77.
[19] M. Egelhaaf, A. Borst, A look into the cockpit of the fly: visual orientation, algorithms, and identified neurons, J. Neurosci. 13 (1993) 4563–4574.
[20] K. Hausen, The lobula-complex of the fly: structure, function and significance in visual behaviour, in: M.A. Ali (Ed.), Photoreception and Vision in Invertebrates, Plenum Press, New York, London, 1984, pp. 523–559.
[21] K. Hausen, M. Egelhaaf, Neural mechanisms of visual course control in insects, in: D.G. Stavenga, R.C. Hardie (Eds.), Facets of Vision, Springer, Berlin, Heidelberg, 1989, pp. 391–424.
[22] A. Borst, J. Haag, Neural networks in the cockpit of the fly, J. Comp. Physiol. A 188 (2002) 419–437.
[23] M. Egelhaaf, R. Kern, H.G. Krapp, J. Kretzberg, R. Kurtz, A.-K. Warzecha, Neural encoding of behaviourally relevant visual-motion information in the fly, Trends Neurosci. 25 (2002) 96–102.
[24] M.J. Berridge, Elementary and global aspects of calcium signalling, J. Physiol. 499 (Pt 2) (1997) 291–306.

[25] R. Hengstenberg, Common visual response properties of giant vertical cells in the lobula plate of the blowfly *Calliphora*, J. Comp. Physiol. A 149 (1982) 179–193.
[26] R. Hengstenberg, K. Hausen, B. Hengstenberg, The number and structure of giant vertical cells (VS) in the lobula plate of the blowfly *Calliphora erythrocephala*, J. Comp. Physiol. A 149 (1982) 163–177.
[27] A.K. Warzecha, R. Kurtz, M. Egelhaaf, Synaptic transfer of dynamic motion information between identified neurons in the visual system of the blowfly, Neuroscience 119 (2003) 1103–1112.
[28] A. Hopt, E. Neher, Highly nonlinear photodamage in two-photon fluorescence microscopy, Biophys. J. 80 (2001) 2029–2036.
[29] H.J. Koester, D. Baur, R. Uhl, S.W. Hell, Ca^{2+} fluorescence imaging with pico- and femtosecond two-photon excitation: signal and photodamage, Biophys. J. 77 (1999) 2226–2236.
[30] T. Nielsen, M. Fricke, D. Hellweg, P. Andresen, High efficiency beam splitter for multifocal multiphoton microscopy, J. Microsc. 201 (2001) 368–376.
[31] J. Haag, A. Borst, Spatial distribution and characteristics of voltage-gated calcium signals within visual interneurons, J. Neurophysiol. 83 (2000) 1039–1051.
[32] K. König, T.W. Becker, P. Fischer, I. Riemann, K.-J. Halbhuber, Pulse-length dependence of cellular response to intense near-infrared laser pulses in multiphoton microscopes, Opt. Lett. 24 (1999) 113–115.