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A simple and practical method to acquire geometrically correct images with resonant scanning-based line scanning in a custom-built video-rate laser scanning microscope

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Summary

Most currently available confocal or two-photon laser scanning microscopes (LSMs) allow acquisition rates of the order of 1–5 images s^{-1}, which is too slow to fully resolve dynamic changes in intracellular messenger concentration in living cells or tissues. Several technologies exist to obtain faster imaging rates, either in the video-rate range (30 images s^{-1}) or beyond, but the most versatile technology available today is based on resonant scanners for horizontal line scanning. These scanning devices have several advantages over designs based on acousto-optical deflectors or Nipkow discs, but a drawback is that the scanning pattern is not a linear but rather a sinusoidal function of time. This puts additional constraints on the hardware necessary to read-in the image data flow, one of which is the generation of a pixel clock that varies in frequency with the position of the pixel on the scanned line. We describe a practical solution to obtain a variable pixel clock add-on that is easy to build and is easy to integrate into a custom-built LSM based on resonant scanning technology. In addition, we discuss some important hardware and software design aspects that simplify the construction of a resonant scanning-based LSM for high-speed, high-resolution imaging. Finally, we demonstrate that the microscope can be used to resolve calcium puffs triggered by photolytically increasing the intracellular concentration of inositol trisphosphate.

Introduction

Commercially available laser scanning microscopes (LSMs) are characterized by low image acquisition rates that are typically of the order of 1–5 full images s^{-1} at best. These slow acquisition rates are limited by the raster scanning system that sweeps the specimen point-by-point and line-by-line in a sequential manner. Raster scanning is usually via relatively slow servo-controlled closed-loop galvanometer scanners for both horizontal and vertical scanning (a historical account of laser scanning technology can be found in Amos & White, 2003). Fast LSM imaging for real-time live cell imaging applications can be obtained through various adaptations to the scanning procedure, e.g. by scanning the field line-wise instead of point-wise (slit scanning), by scanning with ultra fast acousto-optical deflectors (AODs) (Goldstein *et al.*, 1990), with an array of pinholes and microlenses placed on a set of rotating Nipkow discs (Tanaami *et al.*, 2002), or with a solid-state digital mirror device (acting as a spatial light modulator) in a programmable array microscope (Hanley *et al.*, 1999; Heintzmann *et al.*, 2001). The best image quality and system versatility can, however, be obtained by replacing the slow servo-controlled device for horizontal line scanning with a resonant scanning mirror instead. These scanners, which have been available for many years, oscillate at their natural frequency, which is of the order of several kilohertz (typically 7910 Hz). Acquisition of 512 lines, progressively scanned in a bidirectional manner, e.g. odd lines from left-to-right and even lines from right-to-left, yields acquisition rates of the order of 30 frames per second (fps). Resonant scanners have some inherent drawbacks however. One major problem is related to the fact that the velocity of the scanned laser beam projected onto the specimen changes in a cosinusoidal manner, with slowing at the edges and maximal speed in the middle of the scanned field. Thus, when the image data flow derived from a resonant scanning system is acquired with a frame grabber at a constant pixel rate, the images appear stretched at the edges. Several solutions have been proposed to circumvent this

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problem. The most simple option is to limit the image width to the portion where the scan speed is more or less linear (approximately two-thirds of the line scan width) (Callamaras & Parker, 1999). An algorithmic approach has been proposed by Sanderson & Parker (2003), which implies linearization of the image according to an *a priori* calculated look-up table (explained in detail in Sanderson, 2004). The most obvious solution is to read-in the image data flow with a pixel clock that varies as a function of the scanning mirror position. Some manufacturers of resonant scanners offer a variable pixel clock output option in their control electronics, but these signals are also based on look-up tables that do not allow for small additional variations in the scanning pattern due to the mechanical nature of the scanning device. The best solution to the problem has been provided by Tsien & Bacskai (1995), who described a variable pixel clock based on re-direction of part of the horizontally scanned laser excitation beam to a grating of clear and opaque stripes (a Ronchi grating) and measuring of the intensity variations of the light that was transmitted or reflected. A drawback of this approach was its technical complexity, which increased if switching between different excitation wavelengths was necessary. A somewhat simplified approach was used in the Nikon RCM-8000 video-rate confocal microscope, but this instrument is no longer commercially available. Here we present a simple custom-built solution to obtain a variable pixel clock that is based on the Ronchi grating approach and a limited number of additional standard optical components. This basic geometrical image correction set-up is integrated into an up-to-date system that is reduced to its minimal essential configuration and that is integrated with the control circuitry and software that is necessary to obtain a functional and flexible LSM combining both high-speed and high-resolution imaging. Combined with the detailed LSM description provided by Sanderson & Parker (2003), this allows a custom-tailored LSM, for confocal or two-photon use, to be built that fully exploits the fast scanning possibilities of resonant scanners.

Materials and methods

A custom-made confocal LSM was built around a Nikon Eclipse TE300 inverted miscroscope as described by Callamaras & Parker (1999). The vertical scanner was an M-series closed-loop galvanometer type M3H with paddle mirror (GSI-Lumonics, Bedford, MA, U.S.A.); the horizontal scanner was a counter rotation scanner (CRS) resonating at 8 kHz (GSI-Lumonics). The excitation lasers were an argon ion laser for blue excitation (type 543R-AP-A01, 488-nm line) and an HeNe for green excitation (type 25-LGR-393-230) both from Melles Griot (Carlsbad, CA, U.S.A.). Extended details on the other components used in this microscope can be found in Sanderson & Parker (2003). The additional optical components used for the variable pixel clock add-on were a 3-mW infrared (670 nm) diode laser (cat. no. 194-026, RS Components, Anderlecht, Belgium), an old monocular microscope tube with a ×3 objective and a ×5 ocular lens (Meopta, Prerov, Czech Republic), a Ronchi grating with a line width of 10.24 μm for both the clear and the opaque lines (corresponding to 48.83 line pairs mm^{-1} – sample model from Dotrix, Ghent, Belgium, also available from Edmund Optics, Barrington, NJ, U.S.A.) and a photodiode type OSD15–5T (RS Components). The transconductance amplifier was an NE5210 (Phillips Semiconductors, Eindhoven, The Netherlands); the frequency doubler used to double the pixel clock frequency was based on a circuit built around the AD 835 analog multiplier (Analog Devices, Northwood, MA, U.S.A.).

Other components used in the set-up were a frequency doubler used to deliver line trigger pulses for bidirectional scanning, which was based on a digital circuit with two one-shot monostable multivibrators (type 74LS123, Texas Instruments, Dallas, TX, U.S.A.). The preloadable counters in the vertical scanning triangle-wave generator were based on a cascade of three type 74LS169 counters (Texas Instruments). The intermediate digital I/O – analogue I/O board to control the scanning parameters was a DT331 (Data Translation, Marlboro, MA, U.S.A.); analog output lines of this board were used to command the horizontal and vertical scan width and position, and 12 digital I/O lines provided the 'number of lines per frame' information for the preloadable counters. The counter timer board for triggered image acquire operations was a PCI-6601 (National Instruments, Zaventem, Belgium). The photomultiplier tube (PMT) was an R3986 from Hamamatsu (Louvain-La-Neuve, Belgium) and its integrated analog output (photon counting was almost impossible because of the small pixel dwell time, which was of the order of 100 ns $pixel^{-1}$) was connected, together with the pixel clock, line trigger, frame trigger and external trigger, to a frame grabber board in a PC (type DT3162, Data Translation). The electronic blanking circuit interposed between PMT output and frame grabber luminance input was built around an AD 835 analog multiplier (Analog Devices). The software to control the LSM set-up was written in Microsoft Visual C ++6.0 and consisted of separate windows that control basic parameters such as laser excitation energy, shutter opening, pinhole size, detector gain and raster scanning properties, a modified image acquisition routine based on the sample program 'Acquire.cpp' (Data Translation) and a custom-developed analysis module called 'Fluoframes'. Saving a high-resolution video stream to hard disk required high-performance hard disk drives. We used an ultra 160 SCSI with 10 000 r.p.m. disks to achieve image data flows up to 25 Mbyte s^{-1}. As an alternative to fast hard drives, we also used a software emulation hard disk or so called RAM disk (available as freeware for Windows 2000 from AR Soft, http://www.arsoft-online.de). The size of a RAM disk was limited in practice, however, to typically 100 Mbyte in a desktop PC with 1 Gbyte RAM under Windows 2000.

The optical filters in the set-up were the dual-wavelength dichroic XF2043 (490-550DBDR) with corresponding emission

bandpass XF3056 (520-580DBEM), both from Omega Optical (Brattleboro, VT, U.S.A.). This filterset allowed beam splitting and emission bandpass filtering in the classical FITC (blue excitation, 488 nm) and TRITC (green excitation, 564 nm) channels. The beam splitter used to couple-in the excitation lasers was either an XF122 (70 : 30 transmission-to-reflection ratio) or an XF2010 (505DRLP) dichroic; the beamsplitter at the emission side was an XF2017 (560DRLP), all from Omega Optical.

The pollen grain specimen shown below in Fig. 3 was a mixed pollen grain preparation stained with various fluorochromes from the Carolina Biological Supply Company (Burlington, NC, U.S.A.; cat. no. WW-30-4264). The experiments illustrating calcium puffs (Fig. 4) were performed on ECV304 cell cultures, as used in previous studies (Braet *et al.*, 2001, 2003). Cultures were loaded with fluo-3 by incubation for 1 h at room temperature in Hanks' balanced salt solution buffered with 25 mM HEPES (HBSS-HEPES) containing 10 μM fluo-3-AM (Molecular Probes, Eugene, OR, U.S.A.), 0.05% pluronic and 1 mM probenecid. This was followed by 1 h of de-esterification in HBSS-HEPES at room temperature. After fluo-3 loading, the cells were loaded with D-myo-inositol 1,4,5-trisphosphate, P4(5)-(1-(2-nitrophenyl)ethyl)ester trisodium salt (Molecular Probes, or Calbiochem, La Jolla, CA, U.S.A.) by *in situ* electroporation of the monolayer, as previously described (Braet *et al.*, 2004). Cells were viewed with the confocal LSM set-up equipped with a ×40 oil-immersion lens and using the FITC settings described above. Flash photolysis experiments were started 30 min after electroporation and were performed with spot ultraviolet excitation as described in detail in Braet *et al.* (2004).

Results

A custom-made confocal LSM was built around a Nikon Eclipse TE300 inverted miscroscope equipped with a resonant scanning device for horizontal scanning and a servo-controlled vertical scanner, as described previously by Callamaras & Parker (1999) and Sanderson & Parker (2003). An overview of the set-up is illustrated in Fig. 1. The additional optical components necessary to obtain a variable pixel clock signal include an additional low-power semiconductor laser source, a Ronchi grating, intermediate optics to focus the laser light onto the grating and a photodiode. These components are arranged as follows: the light beam from the extra laser source is directed to the reflecting backside of the resonant line scanning mirror; the front side of this mirror receives the laser light for specimen excitation (Fig. 2). The light beam from the extra laser is reflected off the backside of the resonant scanner and the sweeping beam is projected onto the grating by means of an intermediate optical system that consists of a classical 160-mm microscope tube, an objective lens focused at the grating and an ocular lens facing the backside of the scanning mirror. At the level of the grating, the laser beam is scanned over a sequence of alternating opaque and clear lines of equal width, such that the light is either blocked or passes through in an alternating manner. The fluctuations in light intensity are detected by a photodiode positioned immediately behind the grating. During the horizontal scan the photodiode will register pulses, which, after amplification and processing, can be used as a pixel clock with variable frequency. The slowing of the mirror at the edges will indeed result in larger pulse intervals as compared with those generated when the mirror accelerates towards the middle. Figure 2 illustrates the variable pixel clock signal as a function of the position of the resonant scanning mirror and Fig. 3 summarizes the overall set-up.

The following practical considerations are important to obtain a simple and properly working variable pixel clock set-up. For the additional laser source we used a semiconductor infrared laser, but a cheap low-power laser diode from a standard laser pointer gives equally good results. The diameter of the laser beam spot at the level of the grating should be significantly smaller than the grating line width or a diffraction pattern will be produced. The intermediate optical system between the scanning mirror and grating should therefore be of sufficient quality, i.e. its modulation transfer function should allow distinction of approximately 50 line pairs mm^{-1}. We tested several optical arrangements but the cheapest and most simple solution is to use an objective lens and ocular from an old microscope positioned as described above. A ×5 ocular lens in combination with a ×3 objective lens does well with the grating used in our set-up, which had a line width of 10.24 μm (20.48 μm per line pair). The photodiode, placed immediately behind the grating, must be able to handle the required pulse frequency and its surface must be large enough to receive the entire line sweep. The maximal frequency expected for generating 512 pulses per line is of the order of 10–15 MHz (in the middle of the scan). In our experimental set-up a photodiode with an active area size of 5.5 mm and a −3-dB bandwidth of 29 MHz was used. The photodiode is connected to a transconductance amplifier, which transforms the diode current into a voltage; the amplified signal is then fed to a frequency doubler circuit based on an analog multiplier integrated circuit, thus allowing the number of pixels per line to be doubled (Fig. 2). The dimensions of the optical and electronic parts of the variable pixel clock interface are such that the output of the photodiode delivers 256 pulses per line at a maximal horizontal scanning amplitude (angular displacement of the order of 25°). The frequency doubling circuit transforms this to 512 pulses per line, resulting in 512 pixels per line acquired by the frame grabber, and this can be further increased by an additional frequency doubling circuit. When line scanning is performed in a bidirectional manner, acquisition of 512 lines in principle yields an image acquisition rate of around 30 fps. Halving the number of lines will double the acquisition rate to 60 fps. Owing to the slow flyback of the vertical galvanometer–scanning mirror assembly, the number of effective lines is usually smaller than the scanned number of lines, generally of the order of 10–20% lower.

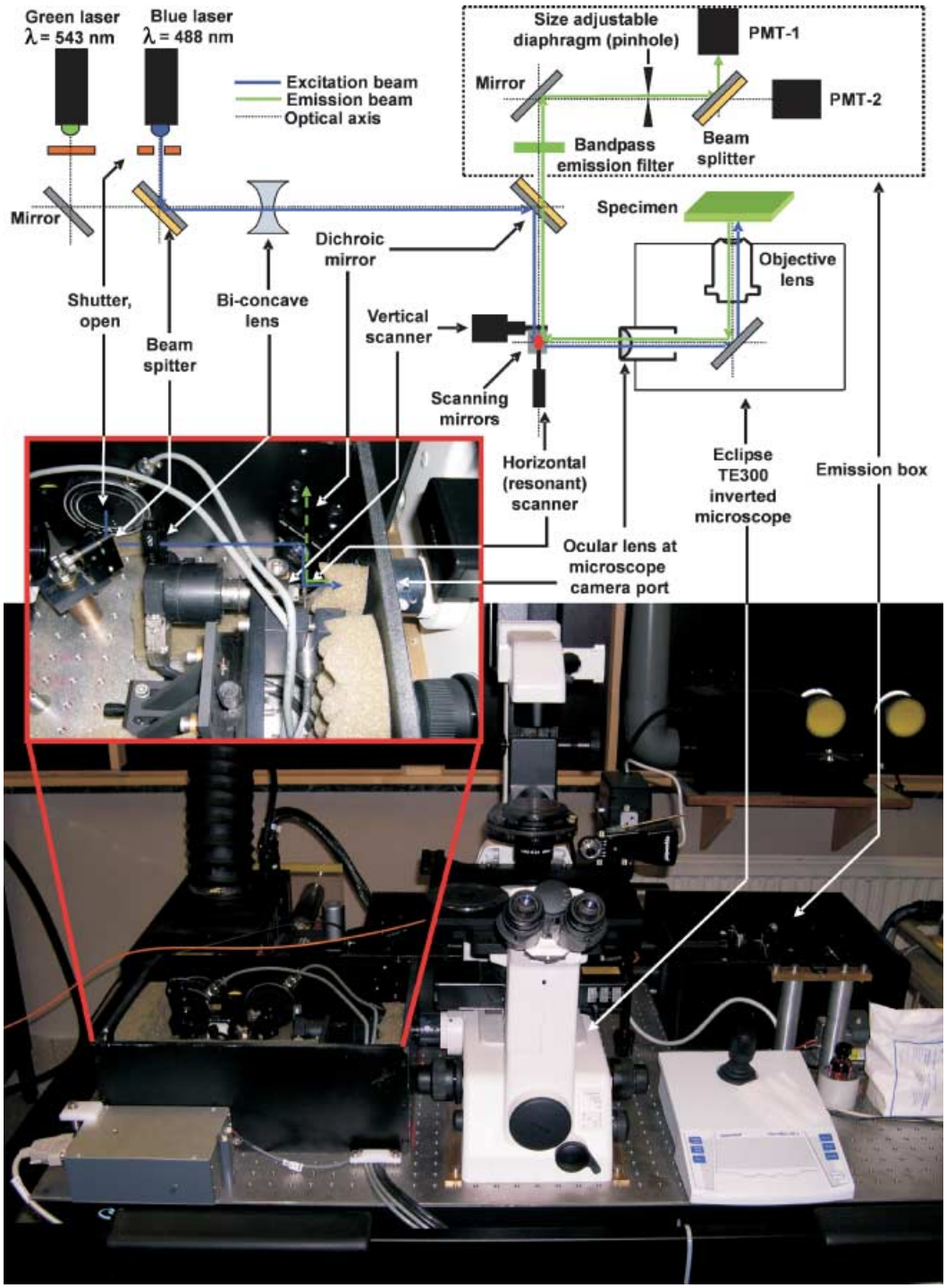

Fig. 1. General set-up of the confocal LSM. The excitation laser beam traverses a biconcave lens (beam expander) that forms a virtual point-image of the excitation source, which is scanned into the microscope by means of vertical and horizontal (resonant) scanning mirrors and an intermediate ocular lens attached to the microscope camera port. The emission light coming from the specimen is de-scanned and relayed to the detector by a dichroic mirror, a bandpass filter, a mirror and an externally adjustable diaphragm that acts as the confocal aperture. The aperture is placed at equal distance from the scanning mirors as the virtual point-image of the excitation laser (the position of the virtual point-image may not be exactly at the focal distance of the beam expander lens, due to a focal length shift introduced by argon-ion or HeNe lasers with a Gaussian intensity profile). After passing the pinhole, the emission signal is picked up by a photomultiplier tube (PMT). The excitation wavelength is determined by the laser shutters in combination with the beam splitter; the corresponding emission light is directed to the correct PMT by means of another beam splitter. The luminance signal of both PMTs is sent to a frame grabber board, allowing selection of the appropriate input signal.

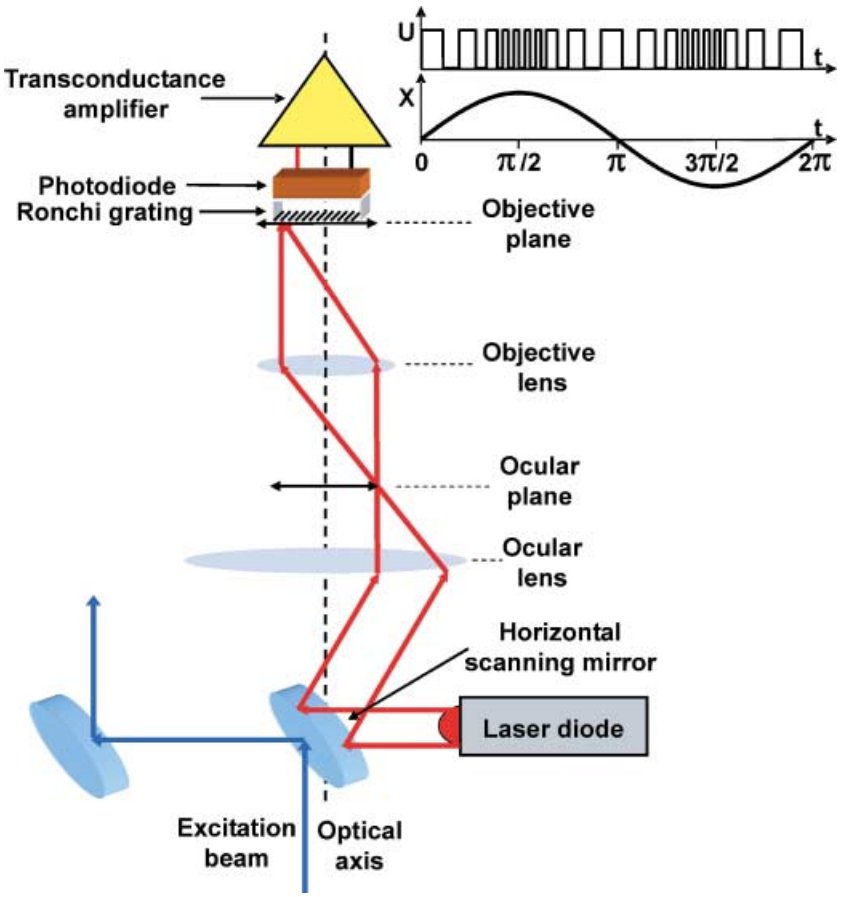

Fig. 2. Additional components necessary to generate a variable pixel clock. An extra laser source (laser diode) is directed to the backside of the resonant scanning mirror; the front side of this mirror receives the excitation laser beam. The sweeping light beam is focused onto a grating of clear and opaque stripes (Ronchi grating) by means of an intermediate optical system that consists of a microscope ocular and objective lens. The light traversing the grating is detected by a photodiode and converted to a voltage output (U) by means of a transconductance amplifier. The output signal is composed of square pulses with a period that changes with the position (X) of the scanning mirror.

Some specific improvements have been added to the original LSM set-up as described by Callamaras & Parker (1999). These include a vertical scanning driver circuit that allows the number of lines per frame to be set to any chosen value. This is achieved by using preloadable counters that can be programmed from the PC by means of an intermediate digital I/O board. All other scan parameters, such as horizontal and vertical scan width and mirror position, can also be set from the PC, via an analog I/O board. This allows easy control of the raster scanning parameters, e.g. to decrease the scan to a small area in order to perform local photobleaching or photoactivation, or to increase the number of lines per frame and pixels per line to accomplish fast imaging at high spatial resolution (1024×1024 pixel images at 15 fps). The actual image acquisition rate is determined by the natural frequency of the resonant scanner, which is fixed, and the number of lines per image. For lower acquisition rates, images are collected in a triggered fashion, with an additional counter/timer board delivering precisely timed pulses that drive the trigger input of the frame grabber board in the PC. It is preferable that the luminance output of the PMT is connected to a d.c. coupled input of the frame grabber, but not all frame grabbers offer this possibility. If a.c. input coupling is used, the frame grabber needs a reference black level at the start of each line. This can be achieved in an optical manner by blinding the lateral limits of the scanned field as it enters the microscope, but an electronic blanking circuit (see Fig. 3) has the advantage that it limits the number of optical components and thus simplifies the set-up. In order to display images scanned in a bidirectional manner in the correct way, it is necessary to invert the lines scanned in reverse order. A simple approach is to limit live images displayed on the PC monitor to the lines scanned left-to-right and to invert alternate, right-to-left, scanned lines after the image sequence has been acquired, thus allowing off-line image inspection at full line resolution.

Development of software to control a complex instrument such as an LSM is often a point of major concern, mainly because of the large time investment this demands. Our approach has been to keep the software as modular as possible, with separate stand-alone Windows-based programs to control raster scanning, laser shutters, filter wheels and photomultipliers. Image acquisition is achieved by using slightly modified sample programs that come with most frame grabbers. These allow 512×512-pixel images to be relayed directly to the hard disk at a rate of 30 fps on currently available standard desktop PCs equipped with a serial ATA drive. For higher resolution images (1024×1024) acquired at 15 fps it is recommended to use SCSI drives. Following the acquisition of an image data sequence, a call from the acquisition program to an external

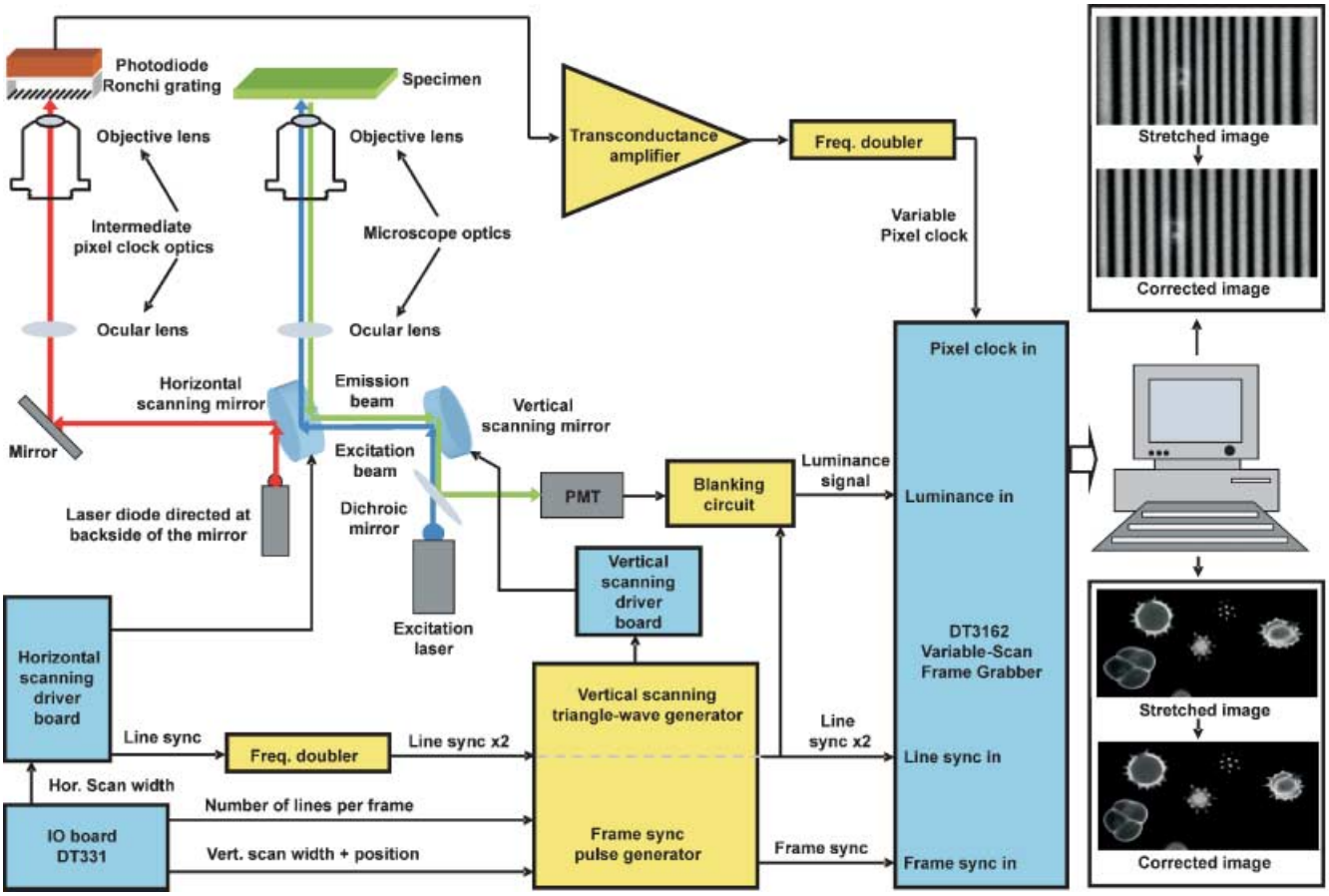

Fig. 3. Schematic diagram of the overall set-up. The LSM is built around the microscope, of which only the objective and ocular lenses are depicted (Microscope optics). The objective and ocular lenses shown left make up the intermediate optics for the variable pixel clock, as described in Fig. 2. Images are acquired by the frame grabber, by clocking in the luminance signal from the PMT at the rate of the variable pixel clock. Line and frame synchronization (sync) pulses derive from an intermediate circuit that contains presettable line counters and a digital-to-analog converter that controls the vertical drive board. The line sync signal is also used to drive the blanking circuit that produces a black reference input to the framegrabber at the start of each line. The horizontal drive board produces a square wave at line frequency that is doubled in frequency to accomplish bidirectional line scanning. All drive electronics are controlled by an I/O board interfacing with the PC, allowing software control of the raster scanning parameters. The entire set-up acquires geometrically correct images at video rate. The inserts illustrate non-corrected and corrected images of a line grating and a pollen grain specimen placed on the microscope stage.

program automatically loads up the images for further evaluation, analysis and final storage. Application-specific routines can easily be added to this external analysis software module. Finally, optical alignment is also often a major point of concern. In order to simplify alignment, it is important that the number of optical components (mirrors, filter wheels, etc.) are reduced to an absolute minimum. If switching between two or three excitation or emission wavelengths is necessary, use of dual- or triple-wavelength filter sets (dichroic mirror and emission filter) greatly simplifies the design and alignment; in addition, it allows fast control of excitation/emission wavelength switching with a minimum of moving parts.

LSM imaging at high temporal and spatial resolution is a prerequisite to resolve the dynamics of fast vascular responses to neuronal or glial activation in the brain (Chaigneau *et al.*, 2003; Mulligan & MacVicar, 2004), or, at the subcellular level, of elementary calcium signalling events such as calcium sparks, calcium blips or calcium puffs (the last two being typically observed in electrically non-excitable cells; Bootman *et al.*, 1997; Thomas *et al.*, 1998). We have used the present set-up to demonstrate its capabilities to monitor calcium puffs in ECV304 cells. Figure 4 illustrates calcium puffs in response to photolytic elevation of the intracellular inositol trisphosphate ($InsP_3$) concentration by flash photolysis of caged $InsP_3$. The calcium puffs were localized in the perinuclear zone and their repeated activation recruited a global increase of calcium all over the cell.

Discussion

Our work illustrates that a custom-built LSM based on resonant scanners can, with a minimal number of additional

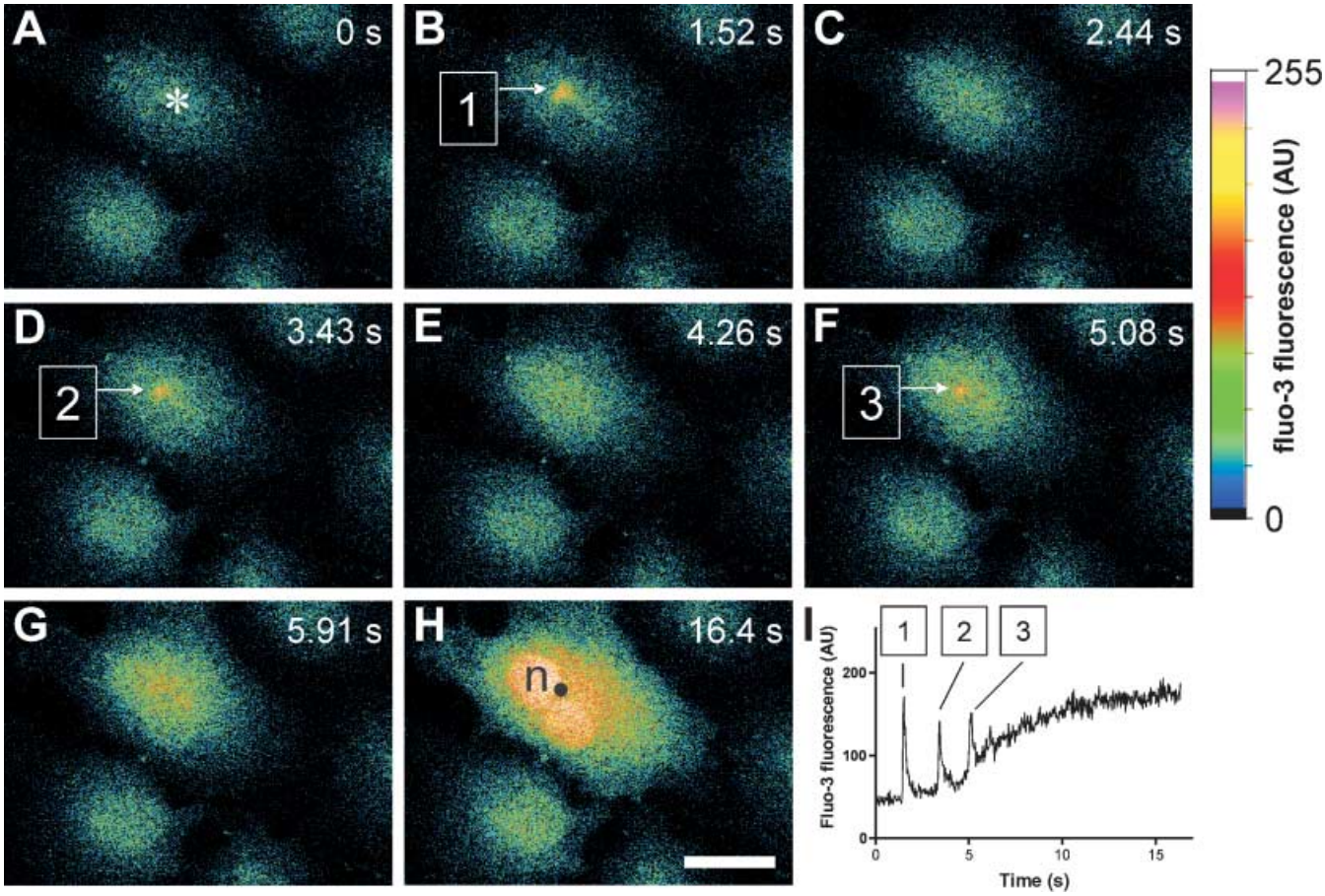

Fig. 4. Calcium puffs in ECV304 cells. (A–H) Time series of calcium images acquired at 30 Hz in bidirectional scanning mode. Scale bar = 50 µm. The images were selected from representative time points within the entire image sequence. The times indicated in the upper right corner refer to time zero at which $InsP_3$ was photolytically liberated in the cell marked with an asterisk (A). The sequence illustrates the appearance of a localized increase of intracellular calcium concentration (occurring at point 1) that recovers and repeatedly comes up again afterwards (indicated by 2 and 3). The repeated actvity at this spot recruited a global increase of calcium in the cytoplasm that was also apparent in the nucleus. The last image demonstrates that the site of calcium signal activity (black dot in H) was localized in the perinuclear zone (nucleus indicated 'n' in H) where the endoplasmic reticulum is located. The graph in I illustrates the time course of the calcium signal at the centre of the calcium hot spot. The calcium signal is represented as fluo-3 fluorescence intensity expressed in arbitrary units (AU), according to a pseudocolour scale shown at the right. The peak amplitude within the spot of calcium activity was reached within approximately 100 ms, the lifetime was of the order of 400 ms and the diameter of the spot averaged 7 µm. These values are indicative of calcium puffs (Bootman *et al.*, 1997; Thomas *et al.*, 1998).

components, be easily adapted to acquire geometrically correct images acquired at high speed. The current set-up allows acquisition of images composed of 440 lines with 512 pixels per line at a speed of 30 fps, and higher rates can be obtained by decreasing the number of lines. Equally fast and even faster imaging is attainable with the spinning disc design offered by some manufacturers (Tanaami *et al.*, 2002), but this is at the cost of reduced flexibility. These devices do not allow the size of the confocal pinhole to be altered, yet when imaging living cells or tissues, a balance has to be made between laser excitation energy, pinhole size (if working in confocal mode) and detector gain. The LSM described here uses the confocal principle to obtain high-quality low-background images, but the system can be adapted easily to use a pulsed laser excitation source to generate two-photon excitation, providing the tissue penetration that is needed to perform live cell imaging in the living brain or other organs (Nguyen *et al.*, 2001). LSM designs based on AOD scanning do not have this flexibility as they cannot be used in conjunction with such lasers (Fan *et al.*, 1999). Resonant scanners therefore have significant advantages over the other technologies for high-speed LSM imaging. In combination with spot photolysis instrumentation (Braet *et al.*, 2004) and fast strategies to obtain ratiometric images (Leybaert *et al.*, 1998), resonant scanning-based LSMs are promising instruments to determine the complex signalling acting at the centre of the brain: the neurovascular unit with its neurones, astrocytes and vessel cells all linked up in an intricate communication network (Leybaert, 2005).

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