Fast scanning and efficient photodetection in a simple two-photon microscope

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Abstract

Two-photon laser scan microscopy carries many advantages for work on brain slices and bulk tissue. However, it has very low signal levels compared to conventional fluorescence microscopy. This is disadvantageous in fast imaging applications when photon shot noise is limiting. Working on brain slices with excitation powers of 8–10 mW at the specimen plane, the resting signal from cerebellar Purkinje cell somas loaded with 10 µM Oregon Green 488 BAPTA-1 averaged 4 detected photons ms⁻¹; axons of interneurons loaded with 200 µM of this indicator yielded about 1 photon µs⁻¹. To obtain satisfactory images at high time resolution, long pixel dwell times are required and data collection should be restricted to as few pixels as necessary. Furthermore, a large proportion of total measurement time (duty cycle) should be available for data collection. We therefore developed a method for scanning small regions of interest with line repetition rates two to four times higher than conventional ones and a duty cycle of 70%. We also compared the performance of several photodetectors and found the optimum choice to depend strongly on the photon flux during a given application. For fluxes smaller than 5 photons µs⁻¹, the photon counting avalanche photodiode shows the best signal to noise ratio. At larger fluxes, photomultipliers or intensified photodiodes are superior. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Two-photon excitation of fluorescence (Göppert-Mayer, 1931; Kaiser and Garrett, 1961) was suggested as a method of particular interest for the study of biological material in the scanning optical microscope as early as 1978 (Sheppard and Kompfner, 1978). It was introduced experimentally into laser scan microscopy by Denk et al. (1990), who pointed out its various advantages over conventional methods. These include larger penetration depth into scattering tissue and less photodamage. Meanwhile, numerous applications on brain slices (Denk et al., 1995a; Yuste and Denk, 1995; Svoboda et al., 1996, 1997, 1999; Denk and Svoboda, 1997; Köster and Sakmann, 1998) and on developing embryos (Potter et al., 1996) have documented these advantages. Nevertheless the method suffers from relatively low signal level, which is particularly severe for fast imaging applications where photon shot noise is limiting. Part of the reason for low signal level results from basic physical principles. Some of the advantages of two-photon microscopy (TPM) result from the square law of fluorescence excitation which holds only at excitation energies and fluorescence levels substantially below fluorescence saturation. Combined with present technology, which employs femtosecond laser pulses at ≈80 MHz repetition rate for excitation, this restriction limits the photon flux that can be obtained from a given dye molecule to 8 × 10⁶ photons per second (at about one photon every ten pulses; higher excitation rates would prevent the desired square law dependence). In contrast, single-photon excitation is only limited by fluorescence saturation, which occurs at typically 5 × 10⁸ photons/s. In addition, the appearance of non-linear photodamage (König et al., 1997) in
biological tissue further restricts the tolerable excitation to levels substantially lower than this theoretical maximum.

For practical applications this means that the photons generated should be used as efficiently as possible and scanning should be restricted to as few pixels as possible (without spending a substantial proportion of time for turnaround and flyback of the laser beam). A commonly used solution to this problem is the line scan or contour scan (Bullen et al., 1997). For many applications, however, information restricted to a single line is not sufficient to characterize the processes under study. Another solution is multifocal imaging (Straub and Hell, 1998; Verveer et al., 1998). This, however, does not allow to utilize scattered photons, which is a disadvantage in brain slice work. We therefore developed a technique for scanning small patches (regions of interest of typically 20-50 pixels) within times smaller than 10 ms using standard galvanometric mirror scanners. This method anticipates high frequency attenuations and phase shifts of the mirror movement to produce mirror deflections which are linear over 70–80% of the duty cycle for scan speeds close to the roll-off frequency of the scanner.

We also made an effort to develop criteria for choosing an optimal photodetector for a given application. To this end we defined and measured an ‘apparent quantum efficiency’ (AQE) as a figure of merit for a detector system in a given application. Measurement of the AQE is based on the signal to noise ratio for a uniform photon flux. Our definition of the AQE results in a value of 100% for an ideal photon counter; the AQE can reach a maximum value equal to the quantum efficiency of the given detector, but it is usually lower than that due to noise sources in addition to the photon shot noise. We find that for very low photon fluxes and pixel dwell times of 10 μs the photon counting avalanche photodiode is the best photodetector presently available. It reaches an AQE equal to its rated value of quantum efficiency (≈ 50%) for photon fluxes of about 1 photon/μs (or 0.5 detected photons/μs). AQE values, however, drop both at lower fluxes (due to dark counts) and at higher ones (due to dead time of the counting device). Thus, at fluxes higher than 5 photons/μs, photomultiplier tubes (PMTs), intensified photodiodes (IPDs) and intensified cameras are better. For fluxes higher than 50 photons/μs (outside the range of TPM) CCDs may be an option. It should be noted that, in the framework of this analysis, simple photodiodes, which reach quantum efficiencies of 80%, are superior to all other devices at very high photon fluxes. But with present technology of photocurrent recording, this will only occur at fluxes > 10 000 photons/μs due to noise in the current measurement. Some of this work was published in abstract form (Neher et al., 1997).

2. Methods and results

2.1. The laser scan microscope: principle of scanner operation

The aim of our scanning scheme is to scan short (about one-tenth of the field of view) lines at high repetition rates, wasting little time for turnaround or flyback of the beam. A limitation in this respect is the inertia of the scanning system, which sets an upper limit to the angular acceleration of the mirrors. Another limitation is the bandwidth of the scanner controller, which together with the galvanometer and position sensor forms a feedback loop with a characteristic roll-off frequency. If the mirrors are operated close or slightly above the roll-off frequency, the response of the system is distorted and an appreciable fraction of time has to be spent to revert the scan direction. In such a situation, substantial time will be spent for flyback, if standard sawtooth (or ramp) waveforms are used for line deflection. Higher line frequencies will be obtained if waveforms close to triangular ones are employed, using both directions of mirror movement for data collection. In both cases the mirror movement will not follow exactly the command signal supplied to the scan controller input and it will display distortions and time lags if repetition rates are comparable to the roll-off frequency of the system. In the following we discuss a strategy for selecting optimum waveforms for the desired mirror movement in such a situation, and present a method to calculate frequency- and phase-corrected command signals, which, when applied to the input of the controller, will result in the targeted mirror movement.

2.2. Selecting optimum scan waveforms for the line deflection

The frequency response of scanning systems drops very rapidly above the roll-off frequency ($f_c$) such that basically no deflection (above noise) can be obtained beyond $5f_c$. The aim, therefore, must be to define a periodic waveform for the line deflection which does not require frequency components $> 5f_c$. For the following it will be assumed (for simplicity) that the fundamental frequency of the mirror movement is about equal to $f_c$, such that we are limited to five harmonics. As argued above, highest line frequencies can be obtained for triangular waveforms, using both forward and backward movements as lines. For such waveforms, which are inversion symmetric around zero crossings and maxima, the second and fourth harmonic is zero, such that we are left with the problem of selecting the best values for the relative amplitudes of the third and fifth harmonic. Phases for such waveforms are zero, if the zero crossing is chosen as the time
origin. An obvious choice would be to use the coefficients of a Fourier expansion of a triangular waveform as amplitudes. However, examination of such a wave (Fig. 1A) shows that it has substantial deviations from linearity over most of the range. We therefore used a least square fit routine (using IGOR-PRO, Wavemetrics, Lake Oswego, USA) to minimize the deviations from linearity, i.e. from \( y = x/(\pi/2) \) of the function:

\[
y = y_0 + \sum_{i=1}^{n} a_i \sin v \cdot x
\]

in the range \(-a < x < a\). For \( a = 0.78 \cdot \pi/2 \) (i.e. a 78% duty cycle) and \( n = 5 \), we obtain \( a_3/a_1 = -0.086 \) and \( a_2/a_1 = 0.017 \) (\( a_1 \) and \( a_4 = 0 \)). This curve is plotted in Fig. 1B, together with the deviation from \( y = x/(\pi/2) \), the latter at ten times expanded scale. It is seen that deviations from linearity within the specified range of \( x \)-values are very small (0.33% compared to 1.4% in Fig. 1A, when maximum deviation from linearity is related to the specified \( y \)-range, i.e. \( 4a/\pi \)). If the same calculation is done for \( a = 0.9 \cdot \pi/2 \), the usable scan range extends to about 90% of the cycle. However, the deviations within the scan range are again larger (\( \approx 0.66\% \)). Thus, depending on requirements, a compromise has to be found between width of the duty cycle and the deviation from linearity within it. For this it should also be considered that a lower value for the duty cycle requires less angular acceleration during turnaround. This has the advantage, in practice, that larger amplitudes are allowed.

For some of our fastest scans (\( f_0 > f_c \)) we had to restrict ourselves to the fundamental frequency and the third harmonic. In that case a duty cycle of 75% requires \( a_3/a_1 = -0.073 \), resulting in deviations from linearity of about 0.7%. For slower scans, more components can be used.

A similar approach can be used to generate ramp- or sawtooth-like waveforms. When the linear portion is centered around \( t = 0 \), all phase values are zero in such scans and amplitudes and deviations from linearity are obtained, as given in Table 1.

### 2.3. A waveform for fast line advance

Advancing lines can be accelerated by using a step function with an overshooting rising phase. This procedure is very similar to the ‘supercharging’ technique, employed by Armstrong and Chow (1987) for obtaining fast step deflections in voltage clamping. We obtained best results (by trial and error) when using a waveform for the command signal at the \( y \)-scanner input which actually employed both an overshoot and an immediately following undershoot before it settled to the new position. Amplitudes and durations of the over- and undershoot varied considerably between scanners. For a particular one, they were 100% and 44% with durations of 60 and 40 \( \mu s \), respectively. Given the delay in the electronics of the scanner controller (General Scanning 120 GTD), the overshoot could already be initiated about 60 \( \mu s \) before the end of the previous scan line. Settling times (10–90% of full deflections) of 100 \( \mu s \) could be reached.

### 2.4. Frequency and phase compensation of the line deflection

Eq. (1) together with the coefficients (Table 1) describe the target wave for the \( x \)-mirror movement (line deflection). In practice, a command signal was applied to the input of the scanner controller, which anticipates the frequency-dependent attenuation \( A_v(\omega) \) and \( \varphi_v(\omega) \) of the scan system, where \( \omega \) is the angular frequency of the periodic waveform:
Table 1
Fourier coefficients for exemplar waveforms

<table>
<thead>
<tr>
<th>Wave description</th>
<th>Range of linearity</th>
<th>$a_2/a_1$</th>
<th>$a_3/a_1$</th>
<th>$a_4/a_1$</th>
<th>$a_5/a_1$</th>
<th>$a_6/a_1$</th>
<th>$a_7/a_1$</th>
<th>Duty cycle$^a$ (%)</th>
<th>Linearity$^b$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two components, triangular</td>
<td>0</td>
<td>-0.073</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>75</td>
<td>0.7</td>
</tr>
<tr>
<td>Three components, ramp</td>
<td>-0.287</td>
<td>+0.062</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>58</td>
<td>0.4</td>
</tr>
<tr>
<td>Three components, triangular</td>
<td>Narrow</td>
<td>0</td>
<td>-0.086</td>
<td>0</td>
<td>0.017</td>
<td>0.0283</td>
<td></td>
<td>78</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Wide</td>
<td>0</td>
<td>-0.1</td>
<td>0</td>
<td>0.0283</td>
<td></td>
<td></td>
<td>90</td>
<td>0.66</td>
</tr>
<tr>
<td>Five components, ramp</td>
<td>Narrow</td>
<td>-0.355</td>
<td>0.129</td>
<td>-0.037</td>
<td>0.0064</td>
<td></td>
<td></td>
<td>59</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Wide</td>
<td>-0.394</td>
<td>0.172</td>
<td>-0.0658</td>
<td>0.0177</td>
<td></td>
<td></td>
<td>70</td>
<td>0.2</td>
</tr>
<tr>
<td>Four components, triangular</td>
<td>Narrow</td>
<td>0</td>
<td>-0.0923</td>
<td>0</td>
<td>0.0221</td>
<td>0</td>
<td>-0.0052</td>
<td>78</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Wide</td>
<td>0</td>
<td>-0.103</td>
<td>0</td>
<td>0.0313</td>
<td>0</td>
<td>-0.012</td>
<td>88</td>
<td>0.4</td>
</tr>
<tr>
<td>Seven components, ramp</td>
<td>Narrow</td>
<td>-0.3966</td>
<td>0.177</td>
<td>-0.0738</td>
<td>0.0259</td>
<td>-0.0068</td>
<td>0.001</td>
<td>62</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Wide</td>
<td>-0.428</td>
<td>0.218</td>
<td>-0.11</td>
<td>0.0504</td>
<td>-0.019</td>
<td>0.005</td>
<td>72</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Wide$^c$</td>
<td>-0.429</td>
<td>0.22</td>
<td>-0.112</td>
<td>0.0522</td>
<td>-0.02</td>
<td>0.006</td>
<td>74</td>
<td>0.06</td>
</tr>
</tbody>
</table>

$^a$ See text for explanation.
$^b$ See text for explanation.
$^c$ Coefficients were further optimized by introducing weights in the fitting routine, which emphasize points at the edge of the specified range.

$x(t) = Y_0 \sum_{r=1}^{n} \frac{a_r}{A_r} \sin(v \cdot \omega_0 t - \phi_r)$ (2)

We determined the coefficients $A_r$ and $\phi_r$ by an iterative procedure, in which we applied the desired number of cycles according to Eq. (2) to the scanner and sampled the resulting sensor signal. The latter was then Fourier analyzed, starting with the second or third cycle (to allow for settling of the movement) and the resulting Fourier coefficients $a_r$ were compared with the values $a_r$ as given in Table 1 (after proper scaling). Values for $A_r$ were then adjusted by linear scaling. Phases, obtained in the Fourier analysis, were used for an additive correction of $\phi_r$. Initial guesses for $A_r$ and $\phi_r$ were obtained by applying a small signal for which $a_r$ rose linearly with $r$ and analyzing the resulting sensor signal.

If the total amplitude of the scan signal was sufficiently small, the iterative improvement of the signal converged after three to five cycles. If too large deflections or too high scan frequencies were requested, non-linearities of the scanning system prevented convergence. The sensor signal for a triangular wave after convergence is displayed in Fig. 2 together with the desired waveform and the command signal, which was applied to the scan controller. It is seen that after an initial settling period the mirror movement follows closely the desired waveform, both with respect to amplitude and with respect to phase. The sensor signal, however, may have a small phase lag with respect to the actual mirror movement. Therefore, a software option was provided to introduce a compensating time shift in the command signal. In practice, this parameter was determined by scanning fluorescent beads with triangular waveforms and minimizing small shifts between alternating lines in the image.

2.5. Two-photon excitation path

Fig. 3 presents a schematic of the optical path. The excitation light for two photon imaging was provided by a Ti–sapphire laser (Tsunami Spectra Physics, Mountain View, CA, USA), which was pumped by a frequency doubled Nd:YVO$_4$ laser (Millennia, Tsunami Spectra Physics). This all solid-state configuration avoids the excessive amounts of cooling water requirements associated with argon lasers albeit of slightly higher powers. At a repetition rate of 82 MHz with pulses of 70 fs duration, the laser provides around 700 mW of average output power at 820 nm when pumped with a power of 5 W.

A small fraction of the beam was reflected from a microscope cover slip for diagnostic purposes as explained below. An electronically controlled shutter (Melles Griot) was placed on the laser path to block the beam when it was not required. Neutral density filters, up to 3.0 OD units (New Focus, Santa Clara, CA, USA) were used to reduce beam intensity in steps of 0.1 OD units. Working with cerebellar slices the beam intensity was typically reduced tenfold to yield $\sim 8–10$ mW of average power onto the specimen plane. In typical whole-cell recordings from neuronal cells in brain slices, we find that this intensity level allows up to 1000 scans without causing any detectable changes in the cell’s electrical properties or in the peak and time course of depolarization-induced intracellular Ca$^{2+}$ rises.
The beam expansion/compression unit, built using two 10 × objectives (Achromat, Zeiss, Goettingen, Germany), had a dual purpose. A 100-μm pinhole placed at the focal plane of the first objective served as a spatial filter to provide an optically cleaner beam. This pinhole removed the high frequency components, which might have been introduced by dust particles either in air or on optical surfaces. The second objective, which was mounted on a micrometric drive, allowed some adjustment of the beam shape in order to compensate for the chromatic aberrations when a low noise cooled CCD (Imago T.I.L.L. Photonics, Planegg, Germany) camera was used as the imaging device (see below). In that case differences in the focal width of the objective between excitation and detection wavelengths led to serious degradation of the image quality. Making the beam slightly convergent and underfilling the objective’s back pupil allowed the compensation of up to 4 μm of chromatic aberration in the Z-direction (Keller, 1995). This, however, resulted in changes in the focus spot size. The beam was then directed to the scan head of the microscope (Axioplan 2, Zeiss). The galvanometers (G120DT) and the Minisax drive electronics (General Scanning MA, USA) were mounted within the scanhead. A dichroic mirror (KP600, Zeiss) was used to reflect the beam down through the microscope optics. In most of the experiments, a Zeiss achroplan 63 × , 0.9 NA water immersion objective was used.

2.6. Fluorescence emission path

Care was taken so that fluorescence emission followed the simplest possible optical path, with the minimal amount of optical components between the objective and the detector. The fluorescence emission was directed to one of the three detectors by placing a mirror into the emission path. The optical path to the avalanche photodiode (APD; type SPCM-AQ-231 photon counting module; EG&G, USA) and to the prismatically enhanced photomultiplier (PEPMT; Thorn EMI Model 9658A, UK) contained 1-mm thick BG39 (Schott) filters to block any laser reflections from the glass surfaces. The PEPMT and the APD were mounted on the epi-ports of the microscope. The PEPMT was mounted with a spacing of 30 cm from the intermediary image plane, in order to blur the image on the photocathode, and thus reduce the effect of photocathode irregularities. The 40 × long working distance objective (LD Achromat NA 0.6, Zeiss) and the APD (with 500-μm diameter sensitive area) were prepositioned with respect to each other by the use of three-dimensional manipulators such that the photodiode’s active area was centered in the focus of the objective. The objective was then cemented to the APD mount and the back pupil of the objective was placed in the intermediary image plane on the epi-luminescence port of the microscope. This configuration allowed the observation of an area corresponding to a diameter of 55 μm in the specimen plane.

For detection with a low noise cooled CCD camera, the standard fluorescence excitation port of the microscope was used. The relay optics and an inner dichroic mirror turret for selection of the appropriate excitation/roll-off wavelengths were manufactured locally and replaced the optics provided by the manufacturer.

Fig. 2. Signals for a fast scan. (A) An approximation to a triangular waveform with just two Fourier components \(a_3,a_0 = -0.073\) which is the waveform designated as ‘two components, triangular’ in Table 1. The fundamental frequency of the waveform is 2 kHz, resulting in four scan lines per millisecond. (B) The command signal, calculated according to Eq. (2), as applied to the input of the scan controller. (C) The sensor signal, as measured at the scan controller output.
Fluorescence emission emanating either from the excitation by the Ti–sapphire laser or by the monochromatic light beam (polychrome-II, T.I.L.L. Photonics) coupled through a locally added excitation port, could be diverted to the camera by the use of the appropriate dichroic mirror selected from the front turret.

2.7. Beam diagnostics

The two-photon advantage factor, $\zeta$, is found to depend on $\tau_{p}^{-1}$, the width of the pulse at half maximal intensity (reviewed in Denk et al., 1995b). It is therefore crucial to measure and monitor the pulse width of the laser beam. The use of a commercial autocorrelator is cumbersome and is not well suited to monitor pulse width throughout a long lasting experiment. Therefore, a more flexible and cheaper method was implemented. Approximately 4% of the laser beam was reflected from a coverslip into the entrance slit of a monochromator (Model 7748 UV, Jobin-Yvon, France), whose exit slit was replaced by a cheap CCD camera (TV-CCD200 Monacor, Bremen, Germany). The 70-fs pulses typically generated by our system are spread over 13 nm (see Denk et al., 1995b) and the camera is placed such that the dispersion from one end of the CCD chip to the other end corresponded approximately to twice this value, i.e. 26 nm. This set-up allowed us to measure the center wavelength (cw) and to monitor the bandwidth of the pulse during an experiment (Fig. 4A1). The image of the pulse gave a rough estimate of its bandwidth without having to measure it by the time-consuming autocorrelation technique. Furthermore a simple glimpse to the monitor screen allowed detection of any tendency of the laser to go out of mode-locking, which appeared initially as the shift of the center frequency to shorter wavelengths, the appearance of a cw component (Fig. 4A2) followed by complete loss of pulsing (Fig. 4A3). It is worth noting here that the cw component does have a bandwidth of approximately 0.1 nm (Fig. 4B3). Therefore, it was possible to detect a cw component smaller than 1%, which might break in due to excessive pump power or incorrect setting of the intracavity dispersion adjustment prisms.

Although the Ti–sapphire laser is quite stable and will remain mode-locked for a long time, air drafts or changes in the room temperature were found to have a profound effect on the laser’s stability. It was found out that the aforementioned way of beam diagnosis was more useful than many commercially available tools.

The average intensity of the laser beam was constantly monitored with a slow, calibrated photodiode using a small fraction of the beam split with another coverslip. With these two provisions it was possible to run the experiment for up to 6 h, mostly limited by the health of the biological sample.

To measure the group velocity dispersion (GVD) introduced by the glass of the microscope optics, a Michelson-type interferometer as depicted in Fig. 5A was constructed. The interferometer could be inserted into or taken out of the optical path by placing mirrors mounted on precision flipping kinematic mounts (Flipper, New Focus, Santa Clara, CA, USA). When the interferometer was included in the optical path, the beam was split into two by the use of a beamsplitter. The relative time delay in one of the arms was varied by...
changing the position of the mirror glued onto a PC speaker coil and its periodic motion was controlled by a function generator. For coarse distance adjustment one of the mirrors was mounted on a micrometer stage. The two beams were initially combined at the back pupil of the objective, and the position of the movable mirror was adjusted until the autocorrelation was observed. The second order autocorrelation function was observed as the variation of two photon fluorescence intensity as a function of position of the mirror. Fluoresbrite beads (Polysciences Warrington, PA, USA) were used at the specimen plane as the source of the second order fluorescence emission. As depicted in Fig. 5B, the pulse broadening introduced by the microscope optics resulted in a pulsewidth of 150 fs at the specimen plane. We considered it was unnecessary to implement the negative GVD compensation techniques in our optical path (Fork et al., 1987). The second order autocorrelation function from these measurements deviated from the expected 8:1 ratio (Diels et al., 1985). This was due to the non-equal distribution of the light intensities in the two arms of the interferometer. One might argue that the glass contents of the two optical paths are not equal and, therefore, call for the four-mirror, two-beam splitter version of an interferometer (see Diels et al., 1985; Brakenhoff et al., 1995). We believe that this is an unnecessary complication and the simplest form of a Michelson interferometer is quite satisfactory for measuring pulse width at the specimen plane.

At various stages of our experiments, it was necessary to observe the shape or the position of the laser beam at the back pupil of the objective. For this purpose one of the faces of an isosceles right angle prism was frosted by rubbing it with coarse grit carborundum. The prism was mounted on the microscope’s revolver with the frosted face placed at a position equivalent to that of the objective’s back-pupil. The total internal reflection from the hypotenuse face rotated the beam by 90°, providing a convenient way of observing the back-pupil through the non frosted face. The frosted surface being a diffuser, the shape of the laser beam could be safely observed even at full power by this simple technique.

2.8. Mechanical stage

In order to optimize the stability and repeatability of the scans, care was taken to choose non-moving optical parts throughout. As the system was designed with the possibility of double patch clamping which demands high levels of mechanical stability, a computer-controlled mechanical stage was constructed. The motion on the three axes was commanded with a C-842 four-axis motor controller module (Physik Instrumente, Waldbronn, Germany). This card implemented the PID (proportional integral derivative) control algorithms in hardware, providing smooth acceleration and deceleration, essential when moving the stage with a cell already in patch-clamp configuration. Once the optimal PID

Fig. 4. Monitoring mode locking. Different modes of operation of the laser. (A1) The mode-locked configuration. The interference fringes are the result of spatial sampling by the CCD of the beam dispersed by a grating. (A2) The appearance of a cw component on a laser with a tendency to get out of mode-locking. (A3) Pure cw mode of operation. (B1, B2, B3) The intensity profiles across a line drawn through the center of the images.
Fig. 5. Interferometric pulse width measurement. (A) A relatively simple Michelson interferometer could be integrated into the system by moving two flipping mirrors (FLM) out of the optical path. Mirror MM, which was glued on a speaker coil, could be moved back and forth \(\pm 400\) \(\mu\)m by applying a sinusoidal wave from a frequency generator. KP600, dichroic mirror; BS, beam splitter. (B) The second order autocorrelation function, measured as the fluorescence of the fluoresbrite bead by the PMT with a Gaussian fitted to the envelope of the autocorrelation trace. See text for details.

Fig. 7. (A) Plot of the apparent quantum efficiency (AQE) of three photodetectors as a function of photon flux. A 10 \(\mu\)M fluorescein solution was excited at different powers (\(\lambda = 840\) nm) and the emitted light was collected at 10-\(\mu\)s sample interval by one of the three detectors. APD, avalanche photodiode; PMT, prismatically enhanced photomultiplier; CCD, IMAGO cooled charge-coupled device camera. The photon flux for a given sample and excitation power was determined as described in the text. AQE is plotted against the number of photons/\(\mu\)s impinging on the detector (one-tenth of the number given by Eq. (8)). The lines on the right indicate the QE for the PMT and the CCD at maximum photon flux. (B) Comparison of the signals obtained with the APD (left image) and the PMT (right image) from the axon of a cerebellar basket cell loaded via a patch pipette with the Ca\(^{2+}\) sensitive indicator Oregon Green BAPTA-1 (OG1; 200 \(\mu\)M). The excitation power and scan parameters were the same for both images. The pseudo-color scale corresponds to 0–2.3 MHz for the APD image; 0–400 mV for the PMT image.

parameters were found, movement of the stage was never the cause of the loss of a cell. A M525.22 (PI) linear positioning stage was used for the Z movement; this stage carried an \(XY\) cross table. For the \(XY\) movement, linear positioning stages from Micos (Umkirch, Germany) were chosen for their double, crossed, cylindrical bearings, and these proved to be very reliable on the long run. The controller card was driven by a joystick interfaced by routines written in LabView G language. This allowed us to move the specimen smoothly and to display the coordinates of the cells in real time, a very useful feature especially for returning to a specific cell or a specific position. The repeatability of the system was estimated to be better than 1 \(\mu\)m.
2.9. Scan control electronics, data acquisition and analysis

Waveforms as described in the section above were calculated in IGOR-Pro and connected to the X- and Y-inputs of the scanner control circuit, after DA conversion on two channels of an ITC-16 laboratory interface (Instrutech Corp., Port Washington, NY, USA), using ‘Pulse Control’ software modules, provided by Dr. R. Bookman, University of Miami.

During the course of this work we used two different types of scan controllers, both driving general scanning, G120 DT galvanometric scanners: An AMP2 (Lobo Electronic, Aalen, Germany) and a Minisax (General Scanning Inc., Watertown, MA, USA) unit. The two types of controllers gave comparable results, with the exception that the Minisax drivers, at a given frequency, allowed larger scan amplitudes before signs of amplifier saturation (lack of convergence; distortions in the recorded waveforms) became apparent. It was possible to drive galvanometers up to fundamental frequencies of 2 kHz. The line deflection signal was either triangular, where both directions of the scan were used for data acquisition (up to 4 lines/ms) or had a sawtooth shape where only one direction was used for data sampling. Either of the signals was expressed as a Fourier series including components up to the fifth harmonic (see above). Once the calibration and compensation parameters had been determined for a given scan pattern according to the procedure described above, the waveforms for driving the scanners were stored on disc and used for periods of weeks. They were updated after all major changes in the optical path or the mechanical set-up of the microscope. Provisions were made to rotate the scans to an arbitrary degree, to generate line scans, or to compress images (zooming) in order to increase the effective pixel dwell time.

The sensor signals provided by the scan controller (or else fluorescence signals) were preamplified, filtered and digitized on the ITC-16 interface at 10-μs sample intervals, using Igor Pro and Pulse Control. Signals from the APD (see below) were linearized, as suggested by the manufacturer, to correct for the dead time of the device. A minimal set of analysis routines was implemented to display the images on line. To generate images, the parts outside the linear scan range, including the data corresponding to turnaround points, were stripped from the continuously acquired data. Fig. 6 illustrates the images constructed from two types of scans of a cerebellar basket cell axon loaded with the Ca²⁺ sensitive indicator Oregon Green BAPTA-1 (OG1). In one of them an area of 8.25 × 4.75 μm² (19 lines) was scanned in less than 10 ms.

Most of the analysis was performed off-line. Typical analysis routines consisted of the measurement of intensities of regions of interest (ROIs), and the calculation of ΔF/F₀ ratios with provision of subtracting background intensities from the selected areas of the image (example in Fig. 6C). In the cases where ΔF/F₀ images were generated (ΔF is the difference between the fluorescence intensity at any given time and the prestimulus intensity F₀; see Fig. 6B3), the images were median-filtered to smooth out the noise inherent to divisions with integers close to zero (Llano et al., 1997).

2.10. Analysis of photodetector performance

We compared the performance of several types of photodetectors, including a type SPCM-AQ-231 (EG&G Optoelectronics, Vandreuil, Canada) photon-counting APD, a Thorn Emi, type 9658A photomultiplier tube (PMT), a cooled CCD camera (Imago CCD, TILL photonics) and several types of photodiodes. In order to handle all these signals (except for the camera signals) with one type of data acquisition system we had to reconvert the digital counting pulses provided by the APD to analog signals, such that they could be sampled, as described above. This was done simply by low pass filtering of the pulse stream. Since the noise of the recording, under all conditions, was dominated by photon shot noise this did not lead to deterioration of the signal to noise ratio. In order for the noise, after such conversion, to be identical to that of a counter set to a counting window of length T (equivalent to a sample interval of T) the analog signal had to be filtered with a −3 db frequency of (Hopt, 1998):

\[ f_c = \frac{1}{T} \frac{\ln 2}{\pi} \]  

(3)

This equation holds for a Gaussian and also for a higher order Bessel filter. The criterion constitutes a small amount of oversampling with respect to the Nyquist criterion.

For a quantitative measurement of fluorescence, we first calibrated our APD in terms of its quantum efficiency. For these measurements, we counted photon pulses, using a PM 6671 counter (Phillips, Eindhoven, The Netherlands). Light from a green light emitting diode was filtered with a 540/30 (center wavelength/ halfwidth) bandpass and the light intensity P was measured at a distance of 4.3 cm with a type 13PDH001 (Melles Griot) power meter. The photon flux was calculated according to ϕ = P·λ/(hc) where λ is the wavelength of 547 nm, h is Planck’s constant and c is the speed of light. Subsequently the power meter was replaced by the APD, and the measured counting rate compared to the expected rate, based on ϕ and the light sensitive area of the APD. This resulted in a value η of the quantum efficiency of 64%, which was later used during measurements on the microscope for converting count rates into photon fluxes. When required (for counting rates above 1 MHz) a dead time correction
Fig. 6.

A. "Large field scan"
26 by 31 µm in 195 ms [pixel size: 250 nm]

B1. basal image

B2. peak image

B3. ΔF/Fo image at peak

C. ΔF/Fo (%) vs. time (ms)

"Fine patch scan"
8.25 by 4.75 µm in 9.9 ms [pixel size: 250 nm]
was applied to the count rate as specified by the manufacturer. The validity of this correction was verified by measuring light fluxes with a series of calibrated neutral density filters. This showed that the corrected count rate was linear for light intensities between 10 kHz and 10 MHz (or 0.006–5.7 pW), apart from a constant dark count of 1.5 kHz.

2.11. The apparent quantum efficiency

For comparing the performance of different photodetectors under a variety of experimental conditions we measured the signal to noise ratio while imaging uniform fluorescence emanating from a fluorescein solution (10 μM). We derived a figure of merit of a given detector, which we call the ‘apparent quantum efficiency’ (AQE) according to the following rationale. For a photon counting detector with quantum efficiency η, the mean number of counts \( \bar{n} \) during a time interval \( T \) will be given by:

\[
\bar{n} = \eta N + N_D
\]

where \( N \) is the number of photons hitting the detector and \( N_D \) is the number of dark counts. The variance \( \sigma_n^2 \) in the number of counts will also be \( \eta N + N_D \). Therefore, the following identity holds:

\[
\frac{(\bar{n} - N_D)^2}{\sigma_n^2} = \frac{\eta}{1 + (N_D/\eta N)}
\]

(4)

The left side of this equation involves ratios of measurable quantities (background corrected mean, variance and photon flux) and the expression on the right will be equal to \( \eta \) for \( N_D = 0 \). In the presence of dark counts (extra noise) it will be smaller than \( \eta \). We call this quantity the apparent quantum efficiency \( \hat{\eta} \) (with respect to noise performance), because a noiseless photon counter with \( \eta = \hat{\eta} \) would provide the same signal to noise ratio.

For an analog detector, such as a photomultiplier, an equivalent equation can be readily derived:

\[
\hat{n} = \eta \bar{N} + N_D
\]

where \( \bar{N} \) is the digitized, background corrected analog signal, \( q \) is the mean contribution of a single detected photon to it, and \( Z \) is an extra noise factor (Hopt, 1998). This factor reflects the amplitude distribution of the quantal events. It is 1 for the photon counting APD and the CCD and approximately 1.4 for a PMT (Jones, 1971). The equals sign holds when the read-out noise is negligible. When the photocurrent or analog PMT signal is digitized with a standard AD converter, a filter criterion as specified above (Eq. (3)) has to be used for a correct evaluation of \( \hat{n} \). All noise sources in excess of shot noise, such as read-out noise or some dispersion in the amplitude distribution of photon generated pulses (\( Z > 1 \)), tend to decrease \( \hat{n} \).

For the photodetectors under consideration we evaluated variance and mean value of the fluorescence signal which was sampled by the ITC-16 (see above) while scanning homogeneous regions of interest at various illumination intensities and plotted \( \sigma^2 \) versus mean. It is expected that the slope of such a plot is equal to \( qZ \). For this analysis it is important to use the filter criterion, given above, which, for 10-μs sample interval, requires a filter setting (−3 dB point) of 47 kHz. Also, in the case of the APD signal, the amplitude had to be restricted to the linear range of the device. From the slopes of such plots we obtained \( q \)-values for the various photodetectors, using \( Z \)-values as specified above. The number of detected photoelectrons was obtained by dividing the mean dark count corrected signal by the quantity \( q \), derived from the slope value. The photon count, measured this way, agrees within 10% with the value obtained by directly counting the output of the APD. Given the quantum efficiency of the APD (64%) we converted the count rates to photon fluxes. Using 10 μM fluorescein and an excitation wavelength of 840 nm, we obtained a flux of \( 1.13 \times 10^6 \) photons/s for 1.16 mW of excitation power, as measured in the focal plane. By variation of the excitation power, we confirmed the square law of two-photon fluorescence excitation. Thus, for a given measurement, we calculated \( \bar{N} \), the mean number of photons reaching the detector in a 10-μs measuring window as:

\[
\bar{N} = 11.3 \left( \frac{p}{1.16 \times 10^{-3}} \right)^2
\]

(8)

Fig. 6. Illustration of single scan images obtained with two different scan protocols from the axon of a cerebellar basket cell loaded via a patch pipette with the Ca2+ sensitive indicator OGI (200 μM). The scan in A covers 26 × 31 μm² in 195 ms, with an effective pixel size of 250 nm. This type of scan was used to select a region of the axon suitable for the study of Ca2+-dependent signals with fast scanning. The two upper panels in B show pseudocolor images of the fluorescence measured in a small subfield of this axon with scans of 8.25 × 4.75 μm² (9.9 ms scan time; effective pixel size of 250 nm) at rest (B1) and at the peak of the response to a 50-ms depolarizing pulse to 0 mV (B2). The scan was rotated 135° with respect to the large field scan shown in A. B3 shows the ΔF/ΔF₀ image at the peak of the Ca²⁺ rise. (C) Plot of the temporal evolution of the relative changes in fluorescence for a region in the branching point, whose position is given by the square drawn in B3. The arrow marks the onset of the depolarizing pulse.
where $P$ is the power in watts measured in the focal plane after replacing the fluorescein solution by the power meter. Performing measurements with different photodetectors under a variety of illumination conditions, we calculated $\hat{\eta}$ from Eqs. (5) and (6) and plotted it versus $\bar{N}$ (see Fig. 7A).

It is seen that the APD reaches an apparent quantum efficiency of about 50% for photon fluxes between $10^5$ and $10^6$ s$^{-1}$. This is close to the directly measured quantum efficiency of 64%. $\hat{\eta}$ drops both for smaller photon fluxes (due to the extra noise of dark counts) and for larger fluxes (due to saturation of the APD). It should be noted that dead-time corrected readings were used for calculating $\sigma_2$ and $\bar{y}$ in the case of the APD.

The PMT reaches $\hat{\eta}$-values close to its specified quantum efficiency (29%) for fluxes larger than $3 \times 10^6$ s$^{-1}$. At lower fluxes its performance is degraded due to its relatively high dark count ($0.3 \times 10^6$ s$^{-1}$) under the conditions of operation in our microscope. The performance of the CCD is severely compromised at the low photon fluxes used here by its readout noise corresponding to approximately 16 photoelectrons (rms-value). It does, however, reach $\hat{\eta}$-values close to 20% for fluxes of $10^8$ s$^{-1}$ and higher.

The analysis shows that the choice of the best detector, in terms of the achievable signal to noise ratio, very much depends on the photon flux under study. For fluxes lower than $5 \times 10^6$ photons/s, which is obtained for a 10 µM fluorescein solution at about 2.5 mW of excitation power in the focal plane, as measured using a 63 × 0.9 NA water immersion objective, the APD is the photodetector of choice. For larger fluxes, photomultipliers are superior. It should be noted that the numbers given refer to a measurement interval (or pixel dwell time) of 10 µs. Effects of read-out noise, which are very limiting for the CCD, will be less severe for longer pixel dwell times.

As mentioned above, our experience using this system for the study of intracellular Ca$^{2+}$ dynamics in neurons of brain slices, indicates that maintaining the excitation power at the specimen plane below 10 mW (with $\lambda = 840$ nm) significantly improves the survival of the preparation. For this excitation power, the average photon fluxes obtained from the center of cerebellar Purkinje cell somas loaded via patch pipettes with 10 µM OG1, at resting Ca$^{2+}$, levels, ranged from 2.4 to 7 MHz (mean ± S.E.M.: $4.1 \pm 0.53$ MHz (10 cells)). Similar photon fluxes are reached in the soma rim of Cs-loaded cells, following a 50-ms depolarization to 0 mV. In axons of cerebellar interneurons, after 20–30 min of whole-cell recording with pipettes containing 200 µM OG1, basal photon fluxes averaged $1.3 \pm 0.13$ MHz (20 cells; see example in Fig. 6). As shown in Fig. 7B, the low signal levels in the axonal structures require the highest AQE provided by the APD.

An analysis of apparent quantum efficiency, as presented here, is quite labor intensive, because it requires absolute calibration of a photodetector, and measurement of the slopes in a $\sigma^2$ versus mean plot (for calculating $\bar{N}$). It should be noted, however, that two detectors can be readily compared in this approach by measuring the signal to noise ratios (SNR) with each of the detectors on a region of a uniform fluorescence with identical illumination conditions. If SNR$_2$ and SNR$_1$ are the SNRs of detector 2 and 1, respectively, SNR$_2$/SNR$_1$ will be equal to $\hat{\eta}_2/\hat{\eta}_1$, and will be a good relative measure of performance under the given conditions.

3. Discussion

We have described the construction and operational performance of a two-photon fluorescence scanning microscope built from commercially available components. As the main interest was the measurement of Ca$^{2+}$ dynamics in small patches, we have developed algorithms that allowed us to scan at the maximum permissible rate. The 250-nm effective pixel size used in
such scans, allowed us to visualize small structures like spines on the CA3 pyramidal cells (Fig. 8). The effective pixel size could be reduced by an arbitrary amount to have an electronic zoom effect as exemplified in Fig. 8B,C.

Most of the two-photon scanning systems are based on coupling a pulsed laser into a standard confocal microscope. This approach has the advantage of requiring a minimum of installation effort and allows the use of commercially developed, user-friendly software right away. The main disadvantages are the non-accessibility of the software, and the necessity to use a very complicated optical path for the excitation and the descanning of the fluorescence through the same optics. In most of the commercial systems, line-scan mode is used as the solution to the study of fast processes, but it is mostly restricted to either the X- or the Y-axis.

The strength of the system described here lies in the fact that it was designed with two-photon fluorescence in mind: The excitation and emission paths are optically very simple, the system employs a standard laboratory interface for beam deflection and data acquisition, and the scanning software is fully accessible to the experimenter. Very small areas can be scanned at about 100 Hz, and scan patterns can be oriented rather than orienting the biological sample. Imaging at those high rates, however, is severely limited by the relatively low quantal content of two-photon excitation at biologically permissive excitation levels. Therefore, an APD with its 50% apparent quantum efficiency was implemented as the detector of choice.

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References


