A modified confocal laser scanning microscope allows fast ultraviolet ratio imaging of intracellular Ca$^{2+}$ activity using Fura-2

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Abstract A confocal, ultraviolet laser scanning microscope (LSM) for reliable ratio measurements of localized intracellular Ca$^{2+}$ gradients using the Ca$^{2+}$-sensitive dye Fura-2 was developed. In a commercial LSM, the filter wheels for the excitation band-pass filters and the grey filters were replaced by acousto-optic tunable filters (AOTF) for rapid switching (<1.5 μs) of the ultraviolet (351 and 364 nm) and the visible (457, 476, 488, 514 nm) excitation light. This enabled dual wavelength excitation of Fura-2, or 2′,7′-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF) for pH measurements. Changing to a transmitted-light detector of high sensitivity allowed for simultaneous recording of differential interference contrast images of the preparation with the excitation light. The AOTF fine control of the intensity of the excitation light and improvements in the emission detector sensitivity enabled the acquisition of up to 120 ratio pairs of high-quality images from a single cell. The optical capabilities and limitations of the instrument were evaluated with fluorescent beads and dye-loaded cultured cells. Agonist-induced intracellular Ca$^{2+}$ transients in HT29 cells were recorded to test for the instrument's ability to measure changes in [Ca$^{2+}$]$_i$. Ratio z-sections from Fura-2-loaded cells showed an inhomogeneity of the Fura-2 loading with an accumulation of the dye mostly in the mitochondria. We show, as an example of the microscope's achievable resolution, the spatial and temporal heterogeneity of [Ca$^{2+}$]$_i$ signals in mitochondria and the cytosol in response to agonist-evoked stimulation of HT29 cells. In addition, we show that the lipophilic, membrane-bound Fura-2 derivative Fura-C18, for measurements of near-membrane Ca$^{2+}$ changes, can be used with this confocal microscope. This new LSM is expected to deepen our understanding of localized [Ca$^{2+}$]$_i$ signals; for example, the nuclear Ca$^{2+}$ signalling or the [Ca$^{2+}$]$_i$ changes that occur during stimulation of ion secretion in polarized epithelial cells.

Key words Confocal microscopy • Acousto-optic tunable filter • Fura-2 • Ratio imaging • HT29 cells

Introduction
Confocal microscopy is now established as a technique that is used by a great number of laboratories. The number of groups using these techniques for intracellular Ca$^{2+}$ imaging in different biological preparations is rapidly increasing. However, due to technical and optical limitations in the use of UV excitation in commercially available confocal microscopes and also due to the lack of ratioable Ca$^{2+}$-sensitive dyes in the visible range, so far only pseudo-confocal [36] and confocal [11] UV excitation ratioing using the dual-excitation dye Fura-2 have been attempted. From these two publications, it is clear that the use of UV excitation in confocal microscopy is a technical challenge. The latter publication [11] also raised some doubts about the usefulness of the available laser wavelengths for Fura-2 excitation, as no inverse fluorescence emission behaviour of Fura-2 was observed.

As an alternative to Fura-2, the dual-emission Ca$^{2+}$-sensitive dye Indo-1 has been used in confocal microscopy [5, 12, 13, 18, 22, 23]. However, the physicochemical properties of Indo-1 seem to change, once it is inside living cells [16, 20, 30]. Optically, Indo-1 is even more difficult to handle than Fura-2 in a confocal microscope [1, 35]. As one of the emission maxima is in the UV range, focal differences between the emitted light can occur and, in addition, a low overall transmission of UV light along the confocal emission path can be a problem.

In the last few years a number of groups have used a mixture of Fluo-3 and Fura-red for confocal dual-emission Ca$^{2+}$ ratio imaging [3, 8, 14, 19, 31, 33]. This has...
been reviewed recently in great detail [32]. The advantage of this approach is that only one excitation wavelength is needed, which should avoid focal differences between the two emission images, as long as the two emission wavelengths are no more than 150 nm apart. However, there are also several disadvantages of this method: (1) both dyes bleach relatively quickly with possibly distinct kinetics; (2) the quantitative distribution of the two dyes inside the cell might be different; (3) the K_D values for Ca^{2+} of the dyes are not the same; (4) the fluorescence emission of Fura-red is inversely related to [Ca^{2+}]. Taken together this might result in falsely reported [Ca^{2+}] changes.

We have previously used an acousto-optic tunable filter (AOTF) for fast switching of the wavelengths of visible light in a confocal microscope. This permitted ratio imaging of 2'7'-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF), as described recently [26]. We have now developed a microscope, that enables the use of Fura-2 and other related dyes for confocal dual-wavelength ratio imaging of Ca^{2+}. This was achieved by the use of a UV-AOTF for rapid switching of the excitation wavelength, a UV-optimized confocal optical path, and the development of a new UV-optimized water-immersion objective.

Materials and methods

Cell culture

HT-29 cells were grown in Dulbecco's modified Eagle's medium as described previously [17, 28]. The cultures were kept at 37°C in an atmosphere of 5% CO_2 and 95% air. For fluorescence measurements cells were seeded on glass cover slips (diameter 30 mm) and used after 3–7 days in a subconfluent state.

Measurements of [Ca^{2+}]_i and pH_i

Fura-2 was used to measure [Ca^{2+}] as described previously [2]. In brief, cells were loaded for 40–60 min at room temperature with Fura-2/AM (5–10 μmol/l). Pluronic F 127 (2 · 10^-6 mol/l) was added to improve the loading, and the dye mixture was sonicated for 5 min before the incubation. Incubation was stopped by superfusing the cells with control solution at 37°C for 10 min. The experiments were performed at a bath exchange rate of 0.5–1 Hz and at 37°C.

Fura-2 was excited alternately at 351 and 364 nm using a confocal microscope (LSM 410 invert, Zeiss, Jena, Germany), modified as described in detail in the Results. The fluorescence images were usually collected with the scan rate set to 1.08 s for a 512x512 pixel full-frame image. For Fura-2 measurements a dichroic mirror, BSP 380, together with a long pass filter LP 397, was used. The filters were especially designed from Delta Light and Optics (Lynby, Denmark). The emission ratio of the 351/364 nm excitation was used as a measure of [Ca^{2+}]. Autofluorescence of the cells was around 1–4% at the photomultiplier (PMT) gain normally used for the measurements. A time series of images at alternating 351 nm and 364 nm excitation could be recorded in fast mode directly into the RAM of a 486/66 MHz EISA computer (ALR, Bad Homburg, Germany). The 80-Mb RAM allowed acquisition of around 280 full-frame images, which covered an experimental time of at least 300 s at the highest possible image acquisition rate. Series of images were later transferred to hard disk and were analysed using Metamorph/Metafluor 2.0 (Universal Imaging, West Chester, Pa., USA). A special software add-on for confocal images from the Zeiss LSM 4 was used to read the information of the TIFF file image header.

Solutions and chemicals

The standard solution contained (in mmol/l): 145 NaCl, 1.6 K_2HPO_4, 0.4 KH_2PO_4, 1.3 Ca-gluconate, 1 MgCl_2, 5 d-glucose, and had a pH of 7.4. The Ca^{2+}-free solutions contained no Ca-gluconate and 5 mmol/l EGTA [ethylene-glycol-bis(beta-aminoethyl)-N,N,N',N'-tetraacetic acid). The osmolality of the solutions was measured with an osmometer (Knauer, Berlin, Germany) and was between 290 and 300 mosmol. The following compounds were used: fluorescent latex beads, iodonium, thapsigargin, pluronic F127, Fura-2/AM and BCECF/AM (Molecular Probes, Eugene, Ore., USA). All other chemicals were of the highest grade of purity available and were obtained from Sigma and Aldrich (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

Results

Modifications of the confocal microscope

Fast switching of excitation wavelength and infinitely variable light intensity control

An inverted, confocal laser scanning microscope (Zeiss LSM 410 invert) was modified to allow for fast UV and visible excitation ratio imaging. A schematic drawing of the LSM before and after the modifications is shown in Fig. 1. As the UV light source, a high-power argon ion laser with a closed loop cooling device was used (Enterprise 653, Coherent, Dieburg, Germany). This laser has two main UV wavelengths at 351.1 and 364.4 nm and no significant visible light emission. The visible laser light source was a multiline argon laser (Omnicrome, Chino, Calif., USA) with special laser mirrors to optimize blue emission [26], also giving some weaker lines, mainly four visible wavelengths at 457, 476, 488 and 514 nm. The maximal light output from both lasers was computer controlled via tube current control from within the LSM software through an RS232 interface box for the UV laser (Coherent) and through a built-in remote interface for the visible laser source. These controls were used to set a defined maximal usable light intensity range for the UV and the visible light excitation. Usually the controls were set to 12% of the maximal UV laser power and 20% for the visible laser power. This always gave more light intensity than needed and assured a reasonably low laser beam noise. Furthermore, the tube currents at these settings were still relatively low, which extended the mean life time of the laser tubes.

The usual filter wheels for wavelength switching with band-pass filters and intensity control with neutral density filters were replaced by two AOTFs, one for the UV light (AA, Opto-Electronique, St-Remy-Les-Chevreuse, France) and one for the visible light from 450 nm to 700 nm (Crystal Technology, Palo Alto, USA). Both AOTFs allowed, if needed, the simultaneous use of all wavelengths. As well as wavelength control, the use of the AOTFs allowed independent, variable light intensity control (12-bit resolution) for each single wavelength.
The measured transmission efficiency of the AOTF was 95% for the visual AOTF (VIS-AOTF) and 75% for the UV-AOTF, due to the use of TeO₂ as the crystal material. The diffraction efficiency of the UV-AOTF was 94%, which resulted in a net UV efficiency of 71%. The extinction of the redundant laser line was 0.23% for the 351 nm line and 0.15% for the 364 nm line. The on and off rates for the laser light switched by the UV-AOTF (rise time) were 0.86 µs and 0.74 µs, respectively, for the VIS-AOTF. This time was measured as the time between 10% and 90% of the maximal signal.

Improved sensitivity in emission and the transmitted light path

The original PMT detectors in the emission light path were replaced in one channel with a detector of higher quantum efficiency but slightly higher noise (R 3896, Hamamatsu, Herrsching, Germany), and in the other channel with a PMT (R4632, Hamamatsu) of comparable quantum efficiency but a 10-fold to 20-fold lower dark current. The photodiode detector in the transmitted light path was replaced [26] by a PMT of high sensitivity (R928, or R3896 Hamamatsu), allowing for simultaneous, differential-interference contrast (DIC) imaging even at the low light levels used for fluorescence ratio measurements with ion-sensitive dyes. Figure 2 shows typical fluorescence images at 351 and 364 nm excitation and a DIC-image taken simultaneously with the 364 nm fluorescence image. At 351 nm excitation a reasonable DIC-image could not be obtained due to the low signal-to-noise ratio.

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**Fig. 1** Schematic drawings of the optical path of the LSM 410 invert before (A) and after (B) the modification for Fura-2 ratio imaging. Two acousto-optic tunable filters (AOTFs) were implemented in the UV and the visible light paths instead of the filter wheels with interference filters for wavelength and grey filters for light intensity change. A motorized lens was implemented in the UV light path for compensation of the UV-VIS beam shift due to chromatic aberration of the objectives. The transmitted-light detector was replaced by a photomultiplier (PMT) and the emission detectors were replaced by PMT of higher sensitivity.

**Fig. 2** Images of a Fura-2-loaded HT³⁵ cells. Excitation at 351 nm (left), 364 nm (middle) and fluorescence emission >380 nm, and 364 nm DIC image (right). Both fluorescence images show remarkable details of the subcellular distribution These images were taken with a Plan-Neofluar 100x/1.3 oil-immersion objective (objective B). The difference in the focal plane between the left and middle images can be clearly seen when comparing the different shapes of the cellular structures with high Fura-2 concentrations. These structures are mostly mitochondria (unpublished results Ricken et al.). Such focal differences were not detectable with objectives A and C.
overall transmission of this wavelength in the transmission light path. The sharp and crisp fluorescence emission images show a clear inhomogeneity of the Fura-2 loading, which has been described previously [21, 34]. We have characterized most of the bright structures surrounding the nucleus by co-staining with specific markers for intracellular organelles, as mitochondria (Ricken S., Greger R., Nitschke R., manuscript in preparation).

Figure 3 shows the Fura-2 fluorescence bleaching as a function of acquired images using different excitation intensities chosen with the AOTF. These curves differ with the type of objective, the objective magnification and the zoom setting of the LSM. At an AOTF setting of around 30% at 351 nm and around 20% at 364 nm the rate of bleaching was down to 20% for 100 recorded images, which is acceptably low for the experiments. However, the S/N (signal to noise) ratio, and therefore the image quality, was poor at these settings. We have chosen for most of the present experiments AOTF settings of 60% and 30% for 351 and 364 nm, respectively. This resulted in a similar rate of bleaching of both wavelengths and allowed for example, the recording of up to 80 image pairs from a single cell at a zoom factor of 1.7x with the Ultrafluar 100/1.2 glycerin objective. With the C-Apochromat 63/1.2 W objective (objective C) even longer series of images (120 images) can be recorded, due to the higher transmission at 505 nm.

Parafocality of the fluorescence images obtained with visible- and UV-light excitation

Maintenance of parafocality is a major problem of confocal microscopy in general, even in the visible range. When using UV-light excitation the problems are even greater. Most optical elements in commercial microscopes, including many of the high-quality objectives, are usually built and corrected for the main visible light range (480–650 nm). Consequently optical image quality and parafocality will often start to decrease below 470 nm. Transmission will dramatically decrease at around 360 nm due to the types of glass used. To achieve parafocality of the excitation light from the visible to the UV range, an adjustable, motorized lens system was integrated in the UV excitation light path. This allowed for the adjustment of parafocality for different objectives, with one or two visible wavelengths together with one UV wavelength (351 or 364 nm). However, for Fura-2...
Fig. 5A–C  Intensity profile of z-image sections through Fura-2-loaded HT29 cells recorded with the objectives A, B and C. The data are normalized to the maximal fluorescence signal. The z-step size was 0.2 μm. The overall magnification was set to equal values with the help of the LSM zoom function.

As the optical parameters – magnification, UV transmission, visible light transmission, immersion medium, numerical aperture (NA) of the objectives – differed, we tried to standardize the measuring conditions. Table 1 summarizes some of the optical parameters of the objectives. However, other optical characteristics, such as the axial and lateral chromatic aberrations as well as spherical aberration, are more important for the image quality. The pinhole size was set to achieve a theoretical full-width, half-maximum of around 1–1.3 μm in the z-direction (this was a size equivalent to 0.9–1.2 times the size of the Airy disk). The scan zoom was set to values equalizing the overall magnification and the light intensity was regulated with the AOTF to achieve comparable fluorescence intensities in the recordings.

Objective A gave, within the limits of this set-up and our detection sensitivity, parfocal image pairs without moving the motorized lens system. Objectives B and D gave, in comparison to objective A, a much more detailed image of those cellular structures that had accumulated higher Fura-2 concentrations. However, these two objectives showed a measurable (only shown for objective B) z-shift (see also Fig. 2) between the corresponding two UV images (around 0.8–1.2 μm). This shift, as focus corrections would have to be done between obtaining ratio image pairs, would considerably slow down the ratio image acquisition time. Other problems may be caused by a mismatch of image congruity between the images generated by the two UV wavelengths and by a movement of the optical axis when focusing through the bead. Image congruity is first of all influenced by the collinearity of the AOTF. The separation of the two wavelengths is achieved by different acousto-optic gratings (RF frequencies). To get rid of the different beam propagation behind the AOTF, the AOTF has a prismatic win-

cially developed for UV imaging in watery media and objects, which have a refractive index close to that of water. Figure 4A-C shows normalized intensity profiles of z-sections recorded from fluorescent latex beads (4 μm diameter, refractive index of the mounting medium 1.473 and 1.6 for the bead, emission maximum 430 nm; Molecular Probes, personal communication) at 351 and 364 nm excitation. Figure 5A–C shows an intensity profile recorded from a z-scan through Fura-2-loaded HT29 cells. All data were scaled to correct for distortions caused by mismatches of the refractive indices of the immersion media, the embedding material and the object [10].

Table 1  Summary of some of the optical specifications of the four objectives tested for use in confocal imaging of Fura-2. The C-Apochromat is the preferable objective since almost no shift between the ratio images, the high transmission at 505 nm, the greater working distance and the good image quality. (NA Numerical aperture, IM immersion medium, WD working distance)

<table>
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<th>Magnification</th>
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<th>WD (mm)</th>
<th>Absolute transmission (%)</th>
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<td>1.2</td>
<td>Glycerin</td>
<td>0.1</td>
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<tr>
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<td>Oil</td>
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<td>Water</td>
<td>0.25</td>
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<td>Water</td>
<td>0.22</td>
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Fig. 6 Mosaic of z-image series from Fura-2-loaded HT29 cells. The images were taken with objective C (C-Apochromat 63/1.2 water). The z-step size was set to 0.1 μm. Only every second image step is shown. The z-scan started from the cover glass side of the cells. The pinhole size was set to a theoretical full-width, half-maximum of 0.8 μm, by use of the pinhole set-up function of the LSM program.

It was evident from our measurements with objectives A, C and D that the z-profile of the latex beads was asymmetrical. This was most obvious for objective A and even more marked for the z-scans through HT29 cells. Another disadvantage of objective A is the limited transmission in the visible range, which further reduced the measured, weak Fura-2 emission signal. As Table 1 and Figs. 4C and 5C show, the objective C gave the best overall results: relatively high UV and visible transmission, almost no focal shift between 351 and 364 and, in addition, the lateral image quality was comparable or superior to that for objectives B and D. Figure 6 shows a series of z-sections taken with objective C through Fura-2-loaded HT29 cells to illustrate the good sectioning quality of the LSM for measurements of [Ca²⁺], as one can easily recognize the details of the compartments in adjacent images.

Changes of the Fura-2 signal in response to [Ca²⁺]c changes

The two wavelengths, 351 nm and 364 nm, delivered by our UV laser are both relatively near to the isosbestic point of 360 nm described for Fura-2 in vitro [9] and
vivo [29]. Therefore, we expected a limited maximal possible ratio change and it was also unclear whether the 364 nm excitation would give a change in fluorescence intensity at all with changes of \([\text{Ca}^{2+}]_i\). Figure 7 shows a typical fluorescence emission intensity trace for both excitation wavelengths recorded from HT29 cells in response to stimulation with an agonist (carbachol 10 μmol/l). It is quite obvious that the two excitation wavelengths give contrasting changes when an increase of \([\text{Ca}^{2+}]_i\) was stimulated. Figure 7 gives the respective calculated ratio curve. The maximal achievable ratio change from 0 Ca²⁺ (R_{min}) to 2 mmol/l Ca²⁺ (R_{max}), measured by ionomycin calibration as described previously [27], was 0.98 ± 0.08 (n=12). To illustrate the enhanced spatial resolution achieved with this new microscope, Fig. 8 shows a sequence of ratio images recorded from HT29 cells loaded with Fura-2/AM. In the first image at 10 s in the non-stimulated cells \([\text{Ca}^{2+}]_i\) is slightly lower in some areas mostly located around the nucleus. We have characterized these structures by specific markers for mitochondria (Ricken S., Gregor R., Nitschke R., manuscript in preparation). Upon stimulation with carbachol, \([\text{Ca}^{2+}]_i\) rose quite homogeneously throughout the cytosol, whereas the increase in the mitochondria was delayed (40–65 s). Upon removal of carbachol cytosolic Ca²⁺ returned near to the resting value, whereas mitochondrial Ca²⁺ stayed elevated for more than a minute (image 95 s in Fig. 8).

**Changes of the Fura-C18 signal in response to \([\text{Ca}^{2+}]_i\) changes**

By incubating the cells from the basolateral side with the highly lipophilic Fura-C18 [6] (non-AM, 10 μmol/l for 4 min) the outer cell surface was labelled with the dye. The fluorescence signal was totally quenchable with 5 mmol/l NiCl₃, indicating that Fura-C18 stayed on the cell surface.
outside of the membrane (data not shown). As Fura-C_{18} is very lipophilic we could change the bath solution without washing the dye away. This allowed us, for example, to measure the Ca^{2+} activity near the outer membrane in response to changes of the Ca^{2+} activity in the perfusion solution.

Figure 9 shows the two raw fluorescence emission traces recorded from a region of interest at the cell membrane; the resulting calculated ratio is also shown. Changing the outside Ca^{2+} activity nominally to zero by including 5 mmol/l EGTA in the bath buffer resulted in a mean decrease of the ratio by 0.38 ± 0.04 units (n=4). This change is quite small compared to the maximal change obtained in a conventional imaging system at optimized excitation conditions (345 nm/373 nm; 2.91 ± 0.06 units, n=7). However, as the S/N ratio was not too low, it should still be sufficient to detect cellular Ca^{2+} export sites.

Discussion

Up until now, Fura-2, the widely used ratioable Ca^{2+}-sensitive dye, has not been used in confocal microscopes due to technical and optical limitations. The modification of a commercial LSM with the implementation of an AOTF for fast switching of UV wavelength, an additional lens in the UV light path, a new high-NA water immersion lens, and the use of PMT detectors with higher sensitivity have enabled Fura-2 ratio measurements at a reasonable speed and superior lateral and axial resolution compared to conventional fluorescence microscopy.

The use of the AOTF has several advantages compared with conventional laser-line interference filters. Although problems with interference filters used in the UV, such as transmission efficiency (due to the necessary block of neighbouring laser lines) and life-time (due to the high power density of the UV laser), have been solved by some manufacturers, the most important advantages of the AOTF are the speed of the wavelength switching and the fine variable control of the excitation light intensity. The fine tuning of the laser intensity allowed for the exact setting of excitation light intensities for the two wavelengths, which resulted in a similar bleaching rate of the Ca^{2+-free} and the Ca^{2+-bound} dye form (Fig. 3). This feature of the AOTF avoided an artefactual change of the ratio due to different bleaching rates during the time course of the experiment. We also used the fine excitation light intensity control in quantitative measurements of fluorescent protein labels (R. Nitschke and K. Kunzelmann, unpublished results). This was easier and more reliable compared to changes of the PMT gain, which required a careful PMT calibration procedure. Up until now the maximal achievable number of ratio images (3 per second) has not been limited by the AOTF speed or the scanning speed, but has been limited by other hardware and software limitations. A further and tighter integration of the AOTF into the software of the LSM should easily enable high-speed, single-line-scan ratioing (200 Hz), where the wavelength switching occurs during the retracing of the y-scanning mirror. However, the AOTF switching speed is currently not sufficient for a pixel-by-pixel change of the wavelength at the usually used scanning speed of around 1 s for 512×512 pixels.

Ratio imaging is very sensitive to lateral and axial chromatic aberrations. A focal shift or unequal lateral chromatic aberrations between the two Fura-2 images would lead to a falsely calculated ratio. A correction of the axial chromatic aberration with the motorized lens in the UV light path is possible; however, this would considerably slow down the image acquisition speed.

In our experiments we always set the pinhole to a value that should result in a full-width half-maximum for the z-axis of around 0.8–1 μm. This was a compromise between the maximal possible z-resolution, the S/N ratio and dye bleaching. At these pinhole settings the objectives B (Fig. 2) and D showed a measurable focal shift, whereas the images obtained at the two excitation wavelengths with objectives A and C looked very similar in terms of the focal point (Figs. 4 and 5). However, the image quality (in terms of the resolution of subcellular structures) of the 351 nm and the 364 nm excitation images was much higher with objective C and nearly indis-
tistinguishable from the resolution of fine cellular structures obtained with objective B (Figs. 2 and 6). The better image quality of the water-immersion objective compared to the glycerin immersion objective is based on several facts: (1) the refractive index values of the immersion medium and the specimen almost match; (2) the higher overall transmission (Table 1) of the water-immersion objective C and the reduced loss of fluorescent light in the deeper sections of the specimen (Fig. 6) influence the S/N ratio positively (for a detailed discussion see [10]); (3) the lateral chromatic aberrations, which will result in a decrease of the fluorescence signal towards the edge of the field, were corrected satisfactorily (data not shown).

Measurements of [Ca\textsuperscript{2+}]\textsubscript{i} and [Ca\textsuperscript{2+}]\textsubscript{o} with Fura-2 and Fura-C\textsubscript{18}

From the wavelengths 351 nm and 364 nm available for UV excitation and the fluorescence excitation spectra of Fura-2 [29] and Fura-C\textsubscript{18} [6], it might be expected that the achievable fluorescence changes would be small with 351 nm excitation. For the 364 nm excitation we expected almost no change in response to Ca\textsuperscript{2+} changes, as this is only 4 nm away from the isosbestic point. Our measurements showed, for a typical, agonist-induced [Ca\textsuperscript{2+}]\textsubscript{i} elevation, an increase of the 351 nm signal by about 30% and a decrease of the 364 nm signal by around 20%. As the typical noise within a smaller pixel area is only around 2–3%, the S/N ratio at the beginning of an experiment is 10–20. During the time course of an experiment, however, the S/N ratio halved due to significant dye bleaching. For Fura-C\textsubscript{18}, the respective values for maximal Ca\textsuperscript{2+} changes were around 15% for both wavelengths, so that the S/N ratio was about only half of that for Fura-2, but still sufficiently high for reliable measurements. The good resolution of fine intracellular structures such as mitochondria in the raw images (Fig. 2) as well as in the ratio images (Fig. 8) is far superior to conventional fluorescence microscopy and, with this relatively high time resolution, also superior to deconvolution techniques [4, 24].

A more general problem with excitation ratio imaging is the time difference between the images recorded at the two wavelengths. For the standard scanning speed the ratioed pixels in a full size 512×512 image were 1.08 s apart, which means that transient and fast [Ca\textsuperscript{2+}] changes could be under- or overestimated or even be missed. However, in practice, the scanned image area was often only between 256 and 64 pixels square, which reduced the time difference to 0.5–0.1 s. To improve time resolution further, a line-wise ratio acquisition, which requires major software programming efforts, would be necessary.

The confocal microscope described enables fast and reliable Fura-2 ratio measurements from single cells and subcellular structures. The advantage to other known methods of [Ca\textsuperscript{2+}] measurements using confocal microscopes can be summarized as follows. Ca\textsuperscript{2+} dyes such as Fluo-3, Fura-red, calcium-green, Fura-2 and Indo-1 are known to be unevenly distributed in many cells. For the non-ratioable dyes this is a major problem, even in a confocal microscope. As discussed in the Introduction, some groups tried to overcome the problem by co-loading two dyes such as Fura-red and Fluo-3 [3, 8, 14, 19, 31, 33]. For the two most widely used ratioable dyes, Fura-2 and Indo-1, the cellular non-uniformities are cancelled out by the ratio method; unfortunately both dyes require UV excitation. An elegant approach has recently been described, which consists of the modification of an LSM for Indo-1 emission ratio imaging [25]. However, in this and several other reports, major problems [15, 16, 20, 22, 30] with the detection of the shorter-wavelength Indo-1 emission signal were described. In addition, Indo-1 is more sensitive to bleaching and requires, not only for the excitation light path but also for the collection of the lower fluorescence emission peak (370–430 nm), UV-optimized optics. This is not, or might not, be standard in many confocal microscopes.

In the current software version the maximal speed of around 3 ratios/s was reached at an image size of 512×8 pixels (xxy). A further reduction of the γ-scanning lines did not result in an increase of the possible number of ratios per second. For a 512×64 pixel image size 2 ratios/s can be acquired. Up until now the AOTF wavelength switching is synchronized to the start command for the scanning mirrors. The possible number of ratios per second could be much higher by synchronizing the AOTF switching with the retrace signal of the γ-scanning mirror, so that the scanning mirrors could scan continuously. This would allow around 200 ratios/s in the line-scan mode. The synchronization would also allow a much better use of the fast light intensity modulation of the AOTF for experiments with caged compounds such as caged Ca\textsuperscript{2+} or caged inositol 1,4,5-trisphosphate. A software addition allowing the use of the AOTF in such experiments is currently being tested in the author’s laboratory.

The intensity of the fluorescence signal collected from Fura-2-loaded cells is dependent on: dye concentration; excitation light intensity; the NA of the objective; scanning speed; transmission efficiency of the optical parts in the emission light path; pinhole diameter; and detector quantum efficiency. Most of these parameters are already optimized to their physical limits or are a compromise between cell damage by excessive UV light, dye bleaching and optimal signal and image quality. There are only two parameters that can still be improved further, i.e. transmission efficiency and the detector sensitivity. By reducing the number of optically active elements in the light path and reducing the detector noise by cooling or increasing the quantum efficiency by using new techniques a further increase of the S/N ratio or longer experiments with a higher number of acquired images might be possible.

The modifications described for changing a conventional LSM to an instrument allowing for visible and/or
UV ratioing can not easily be done by a "normal" user. The major problem would lie with most commercial confocal microscopes not the AOTF installation itself. However, the AOTF changes the laser beam polarization as well as the output angle of the laser beam. This makes a careful realignment of the optical pathway necessary. A direct contact between the user and the LSM manufacturer is also important for the tight integration of the AOTF into the user software. A custom-sized UV-solution for the LSM used in our study will soon be available (Carl Zeiss, Jena, Germany) and previously we have described a fairly similar modification for visible ratioing on a video-rate CLSM (Noram, Middletown, USA) [26].

Fura-2 has been successfully used in more than 2000 studies over the last 4 years and is now available in different forms - bound to dextrans or coupled with lipophilic side chains [6, 7]. The power and the pitfalls of this dye have been described extensively. Therefore, the new confocal microscope described here will greatly improve the detailed examination of subcellular Ca2+ signals - such as near-membrane, nuclear, or mitochondrial Ca2+ signals - compared to the measurements with single-wavelength dyes.

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