Use of the Digital Signal Processor and Acoustico-Optic Tunable Filters in Ratiometric Confocal Calcium Imaging

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Since the development of ratiometric fluorescent calcium indicators like Fura-2 and Indo-1 in the mid-eighties, calcium imaging has become a major approach to studying life cell physiology. The ability to calibrate the spectral properties of the dye as a function of the calcium concentration led to measurements of nearly absolute intracellular calcium concentrations in a large variety of preparations. However, the use of ratiometric calcium indicators in confocal calcium imaging is limited, since the dyes are excited by UV light while the lasers of most confocal microscopes provide visible light only. In addition, high energy UV light may cause rapid dye bleaching and photodamage. Thus it appears advantageous to reduce the illumination of the preparation to the minimum possible minimum. The most frequently used dual-excitation dye Fura-2 requires rapidly alternating excitation at 340 nm and 380 nm, respectively, to permit ratiometric calcium measurements with a maximal efficacy. This demands a high technical standard and fast combined control of the hardware components. The introduction of an acoustico-optic tunable filter (AOTF) into the illumination light path enabled the rapid switching of the two excitation wavelengths and thus ratiometric imaging of the pH-dye BCECF (Spring and Nitschke, 1995) and the calcium-dye Fura-2 (Nitschke et al., 1997). These developments were integrated into a new type of confocal microscope (Zeiss LSM 510), equipped with fiber coupled UV and VIS-lasers in combination with a digital signal processor (DSP), which allowed a coordinated advanced control of the confocal x- and y-scanner and the AOTF. This type of confocal microscope enables fast ratiometric imaging in a fully user controllable mode, making it suited for physiological experiments in general and ratiometric imaging in particular.

Illumination of fluorescent calcium indicators results in bleached or chemically destroyed dye molecules, which also can be toxic for the cell. In addition, UV illumination can cause cell photodamage. Hence, a reduction of the illumination to a minimum is desirable in all physiological experiments. Usually, the whole field of view is illuminated during a calcium imaging experiment, although only a few sub-cellular compartments are of interest, *e. g.*, some dendritic processes of a neuron. Thus, the unwanted side effects of illumination discussed above occur throughout the whole cell. This problem is diminished by the advanced DSP control of the AOTF in the LSM 510, which switches on the laser beam only in region of interest (ROI) areas, but leaves all other parts of the scanning field unexcited. This prevents direct bleaching outside the ROIs. The effect of bleaching and photo-toxicity inside the ROIs is reduced, because non-bleached dye molecules from outside the ROIs diffuse, replacing bleached dye molecules and toxic dye derivatives are diluted by diffusion. Altogether, this leads to a strong reduction of the overall cell photodamage.

The dual-excitation ratiometric calcium indicator Fura-2 has to be excited alternately at 340 nm and 380 nm (or 351 nm and 364 nm using an UV Argon laser in a confocal system, respectively). The fluorescence emission is detected by a photo-multiplier tube (PMT) between 420 and 620 nm. Like CCD camera-based cal-

cium imaging systems, the confocal systems record one image after excitation with the shorter wavelength, and subsequently one image after excitation with the longer wavelength, calculating the ratio of this image pair. Consequently, both images are separated by the time period needed to record one image, *i.e.*, about 0.5 to 2 seconds depending on the number of pixels in the images and the pixel time. During this time, the amplitude and the location of the calcium signal may have changed, resulting in faulty ratio values. To overcome this problem, in the Zeiss LSM 510, the DSP changes in the *'Multi Track'* mode the excitation wavelength via the AOTF after each line of each image. Thus, the corresponding pixels of the 351 nm image and the 364 nm image are now only separated by a time period in the range of hundreds of microseconds. This time can be reduced even by half using the *'Bi-directional'* scan function, which enables the recording of images with the second excitation wavelength during the fly back of the confocal scanning mirror. Now the image pair is almost recorded simultaneously. A problem which arises from this advanced recording modes can again be solved by the tight coupling of AOTF and DSP, which allows a pixel-wise control of the excitation light intensities. The wavelength switching time by the AOTF is approximately 1 µsec; this is much faster than the time needed to change the PMT sensitivity. Therefore, in the fast scanning modes, the 351 nm and the 364 nm image have to be recorded with the same PMT settings. Now the AOTF can be used to tune the excitation light intensities in a wide range to fit both emission signals to the same PMT settings.

To measure fast calcium signals, *e.g.*, those accompanying excitation of neurons, the *'Line scan'* mode is used to record at frequencies of up to 1000 Hz. In a line scan image, the ROI consists of a line with a width of 1 pixel and definable length. This line is scanned 1 to *n* times, and the scans are saved as the first to n^{th} row of the image. By using this recording mode one has to give up most of the spatial information for gaining the high temporal resolution, as unfortunately most of the confocal systems only provide a straight line to be scanned. A straight line, however, often hits a curved structure like neuronal processes twice or three times only, giving very limited information of the spatial distribution of the calcium signal. The DSP control of the confocal scanners enables the scan of a user-defined, spline-fitted curve that more or less follows the structure of interest, depending on the complexity of the structure. In this case, the line scan image now contains the spatial information of the calcium signal over the whole length of the structure recorded at high time resolution. It should be noted that the scan speed decreases with the complexity of the curved line.

Ratiometric line scan images can be obtained using the combination of the line-based *'Multi Track'* function in the *'Line scan'* mode, where one line scan image is recorded with excitation at 351 nm, the other image at 364 nm. The ratiometric line scan image is calculated from this image pair. The corresponding lines (rows) of the original images diverge by some milliseconds only. Some cellular events may cause calcium signals with onset time constants even faster than this, resulting in a somewhat distorted onset of the calcium signal, but the amplitude and the recovery time constants are usually not affected. ■

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