Faster than real-time: confocal linescan systems provide ideal conditions for millisecond-resolution physiological imaging

The LSM 5 *LIVE* is a new design for fast confocal imaging, which acquires images at extremely high frame rates and allows experiments not possible in the past, while maintaining the advantages of conventional scanning systems, such as adjustable confocal apertures and scalable frame formats with pan and zoom. Additionally, the LSM 5 *LIVE* offers fast and sensitive parallel acquisition with charge-coupled device (CCD) detectors for high quantum efficiency.

Today's fluorescent markers are versatile tools used to monitor cell function in various research experiments. Local variations in intracellular calcium concentration are important in regulating a variety of physiological functions, such as muscle contraction, the release of neurotransmitters or hormones, cell death or degeneration, and gene transcription. Ion-sensitive fluorescent dyes have become established as useful tools in recent years for monitoring these changes.

With the LSM 5 *LIVE*, Carl Zeiss offers components optimized not only for kilohertz-speed imaging and analysis of these processes, but also for accurate control of physiological parameters and precise communication with external devices. Users also benefit from imaging arithmetic functions for online ratio calculations in single wavelength (for example, Oregon green, Fluo-4, Fura red) and ratiometric (for example, Fura-2, Indo-1, Pericam) indicators. Extensive calibration tools simplify the task of evaluating and quantifying time-series experiments.

Very recently, new indicators based on fluorescent proteins have also been introduced, like variants of Cameleon, Pericam and Camgaroo. Such dyes have many uses, ranging from measurement of intracellular ion concentrations, for example, by pH determination (using dyes such as SNARF) to verification of physiological regulatory processes with fluorescence resonance energy transfer (FRET)- or translocation-based biosensors (such as Yellow Cameleon). As neuronal communication occurs on a millisecond scale, the monitoring of events in the nervous system is an enormous challenge. For example, to visualize ion changes at spike rate, one needs dyes and imaging equipment capable of kilohertz-speed frame rates. Common dyes for such experiments are the voltage-sensitive dyes.

CCD camera–based multifocal confocal systems are the future

Until now one had to compromise on spatial resolution, accept restricted scanning field size or make trade-offs in sensitivity (low signal-to-noise ratio) to obtain microscopic evidence of fast cell processes. The reason is that just speeding up the scanning and reducing the number of pixels or shortening the pixel dwell time linearly reduces resolution and/or sensitivity.

The only way to overcome this problem is to illuminate multiple locations at the same time and to acquire the image in parallel. The result is the ideal combination of long pixel dwell times and short frame-acquisition time, resulting in fast frame rates and good sensitivity. This, for a long time, was the benefit of the classical confocal spinning disk systems—although this was unfortunately combined with the inability to adjust the confocal aperture opening, the need to illuminate every location multiple times to avoid statistical inhomogenities, and the need to synchronize with the frame CCD readout. Such synchronization reduces the theoretical frame acquisition speed dramatically from, for example, 300 frames per second to typically 50 frames per second, which is not sufficient for certain physiological events, such as spike-rate analysis.

The LSM 5 *LIVE* offers a unique combination of superior fast scanning rate, outstanding image quality and exceptional sensitivity (**Fig. 1**). The trick to achieve this is the use of a line CCD, and the parallel illumination and acquisition mode. The LSM 5 *LIVE* illuminates 512 pixels along a line in the *x* direction, and scans this line in the *y* direction with a scan mirror for up to 2,048 pixels (**Fig. 2**). This is a unique combination, as line CCDs permit readout speeds of up to 60,000 lines per second. Hence, a frame of 512 × 512 pixels can be

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APPLICATION NOTES

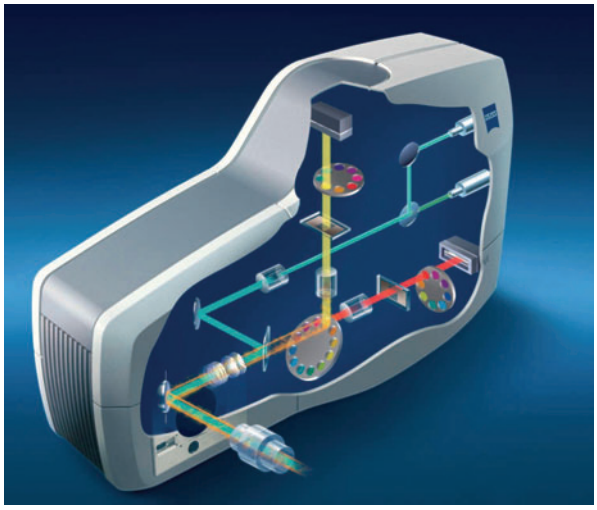

Figure 1 | LSM 5 *LIVE* scanhead design. One or two line CCDs detect the emitted fluorescence light simultaneously 60,000 times per second. The sample is excited by a scanned laser line 512 pixels in length. A set of slit-shaped confocal apertures allow freely adjustable confocal three-dimensional sectioning.

acquired at 120 frames per second, with long pixel dwell times for high sensitivity.

But the combination of a parallel detected line and scanning in *y* direction allows more: it allows increased frame-acquisition rates without affecting the pixel dwell time just by shortening the picture in one direction. For example, scanning an area of 512 × 50 pixels (still sufficient to analyze several dendrites or axons of a neuron), one can reach 1,010 frames per second with exactly the same sensitivity as the full-frame acquisition. This not only allows one to monitor events at kilohertz resolution, fast enough for spike-rate events, but also permits high resolution and sensitivity. The LSM 5 *LIVE* system allows one to experience the fundamental mechanisms in living cells and to gain more information than ever before.

Advantages of the LSM 5 *LIVE*

Ultrahigh-speed observation of living cells in confocal light was the idea when creating the LSM 5 *LIVE*. But the LSM 5 *LIVE* will not only shine with its image recording rate—the design offers other improvements over existing approaches.

Increased pixel dwell times mean lower laser power, as photons are collected for longer at every pixel. This allows one to keep cells alive longer and to reduce artifacts caused by light exposure, which can lead to generation of free radicals. Additionally, the design with the line-scanning principle allows for adjustable confocal apertures, to fine-tune the best balance of three-dimensional sectioning, resolution and light efficiency according to the sample transparency and thickness.

Line CCDs offer a high quantum efficiency of more than 75%, even 80% at the most frequently used wavelengths (in the range of 550 nm). This is a dramatic improvement compared to photomultiplier tubes, which have a typical quantum efficiency of 25–30%. Also, the fact that only a single line needs to be directed to the sample for excitation allows the use of a unique main beam–splitting concept, the new AchroGate beam splitter. This element separates the coherent (line) excitation light from the noncoherent fluorescence emission, as each fluorochrome emits photons in all directions. The Achrogate is a wavelength-independent separation device, with no mechanical or electrical switching necessary. Virtually 100% excitation efficiency and high emission efficiency yields improved imaging performance, even on thick or weakly fluorescent specimens. Color-independent beam splitting allows, for the first time, a universally flexible and sensitive imaging system.

Further advantages of the line-scan design are the ability to shift and enlarge the scan region to target the most interesting areas of the sample. The *y*-directional scanning is an inherent capability of the design, and the addition of an X-scanner allows the movement of the scanned line also in the *x* direction. We are presently developing a unique microscanning mode with this X-scanner, in which three shots will give 1,536 × 1,536 pixels, for the highest resolution of tiny details.

Additionally, an optical zoom allows one to enlarge the field to 18 mm in the intermediate plane, a size not possible with most frame CCD or spinning-disk configurations. Because the LSM 5 *LIVE* illuminates only the scanned rectangular frame, bleaching effects are not observed outside of the scan regions. In contrast, spinning-disk systems illuminate (and bleach) a large circle, with the rectangular frame of the CCD oriented on the center of this circle.

Millisecond resolution with full-cell coverage is not a dream

Dynamic analysis of ion concentrations in time and space is crucial for throwing light on mechanisms that modulate neuronal activity. That is exactly what the LSM 5 *LIVE* allows (**Fig. 3**). Living nerve cells communicate at a lightning kilohertz speed by electrochemical transport mechanisms, for instance, at neuronal switches in the brain. These processes coordinate our motions, direct our senses, allow us to learn, determine our creative potential and form our memories.

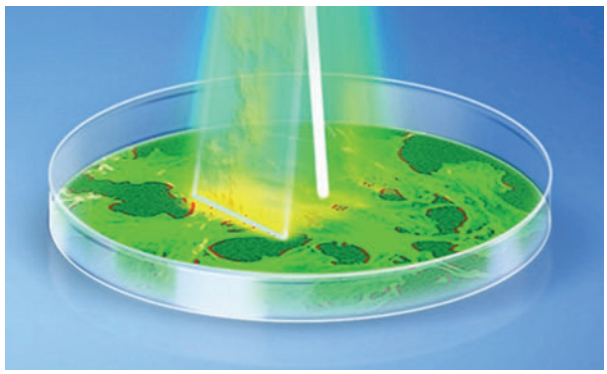

Figure 2 | The scanning principle of LSM 5 *LIVE*. The excitation line is swept over the frame along the *y* axis. A photomanipulation or uncaging device (LSM *DuoScan*) is able to scan any combination of one or multiple regions of interest in addition to the fast image acquisition.

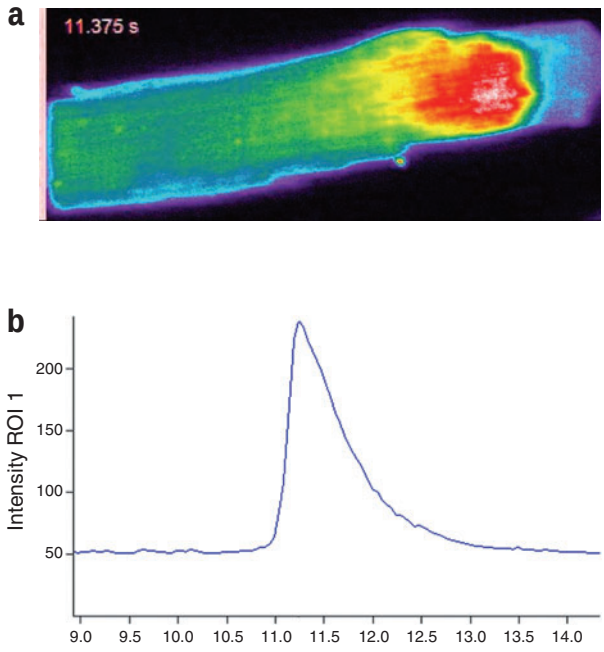

Figure 3 | Imaging and analysis with the LSM 5 *LIVE*. (**a**) Rat ventricular cardiomyocytes stained with Fluo-4, a Ca^{2+} ion indicator. (**b**) Mean intensity region of interest (ROI) plot, captured at 300 frames per second.

The LSM 5 *LIVE* allows one to analyze when neuronal signals throw the switches of the brain. But often passive observation is not enough for a scientific experiment. To analyze the role of transmitters or damage in the cellular physiology, for example, manipulations need to be done in the living tissue. The LSM 5 *LIVE* can be combined with the Zeiss LSM *DuoScan*, a photomanipulation device controlled by the same software and electronics and powered by the same laser module as the LSM 5 *LIVE*. This unit allows photoactivation or UV-uncaging of pharmacological substances at any region on the sample, without mechanical penetration of the tissue.

The LSM 5 *LIVE* acquires confocal images at extremely fast frame rates and allows new experiments not possible in the past. But a flood of high-resolution and multidimensional data also calls for new strategies in data recording, management and visualization. The LSM 5 *LIVE* navigates and analyzes these data streams reliably and effectively to allow the researcher to concentrate on research. For example, one thousand 512 × 512 pixel images collected in 10 s means 250 megabytes of data in 10 s—more than a CD-ROM full of data every half a minute. This is not an unusual amount of data with the LSM 5 *LIVE*. Thanks to new real-time electronics and real-time computational analysis, the LSM 5 *LIVE* can efficiently process these huge four-dimensional data quantities (*x, y, z* and *t*) with data rates of up to 100 megabytes per second.

The design of the LSM 5 *LIVE* was inspired and accompanied by scientists, and indeed every detail in the system reflects a need and a solution for new scientific experiments.

Additional information is available on our company website (http://www.zeiss.de/lsm).

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