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TRENDS

Spectrally resolved fluorescence lifetime imaging microscopy (SFLIM)—an appropriate method for imaging single molecules in living cells

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Ever since the first examination of cellular structures, scientists have been fascinated by investigations of single cells. Over the last decade, many methods (e.g., capillary electrophoresis, electrochemical detection, mass spectroscopy, and optical spectroscopy) have been proposed for analyzing single cells [1]. Many of these methods are appropriate for in vitro experiments, which provide only a snapshot view of cells. However, some optical methods, like fluorescence spectroscopy, are also advanced enough for investigations of living cells. This is due to their simple application without the need for fixation or lysis of the respective cell. A variety of light microscopy techniques for imaging living cells, such as widefield excitation, confocal scanning and total internal reflection excitation, have been reviewed by D. J. Stephens and V. J. Allan [2]. The fluorescently labeled components required (e.g., proteins or DNA) can be added to the cell culture medium, from where they are taken up by the cells if the components are able to pass through the cell membrane. Alternatively, they can be directly injected into living cells via micropipets. Due to their toxicity, the concentration of the fluorescent dye should be as low as possible. This can be achieved by using single-molecule detection techniques that enable the measurement of low concentrations (approximately 10^{-11} M) of chromophores in homogeneous solutions and of individual immobilized molecules. In 2001, Sauer at al. established spectrally resolved fluorescence lifetime imaging microscopy (SFLIM) [3], which uses a standard confocal microscope set-up (Fig. 1a). For this technique, a pulsed laser diode with a wavelength of usually 635 nm and a repetition rate of 40–80 MHz is used. The laser beam is coupled into an oil immersion objective and focused onto the sample surface on a scanning table. Generally, the average excitation power is adjusted to 0.25–5 kW/cm^2. The fluorescence light emitted by the sample is collected by the same objective and focused onto a pinhole with a diameter of 50–100 μm. Fluorescence light passing through the pinhole is spectrally separated by a dichroic beam splitter and detected by two avalanche photodiodes. To generate fluorescence lifetime images, the signals from the two avalanche photodiodes are recorded by a time-correlated single-photon counting interface card and the data are analyzed by applying a maximum likelihood estimator (MLE) algorithm, which is an appropriate method of determining monoexponential fluorescence lifetimes from low photon count statistics.

Figures 1b–d show images of an untreated living cell (3T3 mouse fibroblast) in RPMI 1640 medium recorded by SFLIM [4]. The first image (Fig. 1b) shows the overall fluorescence intensity. The cell culture medium exhibits a constant background with a count rate of approximately 4 kHz, which is due to autofluorescence of the fetal calf serum (which is in the medium), whereas the cell itself shows lower autofluorescence. Some more intense signals occur in the cytoplasm arising from the

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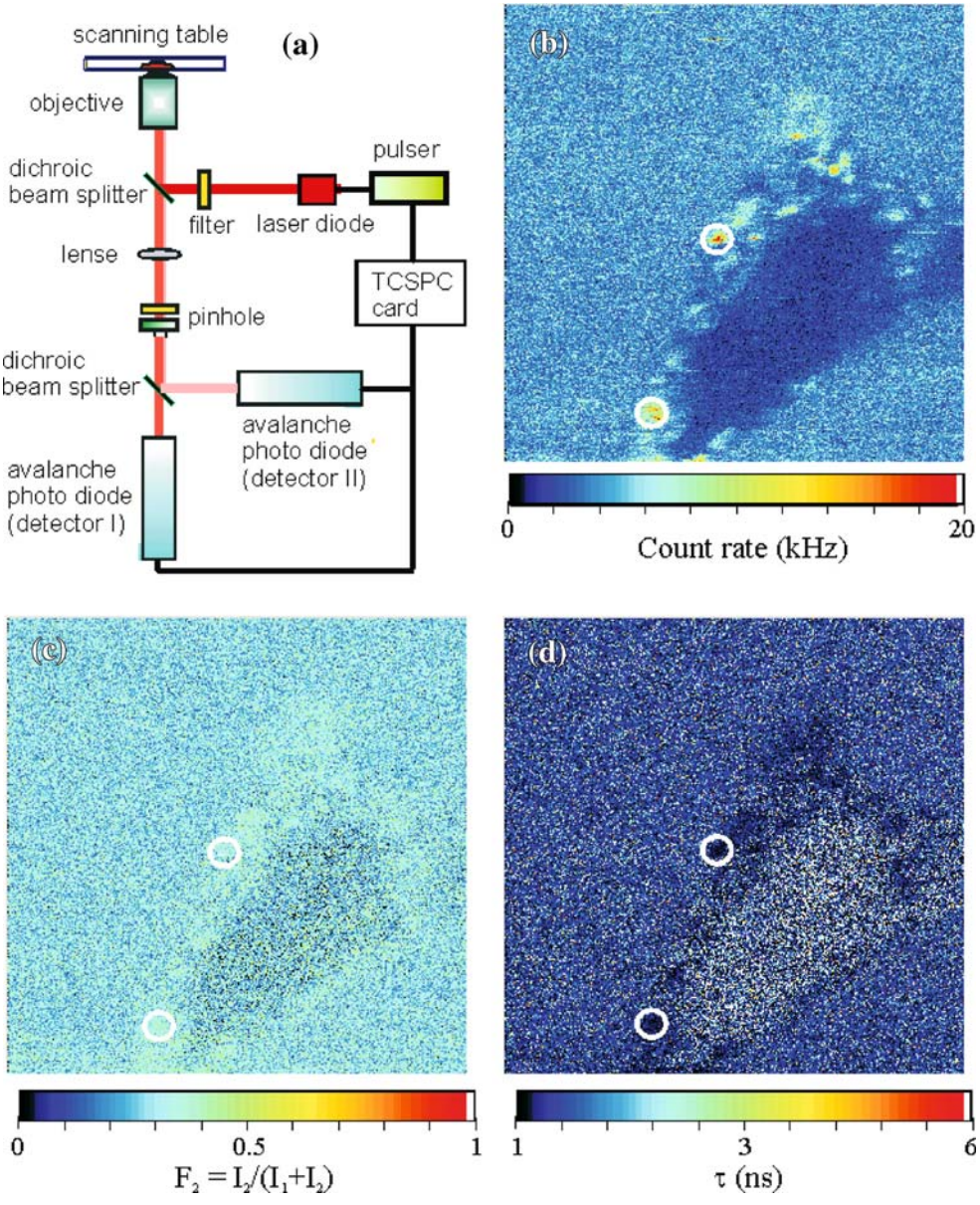

Fig. 1a–d **a** Set-up for spectrally resolved fluorescence lifetime imaging microscopy. **b** Overall fluorescence intensity (detector I_1 + detector I_2) image. **c** Fractional intensity (F_2) image recorded at the detector I_2, $F_2=I_2/(I_1+I_2)$. **d** Fluorescence lifetime image calculated from the overall photon counts. All scanning confocal fluorescence images (20×20 μm) show an untreated 3T3 mouse fibroblast cell in RPMI 1640 cell culture medium containing 10% fetal calf serum and 1 mM glutamine. The cell was scanned from top left to bottom right with a resolution of 50 nm/pixel. All measurements were carried out with an excitation wavelength of 635 nm, and the fluorescence light was split at 670 nm

mitochondria. Figure 1c is a false-color image of the F_2 values that are calculated from the fluorescence intensities measured at both detectors I_1 and I_2 ($F_2=I_2/(I_1+I_2)$), and provides information about the emission wavelength. The image on the right-hand side represents the fluorescence lifetime τ calculated for each pixel from all detected photons using a monoexponential MLE algorithm. The autofluorescence resulting from the medium and from the cell has short fluorescence lifetimes of around 1.3 ns and F_2 values of ~0.5, which corresponds to an emission maximum of 670 nm. Therefore, the areas in the cell with high autofluorescence tend to result in slightly higher F2 values and shorter lifetimes. Both parameters can be used to discriminate between the autofluorescence signals and signals arising from the fluorescently labeled probes applied. Here, MR121 labeled poly-T oligonucleotides were injected into the cell nucleus to mark the poly-A tail of RNAs. MR121 is an oxazine derivative with an emission maximum of 685 nm and a fluorescence lifetime of 2.8 ns, if it is attached to DNA. The microinjection technique is based on applying overpressure for a predetermined time while the micropipet penetrates the cell membrane. Unfortunately, the reproducibility in terms of transferring only a few molecules into the cell is very poor. However, in order to control the amount of probe molecules injected, the laser can be focused at the end of the micropipet where the inner diameter is approximately 500 nm and thus smaller than the laser focus (Fig. 2a). Consequently, after the application of an overpressure, almost all molecules passing the micropipet are detected and can be counted (Fig. 2b). In this case,

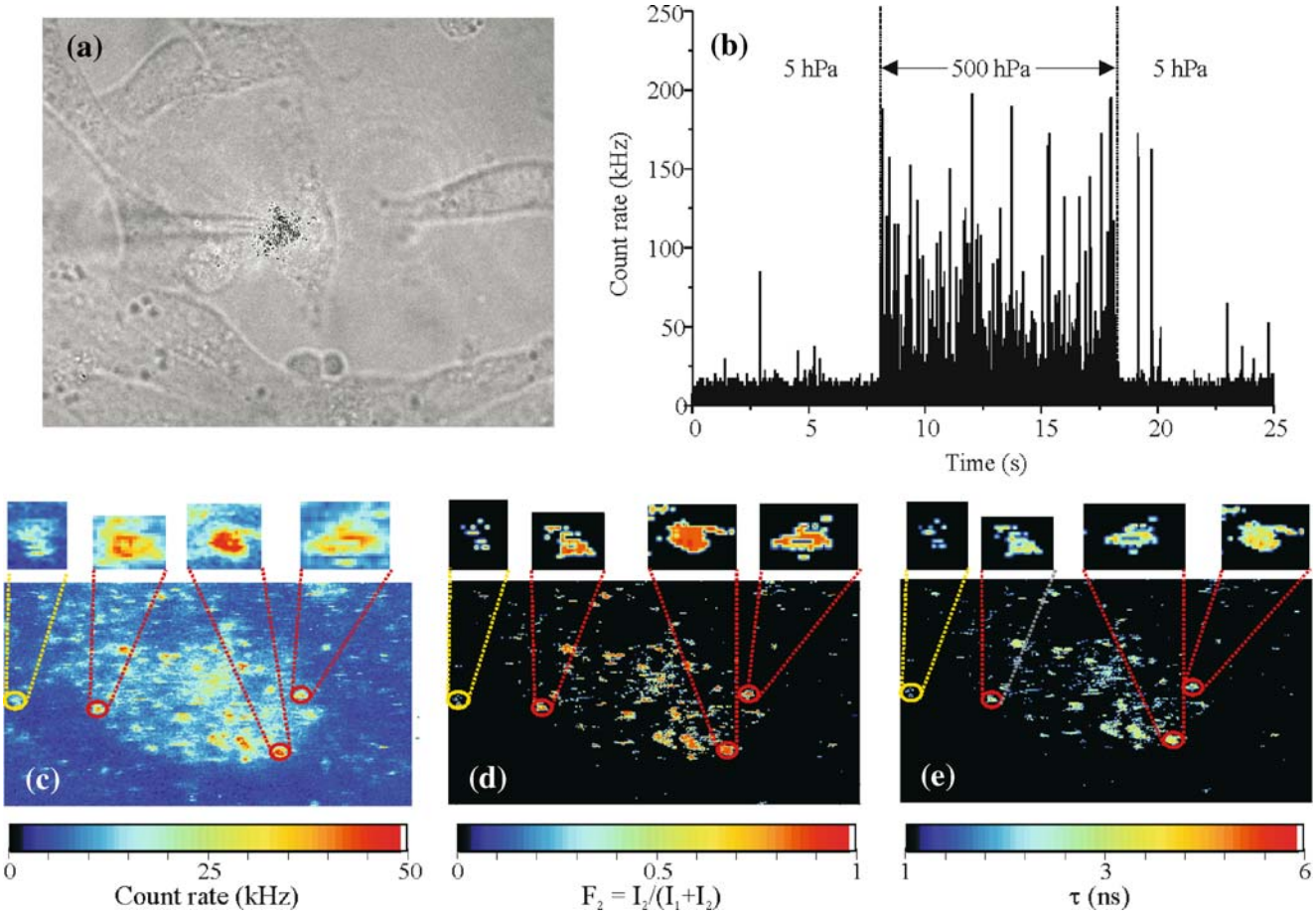

Fig. 2a–e **a** Phase-contrast image of a micropipet penetrating a 3T3 mouse fibroblast cell. The laser is focused into the end of a micropipet filled with a 10^{-9} M solution of a dye-labeled oligonucleotide. **b** Fluorescence signals observed in the micropipet during application of an overpressure for 10 s. **c** Confocal scanning fluorescence intensity image (25×12 μm), **d** fractional intensity image, and **e** fluorescence lifetime image of a 3T3 mouse fibroblast in cell culture medium after microinjection of ~100 fluorescently labeled oligo(dT) molecules. For **d** and **e**, the F_2 value and the fluorescence lifetime for pixels with photon counts of less than 30 are set to zero

approximately 100 fluorescently labeled oligo(dT) molecules pass the micropipet. Assuming a total cell volume of ~4 pL, a concentration of ~0.5×10^{-10} M of the probe molecule in the cell can be calculated, which is sufficient to detect fluorescence signals for freely diffusing molecules in homogeneous solution as well as those for immobilized molecules. Figure 2c shows the respective scanning fluorescence intensity of a 3T3 fibroblast cell after injection of the probe molecules that are supposed to hybridize to poly-A RNA in the cell nucleus. It can be seen that the fluorescence intensity in the nucleus is significantly higher than in the cytoplasm and the surrounding cell medium. Some dye-labeled oligo(dT) molecules are immobile and diffraction-limited fluorescence spots were observed (e.g., Fig. 2c, circles). The scanning images were recorded with an integration time of 6 ms/pixel and a resolution of 50 nm/pixel, and thus it can be calculated that these molecules must be immobile for at least several seconds to be imaged as a regular spot on a 25×12 μm image. Accordingly, the detected fluorescence stripes are due to molecules that stay in the same area for 10–2000 ms before they move away or photobleach. Fluorescence blinking behavior was found within most of the spots, indicating that many of the spots detected are generated from single immobilized fluorophores. Considering that approximately 100 fluorescent molecules were injected into the cell and that most spots are due to the fluorescence of single immobile molecules, 10–30% of these molecules cannot freely diffuse in the nucleus but are rather bound to immobilized elements of the cell. Figure 2d and e clearly show which fluorescence signals are caused not by autofluorescence but by MR121-labeled oligonucleotides. For example, the red circled spots in the images show F_2 values of ~0.8 and fluorescence lifetimes of about 3 ns, corresponding to the spectroscopic properties of MR121 (λ_{em}=685 nm, τ=2.8 ns). In contrast, the yellow circled spot shows a short lifetime and F_2-values below 0.5, indicating autofluorescence.

Summarizing, SFLIM is an appropriate method for identifying single fluorescently labeled molecules in living

cells and separating the fluorescence signals from the autofluorescence of the cell or cell culture medium. Due to the very high sensitivity only a few probe molecules are required. One disadvantage of using confocal scanning microscopy is that taking one image requires several minutes or hours, depending on the size and resolution. Consequently, it is not possible to investigate dynamic processes in the cell. For such investigations the fluorescence must be recorded by fast and sensitive charge-coupled device (CCD) cameras. With such methods, for example, the infection pathways of individual viruses in a living cell have been monitored [5]. However, the technique of SFLIM will have a significant impact on future methods of analyzing single molecules in cells, because for such methods it will be always be important to separate the fluorescence light of the probes from autofluorescence. In particular, the implementation of SFLIM in CCD camera-based systems would also be a promising method for investigating the dynamics in living cells. In this case, time resolution could be achieved via time-gated CCD cameras.

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