Zoological Studies 34, Supplement I (1995)

Zoological Studies

Fast Confocal Beam Scanning Laser Fluorescence Microscope for Single-photon and Two-photon Excitation

Ernst H.K. Stelzer, Nicholas J. Salmon and Clemens Storz

European Molecular Biology Laboratory (EMBL), Light Microscopy Group, Cell Biophysics, Programme Postfach 10.2209, Meyerhofstrasse 1, D-69012 Heidelberg, Germany

A fast confocal fluorescence microscope which is based on the following technologies was designed and built at EMBL:

(1) conventional inverted fluorescence microscope as the basic unit
(2) Argon ion laser as primary light source for single-photon excitation
(3) Titanium sapphire laser as light source for two-photon excitation
(4) resonant galvanometer for fast movement of beam along first axis
(5) conventional galvanometer for slow movement of beam along second axis
(6) piezo-electrically mounted objective lens for focusing
(7) photomultipliers operated in counting mode
(8) multiple processors to record and process the data

The instrument was built for applications in modern cell biology. One of its specific interests is the analysis of processes such as mitosis,endocytosis, exocytosis, and phagocytosis as a function of time not only in two (as e.g. in video microscopy) but in three dimensions. A prerequisite is that the object provides a specific contrast. This is accomplished by vital fluorescent markers that are incorporated into the cell body. At least one marker is specific for the problem that is investigated. The other marker is usually less specific and provides a view of the cell body, the plasma membrane or the nucleus.

The three-dimensional dye distribution is observed as a function of time using two photomultipliers that register the fluorescence emissions of two different dyes. The fast scanner is capable of producing 8000 lines per second which results in about 15 frames (two channels) per second assuming an image size of 512 lines and 512 picture elements per line. The photomultipliers can resolve up to 200 million counts per second. This means, that at most 8 photons will be registered per picture element. The number of photons per picture element is recorded by a computer, stored in main memory during the recording process and finally copied to disk. Effectively the instrument is able to record a complete 15-sections-deep data set every 4 seconds.

For an evaluation of the instrument's performance, latex beads immersed in water were observed to determine their diffusion rate. Diffusion might not be an efficient way for distributing e.g. proteins inside cells (low group velocity), but individual particles tend to move quite a large distance within a short period of time. Being able to determine the diffusion rate of a single particle will therefore provide us with an upper limit for the speed that can be recorded with this instrument.

A biological application is the determination of phagocytic pathways in macrophages. These experiments are an extension of similar experiments performed with video microscopy: phagocytosed fluorescent latex beads are observed in three dimensions while they move through the cell. The experiments determine the transport rate and the exact paths of the beads. The main goals of these investigations are the determination of the different steps of phagocytosis and of the involved intracellular compartments.

All of these experiments have been performed using single-photon and two-photon excitation. Naturally, two-photon excitation is less efficient and results in a lower resolution than single-photon excitation when used with the same dye. However, it does not require the use of a pinhole and the high excitation wavelength is less likely to affect the viability of the biological system that is observed

in the microscope.

The instrument has not been designed to produce "pretty pictures". Although an image can be averaged 16 or more times to increase the dynamic range the goal is to use three-dimensional fluorescence-intensity distributions that have been acquired at high speed. High speed means that the sample does not change while the data is being recorded. Effectively the difference in age between the top left and lower right picture element is reduced as far as possible. The three-dimensional data set will then provide a flash view of the specimen.

An important question in high speed confocal fluorescence microscopy is, how many photons are required to produce an image? Another question is, how relevant are the intensity variations in fluorescently labeled specimens? Are they relevant or are they just an artifact of the labeling process? Our instrument is designed to provide the answer to a very simple question: "Is a fluorophore at this location, Yes or No?" By recording three-dimensional intensity distributions as a function of time we are able to observe changes in the distribution and by careful analysis assign a function to the changes.