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(Eds.)

Imaging Living Cells

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Lab Manual

Rosario Rizzuto · Cristina Fasolato (Eds.)

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With 97 Figures

Springer

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Preface

In the past few years, the direct imaging of living cells has become a fundamental approach in the study of biological events. Indeed, the impressive advances of molecular biology have allowed the fine dissection of complex cellular processes, such as those controlling growth, differentiation, adhesion, etc., identifying the molecules involved in these events. This molecular characterization, together with the possibility of recombinantly expressing native or modified molecules in living cells, opened the challenging task of investigating these complex functions in vivo, i.e. to observe the behaviour of cells with normal or modified molecular repertoires. In this respect, the rapid development of optical, electrical, and computational technologies has dramatically improved our insight into the intimate life of a living cell. Nowadays it is possible to follow, in real time, with high temporal and spatial resolution, not only single cell responses and cell-cell communications, but also different aspects of the intracellular microenvironment. Indeed, it's now a common procedure to monitor a cell, after marking a specific ion, molecule or organelle, while it spreads, secretes, contracts, engulfs, divides or simply dies.

At least two different methodologies have pioneered the route of "imaging living cell": the advent in 1976 of the patch-clamp technique,[1] which allowed to follow the electrical activity of virtually all cell types, and the synthesis in 1982 of the first calcium-sensitive dye, which could be easily trapped by intact cells.[2] The merging of these two techniques by microspectrofluorimetry is one of the first example of a combined approach which has successfully been employed and further refined in many laboratories. The development of fluorescent probes with different ion sensitivities,[3] and the improvement of those already available (in terms of higher fluorescence and different spectral properties)[4] from one side, and the advances in video-imaging technologies, on the other side, allowed to zoom inside the cell, leading to the

discovery of the spatial and temporal heterogeneity of many signalling processes. The widespread diffusion of molecular biology has further boosted the imaging field. In fact, using naturally occurring fluorescent and chemiluminescent proteins, probes can be designed and produced by recombinant techniques, which have a specific intracellular localization and allow to label intracellular structures and/or monitor physiological parameters.[5] A striking example of the potential, and interest, of protein probes is green fluorescent protein (GFP) of Aequorea victoria, which soon after the first report of its heterologous expression[6] has attracted an explosive interest and is now used by hundreds of laboratories across the world for monitoring in vivo a large variety of physiological processes. Finally, the study of cell function with chemical or recombinant probes is not limited to isolated, cultured cells. Cells in slices, or even in the whole organ, without the disruption of the tissue organization, can now be visualized in real time by means of epifluorescence and confocal laser scanning microscopy.

The rapid advance of the imaging field is clearly evident from a quick glance to the recent scientific literature. A simple bibliography search on some of the approaches mentioned in the previous paragraph shows an impressive increase, during the last decade, of the papres published in this field (see figure 1).

For this reason, following the suggestion of numerous colleagues, we decided to accept the invitation of the publisher, and edit a book with the explicit goal of explaining the rationale and

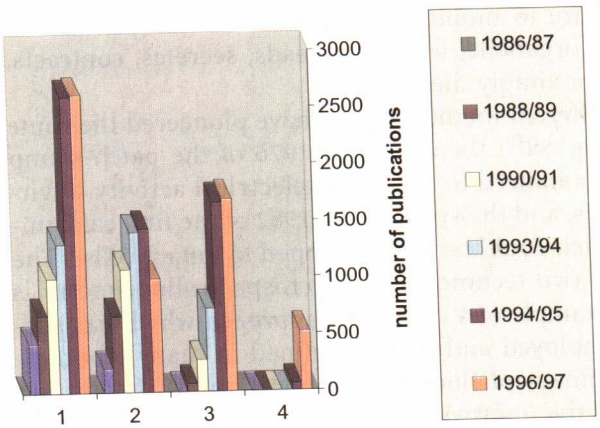

Fig. 1. Number of publications in the past 10 years on some of the experimental approaches discussed in this book. 1, patch-clamp; 2, calcium dyes (fura2, indo-1, fluo3); 3, confocal microscopy; 4, green fluorescent protein.

the state of art of some imaging techniques, providing, at the same time, detailed experimental protocols. Covering the whole imaging field in a single book is impossible; we thus decided to discuss extensively a limited number of broadly different applications, which cover the most common imaging tools and tasks. The following contributions describe the techniques based on the direct experience of the researchers, with a detailed discussion of the advantages and pitfalls of the experimental approach. Obviously, some researchers will find that their specific application of interest is not discussed in the book, but we hope that they will derive useful information for their experimental need from the thorough description of a related topic.

The book is divided in three parts. The first one introduces the instrumentation. It is now clear that confocal and wide-field microscopy are complementary techniques in cell imaging, with advantages and disadvantages which depend on the experimental conditions. Although the principles of confocal and traditional fluorescence microscopy are common knowledge among biologists, Section 1, by Mason and coworkers, will provide a deeper insight and thoroughly update of the most recent technologies, now available to imaging cells with different probes and combined approaches. In Section 2, Carrington and coworkers describe the algorithms used for processing wide-field images, an approach which, by removing the out-of-focus haze, allows to increase the resolution below the optical limits of the microscope.

The second part deals with fluorescent dyes. An extensive overview of both classical and new fluorescent calcium probes is presented in Section 3 by Hofer and Scheenen. The section describes the main strategies up to now developed to monitor calcium changes in the cytosol, in organelles and specific microenviroments such intra- or -extracellular sites near the plasma membrane. The following sections analyse in detail some specific applications of fluorescent dyes. In Section 4, Bolsover shows the advantages, pitfalls, and risks in data interpretation while using different specimens and confocal setups. Robb-Gaspers and coworkers (Section 5) extends the imaging field from isolated cells to cells in the whole organ. By means of either confocal and epifluorescence-deconvolution techniques it is shown how intracellular and intercellular events are finely tuned and differently modulated.

One of the most powerful approach is based on the combination of microspecrofluorimetry with electrophysiology. Zweifach

and coworkers (Section 6) describe the characteristics of the most frequently used setups, those based, respectively, on fura-2 and indo-1 photometry. The potentials of this approach is exemplified by the identification, in non excitable cells, of the store-operated-calcium channel, and the characterization of its permeability properties by calcium flux measurements. In Section 7, Garaschuk and Konnerth extend this approach to the imaging of brain slices, combined with neuronal afferent stimulation and whole-cell current recordings. By measurements of transmembrane calcium fluxes the calcium permeabilty of somata and dendritic receptor channels can be estimated under physiological conditions.

In Section 8, the use of fluorescent probes for other two relevant intracellular ions, Na^+ and H^+ is described, and exemplified in endothelial and smooth muscle cells, by Simpson and Sharma. Nicoletti and coworkers (Section 9) show how to combine fluorescent probes and antibodies to track complex phenomena such as apoptosis and cell death by flow cytometry. Finally, optical techniques and fluorescent dyes have been successfully employed to simultaneously monitor exo- and endocytosis as described in Section 10 by Ingrassia and coworkers.

The final part of this book presents the emerging field of recombinant proteins, by discussing the uses of aequorin, luciferase and GFP in cell biology. Aequorin will open the way, respecting its role of "pioneer" among the protein probes. Indeed, for over 3 decades, aequorin has been an important Ca^{2+} probe in living cells, since not only allowed pivotal discoveries, such as that of $[Ca^{2+}]i$ oscillations,[7] but also, it has recently proved that "subcellular" probes for physiological parameters can be constructed by specifically targeting a reporter protein.[5] Brini and coworkers summarize this work in Section 11, and discuss the principles of use of recombinant aequorin as a Ca^{2+} probe. In Sections 12 and 13, Rutter and coworkers then not only present the exciting extension of this approach to the single cell level, but also, along the same line, discuss in detail the use of recombinant luciferase for monitoring with high sensitivity gene expression. Finally, the last three sections refer to GFP. After a brief overview by Murgia and Rizzuto (Section 14) on the instrumental and technical requirements for imaging GFP in mammalian cells, De Giorgi and Rizzuto discuss a number of applications of GFP mutants in cell biology studies (Section 15). The last section by Haseloff presents the successful application of the GFP technology in plants, thus allowing to open a window on the

rapidly developing field of plant cell biology. The recent demonstration that GFP chimeras, with sensitivity to key intracellular parameters, can be successfully constructed,[8] indicates that these techniques have the potential to be extended to new exciting applications. In the very next future, also plants, which are notoriously difficult to load with chemical probes, will become easily accessible to the cell signalling field.

Finally, we would like to thank Prof. Tullio Pozzan, for his invaluable comments and suggestions through our editorial work and, together with the other excellent contributors, our younger collaborators, who accepted the task of writing "in-house" the introductory chapters on the techniques employed in our laboratory.

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