

A microscopic setup for combined, and time-coordinated electrophysiological and confocal fluorescence microscopic experiments on neurons in living brain slices

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In this paper, a microscopic system for cell physiological research is presented. The setup which is to a large extent based on commercially available products was designed to establish a platform for time-coordinated electrophysiological and fluorescence optical compound experiments on living neurons in brain slices. Instruments for infrared differential interference contrast video microscopy (IRDICM), confocal scanning laser microscopy (CSLM), and for patch clamp studies have been assembled into one unit. Using the IRDICM equipment, a neuron can be patched somatically and dendritically. Loading the neuron with a Ca^{2+} indicating dye substance can be examined epifluorescence optically using the Hg lamp or Xe lamp of the microscope. A stimulus initiating the propagation of an action potential through a dendrite can be synchronized to the electronic control unit of the CSLM, and changes in the concentration of Ca^{2+} in the dendrite can be recorded in a time-coordinated way. The setup has been used successfully in order to study *in vitro* the dynamics of intracellular Ca^{2+} in the dendritic system of living neurons in brain slices. © 1996 American Institute of Physics. [S0034-6748(96)01502-3]

I. INTRODUCTION

The coordination of the information flux in neuronal systems, in particular the physiology of signaling between neurons, and the signal conductance via a single neuron are subjects of intensive research.

A variety of techniques is used in order to experimentally study those events, which are measurable manifestations of links in the complex chain of molecular-physiological processes that establish neuronal communication:

Electrical cell signaling in neurons can be recorded by means of the patch-clamp method (PCM).¹⁻⁴

The infrared differential interference contrast video microscopy technique (IRDICM)^{5,6} considerably enlarges the variety of experimental possibilities for electrophysiological research^{7,8} by increasing the image contrast and reducing light absorption and scattering, hence making visible tiny structures in the brain slice and allowing for, e.g., patching a dendrite.

Neuronal activity generally coincides with intracellular Ca^{2+} fluxes and changes in the concentration of intracellular Ca^{2+} , $[\text{Ca}^{2+}]_i$. Applying fluorescent Ca^{2+} indicators,⁹⁻¹² neuronal activity can be imaged by means of a confocal scanning laser microscope (CSLM)^{13,14} either confocally¹⁵⁻¹⁷ or nonconfocally.¹⁸ In some cases, compared to conventional fluorescence microscopy, confocality might set a lower limit to the speed at which image acquisition can be done.

The methods mentioned are complementary. Cell physiological experiments can benefit from an arrangement com-

binning the three individual techniques, PCM, IRDICM, and CSLM, into one instrument,¹⁹ especially if this would offer the possibility to time coordinate electrophysiological events and their fluorescence-optical manifestations (see Fig. 1).

Here, the realization of such a setup is reported. It was assembled by mostly using commercially available products. No modifications have been made of the internal electronics of the CSLM, and the results have been achieved employing the available basic functions of the instrument.

The main purpose of the described instrument is the possibility to simultaneously, and in a time-coordinated way combine confocal imaging of biochemical events (e.g., changes in $[\text{Ca}^{2+}]_i$) in a living neuron and electrophysiological recording of electrophysiological events in this neuron. An account is given on using the setup to measure the increase of $[\text{Ca}^{2+}]_i$ as a function of time at different positions of the dendrite.

II. DESCRIPTION OF THE INSTRUMENT

A. Mechanical and optical construction

The setup which includes in one instrument the three named techniques is built around a Zeiss "Axioplan" microscope (see Fig. 2). In order to be able to use it for two optical purposes, namely fluorescence studies using a CSLM, and IR studies using DIC optics and an IR video camera, the microscope was fitted with a phototube with two photo outputs including a flapper mirror, which allows for switching from one photochannel to the other.

The IR video camera (model C-2400-07, Hamamatsu Photonics, Hamamatsu City, Japan) was mounted to the horizontal photo output of the phototube. Far-red and near-IR light was selected by suitable infrared filters (see Sec. IV).

The CSLM used was a "Phoibos 1000" (Molecular Dynamics, Sunnyvale, CA). It is fitted with a three-wavelengths

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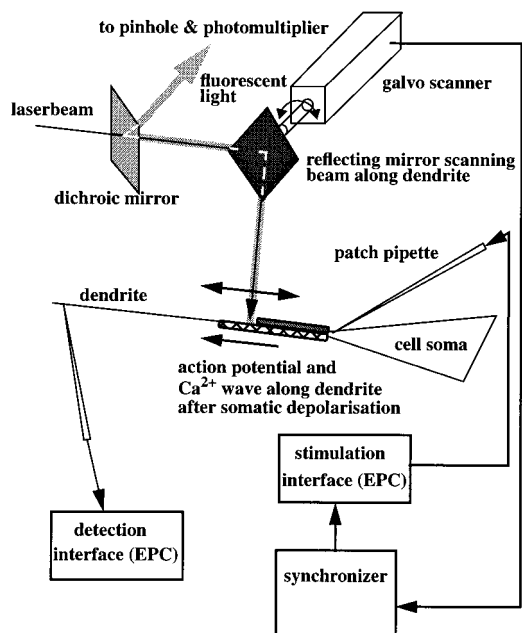


FIG. 1. Somatic depolarization causing an action potential is synchronized to one of the scanning mirrors of a confocal scanning laser microscope. The event induced by the stimulus to the cell (a Ca^{2+} transient expanding along the dendrite) is hence time coordinated to the scanning procedure. The rise of $[\text{Ca}^{2+}]_i$, resulting in a change of the intensity of the fluorescence of a Ca^{2+} indicating dye, can be recorded at any coordinate of the dendrite by means of the CSLM while the arrival of the action potential at the end of the dendrite can be recorded as an electrical signal by means of a patch pipette.

(456, 488, and 514 nm) air-cooled argon-ion laser (model 60X, American Lasers Corp., Salt Lake City, UT) This CSLM has two photomultiplier detector channels so that, using appropriate dichroic filters and detector filters, simultaneous dual color recording is possible. The optical cabinet of the CSLM was mounted onto four X48 profiles (Newport-Micro Contrôle, Évry, France) by means of CX48A assembly carriages and optically connected to the upper output of the phototube. In order to further stabilize the setup mechanically, the upper ends of the X48 profiles were interconnected by means of 10-mm-thick aluminum bars. This construction has several advantages, compared to the original mounting:

First, the CSLM unit is easily adaptable to any upright microscope irrespective of the different mounting heights which are demanded by different types of microscopes and microscope components.

Second, in the original setup mechanical scanner vibrations were transmitted to the microscope stage so strongly that the tip of a patch pipette controlled by an $x-y-z$ micromanipulator, which was screwed to the stage, was showing resonance vibrations. The strong X48 profiles helped to provide an improved mechanical stability thereby suppressing these vibrations.

Third, by appropriately placing the left front support of the optical cabinet of the CSLM, it became possible to fix a set of two micromanipulators with micropipettes to the stage, one to the left, another to the right, as was demanded to perform the planned, cell physiological experiments. This

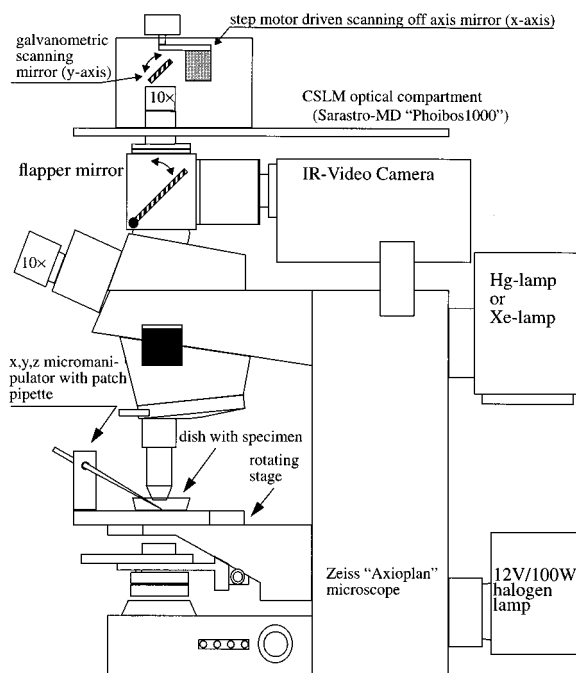


FIG. 2. The components of the instrument, consisting of three functional units: the microscope, including the DIC optics, and IR video camera, the patch pipettes mounted to a rotation stage, and the CSLM. It is possible to simultaneously perform time-coordinated electrophysiological measurements and recordings of fluorescence images with the CSLM. Alternatively, electrophysiology and IR-DIC video microscopy can be combined. Alteration between both optical pathways is done using the flapper mirror in the binocular phototube (model 452925, Zeiss). The used objective (Achromplan $40\times/0.75$ W, Zeiss model 440091) and ocular (Photo ocular S-P1 $10\times/20$, Zeiss model 444039) were suitable for both fluorescence imaging in the visible wavelength region and IR-DIC video imaging. The confocal microscope has been described in detail by Carlsson and Liljeborg (Ref. 14). Information is also available by the www (Ref. 31).

had not been possible on the original setup due to space limitations.

Finally, it was possible to replace the x/y cross stage by a larger, combined x/y cross and rotation stage which was necessary to orient the dendrite of a neuron in study parallel to the scanning direction of the galvo mirror. Also, to achieve an optimal DIC image of the specimen, this must be oriented according to the shadowing performed by the Nomarski optics in the DIC image.

B. Simultaneous, time-coordinated electrophysiology and imaging of biochemical events

When monitoring changes of the concentration of free intracellular Ca^{2+} fluorescence optically by means of Ca^{2+} indicating dye substances, a problem is induced because different intracellular Ca^{2+} buffers, among which the dye is only one, compete in binding Ca^{2+} ions. A steady state regulated by the different dissociation constants (K_d) of the different buffers is not established instantaneously during the change of the concentration of free intracellular Ca^{2+} . Rather, during a short period of time not the K_d values but the reaction speeds of binding or releasing Ca^{2+} by different buffers govern the distribution of the Ca^{2+} ions to the different buffers. In general, several intracellular processes which

happen on different time scales are involved in the binding of free intracellular Ca^{2+} ions or the release of Ca^{2+} ions. Detailed studies have been done, e.g., by Zhou and Neher.²⁰ For the following technical explanations, these problems are not relevant.

The rise of $[\text{Ca}^{2+}]_i$ in a dendrite is induced by an action potential and normally takes place within milliseconds. The rise of calcium can be studied at any point of the dendrite by means of separate measurements for each individual point. However, such measurements can also be performed with a CSLM which, like the Phoibos 1000,¹⁴ does not allow measurements at a fixed point but which offers a line scan mode.

When using the "line scan mode," only the fast galvanometric mirror is engaged, but not the slow mirror which performs scanning in the perpendicular direction. The scan speed of the galvanometric mirror is 50 Hz, i.e., it takes about 20 ms to scan one line.

The present approach is limited to the case when the dendrite is approximately straight. It can then be oriented by means of the rotating stage (see Sec. II A) to coincide with the line covered by the laser beam in the line scan mode. To obtain the rise time curve at a selected point along the dendrite a series of measurements is performed. Each measurement constitutes the recording of a scan line, where the delay between the start of the scan and application of the action potential is accurately controlled. A certain pixel along the line is selected, representing the desired position along the dendrite. The measured quantity obtained at each measurement is the value of this pixel. It represents the fluorescence intensity at this position at a certain time. By varying the delay time from one measurement to another the variation of the fluorescence intensity with time is studied. The time interval between consecutive measurements must be long enough that all transients after the previous measurement have died out. A time interval of 1 min or more will assure that the dendrite has recovered completely. Rather than selecting the value of a single pixel at each measurement, the value of a series of consecutive pixels can be selected and the mean value calculated. This is the technique referred to in the results described later (see Fig. 4). Each measurement supplies one point of the rise time curve. A transistor-transistor logic (TTL) signal provided by the electronics driving the scanning mirror is used to trigger a delay circuit which in its turn releases the action potential (see Fig. 3). By delaying the stimulus to the cell, the laser beam which moves slower along the dendrite than the transient, is given a head start. At the actual experiments the delay time has been changed in steps of 500 μs . In the practical realization, a negative TTL flank indicates the start of a scan of the mirror of the confocal microscope. The stimulus to the cell is synchronized to this negative TTL flank which is fed into a manually or automatically controllable NIM slot ("delay unit"). Depending on the coordinate x of the dendrite just being measured, at time $t_{\text{delay}}(x)$ the delay unit forwards a pulse to an Apple Macintosh Computer (model Quadra 8400 AV) which is used to drive the DA/AD board and amplifier for the pipette stimulation and recording of the electrical signals from the cell (an EPC9 unit, List Electronic, Darmstadt, Germany). The software controlling the stimulation and re-

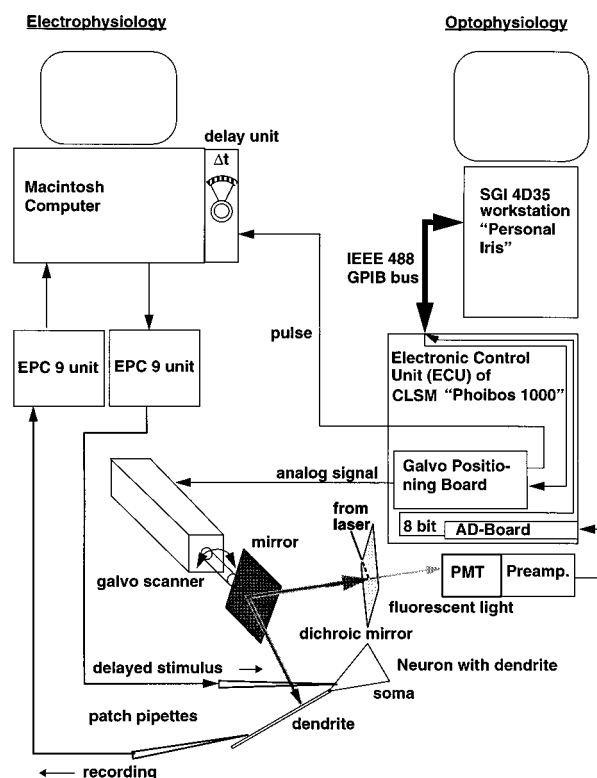


FIG. 3. Simultaneous and time-coordinated electrophysiology and CSLM imaging of cell physiological processes. Acquisition of the optical signal is done by the CSLM, the electrical stimulation of the cell and the recording of the electrical response is performed by means of EPC9 units and a Macintosh computer. It is possible to synchronize the scanning of the laser beam along the specimen, controlled by an analog ramp signal, and the electrical stimulus to the cell via a patch pipette. For any randomly selected but fixed point of the dendrite, x , the stimulus can be delayed. Performing a series of experiments during which the delay is varied systematically in steps which are not any longer than 500 μs , it is possible to record the rise of $[\text{Ca}^{2+}]_i$ at any point of a dendrite with a resolution of at least 500 μs .

coding of the electrophysiological data is a commercial product ("Pulse" by HEKA Elektronik, Ratingen, Germany). Communication between the Macintosh computer and the EPC9 unit is done by means of a Mac-23 board (HEKA Elektronik, Ratingen, Germany).

III. TEST AND APPLICATION OF THE INSTRUMENT

The instrument has been successfully used for the purpose described above (Markram, H., Heidelberg, 1993, 1994, 1995, personal communication). The physiological results obtained have been published elsewhere.^{21,18}

Briefly, pyramidal neurons in the rat neocortex were stained by injection with a membrane impermeable Ca^{2+} indicating dye substance, e.g., Calcium-Green or Fluo-3 (Molecular Probes, Inc., Eugene, OR) and their reactions to stimulations have been recorded.²² It was possible to image the rise of $[\text{Ca}^{2+}]_i$ at individual points of the dendrite with a time resolution of 500 μs (see Fig. 4). In order to simplify the experimental procedure, most of the measurements were done using the CSLM in its nonconfocal mode. This was possible because the autofluorescence of the specimen was low ($\approx 10\%$ of total fluorescence) and the dye was membrane

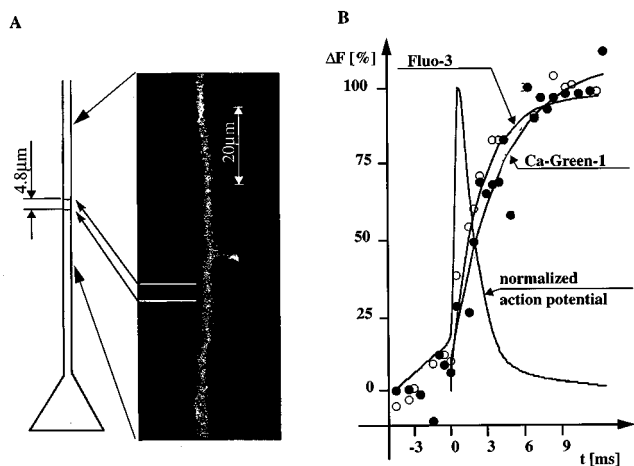


FIG. 4. Example (Ref. 22) for the results of measurements done on the instrument. (A) The right-hand part shows the confocal three-dimensional image of a part of the dendrite of a layer V pyramidal neuron from the neocortex of a rat, a schematic representation of the entire dendrite is shown to the left-hand side. Cells of this type were either loaded with Calcium-Green I or Fluo-3 (Molecular Probes, Eugene, OR). After electrical stimulation, a rise in intracellular concentration of Ca^{2+} ions was detected using the described sampling technique. The region of interest is marked by the two parallel arrows. Rather than selecting the value of a single pixel, the mean value of fluorescences of consecutive pixels have been used. (B) Curves showing the stimulation pulse as well as the rise in intensity of the fluorescence signal as measured in the short region of the dendrite indicated to the left. $[\%]\Delta F = F - F_{\text{basal}}/F_{\text{basal}} \times 100$, where F_{basal} is the initial fluorescence intensity before the stimulus is applied to the cell. The slight difference between the rise time curves of Fluo-3 and Ca-Green-I is probably due to different speeds of the chelating reactions and interactions with other intracellular Ca^{2+} buffers.

impermeable. Therefore, one could be sure that most of the fluorescent light ($\approx 90\%$ of total fluorescence) emanated from the dendrite, only, and not from its surroundings. Since the dendrite was only few microns in diameter, no confocal axial resolution was necessary in this study and the lateral resolution of nonconfocal microscopy was sufficient.

IV. DISCUSSION

Electrophysiological setups often use inverted microscopes. If long distance condensers are used, the specimen is more or less freely accessible for pipettes. The use of an inverted microscope was also considered for the apparatus described in this paper. However, this idea was rejected for three reasons.

First, tests with the IR video camera on a Zeiss Axiovert 135 using a long distance objective LD-Achroplan $40\times/0.6$ Korr, Zeiss model 440864 (necessary due to the thicknesses of both bottom glass of the dishes used for keeping the specimen, and the brain slice itself), and a long distance condenser (necessary to have free access to the specimen for patching) have revealed reduced resolution and light collection efficiency compared to upright microscopes as could be expected due to the comparatively small numerical aperture which a LD objective, by construction optimized for long working distance enforcing a reduced numerical aperture, has.

Second, cleaning the cell from covering tissue as described by several authors^{23,24,7} which always has to be done by accessing the cell from the upper side of the slice while viewing it microscopically is more difficult on an inverted microscope.

Third, with the inverted microscope it would not have been possible to view the patch pipette at the same time as the cell in the study before the patch was done since it has proved impossible to focus the objective (which is underneath the slice) to the patch pipette through the slice (Schiller, Y., Heidelberg, 1993, personal communication).

The use of IR light is not only advantageous for doing DIC microscopy on specimens as thick as brain slices, for many purposes it is unavoidable to work with IR light. However, there are also some problems.

The amount of light available with the 12 V/100 W halogen lamp is not always sufficient on specimens as thick as brain slices to produce a good video image. A compromise had to be accepted between sufficiently high transmission of the combination of heat protection filter ($T \approx 80\%$ at 600 nm, $T \approx 40\%$ at 700 nm, $T \approx 10\%$ at 750 nm) and IR filter on the one side and selection of a band of possibly long wavelengths on the other side. Depending on the used objective magnifications requiring different light intensities, either an RG9 filter (Schott Glaswerke, Mainz, Germany) or a 700BP100 filter (Chroma Technologies or Omega Optical, Inc., both Brattleboro, VT) was applied.

The microscope optics used during the experiments (objective: Achroplan $40\times/0.75$ W, Zeiss model 440091, ocular: photo ocular S-P1 $10\times/20$, Zeiss model 444039) was applicable without any problems over the entire wavelength range used. However, no confocal imaging has been done in the infrared.

The described instrument has proved to be a useful tool for cell physiological research; it is applicable to both confocal and nonconfocal scanning optical microscopy and the time resolution which can be reached is at least $500 \mu\text{s}$ ¹⁸ (see Fig. 4).

To further increase time resolution, single point recording would be useful.²⁵ Limiting the recorded region to a single point, it is possible to sample at rates of up to 200 kHz confocally or nonconfocally.

In order to improve the optical possibilities, a new water immersion objective for long working distance and high numerical aperture is currently being designed (Maxwell, J., London, 1993, 1994, 1995, personal communication). It will be of the catadioptric type,²⁶⁻³⁰ have a numerical aperture of at least 1.1 at $50\times$ magnification, and a clearance of at least 2 mm.

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