DEVELOPMENT OF A VERSATILE SPECTRO-FLUOROMETER FOR KINETIC STUDIES OF SINGLE WHOLE-CELL CLAMPED CELLS

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Ratiometric tecniques using fluorescent indicator dyes have been widely used to measure ion concentrations ($[Ca^{2+}]_i$, $[Mg^{2+}]_i$, $[H^+]_i$, $[Na^+]_i$) in single voltage clamped cells. To fully explore the pitfalls of the conventional dual wavelength technique (bleaching, auto fluorescence, compartmentation), to compare the kinetics of different indicator dyes, and to resolve signals from different dyes used simultaneously, we have developed a new cellular spectro- fluorometer. This computer controlled equipment has the ability to change rapidly between different modes of operation and to collect data either with high spectral resolution or with emphasis on rapid kinetic studies.

The spectro-fluorometer forms an integral part of a whole-cell voltage clamp setup (Dagan amplifier) built around an inverted microscope (Nikon). The optical path is shown in Figure 1A. The voltage clamped cardiomyocytes (C) were epi-illuminated by an xenon arc lamp (AL) via a monochromator (S1-VG1-S2), a dichroic mirror (DM, 450 nm longpass) and an objective (OB, NA = 1.3, x40). The fluorescent light was separated from the excitation beam by the dichroic mirror and was measured by a photomultiplier detector (PM) after passing through a second monochromator (S3-VG2-S4). The adjustable slits of the monochromators (S1, S2, S3 and S4, 0.4 to 3.2 mm) determined the spectral resolution (roughly 2 to 16 nm), and were used also to match the fields of illumination and detection (10 to 80 um wide by 160 um long). The excitation and emission wavelengths were determined by the custom built "vibrating gratings" (VG1 and VG2), each of which was composed of two focussing mirrors, a diffraction grating (1200 lines per mm, blazed for 400

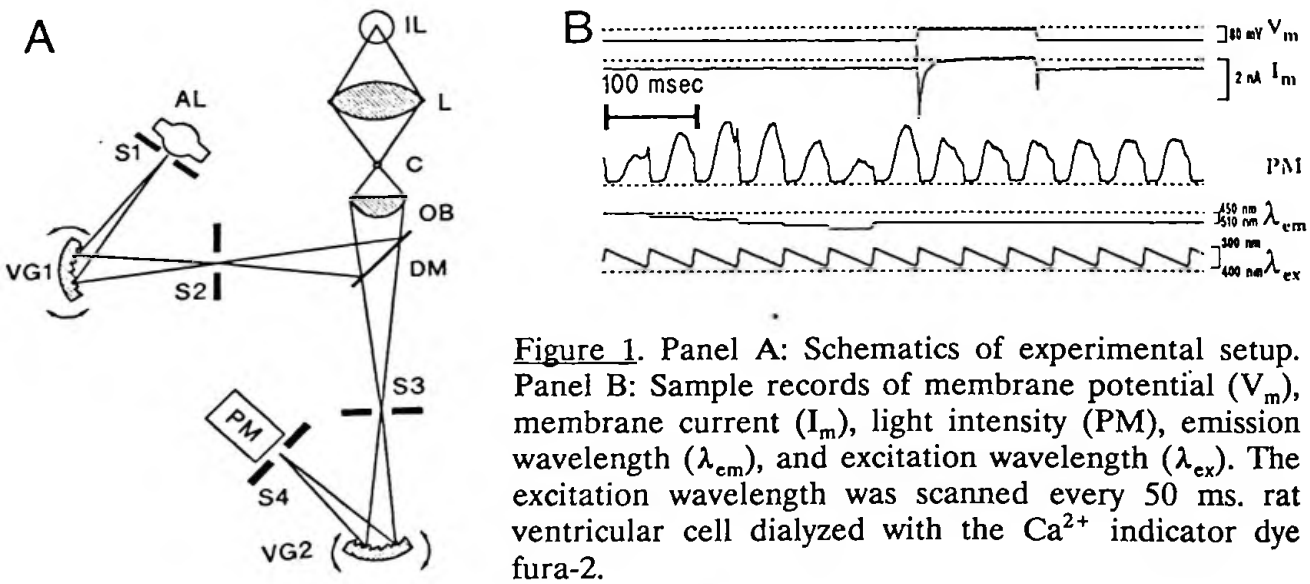
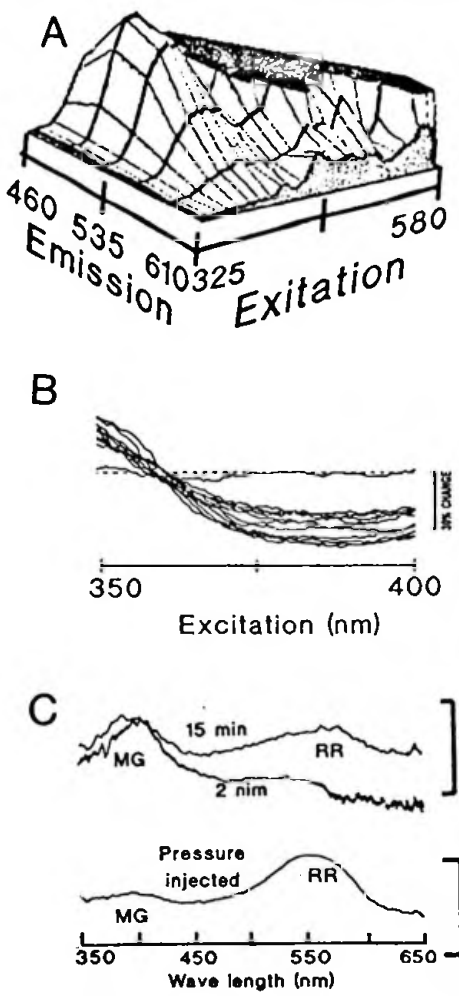


Figure 1. Panel A: Schematics of experimental setup. Panel B: Sample records of membrane potential (V_m), membrane current (I_m), light intensity (PM), emission wavelength (λ_{em}), and excitation wavelength (λ_{ex}). The excitation wavelength was scanned every 50 ms. rat ventricular cell dialyzed with the Ca^{2+} indicator dye fura-2.

or 500 nm) and a servo controlled mirror. An IBM 386 compatible computer, equipped with two Labmaster AD/DA converter boards, was used to control the membrane potential and the angular positions of the mirrors and to sample the membrane current and the light intensity (Fig. 1B). Custom designed software provided a flexible tool for design and execution of complex protocols.

Fig 1B shows an example where the acquisition of a fluorescence spectrum (fura-2 fluorescence vs. excitation and emission wavelengths) is immediately followed by the repeated measurement of excitation spectra during a depolarization induced Ca^{2+} transient.

The measurement of the detailed fluorescence spectrum has the potential for distinguishing several fluorescence sources based on their characteristic excitation and emission properties. Figure 2A shows a 3-dimensional projection of the spectrum recorded from a cell dialyzed with both fura-2 (smooth nearby hill) and fluorescein (ridges in background corresponding to 3 or 4 excitation peaks around 500 nm). Figure 2B shows changes in the relatively measured excitation spectrum of fura-2 during a depolarization induced $[Ca^{2+}]_i$ transient. The isosbestic point is stable close to the <u>in vitro</u> value, 360 nm. The measurements of fluorescence were supplemented with measurements of absorption. In this case an incandescent light bulb (IL, fig. 1A) and a lense (L) were used to trans-illuminate the cell while the photomultiplier-based detection system was unchanged. Fig. 2C shows that such measurements can be used to verify the presence of a non-fluorescent drug like ruthenium red (RR) inside a cell. The pentration of the dye was slow (top traces) in the absence of pressure injection (bottom traces).



It is concluded that this equipment can be used to rapidly measure the fluorescence and absorption of single cells with high spectral resolution.

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<u>Figure 2</u>. Spectra . Panel A: Fluorescence spectrum recorded from rat ventricular cell dialyzed with 100 μM fura-2 and 20 μM fluorescein. Emission and excitation wavelength are in nm. Panel B: Relative excitation spectra measured repeatedly during a depolarization induced Ca^{2+} transient in a myocardial cell dialyzed with 200 μM fura-2. Panel C: Absorption spectra in myocardial cells loaded with ruthenium red (RR) via the patch pipette either by diffusion (top) or pressure injection (bottom).