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Coupling of Simultaneous Fluorescence and Electrophysiology: Historical Survey, Contributions, and Prospects[1]

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The coupling of simultaneous fluorescence and electrophysiology measurements paved the way for the development of potentiometric and intracellular free ion-sensitive probes, although the basic and initial aim of these combined studies remains to record fast conformational changes during ion channel activation. Recent high-resolution studies of some channels put back on the agenda challenging methods that combine spectroscopy and electrophysiology. Fluorescence is certainly the most versatile and sensitive spectroscopy in this kind of experiment, and we have recently witnessed significant breakthroughs, at the level of whole cells with intact or mutated channels or with planar lipid bilayers doped with channels or their peptide models. After our initial study of membrane dynamics associated with excitability, following transient pyrene excimer signals during action potentials or voltage-clamp of nerve fibers, we tested the feasability of FRAP (fluorescence recovery after photobleaching) experiments on planar lipid bilayers. This technique was later applied to lateral diffusion measurements of channel forming peptides (alamethicin labeled with fluorescein and the voltage-sensitive segment S4 of the *Shaker* potassium channel labeled with NBD) under applied voltage. We provide independent evidence for voltage-dependent partitioning and transmembrane insertion and propose renewed experimental avenues to reveal movements and conformational changes associated with ion channel gating and opening.

KEY WORDS: Excitable membranes; planar lipid bilayers; ion channel gating; extrinsic fluorescence; lateral diffusion.

INTRODUCTION AND HISTORICAL SURVEY

In the late 1940s, while the seminal electrophysiological experiments of Hodgkin and Huxley on the squid giant axon were carried out at the Marine Biological Laboratory in Plymouth (UK), the Cambridge biophysicists A. V. Hill and R. D. Keynes showed that repetitively stimulated crab leg nerves developed accumulating light scattering changes [1]. In the midst of the heyday of classical electrophysiology (dissection of the membrane ionic currents involved in action potentials, pharmacology of specific blockers . . .), and also at a turning point (1972) when the "fluid mosaic" model was proposed, it was soon realized that electrophysiological recordings alone could not give much indication about the membrane structural changes that ought to be associated with "activation" and the fast and huge albeit specific ion fluxes. In those years, and although Hodgkin and Huxley put forward the hypothesis of gates that control the ion path-

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ways, the latter were not formally identified as channel proteins until well into the 1970s.

Early Intrinsic Optical Studies of Nerve Activity

Twenty years after the first optical experiments on nerves (see Table I for a summarized historical sketch), the technology available, especially signal averaging, put coupled optical–electrophysiology back on the agenda. The stakes also had been raised: not only should an "optical spike" be demonstrated, but it should be put to the test of voltage-clamp conditions. Indeed, the quest was not only for signals simply or linearly correlated with membrane potential changes but, instead, for those that could reflect conductance kinetics or channel gating transitions. The comprehensive full analysis by Cohen *et al.* in 1972 [2] revealed that the interesting light scattering signal developing upon depolarization, i.e., during channel opening, was in fact reflecting the accumulation of potassium ions in the "periaxonal space" and the ensuing fiber swelling [2].

The preceding year, Cohen *et al.* [3] had demonstrated another optical signal, a transient birefringence change that was synchronous with the propagated action

Table I. The Place of Coupled Fluorescence–Electrophysiology Experiments in Ion Channel Research

Channel proteins		Channel forming peptides
	1952	
Hodgkin & Huxley Voltage-clamp, giant axon Ionic theory of nervous impulse Gates hypothesis		***/*** Langmuir
	1968	
Intracellular perfusion & pharmacology . . .		Excitability inducing material, alamethicin: action potentials in planar lipid bilayers
	1972	
Coupling of spectroscopy + electrophysiology	"Fluid mosaic" membrane dynamics	Single-channel recordings (gramicidin)
	1974	
Gating currents		"Barrel-stave" model (alamethicin)
	1978	
Purification & biochemistry of channels		Kinetics of single channels
	1982	
Improved patch-clamp		Crystallographic structure of alamethicin
	1984	
Amino acid sequences Molecular Biology		Macroscopic I–V analysis (alamethicin)
	1988	
Site-directed mutations	"Key" residues & segments	Peptide approach strategy
	1992	
"Structure–function" models & channelopathies	Molecular modelization dynamics	"New" antimicrobial peptides
	1998	
	Crystal structure of a minimal (i.e., non-voltage-gated) bacterial K^+ channel & Comeback of combined fluorescence–electrophysiology	

potential in single squid giant axons. The voltage-clamp analysis of this signal showed that it was dependent predominantly on the square of membrane potential changes [3] and thus difficult to correlate with gating. However, in a much later study by Landowne (1985), the time course of the birefringence change was found to share many of the features of gating currents [4]. The latter represent the movement of charged "particles" within the channel that control the gates and the subsequent opening and can be put into evidence only after all ion currents are blocked and incidentally also with averaging. They were discovered in 1974 and in fact took the relay of optical experiments (see Table I). Pharmacological agents such as internally applied colchicine, disrupting the cytoskeletal network and possibly its binding to sodium channels, and externally applied chloramine-T, removing inactivation, specifically altered the birefringence signal during depolarization [5,6]. Landowne's findings were consistent with a change in the alignment of several hundred peptide bonds per sodium channel and a "rocking helix model" was proposed [6]. Fifteen years later, this model is still one of the most plausible overall mechanism and indeed begins to be confirmed in quite recent experiments, albeit for a different channel, K_{CSA}, a bacterial potassium channel with the same central permeation motif as the eukaryotic seven-transmembrane segments channels, gated not by voltage but by pH changes. Its crystallographic structure was resolved in 1998 [7] and its opening–closing scheme studied through spin-labeling and electron paramagnetic resonance involves helix tilting and rotation [8]. Even with the much more complex sodium channel, the same mechanism might hold [60].

Another earlier study took advantage of the high density of axonal membranes in nerve trunks such as the olfactory nerve in the pike and managed to record an optical spike, i.e., a high signal/noise ratio (S/N) bifringence signal during a single stimulation sweep [9]. Detailed kinetic comparisons with the superimposed electrical spike, and especially with thermal spikes [10], allowed interpretation of these retardation changes as a decrease in entropy, an increased ordering of the radially oriented molecules in the excitable membranes, mainly phospholipids. This was partly consistent with our own investigations using the fluorescent probe pyrene (see below). Finally, with a related technique, changes in optical activity arising from chiral molecules were recorded from nerve fibers using a photoacoustic modulator [11]. Reversing the nerve impulse direction on the same nerve fiber produced an astonishing reversal of optical activity changes, suggesting a macroscopic ordering of ion channels along the fiber [12,13].

Faced with the complexity of these intrinsic signals that could not be correlated with conductances or, better, with gating in a straightforward way, investigators soon shifted to extrinsic fluorescence of labeled nerves. It is to be noted that other physical and nonelectrical experimental alternatives were also assayed including an infrared study which concluded that the electric field-induced shifts of some the infrared peaks during nerve stimulation were due to phospholipids [14].

General Principles and Experimental Considerations in Experiments Combining Electrophysiology and Fluorescence

Compromises must be found between the two kinds of measurements that ought to be recorded simultaneously on the same preparation. In particular, one has to allow easy access for electrodes and optimize the light excitation (stabilized) and emission pathways and collecting system to improve the often inherently low S/N and avoid light scattering. It is quite often necessary to have recourse to signal averaging while avoiding preparation fatigue (during many sweeps of stimulation), photoinactivation (see, e.g., Ref. 15), and photodynamic damage. Of course, the labeling itself should not modify the essential channel functional properties. Due to light sources and photodetection efficiencies, the S/N increases with the wavelength and thus studies have been biased toward dyes absorbing and emitting at longer wavelengths. The squid giant axon has been the favorite preparation in this kind of experiment since, apart from its ease of dissection, membrane conductances are highly reproducible, membrane potentials are easily measured and controlled (both spatially and temporally), and its relatively large axonal membrane surface tends to increase the S/N [16].

Classical Experiments Combining Fluorescence and Electrophysiology

The first attempt at measuring fluorescence changes during action potentials of stained axons was pioneered by Tasaki *et al.* in 1968 [17], that is, concomitantly with the onset of studies devoted to the above-summarized intrinsic optical signals. The fluorescence intensity of nerve fibers stained with 1-anilinonaphthalene-8-sulfonate (1,8-ANS; a probe that was later widely used) changed during a single nerve impulse [17]. An extension of this study with the same probe as well as with rhodamine B and pyronin B, focusing on the voltage-clamp assay of the fluorescent response, demonstrated voltage-dependent behavior, although the response associated with depolarization was larger but slower than with an equivalent hyperpolarization [18]. This conclusion was substantiated in similar experiments on planar lipid bilayers [19], convenient artificial membranes, especially

for electrical measurements with or without added channel protein or channel forming peptide (see Table I), that were coming of age in the late sixties–early seventies. The same output still held with polarized signals issued from other polarity-dependent naphthalene-derived probes located near the membrane interface (as more recently reinvestigated with the "parallalax" method [20]), such as polarized fluorescent signals with 2-*p*-tolindinyl-6-naphthalene sulfonate (2,6-TNS) [21]. The authors postulated that longitudinal elements on the intracellular face of the axonal membrane were moving during excitation.

There followed an extensive study of more than 300 dyes by Cohen *et al.* (1974) in [22]. With the exception of malachite green and stearic anthroyl acid, whose responses were similar to light scattering signals, all these molecules induced purely voltage-dependent signals [22] and, hence, were of little use to probe conformational changes associated with gating. In 1978 R. D. Keynes [23] acknowledged that the channel density was too low to yield any meaningful specific optical response. For alternative views, see the reviews by Conti [24] and by Cohen *et al.* [25]. Nevertheless, the study by Cohen *et al.* [22] had an interesting pratical output: a single fluorescent "spike" could be recorded with a high S/N on a single sweep with a single giant axon and this was to prove quite useful much later in monitoring the activity of small excitable cells and neural networks (see below), where the use of microelectrodes or patch-clamp was impractical.

On Planar Lipid Bilayers

In parallel with experiments on the squid giant axon, and in a few instances by the same authors, were published coupled fluorescence–electrical measurements on planar lipid bilayers. In those "early years," bilayers were made by the "painting" method over a hydrophobically coated hole with lipids (often monoglycerides) dissolved in a nonvolatile organic solvent (decane), with the associated problems of annulus, "fat" lenses, and enhanced electrostriction.

One of the first meaningful studies involved gramicidin, a 15-amino acid antibiotic peptide with alternating R- and L-handed amino acids forming cation-specific channels (albeit voltage-independent) in planar lipid bilayers, which was "dansylated." The analysis was consistent with an "in-line" dimerization [26]. Earlier on, in 1972, Conti and Malerba [19] had shown that fluorescence signals arising from ANS-labeled bilayers were stricly voltage dependent.

OFFSHOOTS: VOLTAGE MONITORING AND FREE ION IMAGING

Following the discovery of high S/N potentiometric dyes, a lot of studies took advantage of this new methodology to investigate the activity of either individual cells too small to be impaled or patch-clamped or neural networks to record spreading of activity where the use of multiple microelectrodes is impratical. In most cases, care was taken to calibrate ΔV_M from fluorometric and voltammetric measurements. The search for potentiometric dyes continued (see, e.g., Ref. 27) and the physicochemical basis of cyanine dyes (the first high S/N probes being screened by Cohen *et al.* [22]) were more recently reinvestigated [28,29]. Recent examples of applications of such dyes include "regional" activity recordings of arborizations of identified molluscan neurons [30] and, quite recently, of ganglions for leech swimming using fluorescence resonance energy transfer [31].

As for calcium and other intracellular free ion imaging, the literature abounds with applications in excitable as well as in nonexcitable cells, thanks to the chemical design of specific probes. An inexpensive dual-ratioing excitation apparatus has been described [32], as well as new probes for noncalcium signaling, e.g., for sodium [33]. Optimally, calcium signals also have to be correlated with another independent response (e.g., pH_i) or with a stimulus, such as odorants in human olfactory neurons [34]. Recently, calcium sparks were shown to occur in quiescent ventricular myocytes [35]. The challenge is now to monitor calcium signaling/imaging in intracellular stores and mitochondria [36].

LIPID DYNAMICS AND MEMBRANE EXCITABILITY

The photophysical properties of pyrene, especially the formation of excimers or excited dimers (D* or E) via a diffusion-dependent process (excited monomer + ground state pyrene collision), make this aromatic hydrocarbon an interesting membrane fluidity probe. Indeed, the ratio of excimer (I_e)-to-monomer (I_m) emissions is

$$I_e/I_m = [P] \cdot TK/\eta$$

where [P] is the local pyrene concentration, η is the medium microviscosity, and T and K have their usual meaning.

Transient and opposite monomer and excimer fluorescence signals synchronous with the propagated action potentials were recorded in pyrene-labeled pike olfactory nerves (50 sweeps averaged). The positive monomer sig-

nal together with the negative excimer signal meant a transient reduction in axonal membrane fluidity [37]. Was this response arising from the bulk of the lipid bilayer or could it be specifically correlated with channel gating? In other words, were the signals simply voltage dependent or not? An analysis of pyrene excimer fluorescence signals in voltage-clamped giant axons showed signals only during depolarizing pulses [38] (with which are associated inward sodium I_{Na} and outward potassium I_K currents). These signals were additive during two successive depolarizations even when ionic currents were blocked by tetrodotoxin (I_{Na}) and 4-aminopyridine (I_K). There was thus a fluidity reduction specifically associated with channel gating. It was proposed that, during channel opening and "activation" volume change, the surrounding lipid layers around the proteins were laterally compressed.

Finally, pyrene, and especially its derivatives (e.g., linked to peptides [39]), remains a favorite probe. It also allows FRET (fluorescence resonance energy transfer) with a downward wavelength such as the protein intrinsic fluorophore typtophan [40] or upward with the common probes NBD and FITC (see below).

IMPLEMENTING FRAP IN PLANAR BILAYERS: DIFFUSION OF PORE FORMING PEPTIDES UNDER APPLIED VOLTAGE

Fluorescence recovery after photobleaching (FRAP) is another powerful method to measure membrane dynamics, at least one component, namely lateral diffusion of either lipids or proteins, according to which is labeled. In FRAP experiments, the fluorescence of a microscopic spot of the membrane is monitored with an attenuated laser beam. At time t, the full power of the incident light is delivered for a short time, leading to probe quenching within the spot. The return of labeled molecules replenishing the spot via lateral diffusion translates into an exponential recovery of the emitted fluorescence intensity. The fit of the revovery curve yields the lateral diffusion coefficient (D). Although this method has been quite popular with cells for about 20 years [41], it has not yet been applied to planar lipid bilayers, which are so convenient for ion channel reconstitution and measurements of their conductance properties, apart from the very early study by Fahey and Webb [42] using electron microscopy grids as "unconventional" bilayer support [42].

In collaboration with Alan Mackie and Shab Ladha (Institute of Food Research, Norwich, UK), a FRAP apparatus with an inverted microscope (reflection mode) arranged horizontally on an optical rail was adapted to a specially designed chamber amenable to both optical and electrical measurements on planar lipid bilayers [43]. The sytem was assayed with lipids labeled with *N*-(7-nitrobenzoyl-2-oxa-1,3-diazol-4-yl) or (NBD), 1% in the bilayer forming solution, and with conventional membrane fluidity modifiers (cholesterol, calcium with negatively charged phospholipids, etc.). Interestingly, it was found that the incorporation of a channel forming peptide did slightly reduce the lipid lateral mobility [43].

We then measured D for two labeled channel forming peptides: alamethicin and the S4 transmembrane segment of the *Shaker* K^+ channel. The former is a well-known antibiotic peptaibol whose macroscopic conductance is highly voltage dependent and which functions according to the "barrel stave" model, i.e., through intramembrane aggregation of helical monomers with a hydrophilic pore in the center of the bundle (for a review, see Ref. 44). As for S4, it presents Arg every three residues and constitutes the main voltage sensor underlying the gating of all physiologically important voltage-dependent ion channels [45]. Alamethicin and S4, labeled, respectively, with FITC (fluorescein isothiocyanate) at the C terminus [46] and NBD at the N terminus (with little influence on their conductances), were added to the *cis* side of neutral planar lipid bilayers in the above-described FRAP apparatus. Applied voltage (and hence the conducting state) reduced lateral diffusion, reflecting transmembrane insertion for alamethicin or a greater embedment/aggregation for S4 (see Fig. 1).

COMEBACK TOWARD THE BASIC AIMS

It took over 20 years to witness the comeback of fluorescence signals simultaneously coupled to electrophysiology with the initial basic goals: *to record conformational changes during channel gating or opening*. This is illustrated by two recent instances.

Specific Labeling of Amino Acids in Voltage Sensors of the Sodium Channel Expressed in Oocytes

In 1996 Mannuzzu *et al.* [47] reopened the field with the homotetrameric K^+ channel. Then Cha *et al.* [48] labeled specific residues in the voltage sensor S4s of all four homologous domains of the action-potential sodium channel. To these residues, most often comprising the first arginine of S4 segments mutated to cysteine, was attached tetramethylrhodamine-5-maleimide (TMRM). cRNA for these channels was expressed in *Xenopus* ooo-

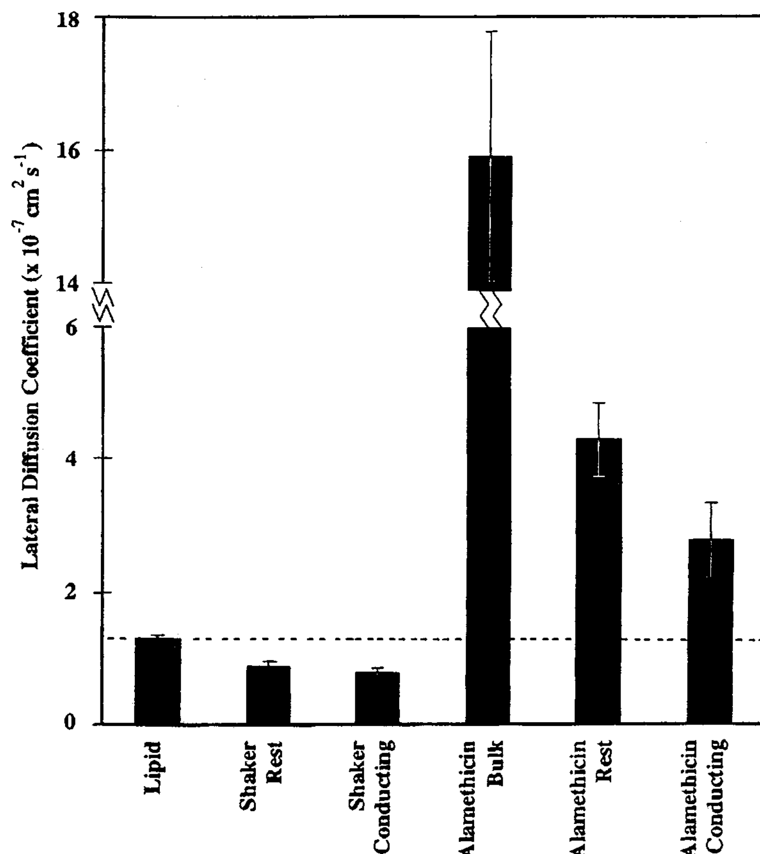

Fig. 1. Lateral diffusion of peptides in the membrane-associated state, at rest (0 mV applied) and in the conducting state (about 100 mV: 10 nA for alamethicin–FITC and 2 nA for the NBD–S4 *Shaker* peptide). Lateral diffusion of the bilayer lipids obtained with the NBD–PE probe [43] is shown for reference (dashed line), as well as the diffusion coefficient of alamethicin–FITC in the bulk.

cytes that were then "cut-opened" (for electrophysiological convenience and internal perfusion) and transferred to an epifluorescence setup allowing voltage-clamp. Comparing the fluorescence changes during voltage pulses of increasing amplitude with the macroscopic conductances for each of the labeled domains, the authors demonstrated a functional asymmetry [48] already proposed by Helluin *et al.* [49] from the peptide approach in planar lipid bilayers: domain III rises earlier than domain IV.

Movements or Conformational Changes of Model Peptides in Planar Lipid Bilayers Under Applied Voltage

In 1998 a study was published entitled "Simultaneous Measurement of Spectroscopic and Physiological Signals from a Planar Bilayer System: Detecting Voltage-Dependent Movement of a Membrane-Incorporated Peptide" [50], which nicely complements both investigations of fluorescence changes associated with whole-sodium channel gating (see above) and our own studies, specifically conductances induced by the four isolated voltage sensors (S4L45 segments) of the electric eel sodium channel [49] and FRAP measurements of lateral diffusion [46].

Indeed, Hanyu *et al.* [50] synthesized the voltage sensor S4 (domain IV) of the eel sodium channel and labeled its N terminal with tetramethylrhodamine isothiocyanate. Using a special chamber and an optical setup quite similar in design to ours, they were able to record fluorescence signals during a single sweep. As the peptide was initially in the *cis* side, whereas a quencher was added to the *trans* side of the bilayer, these experiments demonstrate bilayer crossing of the peptide or at least *trans* exposure of the labeled terminus [50].

CONCLUSIONS AND OUTLOOK

So far, experiments combining fluorescence and electrical measurements both in cell membranes and in

reconstituted systems have relied on extrinsic probes labeling either lipids or proteins rather than monitoring intrinsic fluorophores. The latter, mainly tryptophan (Trp) in proteins, may be too numerous in a given channel (even if not randomly localized) to yield any specific signals really associated with gating or the subsequent opening. However, in the sodium channel, Trp occurs at specific positions, especially in the pore lining segments, where they may be critical since the photodegradation of very few of them completely deactivate the channel [15]. Thus, the implementation of "tryptophan imaging" through energy transfer with extrinsic probes [51], especially during channel activity, might prove valuable, as well as the study of fluorescence decay recently revisited with model peptides [52]. It must, however, be acknowledged that some fluorescence techniques are more prone than others to be useful in the present context, either because of practical considerations in the coupling with electrophysiology or because of limitations imposed by the preparation. For instance, fluorescence energy transfer within the protein (see, e.g., Ref. 53) or in interaction with the immediate lipid vicinity is more likely to yield meaningful information than the otherwise elegant "single-particle tracking" methods [54,55]. Specific experimental approaches (including resolution at the single-channel level) have been proposed in the inspiring review by Macdonald and Wraight [56].

Site-directed mutagenesis may be a solution to an excess of intrinsic fluorophores but there remains the still outstanding problem of reaching a sufficiently high expression level. This will possibly be overcome by the application of two-photon excitation, not yet applied in these systems, and/or, when it comes of age, near-field optics. Note that Szmacinski *et al.* compared one-and two-photon excitation and lifetime analysis in calcium imaging using the dye Indo-1 [57]. This could prove useful when it comes to monitoring calcium in cellular compartments (for a higher S/N) such as mitochondria [36].

Even though voltage monitoring with optical and fluorescence methods continues to be an active field even in complex preparations such as *in vitro* brain slices [58], the comeback of coupled fluorescence (foremost, but other spectroscopies as well)–functional experiments with the basic aims in mind is most welcome after an eclipse of more than 20 years. This return to favor happens at the right time, i.e., when we begin to have a glimpse of channel and receptor structure in either their closed or their open states, thanks to high-resolution electron microscopy or three-dimensional crystallography. The present (and long-standing) challenge is thus recalled: "*The resulting picture is incomplete, however. We still do not understand the nature of the structural transitions involved in channel gating. To complete the picture, we will need . . . also snapshots of such a channel in its various conformational states*" [59].

With model peptides, we have provided independent evidence (other than electrical measurements) for the earlier steps in pore or channel formation: voltage-dependent partitioning and insertion. As for the final steps, we shall be relying on internal self-quenching within the bilayer when monomeric labeled peptides are brought together and, possibly, on FCS (fluorescence correlation spectroscopy). No doubt the latest developments in fluorescence, whether in new probe design or improvements of opto-electronic devices, together with the implementation of sophisticated and combined experiments, will further decisive outbreaks in ion channel research and help to solve that still-elusive gating.

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