

Voltage-sensitive dye recording of action potentials and synaptic potentials from sympathetic microcultures

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ABSTRACT Given the appropriate multicell electrophysiological techniques, small networks of cultured neurons (microcultures) are well suited to long-term studies of synaptic plasticity. To this end, we have developed an apparatus for optical recording from cultured vertebrate neurons using voltage-sensitive fluorescent dyes (Chien, C.-B., and J. Pine. 1991. *J. Neurosci. Methods*. 38:93–105). We evaluate here the usefulness of this technique for recording action potentials and synaptic potentials in microcultures of neurons from the rat superior cervical ganglion (SCG). After extensive dye screening and optimization of conditions, we chose the styryl dye RH423, which gave fast linear fluorescence changes of $\sim 1\%/100$ mV for typical recordings. The root mean square noise of the apparatus (limited by shot noise) was typically 0.03%, equivalent to 3 mV of membrane potential. Illumination for at least 100 flashes of 100 ms each caused no noticeable photodynamic damage.

Our results show that voltage-sensitive dyes can be used to record from microcultures of vertebrate neurons with high sensitivity. Dye signals were detected from both cell bodies and neurites. Signals from presumptive dendrites showed hyperpolarizations and action potentials simultaneous with those in the cell body, while those from presumptive axons showed delayed propagating action potentials. Subthreshold synaptic potentials in the cell body were occasionally detectable optically; however, they were usually masked by signals from axons passing through the same pixel. This is due to the complex anatomy of SCG microcultures, which have many crisscrossing neurites that often pass over cell bodies. Given a simpler microculture system with fewer neurites, it should be possible to use dye recording to routinely measure subthreshold synaptic strengths.

INTRODUCTION

Neuronal microcultures, by which we mean networks of < 10 neurons grown in cell culture, making synapses with each other but isolated from the outside world, are an elegant electrophysiological preparation for studying synaptically connected neurons. The neurons are easy to penetrate with electrodes; the small size and low cell number of the cultures make it easy to find synaptically connected pairs; the liquid environment can be manipulated at will; and development can be followed over weeks. Invertebrate microcultures have been used to study control of synapse formation (Ready and Nicholls, 1979; Hadley et al., 1985) and the properties of small networks (Parsons et al., 1989, 1991; Kleinfeld et al., 1990), and to reconstruct the in vivo behavior of a three-cell central pattern generator (Syed et al., 1990). Microcultures of neurons from the rat superior cervical ganglion (SCG) have been used to study transmitter plasticity (Furshpan et al., 1976; Higgins et al., 1984; Potter et al., 1986). Optical recording with voltage-sensitive dyes can record from many cells at a time. Given sufficient sensitivity, it should be possible to make a small network in culture, then use dye recording to monitor all activity (action potentials and synaptic poten-

tials) in the microculture. We set out to record from microcultures of rat SCG neurons in this way. We chose these neurons because they form an apparently homogeneous population, have previously been grown in microculture, and make fast cholinergic synapses with each other (Higgins et al., 1984; Furshpan et al., 1986).

Our eventual aim is to study short- and long-term synaptic plasticity. Changes in synaptic strengths are widely thought to underly at least some forms of learning and memory (e.g., Hebb, 1949; Brown et al., 1988). By analogy to the matrix of connection strengths T_{ij} commonly used in theoretical neural network models (e.g., Hopfield, 1982), one can define the “synaptic map” of a microculture: the synaptic strengths of all N^2 direct connections possible in an N -cell culture (this includes the possibility of cells synapsing back on themselves), where “synaptic strength” is defined as the amplitude of the sub- or suprathreshold postsynaptic potential (PSP). Given the appropriate electrophysiological techniques, it should be possible to measure this synaptic map, stimulating each cell in turn while recording synaptic responses from all the other cells. After applying an electrophysiological or biochemical stimulus, the resulting changes in synaptic strengths (ΔT_{ij}) could be measured. For instance, one could test for a Hebbian learning rule by synaptically mapping a culture, stimulat-

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ing neurons *a* and *b* for a time, and then looking for specific enhancement of connections T_{ab} and T_{ba} .

Such synaptic mapping experiments require stimulation and recording techniques that are (*a*) multicell and (*b*) noninvasive. First, one must be able to stimulate any cell and record from all the cells at once. Second, stimulation and recording must be noninvasive: to study plasticity, one must synaptically map the same culture at several successive times. Conventional intracellular electrodes are ill suited to these tasks because it is usually impractical to penetrate more than two or three neurons at a time, and vertebrate neurons rarely survive multiple penetrations. Voltage-sensitive dyes, whose absorption or fluorescence changes with membrane potential, can be used for noninvasive multicell recording (for reviews see Salzberg, 1983; Cohen and Leshner, 1986; Grinvald et al., 1988). Combined with extracellular stimulation, using either conventional glass electrodes, or microfabricated dishes with built-in electrode arrays (Regehr et al., 1989), it should eventually be possible to synaptically map microcultures without any intracellular penetrations.

This paper describes the development of fluorescence dye recording for SCG microcultures, with the aim of optically detecting both action potentials (APs) and subthreshold PSPs. It falls into three parts: improvements in dye-recording technique, development of the SCG microculture system, and results of dye recording from microcultures. First, we describe extensive efforts to optimize the dye-recording procedure for maximum signal-to-noise ratio from these cells. The dye-recording apparatus is described in detail elsewhere (Chien and Pine, 1991). Over 25 dyes were screened, staining procedures and filter sets were optimized for maximum fluorescence and dye sensitivity, and the dye RH423 was chosen for further use. Fluorescence signals from RH423 were verified to be fast and linear in membrane potential, and phototoxicity was shown to be low enough for synaptic mapping of simple cultures. Second, we describe a simple method for patterning for microcultures that should be generally applicable to many neuronal types. Third, we present the results of dye recording from SCG microcultures while stimulating cells with intracellular electrodes, comparing the resulting dye signals with simultaneous intracellular recordings.

Early parts of this work have been described in abstract form (Rayburn et al., 1984; Chien et al., 1987), and it makes up part of a Ph.D. thesis (Chien, 1990).

MATERIALS AND METHODS

Cell culture

Rat SCG neurons were cultured using standard techniques. Rat pups (0–3 d old; pregnant Wistar females obtained from Simonsen Labs,

Gilroy, CA) were killed by decapitation. Their SCGs were removed to sterile saline, cleaned of extraneous tissue, enzymatically dissociated using trypsin, shredded, triturated, and the resulting cell suspension diluted to plating density. Cells were plated either in a single 100 μ l drop, or inside a glass cloning ring in a dish flooded with medium. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere and regularly fed by partial replacement of medium. Nonneuronal cells were killed by treatment with the antimitotic agent 5-fluoro-2-deoxyuridine (FUDR). 20 μ M FUDR (Sigma Chemical, St. Louis, MO) and 20 μ M uridine (Sigma Chemical Co.) were added 1 or 2 d after plating, then diluted out by subsequent feeding; this treatment was usually sufficient to completely remove all nonneuronal cells.

The culture medium used was essentially the L-15-CO₂ of Mains and Patterson (1973), modified by omitting Methocel and bovine serum albumin, and replacing rat serum with horse serum. Each 100 ml of liquid Leibovitz's L-15 medium (Whittaker MA Bioproducts, Walkersville, MD) was supplemented with 2.9 ml of 7.5% NaHCO₃ (Gibco, Grand Island, NY), 0.5 ml stable vitamin mix, 10 ml horse serum (Whittaker), 1 ml of 200 mM L-glutamine (Gibco), 1 ml of penicillin/streptomycin solution (10,000 units/ml penicillin, 10 mg/ml streptomycin; Sigma Chemical Co.), 2 ml of 30% (wt/vol) glucose, 1 ml fresh vitamin mix, and 50 μ g 7S nerve growth factor (NGF).

NGF was prepared from mouse saliva using the Sephadex G-100 step of Burton et al. (1978), then concentrated to 1 mg/ml with a YM10 filter (Amicon Corp., Lexington, MA). Stable vitamin mix was made by adding 0.3 g L-aspartic acid, 0.3 g L-glutamic acid, 0.3 g L-proline, 0.3 g L-cystine, 0.4 mg d-biotin, 0.1 g *p*-aminobenzoic acid, 8 mg coenzyme A, 0.1 g β -alanine, 40 mg vitamin B-12, 0.2 g myo-inositol, 0.2 g choline chloride, 10 mg DL-6,8-thioctic acid, and 0.5 g fumaric acid (coenzyme A from Pharmacia, Milwaukee, WI; all others from Sigma Chemical Co.) to 100 ml H₂O. Fresh vitamin mix was made by adding 1 mg 6,7-dimethyl-5,6,7,8-tetrahydropterine, 5 mg glutathione, and 100 mg ascorbic acid (all from Sigma Chemical Co.) to 20 ml H₂O, then adjusting to pH 5–6 with KOH.

Culture substrates

Fig. 1 shows the lift-off method used for making microculture dishes. Each plastic 35-mm tissue culture dish was prepared by drilling a 6-mm hole through its bottom, then glueing on a glass coverslip (Fig. 1*a*) using Sylgard 184 silastic adhesive (Dow Corning, Midland, MI). Inside the central well thus formed were patterned 16 polylysine-laminin islands (other substrates were tried in earlier experiments), each roughly 70 μ m in diameter, surrounded by a sea of bare glass. SCG neurons adhered only to the islands, and not to the surrounding sea.

The first step in patterning the substrate was to lay a pattern of silastic adhesive on the bottom of a dish (Fig. 1*b*). Under a dissecting microscope, a "rubber stamp" (a small square of plastic with 16 holes drilled through it) was coated by pressing it lightly against a glass slide coated with a thin film of silastic (MDX4-4210 medical grade elastomer, Dow Corning). The silastic pattern was transferred to the coverslip from the rubber stamp, then cured for 1 h at 70°C.

The second step was to coat the dish with polylysine and laminin. The dish was UV-sterilized, then flooded with a 0.5–1 mg/ml solution (all solutions were sterile-filtered) of poly-DL-lysine (roughly 20 kD; Sigma Chemical Co., St. Louis, MO) in Dulbecco's phosphate-buffered saline (DPBS; Flow Laboratories, McLean, VA) and left overnight at 4°C. The next day, the dish was rinsed twice with distilled water, then air dried after aspirating off the water. The central 6-mm well was then coated with laminin by filling it with 10–20 μ g/ml laminin (Sigma Chemical Co.) in DPBS, letting this solution sit for at least 45 min, then rinsing twice with DPBS.

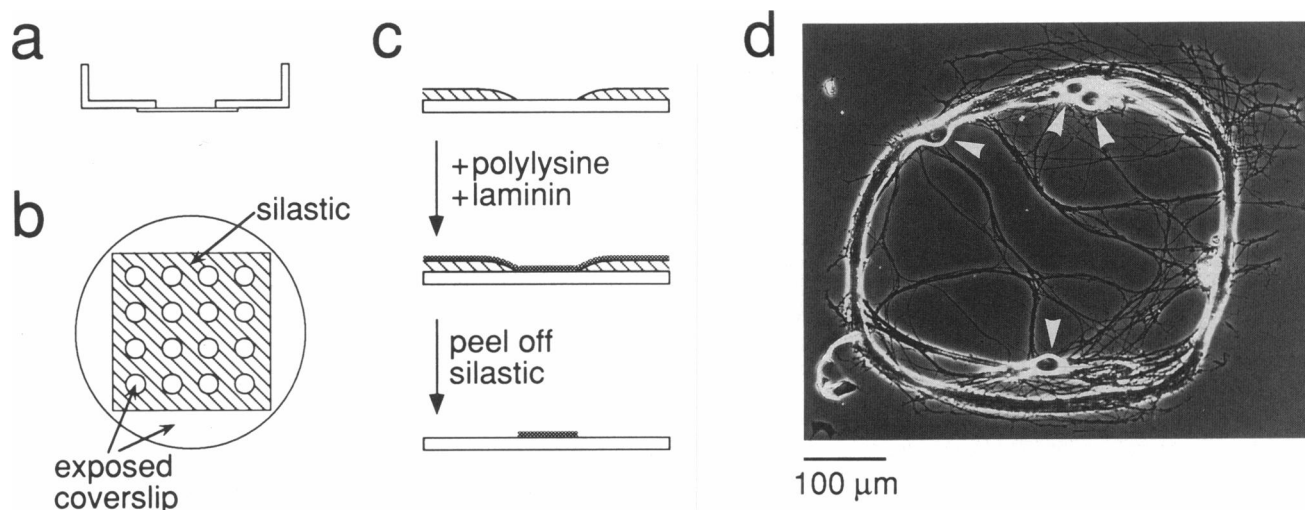


FIGURE 1 Lift-off method for making SCG microcultures. (a) Side view of drilled 35-mm dish with attached coverslip. (b) Pattern of silastic adhesive that defines island boundaries. (c) Procedure for patterning polylysine-laminin. (d) An SCG microculture grown on polylysine-laminin for 22 d. Arrowheads indicate the four cell bodies. (The phase-bright fishhook in the lower left corner is a scrap of silastic.)

The third step was to use sterile forceps to peel the silastic off in one piece (Fig. 1 c), leaving only the substrate on the islands and lifting off the rest. (It was important to use coverslips straight out of the box: if they were cleaned with ethanol, or even sonicated in water, the silastic adhered too tightly and would not peel off. Cleaning apparently removed some surface film left from their manufacture.) The laminin was kept wet, as drying made it a poor substrate. Neurons were plated at a density calculated to give 1–10 cells per island.

Fig. 1 d shows a four-cell microculture 22 d after plating on polylysine-laminin. It has a typically complex anatomy, with anastomosing neurites and a thick fascicle that runs around the outside of the island. The area of neurite growth shows the extent of the polylysine-laminin island, which is otherwise invisible.

Electrophysiology

Early experiments used a modification of Freschi's high-calcium recording solution (Freschi, 1983): 140 mM NaCl, 5 mM KCl, 6.25 mM CaCl_2 , 0.5 mM MgCl_2 , 11 mM glucose, and 10 mM Hepes buffer, adjusted to pH 7.3 with NaOH and to 340 mOsm with sucrose. Most experiments described here used high- Ca^{++} L15/Air, made by adding stable vitamin mix, fresh vitamin mix, glucose, glutamine, and penicillin/streptomycin (all in the same proportions as for L-15- CO_2 culture medium; see above) to L15, adding 5 mM CaCl_2 for a final Ca^{++} concentration of 6.3 mM, and setting the pH to 7.3. Both modified Freschi's solution and L15/Air had elevated calcium to facilitate stable penetrations. No difference in dye properties was noticed between the two solutions.

For experiments using the cholinergic blocker hexamethonium, a 1-mM stock solution of hexamethonium chloride (Sigma Chemical Co.) was made up in modified Freschi's solution, then diluted to the desired concentration. The culture dish was perfused using a gravity-fed input, and vacuum-driven aspiration for the output.

Conventional intracellular electrodes were filled with 3 M KAc and had resistances of 80–100 M Ω . Whole-cell patch electrodes were pulled from borosilicate glass to a bubble number of 4, and had resistances of ~ 15 M Ω when filled with intracellular solution: 140 mM KCl, 2 mM MgCl_2 , 11 mM EGTA-KOH, 1 mM CaCl_2 , and 10 mM

Hepes, pH adjusted to 7.2 with KOH and osmolality adjusted to 340 mOsm with sucrose (modified from Marty and Neher, 1983). Used without fire polishing, they gave seal resistances of several gigohms.

Standard current-clamp methods were used with both intracellular and whole-cell patch electrodes. Healthy SCG neurons typically had resting potentials of 55–60 mV when penetrated. Most experiments were done at room temperature (22–25°C), but some were done at 30–33°C. No dependence of dye properties on temperature was noticed.

Dye staining

The voltage-sensitive styryl dye RH423 was routinely used. Other dyes tested included RH160, RH237, RH292, RH293, RH295, RH332, RH376, RH414, RH415, RH421, RH425, RH429, RH437, RH461, RH477, RH528, RH687, RH725, RH743, and RH749 (kindly supplied by Dr. Amiram Grinvald; see Grinvald et al., 1982a, 1983, 1984, and 1987 for some structures); and di-4-ASPBS, di-4-ASPPS, di-6-ASPPS, and di-4-ANEPPS (kindly supplied by Dr. Leslie Loew; see Fluhler et al., 1985, for structures). Many of these dyes are available from Molecular Probes (Eugene, OR). Dye stocks were typically 1 mM solutions in ethanol, kept refrigerated or at room temperature in tightly capped tubes, and diluted in recording solution to a working concentration of 0.5–2 μM just before use. Cultures were stained by washing thrice with recording solution to remove culture medium (it was important to remove all traces of serum; see Results), adding the desired dye concentration for 5–10 min, then washing thrice more to remove unbound dye. Ethanol reached concentrations as high as 0.2% during staining, but was diluted out during subsequent washing.

Phototoxicity was assayed in two ways. First, dye-recording experiments provided anecdotal electrophysiological data on acute toxicity. When a penetrated cell was illuminated for a long time, its action potential broadened and it became less excitable, until finally with continuing illumination it stopped firing altogether. Second, more rigorous experiments tested long-term viability. In mass cultures grown on dishes marked with a reference grid, the positions of a set of healthy cells were recorded, the culture stained, and the cells exposed with a single long flash or a series of flashes. The health of the cells was then

assessed over several days by observation in phase contrast. Heavily illuminated cells usually showed severe damage at one day (losing their smooth rounded shape and looking shrunken), then disintegrated over the next few days. In contrast, control cells (stained but not illuminated) in the same dishes had essentially perfect survival over the observed period.

Dye recording

The dye-recording apparatus, data acquisition system, and software are described in detail elsewhere (Chien and Pine, 1991). The apparatus was built around an Olympus IMT-2 inverted epifluorescence microscope, using the Olympus "G" filter cube supplemented by an LP515 excitation filter. This filter set passed excitation wavelengths from 520–550 nm and emission wavelengths longer than 610 nm. (In one set of experiments, different filter combinations were tested for improvements in dye sensitivity.) Fluorescence measurements usually used the Nikon Fluor 20× Ph3DL air objective (numerical aperture 0.75), though this was sometimes replaced by the Nikon Fluor 40× Ph3DL (air, NA 0.85). Illumination came from a HBO-100/W mercury arc lamp (Osram, Berlin, FRG) stabilized by an optical feedback regulator, and could be turned on and off by a fast electrochemical shutter. With the standard IMT-2 epiilluminator and the 20× objective, the illumination intensity at the specimen was 10 W/cm², mostly at the 546 nm mercury line.

A fluorescent image of the culture was projected through the side port of the IMT-2 onto a 256-pixel fiber-optic camera detector. With a 20× objective, the pixels were 45 μm squares. An optical fiber carried the light from each pixel to a separate photodiode-preamplifier combination, where it was converted to a voltage and sent to a data acquisition system controlled by an IBM AT personal computer. The data acquisition system could digitize up to 90,000 samples/s with 11-bit resolution; the software could address individual pixels at random, up to 16 pixels at a time. The trinocular headpiece of the IMT-2 was fitted with a video camera; in addition to acquiring optical signals and conventional electrophysiological signals, the computer used a frame-grabber board to take video pictures of the culture.

Fluorescence from stained cultures typically gave detected photocurrents that fell within the useful range of the detector, 0.2–2 nA per pixel. The fundamental noise limit for photodetection is due to statistical fluctuations (shot noise) in this photocurrent. The root-mean-square (rms) shot noise is given by $i_{\text{rms}} = (2qIB)^{1/2}$, where $q = 1.6 \times 10^{-19}$ C is the charge of the electron, I the detected photocurrent, and B the bandwidth. The signal-to-noise ratio for a shot-noise-limited measurement is therefore proportional to $I^{1/2}$. For a typical fluorescence level of 1 nA, and a standard bandwidth of 300 Hz, $i_{\text{rms}} = 300$ fA, or 0.03%. For frequencies above ~10 Hz, shot noise dominated instrumental sources of noise. The optical feedback circuit suppressed fast illumination fluctuations to ~0.02%, while the preamplifiers had an average noise of 80 fA rms, or 0.008% for $I = 1$ nA. Digitization noise was noticeable, but always <0.03%. The only significant deviation from the shot noise limit came from slow illumination fluctuations (apparently due to arc wander), which sometimes caused sloping or curved baselines in the optical signals.

A typical experiment for dye recording from a microculture proceeded as follows. After the culture was washed, stained, and placed on the stage, its alignment was checked and adjusted by comparing a video picture to the computer's stored diagram of the detector array. A set of pixels for recording optical signals was chosen, a video alignment picture was stored to disk, and the culture was not moved from then on. The chosen set always included pixels over all of the cell bodies in the culture, and also usually pixels over presumptive dendrites or axons. After impaling one or two neurons with intracellular electrodes, dye signals were recorded by opening the illumination shutter,

stimulating a cell, and closing the shutter after an illumination time of 50–200 ms. The stimulus was sometimes repeated to reduce noise by signal averaging, and from time to time the culture was flashed without a stimulus to record a pure bleach trace. (To reduce bleaching and phototoxicity, light exposure was kept to a minimum whenever possible.) Intracellular signals and dye signals from all the cells were recorded to disk. Optical signals were analyzed offline by correction for bleaching, followed by digital low-pass filtering at 300 Hz. For bleach correction, we used a simplified version (Chien and Pine, 1991) of the procedure of Grinvald et al. (1982b): each experimental trace, recorded with stimulation, was corrected by subtracting a quadratic curve fitted to a pure bleach trace, recorded from the same pixel without stimulation.

RESULTS

Dye screening and dye sensitivity

Because of the well-known variability of dye responses between cell types (Ross and Reichardt, 1979; Loew et al., 1985), it was necessary to screen dyes to find one that worked well on our particular preparation. As a general principle, fluorescence measurements become more sensitive than absorption measurements as the specimen becomes more transparent. Because SCG neurons are relatively small and have little surface area to bind dye, we decided to use fluorescence dyes rather than absorption dyes (this choice is dealt with further in the Discussion and Appendix). In particular, we chose the styryl class of fluorescent dyes, which had given large signals on other vertebrate cells (Grinvald et al., 1983; Gross et al., 1986). About 25 styryl dyes (see list in Materials and Methods) were screened by staining mass cultures of SCG neurons, observing and quantifying their fluorescence, and measuring dye signals in response to intracellular stimulation. The two main screening criteria were (a) good staining: a dye that gave bright fluorescent rings upon focussing on the equators of stained cells, and entered cells slowly (over hours) if at all, and (b) large dye signals. Dye bleaching and phototoxicity were not assayed at this time.

From this screening emerged a set of four good dyes with roughly equal properties: RH237, RH421, RH423, and di-4-ANEPPS. All four gave fluorescence $F = \sim 1$ nA detected photocurrent per cell, and dye signals $\Delta F/F = \sim 1\%/100$ mV (a 1% change in fluorescence for every 100 mV change in membrane potential). Though these properties were relatively consistent from cell to cell and culture to culture on the same day, they were variable over longer periods (weeks), with voltage sensitivity sometimes changing by a factor of 3 or 4 for no clear reason. Presumably this was due to changes in the cells rather than the dyes, since the absorption spectra of the dye stocks remained unchanged.

Several variables were tested for their effects on dye

staining. The length of the styryl's double alkyl tail was important: dyes generally did not stain well unless the tails were at least four carbons long (those of RH423 are six carbons long). There were no obvious changes in dye properties with variations in dye staining time (5–10 min) or staining concentration (0.5–2 μM). However, cell fluorescences were greatly reduced when serum was present in the medium during staining. This was presumably due to binding of the dye by serum because incubating stained cells in serum-containing medium for an hour effectively destained them, reducing their fluorescence by a factor of 10. It was therefore important to completely remove all serum before staining. Carefully washing the cultures was sufficient; culturing in serum-free medium for days (as done in the preliminary report of Chien et al., 1987) was not necessary. We did not experiment extensively with longer staining times or higher dye concentrations. The pragmatic reason was that our preamplifiers are already saturated for bright cells (the limit is ~ 2 nA); also, bleaching and phototoxicity will increase with greater staining. We estimate (see Appendix) that in SCG neurons stained with RH423, there is of the order of 1 dye molecule for every 100 membrane lipids. A large increase in dye staining is clearly impossible.

Exciting with the 546 nm Hg line and detecting fluorescence above 610 nm, RH423 and di-4-ANEPPS gave somewhat higher voltage sensitivity than RH237 and RH421. To check that this was not peculiar to these wavelengths, the wavelength dependence of dye sensitivity was tested for all four dyes, using excitation filter sets with passbands ranging from 415–485 nm to 540–550 nm, and using long-pass emission filters with cutoff wavelengths ranging from 610 to 790 nm. For all four dyes, the 520–550 nm excitation passband gave the highest signal-to-noise ratio, while shorter-wavelength excitation often gave no detectable signal. For all four, the longer-wavelength emission filters merely reduced the detected fluorescence with little effect on $\Delta F/F$. Thus our choice of standard filters: excitation from 520–550 nm (effectively selecting the 546 nm Hg line) and emission above 610 nm.

Of the two dyes with the best signals, RH423 and di-4-ANEPPS, we arbitrarily chose to concentrate on RH423, and used this dye for all further experiments. Fig. 2 *a* shows its structure. Fig. 2 *b* shows intracellular and optical signals recorded from an SCG neuron in a mass culture, stained with RH423. The optical signal has had the bleach artifact subtracted, and has been low-pass filtered at 300 Hz. It shows a dye sensitivity $\Delta F/F = 0.92\%/100$ mV: the fluorescence decreases by 0.92% for every 100 mV depolarization. The total fluorescence of this cell was $F = 0.86$ nA; the observed noise is a good

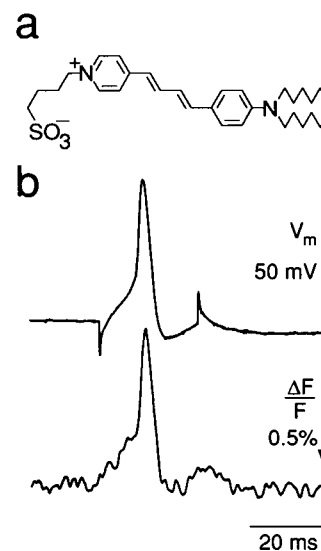


FIGURE 2 (a) Structure of the styryl dye RH423. (b) Intracellular (V_m) and fluorescence ($\Delta F/F$, bleach-subtracted, and filtered) recording from an SCG neuron in a mass culture stained with RH423. The cell was stimulated intracellularly by injecting a square current pulse through a bridge circuit. This is a single, unaveraged record. The direction of the arrow indicates increasing fluorescence.

match for the predicted shot noise of 0.033% rms. This translates to $(0.033)/(0.0092) = 3.6$ mV rms in the measured membrane potential. A large PSP should be just detectable with this signal-to-noise ratio, and easily detectable after signal averaging a few trials.

As to the light dose that could be administered before causing photodynamic damage, casual observations during dye-recording experiments and more rigorous long-term viability studies were in rough agreement. During experiments (standard illumination, standard RH423 staining), deterioration of the action potential typically set in after a total illumination time of a few tens of seconds. In long-term toxicity experiments, the half-lethal dose varied between cultures, but was typically ~ 20 s. The phototoxicity of RH421 was roughly comparable to that of RH423; we did not study the other dyes in any detail.

With both kinds of observations, cells usually did not show any ill effects until a threshold of phototoxicity was crossed, and deteriorated rapidly thereafter. Though it is possible that even low light doses cause subtle photodynamic damage that we cannot detect, it seems that at our standard level of illumination, experiments may be safely carried out with total exposures of up to 10 s. It should be possible to map a small microculture within this time, using for instance 100 flashes of 100 ms each. All of the dye signals shown here were measured well before 10 s total exposure.

Speed and linearity of dye signal

For voltage-sensitive dye signals to be interpretable, it is necessary that they faithfully reproduce the membrane potential. "Fast" dyes respond quickly compared with membrane time constants: those that have been tested on squid giant axon or artificial bilayers (Cohen et al., 1974; Waggoner, et al., 1977; George et al., 1988) respond within tens of microseconds and give signals linear over hundreds of millivolts of membrane potential. Styryl dyes such as RH423 are generally considered to be fast dyes.

To check the speed and linearity of RH423, dye signals were measured from SCG neurons within a few hours after plating on bare glass. These cells were nearly spherical, having had neither the time nor the appropriate substrate to grow neurites. (It is hard to interpret dye signals from cells with substantial neurites because their membranes may not be isopotential.) A whole-cell patch electrode measured the membrane potential while simultaneously injecting current to drive it through a range of hyperpolarized and depolarized potentials. Fig. 3 *a* shows the intracellular and optical signals from one such cell. The filtering of the two signals has been carefully matched so that their time courses may be compared directly. The dye signal has a submillisecond response time (no detectable delay), and is linear over more than 100 mV. The linearity and lack of hysteresis are better shown by the scatterplot *b*. (No quantitative analysis was attempted, because values at successive time points are not statistically independent.) Five other cells gave similar linear responses.

Microcultures

Other groups have seen extensive neuron–neuron synapses in SCG microcultures: Furshpan et al. (1986) reported that cholinergic autapses (synapses made by a cell on itself) were common in single-neuron microcultures grown on islands of heart cells, and Higgins et al. (1984) reported cholinergic synapses in microcultures grown on collagen dots. Preliminary experiments with intracellular electrodes characterized the synaptic properties of our SCG microcultures to show that they were similar. Single-cell microcultures often had autapses such as that shown in Fig. 4 *a*. Larger cultures often showed complex reverberatory activity such as that seen in the five-cell culture of Fig. 4 *b*, where stimulation of cell 2 resulted in as many as three subsequent APs in cells 1 and 2, presumably through a chain of suprathreshold synapses. In both these cultures, synaptic activity was suppressed when the nicotinic antagonist hexametho-

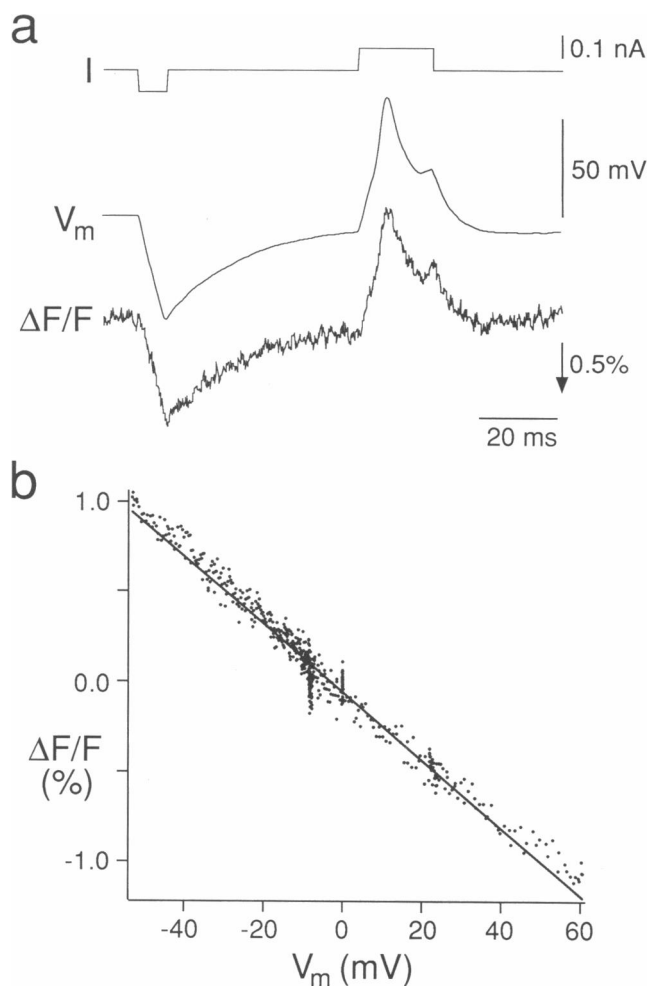


FIGURE 3 Linearity of the RH423 signal from a freshly plated neuron stimulated with a whole-cell patch electrode. (*a*) Injected current (I), membrane potential (V_m), and fluorescence signal ($\Delta F/F$). Average of four trials; filtering of V_m and $\Delta F/F$ has been matched. (*b*) Scatterplot of $\Delta F/F$ versus V_m (same data as *a*). $V_m = 0$ at the cell's resting potential. The fitted line has a slope of $-1.89\%/100$ mV.

nium was perfused in, and returned when the drug was washed out.

Under our culture conditions, the vast majority of connections seen were fast EPSPs, presumably cholinergic since hexamethonium reduced or eliminated in PSPs in the four cultures tested. Electrical synapses occurred very rarely. PSP amplitudes varied from the limit of intracellular detection (~ 1 mV) to well above threshold. At room temperature, synaptic latencies varied from 5–30 ms, measured from the peak of the presynaptic AP to the onset of the PSP. These long latencies presumably reflected propagation delays in the small unmyelinated axons.

The procedure for patterning microculture substrates

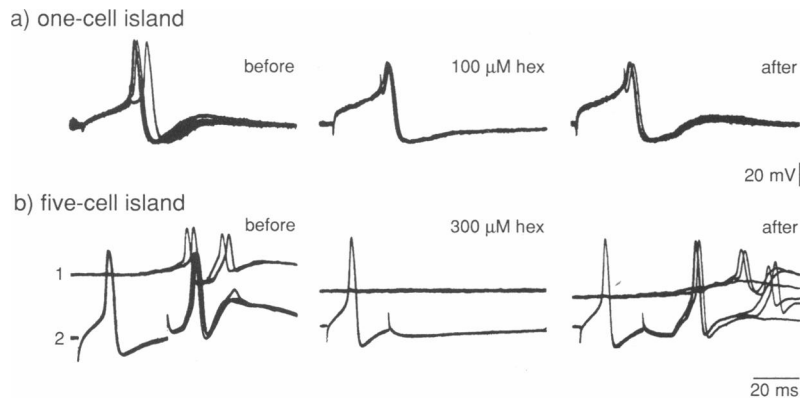


FIGURE 4 Fast cholinergic PSPs in microcultures grown on ECM, measured with intracellular electrodes after intracellular stimulation of one cell. Responses are shown before, during, and after addition of hexamethonium. Each panel shows several superposed traces. (a) A one-cell microculture with a subthreshold autapse. (b) Two cells from a five-cell microculture, with complex suprathreshold connections.

is described in Materials and Methods. Of several different substrates that were tried, SCG neurons attached well and gave healthy microcultures on two: dried polylysine coated with laminin, and extracellular matrix (ECM) made by lysing bovine endothelial corneal cells (MacCallum et al., 1982). Cultures survived longer on ECM: they were often still healthy after 2 or 3 mo, whereas polylysine-laminin cultures were best used after 2 or 3 wk, and usually deteriorated after 4 wk. ECM also seemed a better substrate for developing synapses: when penetrated intracellularly, almost all ECM cultures showed evidence of synaptic connections, while only about half the polylysine-laminin cultures did. On the other hand, polylysine-laminin was far simpler to process, gave more consistent results, and gave less fluorescent background upon staining because it lacked the cellular debris found in ECM. Except for Fig. 4, all data shown is from microcultures grown on polylysine-laminin, and ECM will not be discussed further.

Recording from a single-cell microculture

After optimizing dye-recording conditions and developing a microculture protocol for SCG neurons, we made multiple-site dye recordings from SCG microcultures to explore the usefulness of dye recording for synaptic mapping of microcultures. Intracellular electrodes served both to stimulate cells and to provide reliable records of membrane potential against which the dye signals could be checked. The present study includes dye recordings from 24 islands grown on polylysine-laminin, comprising 68 cells of which 49 were penetrated. In each culture, dye signals were usually recorded from pixels over all of the cell bodies, pixels over any obvious presumptive

dendrites, and at least a few pixels over the background network of presumptive axons. Processes were classed as presumptive dendrites if they tapered, were wider than $\sim 5 \mu\text{m}$ at the base, and were visibly hollow when viewed in RH423 fluorescence. (This definition may have included some proximal axon segments.) Presumptive dendrites were typically $\sim 100 \mu\text{m}$ long, but were occasionally as long as $200 \mu\text{m}$.

The dye signals were often very complex, so it is best to first examine the signals from the simplest possible culture: one with a single cell. Fig. 5 shows phase-contrast and fluorescence micrographs (*a*, *b*, and *c*) of a single-cell island, and a culture schematic (*d*) showing the locations of 12 pixels from which dye signals were recorded. Viewed in phase contrast, the cell had several presumptive dendrites; the membrane staining of RH423 (*c*) showed these as hollow cylinders, and "dendrite 1" appeared to actually be a pair of adjacent dendrites. The culture also had a complex network of fine neurites, presumably branched and fasciculated axons. Dye signals were recorded from three pixels over the soma (SA-SC), five over presumptive dendrites 1 and 2 (pixels 1A-1C and 2A-2B), and four pixels over the network (N1-N4). Brightly-stained debris was attached to some parts of the network, and some dye entered the cell during the experiment (the fluorescence picture was taken after dye recording was completed).

After the culture was stained for 7 min with $2 \mu\text{M}$ RH423, the neuron was penetrated intracellularly, stimulated, and dye signals recorded from the pixels shown (because the culture was small, a $40\times$ objective replaced the normal $20\times$). The figure shows an average of the data from four trials. The dye signals fell into two classes: those from the soma and presumptive dendrites

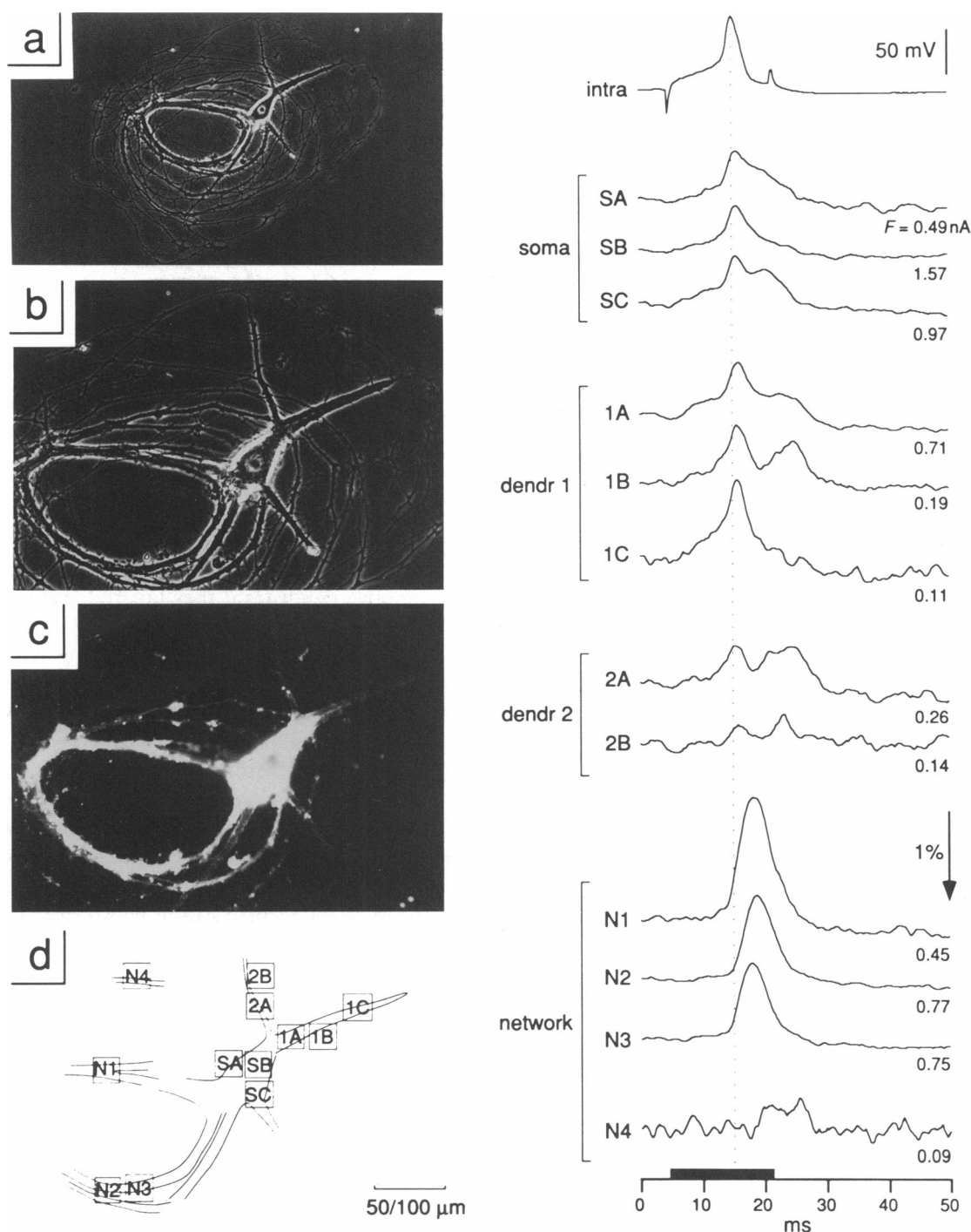


FIGURE 5 Dye recording from a single-cell microculture grown in culture for 21 d on polylysine-laminin. (a) Phase contrast, 20 \times objective. (b) Phase contrast, 40 \times objective. (c) RH423 fluorescence, 40 \times . (d) Culture schematic made by tracing stored video image. Squares show the locations of pixels used for dye recording: over the soma (SA-SC), presumptive dendrites (1A-1C, 2A-2B), and thinner neurites (N1-N4). The scale bar represents 100 μm for a, 50 μm for b, c, and d. The cell body was penetrated and stimulated intracellularly; the traces show the average of four successive stimuli. The membrane potential from the intracellular electrode is labelled *intra*; dye signals are labeled on the left by pixel, and on the lower right by the photocurrent (F , in nA) from that pixel. The arrow is the scale for all dye signals; it shows a 1% increase in fluorescence. The solid bar on the time axis shows the period of intracellular current injection; the vertical dotted line is drawn for reference through the peak of the intracellular AP.

(SA-SC, 1A-1C, 2A-2B), and those from presumptive axons (N1-N4).

Soma and dendrites

In contrast to neuriteless cells (Fig. 3) or neurons in mass culture (Fig. 2), the signal from the cell body (SA-SC) of this cell did not follow the membrane potential recorded intracellularly (*intra*). This seems to be due to superposition of signals from different cellular elements (soma and axons) in the same pixel. The signals from the soma may be divided into early responses (the period before the peak of the intracellular AP) and late responses (the period afterward). All of the early responses faithfully followed the potential measured by the intracellular electrode, showing an initial slow rise before the cell reached threshold, followed by a fast rise once the cell crossed threshold. (The delay of 0.5–1.0 ms between the peak of the intracellular AP and the initial peaks of the optical signals is attributable to the time constants of the optical detector's preamplifiers.) In contrast, the late responses deviated significantly from the electrode potential: after the initial AP, some pixels had late peaks, and others had shoulders. Signals from dendrites followed the same pattern. We interpret the late responses as signals from APs propagating in axons that curved back through the pixels used to record from the cell body and dendrites.

Axons

Pixels N1 through N3 were situated over axon fascicles. The dye signals from all three pixels showed a single peak with the characteristic shape of a propagating AP. This peak was quite broad, as would be expected since these pixels covered many different axons whose propagation velocities may have differed. The three peaks lagged the peak of the intracellular AP by ~3 ms; the delay cannot be specified more accurately without careful accounting for the time constants of the preamplifiers. Pixel N4 covered several thin processes in the periphery of the network. Its dim fluorescence made the signal quite noisy, but there was a small bump, lasting from 3 to 13 ms after the peak of the intracellular AP, that repeated from trial to trial. This was presumably the sum of several small AP signals with different propagation delays.

These signals, though complex, can be explained in terms of the observed anatomy and expected electrophysiology of this neuron. When the neuron is stimulated, one expects that its soma and dendrites should fire essentially simultaneously because the dendrites are short and electronically very close to the soma. The action potential should then spread out through the

branching axonal network at some finite propagation velocity, which will be quite slow for these small unmyelinated axons. Thus, the expected signature of a dye signal from the soma or a dendrite is a peak simultaneous with the intracellular AP, while the expected signature of an axonal signal is a delayed peak. Allowing for the axons that curve back over the cell body and dendrites, the dye signals observed from this cell fit these predictions very well. The variations in signal size from pixel to pixel corresponded well with the amount of background fluorescence (Fig. 5c). Pixel N1 had less background than N2 and N3, and had a correspondingly larger $\Delta F/F$, while the pixels over the cell body had high background staining and correspondingly mediocre signals.

This explanation for the dye signals was further tested by injecting a negative current pulse into the cell, hyperpolarizing it by ~30 mV (data not shown). Such a current should hyperpolarize the soma and the electronically close dendrites, but not the axons. This was borne out by the dye signals: in an average of four trials, distinct signals were seen in pixels SA-SC and 1A-1C, and there was no signal in N1-N3. (Pixels N4, 2A, and 2B were quite dim, so their signals were noisy and no conclusion could be made.)

Optical detection of PSPs

It was important to test whether dye recording could give useful signals from a multiple-cell culture. For a shot-noise-limited recording with detected photocurrent 1 nA and dye sensitivity 1%/100 mV, theory predicts that the rms noise for measurement of changes in membrane potential should be a few millivolts. This was confirmed experimentally (Fig. 2). Given a favorable geometry where axons do not contribute significantly to the dye signal from the cell body's pixel, this noise level should allow detection of a large PSP.

Fig. 6 shows recordings from a five-cell culture in which a PSP was visible in the optical signal. After intracellular penetration of cells 1 and 3, stimulation of cell 1 resulted in a large complex PSP in cell 3 (Fig. 6, *b* and *c*) that was occasionally suprathreshold. Only the dye signals from two pixels over cell 3's soma (3R and 3L) are shown. The subthreshold and suprathreshold PSPs are clearly visible in both pixels. (*b* is the average of three consecutive trials, while *c* is a single, unaveraged trial.) The dye signals from the suprathreshold PSP in *c* had the same amplitude as those from the directly stimulated AP in *a*. The PSP had two distinct components; dye signals from the other cells (data not shown) suggested that the first component was due to a monosynaptic connection 1 → 3, and the second component due

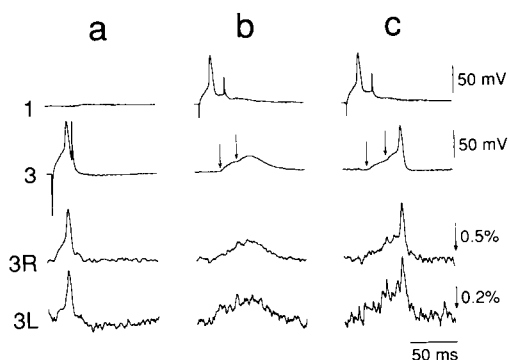


FIGURE 6 Optical detection of PSPs in a five-cell microculture, grown in culture for 20 d on polylysine-laminin. 1 and 3 are intracellular recordings from cells 1 and 3; 3R and 3L are dye signals from two pixels over the cell body of cell 3. Pixel 3R had photocurrent $F = 0.75$ nA; 3L had $F = 0.58$ nA. (a) Stimulating cell 3 (average of 4 trials). (b) Stimulating cell 1, subthreshold PSP in 3 (average of 3 trials). (c) Stimulating cell 1, suprathreshold PSP in 3 (single trial). In b and c, the arrows indicate the onsets of early and late components of the complex PSP.

to a disynaptic connection $1 \rightarrow 2 \rightarrow 3$. Though close examination shows slight deviations between the optical and intracellular signals (presumably due to small axonal signals), the PSP was clearly detectable in the optical signal.

Recording from a five-cell microculture

It was more typical that only APs and not PSPs could be detected in the optical signals from multiple-cell cultures. Fig. 7 shows phase contrast and fluorescence micrographs (a and b) of a five-cell culture (different from that of Fig. 6), and a culture schematic (c) showing the 12 pixels from which dye signals were recorded. Seven pixels (1A, 2A-2C, 3-5) were over cell bodies, four were over the perimeter fascicle (F1-F4), and the last (1B) was over cell 1's presumptive dendrite. Viewed in fluorescence, cells 1 through 4 showed nice rings of staining, but cell 5 filled with dye during the experiment. The presumptive dendrites of cells 1 and 2 showed as hollow cylinders, though this is not visible at the exposure shown. After the culture was stained for 8 min with $2 \mu\text{M}$ RH423, cells 1 and 2 were penetrated, and stimulation of cell 2 gave the signals shown. Again, the cell bodies and axons gave distinctly different signals.

Processes

The shapes of the signals shown here for pixels F1-F4 and 1B, broad humps lasting about 30 ms, repeated from trial to trial, and presumably represent sums of several

APs with different latencies. When cell 1 was stimulated intracellularly, the signal in pixel 1B was $<0.2\%$ (data not shown), so the presumptive dendrite did not contribute much signal. The signals from the processes were too small and complex to be interpreted in terms of specific neuronal firing patterns.

Cell bodies

The dye signals from cell bodies showed distinct sharp peaks. All of the pixels over cells 1 and 2 have peaks corresponding to the intracellularly recorded APs; in 2A and 2C, these optically recorded APs are clearly visible above the signals from passing axons. Cells 3 and 4 also showed large peaks; later intracellular penetrations of these cells confirmed that these signals were indeed due to their respective APs. Fig. 7 shows a large peak in the dye signal from cell 5 which was never seen in subsequent trials. Though never confirmed by intracellular penetration, it seems likely that this peak indicates an action potential in cell 5 due to a synaptic potential that was just barely suprathreshold for this trial, then fell below threshold.

Thus, the dye signals from cell bodies are good indicators of APs in those cells: they show that the culture was strongly connected, with suprathreshold synapses. The cell-body dye signals are, however, poor indicators of subthreshold responses: compare the late PSPs recorded intracellularly from cells 1 and 2 with the corresponding dye signals. This lack of correspondence between optical and intracellular recording seems again to be due to signals from passing axons: for instance, pixel 2B straddled the perimeter fascicle, and had a particularly muddled signal.

Summary of dye recording

Dye signals recorded from mass cultures during dye screening and preliminary experiments corresponded well to the membrane potential recorded by the intracellular electrode. Upon first recording from microcultures and observing complex signals such as those in Figs. 5 and 7, we were taken aback, and suspected that the dye was exhibiting some nonlinear behavior. We now believe that this is not so: the dye signal from any small patch of membrane is strictly linear in its membrane potential, and complex signals arise purely from the superposition of signals from cellular elements with differing membrane potentials. The signals resulting from firing cells in a microculture corresponded to the expected electrophysiology: the soma and dendrites, being essentially isopotential, had early responses that followed the intracellular electrode potential. The axons showed delayed, sometimes complex signals, due to propagation delays of

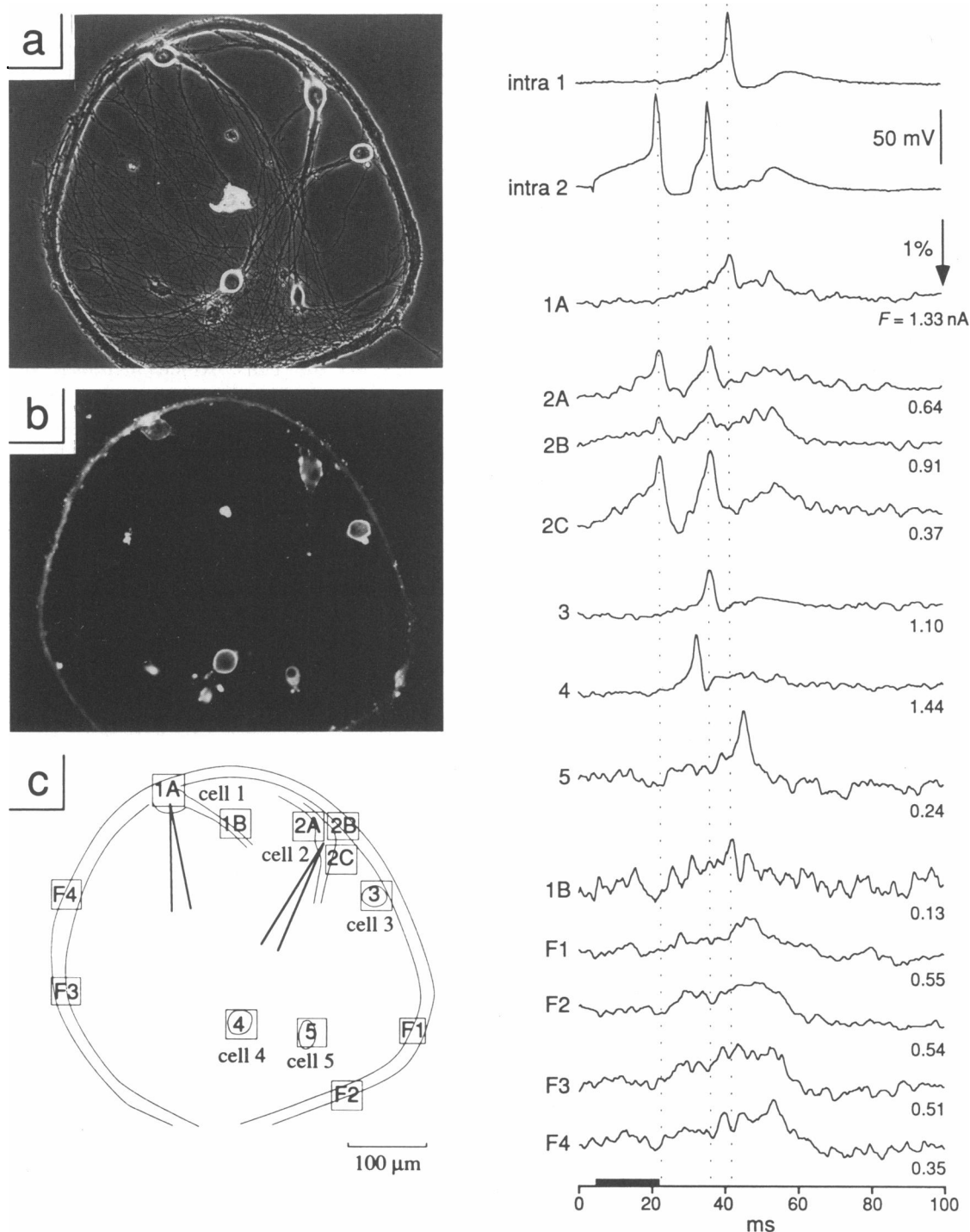


FIGURE 7 Dye recording from a five cell microculture grown in culture for 21 d on polylysine-laminin. (a) Phase contrast. (b) RH423 fluorescence. (c) Culture schematic. Cells are numbered 1 through 5. Squares show the locations of pixels used for dye recording: over the cell bodies (1A, 2A-2C, 3-5), presumptive dendrite (1B), and girdling fascicle (F1-F4). Cells 1 and 2 were penetrated intracellularly; the traces show a single unaveraged trial in which cell 2 was stimulated. Intracellular membrane potentials are labeled intra 1 and intra 2; dye signals are labeled on the left by pixel, and on the lower right by the photocurrent (F , in nA) from that pixel. The arrow is the scale for all dye signals; it shows a 1% increase in fluorescence. The solid bar on the time axis shows the period of intracellular current injection; the vertical dotted lines are drawn for reference through the peaks of the three APs recorded intracellularly from cells 1 and 2.

the APs. Late signals from the soma and dendrites looked like the axon signals.

In the 24 islands studied here, 49 cells were penetrated and stimulated. The cell bodies of 46 (94%) had clear dye signals simultaneous with the intracellular APs. Of 37 pixels over presumptive dendrites, 34 (92%) showed dye signals simultaneous with the intracellular AP. (Though it is not possible to interpret definitively the cases where signals were not detected, they were presumably due to insufficient signal-to-noise ratio.) Of 51 pixels that covered axons only, dye signals above background were seen in 45. Only 9 of these (20%) had dye signals simultaneous with the intracellular action potential, and most of these pixels were within 100 μm of the soma. In 15 cells that were hyperpolarized intracellularly, the hyperpolarization was detected optically in 23 of 26 (88%) pixels over presumptive dendrites, but in none of the 32 pixels over presumptive axons.

While APs were detected routinely in the dye signals (46 of 49 cells), PSPs were not. Of the nine cases in which a subthreshold PSP was detected intracellularly, in only two cases (one shown in Fig. 6) was it detectable optically as well. (There were many more cases where a *suprathreshold* PSP was detected intracellularly or optically.)

DISCUSSION

We set out to determine whether synaptic mapping of vertebrate microcultures with voltage-sensitive dyes was possible, using SCG neurons as a test case. While several experiments have been able to optically detect subthreshold PSPs in vivo (Salzberg et al., 1977; Grinvald et al., 1987), to the best of our knowledge previous dye-recording experiments in culture have not. The dye signals obtained by Grinvald et al. (1983) were very large, but their neuroblastoma cells made no synapses; Parsons et al. (1989) also had very good signal-to-noise ratio, but their AC-coupled amplifiers could not detect the very slow PSPs of their cultured *Aplysia* neurons. Vertebrate neurons have considerably smaller cell bodies than either fused neuroblastoma cells or identified *Aplysia* neurons, and dye recording is correspondingly harder. Our main conclusion is that dye recording can indeed be sufficiently sensitive to detect subthreshold PSPs from vertebrate neurons; we were able to detect PSPs optically in two cases. The SCG microcultures are not the best preparation because of their anatomical complexity, which causes overlap between signals from cell bodies and axons. However, a different microculture system may alleviate this problem.

Dye-recording sensitivity

The dye signals measured here were disappointing, and once again point up the variability of dye sensitivity between preparations. On other cultured cells, RH421 has given signals of 21%/100 mV (Grinvald et al., 1983), and di-4-ANEPPS has given $\sim 9\%$ /100 mV (Gross et al., 1986). After extensive optimization, the best signals routinely obtained from SCG neurons were $\sim 1\%$ /100 mV (RH237, RH421, RH423, di-4-ANEPPS), though larger signals were occasionally seen and there was a single instance with $\Delta F/F = 7\%$ /100 mV (RH423 on a freshly plated neuron; data not shown). The cause of this variation is unknown. Some of it may be due to debris adhering to the cells, which increases fluorescence without adding to the signal; note that the single case of $\Delta F/F = 7\%$ /100 mV was from a neuron that was freshly plated, and perhaps especially clean. It would be interesting to take high-resolution images of stained cells to resolve true membrane fluorescence from adherent debris and correlate this with $\Delta F/F$; it might also be useful to try various cleaning treatments. An increase in dye sensitivity to 2 or 3%/100 mV would make detection of PSPs a much simpler matter.

One may wonder whether absorption-mode measurements might give better sensitivity. The choice between absorption and fluorescence measurements depends mainly on the fraction of incident light absorbed by the stained specimen (Hirschfeld, 1977). It is useful to compare the present cells with the cultured *Aplysia* neurons of Parsons et al. (1989). These invertebrate neurons are large and have heavily invaginated surfaces, and therefore bind large amounts of dye and absorb a large fraction of the incident light, so that absorption measurements can give very good signal-to-noise ratios. Vertebrate neurons have vastly less surface area to bind dye and absorb light, so the use of fluorescence is favored. Our SCG neurons, heavily stained with RH423, absorb only $\sim 10^{-3}$ of incident light, so it seems unlikely that an absorbance dye could give better signal-to-noise (see Appendix). It is difficult to make a direct comparison with *Aplysia* neurons because Parsons et al. (1989) did not measure the fraction of incident light absorbed by their stained specimens; as a general rule, however, we expect that absorbance dyes are likely to be more useful for large (i.e., invertebrate) cultured neurons, while fluorescence dyes are better for small (vertebrate) neurons (see Appendix).

Microcultures

Our method for patterning microcultures using a silastic lift off is simple and reliable. In our hands, it was much more consistent than the collagen-dot method of Furshpan et al. (1976), and it should be adaptable to other cells and substrates. In particular, it may be possible to

grow microcultures with shorter, less complex axons by switching cell types, adjusting the adhesivity or the pattern of the substrate, or using the cultures at an earlier time, when the axons are not so long. This should alleviate the problem of obfuscating axonal signals.

Other groups that have grown SCG microcultures in serum-containing medium (Higgins et al., 1984; Furshpan et al., 1986) report a high incidence of fast cholinergic PSPs; intracellular recordings from our microcultures replicated these findings. The observed PSP latencies (5–30 ms), though unusually long, are similar to those shown in the earlier reports, and presumably reflect propagation delays in the thin, very long SCG axons. For interpretation of dye signals, it is important to be able to classify neurites as dendrites or axons. Furshpan et al. (1986) used electron-micrographic serial-sectioning and reconstruction of their SCG microcultures to show that the short, thick neurites seen in phase contrast had ultrastructure characteristic of dendrites or proximal axons, while thin neurites had axonal ultrastructure. Thus, our classification of neurites as “presumptive dendrites” or “presumptive axons” on the basis of phase-contrast morphology seems justified, though the former class may also include proximal axons.

Interpretation of dye signals

We were at first surprised by the complexity of dye signals from microcultures because the dye signals from cells in mass culture had usually been linear in membrane potential. It is now clear that the dye response is always linear, but complex signals can arise when several cellular compartments overlap in the same pixel, as happens frequently when long axons are confined to the small area of a microculture. The evidence for this interpretation is as follows: (a) dye signals from neuriteless (therefore isopotential) cells were without exception fast and linear. (b) In mass cultures, where axons had room to grow and were much less likely to curve back over the cell body, dye signals almost always followed the membrane potential. Occasional deviations were presumably due to the rare returning axons. (c) The signals resulting from firing a cell in a microculture corresponded to the expected electrophysiology. The soma and dendrites, being essentially isopotential, had early responses that follow the intracellular potential, plus delayed signals from returning axons. The axons showed delayed signals, either single peaks or more complex shapes, from propagating APs. (d) When a cell was hyperpolarized, dye signals were visible only in the soma and dendrites, never in the axons.

Due to considerations of membrane area, we originally expected that dye signals might be visible from dendrites, but not from axons. In phase contrast, typical diameters were 30 μm for SCG cell bodies and 5 μm for

dendrites, while the electron micrographs of Furshpan et al. (1986) show axons with diameters of $\sim 0.5 \mu\text{m}$. Despite this difference in size, pixels over fascicles often give as high a detected photocurrent as pixels over cell bodies. This must be due to the great number of axons per fascicle.

Implications for synaptic mapping

Our results show that dye recording can be very sensitive. Given that the apparatus is shot-noise limited, the detected photocurrent from a cell body is $\sim 1 \text{ nA}$, and the dye sensitivity is $\sim 1\%/100 \text{ mV}$, theory predicts that membrane potential changes can be measured with an rms noise of $\sim 3 \text{ mV}$ (see Materials and Methods), with no signal averaging. When there were no interfering axonal signals, this noise level was achieved in practice, as shown in Figs. 2 and 6.

When superposed axonal signals were present, APs in the cell body were still usually detectable: the dominant peak in the somatic dye signal was almost always simultaneous with the intracellular AP. However, PSPs were detected optically only very rarely, and it is clear that the original program of synaptic mapping is not practical for these SCG microcultures. In another microculture system with less exuberant processes, it may well be possible to record PSPs routinely. The key criterion is that the cultures should have little physical overlap between axons and cell bodies. In such cultures, it should be possible to combine dye recording and extracellular stimulation to achieve noninvasive synaptic mapping.

In addition to plasticity studies, dye recording from anatomically simple cultures would be useful for studying the properties of small defined networks of neurons, and for studying cable properties of cultured neurons. Parsons et al. (1989, 1991) have studied small networks of *Aplysia* neurons in vitro; Krauthamer and Ross (1984) have used dye recording and extensive signal averaging to measure the cable properties of barnacle neurons in vivo. It is most tempting to imaging extending the work of Syed et al. (1990) by attempting to reconstruct larger central pattern generators in culture, and studying their behavior using dye recording.

APPENDIX

To compare the relative merits of absorption and fluorescence measurements, it is instructive to estimate the concentration of dye in the membrane (to see if staining can be increased), and the fraction of incident light absorbed by the stained cell. The membrane concentration of dye can be calculated in two ways: from the binding properties of the dye or from the observed fluorescence. First, from binding properties. Since we did not directly measure these properties for RH423, we assume that it binds to SCG neurons with the same affinity

as that measured by Fluhler et al. (1985) for a very similar dye, di-6-ASPPS, on phosphatidylcholine vesicles. This is a crude approximation, but should be good to within an order of magnitude. Fluhler et al. (1985) found that 0.015 mg/ml of lipid bound half the dye in a 1- μ M solution. Taking the molecular weight of phosphatidylcholine as 700, this corresponds to roughly 1 dye molecule per 42 lipid molecules (a molar fraction of 2.4%). Our usual staining protocol uses dye concentrations of 0.5–2.0 μ M, and does not necessarily proceed to equilibrium, but 1% seems a good order-of-magnitude estimate for the molar fraction of RH423 in the stained SCG neuron's membrane.

Next, from detected fluorescence. We did not directly measure the extinction coefficient ϵ and quantum efficiency q for RH423 on SCG neurons, and so again take values found by Fluhler et al. (1985) for di-6-ASPPS on lipid vesicles: $q = 0.3$, $\epsilon = 3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. Starting from a typical detected photocurrent of 1 nA, and taking into account the quantum efficiency of our photodiodes (~ 0.85), the collection efficiency of the objective (~ 0.87 for NA 0.75), the known illumination intensity (10 W/cm² at 546 nm), and $q = 0.3$, we estimate that of the light incident on a 45 μ m square pixel (5.5×10^{14} photons/s), roughly 5×10^{-4} is absorbed. For a spherical cell of diameter 30 μ m, taking $\epsilon = 3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and assuming that each lipid takes up an area of 0.7 nm², this gives a dye molar fraction of 2.2% in the outer leaflet. This agrees well with the first estimate.

Thus, the molar fraction of RH423 in the membrane of an SCG neuron stained according to our standard protocol is of the order of 1%, and the fraction of incident light absorbed is of the order of 10^{-3} . Though we have not seen any electrophysiological effects from the present level of staining, it seems unlikely that staining can be much increased without seriously affecting the cells' membrane properties.

To compare the efficacy of fluorescence and absorption measurements for vertebrate neurons, let us imagine an absorption dye (call it AD423) that has the same staining properties, extinction coefficient, and voltage sensitivity as RH423, and calculate for both dyes the signal-to-noise ratios for a 10-mV synaptic potential in the typical SCG neuron. For RH423, the total detected fluorescence is $F = 1 \text{ nA} = 6.3 \times 10^9$ photons/s; with a 300 Hz bandwidth, the rms shot noise will be $i_{\text{shot}} = 1.9 \times 10^6$ photons/s. With $\Delta F/F = 1\%/100 \text{ mV}$, the 0.1% signal from a 10 mV PSP will be $\Delta F = 6.3 \times 10^6$ photons/s, for a signal-to-noise ratio $S/N = 3.3$.

A cell stained with AD423 will still absorb 5×10^{-4} of incident light. The voltage sensitivity of AD423 is such that its absorption changes by 1% for every 100 mV of membrane potential; this gives an overall intensity change $\Delta I/I = 5 \times 10^{-6}/100 \text{ mV}$. Signals of this size would be completely masked by the intensity fluctuations from even a well-stabilized mercury arc lamp. More stable illumination requires switching to a tungsten-halogen lamp, at a cost of roughly a factor of 10 in illumination intensity. Taking the illumination intensity of 1.6 W/cm² (8.8×10^{13} photons/s per pixel) used by Parsons et al. (1989), and multiplying by a detection efficiency of 0.85 gives $I = 7.5 \times 10^{13}$ photons/s and $i_{\text{shot}} = 2.1 \times 10^6$ photons/s. For a 10-mV PSP, $\Delta I/I = 5 \times 10^{-7}$, so $\Delta I = 3.8 \times 10^7$ photons/s, and $S/N = 0.18$.

The signal-to-noise ratio for the hypothetical absorbance dye AD423 is thus twentyfold worse than that for the fluorescence dye RH423. This difference could perhaps be made up by finding a dye with a higher extinction coefficient and very high voltage sensitivity, but the effort would probably be better spent in searching for a better fluorescence dye. In general, it seems that fluorescence is preferable to absorption for small cultured vertebrate neurons, mainly because of their small membrane area.

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