

## Marking microelectrode penetrations with fluorescent dyes

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### Abstract

Fluorescent dyes were used to mark and identify the tracks left by extracellular microelectrodes in neurophysiological experiments. Forty-two penetrations were made into the postcentral gyrus of 3 Macaque monkeys with electrodes coated with 1 of 5 fluorescent dyes (DiI, DiO, DiI-C5, PyPO, and Fast Blue). The electrodes were driven at rates ranging from 10 to 1000  $\mu\text{m}/\text{min}$ , to a depth of about 4000  $\mu\text{m}$ , where a small electrolytic lesion was made. Histological sections were viewed under fluorescent optics and the electrode tracks were reconstructed from the dye traces. Fluorescent traces (width 50–400  $\mu\text{m}$ ) were observed in 41 of 42 penetrations with 24 traces extending to the lesion site. Of the electrodes driven in less than 3 h, those coated with DiI (8/8) and DiI-C5 (8/8) left a trace to the lesion site, while 57% (4/7) of the DiO, 40% (2/5) of the Fast Blue and only 11% (1/9) of the PyPO tracks were fully marked.

This method of marking penetrations can be used with any extracellular recording configuration, does not require tissue sections to be processed or stained, does not require electrical lesions, and causes no detectable tissue damage. Because the dyes fluoresce at different wavelengths, closely spaced tracks can be uniquely identified.

*Keywords:* Reconstruction; Mapping; Fluorescence; Microelectrode; Cortex; Lamina; Neural recording; (Monkey)

### 1. Introduction

A significant problem in neurophysiology is correlating neurophysiological responses with neuroanatomical structures. This problem is especially difficult in experiments that last several weeks or more and involve many penetrations. Although a variety of methods have been used, none of them reliably identify individual tracks from multiple penetrations densely placed in a small region of cortex. Because topography (e.g., somatotopy) and lamination are key organizational features of the cortex, it is important to know the precise locations of recording sites to understand the functional significance of the recorded signals.

The most widely used method for reconstructing electrode penetrations is to place small electrolytic lesions (e.g., 5–10  $\mu\text{A}$  for 20 s) at points along each recording track. Recording sites are then identified by finding the lesions on histological sections and interpolating between them. The lesions are visible in unstained sections and are even detectable after several months by staining for markers that are sensitive to glial activation such as cytochrome oxidase. The lesion method is limited because it damages cortex and closely spaced lesions from adjacent tracks are difficult to identify.

Alternative methods that rely on tissue damage resulting from the electrode passing through the tissue have only been partially successful. Marshall (1941) identified electrode tracks using an iron hematoxylin stain but his electrodes were very large (600–800  $\mu\text{m}$  in diameter) and caused a large amount of easily detected tissue damage. Other methods involving more sensitive stains such as thionin (Nissl stain) (Powell and Mountcastle, 1959), cytochrome oxidase (Livingstone and Hubel, 1984), and immunological staining (Benevento and McCleary, 1992) can mark tracks made by smaller electrodes (100–200  $\mu\text{m}$  in diameter). Immunological staining for tracks relies on antibodies to glial fibrillary acidic protein (GFAP) and is sensitive to small amounts of glial activation, such as that occurring after damage associated with electrode penetrations. These methods have two problems: they are less effective when the time between penetration and perfusion is long (presumably because the tissue damage caused by the electrode is repaired) and closely spaced tracks are difficult to identify.

In this paper, we describe a method for marking electrode tracks based on coating the electrodes with fluorescent dyes. This method requires only that the electrode be coated with a commercial dye before the penetration; it

involves no treatment of the tissue other than standard perfusion and sectioning. Coating the electrodes with dyes allows each penetration to be easily located and, because the dyes have unique fluorescent absorption/emission signatures, the identity of each penetration is imbedded in the tissue.

## 2. Materials and methods

### 2.1. Animals and surgery

Three monkeys (*Macaca mulatta*, 3–4 kg) were used in this study. All surgical procedures were done under sterile conditions according to the rules and regulations of the Johns Hopkins Animal Care and Use Committee. Recording chambers, centered over the post-central gyrus were fixed to the skull of all 3 animals and a 10-mm-diameter hole was drilled through the skull at the center of the chamber to expose the dura mater. Two animals were initially anesthetized with ketamine HCl (33 mg/kg, i.m.) and kept under anesthesia with sodium pentobarbital (10 mg/kg/h, i.v.) while the penetrations were made. The third animal was awake and doing a behavioral task during all penetrations. Since the anesthetic state had no effects on how well the electrode tracks were marked, the results from the 3 animals were combined.

### 2.2. Fluorescent dyes

Five fluorescent dyes were tested in these experiments. These dyes were chosen because they are readily available, uniquely identifiable by their spectral characteristics (Table 1), and thought to be non-toxic (Honig and Hume, 1986, 1989; Kuffler, 1990; Chen and Schofield, 1992). In addition, they successfully marked electrode tracks in neurophysiological recordings from rat cortex (unpublished

observations). DiI, DiO, DiI-C5, and PyPO were obtained from Molecular Probes and Fast Blue was obtained from Dr. Illing, GmbH, Germany and Sigma. Concentrated solutions of the dyes (Table 1) were made by dissolving the dye crystals in ethanol or distilled water (Fast Blue, one penetration).

### 2.3. Electrode preparation

A multielectrode microdrive (Mountcastle et al., 1991) was loaded with 7 quartz-coated platinum/tungsten (90/10) electrodes (diameter: 80  $\mu\text{m}$ ; tip diameter: 4  $\mu\text{m}$ ; impedance: 1–5  $\text{M}\Omega$  at 1000 Hz). The electrodes were positioned in the microdrive in a linear array (400  $\mu\text{m}$  inter-electrode spacing).

Two different procedures were used to coat the electrodes with dye. The first involved orienting the electrode vertically, dipping it repeatedly (10 times) into one dye solution, and allowing it to dry in air for about 5 s between dips. The second procedure involved orienting the electrode horizontally 1 mm above a flat metal surface and placing a drop of dye solution over its tip so that the distal 10 mm was submerged. The dye solvent was then allowed to evaporate leaving the electrode tip coated with dye. After both procedures, the electrode was inspected under a microscope to confirm that it was undamaged.

### 2.4. Penetrations

Electrophysiological recordings were made in the post-central gyrus of the monkeys using standard techniques (Phillips et al., 1988; Mountcastle et al., 1991). The recording chamber was filled with physiological saline and the microdrive was mounted normal to the dura. The electrodes were then individually advanced through a latex seal that protected the end of the microdrive, through approximately 1 mm of saline, through the dura, and into

Table 1  
Summary of the fluorescent dyes and optical filters used

Dye	Chemical name	Dye peak absorption/emission (nm)	Filter set used (excit/dicroic/emiss) (nm)	Dye solution conc. (mg/ml)
Fast Blue	not available	382/ *	broadband (320–380/395/420)	16
PyPO	5-phenyl-4-pyridyl-2-oxazole octadecyl bromide	388/476	broadband (320–380/395/420)	42
DiO	3,3'-dioctadecyloxycarbocyanine perchlorate	484/501	fluorescein (450–490/510/520)	42
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate	550/565	rhodamine (540–552/580/590)	50
DiI-C5	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate	644/665	long wave (590–630/645/660)	50

Dye absorption/emission data are peak values as measured in ethyl alcohol (Molecular Probes, Sigma). Excitation filter values are half-peak wavelengths. Emission and dicroic filter values are half-cut-on wavelengths and all emission filters are long pass (Zeiss, Omega). Dye solutions are in ethanol.

\* Peak emission not published (Sigma).

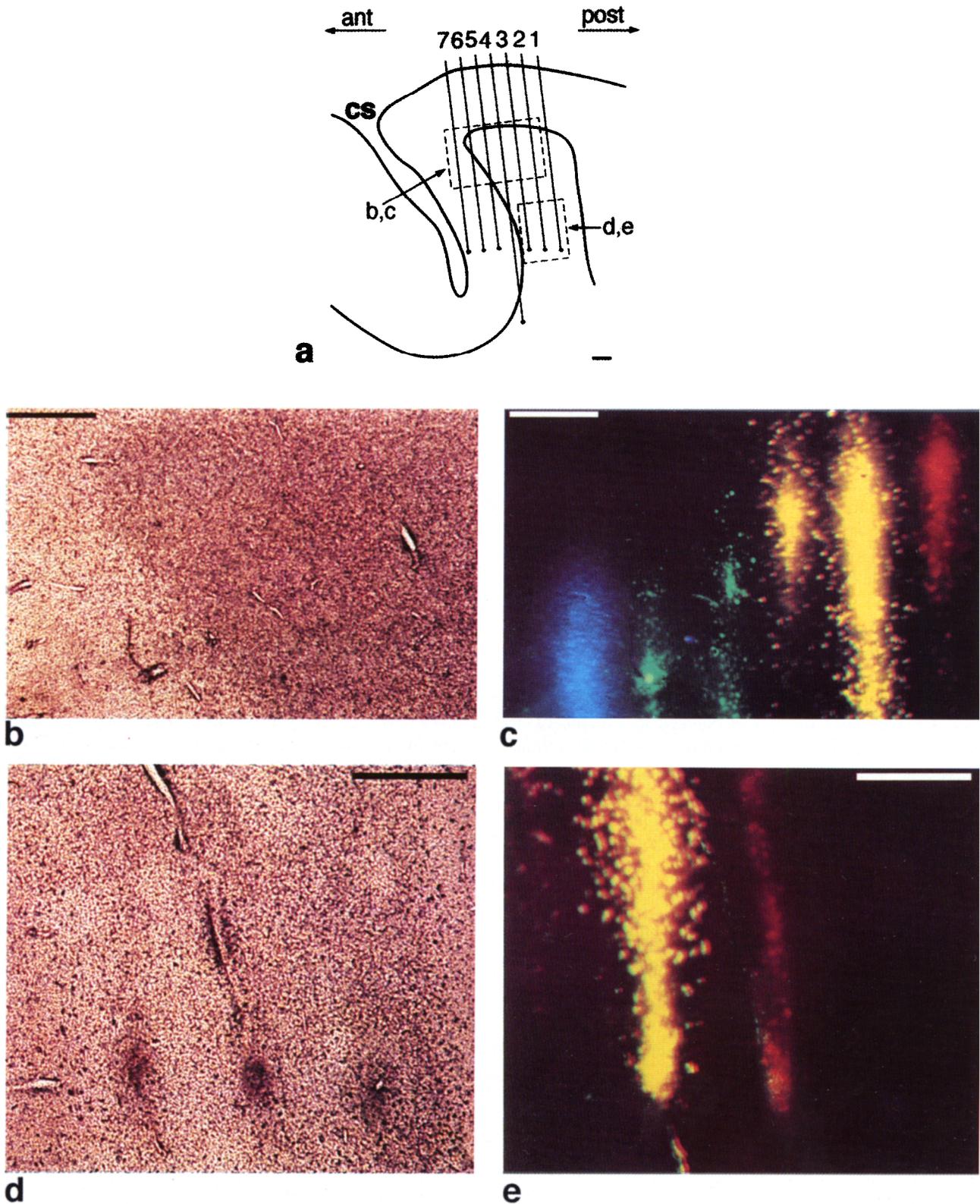


Fig. 1. a: Sketch of a typical section with approximate locations of the numbered electrode penetrations and lettered micrographs (CS = central sulcus). b: Bright-field micrograph of region indicated in (a). Note the lack of tissue damage. c: Multiply exposed fluorescent photomicrograph of the same field and section in (b). Dye traces left by 6 electrodes. From left to right, Fast Blue, DiO, DiO, DiI, DiI, DiI-C5. The punctate areas of high fluorescence are cell bodies that have incorporated dye. d: Bright-field micrograph of region specified in (a), showing the end-point lesions of electrodes 3, 2, and 1 (left to right). e: Multiply exposed fluorescent micrograph of the same field and section in (d). The left 2 electrodes (3,2) produced traces that extended to the lesions, whereas the right-most electrode (1) left no trace (PyPO). a–e: Sections are from an animal perfused 4 weeks after the penetrations were made. Anterior is left. Scale bars: 500  $\mu\text{m}$ .

the cortex. For each penetration, detailed information concerning the top of neural activity (TONA), neuronal receptive fields, spontaneous activity, and driving rate were stored in a microcomputer database. Single units were isolated and recorded in response to scanned, embossed tactile stimuli (Phillips et al., 1988).

The electrodes were driven into the cortex at rates ranging from 10 to 1000  $\mu\text{m}/\text{min}$ . Each electrode was driven to a total depth of approximately 4000  $\mu\text{m}$  below TONA, and at the deepest position a small electrolytic lesion was made (5  $\mu\text{A}$  for 20 s). After all of the electrodes had been withdrawn, two drops of dexamethasone phosphate (0.1%) and gentamicin (0.002%) were applied to the dura, Gelfoam (Upjohn) soaked in sterile saline inserted into the recording chamber, and the chamber sealed.

### 2.5. Histology

Three hours to 4 weeks after the last electrode penetration, each animal was perfused and its brain sectioned. Perfusing the animal consisted of anesthetizing with ketamine HCl (33 mg/kg, i.m.) and inserting five 28-ga pins 10 mm into the cortex at locations surrounding the penetration sites. The animal was then deeply anesthetized with sodium pentobarbital (65 mg/kg, i.v.) and perfused transcardially with phosphate buffer followed by cold, phosphate-buffered 3% paraformaldehyde (pH 7.4). After extracting the brain, a 15  $\times$  10 mm region of cortex surrounding the penetrations was blocked to a depth of 10 mm. The tissue block was postfixed in 3% paraformaldehyde at 4°C for 24 h, and then transferred to 20% sucrose at 4°C for another 48 h for cryoprotection.

The tissue was then frozen and cut into 50- $\mu\text{m}$ -thick sections parallel to the electrode tracks. Sections were collected into cold phosphate buffer and mounted on gelatin-coated slides; they were not cover slipped. The sections were allowed to dry for several minutes before being placed in a covered container at 4°C.

Each section was viewed under a mercury lamp (100

W) fluorescent microscope (Zeiss) equipped with 4 different fluorescent filters tuned to visualize different spectral ranges (see Table 1): a broadband filter was used to visualize PyPO and Fast Blue, a fluorescein filter for DiO and DiI, and a rhodamine filter for DiI. In addition, a long wavelength filter (Omega) was used to visualize DiI-C5 (Roe et al., 1990). Combination fluorescent photomicrographs were taken by exposing color film under all 4 filter sets.

Cytoarchitectural boundaries and cortical lamina were determined by applying a few drops of phosphate-buffered (0.1 M, pH 7.4) Acridine Orange (0.001%, Sigma) or bisbenzamide (0.001%) to the sections.

### 3. Results

Forty-one of 42 electrode tracks were found and identified by reconstructing the fluorescent dye traces. Only one track made by an electrode coated with PyPO was not marked by the dye. Typically a penetration crossed 5–10 sections and occasionally more than 2 mm of continuous track was visible on a single section. Fig. 1c shows an example of a combined fluorescent photomicrograph of one such section where 6 out of 7 tracks from a single recording session are visible. The electrode coated with Fast Blue (Fig. 1, electrode 7) left a track that is clearly visible using the broadband filter and is only barely visible under the fluorescein filter. The electrodes coated with DiO (Fig. 1, electrodes 5 and 6) were only visible under the fluorescein filter. The electrodes coated with DiI (Fig. 1, electrodes 3 and 4) were visible under the rhodamine filter (orange) and fluorescein filter (green). Because photomicrographs 1c and 1e were exposed to all 4 filter sets, the traces for these 2 electrodes appear as yellow. The electrode coated with DiI-C5 (Fig. 1, electrode 2) was only visible under the long wavelength filter (red). Except the uppermost section (not shown), no trace was left by the electrode coated with PyPO (Fig. 1, electrode 1).

Lesions were identified for 41 of the 42 penetrations,

Table 2

Summary of the percentage of track marked by dye type for the 41 penetrations in which the lesion was located

Dye	Driving time (h)	Number of penetrations marking the indicated percentage of track length(%)			Total number of penetrations
		< 80	80–99	100	
DiI	< 3	0	0	8	8
	> 3	0	0	1	1
DiI-C5	< 3	0	0	8	8
	> 3	0	3	0	3
DiO	< 3	1	2	4	7
Fast Blue	< 3	1	2	2	5
PyPO	< 3	4	4	1	9
Total		6	11	24	41

See text for details.

and the percentage of each track marked by the dye computed (Table 2). The dye traces from 23 of these 41 penetrations marked the entire length of the track up to the lesioned endpoint (e.g., Fig. 1d,e, electrodes 2 and 3). When the dye failed to mark the entire track, the trace was always clear from the surface of the cortex down to some variable distance above the lesioned endpoint. DiI and DiI-C5 consistently marked the entire length of track when the driving time (time to reach maximum depth after passing through the dura mater) was less than 3 h, with DiI-C5 showing a decrease in the amount of dye deposited at the deeper locations for longer drive times. DiO and Fast Blue usually marked at least 80% of the penetration. PyPO performed least reliably, being the only dye that did not leave a trace (1 penetration) and in the other 8 penetrations only once reached the lesion site.

The width of the tracks left by the 41 penetrations varied from 50 to 400  $\mu\text{m}$ , with an average width of about 150  $\mu\text{m}$  (e.g., Fig. 1c). For all of the tracks, the center of the trace was brighter than the edges allowing the center of the electrode track to be identified. The bright punctate spots at the edges of the traces are single cells labeled by the dyes (Fig. 1c).

No tissue damage was evident on any of the sections. For example, Fig. 1b is a bright-field photomicrograph of the same section shown in Fig. 1c. This figure, in which the white matter appears dark, reveals no tissue damage although 7 electrodes covered with dyes passed through this section. GFAP staining of sections containing electrode tracks with and without dye revealed nearly identical staining density and width (not shown).

#### 4. Discussion

In this study we show that microelectrodes coated with fluorescent dyes leave clear traces in cortical tissue where electrode penetrations have been made. Five different dyes were tested and all of them (except PyPO, 1 case) were clearly visible and showed minimal lateral diffusion even after 1 month. A significant length of track was marked by the dyes in 41 of 42 penetrations. All of these penetrations were uniquely identified based on the fluorescent absorption/emission signatures of the dyes and on the relative positions of the traces.

Of the 5 dyes, DiI, and DiI-C5 consistently marked the entire penetration track. PyPO, DiO, and Fast Blue usually marked a significant portion of the penetration, but did not consistently mark the entire track. One possibility is that these dyes did not adhere as well to the electrode and thus the electrodes were not as well coated. Another possibility is that these dyes may have a higher solubility or affinity to cortical tissue and thus may have diffused off the electrode before it reached its maximum depth. PyPO is less fluorescent and less photo-stable than the other dyes (Molecular Probes) which may partially explain why its

trace was not consistently observed near the bottom of the penetration.

These experiments suggest that the types of dye and how quickly an electrode is driven into the cortex are important determinants of whether the entire penetration is marked. DiO, PyPO, and Fast Blue showed variable results independent of driving rate and DiI and DiI-C5 always marked the entire electrode track over a wide range of driving rates. Therefore, a critical parameter is the type of dye used to coat the electrode, presumably because some dyes coat better than others. Driving rate seems to have a small but significant effect especially for experiments that last many hours. For driving times greater than 3 h, the amount of electrode track marked by DiI-C5 appeared to decrease with driving time (Table 2).

Although we made electrolytic lesions to mark the bottom of these electrode penetrations, it is desirable that no lesions are made and that the bottom of the penetration is marked by the presence of dye. Since DiI consistently marked the entire length of the track for driving times up to 5 h, penetrations made with DiI-coated electrodes driven in less than 5 h do not require lesions to mark the bottom of the track. Similarly, DiI-C5 may be used to mark the bottom of the track for electrodes driven in less than 3 h. The results for the other dyes were less reliable and thus another method must be used to mark the bottom of the track. One method is to coat an adjacent electrode with DiI or DiI-C5 and to use the relative depths from this electrode as a guide. Another is to modify the present method and control the diffusion rate of dyes or other markers off the electrodes. By dissolving any marker in an electrode coating polymer with a 5 h half-life in cortical tissue, the marker could be deposited along the entire track during a 5 h penetration. Alternatively, the marker diffusion rate could be decreased by treating the electrode (e.g., with silanes) so that the surface has a greater affinity for the marker. It may even be possible to bind the marker to the electrode surface until the electrode is driven to its final depth, where the marker could be released by slightly vibrating the electrode or delivering a high voltage/low current electrical pulse.

Increasing the number of uniquely identifiable markers would also be desirable. Other dyes that might be used include DiA and DiS, which are structurally similar to DiI, DiO, and DiI-C5 (Molecular Probes), and have differentiable fluorescent spectral properties. Dyes could also be mixed, resulting in electrode tracks with mixed absorption/emission signatures. If each mixture could be uniquely identified, then the number of effective markers would be increased to  $2^n - 1$ , where  $n$  is the number of independent dyes. Thus even if just 4 dyes are used, the number of uniquely identifiable tracks would be increased to 15. We successfully tested a simple version of this method in two penetrations using electrodes coated with a mixture of DiO and DiI-C5 which have well-separated absorption/emission spectra (not shown). However, identifying dye mix-

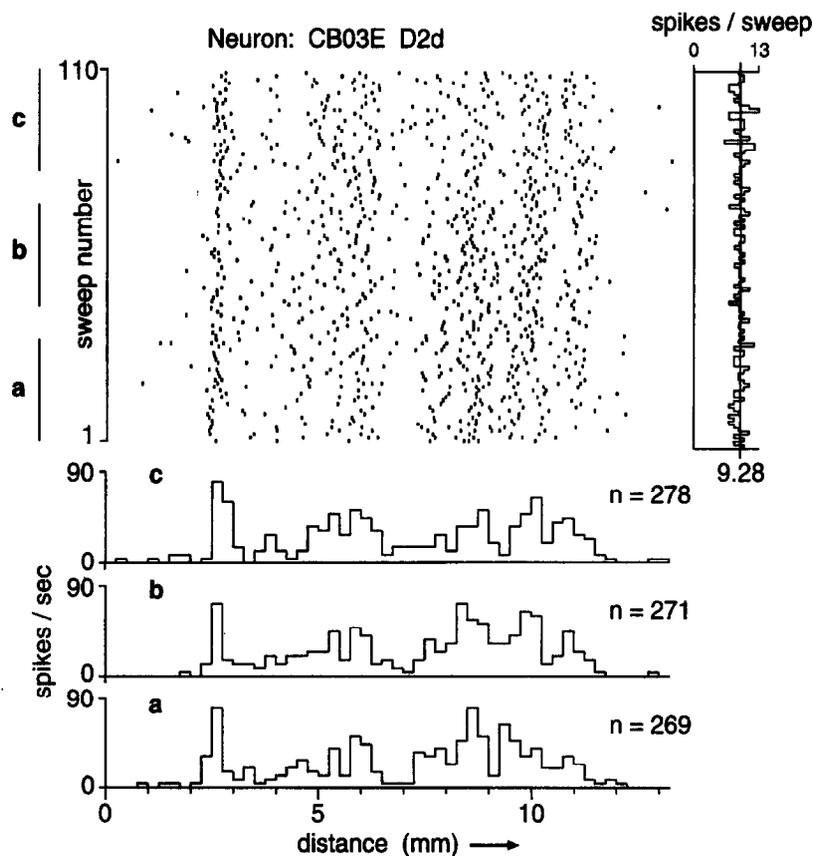


Fig. 2. Typical response of a neuron in area 3b of SI cortex to an embossed bar scanned repeatedly across the neuron's receptive field on a distal finger pad (40 mm/s, 30 g force, arrow indicates scan direction). This neuron was recorded along a fully marked penetration made by an electrode coated with DiI-C5. Each row of tick marks represents the action potentials recorded from 1 sweep of the bar (110 sweeps total). Action potentials from the first sweep were recorded 15 min before the action potentials from the last sweep. Histograms computed from the first, middle, and last 30 sweeps (a,b,c) are not significantly different (Kolmogorov-Smirnov,  $P > 0.1$ , all pairs). The number of spikes per sweep (right side) is independent of sweep number (linear regression, intercept = 9.28 spikes/sweep, slope = 0.0029, SE of slope = 0.0038,  $P = 0.452$ ).

tures that have close absorption/emission spectra requires quantitative measurement of the combined spectrum.

A potential problem with using dyes to mark electrode tracks is that the dyes may affect the neuronal responses. However, previous neuroanatomical studies, as well as the results reported here, suggest that even after long periods of time, the dyes do not cause significant tissue damage. Of the dyes that we tested in this study, DiI and DiO, have no obvious adverse effects on the growth, survival, or electrophysiological properties of neurons in culture (Honig and Hume, 1986, 1989; Kuffler, 1990). DiI-C5 and PyPO have molecular structures and membrane insertion/diffusion behavior similar to DiI and DiO (Molecular Probes) suggesting that they also do not cause tissue damage. Although the evidence that Fast Blue is non-toxic is less secure, Chen and Schofield (1992) show that calcium currents appear normal in Fast Blue-labeled neurons.

We have searched for both anatomical and physiological signs of dye-induced neuronal alteration. Anatomically, dye-marked penetrations demonstrate no tissue damage beyond that found in unmarked penetrations in un-

stained (Fig. 1), Acridine Orange/bisbenzamide-stained or GFAP-stained sections (not shown). Physiologically, neurons recorded from dye-marked penetrations placed less than 300  $\mu\text{m}$  apart on successive recording days exhibited normal somatotopic progression, responsiveness, and firing rates. Although there is no evidence of long-term damage, it is possible that neuronal function is altered transiently; for example, by disruption of local ionic concentrations or osmolality. However, the responses of neurons to repeated presentations of the same stimulus show no such disturbances (see Fig. 2). Although we have found no evidence of long- or short-term adverse effects of these dyes, the possibility of such effects cannot be ruled out.

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