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# Improvements in the Optical Recording of Neuron Activity Using Voltage-Dependent Dyes

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Studies of the electrical activity and plasticity of neural networks, individual nerve cells, and their subcellular compartments are now impractical without using optical methods to visualize functional signals which allow electrical events to be recorded both in many neurons simultaneously and in individual dendrites and axons and provide for linking these data to precise morphological images. Use of voltage-sensitive dyes (VSD) is the only of the small number of currently available methods which combines high spatial resolution with the ability to record ultrafast signals (<0.1 msec) in real time with a high signal:noise ratio. Significant progress in applying VSD has been made in the last decade, particularly in analysis of the occurrence and encoding of electrical signals in dendrites and axons, and has resulted from improvements in the method associated with using the best dyes in combination with sequential improvements in apparatus and optical instruments. This has allowed the method to demonstrate its value and effectiveness and advance from purely technical areas to the arsenal of major equipment in the world's leading laboratories working at the frontiers of cell biology and neurophysiology.

**Keywords:** neuron, voltage-dependent stain, visualization, optical recording.

Optical recording using VSD has been used successfully since the end of the 1980s by numerous laboratories to follow neuron activity in real time. Optical methods are often used to obtain data which are difficult or impossible to obtain by other methods. The range of applications of VSD varies from recording of overall potentials, for example to map directionally sensitive columns in vivo to simultaneous recording of the spike activity of networks of many individual neurons or to record potentials in axon compartments and dendrite spines. Despite decades of successful applications [22], the method remains methodologically and technically complex and has high demands for apparatus, clear organization of experiments, and other conditions required for an acceptable final result. In addition, successful studies require constant improvements to methods for adaptation to particular tasks or study systems and introduction of technical advances and improvements – which often lead to qualitatively new results and visualization of finer details of physiological processes which had remained unknown because of lack of methodological sensitivity. In these conditions, technical progress in the application of VSD as reflected in the increasing number of publications from the world's leading research groups can be followed particularly clearly. This review addresses new state-of-the-art aspects of the application of optical recording with VSD in relation to the most relevant study systems and objectives of contemporary neurophysiology.

**Selection of the Optimum Design of Instrumentation for Optical Recording.** The key point in the process of determining the specific task of an experiment using VSD is to select the appropriate design of the instrumentation for addressing the main problems of a study. Current experimental practice mostly uses two main systems for optical recording, which have defined non-overlapping spectra of applications and benefits, which are difficult to change after the choice has been made. The first scheme is based on the use of photodiode arrays with parallel and

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independent amplification of the signals from each element. In practice, an instrument based on photodiode arrays consists of a set of independent photodiodes formed into an array and sending signals from each photodiode to its amplifier, connected via a multichannel analog-to-digital converter (ADC). This type of scheme has high time resolution (0.2 μsec) but low spatial resolution (124 or 464 light-sensitive elements), along with intermediate sensitivity with a high photodynamic range (Fig. 1, *A*). Time resolution is limited by the properties of the dyes, and also by the rate of photodiode interrogation giving an acceptable signal:noise ratio [20]. The signal from each photodiode is amplified in parallel, so, for example, an instrument with 124 diodes requires 124 two-element cascade amplifiers, making construction and servicing complex. Because of high sensitivity to vibration noise (up to 20 Hz) and mechanical interference modulating the intensity of transmitted light, the microscope must be rigidly attached to the anti-vibration mounting of the micromanipulator or have a pneumatically activated platform.

The signal obtained by recording the electrical activity of neural structures using absorption dyes in transmitted light is the sum of the membrane potentials of the neurons projected onto the light-sensitive elements of the array [20, 55]. The light illuminating the specimen is initially passed through a heat filter and then through a multilayer narrow-band filter with a bandpass of ~720 ± 20 nm to obtain the greatest response. Increases in the quantity of transmitted light correspond to neuron depolarization and decreases to hyperpolarization. The system used for recording in transmitted light does not require high sensitivity as transmitted light has high intensity; however, parameters such as a wide linear range and low intrinsic noise from the diode array, amplifiers, and light source are critical.

The second scheme is based on use of a CCD camera (coupled charged device, Fig. 1, *B*) followed by interrogation of the photoelements. This scheme has high spatial resolution (256,000 or more elements), high time resolution (~1 msec [18], ~0.1 sec [50]), and high sensitivity to weak fluorescent signals. When fluorescent dyes are used for recording with a CCD camera, the useful signal reflects relative changes in fluorescence ($\Delta F/F$). The greatest time resolution with the best signal:noise ratio currently available is provided by the RedShirtImaging Neuro CCD SMQ 80 × 80 pixel integrated system (USA), which provides for recording of 10000 frames/esc in a regime with frame cropping to 12 × 80, which is in fact the most widely used standard instrumentation in many laboratories because of the convenient programming system (Neuroplex, OptImaging LLC, USA) and the integrated instrumentationand programming solutions for simultaneous control and synchronous recording of optophysiological and electrical signals and stimuli. The system includes a camera, an optical adapter for attachment to the microscope (depending on model), and a processing computer with an ADC, software, and control and synchronization unit.

Apart from a CCD camera, fluorescence can also be recorded using a confocal microscope. However, classical confocal microscopes are not particularly suitable for recording VSD signals, as they cannot record sufficiently strong signals with sufficient speed, because of loss of intensity on passage of the excitatory and reflected light through the scanning aperture of the diaphragm. In addition, confocal scanning involves loss of time resolution of the signal, because it runs point by point (or line by line) with physical displacement of the point (or line, if the detector is a line detector) of the scanning mirror such that no signal consisting of other points of the image is recorded at this time. In comparison with CCD cameras, massive benefits in the rate of image visualization can be obtained from the fact that each pixel continuously receives light projected from the image; it is only the charge reading point formed by the photocurrent of a single cell that moves, while the charge on other pixels continues to accumulate until the next interrogation cycle. Some investigators combine the confocal scanning device with a fast CCD camera with lower resolution using an addition beam splitter in a single instrument. This provides for comparison of high-resolution pictures obtained using the confocal device with fast recording at lower resolution with the CCD camera [29, 30]. Alignments of the images are approximate, especially on the Z axis, because of the limited resolution of the CCD camera (~4–5 μm). The additional confocal system also allows genetically modified rats/mice with neurons labeled with the fluorescent probe GFP (green fluorescent protein) to be used, such that neurons of the type and morphology of interest can be selected for subsequent staining with VSD and used in experiments [30, 52].

Equivalent or better results using confocal microscopy can only be obtained in continuous scanning regimes not using a scanning point. A recently developed type of line-scanning confocal microscope, the LSM 5 Live (Zeiss, Germany), provides significantly better characteristics for optical recording using VSD in high-speed line scanning, as its sensor is a high-resolution linear CCD detector consisting of 512 elements [27]. While line scanning has limited potentials compared with full-frame CCD cameras, much finer spatial structures can be resolved (resolution 0.5 μm), such as the central synapses of mammals, and high-precision three-dimensional reconstruction of images can be performed, as the same detector and optical path are used for both recording and three-dimensional scanning with a special adapter, as there is no requirement for a separate camera and confocal attachment [29, 52].

Recent studies have demonstrated that voltage-dependent signals can be observed using the known lipophilic marker FM4-64, which is widely used to visualize presynaptic transmitter release [15] using the second harmonic of the excitation. The signal is recorded in transmitted light with infrared laser transmission using twice the wavelength of the absorption spectrum peak and a photomultiplier as

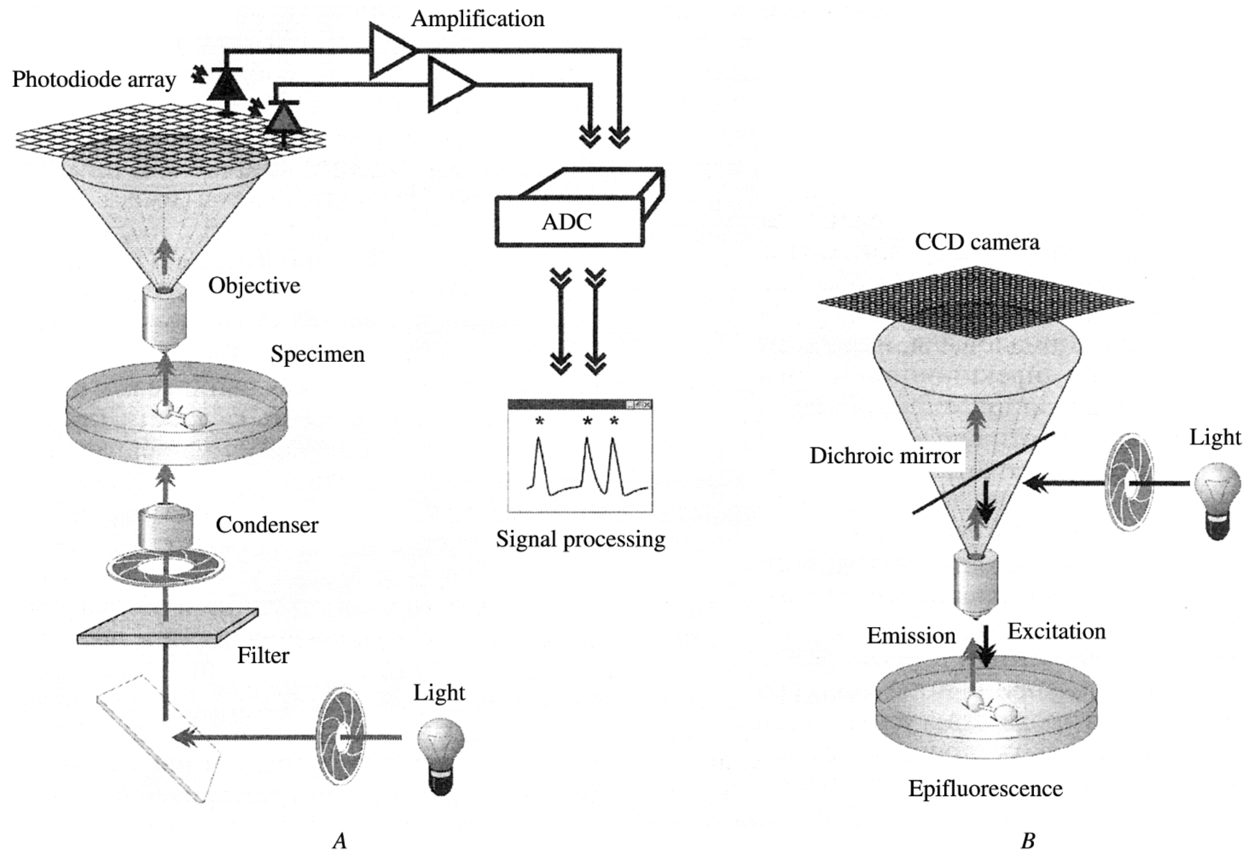


Fig. 1. Diagram of the apparatus used for optical recording of changes in the passage of light through the specimen ($\Delta T/T$, relative change in transmission) using a photodiode array (*A*) and using epifluorescence ($\Delta F/F$, relative change in fluorescence) and CCD camera as detector (*B*). The signal from the CCD camera is also amplified and digitized (not shown in diagram). Labels show components of the light pathway of the microscope and detection attachments.

detector [26]. Despite the fact that optical recording of potentials using the second harmonic has the important advantages of suppressing interference due to internalization of dye into the cell (as the signal is produced exclusively by dye molecules in an ordered orientation in the membrane, and not by molecules in the cytoplasm) and a low signal:noise ratio, this method cannot be used to address any significant tasks.

**The Range of Objectives for Optical Recording.** Selection of the objective is determined by the scaling factor required for the area of the camera/sensor chip for optimal projection and resolution of fine details of biological structures by optical methods. Additional requirements are also imposed by the type of dye used, which determines the particular conditions needed to obtain a satisfactory signal. Recording of multiple-cell activity with fluorescent or absorption VSD is performed using contact (LK ×25 0.4 NA, W.D. < 0.025, LOMO [3, 46, 48]) or water immersion (Olympus UMPlanF1 20×, 0.5 NA, W.D. 3.3 or similar ×10–20 [13, 16, 47] objectives, which can be combined with intracellular recording and stimulation at a suitable working distance [5, 48]. For recording using a contactless objective, stained specimens are sometimes embedded in 1–1.5% agarose [6] prepared in normal physiological saline. Air objectives are practically unsuitable for recording with absorption stains because of the varying water surface, which alters the intensity of transmitted light to a much greater extent than the useful signal.

When fluorescent VSD are used for recording individual neurons, electrically insulated water immersion objectives with high numerical apertures are generally used (N.A. 0.4–1.0), on which the fluorescence brightness of the picture and the sensitivity of the method directly depend [23]. Single neurons in invertebrates are recorded using ×10–20 objectives (N.A. 0.4–0.5) [10, 11], while single neurons, axons, and dendrites in mammals are recorded using ×60–63 (N.A. 0.9–1.0; W.D. 2–2.2) objectives manufactured by Olympus, Zeiss, and Nikon [14, 25, 29, 35, 52] and, less often, ×40 (N.A. ~0.8) for dendrites [9, 25]. When a CCD camera is combined with a ×60 objective, the recording field is about 200 μm, which is sufficient for recording the initial segment and the first two nodes of Ranvier of axons in large mammalian neurons such as Purkinje cells and pyramidal cells in layer 5 of the somatosensory co-

rtex, where the first node of Ranvier is located 80–130 μm from the neuron body [29]. In addition, the most widely state-of-the-art dyes (ANEP derivatives) require the objective to have good transmittance (85–90%) in the near infrared range at 700–800 nm, which overlaps the emission peak of the dye [17]. As recording usually involves simultaneous use of the glass electrodes used for dye delivery, the optical characteristics of the objective represent a compromise with the need for a significant working distance (>2 mm) to allow the electrode to be positioned beneath the objective. Zeiss recently released the new W Plan-Apochromat 40×/1.0 DIC objective, whose stated specifications make it an improvement over previously used objectives and may provide further enhancements to the useful optical signal, though reports as to how well it behaves in real experiments remain to be published.

Apart from the objectives described above, low-magnification air objectives are sometimes used for recording large neurons in invertebrates or local population signals [24], and also for recording population signals in vivo, for example overall responses of neurons in the olfactory bulb [39]. The air objectives of binocular microscopes are used when there is a need to combine optical recording using two (or more) electrodes, as this provides sufficient depth of visibility [36].

**Improvements in Light Sources.** Selection of a light source matching the selected optical recording method plays a very important and sometimes decisive role. Optical recording almost always requires recordings to have high time resolution (up to 10000 frames/sec [50]), such that stabilized power supplies must almost always be used for the light source to minimize noise (ripple noise), especially when individual action potentials are to be recorded. The only exceptions are, for example, experiments recording neuron processes in conditions of stimulation with long-duration depolarizing steps, when high time resolution is not needed [36].

Recording in transmitted light is usually performed using halogen lamps of 100–150 W with stabilized power supplies [13, 46, 47], with sufficient output to ensure a uniform light beam for transmission. Recording of fluorescent signals requires more powerful light sources, fitted with stabilized high-voltage mercury-xenon arc lamps of 150–250 W) (e.g., Opti-Quip, USA, or Crain Research Optosource, UK [8, 25, 51]). As compared with classical mercury lamps, this type of lamp has virtually no influence on the size or brightness of fluorescing objects, though it has a flat spectrum without marked peaks in the ultraviolet range and increased arc stability.

More recently, it has become routine for the most demanding tasks, such as those linked with ultrafast recording of action potentials in the initial segments of axons in mammals, to use highly stable semiconductor lasers delivering 200–500 mW at 532 nm [29, 30, 52, 59], whose emissions are projected via a light condenser and waveguide or a system of independent mirrors. Use of a laser avoids the need for an excitation filter, and a single laser can generally excite only one dye. Despite the lower flexibility, systems based on lasers allow, for example, simultaneous recording of signals from the voltage-dependent dye JPW-1114 or JPW-3028 excited with a 532-nm laser, with simultaneous recording from the same neuron of intracellular calcium levels using the probe Oregon Green BAPTA 1 (hydrazide form) excited with a 488-nm laser and linked with the first laser with a semi-transparent dichroic mirror. Use of two-photon excitation in the infrared spectrum at 1100–1300 nm has also been proposed [56], though this has yet to be employed in actual experiments.

**Optimum Types of Dye and Means of Application.** Recording using absorption methods is performed using RH-155 vital pyrazo-oxonol voltage-dependent dyes (NK-3041, (Nikon), JPW-1131 and JPW-1150 (Wuskell and Loew, University of Connecticut)). Dye solubility is sometimes improved using the non-ionic detergent Pluronic F-127 (20% solution in dimethylsulfoxide, Molecular Probes) at a ratio of 1/1–2 to the dry weight of dye. These dyes are members of the pyrazo-oxonol group, whose molecules bind to cell membranes [19]. All three dyes are isomers with different numbers of carbon atoms in the alkyl substituent. Depending on this number of carbon atoms, hydrophobicity in the pyrazo-oxonol series increases from the least hydrophobic RH155 (in which the substituent is a methyl group) towards the most hydrophobic JPW-1150 (*n*-propyl). This property determines the ease of dissolution in water and the rate at which the dye leaches from cell membranes after staining [13, 46]. In absorption methods, the dye is generally added to neurons by application to the bathing physiological saline. Changes in membrane potential cause pyrazo-oxonol dye molecules to change their absorption spectrum, this being the source of the optical signal. In addition, one of the most important properties of these and other VSD is the linearity of the signal, i.e., the optical signal is proportional to the potential and accurately reproduces its shape (Fig. 2). The greatest changes in absorption occur close to 720 nm [44]. The optical signal reflects changes in absorption of light by the dye as a proportion of the mean level ($\Delta T/T$).

Apart from absorption methods, the most widely used methods in current practice employ fluorescent voltage-dependent dyes for recording the overall potential and for detecting the electrical activity of individual cells and their compartments at the subcellular level. For overall recording from the cortex and intact CNS structures in mammals, the most commonly used dyes are RH414 [32, 39], DI-4-ANEPPS [36], and, much more rarely, RH795, which is also sometimes used simply as a morphological statin for cell membranes, or JPW-1114 [53], which is mostly used for recording individual neurons.

Major contributions to state-of-the-art optical recording science have been made by the use of VSD for optical

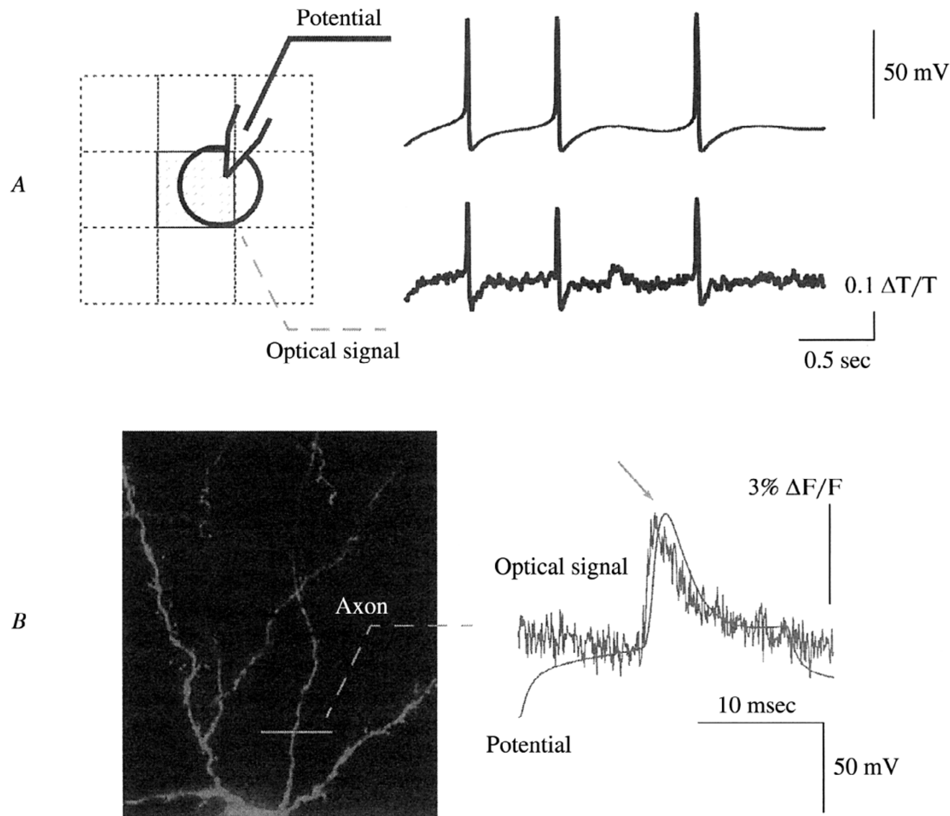


Fig. 2. Comparison of electrophysiological recordings of potential with optically recorded oscillogram. *A*) Simultaneous recording of the potential of a mollusk giant neuron with a sharp electrode (upper recording) and optical recording of the same neuron stained with the voltage-dependent absorption dye JPW-1131 using a photodiode array. *B*) Simultaneous recording of the potential of a pyramidal neuron in the rat neocortex with a patch electrode (below) superimposed on the optical recording of the axon of the same neuron at a distance of 40 μm stained with the fluorescence voltage-dependent dye JPW-1114 using continuous linear scanning at a rate of 20000 lines/sec (mean of 17 repeat scans).

recording of the activity of individual neuron compartments in mammals and invertebrates using intracellular application of VSD with ANEP (aminonaphthylethenylpyridinium) derivatives, such as JPW-1131 (di-2-ANEPEQ) [11, 35, 46] and its close analogs JPW-3028 (di-1-ANEPEQ) [24, 42, 45], and JPW-3027 (di-1-ANEPPQ) [12, 40]. In mollusk neurons, these VSD are applied by injection under pressure of saturated aqueous solutions of dye (solubility is up to 5 mg/liter at room temperature) from sharp glass microelectrodes piercing the cell membranes of neuron bodies [11]. These dyes are injected into mammalian neurons by dissolving them in intracellular solution for patch clamping in the whole-cell configuration and allowing the dye to diffuse passively into the neuron over a period of about an hour at room temperature [29]. After application, the microelectrode is withdrawn and stained neurons are incubated at room temperature before recording for 1.5–2.5 h (mammals [29, 30]) or with cooling to 8–10°C for 8–12 h [46].

Apart from currently routine methods for delivering voltage-dependent dyes, new methods have been developed with the aim of circumventing the limitations of the traditional methods. A method for biolistic delivery was verified recently, by firing gold particles coated with water-insoluble dyes into living slices of cerebral cortex [1, 2, 12]. This method allows individual arbitrary neurons to be stained in a selected area of a slice using the most hydrophobic dyes (such as di-8-ANEPPS and di-12-ANEPPQ) followed by optical recording of activity and propagation of excitation to their dendrite and axon compartments.

Particular note should be made of the rapid progress in the development of a new type of voltage-dependent dye encoded by the genes for fluorescent voltage-sensitive proteins expressed in the brains of transgenic animals. The best results to date were reported by Jin et al. [34]. And although the signal:noise ratio of genetically encoded dyes is lower than that obtained with monomolecular organic compounds, another significant relative disadvantage of the method is that these dyes mount a delayed response to voltage, distorting the shape, dynamics, and latency of recording individual action potentials. Despite the great financial support throughout the world for groups developing genetically encoded voltage-dependent probes, the signal:noise

ratio, and the precision with which action potentials can be followed, these methods significantly lag behind low molecular weight ANEP derivative dyes.

**Major Current Experimental Advances Obtained Using VSD.** Optical methods for recording potential have been used with success for in vivo mapping of direction selectivity columns, for monitoring epileptiform activity of neuron ensembles in the mammalian cortex [40, 41], for the simultaneous recording of the spike activity of multiple neurons in invertebrate ganglia [21, 44], for recording overall action potentials in the nerves and commissures of invertebrates [20, 31], and for recording activity in the olfactory tract of vertebrates [18] and mollusks [24, 37].

One of the earliest methods of using VSD was in the multiple recording of individual neurons with absorption methods followed by discrimination of individual action potentials in terms of the position, size, and shape of neurons in the area recorded [13]. Optical recording studies in the marine mollusk *Aplysia* showed that the simplest gill retraction reflex in response to stimulation of the siphon involves the least half the neurons in the abdominal ganglion, while production of the dynamic retraction curve required the activity of at least 40 neurons active at different phases of contraction to be followed [57].

Absorption VSD have also been used for recording a compact group of serotoninergic neurons (up to 56 simultaneously) in the ganglia of the mollusk *Helix* to determine the parameters of action potentials and the positions and morphologies of neurons, illuminating the structural-functional organization of the group [4, 13]. Clear dynamic clustering in the group was seen, with phasic and tonic components in the responses of neurons to electrical and tactile stimuli; these studies showed that identified neuron Pd2 could induce activation of a significant number of neurons in the group, while neuron Pd4 did not induce this activation, though its response was delayed in time in relation to activation of the main group.

Phase shifts in spreading waves of spontaneous oscillations in the olfactory brain of terrestrial mollusks can be detected by analysis of time sequences of data obtained by optical recording with VSD. The shift in the lagging wave is taken as the latency of the half-peak, or cross-correlation analysis of predicted shifts is used, calculated for the wave recorded using adjacent detectors [46, 54]. The calculated mean rate of propagation of the oscillation wave in the mollusk olfactory brain was about 3 mm/sec, though snails and slugs had different amplitude ratios for the ascending (depolarization) and descending (hyperpolarization) phases of oscillation waves [24, 47]. Odor induces changes in the frequency and amplitude of oscillations, with a quite complex temporospatial propagation pattern. Apart from spontaneous oscillations, these studies also detected waves of depolarization in the neuropil (the fibrous layer) of the olfactory brain, induced by presentation of the odor. Evoked potentials were not recorded in the zone showing the greatest amplitude of spontaneous oscillation waves; they appeared from the olfactory nerve propagated across the neuropil towards the cell body layer [4]. The area of propagation of spontaneous olfactory oscillations and the evoked potential, mapped optically, only showed partial overlap.

Studies of the generation and conduction of nerve spikes in the axons and somatodendritic compartments of neurons are impossible without using optical methods for recording membrane potential and functional activity, which permit the activity of any part of an individual neuron to be observed in real time. One of the most important directions in current studies using intracellular application of VSD JPW-1114 and JPW-3028 consist of investigations of the generation and movement of potentials at the level of individual neurons and their compartments in the main central neurons of the mammalian brain, such as pyramidal neurons in cortical layer 5 or Purkinje cells in the cerebellum. VSD have been used for detailed study of the reverse propagation of action potentials (backpropagation) in the basal and apical dendrites and their fine branches [8, 9, 11, 34]. The first measurements were also made of the resistance of the necks of dendritic spines in cortical pyramidal neurons [51]. The dye JPW-3028 was also used successfully for the simultaneous optical recording of potentials and changes in intracellular calcium levels in dendrites in combination with the probe Fura-2, given simultaneously and having distinct absorption and emission spectra [18].

The most important advance in recent years was the application of VSD for the direct superfast recording of action potential initiation in the initial axon segment of central neurons in mammals. A previous incorrect suggestion was that action potentials are generated in the axon hillock, where the gradual postsynaptic potential of the neuron is converted into threshold nerve spikes. Optical recording of the potential at a rate of 10000 frames/sec showed that action potentials are generated in the axon itself, specifically in the initial segment, at a distance of 30–50 μm from the neuron body [29, 49, 50, 52]. These data provide the full picture of action potential propagation and show good consistency with visualization of intracellular sodium in axons [28, 38], molecular biological data on the distribution of sodium channels and ankyrins in the distal axon [33], and electrophysiological recordings of axon activity using patch clamp methods [37].

**Ontogenetic Studies.** Ontogenetic methods have recently come into wide use; these allow finely focused light to activate genetically expressed light-sensitive channels with depolarizing or hyperpolarizing effects on neuron membranes, depending on the type of construct. Combinations of two light-activated channels, NpHR and ChR2, expressed in neurons and activated by two different wavelengths of light and allowing the passage of chloride and ions and nonspecific cations respectively, provide almost complete control over neural networks, switching individual neurons on and off and linking them to artificial patterns

of activity [58]. VSD such as RH1692 [42] are quite often used for recording of the activity of neural networks simultaneously with this methodology. Another approach is to use combinations of genetically encoded voltage-dependent probes expressed simultaneously in the same neurons [7].

**Conclusions**

Despite the fact that recording of neuron potentials with VSD requires apparatus and close following of complex protocols, this method yields major physiological data which are difficult or impossible to obtain by any other method. In comparison with traditional electrophysiological recording methods, optimal approaches are continually undergoing development and improvements, which open up new horizons for obtaining major information on the operation and interaction of neuron populations and individual neurons. The time resolution of optical recording approaches that of electrophysiological methods, while the spatial resolution, linearity, and consistency of interpretation of experiments and their linking with morphological images are much better. Recent improvements in the techniques and technology of optical recording have closed many gaps in our knowledge and corrected our understanding of neuron function. Optical recording is one of the most potential approaches to developing the science of the brain, especially if the search for rapid genetically encoded voltage-dependent sensory can be brought to success.

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