Ion channels are cellular sensors allowing cells to respond to a wide spectrum of stimuli that are either physical (e.g., membrane potential, mechanical force, temperature) or chemical (e.g., neurotransmitters, second messengers) in nature (15). Activated channels open a pore across the cell membrane for selected ions, causing changes in the membrane potential and/or the intracellular concentration of ions such as Ca$^{2+}$ that serve as important cellular signals. To fulfill their functional role as cellular sensors, ion channels generally exhibit exquisite sensitivity to their physiological stimuli; for example, the sensitivity of many voltage-gated channels to voltage changes easily surpasses the sensitivity of man-made transistors (37). As expected for such functional specialization, these nanoscale biological machines exhibit very sophisticated molecular design. Biophysical studies suggest that the Ca$^{2+}$-activated K$^+$ channel has >50 kinetic states (25). Knowledge of channel structures as well as dynamic changes in these structures is crucial to fully understand how an ion channel works under normal physiological conditions and how channel functions are affected under pathological conditions. In the past 5 years patch fluorometry (46, 47) has emerged as a novel tool that sheds new light on ion channels by providing a direct link between the channel structure and its function.

The basic idea behind the patch-fluorometry approach is to use fluorophores attached to the moving part of an ion channel as localized molecular sensors that report real-time structural changes (FIGURE 1). The first step for a patch-fluorometry experiment is to introduce a fluorophore docking site to the channel protein (FIGURE 2). Site-specific cysteine mutagenesis, for example, is a simple way to generate such a docking site in cloned channels expressed in cultured cells. A traditional cell-free, inside-out, patch-clamping configuration is established from cells expressing these channels. Treating the membrane patch with fluorophores specifically recognizing the docking site (for example, fluorophores with a sulfhydryl-reactive group) allows for fluorophore attachment to the site of interest. Emission from the fluorescent probes is sensitive to the local environment, the accessibility to quenching molecules, and the proximity to neighboring fluorophores. Thus recordings of the change in the fluorescence emission allow direct real-time observation of conformational rearrangements in channel proteins during channel activation. In patch-fluorometry experiments, fluorescence recordings are made from the same channels that are recorded electrically using patch clamping. The simultaneous recording of fluorescence and current allows correlation between changes in channel structure and changes in channel function. The power of patch fluor-
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ment of the voltage sensor in Shaker K⁺ channels, Isacoff and colleagues used fluorescein maleimide to label cysteines introduced at various positions of the S4 transmembrane helix. Movements of the voltage sensor, induced by two-electrode voltage clamp, were recorded in Xenopus oocytes as changes in the amount of fluorescence quenching of fluorescein maleimide as the fluo-
rophore moved from buried positions to those exposed to the extracellular iodide-containing solution. Subsequent fluorescence-based experiments using this “voltage-clamp fluorometry” technique have yielded rich information on conformational changes in voltage-gated Shaker K⁺ channels, Na⁺ channels, and transporters.

For example, using two different fluorescence resonance energy transfer (FRET) approaches, Bezanilla and colleagues recently demonstrated that a limited transmembrane movement of the S4 voltage sensor is sufficient to drive the activation of Shaker channels.

Patch fluorometry differs from whole-oocyte voltage-clamp fluorometry in that it records from a cell-free membrane patch. This recording configuration was developed so that higher sensitivities in fluorescence detection can be achieved. The high sensitivity comes from both the preparation and the recording system. The intracellular milieu of a cell not only presents a rich source of autofluorescence (for example, 50% of the intracellular content of Xenopus oocytes is yolk), which is strongly fluorescent in the visible range, its constituents also bind or trap chemical fluorophores. When a membrane patch is isolated from the cell, most of the intracellular milieu is excluded. For patch fluorometry, a membrane patch is observed under an epifluorescence microscope with a high numerical aperture (NA) objective. The image brightness is proportional to the fourth power of the NA value. Thus an increase in the NA value translates into significant improvements in fluorescence detection sensitivity. Although powerful objective lenses have been used in whole-oocyte fluorescence recordings, only a small fraction of the cell surface can be observed through the objective. With patch fluorometry, both fluorescence and current are recorded from the same population of ion channels in the patch membrane, allowing a direct correlation between structural changes and functional states of the channel. The enhanced fluorescence-recording sensitivity of patch fluorometry is further coupled to the high accuracy of the patch-clamp current recording. The improvement in both the fluorescence sensitivity and the current sensitivity should permit the design of many new experiments.

Another advantage of patch fluorometry is that, with a cell-free membrane patch, fluorophore probes can be attached to intracellular as well as extracellular sites. Access to the intracellular fluorophores opens new avenues to investigate the function and modulation of ion channels and transporters.
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For example, the main activation gate of voltage-gated \( K^+ \) channels is formed by the bundle crossing of the sixth transmembrane helices (S6) at the intracellular end of the channel pore (9). The movement of the voltage sensor is coupled to the S6 movement through an intracellular helix linker (8, 17, 18, 23). Fluorophore probes attached to these regions should yield rich information about the conformational changes that underlie voltage-dependent channel activation. The need to access the intracellular side of the channel is not limited to the fluorophore attachment. Many ion channels are either gated by intracellular ligands [e.g., cyclic nucleotide-gated (CNG) channels] or regulated by intracellular factors [e.g., \( \text{Ca}^{2+} \) for large-conductance \( \text{Ca}^{2+} \)-activated \( K^+ \) channels, cyclic nucleotides for hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels, \( \text{Ca}^{2+} \)-calmodulin for CNG channels, and small-conductance \( \text{Ca}^{2+} \)-activated \( K^+ \) channels, etc.]. Direct access to the intracellular side of the channel during experiments permits manipulation of these regulating factors required to study these channels.

How many channels does one need for patch-fluorometry recordings? It turns out that the number is surprisingly small. It was estimated, based on the total patch current and the single-channel conductance, that a few hundred channels would yield enough signals for reliable fluorescence recordings (46). Because each tetrameric channel contains four cysteines, the total number of channel-attached fluorophores in these patches was estimated to be \( \sim 1,000 \). Currently it appears that the number of channels required for patch-fluorometry experiments is limited by the signal-to-background ratio. If background fluorescence signals can be reduced (see below), patch fluorometry should be applicable to patches containing a smaller number of channels until the light detection sensitivity becomes the limiting factor. With a thousand or so fluorophores and the setup described in FIGURE 3, the exposure time for each measurement can be as short as a few milliseconds, allowing many repetitive measurements before photobleaching starts to significantly affect the fluorescence intensity. For comparison, the photobleaching time course for channel-attached Alexa Fluor 488 exhibited a time constant of \( -1.5 \text{ min} \).

Because brighter fluorescence signals are advantageous for practical reasons, we routinely use patch pipettes with a large tip opening to obtain a relatively large piece of membrane patch (46). The pipette is first pulled to a tip size of \( >10 \mu \text{m} \); heat polishing with a microforge reduces the tip opening to a final size of a few micrometers. Such pipettes work very well for patching the oocyte membrane, although they may not be applicable to much smaller cultured cells.

To implement patch-fluorometry recording, the main focus has been to reduce the background light level and to optimize the fluorescence light-collection efficiency. Fluorescence recordings should be done in a dark room or with the recording setup enclosed behind black curtains. We found that the light from the computer monitor can often be picked up by the detector if the screen is directly facing the setup. The fluorescence-recording system is built around an epifluorescence microscope (FIGURE 3). A key consideration for the microscope is the objective. As mentioned above, high NA-value (>1.4) objectives are more efficient in focusing excitation light on the membrane patch, as well as in collecting fluorescence emissions. However, some sophisticated objectives, for example, the Apo category objectives that typically have an NA value >1.4, contain a set of many glass lens elements to achieve superb imaging quality. These objectives may not be as efficient in transmitting light as an objective with the same NA value and fewer glass lens elements. A useful specification for selecting objectives is the “relative brightness” number. The excitation light can be produced by a mercury lamp, a halogen lamp, and—more widely used recently—a laser source having an output power in the range.
of tens of milliwatts. The laser generator and the associated optic parts can be fixed to an optic table and the laser light fed into the microscope through an optic fiber. Such an arrangement helps to isolate mechanical vibrations, e.g., shutter opening and closing, from the fluorescence/current recording unit. For fluorescence detection, we have used photomultiplier tubes (PMTs) and charge-coupled device (CCD) cameras. The configuration of the dual-PMT system shown in FIGURE 3 allows simultaneous detections at two wavelengths for fluorescence ratio measurements. Although CCD cameras used to be a lot slower than PMTs, newer-generation cameras are approaching the same practical rate of light collection. For example, a CCD camera built with the year 2004 technology can already take 128 × 128-pixel images at a rate of 2 ms per image when the data is temporarily stored in the computer memory space. Newer cameras can collect and write data to the hard disk at higher rates, allowing high-frequency fluorescence recordings for an extended time.

Whereas patch fluorometry is still in its infancy, recent studies in CNG channels have demonstrated great potentials for this powerful new approach. One example is the study of channel modulation. Activation of CNG channels in both the visual system and the olfactory system is down-regulated by calmodulin, which can be activated by Ca\(^{2+}\) that permeates through open channels. The negative feedback loop formed between CNG channel activation and Ca\(^{2+}\)-calmodulin modulation plays an important role in sensory adaptation (48). Using patch-fluorometry recordings with fluorescently labeled calmodulin and channel subunits, Trudeau and Zagotta (39) presented the first direct measurement of the calmodulin-binding process (FIGURE 4A). In one set of experiments, calmodulin was tagged with the fluorophore Alexa Fluor 488. Binding of calmodulin to the rod CNG channel was followed by the increase of the fluorescence signal from the patch membrane. In the second set of experiments, the channel subunit was tagged with an enhanced cyan fluorescent protein (eCFP). Binding of the Alexa Fluor 488-labeled calmodulin to the channel was followed by FRET between Alexa Fluor 488 and eCFP. The time course of changes in both fluorescence signals agreed nicely with the time course of the current change. In this study as well as an earlier study by Zheng et al. (45), different channel subunits were tagged with eCFP and enhanced yellow fluorescent protein (eYFP). Binding of fluorophore-free calmodulin caused conformational changes in the channel that altered the proximity between eCFP and eYFP, which was observed as changes in the FRET efficiency.
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between the two fluorescent proteins. These results provided direct evidence supporting the hypothesis that a physical separation of the NH₂-terminal calmodulin-binding domain from the COOH-terminal ligand-binding domain underlies Ca²⁺-calmodulin from the COOH-terminal ligand-binding domain of the NH₂-terminus of the NH₂-terminal calmodulin-binding domain. A physical separation of the NH₂-terminus from the COOH-terminus is supported by the hypothesis that these results provided direct evidence supporting the hypothesis that a physical separation of the NH₂-terminal calmodulin-binding domain from the COOH-terminal ligand-binding domain of the NH₂-terminus of the NH₂-terminal calmodulin-binding domain.

Recordings from channel-attached fluorophores with patch fluorometry not only serve as site-specific event detectors for local conformational changes, they can also provide detailed information about the nature of these conformational changes. When cyclic nucleotides bind to the CNG channel COOH-terminal intracellular cyclic nucleotide-binding domains (CNBD), it is believed that a conformational wave propagates to the membrane-spanning channel pore (44). Much of the molecular mechanism underlying this gating process remains unclear. To elucidate the conformational coupling between CNBD and the pore, Zheng and Zagotta (46) attached chemical fluorophore Alexa Fluor 488 or fluorescein to a cysteine (C₄₈₁) introduced to the CNBD that links CNBD to the channel pore. The C₄₈₁ mutation was made in a channel in which all of the endogenous cysteines had already been removed through mutagenesis (27). This allowed maleimide-derivative fluorophores to be specifically attached to C₄₈₁, the only cysteine present in the channel. Changes in accessibility to the C₄₈₁-attached fluorophore due to activation-conformational changes were monitored through fluorescence quenching by intracellularly applied ionic quencher molecules (FIGURE 4B).

An intriguing observation in these experiments was that, although the positively charged quencher Tl⁺ quenched the fluorescence more efficiently when the channel was activated, the negatively charged quencher I⁻ quenched the fluorescence more efficiently when the channel was closed. A simple interpretation of this dual charge- and state-dependent fluorescence quenching is that conformational changes in the C-linker moved positive charges or dipoles away from C₄₈₁, allowing easier access of the positively charged Tl⁺ in the open state and the negatively charged I⁻ in the closed state. In the structure model based on the crystal structure of the homologous HCN channel intracellular domain (43), several positively charged amino acids can be identified in the vicinity of C₄₈₁. It would be interesting to test whether mutations introducing neutral or negative charges to any of these positions would alter fluorescence quenching.

“Although patch fluorometry is clearly a useful tool for ion-channel studies, improvements are needed to fully realize its potential.”

Although patch fluorometry is clearly a useful tool for ion-channel studies, improvements are needed to fully realize its potential. One such area is the signal-to-noise ratio of the fluorescence signal. In the study by Zheng and Zagotta (46), it was estimated that only half or less of the total fluorescence intensity was from fluorophores attached to the channel. Whereas the emission from these channel fluorophores can be reliably separated from the background, it is highly desirable to reduce or even eliminate background contamination. Background fluorescence may come from fluorophores attached to cysteines in native membrane proteins, fluorophores nonspecifically incorporated into the membrane, and free fluorophores in the recording chamber. Free fluorophores can be almost completely removed after thorough perfusion of the recording chamber. In cases where channel cysteines can be effectively protected, native cysteines can be eliminated before fluorescence labeling of channel cysteines by the application of nonfluorescent thiol-reactive molecules like N-ethylmaleimide. Further improvement of the selectivity of the fluorescence labeling will be advantageous. For example, Tsien and colleagues developed a highly site-specific fluorophore called FlAsH that contains two arsenics per molecule that react with four thiols (11, 12). It recognizes an engineered Cys-Cys-X-X-Cys-Cys motif in which two pairs of cysteines are separated by two amino acids.

Fluorescent proteins as fusion tags to channel subunits are superior in terms of the signal-to-noise ratio; all fluorophores in the cell expressing such tandem fusion constructs should be attached to the channel. There should be no free fluorescent protein in the cell except, perhaps, for endosomes, where partially degraded proteins may exist, and for endoplasmic reticulum, which contains newly synthesized immature proteins. As site-specific probes, fluorescent proteins obviously suffer from their rather large size: these molecules have a roughly cylinder-like shape that is 24 Å in diameter and 42 Å in length (30).

The size is also a concern for chemical fluorophores. Widely used classic fluorophores that emit in the visible range, e.g., fluorescein and rhodamine, contain a three-ring chromophore and a linker between the chromophore and the reactive group (FIGURE 3). The Alexa dyes from Molecular Probes have a larger chromophore and a much longer linker. The size of the terbium chelate fluorophores (36) is also considerably larger than the chromophore they contain. Complications due to the fluorophore size have to be considered. This is particularly true when the measurement involved is highly distance sensitive, as for FRET. Smaller fluorophores are preferable in probing local conformational changes. In general, fluorophores containing a smaller chromophore tend to have an excitation spectrum that is more toward the lower wavelength side. Although many fluorophores are as small as the side chain of an amino acid...
Acid, their excitation peaks are also close to those of the light-absorbing amino acids (phenylalanine, tryptophan, tyrosine), making these fluorophores less useful for labeling proteins. Several fluorophores containing either a naphthalene or a bimane moiety are interesting in that, although they are relatively small in size, their excitation peaks are still within the visible range and well above the excitation range for amino acids. After attaching such a fluorophore to a cysteine, the total adduct is not much larger than the side chain of a tryptophan (see **FIGURE 5**). An attractive approach to further reduce the fluorophore size is the usage of nonnatural, fluorescent amino acids that can be introduced to specific sites in ion channels by engineering an artificial codon at the corresponding position in the channel cDNA (6, 29, 34).

A major milestone in the advance of ion-channel studies was the development of the patch-clamping technique, which improved the sensitivity of the electrical recording to the level that permitted resolution of single-channel events (13). Can the sensitivity of the fluorescence recording of ion channels also be improved to the single-molecule level? Without any doubt it will happen in the near future, and great advances in our understanding of channel gating should come with it. Technologies for detecting optical signals from a single molecule are currently available. Indeed, single-molecule fluorescence recordings have been demonstrated in many purified systems (14, 31, 32). Ion channels are membrane proteins. Because structural integrity as well as enzymatic activities rely on the lipid environment (24), it is preferable that single-channel fluorescence recordings be carried out with the channel existing in its native membrane. Patch-fluorometry recordings from intact ion channels in isolated membrane patches clearly exhibit great potential in this pursuit. Many hurdles still lie ahead of us. To resolve fluorescence signals from a single channel, the background signal has to be reduced to a comparable level. Modifications in the recording configuration may also be required. Nonetheless, recent advances (16, 28) suggest that we have ample reasons to be optimistic that we are not far from achieving single-channel fluorescence recordings.

Much of the recent progress in understanding ion-channel gating has come from structural studies such as crystallography and cryoelectron microscopy. Although these structural studies reveal great details in various channel conformations, such information is available only in snapshots under nonphysiological conditions. As patch fluorometry and its sister approach, voltage-clamp fluorometry, naturally link dynamic structural information to channel functions, they may fill the void between the amazing structural details of the gating machinery and decades of functional studies. It is expected that such efforts will shed new light on ion channels.

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### References


