

Patch-Clamp Fluorometry Recording of Conformational Rearrangements of Ion Channels

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Abstract

Conformational rearrangements are commonly observed in membrane proteins and constitute the molecular mechanisms through which they contribute to signal transduction. Information on structural changes in membrane proteins has mostly been inferred from functional studies. Recently, site-specific fluorescence recordings have made it possible to directly observe such molecular events in real time within the membrane environment. Here, we describe the patch-clamp fluorometry technique that records simultaneously the local structural changes and the functional states of ion channels in isolated cell membranes. Combined with fluorescence resonance energy transfer, the technique should shed new light on ion channel activation, regulation, and interaction with other membrane proteins.

Introduction

Ion channels are one class of membrane proteins that allow cells to communicate with each other and with the cellular environment. In response to electric or chemical stimuli, an ion channel activates by undergoing conformational changes that open a hydrophilic pathway for selected ion species to cross the otherwise impermeable membrane. Channel activities have profound effects on cellular functions that include electrical excitability, gene expression, secretion, and many others (1). Numerous techniques have been applied in past decades to the studies of channel activation. Site-specific fluorescence recording of ion channels in membranes has emerged as a powerful method for directly observing conformational rearrangements occurring in specific channel structures in real time (2). Cysteines are introduced through mutagenesis to sites of interest in the channel. If necessary, additional naturally occurring cysteines are replaced by mutagenesis such that the signal is specific. The cysteine residue serves as an anchor point for sulfhydryl-reactive fluorophores, allowing site-specific fluorophore labeling. Current and fluorescence signals are recorded simultaneously from the same population of fluorescent channels. Current signals, which change as the result of channel opening and ion flux, serve as an indicator of channel functional states. Fluorescence emission changes when protein structural rearrangements in the vicinity of the cysteine affect the fluorophore's quantum yield, mobility, accessibility to quencher molecules, proximity to neighboring fluorophores, and other factors. In this way, the fluorophores serve as localized, highly sensitive reporters of protein conformational changes. Because fluorescence signals do not rely on opening of the channel pore, as do ionic current signals, fluorescence recordings add a new avenue to investigate temporal and spatial information of conformational changes in channel proteins before they reach the open state. Site-specific fluorescence recordings should be generally applicable to any channel type, and even nonchannel membrane proteins (for example, transporters) as a way to investigate local molecular events that underlie their function. It is conceivable that fluorescence recording could also be an efficient substitute for current recordings in high-throughput drug screening (3).

Fluorescence recording was first introduced to studies of functional ion channels in situ with whole-cell preparations of *Xenopus* oocytes (4-6). Messenger RNAs (mRNAs) encoding cysteine-containing channel proteins are injected into oocytes a few days prior to experiments. Oocytes are then soaked in a solution containing cysteine-reactive fluorophores. The cysteine residues exposed on the extracellular face of the oocyte are covalently labeled. The pigment-rich animal pole of the oocyte contributes less autofluorescence from the cellular constituents and is generally chosen for fluorescence recordings. Voltage clamping activates voltage-sensitive ion channels, as well as allows monitoring of the functional states of the channels. This approach, named voltage-clamp fluorometry (VCF) by its inventors, allows direct observations of the real-time motions of protein structures that are exposed on the extracellular side of the cell (4, 5, 7-19). The power of this fluorescence-based approach was demonstrated by the studies utilizing fluorescence resonance energy transfer (FRET), which demonstrated with atomic resolution that the fourth transmembrane domain (S4) of voltage-sensitive Shaker potassium channels undergoes rotational and possibly translational movements during voltage-gated channel activation (20, 21).

We have developed techniques to study channel activity fluorescently using isolated cell membrane preparations (Fig. 1) (22). The driving force behind our efforts was a need to expand fluorescence studies to the intracellular side of channels where the gates and binding sites for allosteric modulators are often located (23). A polished glass pipette with an opening of a few micrometers is sealed tightly against the surface membrane of cells expressing cysteine-containing channels. Withdrawal of the pipette removes a small piece of membrane that sticks to the glass tip. Fluorophore labeling and recordings are carried out with this membrane patch (Fig. 2). This approach, which we named patch-clamp fluorometry (PCF), allows fluorescence recordings to be made on both the intracellular and extracellular side of channel proteins. Because fluorescence signals are collected from channels in a small (~50 μm^2) cell-free membrane, contamination from autofluorescence of intracellular constituents is eliminated. This translates into increased signal-to-noise fluorescence signals and higher resolution. When combined with high-sensitivity, low-noise fluorescence detectors, PCF might eventually lead to recordings of a single protein molecule. With standard detectors like charge-coupled device (CCD) cameras and photomultiplier tubes (PMTs) that are readily available, fluorescence studies can be routinely performed with patches containing a relatively small number of channels. From current amplitude measurements, we estimated that in a typical experiment there were a few hundred to a few thousand channels in the patch. Like VCF, PCF records fluorescence and current signals from the same population of channels simultaneously. Patch-clamp recording offers superior sensitivity and temporal resolution, and provides direct control of the intracellular milieu compared to voltage-clamp recording.

We have applied PCF to the study of cyclic nucleotide-gated (CNG) channels, which are opened or closed in response to changes in the concentration of the intracellular second messengers guanosine 3',5'-monophosphate (cGMP) or adenosine 3',5'-monophosphate (cAMP). For this protocol, we use CNG channels as an example, but the procedure can be equally applied to other channel types. We also limit the discussion to cysteine-tagged fluorophores. Other types of fluorophore tagging, such as genetically tagging with green fluorescent protein (GFP) variants, have been reported (24, 25) and can also be used for PCF (26).

PROTOCOL

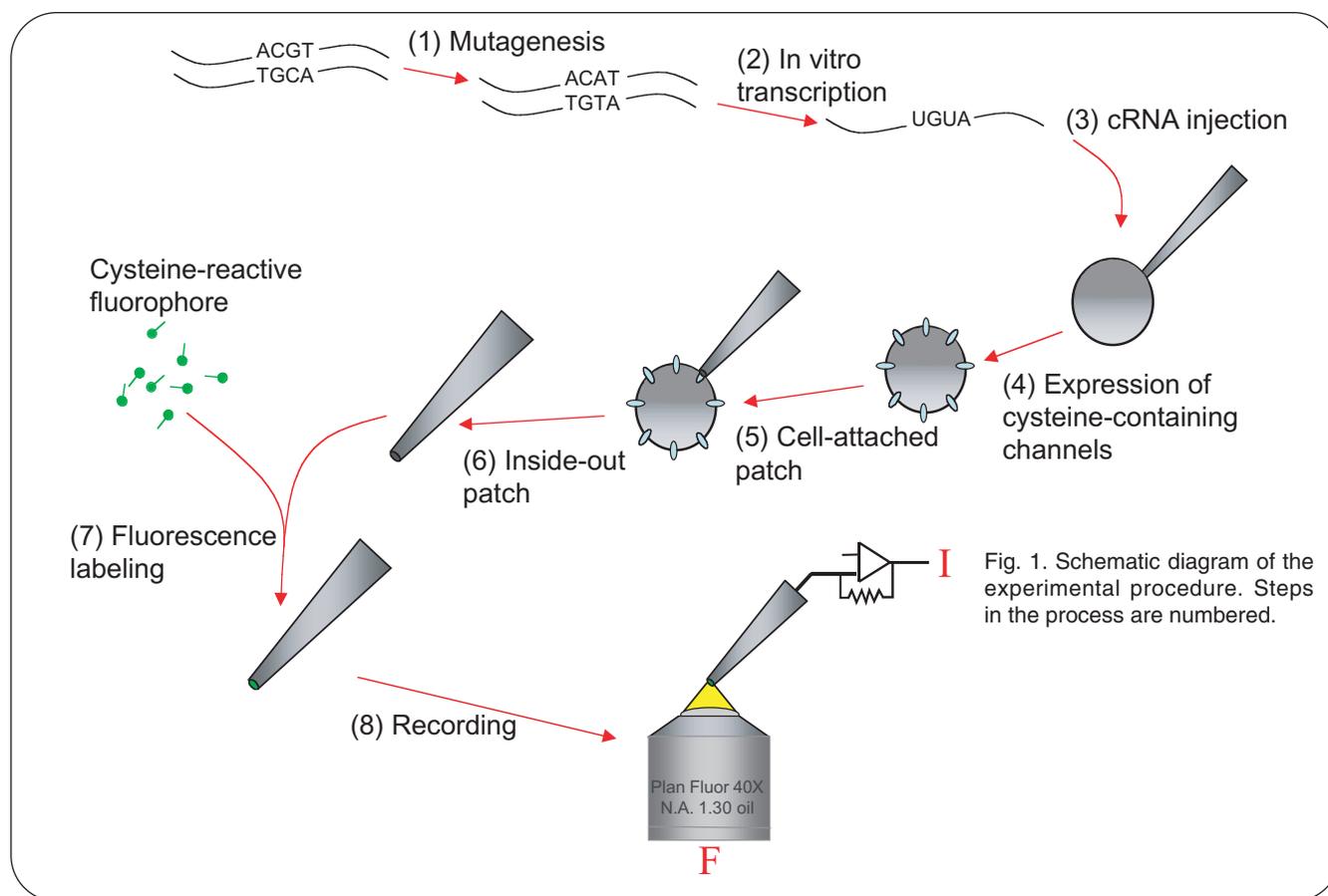


Fig. 1. Schematic diagram of the experimental procedure. Steps in the process are numbered.

Materials

Glass tube (ID, 0.78 mm, length, 8 cm, fire-polished ends)
No. 1 coverglass
Patch pipettes
Syringe
Two-way stopcock
Xenopus oocytes (*Xenopus* One, Ann Arbor, MI)

Chemicals and Reagents

cGMP or cAMP, sodium salt
Dimethyl sulfoxide (DMSO)
Ethylene diamine tetraacetic acid (EDTA)
Hepes
High vacuum grease (Dow Corning)
Immersion oil
Mineral oil or Fluorinert (Sigma-Aldrich)
N-ethyl-maleimide (NEM)
Sulfhydryl-reactive fluorophores, such as maleimide [Alexa Fluor 488 C5 maleimide (Molecular Probes, Eugene, OR)] or iodoacetamide derivatives

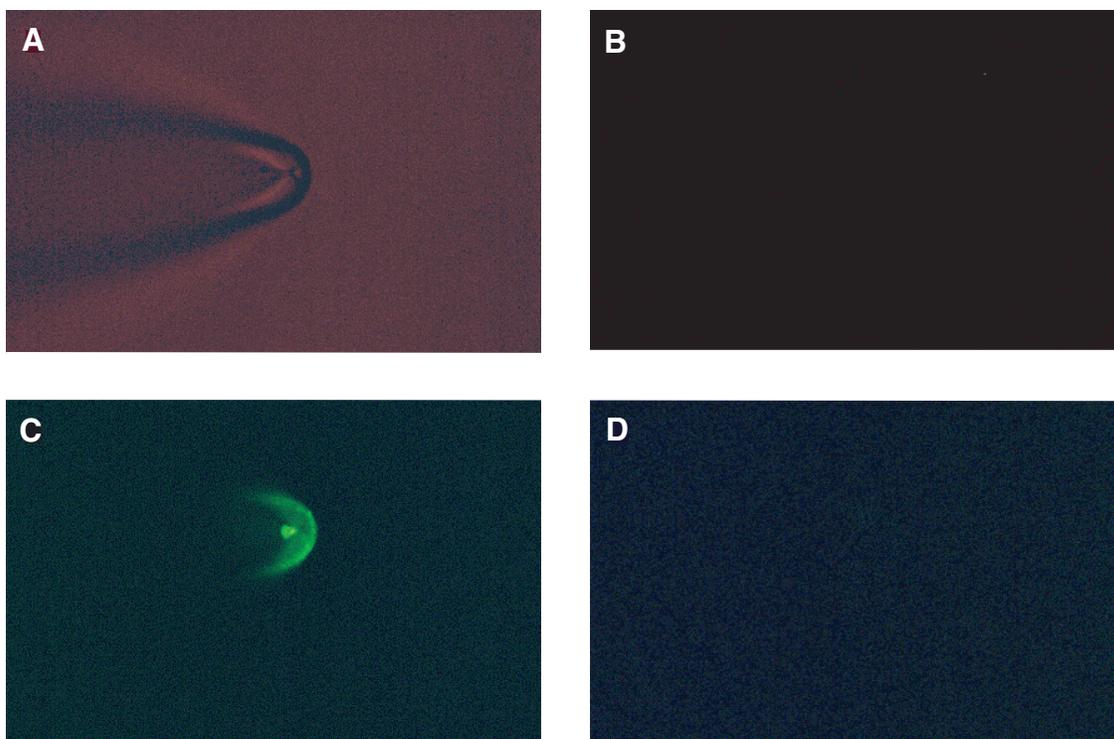


Fig. 2. Example images of a membrane patch. The membrane was isolated from an oocyte expressing CNG channels with a single cysteine in each subunit. **(A)** Bright field showing the position and shape of the patch pipette. **(B)** Fluorescence view before labeling. No autofluorescence is observed. **(C)** After labeling with 2 mM Alexa Fluor 488 maleimide for 2 min. **(D)** After photobleaching. Images shown in B through D were collected under identical conditions. Excitation at 450 to 490 nm; emission at 520 to 550 nm.

Equipment

Princeton Instruments MicroMax CCD camera (Roper Scientific, Trenton, NJ)

Filter wheel (Sutter, Novato, CA)

Note: We use a filter wheel that holds 10 filters. In order to get better defined spectra, we have two wheels fused head-to-head, which yields 18 useful filter positions.

Inverted microscope (Nikon Diaphot 300) with 40× oil immersion numerical aperture (N.A.) 1.3 objective

MetaMorph imaging software (Universal Imaging, Downingtown, PA)

Microforge (Narishige, Tokyo, Japan, #MF-9)

Micropipette puller (Sutter, Novato, CA, #P-97)

Microsoft Excel spreadsheet software

Minimanipulator (Narishige, Tokyo, Japan, #M-44)

Monochromator (Cairn Research, UK)

Patch clamping setup, including Axopatch 200A amplifier (Axon Instrument, Union City, CA), ITC-16 AD converter (Instrutech, Port Washington, NY), and Pulse data acquisition software (HEKA, Lambrecht, Germany)

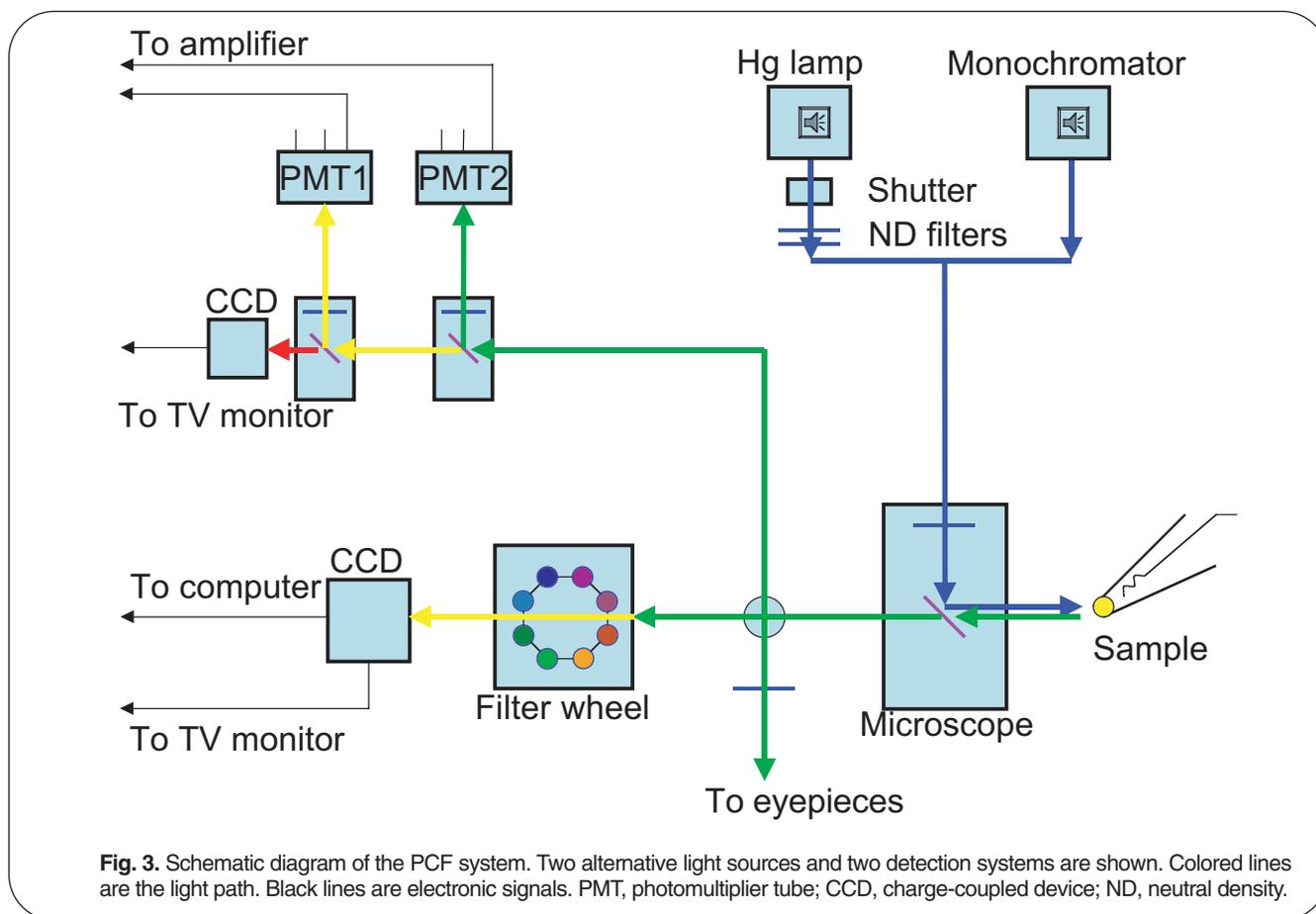
Photomultiplier tube (PMT) (Hamamatsu, Hamamatsu City, Japan, #R6095)

RSC-100 rapid solution changer (Molecular Kinetics, Pullman, WA)

Recording chamber, such as Warner's RC-10 (Warner Instruments, Hamden, CT)

Shutter (Vincent Associates, Rochester, NY)

Note: Fig. 3 demonstrates the configuration of the PCF recording system.



Recipes

Recipe 1: Ringer's Solution

NaCl 130 mM

EDTA 0.2 mM

Hepes 3 mM

Adjust pH to 7.2. Store at 21°C until use.

Recipe 2: Low-Sodium Ringer's Solution

NaCl 114 mM

EDTA 0.2 mM

Hepes 3 mM

Adjust pH to 7.2. Store at 21°C until use.

Recipe 3: Cyclic Nucleotide Stock Solution

Add cGMP or cAMP (sodium salt) to the Low-Sodium Ringer's Solution (Recipe 2) to a final concentration of 16 mM. Store at 4°C until use.

Recipe 4: Cyclic Nucleotide Working Solution

Dilute Cyclic Nucleotide Stock Solution (Recipe 3) with Ringer's Solution (Recipe 1) to a final concentration of up to 1 mM. Store at 4°C until use.

Recipe 5: Fluorophore Stock Solution

Add 14 µl of DMSO to a tube containing 1 mg of Alexa Fluo 488 C5 maleimide (final concentration 0.1 M). Store in dark at -20°C for up to a month.

Recipe 6: Fluorophore Working Solution

Add 1 µl Fluorophore Stock Solution (Recipe 5) to 50 µl of Ringer Solution (Recipe 1) or Cyclic Nucleotide Working Solution (Recipe 4) before use (final concentration 2 mM).

Instructions

Site-Specific Fluorophore Labeling

Fluorophore labeling is carried out on cysteine-containing channels, which are expressed in *Xenopus* oocytes or cell lines (Fig. 1). A cell-attached patch is formed by standard patch-clamping technique (27). To enhance fluorescence and current signals, a pipette with a large opening (2 to 5 µm) is generally used to achieve a "giant patch." The patch pipette is then withdrawn to form an inside-out patch. To accommodate the labeling process, we use a recording chamber with a relatively small volume. The solution entrance and exit ports are located on opposite sides of the chamber, so that the solution flow is unidirectional and less perturbed.

1. Inject oocytes with 50 µl of mRNA encoding the cysteine-containing channel.
2. Maintain the oocytes at 16°C in Ringer's solution (Recipe 1) for 2 to 7 days to allow channel expression.
3. Pull patch pipettes to an initial tip opening of 10 to 20 µm and fire-polish the tip heavily to generate smooth surface, yielding pipettes with tip opening of 2 to 5 µm and resistance in the range of 200 to 400 KΩ.
4. Form a cell-attached patch.
5. Withdraw the patch pipette to form an inside-out patch.

Note: Some patches form with visible intracellular constituents. These patches are not usable because they yield high background fluorescence signals.

6. Prepare the fluorophore perfusion apparatus by attaching a glass pipette with tip opening of 100 µm to a syringe through a flexible tubing (Fig. 4). Fill the whole pathway with fluorinert.
7. Using a stopcock to start and stop fluorophore filling, front-fill a perfusion pipette with 25 µl of Fluorophore Working Solution (Recipe 6) by dipping the tip of the pipette (filled with fluorinert) into a fluorophore-containing microcentrifuge tube and then lifting the tube above the fluorinert level in the syringe (Fig. 4, step 1).
8. With a low-power (10×) objective, position the fluorophore perfusion pipette next to the patch pipette with a minimanipulator.

Note: Lower the patch pipette so that it is close to the coverslip; this helps to limit diffusion of the fluorophore solution.

9. Perfuse the fluorophore solution by gravity for 1 to 2 min (Fig. 4, step 2.). Use patch-clamp recording to monitor changes in current due to fluorophore modification of channel cysteines.

Note: If background labeling of endogenous proteins is determined to be unacceptably high even on patches lacking visible intracellular constituents, cysteines can be modified with 2 mM NEM immediately before fluorophore labeling if channel cysteines can be efficiently protected (see Notes and Remarks).

10. Remove the fluorophore perfusion pipette.
11. Wash the preparation completely with Ringer's Solution (Recipe 1) for 2 to 3 min. After completely washing away free fluorophore, the background level should be close to its original value.

Note: It is very important to wash away excess fluorophore in the bath solution, because excess fluorophore will generate background during the experiment.

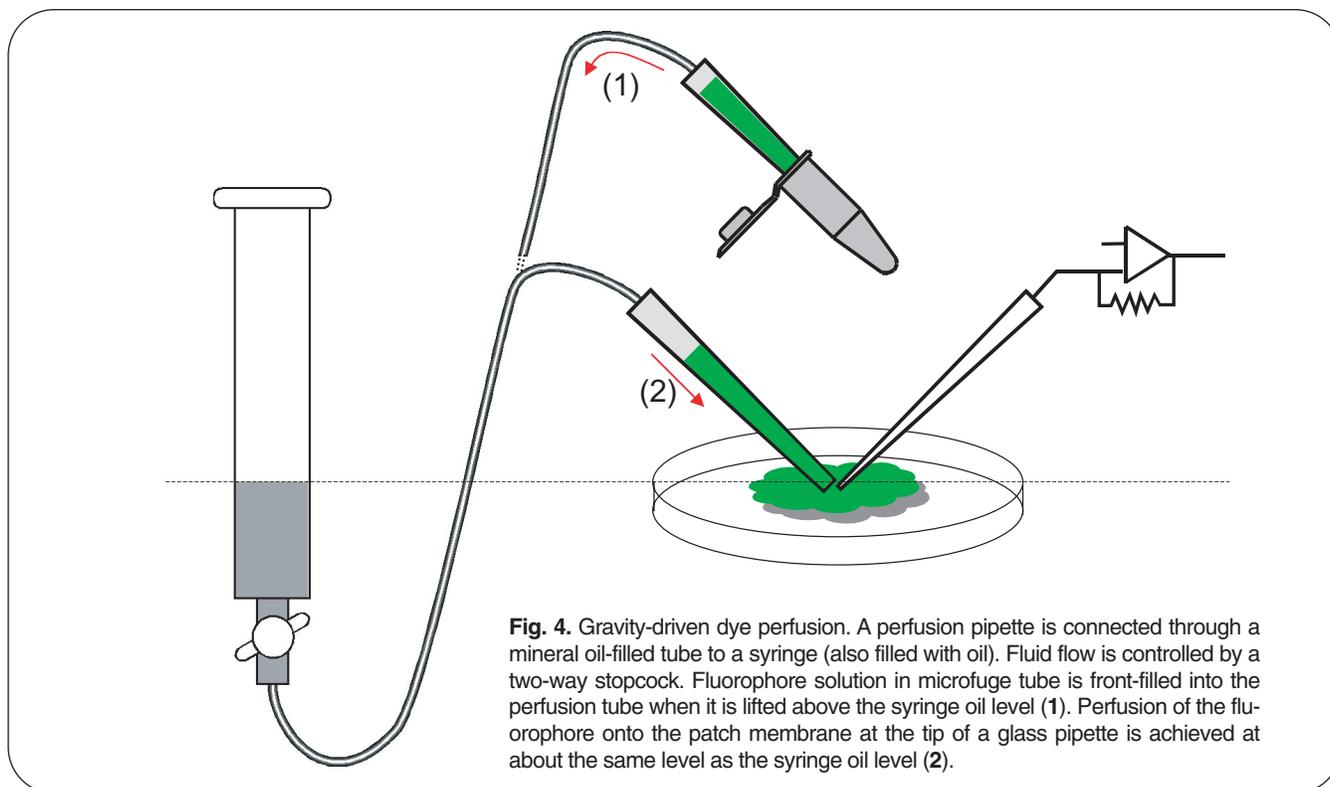


Fig. 4. Gravity-driven dye perfusion. A perfusion pipette is connected through a mineral oil-filled tube to a syringe (also filled with oil). Fluid flow is controlled by a two-way stopcock. Fluorophore solution in microfuge tube is front-filled into the perfusion tube when it is lifted above the syringe oil level (1). Perfusion of the fluorophore onto the patch membrane at the tip of a glass pipette is achieved at about the same level as the syringe oil level (2).

Recording Fluorescence Signals

Recordings of fluorescence emission are acquired with light detectors attached to the microscope's exit ports (Fig. 3). A dark environment must be maintained during light detection. A hood made of black cloth that encloses the Faraday cage is an efficient way to decrease scatter light. The light source of the microscope must be turned off. We have used two kinds of light detectors, a PMT and a CCD camera. A PMT allows fast online light quantification. On the other hand, a CCD camera captures information-rich images, although at a much lower rate. It appears that a PMT is suitable for fast kinetics studies and a CCD camera is preferred for steady-state studies. These steps describe recording from cGMP-dependent channels using MetaMorph software, but can be adapted to channels responsive to other signals using other types of image acquisition software.

1. With low power objective (10×), position the cGMP perfusion tubes close to the patch pipette (50 to 100 μm).
2. Switch to a 40× objective and focus on the patch, using the fine focus to adjust the patch position so that it is in the center of the field.
3. Acquire an image of the patch pipette with white light illumination, using the “digital zoom” function in MetaMorph to collect images from an area that includes the pipette tip. This image registers the position of the patch membrane; it is also helpful in detecting possible drift of the patch pipette.

Note: Do not focus so tightly on the tip that there is no background present. Be sure that some area outside the tip is in the field to use for background subtraction.

4. Adjust excitation light intensity to optimize signal acquisition and minimize photobleaching.

Note: Digital binning helps to detect dim signals and increase imaging speed. Reducing excitation light intensity by blocking light going through the monochromator or using neutral density (ND) filters reduces photobleaching.

5. Open the shutter to shine excitation light on the patch just before recording.

Note: Depending on the excitation light source used, this can be achieved in different ways. When a Hg lamp is used in conjunction with a shutter, a sync voltage trigger signal is generated by the patch amplifier and is fed into the shutter's control unit. When a monochromator is used, the light exit slit of the monochromator is open and closed through MetaMorph.

6. Digitize the fluorescence signal.
7. Close shutter to turn off excitation light.

- Save each individual image file using filenames with incremental numerical endings, for example, EXP001, EXP002....

Note: Incrementally numbered filenames are essential for the semiautomated fluorescence intensity analysis described in the next section.

- Repeat steps 5 to 8 while cGMP concentration is varied.

Quantification of Fluorescence Intensity

Going through numerous fluorescence images and quantifying intensities of multiple regions in each image can be a tedious job. In this example, a semiautomatic procedure for fluorescence intensity quantification is discussed. The software that is used in this procedure includes MetaMorph and Microsoft Excel.

- Load images into MetaMorph using the “stack” function (under File Menu/build stack/numbered names).

Note: This is possible only when images are saved under incrementally numbered filenames .

- Define the ROI (region of interest) (under Regions/Region Tools).

Note: Two ROIs are normally defined, ROI 1 for the patch fluorescence that excludes fluorescence from the tip and ROI 2 for the background (Fig. 5).

- Connect to Excel through Dynamic Data Exchange (Under Measure Menu/Region Measurements/Open log/DDE/Excel).

- Choose “all planes” and “all regions” (in Region Measurements window).

- Log data. This will generate a table containing image number, average intensity, and other values as specified in MetaMorph.

Troubleshooting

Background

Various sources contribute to the total background fluorescence under these experimental conditions. Some of them can be reduced during data acquisition. Here, we address ways to subtract background signals. Additional approaches to reducing background are discussed in Notes and Remarks.

First, always record background fluorescence associated with the recording system (for example, CCD dark current) before adding a fluorescent dye. This level of background is expected to be stable throughout the experiment.

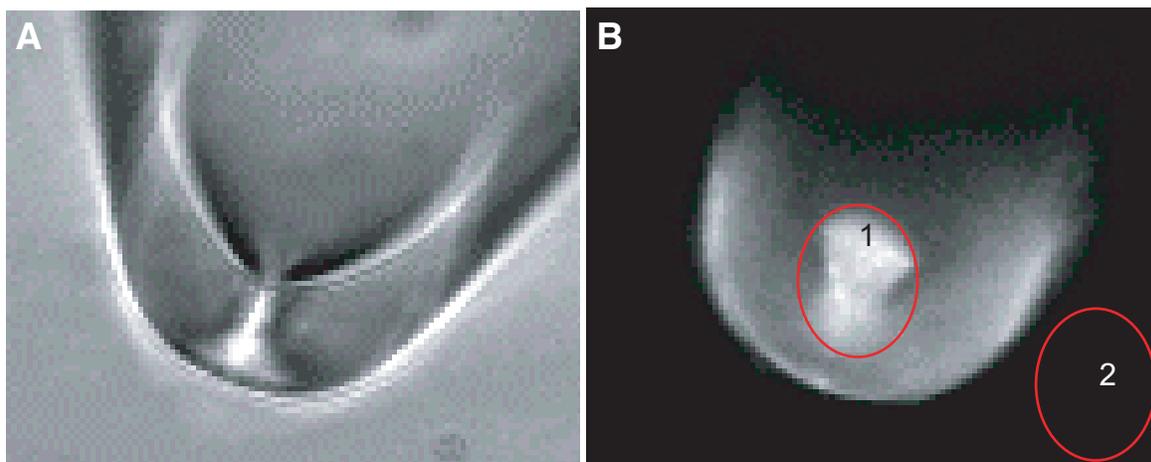


Fig. 5. Quantification of channel fluorescence and the level of background. **(A)** Bright field image of a patch pipette. **(B)** Fluorescence image of the same patch with two regions of interest (ROIs) (1 and 2). ROI 1 represents the patch and ROI 2 represents the blank area.

Second, at the end of the experiment, background signals are measured with the same excitation and emission settings as those used during data acquisition. This background signal can be obtained by either bleaching the fluorophores on the patch or removing patch pipette from the field. This provides an estimate of any possible increase in background that occurred during the experiments, for example, due to free fluorophores.

Third, when conducting data analysis, compare fluorescence signals in the patch area to the neighboring blank area (Fig. 5).

Finally, free fluorophores in the recording system can contribute significantly to background signal. PCF requires adding fluorophore solution directly to the recording chamber during fluorophore labeling. It is thus crucial to remove any free fluorophore molecules before fluorescence recordings. We use a unidirectional flow chamber similar to Warner's RC-10 (Warner Instruments, Hamden, CT). The small volume and unidirectional solution flow facilitate removal of free fluorophores. Fluorophore labeling is carried out near the chamber's exit port. Free fluorophores are then washed away. After extensive washing, the patch pipette is moved to chamber center for fluorescence recording.

Photobleaching

Photobleaching by excitation light does not appear to be a major problem, because the high intensity of fluorescence signals allows for short (milliseconds) exposure times. For comparison, the time constant for photobleaching of Alexa Fluo 488 (Molecular Probes) attached to C481 of the rod CNG channel CNGA1 was measured to be a few minutes under our experimental conditions (22). Exposure times can be much longer when the number of fluorophores reduces significantly. In those cases, antibleaching reagents may be used to decrease the rate of bleaching (13).

Nonspecific Fluorescence Signals

The success of PCF recordings is, to a great extent, a game of "signal-to-noise." Modern technologies of light detection allow observation of a single photon at a reasonable speed. Reliable recording of fluorescence signals from fluorophores attached to specific positions of membrane proteins, however, requires reduction of contamination due to nonspecific fluorophore attachments, as well as system background (light scattering, detector dark noise, and others). Several sources of signal contamination and possible solutions are discussed here.

Endogenous binding sites for chemical fluorophores

The channel-expressing cells may also express various native cysteine-containing membrane proteins that can contribute to nonspecific fluorophore labeling. In most cases, however, channel proteins can be expressed at densities high enough to dominate the signals produced by native membrane proteins. Labeling of native cysteines can be further reduced or eliminated by treatment with nonfluorescent cysteine-reactive reagents. Treatment of oocytes with 3-maleimidopropionic or tetriglycine maleimide about 1 day before experiments successfully reduced background fluorescence in extracellular fluorescence recordings with VCF (4, 17). Alternatively, native cysteines can be modified with NEM immediately before fluorophore labeling if channel cysteines can be efficiently protected by controlling channel open or closed, liganded or unliganded state such that the channel cysteines are inaccessible (22). There is significant nonspecific fluorescence background even when native cysteines have been blocked (22). It is currently unclear where the fluorophores are attached. Our suspicion is that fluorophores incorporated into lipid membrane through hydrophobic interactions might contribute to this type of background. Consistent with this hypothesis, this nonspecific fluorescence appears to exhibit fluorophore specificity (28), giving hope that it can be significantly reduced.

Autofluorescence from membrane and embedded proteins

An advantage of recording fluorescence signals from isolated membrane patches is that it eliminates autofluorescence from intracellular constituents. Nevertheless, many membrane constituents are also known to be fluorescent. For example, tryptophan, tyrosine, and phenylalanine produce intrinsic protein fluorescence. These aromatic amino acids absorb at 260 to 295 nm and emit at 300 to 350 nm. NADH, an enzyme cofactor, is also highly fluorescent, with absorption and emission maxima at 340 nm and 460 nm, respectively. A test should be done to ensure that there is no autofluorescence from the membrane and its constituents (as well as other background fluorescence contaminations) throughout the wavelength range that is used.

Membrane attached to the patch glass

We noticed that some patches exhibited significant fluorescence at the tip beyond the area of patch membrane (Fig. 5). These fluorescence signals apparently came from membrane attached to the surface of the patch glass, as revealed by confocal microscopy (22). Because this membrane is not voltage-clamped and current from these channels is not recorded, it is desirable not to include fluorescence of this region in the analysis. In general, most of the fluorescence from these areas can be excluded during data analysis by selecting only the patch in the ROI (Fig. 5).

Related Techniques

FRET is becoming a widely used technique for studies of protein-protein association, domain-domain interaction, and conformational rearrangements. With FRET, light energy absorbed by a donor fluorophore is transferred nonradiatively to a nearby acceptor fluorophore whose absorption spectrum overlaps the emission spectrum of the donor. When used to monitor channel conformational changes, this occurs when two or more sites of the channel protein are labeled simultaneously with donor and acceptor fluorophores. The efficiency of energy transfer falls off with the sixth power of the distance between the donor and acceptor molecules. Because of this very strong dependence on distance, FRET is a sensitive reporter of proximity, making it suitable for many applications in ion channel studies (20, 21, 24, 29-31).

Quantification of FRET efficiency is, however, not always straightforward. In general, emission at the acceptor wavelength contains three components: (i) acceptor emission due to FRET; (ii) acceptor emission due to direct excitation; and (iii) donor emission. Various approaches have been reported to separate enhanced acceptor emission from contaminations due to donor emission and direct excitation of the acceptor (31, 32). Here, we discuss a modified spectrum-based approach (Fig. 6) that allows accurate separation of FRET emission, as well as elimination of errors arising from variation in the quantum yield of the acceptor or in the concentration of total fluorescence molecules (30-32). The same basic strategy can be applied when fluorescence emissions are measured with bandpass filters (33, 34), although, as discussed below, spectra measurements provide several advantages.

The spectrum-base approach involves recording a donor spectrum collected from a control sample containing donor-tagged channels (Fig. 6, blue trace, panel A). This donor spectrum is used to subtract donor emission from spectra taken from samples containing donor and acceptor-tagged channels (Fig. 6, red trace). This yields a subtracted acceptor emission spectrum, F_d (Fig. 6, green trace), which has two components: one due to direct excitation and one due to FRET (Fig. 7). F_d is normalized by total acceptor

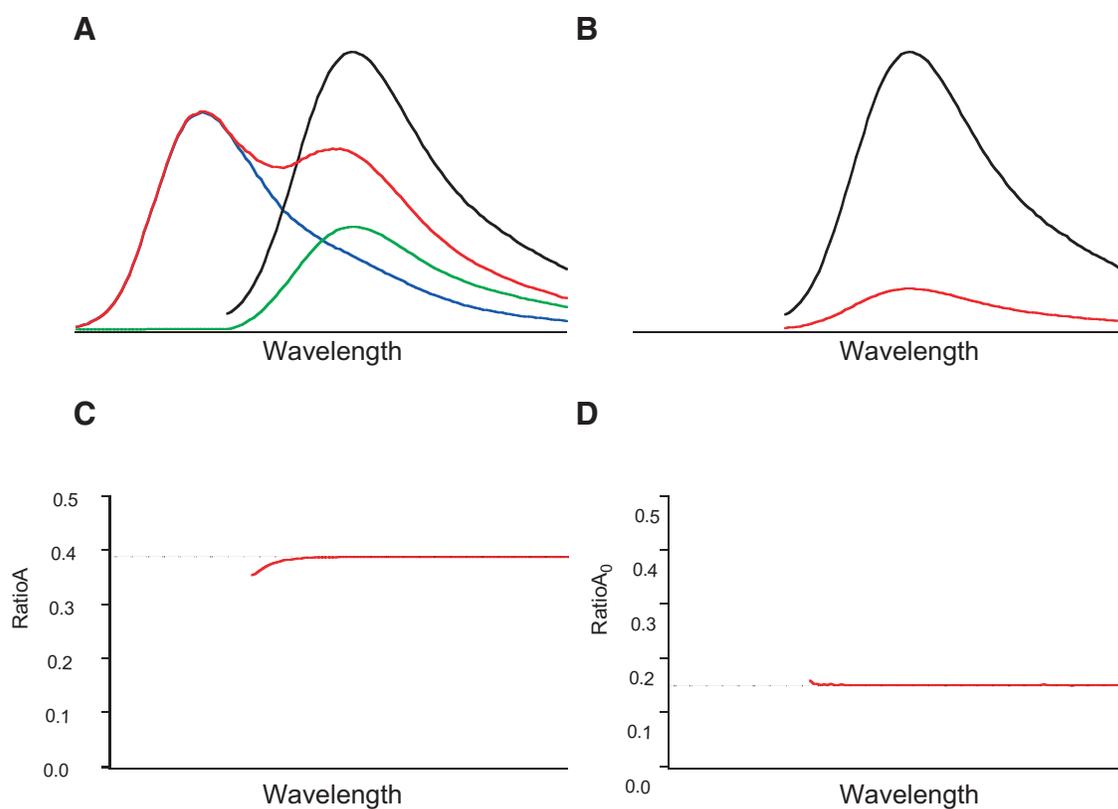


Fig. 6. Spectrum-based FRET efficiency measurement. (A) Spectra from donor and acceptor samples. The spectra are color-coded as follows: red, emission with donor excitation (F_d); black, emission with direct acceptor excitation (F_a); blue, donor emission; green, extracted acceptor emission. (B) Spectra from acceptor alone sample, color-coded as in A. (C) RatioA as a function of wavelength, calculated from spectra in A. (D) RatioA₀ as a function of wavelength, calculated from spectra in C.

Equation 1

Extracted acceptor emission spectrum

$$F_d^{\text{direct}} + F_d^{\text{FRET}}$$

Equation 2

Normalizing the acceptor emission spectrum (RatioA)

$$\text{RatioA} = \frac{F_d}{F_a} = \frac{F_d^{\text{direct}}}{F_a} + \frac{F_d^{\text{FRET}}}{F_a}$$

F_d^{direct} , the direct excitation component of the subtracted extracted acceptor emission spectrum;
 F_d^{FRET} , the FRET component of the subtracted extracted acceptor emission spectrum.

Equation 3

Calculating the FRET Ratio (FR)

$$\text{FRET Ratio (FR)} = \frac{\text{RatioA}}{\text{RatioA}_0} = 1 + \frac{F_d^{\text{FRET}}}{F_d^{\text{direct}}}$$

$\text{RatioA}_0 = \frac{F_d^{\text{direct}}}{F_a}$, the direct excitation component of the normalized acceptor emission spectrum.

Equation 4

Calculating effective FRET efficiency (E_{eff})

$$E_{\text{eff}} = \frac{\epsilon_a}{\epsilon_d} (\text{FR}-1)$$

ϵ_d , the molar extinction coefficient of the donor;
 ϵ_a , the molar extinction coefficient of the acceptor.

Fig. 7. Equations used to calculate the FRET efficiency and plot the RatioA and RatioA₀ graphs.

emission with acceptor excitation, F_a (Fig. 6, black trace); see RatioA (Fig. 6C and Fig. 7). RatioA₀ defines the direct excitation component, which is experimentally determined with samples containing acceptor-tagged channels (Fig. 6B and Fig. 6D). The ratio between RatioA and RatioA₀ is the FRET Ratio, which is used to calculate the FRET efficiency (FR).

One useful feature of this spectrum-based approach is RatioA, which is independent of wavelength. When calculated over a range of high- and low-emission wavelengths, RatioA can be used conveniently to check for linearity of the recording system and for significant contamination by other fluorescence sources (31). (Notice that in Fig. 6C, RatioA deviates from its true value at very low emis-

sions where signal-to-noise is poor. This is different from the nonlinearity discussed above.) By taking ratios to calculate RatioA, the quantum yield and concentration factors associated with the acceptor, as well as the transfer function of the recording system, also cancel out.

Notes and Remarks

Fluorophore Labeling

It is generally desirable to achieve complete fluorophore labeling. Besides maximizing fluorescence intensity, complete labeling ensures that fluorescence changes represent structural changes in the same population of channels from which current signal is simultaneously recorded. With PCF, the degree of fluorophore labeling, as well as its time course, can be monitored with current recordings (22). One of the advantages of PCF is that, when attachment of fluorophore molecules causes changes in a channel's open probability or conductance, the time course of labeling can be conveniently monitored as changes in current during application of fluorophore solution. It is, however, not always necessary to achieve complete fluorophore labeling. Particular caution should be taken when multiple fluorophore molecules are attached to the same channel. If close enough, these fluorophores might exhibit self-quenching, which will reduce the total fluorescence intensity and change fluorescence anisotropy (35). Selective labeling of multiple cysteines with different fluorophores (as required by FRET) can be achieved when the rates of cysteine modification exhibit different state-dependence.

Linearity of the Light Detectors

Most light detectors have a nonlinear input-output relationship: the output lags at low light intensity and saturates at high intensity. For quantitative analysis, it is thus crucial to limit light detection to the linear range. In Related Techniques, we discussed one way to check for linearity of the detector.

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