Measurement of halide efflux from cultured and primary airway epithelial cells using fluorescence indicators

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Abstract

The use of the halide-sensitive fluorescent probes (6-methoxy-N-(–sulphopropyl)quinolinium (SPQ) and N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE)) to measure chloride transport in cells has now been established as an alternative to the halide-selective electrode technique, radioisotope efflux assays and patch-clamp electrophysiology. We report here procedures for the assessment of halide efflux, using SPQ/MQAE halide-sensitive fluorescent indicators, from both adherent cultured epithelial cells and freshly obtained primary human airway epithelial cells. The procedure describes the calculation of efflux rate constants using experimentally derived SPQ/MQAE fluorescence intensities and empirically derived Stern–Volmer calibration constants. These fluorescence methods permit the quantitative analysis of CFTR function.

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1. Overview

The use of the quinolinium salt-based halide-sensitive fluorescent probes (6-methoxy-N-(–sulphopropyl)quinolinium (SPQ) and N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE)) to measure chloride (Cl\textsuperscript{−}) transport in epithelial cells has now been established as an alternative to the halide-selective electrode technique, radioisotope efflux assays and patch-clamp electrophysiology. In the assay introduced by Verkman and colleagues, halide secretion is followed by measuring the dequenching of intracellular SPQ/MQAE fluorescence by the effluxing halide in response to stimulation of a specific agonist-mediated cellular Cl\textsuperscript{−} secretion mechanism [1–3]. In general, cells attached to a coverslip are loaded with SPQ/MQAE, the extracellular dye is washed off, and the cells are transferred to a superfusion chamber on a fluorescent microscope and fluorescence imaging performed. Images are saved for offline analysis, and SPQ/MQAE fluorescence intensity for each region of interest (ROI) is compared over time or treatment. Many studies have used the fluorescence indicators to assess actual Cl\textsuperscript{−} channel efflux. Iodide is increasingly used as a Cl\textsuperscript{−} surrogate chiefly because indicator fluorescence is more effectively quenched by I\textsuperscript{−} than by Cl\textsuperscript{−} (halide ion selectivity: I\textsuperscript{−}>Br\textsuperscript{−}>Cl\textsuperscript{−}) thus improving the signal to noise ratio in SPQ/MQAE spectrofluorimetry. To date, SPQ/MQAE spectrofluorimetry has been used in numerous studies to assess halide efflux in adherent cultured cell lines [4,5,7–11]. In

Abbreviations: CCD, charge-coupled device; FSK, forskolin; IBMX, 3-isobutyl-1-methylxanthine; MQAE, N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide; ROI, region of interest; SR, Standard Ringer’s (solution).
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contrast, the use of the technique for measurement of halide channel activity in primary epithelial cells is more technically demanding, reflected by the paucity of examples in the literature \[3,4,7,10–13\]. Below is a description of each step in the procedure to image halide-sensitive fluorescence in (A) adherent cultured epithelial cell lines, and (B) freshly obtained primary epithelial cells.

2. Purpose

This procedure provides guidelines and procedures and the interpretation of the results used for the assessment of Cl\(^{-}\) or I\(^{-}\) efflux, using SPQ/MQAE halide-sensitive fluorescent indicators, from both adherent cultured epithelial cell lines and freshly obtained primary human airway epithelial cells. The operating procedures start with the setting up of the epi-fluorescence digital imaging microscope together with experimental materials (including epithelial cells attached to coverslips), and ends with the calculation of efflux rate constants using experimentally derived SPQ/MQAE fluorescence intensities and empirically derived Stern–Volmer calibration constants.

3. Measurement of iodide efflux from adherent cultured epithelial cell lines\(^1\)

3.1. Materials and solutions

Efflux solution (mM): 135 NaNO\(_3\); 1 Ca\(_2\)SO\(_4\); 1 Mg\(_2\)SO\(_4\); 2.4 K\(_2\)HPO\(_4\); 0.6 KH\(_2\)PO\(_4\); 10 HEPES, 10 glucose; pH 7.4. Iodide solution: 135 mM NaI in the above efflux buffer replaced the NaNO\(_3\). Cyclic AMP elevating agent forskolin (FSK) and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). Quenching solution: Iodide solution. At time \(t=0\) min, the maximal fluorescence is measured over 5 min in efflux solution (Fig. 1A). Intracellular SPQ is then quenched by perfusion for a further 15 min with iodide solution. At time \(t=20\) min, the establishment of basal fluorescence is achieved by substitution of nitrate (an anion transported by CFTR that does not quench SPQ) for \(\Gamma\). Cyclic AMP (20 \(\mu\)M FSK and 100 \(\mu\)M IBMX) agonist stimulation of the cells then follows at time \(t=25\) min and autofluorescence is finally measured by complete quenching of the intracellular SPQ at time \(t=45\) min with quenching solution containing KSCN (150 mM) and valinomycin (5 \(\mu\)M) after 30 min to obtain the minimum fluorescence (Fig. 1A).

3.3. Loading cells with SPQ/MQAE\(^2\)

3.3.1. Metabolic loading

Incubate cells with SPQ solution for 12 h prior to assay (556 \(\mu\)l of 36 mM stock per 1.5 ml cell growth media will give 10 mM final SPQ concentration).

3.3.2. Hypotonic loading

To eliminate any possible fluorescent ‘hotspots’ due to preferential subcellular localisation of SPQ, cells can be loaded with SPQ via hypotonic shock (50% for 4 min). The hypotonic loading buffer contains 0.56 ml of 36 mM SPQ stock with 1 ml of filtered deionised water and 0.44 ml of NaNO\(_3\) solution (2 ml total volume).

3.4. Experimental procedure

Coverslips with SPQ/MQAE-loaded cells are inserted into a perfusion chamber mounted on the stage of a Nikon Diaphot 300 inverted microscope (Nikon) and continual perfusion is initiated (140 \(\mu\)l volume and flow rate of 3.2 ml/min with complete buffer exchange achieved in approximately 2.6 s). Cells are illuminated with a 100 W xenon (Xe) arc lamp with neutral density filter attenuation. The cells are viewed first using a Nikon 10 × fluorescence objective and the fluorescence imaged with a 40 × Nikon fluorescence 1.4 N.A. objective onto an intensified charge-coupled device (CCD) camera (C2400-68, Hamamatsu Photonics) through a D460/50 band-pass filter (Glen Spectra). Once a suitable field of view is found, the main experiment is started by extensive washing with NaNO\(_3\) solution to remove extraneous SPQ/MQAE. The frame acquisition rate for all the data is 30 s with an exposure time of 15 ms/frame. At time \(t=0\) min, the maximal fluorescence is measured over 5 min in efflux solution (Fig. 1A).

3.5. Image analysis

Data are acquired and analysed off-line using Meta-morph™ Image software (Universal Imaging). The average

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\(^1\) Procedure as used in the Department of Gene Therapy, Imperial College London, UK (performed in dark room at room temperature).

\(^2\) At room temperature in the dark.
fluorescence intensity of each region of interest (ROI, drawn along the perimeter of a cell) for each of the frames is determined and directly logged into a spreadsheet. These ROI average fluorescence intensities are then used to assemble fluorescence traces (arbitrary fluorescence units as a function of time) as shown in Fig. 1B and C.

3.5.1. Calculation of iodide efflux rates

Iodide efflux rates \( (J_I, \text{mM/s}) \) are calculated from the observed fluorescence changes as detailed previously [1]. Computation of these rates requires knowledge of the Stern–Volmer constant \( (K_I) \) for quenching of intracellular SPQ by \( I^- \) (calibration of SPQ fluorescence vs. intracellular \( I^- \) concentration). The Stern–Volmer constants \( (M^{-1}) \) for the commonly used cell lines, 16HBE14o- (+/+), C127 (−/−), and C127 (+/−), are 15.1 ± 2.6, 10.2 ± 1.5, and 15.4 ± 6.7 (mean ± SEM, \( n = 6 \)), respectively.

The rate of \( I^- \) efflux \( (dF/dt) \) is calculated as \( J_I \) from Eq. (1).

\[
J_I (\text{mM/s}) = \frac{(F_0/K_IF^2)}{dF/dt}
\]  

(1)

where \( K_I \) is the Stern–Volmer constant for quenching of intracellular SPQ by \( I^- \) as determined above. \( dF/dt \) is the initial rate of change of SPQ fluorescence \( (t = 10 \text{ minus } 2 \text{ min after agonist addition}) \). \( F_0 \) is determined from the difference in fluorescence signal measured in cells in zero iodide buffer and after addition of KSCN/valinomycin (5 \( \mu \)M) after 30 min to obtain the minimum fluorescence.

4. Measurement of halide efflux from primary epithelial cells from human nasal and bronchial brushings—protocol 1

4.1. Specific materials and solutions

Efflux buffer: Standard Ringer’s (SR) solution (mM): 140 NaCl; 5 KCl; 5 HEPES; 1 MgCl\(_2\); 1.5 CaCl\(_2\); 5 glucose; pH 7.4. Chloride-free nitrate buffer (NO\(_3^-\) in the above SR buffer replacing all Cl\(^-\)) contains KSCN (150 mM) and valinomycin (5 \( \mu \)M) at pH 7.4. For the intracellular calibration, a K\(^+\)-rich buffer containing KCl (120 mM) and various concentrations of Cl\(^-\) and NO\(_3^-\) is used at pH 7.4.

Fig. 1. Typical cyclic AMP-induced \( I^- \) efflux from a pair of wild-type CFTR stably expressing (C127 +/+ ) or control (expressing vector alone, C127 −/− ) mouse mammary epithelial cell monolayers (A) Representative pseudo-colour fluorescent images of SPQ-loaded C127 (+/+ ) and (−/− ) cells (original magnification ×600). Colour corresponds to fluorescence intensity in ascending order: blue, green, yellow, red and white. Data represents the mean ± S.E.M., of the relative fluorescence (\( F/F_0 \)) imaged from 10 randomly selected cells, where \( F \) is the observed SPQ fluorescence and \( F_0 \) the fluorescence in the absence of \( I^- \) (maximal fluorescence) measured initially for 2 min in buffer (consisting of 135 mM NaNO\(_3\), 1 mM Ca\(_2\)SO\(_4\), 2.4 mM K\(_2\)HPO\(_4\), 0.6 mM KH\(_2\)PO\(_4\), 10 mM HEPES and 10 mM glucose). Following a 20-min incubation, basal fluorescence was measured for 5 min after substitution of nitrate for \( I^- \). At time \( t = 5 \) min, C127 (+/+ ) (B) or C127 (−/− ) (C) were stimulated with FSK (20 \( \mu \)M)/IBMX (100 \( \mu \)M). Intracellular SPQ was completely quenched by addition of \( I^- \)-containing KSCN (150 mM) and valinomycin (5 \( \mu \)M) after 30 min to obtain the minimum fluorescence.
loaded with dye by incubating for 40 min in 10 mM MQAE (in SR buffer) in a cell culture incubator. The loaded cells are rinsed with fresh SR and centrifuged at 1600 g for 1 min and resuspended in 4 μl SR (37 °C). Dye-loaded cells are seeded directly on to coverslip mounted on the microscope stage.

4.6. Measurement of halide efflux from primary epithelial cells from human nasal and bronchial brushings – protocol 4

The loaded cells are placed on the Cell Tak™-treated coverslip and allowed to attach for a few min and are then bathed in SR. Cell clusters with beating cilia are chosen for analysis. Cells are perfused continually using a peristaltic pump (Ismatec, Zurich, Switzerland) (30 mm³ exchange volume and flow rate of 1 ml/min with complete buffer exchange achieved in approximately 2 s). The frame acquisition rate for all the data is 3–7 s with an exposure time of 16 ms/frame. At the end of the experiment, ROIs corresponding to single cells or clusters are selected and the average intensity of fluorescence cells determined. At 40 × magnification, the fluorescence of the cells is homogenous and the dye does not show compartmentalisation, therefore the average intensity is a good indicator of MQAE fluorescence. The fluorescence is displayed as arbitrary units, after the background subtraction and, if necessary, correction for dye leakage and bleaching.

The experiment is performed sequentially, by exposing the cells to a Cl⁻ gradient under basal conditions, followed by cAMP (100 μM IBMX and 5 μM FSK) agonist stimulation, and subsequent in situ double-point calibration and determination of autofluorescence. The quenching solution consists of 150 mM KSCN in 10 mM HEPES, pH 7.2.

4.7. In situ double-point calibration

MQAE fluorescence is linearly related to intracellular [Cl⁻], according to the Stern–Volmer equation:

$$[\text{Cl}^-](t) = \left( \frac{F_0}{F_{\text{Cl}^-}} - 1 \right) \cdot \frac{1}{K_{SV}}$$

\(F_0\) is the fluorescence in the absence of Cl⁻ after subtraction of background fluorescence, \(F_{\text{Cl}^-}\) is the fluorescence in the presence of [Cl⁻], after subtraction of background fluorescence and \(K_{SV}\) is the Stern–Volmer constant for Cl⁻ quenching. \(K_{SV}\) is determined by double-point calibration, which is carried out as follows.

The cells are exposed to the K⁻-rich buffer described above containing the ionophores tributyltin (10 μM) and nigericin (10 μM). After this treatment, the intracellular

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4 Procedure as used in the Department of Medical Cell Biology University of Uppsala, Uppsala, Sweden (performed in dark room at 37 °C) [7–9].
Cl\(^-\) concentration equals the extracellular Cl\(^-\) concentration. If the experiment is carried out at, e.g., 20 and 80 mM Cl\(^-\), \(K_{SV}\) can be calculated from Eq. (2). Subsequently, this value of \(K_{SV}\) can be used to calculate \([\text{Cl}\(^-\)]_i\) during the experiment.

In practice, it is difficult to obtain \(F_0\), because the cells may be damaged by prolonged exposure to an extracellular Cl\(^-\) concentration of 0. Therefore, \(F_{20}\) (which is the fluorescence at 20 mM [Cl\(^-\)]\(_i\)) is determined experimentally and then \(F_0\) can be calculated from it.

In order to calculate the efflux rate, we use an exponential fitting (Fig. 3) of the experimental data. Due to the electroneutral characteristics of the Cl\(^-\) efflux in non-exitable epithelial cells, the intracellular Cl\(^-\) concentration during the efflux ([Cl\(^-\)]\(_i\)(t)) is described by the equation:

\[
[\text{Cl}^-]_i(t) = \text{Bottom} + (\text{Plateau} - \text{Bottom}) \times \exp(-Kr)
\]

where Bottom is [Cl\(^-\)]\(_i\) at the end of the efflux, Plateau is [Cl\(^-\)]\(_i\) before the efflux, \(t\) is the elapsed time from the
beginning of the efflux, and $K$ is the permeability constant of the membrane for $\text{Cl}^-$. The $\text{Cl}^-$ efflux rate $J_{\text{Cl}}$ is then described by the equation:

$$J_{\text{Cl}} = \frac{d[\text{Cl}^-]}{dt} = -K(\text{Plateau} - \text{Bottom})\exp(-Kt)$$

In addition, the extreme of this function is $-K(\text{Plateau} - \text{Bottom})$, representing the maximal $\text{Cl}^-$ efflux.

The successive exposure of the same cells to different conditions allows a powerful and meaningful comparison of the effects of agonists in a paired-way fashion. This is true especially when cells behave heterogeneously and the variability of responses impairs statistical analyses. At the same time, it offers an advantage when scarce and sensitive material such as cells collected from human donors is to be examined. Although there are reports that cells behave differently after successive exposure to a $\text{Cl}^-$ gradient [14], in our experience, the nasal cells and most of the cells lines are not affected.

5. Additional notes

(a) All buffers/solutions should be filtered through a 0.2-μm pore-size filter prior to use to minimise light scatter. (b) Validated equipment, supplies and reagents are listed in the appendix of the on-line version of the article in the virtual repository. (c) Care should be taken to avoid photobleaching caused by extended periods of illumination. In addition, the deleterious effects of heat from the microscope mercury and/or Xe arc lamp sources are minimised by use of appropriate neutral density filters and use of objective especially modified to correct for optic aberration due to heating. (d) Local ethics and regulatory authority approval should be obtained before the use of human biological material.

References


