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Phosphorimaging detection and quantitation for isotopic ion flux assays

Richard W. Fitch *,[1], John W. Daly

Section on Drug–Receptor Interactions, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20892, USA

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Abstract

A 96-well-microplate-based ion flux method utilizing readily available autoradiographic phosphorimaging detection is described. Nicotinic acetylcholine receptor-mediated ^{22}Na influx in four cultured cell lines provided satisfactory concentration-response data for epibatidine and several other nicotinic agonists. The data were consistent with data obtained using standard 6-well assays. Assays for nicotinic-receptor-mediated ^{86}Rb efflux produced data similar to data obtained with the ^{22}Na influx assay. However, assays for ^{45}Ca influx were not successful, although ^{45}Ca was readily detected and quantified. Voltage-gated sodium channel-mediated ^{22}Na influx in a neuroblastoma cell line allowed assay of the effects of such sodium channel activators as batrachotoxin and a pumiliotoxin B/scorpion venom combination. Phosphorimaging detection allows for reliable beta counting of up to 1200 simultaneous samples with excellent sensitivity and is amenable for application to high-throughput screening.
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Radioisotopic tracing has been used for decades to analyze the passage of ions across membranes in intact cells, synaptosomes, and related systems [1–5]. Such assays utilize radioisotope tracers of the native ions to measure the passage of ions across membranes through ligand- and voltage-gated ion channels. The data obtained from such isotopic flux studies in most cases have been comparable to results obtained by classical electrophysiology. However, for screening purposes of agonists, antagonists, and modulators, ionic flux, like electrophysiology, has been limited by the throughput with which data can be obtained. Although recent developments, such as multichannel electrophysiology instruments and plate-based scintillation counters with multiple counting heads, have improved throughput in these areas substantially, the required instrumentation is often expensive. Moreover, isotopes with significant gamma or high-energy beta emissions can lead to cross-talk problems in plate-based scintillation counting [6].

We have developed a convenient method for measurement of radioisotopic ion flux that is higher in throughput than methods previously described for ion flux assays. The heart of the method relies on carrying out the assay in 96-well plates and measuring the isotopic influx or efflux in the assay plates using storage phosphor screens, which are then read on a standard phosphorimaging gel reader. The use of microtiter plates and the size and number of storage phosphor screens determine the throughput. Simultaneous counting of samples makes the throughput substantially higher than standard scintillation counting. Using this method, between 6 and 18 plates per day (576 to 1728

* Corresponding author. Fax: +1 812 237 2232.
E-mail address: rfitch@indstate.edu (R.W. Fitch).
[1] Present address: Department of Chemistry, Indiana State University, Science S35E, Terre Haute, IN 47809, USA.

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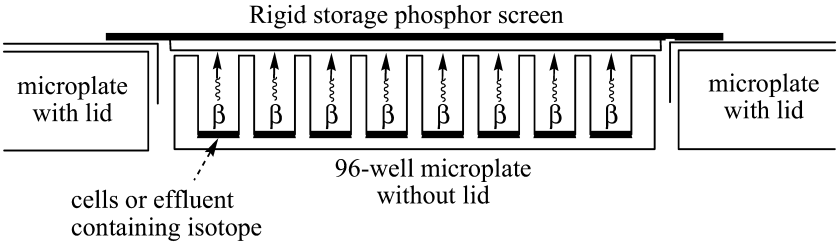

Fig. 1. Phosphorimaging method and principles. A storage phosphor screen is suspended on rigid support above the microplate to be assayed. Typically, this support was another microplate with lid providing 1–2 mm clearance between the plate and the screen. Alternately, the screen may be allowed to rest on the plate surface, provided that the plate has been cleaned of all surface contamination. The screen is exposed by the betas emitted from the sample in each well.

samples) plus decomposition per minute (dpm)[2] standards may be assayed and counted in a single 24-h period by a single operator. Throughput is limited only by the manual assay process and preparation of plates for reading. However, automation could substantially increase this number using commercially available automated washing and dispensing instruments. Such instrumentation typically improves reproducibility over manual processes also. While this method does not allow for dual-label experiments, careful manipulation of cells with thorough but gentle washing and aspiration affords acceptable noise levels and reproducibility.

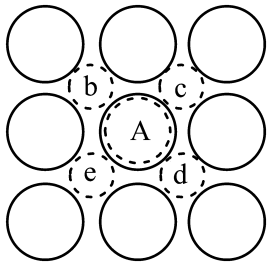

Fig. 2. Diagram of microwells, illustrating the method for background subtraction/cross-talk correction. The density of well A is calculated and the average density of areas a–d subtracted to give a background-subtracted and partially cross-talk-corrected value.

Materials and methods

Instrumentation

Microwell plates (Corning Costar 3596 or equivalent, see **Cell culture**) were imaged and the imaging screens read on either of two systems. Initial studies were performed on a Molecular Dynamics Storm 860 phosphorimaging system (Amersham Biosciences), while later parts of the study were done on a FL-3000IR system (Fujifilm Medical Systems). The two systems gave comparable results. Screen sizes with the Storm 860 ranged from 19×24 cm, which could handle 4 plates with standards, to 35×43 cm, which could handle 12 plates with standards. However, the most commonly used screens for this study were the Fuji 20×40 cm, which handled 6 plates with standards. Plate imaging was performed in a dark drawer either by suspending the storage phosphor screen over the plates (Fig. 1) or by laying the screen on the top of the plate. Typical exposures were overnight (18–24 h) but longer exposures were used for some experiments (see Results and discussion).

Storage phosphor screens were routinely monitored for surface contamination with a Geiger–Muller counter and screens were periodically developed in the absence of isotope and read to ensure that no surface contamination was present. Prior to exposure, screens were erased using a light box. Data analysis was done using the standard instrument software. Each of the systems has image templates of 96-well plates for determining image density for each well. Background subtraction and cross-talk correction was accomplished by subtraction of the average density of the four empty areas surrounding each well (Fig. 2).

In most cases, comparison with unsubtracted data showed no substantial differences in the pharmacological parameters (EC_{50}, E_{max}) obtained except for ^{86}Rb, where cross-talk can amount to around 10% for adjacent wells. However, variation in background intensity across a plate sometimes occurs (see below). While not a "true" cross-talk correction which can be obtained by matrix algebra methods [6], this approach was found to give satisfactory results in our studies.

Buffers

Hanks' balanced salt solution (HBSS) was prepared from 10× stock solution (Invitrogen 14065), supplemented with 20 mM 4-hydroxyethyl-1-piperazinesulfonic acid (Hepes; ICN), and adjusted to pH 7.4 with sodium hydroxide.

Reagents

All solvents and common reagents (e.g., salts) were reagent or analytical grade, obtained from commercial

[2] *Abbreviations used*: dpm, decomposition per minute; HBSS, Hanks' balanced salt solution; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium.