Fluorescence Quenching in Cell-Based Assays Can Be Dye Specific: A Case Study Using Both Fura-2 AM and Fluo-4 AM Calcium Reporter Dyes Using the FDSS6000

Introduction

Fluorescence quenching refers to any process which decreases the fluorescence intensity of a given substance[1]. Quenchers may not affect all fluorophores, allowing for selective quenching. There are two types of quenching: Static and Dynamic. Static quenching occurs when the fluorophore binds to the quencher; upon light absorption (excitation) fluorophore returns to the ground state with no photon emission (fluorescence). Dynamic quenching describes reducing the fluorescence lifetime of the fluorophore, that is the fluorophore emits photons for less time (decrease in 'fluorescence lifetime') than in the absence of a quencher.

Diverse compound libraries used in High Throughput Screening (HTS) for antagonists may contain fluorophore quenchers, generating results apparently indistinguishable from real antagonist hits (false positive). Further, in agonist screening campaigns quenchers may mask true positive agonist hits (false negatives), an all important consideration in assay design.

In this study we screened a panel of 3500 compounds for antagonism using both Fura-2 AM and Fluo-4 AM dye loaded cells. Results reveal some compounds mediate strong inhibition, reported as no change in fluorescence upon agonist addition, against one dye-loaded cell model and not the other, suggesting dye-specific quenching.

Materials and Methods

Adherent cells were washed and then loaded using either Fluo-4 AM or Fura-2 AM, 1.2 μM final concentration, for 1 h, 37 °C. Following washing antagonist was added to cells and incubated 30 min, 37 °C. Agonist was added using the Hamamatsu FDSS6000 and calcium mobilization measured, taken as the peak response during either 26 sec or 53 sec reaction time windows. Negative control correction, subtract bias, Z' factor, and percent specific inhibition were calculated using CeuticalSoft (Hudson NY).

Results

Z' factor ranges for the ten plates assayed using Fura-2 AM and Fluo-4 were 0.5-0.6 and 0.7-0.8, respectively.

The results for all compounds tested using 53 sec reaction time window were plotted as a scatter gram of percent specific inhibition of Fluo-4 Dye loaded cells vs. Fura-2 Dye loaded cells (Figure 1A). Note the four populations. The lower left quadrant represents compounds double negative using both dye loaded cells (no signal inhibition). The upper right quadrant represents compounds double positive for inhibition. Interestingly, two additional populations are present, apparent inhibition using Fluo-4 dye loaded cells only (upper left quadrant) and apparent inhibition using Fura-2 AM dye loaded cells only (lower right quadrant). These results suggest dye specific quenching is responsible for signal inhibition. Further, the upper right quadrant may contain quenchers effective against both Fura-2 AM and Fluo-4 AM dyes. We hypothesized that quenchers will inhibit fluorescence independent of reaction time, where at least for some less potent yet specific antagonists the kinetic trace may evolve over time. To test this hypothesis we used a shorter reaction time window (26 sec) to see if any hits show more inhibition as compared to a 53 sec window. The results, plotted in Figure 1B, indicate a shift to the right (increase) in antagonist potency using Fura-2 AM loaded cells of some compounds (upper right quadrant). These results are consistent with measuring the off rate of antagonists, that is with time antagonists are replaced with agonists and more calcium is mobilized.

Taken together these results show that by combining results using two fluorescent dyes over 300 hits are identified as single dye-specific inhibitors, consistent with fluorescence dye quenching. Fanger et al. reported a compound quenching signal in Fura-2 AM loaded cells but not Fluo-4 AM loaded cells[2], consistent with results reported here.

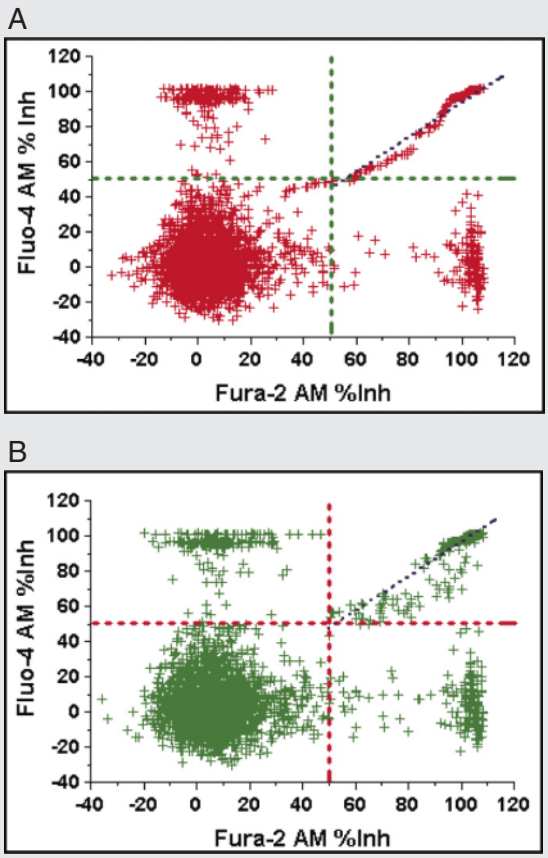

Fig. 1: Scatter gram of specific percent inhibition activity, Fluo-4 AM dye loaded cells vs. Fura-2 AM loaded cells.
A) 53 second reaction window, maximal response,
B) 26 second reaction window, maximal response.

Summary

Fluorescence dye quenching may increase 1) false positive hit rates using inhibition assays and 2) false negative hit rates using agonist assays. This report suggests one way to identify dye specific quenchers is to screen using multiple fluorescence reporter dyes.

References

1. Lakowicz, J.R. (1983). Principles of Fluorescence Microscopy. New York, NY: Plenum Press.
2. Fanger, C and Zhen, X. A dual-dye method for reducing false positives and rescuing compounds of interest in fluorescent calcium assays. Poster. Society for Biomolecular Screening, September 2005, Geneva, Switzerland.

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