Comparison of Orthogonal Technologies to Confirm Agonist Hits Generated Using the FDSS6000

Introduction
In this report we identify a subset of primary hits from a No Wash, fluorescence based agonist campaign using a recombinant cell line. We cherry picked the hits for retesting against a panel of assays to confirm activity.

Materials and Methods
CHO cells stably transfected with the coding sequence of a human receptor and Aequorin (Recombinant) or the parental cell line, stably transfected with Aequorin (Parental) were used in the study. The various assays, Aequorin (Euroscreen), IP-ONE (Cis-Bio), RT-CES (ACEA), Calcium No Wash (BD Biosciences), were used according to manufacturer’s instructions.

Results
A primary screening campaign of 50,000 compounds against BD PBX Calcium Assay Kit loaded Recombinant cells yielded 365 hits, defined as signal greater than 50 % of the positive controls. By contrast, the same library and cell line, prepared for Aequorin assay and analyzed using the FDSS6000 yielded only 1 hit (Table 1).

To test the possibility that the false positive compounds interacted with the fluorescence dye directly, we utilized the dual excitation capability of the FDSS6000. Cells were rendered nonadherent, preloaded with either Fluo-3 AM or Fura-2 AM dyes, washed, and combined into one well (Multiplex). We hypothesized that a compound that affected one fluorescence dye signal might not affect the other dye signal. Thus, if we use the criteria of selecting compounds that were positive using both dyes, we could reduce the false positive rate significantly. As shown in Figure 1, only 2/365 compounds were double positive. Interestingly, 17 compounds were single positive with Fluo-3 AM loaded cells only, suggesting autofluorescence at excitation 488 nm. The remainder were double negative, suggesting that the false positive compounds were not directly interacting with the fluorescent dyes.

Table 1: Fluorescence Based Assay Hits are Non-Specific, not Confirmed Using Orthogonal Technologies

<table>
<thead>
<tr>
<th>Primary Hits</th>
<th>Primary Hit Retests</th>
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<tbody>
<tr>
<td>Library</td>
<td>Fluorescence Aequorin</td>
</tr>
<tr>
<td>50 000</td>
<td>365</td>
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Fig. 1: Correlation of agonist hits using GPR54 cell line loaded using either Fura-2 AM (Y-axis) or Fluo-3 AM (X-axis) (Multiplex). Results are expressed as the minimal fluorescence at ex380 em540 for Fura-2 AM versus maximal fluorescence at ex480 em540 (Fluo-3 AM). Results fall into three groups: (-,-), double negative; (+,-), positive in Fluo-3 AM loaded cells, negative in Fura-2 AM loaded cells; and (+,+), positive in both dye loaded cells. The true positive hit is marked with an arrow, a single false positive hit is present.
Summary

In summary, we suggest screening for GPCR agonists with No Wash dye kits in cell based assays may generate a significant number of nonspecific positive hits. When compared against the parental cell line response all nonspecific hits were identified using both Aequorin and IP-One technologies and nearly completely identified using multiplex technology, washed cells. In conclusion, follow-up assays for No Wash dye agonist campaigns should include orthogonal technology / assays to identify nonspecific hits.