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A Unique High-Throughput Assay to Identify Novel Small Molecule Inhibitors of Chemotaxis and Migration

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Abstract

Chemotaxis and cell migration play pivotal roles in normal physiological processes such as embryogenesis, inflammation, and wound healing, as well as, in pathological processes including chronic inflammatory disease and cancer metastasis. Novel chemotaxis/migration inhibitors are desirable for developing effective therapeutics and probing molecular mechanisms. We describe a fluorescence-based phenotypic assay in a 1536-well plate format for high-throughput screening of novel inhibitors of chemotaxis/migration within complex libraries of multi-1000 compounds. Although the assay utilizes the unique cellular response properties of *Dictyostelium*, the compounds identified are able to inhibit chemotaxis of mammalian cells. In addition, a parallel cell cytotoxicity counter-screen with an ATP content assay is described that eliminates cytotoxic compounds from the screen. This novel compound screening approach enables rapid identification of novel lead compounds that inhibit chemotaxis in human and other cells for drug development and research tools.

Keywords

Cell Migration; Chemotaxis; LOPAC; Acumen ^eX3; *Dictyostelium*

INTRODUCTION

Cell chemotaxis/migration plays pivotal roles in embryogenesis, immunity, and wound healing, as well as, in diseases of cancer metastasis and chronic inflammation. Chemotaxis/migration inhibitors have potential as probes for mechanistic studies and as lead compounds for development of novel therapeutics. However, current cell-based chemotaxis assays are not easily adapted for high-throughput screening for systematic identification of such novel inhibitors.

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Dictyostelium is an excellent model organism which shares similar chemotactic mechanisms with mammalian migratory cells. Upon nutrient depletion, *Dictyostelium* undergo a transition from a unicellular growth state to multicellular development, a program involving secretion and sensing of the chemoattractant cAMP for cell-cell chemotaxis and multi-cell aggregation. Aggregate formation induces a series of developmental-specific gene expression markers.

Utilizing the unique chemotactic properties of *Dictyostelium*, we developed a simple, 1536-well plate format, fluorescent chemotaxis-dependent aggregation assay that can identify inhibitors of mammalian chemotaxis/migration within very large libraries of small molecule compounds. A high-throughput cytotoxic counter screen is also described that eliminates cytotoxic false positive compounds.

STRATEGIC PLANNING

As a chemotaxis model system, *Dictyostelium* and mammalian cells share many conserved pathways involved in chemoattractant/chemokine response (e.g. GPCRs, G proteins, ERKs, PKA, PI3K, TORC2, AKT, PLA2, PDK1, cyclases, Actin; Artemenko et al., 2014; Jin, 2013; Jin et al., 2008; McMains et al., 2008; Swaney et al., 2010; Van Haastert and Veltman, 2007). The actin polymerization inhibitor latrunculin A, the PI3K inhibitor LY 294002, and other chemotaxis/migration inhibitors of mammalian cells also efficiently suppress *Dictyostelium* chemotaxis/migration. Rationally, chemotaxis/migration inhibitors identified from the *Dictyostelium* chemotaxis assay would reciprocally have the potential to similarly inhibit mammalian cell chemotaxis/migration, which are then easily verified in direct chemotaxis/migration assays.

Dictyostelium grow as single cells under nutrient abundant conditions, but upon starvation, they enter a multicellular developmental program; as *Dictyostelium* sense nutrient depletion, centers of cAMP secretion become established and proximal *Dictyostelium* chemotax within the cAMP gradients, relay the cAMP signal outwardly, and, thus, recruit additional cells to participate in chemotaxis and signal/relay. Cells ultimately form tight multi-cell structures at centers of cAMP signaling (Figure 1) and culminate development as a complex organism comprised of highly differentiated cell-types (Jin et al., 2008; McMains et al., 2008; Swaney et al., 2010; Van Haastert and Veltman, 2007; Williams, 2010). A GFP reporter expressed under control of the *cotB* promoter, which is only activated in multi-cell aggregates (Nicol et al., 1999), is used to assess chemotaxis-dependent aggregation (Figure 1). The GFP fluorescence signal is proportional to successful chemotaxis-dependent phenotypic aggregation and is quantified using the laser-scanning Acumen[®]X3 cytometer (Liao et al., 2016).

This *Dictyostelium* aggregation assay has been automated and miniaturized in a 1536-well plate format (Liao et al., 2016), using only 8,000 cells/well (~6 µl), with the GFP fluorescence rapidly assessed, with full data collection of an entire 1536-well plate within 10 min (Figure 2). 1-3 aggregates are usually formed within each of the 1536 wells, while no aggregates are formed when chemotaxis is chemically inhibited (Figure 2). When parameters of the Acumen[®]X3 are set to only collect GFP signals in objects >30 µm

diameter, wells containing chemotaxis inhibitors have a zero GFP signal (at 100% inhibition; Figures 2 and 3). Thus, each well can be robustly classified in a binary fashion, with zero (no GFP expression) values indicative of strong chemotaxis inhibition and non-zero (GFP expression) values as non-inhibitory.

Since cytotoxic compounds that compromise cell viability may also inhibit chemotaxis (Figure 4), a cell-based ATP content assay was adapted to evaluate compound cytotoxicity to eliminate false positives (Miret et al., 2006). *Dictyostelium* cells within aggregates are resistant to lysis, so the ATP content assay uses a plating protocol that is non-permissive to aggregation.

To identify compounds with a wide range of activities and to avoid high false negatives of a traditional single-concentration assay, all compounds are serially diluted at a 1:5 titration ratio over 7 concentrations in a quantitative high-throughput screen (qHTS) for chemotaxis/migration inhibition. Thus, concentration-response curves can be immediately produced with the rich data obtained from a single qHTS experiment. The qHTS method further increases the reliability of our screening assays.

This high-throughput, phenotypic assay can be used as a primary assay for screening chemotaxis/migration inhibitors from large, chemical libraries. The identified candidate compounds are then further examined for inhibitory activity against mammalian migratory cells by low/middle-throughput chemotaxis assays, such as monolayer scratch assay (Liang et al., 2007), transwell assay (Ponath et al., 2000), EZ-TAXIScan assay (Liu et al., 2010), among others (see below).

MINIATURIZED *Dictyostelium* AGGREGATION ASSAY FOR SCREENING OF CHEMOTAXIS/MIGRATION INHIBITORS

On solid substrata, *Dictyostelium* progress through all developmental stages, but the under buffer assay used here promotes arrest at aggregation (Figure 1). In *Dictyostelium* strain [cotB]:GFP, the GFP reporter is controlled by the *cotB* promoter which is only activated after cells have undergone chemotaxis and aggregate formation (Nicol et al., 1999). GFP expression in this strain is used to indicate success of chemotaxis and multicellular aggregation. The chemotaxis/aggregation assay has been miniaturized to a 1536-well plate format for large-scale screening. Latrunculin A is incorporated as an inhibitory control in all screening plates (Figures 2 and 3). Multiple parameters which might impact the assay have been optimized. 48 hrs is chosen as an optimal incubation time to ensure that all aggregates attain peak GFP signals. 8,000 cells/well is the optimal cell density for chemotaxis-dependent aggregation; at lower cells densities, aggregates do not form and at higher densities, aggregates form without chemotaxis. 20-23.5 °C is the optimal incubation temperature. Compound solvent DMSO has no impact on cell chemotaxis under experimental concentrations (*i.e.* 0.38%) and is included as a non-inhibitory control for all plates (Figure 2).

Materials

Dictyostelium strain [cotB]:GFP [dictyBase, DBS0236466; (Basu et al., 2013)]

D3-T medium (see recipe below)

DB starvation buffer (see recipe below)

DMSO (Sigma-Aldrich, #D2650)

Latrunculin A (Sigma-Aldrich, #L5163)

Library of Pharmacologically Active Compounds: LOPAC¹²⁸⁰ (Sigma-Aldrich, #LO1280) or other compound collections

25 cm² Canted Neck Cell Culture Flask, sterile

250 ml or larger glass or plastic Erlenmeyer flask, sterile

1536-well clear bottom plates (Aurora Biotechnologies)

Orbital shaker at 21°C

Incubator with temperature controlled at 21°C

Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific Inc.)

Pintool Station (Kalypsys, San Diego, CA)

Evolution P3 system (PerkinElmer, Wellesley, MA).

Acumen ^eX3 (TTP LabTech, UK)

Reagents and Solutions

D3-T media for Dictyostelium culture—7.15 g of peptone (BD Bioscience, #211693)

7.15 g of thiotone (BD Bioscience, #211921)

7.15 g of yeast extract (BD Bioscience, #212750)

15.4 g of D-glucose

0.48 g of KH₂PO₄

0.525 g of Na₂HPO₄•7H₂O

Bring to 1 liter with distilled water and adjust the pH to 6.5

Autoclave for 20 minutes to sterilize

DB starvation buffer—7.4 mM NaH₂PO₄-H₂O

4 mM Na₂HPO₄•7H₂O

2 mM MgCl₂

0.2 mM CaCl₂

Bring to 1 liter with distilled water and adjust the pH to 6.5

***Dictyostelium* axenic culture [BASIC PROTOCOL 1; see dictyBase (Basu et al., 2013) for details]—**

1. *Dictyostelium* are stored for long-term in screw cap vials at -80°
2. For daily use, *Dictyostelium* are maintained as adherence cells in canted neck culture flasks containing 10 ml of D3-T medium at $\sim 21^{\circ}\text{C}$.
3. When cells are confluent, medium should be changed every several days, for the next two weeks. Healthy cells are required for the experiments; passage the cells with reasonable dilution or thaw a new storage vial when cells are cultured for more than 2 weeks.
4. Wash the cells from canted neck culture flasks, count cell density and inoculate the cells into Erlenmeyer flask containing D3-T medium at $\sim 2 \times 10^5$ cells/ml. Shake cells at 220 rpm, 21°C .
5. When the cell density reaches $\sim 3 \times 10^6$ cells/ml, shaking cultures should be diluted and cells passaged again; at $< 2 \times 10^6$ cells/ml, pellet cells by centrifugation at $1000 \times g$ for 5 min at RT.
6. Wash cells twice with DB buffer and pellet cells again at RT.
7. Resuspend cells in DB buffer at desirable densities (for aggregation assay, 2.67×10^6 cells/ml; for cytotoxicity assay, 2.5×10^5 cells/ml).

Preparation of Compound Plates (BASIC PROTOCOL 2)—

8. All of the compounds (including all compounds in the libraries, the chemotaxis inhibitor control latrunculin A, and the cytotoxic control hygromycin B) are dissolved in 100% DMSO at 10 mM stock solutions.
9. For control compounds, the stock solutions of latrunculin A or hygromycin B are diluted with DMSO in 384-well plates at a 1:3 titration ratio over 12 concentrations, and then formatted to 1536-well compound plates using an Evolution P3 system (Inglese et al., 2006), as detailed below.
10. For the LOPAC or other library compounds, each stock solution is serially diluted with DMSO in 384-well plates at a 1:5 titration ratio over 7 concentrations and then formatted to 1536-well compound plates using an Evolution P3 system. The final concentration of each compound ranges from 2.46 nM to 38.3 μM in the chemotaxis-dependent aggregation assay and from 3.68 nM to 57.5 μM in the cytotoxic assay, as detailed below (with 0.38% DMSO in all wells).

***Dictyostelium* plating, aggregation, and scanning—**

11. Dispense 3 μ l/well of DB into a 1536-well clear bottom plates using Multidrop Combi Reagent Dispenser.
12. Add 23 nl/well of the control compounds to the four left-most rows from the control compound plate using the Pintool Station.

The left-most vertical row of cells should have 12 serially diluted concentrations of latrunculin A with triplicates of each dose (Figure 2). The second vertical row of cells from left has undiluted latrunculin A in all wells, as a negative signal (*i.e.* a full inhibitory) control. The third and fourth vertical rows of cells have 0.38% DMSO, as positive signal controls (*i.e.* without any inhibition).

13. Add 23 nl/well of the candidate compounds to the rest of the wells from the LOPAC or other library compound plate using the Pintool Station. The final concentrations of each compound ranged from 2.46 nM to 38.3 μ M.
14. Disperse 3 μ l/well of *Dictyostelium* (2.67×10^6 cells/ml DB) to a final density of 8,000 cells/well using the Multidrop Combi Reagent Dispenser.
15. Centrifuge the plate at $157 \times g$ for 30 sec and incubate at 21 °C for 48 hrs to allow cells to chemotax, aggregate, and express GFP.
16. Measure the GFP signals in the 1536-well plate using Acumen ^eX3 with excitation at 455-488 nm and emission at 500-530 nm. The object threshold setting for the Acumen ^eX3 plate reader was at $>30 \mu$ m diameter (>3 cell equivalents) to detect signal from aggregates only.

DICTYOSTELIUM CYTOTOXICITY ASSAY FOR ELIMINATING CYTOTOXIC COMPOUNDS

Cytotoxic compounds can also suppress chemotaxis, aggregate formation, and GFP expression and would, thus, be falsely identified as chemotaxis/migration inhibitors. To eliminate these false positive compounds, we adapted an ATP content assay for measuring *Dictyostelium* viability. In the assay, ATP content is proportional to the emitted luminescence produced by the reaction of ATP with added luciferase and D-luciferin. The intensity of the emitted luminescence is measured using the CCD-imager based ViewLux plate reader. Hygromycin B is used as a cytotoxic control. Since it is difficult to lyse cells in tight aggregates, we use 500 cells/well, which is non-permissive for chemotaxis/aggregation and optimal for the ATP-content assay. Hygromycin B exhibited similar IC₅₀ values for inhibition of ATP content and chemotaxis (Figure 4; Liao et al., 2016). We use 3-days as an optimal incubation time for measuring compound cytotoxicity. Some compounds might inhibit chemotaxis at lower concentrations than for cytotoxicity. These compounds can be also identified as chemotaxis inhibitors when careful cutoffs are chosen (*e.g.* at 10-fold differences in IC₅₀ values).

Materials

Hygromycin B (Sigma-Aldrich)

ATPlite assay kit (PerkinElmer, #6016731)

CCD-imager based ViewLux plate reader (PerkinElmer)

***Dictyostelium* plating, ATP assay, and scanning**

1-7. Culture and harvest *Dictyostelium* as described in **Basic Protocol 1**. Resuspend cells in DB buffer at 2.5×10^5 cells/ml.

8-10. Prepare control compound plates with serial dilution of hygromycin B as described in **Basic Protocol 2**.

11. Dispense 2 μ l/well of DB into 1536-well clear bottom plates using Multidrop Combi Reagent Dispenser.
12. Add 23 nl/well of the control compounds to the four left-most rows from the control compound plate using the Pintool Station. The left-most vertical row of cells should have 12 serially diluted concentrations of hygromycin B with triplicates each dose. The second vertical row of cells from left has undiluted hygromycin B in all wells, as a negative signal (*i.e.* a full inhibitory) control. The third and fourth vertical rows of cells have 0.38% DMSO, as positive signal controls (*i.e.* without any inhibition).
13. Add 23 nl/well of the candidate compounds to the rest wells from the LOPAC or other library compound plate using the Pintool Station. The final concentrations of each compound ranged from 3.68 nM to 57.5 μ M.
14. Dispense 2 μ l/well of *Dictyostelium* (2.5×10^5 cells/ml DB) to a final density of 500 cells/well using the Multidrop Combi Reagent Dispenser.
15. Centrifuge the plate at $157 \times g$ for 30 sec, and incubate at 21 °C for 72 hrs.
16. Add 3 μ l/well of ATPlite reagent using the Multidrop Combi Reagent Dispenser and then incubate plates for 10 min at RT.
17. Detect the luminescence signal using the CCD-imager based ViewLux plate reader.

DATA ANALYSIS

The screening data are analyzed by NCATS-developed software, as described in detail (Southall, 2009). IC₅₀ values are obtained by data fit using a residual error minimization algorithm with automatic outlier determination to a 4-parameter Hill. Concentration-effect relationships (CERs) were categorized by fit quality (r^2), response magnitude, and degree of measured activity (Southall, 2009).

RE-CONFIRMATION AND VALIDATION OF CHEMOTAXIS INHIBITION

Once non-cytotoxic inhibitors of chemotaxis are identified in a primary screen, they should be re-assayed to confirm the results. Selected compounds are diluted with DMSO in 384-well plates at a 1:3 titration ratio over 12 concentrations, and then formatted to 1536-well

compound plates (see Basic Protocol 2). *Dictyostelium* aggregation and cytotoxicity assays are performed as described above. This step will further eliminate both false positives and false negatives, and better define IC₅₀ values. Inhibitory compounds are then validated for effects on mammalian cells using various direct assays (see below).

COMMENTARY

Comparative Perspective

Discovering chemotaxis inhibitors is important to further understand the molecular mechanisms of chemotaxis and to develop new therapeutics against cancer metastasis, chronic inflammation etc. (Chan and Mooney, 2008; Djekic et al., 2009; Inamdar et al., 2010; Jin et al., 2008; Mackay, 2008; Su et al., 2009; Wu et al., 2009). Previous *in vitro* drug screens for compounds against inflammatory and metastatic processes mainly focused on specific molecules/pathways involved in cell chemotaxis/migration (Djekic et al., 2009; Inamdar et al., 2010; Mackay, 2008; Su et al., 2009; Wu et al., 2009). Furthermore, compounds identified in these target-based screens have not had high *in vivo* activity and few have been applied in clinical trials. Instead, cell-based assays are more favorable to identify compounds which function well *in vivo*.

The most common chemotaxis assays (*e.g.* monolayer scratch assay, transwell assay and EZ-TAXIScan assay) are not easily adaptable for high-throughput screening. 96-well formats can be modified and automated for microfluidic devices (Berthier et al., 2013; Guckenberger et al., 2014; Irimia and Toner, 2009; Wu et al., 2013), Boyden chambers (Hulkower and Herber, 2011; Mastuyugin et al., 2004), and several image-based assays (Berthier et al., 2013; Guckenberger et al., 2014; Hattori et al., 2010; Hulkower and Herber, 2011; Kato et al., 2008; Mastuyugin et al., 2004; Ponath et al., 2000; Soltaninassab et al., 2008; Timm et al., 2013; Wu et al., 2013), but they are not optimally compatible to screen ~1000 compounds. We are able screen >10,000 compounds over 5-log concentrations, for both chemotaxis and viability.

Critical Parameters and Troubleshooting

We have optimized cell density, incubation time, incubation temperature, mini-well buffer volume, image detection parameters for the Acumen ^eX3, etc., as described above. Accurate temperature control is essential to obtain experimental consistency. The cytotoxicity assay should be performed at non-aggregating cells densities, at 500 cells/well.

The Acumen ^eX3 records numbers of aggregates, total fluorescence intensity, and peak fluorescence intensity of aggregates within each well. The GFP peak intensity was the most consistent and was chosen as the primary readout. We have tried to quantify the GFP signal using a CCD-imager based plate reader (ViewLux, PerkinElmer) and a PMT based plate reader (EnVision, PerkinElmer), but neither were able to obtain consistent signals.

Since the GFP-expressing multicellular aggregates form randomly, with different sizes and numbers, fluorescence signals can vary enormously [coefficient of variation (CV) = 27%] from well-to-well, even without chemical treatment. The Z' factor of the aggregation assay is 0.47, which is normally not favorable for a screening assay. However, when the

parameters of the laser scanning plate cytometer are set to only quantify GFP fluorescence presented in multicellular aggregates, the signals from wells with aggregation completely inhibited is “zero”. Consequently, the signal-to-basal ratio is unique in approaching infinity, emphasizing the extreme robustness and reliability of the assay.

Interestingly, partial inhibition of the GFP signal was rarely observed. Cells either completed aggregation and expressed GFP or remained as individual cells without expressing GFP. These observations can be partly explained by the characteristics of *Dictyostelium* signal-response. Cells both release and respond to secreted cAMP, in a positive feedback loop (Artemenko et al., 2014; Jin, 2013; Jin et al., 2008; McMains et al., 2008; Swaney et al., 2010; Van Haastert and Veltman, 2007). In addition, their networks are cross-regulated. Thus, compounds that inhibit chemotaxis often also inhibit cAMP signaling, which would further suppress aggregate formation and GFP expression, amplifying the inhibitory affect. The wells are best classified in a binary fashion, as aggregation/GFP positive or negative.

Anticipated Results/Validation

In our pioneer screening from 1280 LOPAC compounds library (Liao et al., 2016), 2 out of 22 primary inhibitory compounds were not also cytotoxic (Figure 5), using the parallel ATP content assay.

Other false positives for chemotaxis inhibition (*e.g.* blocking cAMP synthesis, loss of starvation sensing) would not be excluded from the ATP content assay, but they are easily eliminated by direct chemotaxis assay [*e.g.* EZ-TAXIScan assay (Figure 6), transwell assay, and monolayer scratch assay], and none of these were identified in the LOPAC screen.

There are at least 6 intracellular signaling pathway arms that regulate chemotaxis in *Dictyostelium* (Artemenko et al., 2014; Jin, 2013; Jin et al., 2008; McMains et al., 2008; Swaney et al., 2010; Van Haastert and Veltman, 2007). Genetic deficient *Dictyostelium* strains, in which some key components of known chemotaxis pathways have been deleted, might be used to increase the sensitivity of the screen and to screen potent inhibitors for specific chemotaxis pathways. To date, chemotaxis inhibitors have not been identified with nM activities (*e.g.* Figure 5).

The *Dictyostelium* aggregation assay that we developed is simple and robust, and the first cell-based chemotaxis assay compatible with extreme high-throughput screening. Although compounds were identified in a screen that inhibits *Dictyostelium* chemotaxis, they were similarly potent to inhibit chemotaxis of human neutrophils (Figure 6).

In addition, this assay may also be used to screen for small molecules that regulate the chemotactic response, but not inhibit it. 10 compounds (<10 μ M) were identified that increased aggregate numbers/well, thereby reducing aggregate size formation but without blocking GFP expression (Figure 7) and >10 compounds supported GFP expression under conditions that were normally non-permissive (*e.g.* <2,000 cells/well) for *Dictyostelium* chemotaxis and aggregation (Figure 8).

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Figure 1. Strategic design of the *Dictyostelium* chemotaxis/aggregation assay

Growing cells of *Dictyostelium* strain *cotB/GFP* do not express GFP (top). Following starvation, chemotaxis, and aggregation, cells express GFP within 24 hr (bottom).

Figure 2. Protocol for compound screening using the GFP reporter-based *Dictyostelium* chemotaxis-dependent aggregation assay

Each well of the 1536-well plate contains 8,000 cells of *Dictyostelium* strain *cotB*/GFP, with various compounds (top). After 48 hrs, the plates are imaged for GFP expression (middle), with selected individual wells with positive and negative signals enlarged (bottom).

The left-most vertical row has the chemotaxis inhibitor latrunculin A, at sequentially diluted concentrations from top to bottom. The second vertical row (from left) has 38 μ M latrunculin A in all wells, as a full inhibitory control. The next two vertical rows have 0.38% DMSO, as non-inhibitory controls. Different small molecule compounds are in the remaining rows.

Figure 3. Effect of latrunculin A on chemotaxis-dependent aggregation (GFP expression)
Inhibition of GFP intensity (with standard deviations) for different concentrations of the chemotaxis inhibitor latrunculin A in the 1536-well plate assay, compared to uninhibited controls.

Figure 4. ATP content assay as a counter screen for cytotoxicity

Inhibition of GFP intensity and ATP/viability for different concentrations of inhibitor hygromycin B in the 1536-well plate assay, compared to uninhibited controls. The chemotaxis/aggregation and viability curves (with standard deviations) superimpose.

Figure 5. Chemotaxis and ATP assay of LOPAC compounds PD 169316 and SB 525334
PD 169316 and SB 525334 are strongly inhibitory for chemotaxis-dependent aggregation (GFP), but without significant effect on cell viability (ATP).

Figure 6. LOPAC compounds PD 169316 and SB 525334 inhibit chemotaxis of both *Dictyostelium* and human neutrophils

The images show positions of individual *Dictyostelium* or neutrophils in EZ-TAXIScan chambers during chemotaxis within a gradient of cAMP or fMLP, respectively, after treatment with or without of the chemotaxis inhibitors PD 169316 and SB 525334, newly identified by our chemotaxis/aggregation screen.

Figure 7. LOPAC compounds that increase aggregate numbers/decrease aggregate sizes
Each well of the 1536-well plate contains 8,000 cells of *Dictyostelium* strain *cotB*/GFP, with various compounds. Selected individual wells are shown with differences in aggregate numbers/size, corresponding to novel LOPAC compounds.

Figure 8. LOPAC compounds that promote GFP expression under conditions that are inhibitory for chemotaxis/aggregation

Each well of the 1536-well plate contains 500 cells of *Dictyostelium* strain *cotB*/GFP, with various compounds. These conditions do not support chemotaxis/aggregation and GFP expression. After 48 hrs, the plates are imaged for GFP expression and several wells with novel LOPAC compounds exhibit strong GFP expression compared to controls.