

# Biomolecular screening of formylpeptide receptor ligands with a sensitive, quantitative, high-throughput flow cytometry platform

Bruce S Edwards<sup>1,2</sup>, Susan M Young<sup>1</sup>, Tudor I Oprea<sup>3</sup>, Cristian G Bologna<sup>3</sup>, Eric R Prossnitz<sup>4</sup> & Larry A Sklar<sup>1,2</sup>

<sup>1</sup>Cytometry and <sup>2</sup>Department of Pathology, Cancer Research and Treatment Center, <sup>3</sup>Division of Biocomputing and <sup>4</sup>Department of Cell Biology and Physiology, Cancer Research and Treatment Center, University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131, USA. Correspondence should be addressed to B.S.E. (bedwards@salud.unm.edu).

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The formylpeptide receptor (FPR) family of G protein-coupled receptors contributes to the localization and activation of tissue-damaging leukocytes at sites of chronic inflammation. Here we describe a high-throughput flow cytometry screening approach that has successfully identified multiple families of previously unknown FPR ligands. The assay detects active structures that block the binding of a fluorescent ligand to membrane FPR of intact cells, thus detecting both agonists and antagonists. It is homogeneous in that assay reagents are added in sequence and the wells are subsequently analyzed without intervening wash steps. Microplate wells are routinely processed at a rate of 40 wells per minute, requiring a volume of only 2  $\mu$ l to be sampled from each. This screening approach has recently been extended to identify a high-affinity, selective agonist for the intracellular estrogen-binding G protein-coupled receptor GPR30. With the development of appropriate assay reagents, it may be generally adaptable to a wide range of receptors. The total time required for the assay ranges between 1.5 and 2.5 h. The time required for flow cytometry analysis of a 96-well plate at the end of the procedure is less than 2.5 min. By comparison, manual processing of 96 samples will typically require 40–50 min, and a fast commercial automated sampler processes 96-well plates in less than 15 min, requiring the aspiration of 22  $\mu$ l per sample for an analysis volume of 2  $\mu$ l.

## INTRODUCTION

### Flow cytometry for high-sensitivity measurements in homogeneous assays

Modern drug discovery involves the testing of cellular targets against millions of potentially valuable compounds that may bind cellular receptors to effect clinically therapeutic cellular responses. Flow cytometry is a sensitive and quantitative assay for the measurement of particle fluorescence, capable of high-content characterization of a diversity of compound bioeffects at the single-cell level. Sample particles are hydrodynamically focused into a laminar flow so that single particles pass through a laser beam sequentially. The subsequent optical signal characteristics are recorded in real time. A flow cytometer can detect hundreds to thousands of fluorescent molecules on a cell or bead, allowing a fluorescent molecule concentration as low as 10–100 pM. Because of the optical configuration, the laser in a flow cytometer excites only a very small volume of the sample fluid immediately surrounding the cell. That diminishes the background signal by limiting the excitation of excess, unbound fluorescent molecules in solution. Therefore, homogeneous, 'no-wash' assays are possible in which measurements may be made without the need for removing the excess fluorescent tag to distinguish between free and bound probes<sup>1</sup>.

### High-throughput flow cytometry

For the serial analysis of individual cells or beads, the flow cytometer has always been considered a high-throughput analysis instrument. It is now a routine task to analyze from thousands to tens of thousands of particles per second. However, flow cytometry has been severely limited in throughput rates for the analysis of multiple discrete samples of cells. Commercial automated sample-

handling systems typically process about two samples per minute with a maximum throughput of six to seven samples per minute (<http://www.bdbiosciences.com>). That is a significant limitation when the objective is to screen a large collection of compounds against replicate cell samples. We have recently developed a high-throughput flow cytometry platform that uses a peristaltic pump in combination with an autosampler (Fig. 1) to boost endpoint assay performance to rates in excess of one sample per second<sup>2,3</sup>. As the sampling probe of the autosampler moves from one well to the next of a multiwell microplate, a peristaltic pump sequentially aspirates sample particle suspensions from each well. Between wells, the continuously running pump draws a bubble of air into the sample line to generate a tandem series of bubble-separated samples for delivery to the flow cytometer. As described in the protocol below, a sampling rate of 40 samples per minute is routinely used, as the most practical for producing robust assay results while requiring a volume of only 2  $\mu$ l to be aspirated from the assay well. We anticipate the application of high-throughput flow cytometry to many assays compatible with flow cytometry analysis.

### A fluorescence-based high-throughput flow cytometry assay to detect FPR ligands

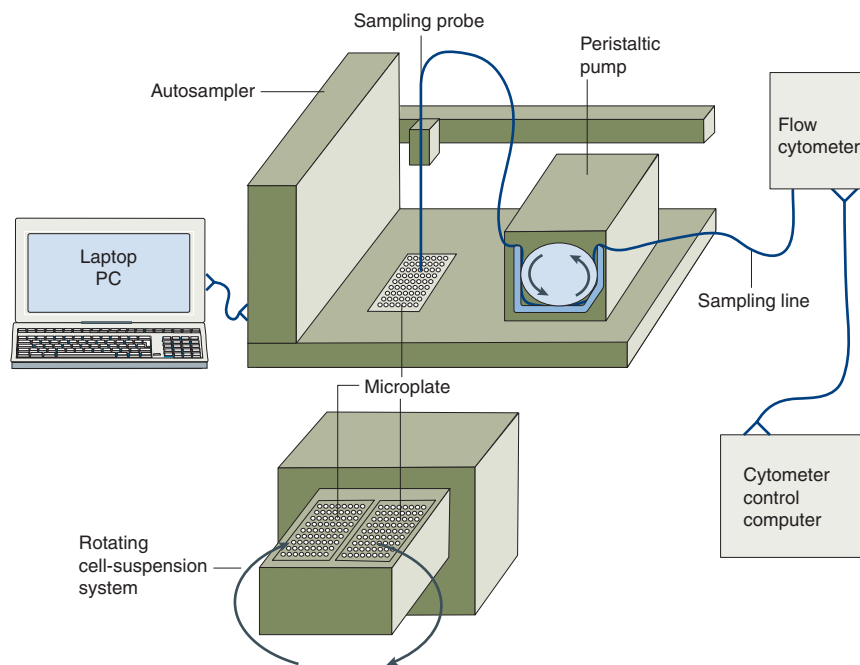
The FPR, a prototypic G protein-coupled receptor in the chemottractant receptor superfamily, has been linked to the mediation of inflammatory responses of clinical significance<sup>4,5</sup>. Known FPR agonists include *N*-formylated peptides of bacterial and mitochondrial origin<sup>6–9</sup>, the glucocorticoid-regulated protein annexin I (lipocortin I)<sup>10</sup> and HIV-1 envelope proteins<sup>11–13</sup>. We describe below a simple, robust assay for detecting previously unknown FPR ligands

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by high-throughput flow cytometry. The assay detects active structures on the basis of their ability to block the binding of a high-affinity ligand to the FPR. As ligand binding is measured directly, the assay is likely to detect active compounds independently of potential complexities in the patterns of the physiological responses of cells. For example, it should detect molecules regardless of whether they act as agonists or antagonists or mediate full, partial or selective (e.g., signaling pathway specific) activity. This is an advantage over high-throughput functional response assays (e.g., those measuring intracellular  $\text{Ca}^{2+}$  flux) that are typically designed to detect exclusively agonists or antagonists and are less sensitive in detecting compounds with selective or partial response activity.

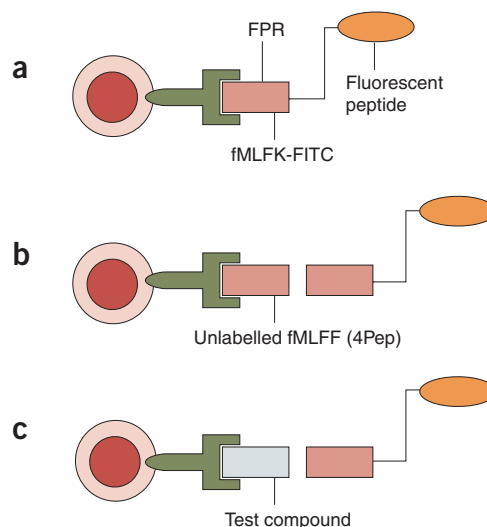
It is worthwhile to compare fluorescence polarization and flow cytometry for high-throughput molecular ligand-binding assays. In fluorescence polarization assays, binding of ligand to membrane-bound receptors is detected as a change in the molecular rotation rate of the fluorescent probe. Because the 'rotational relaxation' time (the time required for unbound molecules to transition from polarized to random orientation) must be comparable to the fluorescence lifetime of the probe, fluorescence polarization is particularly appropriate for small fluorescent molecules. Moreover, for the polarization signal to sense a binding event, a substantial fraction of the probe must be bound to the target. This typically requires that the target concentration be greater than the dissociation affinity constant ( $K_d$ ). By contrast, for flow cytometry with the target on the particle, the actual target concentration can be picomolar and binding is detected for probe concentrations in the vicinity of the  $K_d$ . As a result, the quantities of protein target required for polarization analysis can be thousands of times larger than for flow cytometry. Moreover, the discrimination of free and particle-bound assemblies in flow cytometry does not require a small molecule, as does fluorescence polarization. We have resolved bound ligands in homogeneous conditions with concentrations of free ligands up to hundreds of nanomolar concentration.

The FPR assay measures the ability of test compounds to compete with a high-affinity fluorescent ligand, fMLFK-FITC, for binding to cell membrane FPR (Fig. 2a–c). The assay response range is defined by replicate control wells containing unlabeled fMLFF blocking peptide (4Pep; Fig. 2b) or buffer alone (Fig. 2a). The assay is homogeneous in that cells, compounds and fluorescent peptide are added in sequence and the wells are subsequently analyzed without intervening wash steps. Additions to wells are in sequence as follows: first, test compounds and control reagents; second, cells; third (after incubation for 30 min at 4 °C), fluorescent peptide ligand. After an additional incubation for 30 min (or longer) at 4 °C, plates are immediately analyzed by flow cytometry with the HyperCyt platform. The HyperCyt autosampler is programmed to move the sample uptake probe sequentially into each microplate well while the peristaltic pump that mediates sample



**Figure 1** | The HyperCyt platform for high-throughput flow cytometry.

aspiration runs continuously at 15 r.p.m. The probe is immersed for about 1 s to take up about 2  $\mu\text{l}$  of sample and moves to the next well within about 0.5 s while aspirating an air bubble. The bubble-separated stream of samples is analyzed continuously by the flow cytometer, requiring a total analysis time of about 2.5 min for 96 wells and about 10 min for 384. Time is used as a data-acquisition parameter by which to allow resolution of the discrete clusters of events representing the contents of each well. The time-resolved fluorescence and light-scatter data are saved in a standard flow cytometry data format (flow cytometry standard 2.0 or 3.0 format) and are subsequently analyzed with specialized software



**Figure 2** | Assay design. (a) Unblocked negative control cell with bound fluorescent peptide. (b) Blocked positive control cell with fluorescent ligand displaced by 4Pep peptide. (c) Displacement of fluorescent ligand by test compound.

(IDLeQuery) that allows rapid binning, analysis and export to a spreadsheet of data from each well.

The procedure described below is tailored for single-point screens (only one test compound concentration evaluated) in 96-well plates. The 80 wells in columns 2–11 contain test compounds, whereas columns 1 and 12 are reserved for replicate negative and positive controls, respectively. Dose-response analyses are typically done by arraying serial compound dilutions in triplicate in the central 80-well region. Screens are now being done routinely in 384-well plates using similar assay layout patterns and reagent volumes. We have focused here on the 96-well format because of the lower demand for full automation to achieve efficient plate preparation.

### FPR screening applications

The FPR ligand-binding assay has been used in two recent screening studies<sup>14,15</sup>. The first study documented assay performance characteristics (sensitivity and linearity), the visual ‘signature’ of a ‘hit’ in the time-resolved graphical data display, and types of assay interference encountered in screening of the Prestwick Chemical Library, a collection of off-patent drugs and alkaloids<sup>14</sup>. The second study documented the combination of the FPR assay with a preliminary round of virtual screening *in silico* to enable the discovery of a series of small-molecule ‘chemotypes’ (compounds grouped into sets by virtue of common chemical structure or scaffold) with more potent FPR activity than any previously reported non-cyclosporin drugs<sup>15</sup>. We recently extended this integrated virtual and biomolecular screening approach to identify the first high-affinity and highly selective nonsteroidal agonist<sup>16</sup> for GPR30, a previously unknown, intracellular, estrogen-binding, G protein-coupled receptor<sup>17</sup>.

If reagents with appropriate characteristics are available or can be developed, we expect that the assay approach described below will be generally adaptable to a wide range of receptors. First, there must be a cell or particle that expresses functional target receptors, preferably at densities averaging  $5 \times 10^4$  or more receptors per cell. Our experience is that fluorescent reagents with a  $K_d$  of up to at least 100 nM (assuming ligand and fluorophore are equimolar)

may be used in homogeneous ‘no-wash’ flow cytometry binding assays with good signal-to-background characteristics. For purposes of assay flexibility and reagent cost savings, we prefer to use fluorescent ligands with a  $K_d$  in the low nanomolar range. For the positive control, an unlabeled ligand is used at a concentration 100-fold higher than the inhibition constant ( $K_i$ ) with which it blocks binding of labeled peptide to the target receptor. Important considerations and potential limitations of measurements made with the HyperCyt high-throughput flow cytometry platform are discussed in the ANTICIPATED RESULTS section below.

### Potential limitations

Cell carryover from one well to the next typically ranges between 0% and 2% when the ‘no-wash’ sampling format described above is used. That was documented in the original characterization of the HyperCyt platform<sup>2</sup> and has been confirmed repeatedly in subsequent studies<sup>3,14</sup>. The fluorescent ligand-binding assay described below is insensitive to contaminating cells representing that proportion of the total cell population. Also, a median fluorescence intensity (MFI) statistic is used to characterize the response of the cell population to minimize the potential skewing effects of a small percentage of contaminating cells with widely divergent responses. However, that carryover might prove problematic if evaluation of FPR in cells that represent only a small fraction of total cells in the assay is attempted.

Because the HyperCyt platform uses a relatively high flow rate (about  $2 \mu\text{l s}^{-1}$ ), a slight broadening of the fluorescence intensity distribution of a cell population should be expected relative to what is observed for cells analyzed manually at typically lower flow rates of  $1 \mu\text{l s}^{-1}$  or less. This does not affect accurate calculation of the MFI statistic for a cell population that expresses relatively uniform amounts of the target receptor<sup>3</sup>. However, the potential for systematic errors should be evaluated if a response threshold type of statistic (e.g., percent positive) or a parametric statistic (e.g., mean rather than median fluorescence intensity) is to be used for quantifying ligand binding, particularly in cells with heterogeneous amounts of receptor expression.

## MATERIALS

### REAGENTS

- Test compounds
- Peptide dilution buffer (see REAGENT SETUP)
- Peptides (see REAGENT SETUP for preparation of peptide solutions): fluorescein isothiocyanate-labeled *N*-formyl-methionine-leucine-phenylalanine-lysine peptide (fMLFK-FITC; Bachem, special synthesis) and unlabeled *N*-formyl-methionine-leucine-phenylalanine-phenylalanine peptide (fMLFF; Sigma, cat. no. F2009)
- CytoPlex beads (Duke Scientific, cat. no. FM4CR08)
- Contrad70 (Fisher, cat. no. 04355), diluted with water to 2.5% (vol/vol) for use
- Cells (see REAGENT SETUP)
- Tissue culture medium (see REAGENT SETUP)

### EQUIPMENT

- Compound dilution plate: 96-well conical-bottomed PVC plates (Costar, cat. no. 2897).
- Assay plate: 96-well Greiner Imp@ct plates (cat. no. 673101) ▲ **CRITICAL** Greiner Imp@ct plates are the only commercial 96-well product we have found so far that will allow efficient sampling of volumes of  $15 \mu\text{l}$  or less ( $8 \mu\text{l}$  minimum) with the HyperCyt platform.

- T175 plastic tissue culture flasks
- MAP-C dispensing instrument (Titertek), used for dispensing cells and reagents into microplate wells
- Flow cytometer (see EQUIPMENT SETUP)
- HyperCyt instrument (see EQUIPMENT SETUP)
- Computer with Microsoft Windows 2000 or Windows XP, 512 MB or more of RAM, 500 MB or more of free disk space, and a legacy RS232 serial port or a USB port

### REAGENT SETUP

**Cells** U937 cells transfected with a human FPR internalization-deficient mutant (DeltaST) are used for this assay. Cells are passaged twice weekly in 100-ml suspensions in T175 plastic tissue culture flasks. U937 cells with wild-type FPR have also been used successfully<sup>3</sup>. Cells expressing  $1 \times 10^5$  to  $4 \times 10^5$  FPRs per cell are used in assays. FPR expression is determined by incubation of DeltaST cells with 10 nM fMLFK-FITC for 30 min at  $4^\circ\text{C}$  in the presence and absence of  $1 \mu\text{M}$  fMLFF. Specific peptide binding is computed as the difference in cell MFI in unblocked versus blocked (fMLFF present) conditions; this is ‘transformed’ to the estimated number of receptors (ligand-binding sites) per cell by comparison with an MFI standard curve generated with Quantum FITC.MESF calibration beads (Bangs Labs, cat. no. 825B).



**Peptide dilution buffer (PDB)** Prepare fresh weekly a solution of 110 mM NaCl (Sigma, cat. no. S-9625), 30 mM HEPES (Mediatech, cat. no. MT-25-060-CI), 10 mM KCl (Sigma, cat. no. P-3911), 1 mM MgCl<sub>2</sub> (Sigma, cat. no. M-8266), 10 mM glucose (Sigma, cat. no. G-7528) and 0.1% bovine serum albumin (BSA; Sigma, cat. no. A-3059). Store at 4 °C.

**Peptide solutions** Peptides are stored as aliquots of 1 mM in 100% DMSO at –20 °C. Aliquots are freshly thawed and diluted to final specified concentrations (1% or less DMSO) on each day of assay.

**Tissue culture medium** RPMI-1640 medium (Mediatech, cat. no. MT-10-040-CVMP) supplemented with 10% fetal bovine serum (US Biotechnologies, cat. no. FBU 3195), 2 mM l-glutamine–10 U ml<sup>-1</sup> penicillin–10 µg ml<sup>-1</sup> streptomycin (Mediatech, cat. no. MT-30-009-CI), 10 mM HEPES (Mediatech, cat. no. MT-25-060-CI), 4 µg ml<sup>-1</sup> ciprofloxacin (Bayer Pharmaceuticals) and 500 µg ml<sup>-1</sup> geneticin (Mediatech, cat. no. MT-30-005-CR), heat-inactivated for 30 min at 54 °C. May be stored at 4 °C for up to 3 weeks.

**EQUIPMENT SETUP**

**Flow cytometer** FACScan, FACSCalibur (both BD Biosciences) and Cyan (Dako) flow cytometers have been successfully used in FPR screening applications. Only a single argon laser (488 nm) is required for the basic procedure described below. However, a cytometer equipped with a second red diode laser (635 nm) will be useful in troubleshooting problems of innate test compound fluorescence, as described in TROUBLESHOOTING.

**HyperCyt instrument** The HyperCyt platform now in use is a custom instrument we built at the University of New Mexico. It includes an autosampler, a peristaltic pump, 25-G stainless steel tubing for the sample uptake probe and PVC tubing for sample transport to the flow cytometer. There is also a separate piece of equipment for maintaining cells and particles in suspension, the micro-assay rotational suspension (MARS) system. It consists of a rotisserie motor that rotates a microplate carrier to periodically invert the microplates (4 r.p.m.). A detailed list of parts and assembly instructions are available on our web site (<http://nmmlsc.health.unm.edu/HyperCyt>) or by contacting the corresponding author directly.

**Software for HyperCyt:** The software is written in IDL programming language (RSI) and requires the use of the IDL 6.2 Virtual Machine for operation. The Virtual Machine for Microsoft Windows XP or Windows 2000 is available as a free download from the RSI web site (<http://www.rsinc.com/download/>). Two programs are needed to run the HyperCyt platform: HyperSip controls the HyperCyt autosampler; IDLQuery is used to analyze time-resolved data

files stored in flow cytometry standard 2.0 or 3.0 format (all commercial flow cytometry platforms store data in one of those formats). Compiled versions of HyperSip and IDLQuery that run with the IDL 6.2 Virtual Machine are available through our web site (<http://nmmlsc.health.unm.edu/HyperCyt/SoftwareRequest.php>) or by contacting the corresponding author directly. Also available are software installation and operating instructions.

**HyperCyt setup:** Peristaltic pump speed: We routinely run the pump at 15 r.p.m. to result in a flow rate of about 2 µl s<sup>-1</sup>. Faster or slower is typically suboptimal and can also result in increased particle carryover.

Peristaltic pump clamping pressure: when adjusted properly, there should be uniform air bubbles on both sides of the pump. If the bubbles are broken up on the flow cytometer side of the pump, the clamp has probably been tightened too much. Finding just the right tension will sometimes take a bit of time and patience. Once set, clamping pressure typically remains stable over the course of a day without requiring operator intervention.

Tubing: PVC tubing as recommended by manufacturer ▲ **CRITICAL** No other type of tubing should be substituted for the recommended PVC tubing. All other tubing types suitable for peristaltic pumping that we have tested produce large amounts of carryover. It is also important to minimize any dead volume in the junction where the PVC sample tubing adjoins the flow cytometer sample uptake tube. This is a potential ‘particle trap’ that can lead to significant increases in carryover. This is where most particle carryover problems originate. We recommend slipping the PVC tubing directly over the flow cytometer sample uptake tube. If you try to use Upchurch-type fittings to make connections, dead volume is an important issue that must be addressed carefully. The claim of ‘zero’ dead volume for these fittings is irrelevant if tubing ends do not join properly. If the ends are not perfectly square, if they are not in close physical contact or if there are any flaws in the end of either tube, carryover is exacerbated. Also, any additional tubing junctions you might introduce in the sample line are likely to cause increased particle carryover.

Maintenance routine: before the first sampling run and between subsequent runs, we move the sampling probe to a wash station containing PDB while the peristaltic pump continues to run. The 0.1% BSA in the PDB is an important surfactant that helps flush particles from the sample line. We have found that the presence of at least 0.1% BSA (or some other equivalent protein) in all solutions helps minimize particle carryover. We also routinely run detergent (2.5% Contrad70) through the tubing at the beginning and end of each day.

**PROCEDURE**

**Preparation of the sample**

- 1| Pipet 1 µl of each test compound stock preparation into 49 µl PDB (columns 2–11 of a conical-bottomed 96-well compound dilution plate).
- 2| Pipet 5 µl of diluted test compounds into test wells of a separate 96-well assay plate (columns 2–11). Use three or more aspirate-dispense pipetting cycles to mix before transfer.
- 3| Pipet 5 µl blocking peptide (450 nM fMLFF in 2% (vol/vol) DMSO in PDB) into positive control wells (column 12).
- 4| Pipet 5 µl of 2% (vol/vol) DMSO in PDB into negative control wells (column 1). Alternatively, red fluorescent CytoPlex beads (1 × 10<sup>8</sup> beads per ml in 2% (vol/vol) DMSO in PDB) may be added instead to these wells as an aid to ensure accurate row-by-row registration between observed clusters of events and source wells<sup>14</sup>.
- 5| Dispense 5 µl DeltaST cells into each well. Suspend cells at a density of 1 × 10<sup>7</sup> cells per ml in PDB and prechill on ice before addition. Alternatively, dispensing is done manually. With this option, the plate should be kept cold (e.g., placed on ice) and a manual pipetting mix step should be considered at the end of the dispense cycle.
 

▲ **CRITICAL STEP** Step 5 should be done quickly (in less than 3 min) to minimize warming and cell settling. A Titertek MAP-C dispensing instrument is used to automate and speed this step.
- 6| Incubate plate at 4 °C for 30 min, periodically inverting the plate with a custom-built MARS system rotating at 4 r.p.m. to maintain cells in suspension. Alternatively, incubation can be done using static conditions without continuous rotation of the plate. A pipetting mix step to resuspend cells should be added at the end of the incubation if the latter option is selected.

▲ **CRITICAL STEP** Specialized 96-well plates must be used that allow ‘hanging-drop’ retention of cell suspensions in wells when plates are inverted. Greiner Imp@ct plates, originally designed for crystallization procedures, are suitable for this step.

7| Dispense 5 μl fMLFK-FITC (4.5 nM in PDB) into each well.

▲ **CRITICAL STEP** Follow the same critical step and alternative option considerations as in Step 5.

8| Incubate plate at 4 °C for 30 min with rotation in the MARS.

▲ **CRITICAL STEP** Follow the same critical step and alternative option considerations as in Step 6.

■ **PAUSE POINT** This incubation step can be extended to overnight at 4 °C.

### Flow cytometry analysis of the sample

9| Move plate to the HyperCyt autosampler deck for high-throughput sampling and flow cytometry analysis.

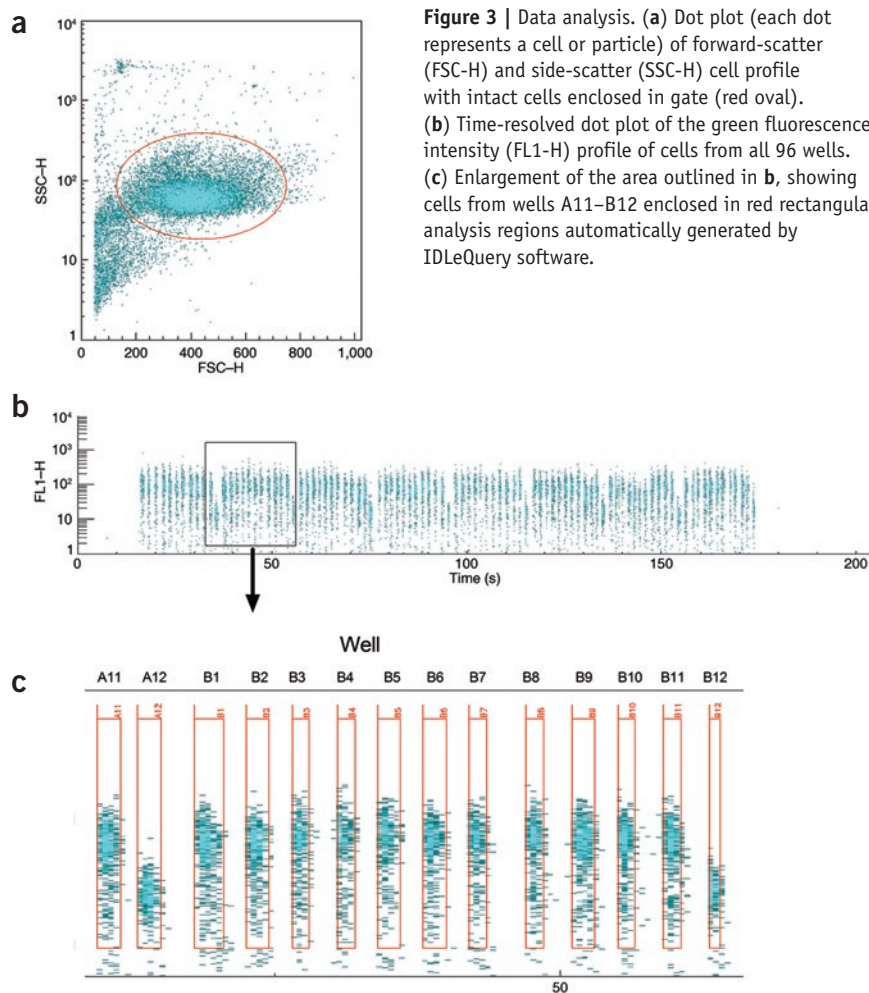
10| Under the control of HyperSip software, the autosampler probe moves from well to well, sampling from each for 1 s and moving to the next well without an intervening probe wash step. The peristaltic pump that mediates liquid transfer runs continuously during sampling of the entire plate so that an air bubble is aspirated as the probe moves from one well to the next.

11| The series of 96 air bubble-separated samples is delivered to the flow cytometer and is analyzed as a single data file that is saved in flow cytometry standard 2.0 or 3.0 format.

▲ **CRITICAL STEP** Time must be recorded as an analysis parameter for correlation with cell-scatter and fluorescence parameters. To optimally resolve clusters of events, you must acquire data with a time resolution of 100 ms. That means that you need a time ‘channel’ of 100 ms so that events are binned at intervals of 100 ms. The time parameter in software from analog data-acquisition instruments (e.g., FACScan and FACSCalibur from BD Biosciences, Elite and FC500 from Beckman Coulter) is typically 10 bits, which means that you are allowed only 1,024 time channels for a single data acquisition session; when you reach the 1,024th channel, data acquisition automatically stops and the data file is stored. At a resolution of 100 ms, you can only acquire data for a total of 102.4 seconds. We use a time channel with a resolution of 200 ms with FACScan or FACSCalibur data acquisition (suboptimal but workable), which allows an acquisition time of 204.8 s, a little more than is required for a 96-well plate at 40 samples per minute.

12| Analyze data with IDLeQuery software. In a two-parameter plot of forward light scatter (FSC) versus side light scatter (SSC), an electronic gate is constructed enclosing healthy cells and excluding apparently dead cells (low FSC) and debris (Fig. 3a). A plot of time versus log green fluorescence intensity ( $530 \pm 15$  nm) allows resolution of the 96 clusters of events corresponding to cells sampled from each of the 96 wells (Fig. 3b). An analysis gate is automatically placed around each cluster for use in determining the corresponding MFI, a measure of the amount of fluorescent ligand bound to cellular FPR in the presence of each test compound. The fluorescence intensity data are exported to a Microsoft Excel spreadsheet template that automatically makes the required calculations.

Alternatively, other commercially available software for data analysis can be used. This option will require more time because of the need to manually enclose each time-resolved event cluster in a separate analysis gate. IDLeQuery software has



**Figure 3** | Data analysis. (a) Dot plot (each dot represents a cell or particle) of forward-scatter (FSC-H) and side-scatter (SSC-H) cell profile with intact cells enclosed in gate (red oval). (b) Time-resolved dot plot of the green fluorescence intensity (FL1-H) profile of cells from all 96 wells. (c) Enlargement of the area outlined in b, showing cells from wells A11–B12 enclosed in red rectangular analysis regions automatically generated by IDLeQuery software.

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a built-in cluster-detection algorithm to allow automated generation and placement of the 96 analysis gates. We are unaware of any commercial software now available that will do this.

**13|** Calculate assay response using the following equations:

Assay response:

$$\% \text{ inhibition} = 100 \times \{1 - [(MfiTest - MfiPCAVG) / (MfiNCAVG - MfiPCAVG)]\}$$

where MfiTest, MfiPCAVG and MfiNCAVG are the MFI of individual wells containing test compounds, the average MFI of positive control wells and the average MFI of negative control wells, respectively.

**14|** Calculate assay quality statistics:

$$Z' = 1 - \{[(3 \times MfiPCSD) + (3 \times MfiNCSD)] / (MfiNCAVG - MfiPCAVG)\}$$

where MfiNCSD and MfiPCSD are the standard deviation of MFI in negative and positive control wells, respectively.

$$S/N = (MfiNCAVG - MfiPCAVG) / MfiPCSD$$

$$S/B = MfiNCAVG / MfiPCAVG$$

where S/N is signal/noise and S/B is signal/background.

### ● TIMING

#### Timeline (per plate):

Steps 1–4: 3 min (automated pipetting by robot) or 10 min (manual pipetting)

Step 5: 2 min (automated dispense) or 10 min (manual dispense and mix)

Step 6: 30 min (MARS incubation) or 35 min (static incubation and manual mix)

Step 7: 2 min (automated dispense) or 10 min (manual dispense and mix)

Step 8: 45 min (MARS incubation) or 50 min (static incubation and manual mix)

Steps 9–11: 3 min

Steps 12–14: 2 min (IDLeQuery software) or 20 min (commercial flow cytometry software)

Total assay time may range from 1.5 h to 2.5 h depending on the options selected.

### ? TROUBLESHOOTING

See **Table 1**.

**TABLE 1 |** Troubleshooting table.

PROBLEM	POSSIBLE REASON	SOLUTION
Small difference in fluorescence intensity between positive and negative controls	Loss of receptor expression	Stain cells with fluorescent ligand and sort in flow cytometer to isolate and propagate cells with high expression
	Concentration of control blocking ligand (fMLFF) is too low	Increase blocking ligand concentration
	Concentration of fluorescent ligand (fMLFK-FITC) is too low	Increase fluorescent ligand concentration, but never more than 2× the $K_d$
Cell fluorescence intensity in a test well is much greater than that of negative control cells	Innate fluorescence of test compound at the 488-nm wavelength used to excite probe fluorescence	Use ligand probe tagged with a dye that has a different fluorescence excitation spectrum
Cell fluorescence intensity is consistently low in wells sampled immediately after positive control wells	Concentration of control blocking ligand (fMLFF) is so high that fluid carryover affects 'downstream' wells	Decrease blocking ligand concentration or use a control blocking ligand with a lower $K_d$
Overlap is observed between clusters of events during post-acquisition analysis of stored flow cytometry data	Resolution of the data acquisition time parameter is suboptimal (>200 ms)	Acquire data at a resolution of 200 ms or optimally 100 ms (may require analysis of fewer wells per round of sampling)
Number of particles aspirated from each well is less than expected	Sample line is clogged	Verify clogging as the problem by replacing the sampling tube set (probe and PVC tubing) with a spare pre-tested set; run 2.5% Contrad70 through the clogged tubing set
	Particles have settled in the wells	Resuspend particles by pipetting or by inverting the plate for 1–2 min



## ANTICIPATED RESULTS

Approximately 2  $\mu$ l is aspirated from each well during a single round of sampling by the HyperCyt platform and a plate is processed in less than 2.5 min. Each resulting cluster of events should consist of  $1 \times 10^3$  to  $3 \times 10^3$  cells. Because of the relatively small volume and time requirements for a single round, we typically do a second round of sampling immediately after the first and average the results.

At a time resolution of 200 ms (each time ‘channel’ contains data collected over a time interval of 200 ms), you should get detectable gaps between samples or minimal overlap of sample tails, and the peaks should be uniform in size and shape. This is best visualized in a graphed display of time versus number of events. We typically get better temporal separation for cell samples than for bead samples. Cells tend to migrate to the front of each sample ‘slug’ as they move through the sample tubing, whereas beads do not. Expect a 10–20% coefficient of variation for particle counts from identical samples, where coefficient of variation =  $100 \times (\text{particle count s.d.} / \text{particle count average})$ .

We have also adapted this assay approach for work with intracellular receptors<sup>17</sup>. This requires the use of a saponin-based permeabilization buffer to allow entry of the fluorescent receptor ligand into the cell cytoplasm (10 min at 37 °C) and a subsequent plate-washing step to remove the saponin before HyperCyt sampling and flow cytometry analysis.

## Assay throughput

When dilution and plate-to-plate transfer of compounds (steps 1–4) have been done manually, throughput has typically been four to six plates per day (384–576 wells). Using commercial automated robots for these plate preparation functions, together with ‘reformatting’ of samples into 384-well plates, it has been very easy to initially process a similar number of plates daily for an increase in total throughput of fourfold or more (1,536–2,304 wells). Further increases in throughput are expected with increasing experience and automation.

## Quality-control statistics

The  $Z'$  factor is our preferred measure of screening assay quality that reflects both assay signal dynamic range and data variation associated with the signal measurements<sup>18</sup>. An assay with  $Z'$  scores ranging from 0.5 to 1.0 is considered excellent, whereas a  $Z'$  score of 0 reflects a ‘yes/no’ class of assay<sup>18</sup>. In a series of 268 96-well microplates analyzed in our published FPR assay studies<sup>14,15</sup>,  $Z' = 0.62 \pm 0.16$  (mean  $\pm$  s.d.). Our experience is that  $Z'$  scores of 0.3 or better are indicative of assays that detect ‘hits’ (70% inhibition or more) with high reproducibility. Anticipated values of the other quality control statistics can be inferred from a 32-plate subset in which the following results were obtained: S/N =  $37.3 \pm 24.9$  and S/B =  $4.1 \pm 1.2$ .

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