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Development Of A Multiplex High Throughput Screening Assay Cascade For Evaluating Receptor Tyrosine Kinase Binding And Activation.

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ABSTRACT

Receptors and other cell surface proteins are attractive targets for biologics drug discovery. Screening for protein based therapeutics that inhibit ligand binding and/or downstream activation events in living cells represents a powerful approach for the discovery of novel drugs. We have developed a series of live-cell based screening assays for the HTFC® Screening System that addresses multiple steps in these ligand-receptor driven pathways. For these studies, interactions between the EphA2 receptor and its ligands were used as a model system. EphA2 is a receptor tyrosine kinase and is known to be involved in growth, migration and invasion of cancer cells in culture, and can affect tumor growth, invasiveness, angiogenesis and metastasis in vivo. Ephrin A1 and Ephrin A3 are natural ligands that bind to the EphA2 receptor with high affinity. Ligand binding induces tyrosine phosphorylation and activates receptor internalization and degradation. Using multiple cell lines that utilize this receptor ligand system, sequential screening assays were developed that assess ligand-receptor binding, receptor internalization, and intracellular tyrosine phosphorylation. Using this high throughput flow cytometry screening platform, it was possible to evaluate multiple cell lines in each well of the screening plates. The results demonstrate that high throughput flow cytometry can be leveraged to improve productivity in the drug discovery process.

INTRODUCTION

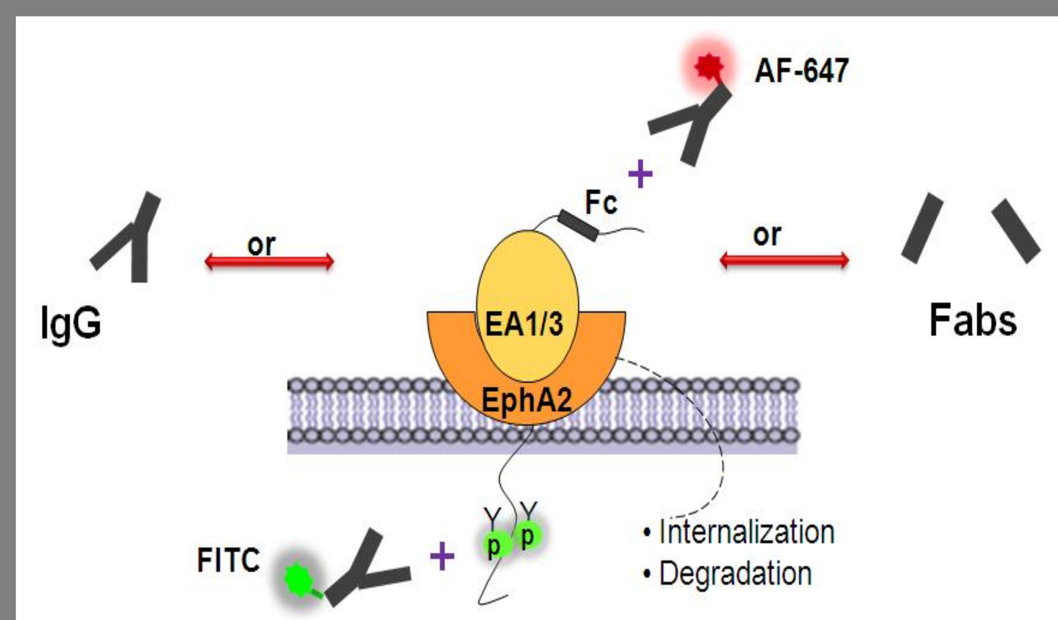
High Throughput Flow Cytometry technology is instrumental in receptor screening assays (1). In protein therapeutic projects, whole-cell binding assays can be applied to hybridoma and phage display library screens, as well as secondary screening assays. It can be utilized to screen monoclonal antibodies (Fab fragments and IgGs), as well as peptides and immuno-conjugates that directly bind to a target antigen. In this poster we present a suite of multiplexed antibody screening assays that include whole-cell binding, receptor phosphorylation and receptor internalization.

EphA2 is a receptor tyrosine kinase and an attractive therapeutic target. It is known to be involved in growth, migration and invasion of cancer cells in culture, as well as tumor growth, invasiveness, angiogenesis and metastasis *in vivo* (2).

Ephrin A1 and Ephrin A3 are natural ligands that bind to EphA2 with high affinity. Ligand or antibody binding induces tyrosine phosphorylation and activates receptor internalization and degradation, making this system a convenient model to demonstrate multiplexing capability. Both endpoints (binding and receptor phosphorylation) share the same exposure time with the ligand.

The HTFC® Screening System is a benchtop screening system that makes multiple, highly sensitive, single cell fluorescence measurements on thousands of cells per second. This ability to distinguish several fluorescent tags simultaneously allowed us to tag two cells lines—PC3 and A549 with fluorescent probes and measure EphA2 binding and activation differentially. Fully optimized, the HTFC system takes less that 3 minutes to read a 96 well plate, and 12 minutes to read a 384 well plate. Flow cytometry-based detection in the system demonstrates higher sensitivity and a wider linear detection range than conventional plate readers. Sample size is typically around 2ul, but can be as much as needed. Total assay volume can range from less than 10ul for screening to as much as 1ml for rare events analysis.

ASSAY CONCEPT



EphA2 Receptor Activation

Ephrin A1 and Ephrin A3 ligand or antibody binding induces tyrosine phosphorylation and activates receptor internalization and degradation. Both binding and internalization were detected using an Fc-conjugated ligand conjugated to AlexaFlour 647. EphA2 phosphorylation was with a FITC labeled anti-phosphotyrosine antibody.

SCREENING CASCADE

Receptor-ligand Binding

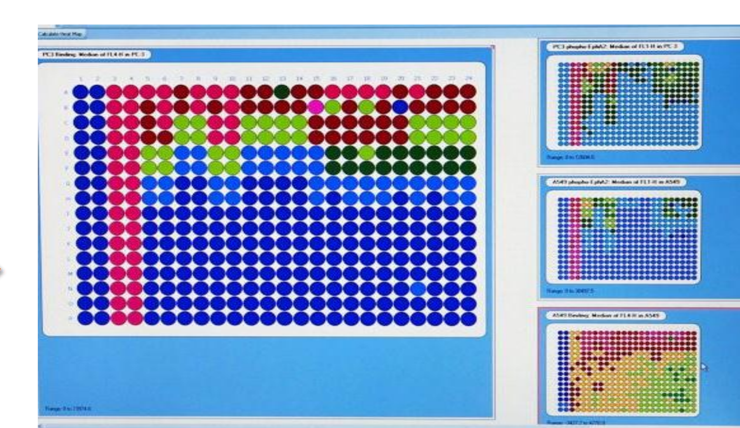
Tyrosine Kinase Activity

Receptor Internalization

HTFC SCREENING SYSTEM AND DATA ANALYSIS

HTFC® Screening System

- High throughput suspension assays.
- 2 lasers and 6 detectors provide 4-color fluorescent and 2 light scatter measurements
- Analyze thousands of cells/second
- Sample a 96 well plate in 3 minutes or a 384 well plate in 12 min.
- Sample 1-2ul/well with no sample waste



IntelliCyt Data Manager (iDM)

- Scalable server-based informatics platform
- Manage data from multiple users and multiple systems
- Identify and color code populations from wells
- Apply multicolor gating to generate statistics and heat maps
- Export data in FCS and Excel formats

CONCLUSIONS

In this study we demonstrated the power of the HTFC Screening System in multiplexing a cell based assay. We developed a screening cascade based on an Ephrin receptor activation model that highlights some significant features and uses of this technology, including:

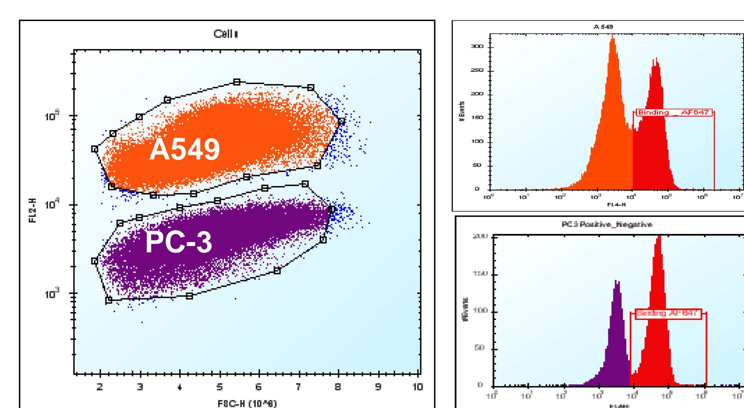
- Multiplexing
  - Through the use of color coding cellular stains, binding and activation of different cell lines expressing the receptor can be tested simultaneously in each well of the screening plate.
- Biological profiling
  - Using the screening scenario described, multiple aspects of a receptor signaling cascade can be performed either simultaneously or sequentially on the HTFC Screening System.
- Productivity
  - The throughput of the HTFC Screening System, combined with the ability to examine multiple cell lines and endpoints per well (multiplexing) can provide significant increases in productivity.

REFERENCES

1. Sklar, L.A., Edwards, B.S., at al; Flow cytometric analysis of ligand-receptor interactions and molecular assemblies, *Annual Rev. Biophys. Biomol. Struct.*, Vol. 31, pp. 97–119, 2002
2. Elena B. Pasquale, Eph receptors and ephrins in cancer: bidirectional signaling and beyond, *Nature*, Vol. 10, 165-180, 2010.
3. Christopher B. Black, Thomas D. Duensing, Linda S. Trinkle, and R. Terry Dunlay: Cell-Based Screening Using High-Throughput Flow Cytometry. ASSAY and Drug Development Technologies, 2011

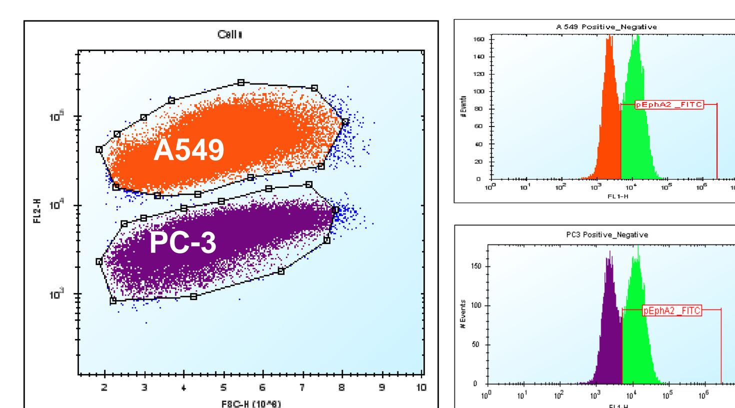
SCREENING ASSAY DATA

Receptor-ligand Binding



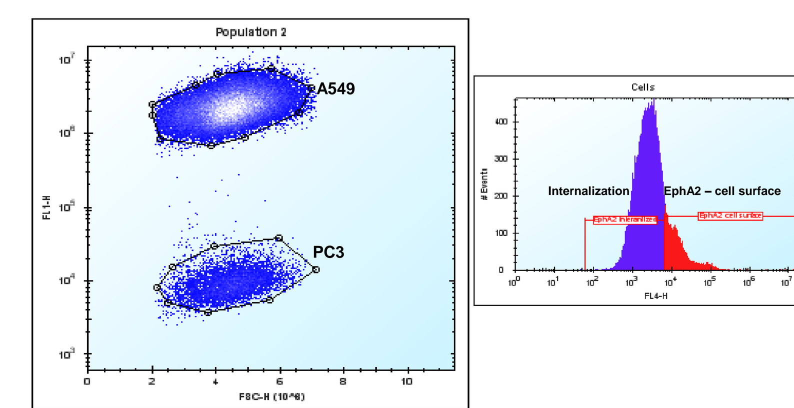
EphA2 Receptor Binding (red peak)

Tyrosine Kinase Activity

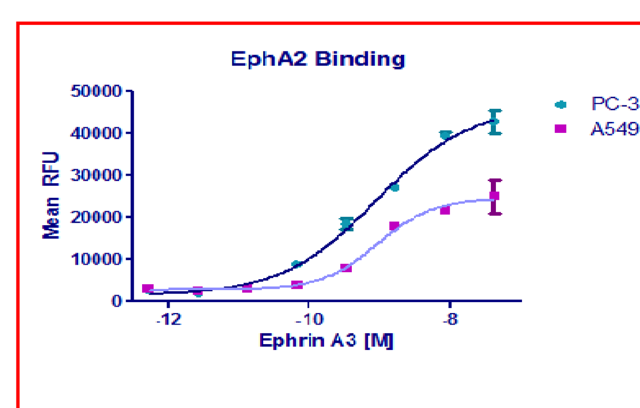


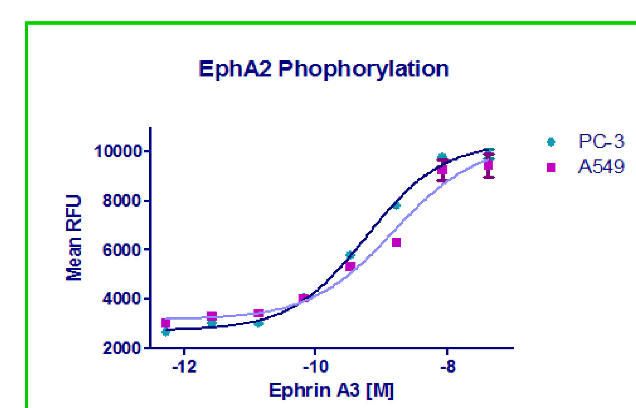
Tyrosine Phosphorylation (green peak)

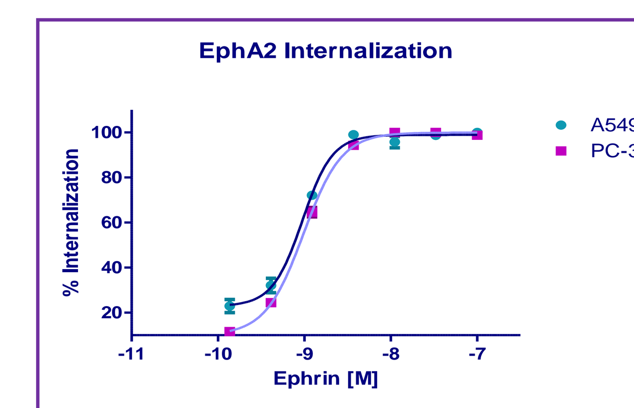
Receptor Internalization



EphA2 Receptor Internalization (purple peak)







Dose response curves were generated for each cell line

PC-3 cells were left unstained (purple), while A549 cells (orange) were labeled with Mito Tracker Orange dye. Receptor binding was measured on the FL4 - channel (red) and EphA2 phosphorylation on the FL1 - channel (green).

The two cell lines were co-incubated in a 384 well plate with hrEphrin A3/Fc ligand or mAbs. After fixing and permeabilizing, the cells were incubated with the goat anti-human AF 647(Life Technologies) and anti- 4G10/FITC (Millipore).

Using a multicolor gating method the cell populations were separated and antibody or ligand binding and EphA2 receptor phosphorylation was measured for each cell line.

PC3 cells remained unstained and A549 cells were labeled with Cell Tracker Green dye (Life Technologies).
Both cell lines were co-incubated with Ephrin A3 ligand or IgGs serial dilutions for 4hr, followed by the ligand treatment at 1ug/ml. EphA2 receptor remained on the cell surface was detected with anti-human AF 647 (red gate). The purple gate measures EphA2 receptor internalization.