Combining FLIPR and Transfluor: A Novel Assay for the Sequential Analysis of Calcium Flux and Receptor Desensitization

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Abstract

Two of the most widely used cell-based techniques for GPCR function are: 1) monitoring intracellular calcium with fluorescent dyes using FLIPR® and 2) image analysis of beta arrestin-mediated GPCR desensitization using the Transfluor® assay. Since the studies of these signaling pathways are complementary, we have developed a novel, sequential and homogenous assay for FLIPR and Transfluor analysis on the same cells. A novel calcium dye (red emission) and FLIPR are used in combination with a Transfluor-enabled cell line and a new imager to analyze calcium signaling and receptor desensitization in the same cells. Experiments with the Angiotensin receptor demonstrate the compatibility and robustness of the two assays and the equivalent pharmacology as measured independently. In addition, the synergy between the two analysis technologies, with respect to the Angiotensin receptor, can be used to reveal ligands that signal and do not desensitize (NDLs) as well as inhibitors of the desensitization.

Introduction

GPCRs are proven targets in a wide range of major disorders. The Human Genome Project identified over 1,000 GPCRs, 200 of which are orphan GPCRs whose ligands have yet to be identified. The physiological relevance of cell-based assays has made them a crucial tool for the study of G-protein-coupled receptors (GPCR) function. Molecular Devices offers complimentary tools for GPCR drug discovery:

- → FLIPR allows the monitoring of intracellular calcium with fluorescent dyes.
- → Transfluor is an image-based assay for the beta arrestin-mediated GPCR desensitization. Transfluor is universal (all GPCRs desensitize regardless of their coupling mechanism), ideal for the de-orphanization of orphan GPCRs (ligand-independent translocation assay), and can be used to investigate compounds which target the desensitization mechanism.

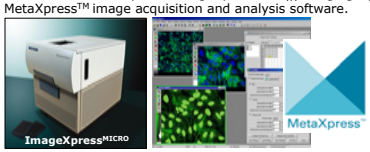
Can GPCR pathway mapping studies be conducted?

- → Measure ligand-induced calcium response on FLIPR, then GPCR internalization and desensitization with Transfluor.
- → Can a protocol be developed where Transfluor enabled cell lines be used on FLIPR and FlexStation®?
  - →GFP and a spectrally compatible calcium probe.
  - →Can the same plates be read sequentially?
- → Reveal ligands that signal and do not desensitize (NDLs) as well as inhibitors of the desensitization mechanisms (DACs)

Materials and Methods

1. Seed cells: Angiotensin Receptor. GPCR (AT1AR-Gq) beta-arrestin-GFP expressing cells.
2. Add Ca++ Red Dye (MDC prototype), add Angiotensin II.
3. Read Ca++ with FLIPR.

4. Add fixative and nuclear dye.
5. Run Transfluor assay with ImageXpress$^{MICRO}_{TM}$ Imaging System and MetaXpress™ image acquisition and analysis software.



6. Analyze results with AcuityXpress™ cellular informatics software.
7. Pathway mapping.

Figure 1: Redistribution of arrestin-GFP

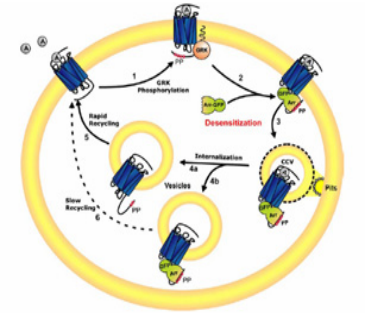


Figure 1. Molecular Devices' Transfluor assay utilizes the redistribution of arrestin-GFP to monitor GPCR activation and inactivation. By attaching a fluorescent label to beta-arrestin, the location of the receptor-arrestin complex may be monitored. Since desensitization only occurs with an activated receptor, monitoring beta-arrestin translocation and subsequent receptor recycling provides a method to detect the activation of any GPCR. Using cell lines genetically engineered to express both the labeled beta-arrestin and the GPCR of interest, the Transfluor technology can be used to screen for natural or synthetic agonists (receptor activators) or antagonists (receptor inhibitors) ligands. Activation of the receptor induces a mass movement of the fluorescence to the cell membrane (pits) within seconds, and to endocytic vesicles within minutes.

Figure 2: Transfluor Assay

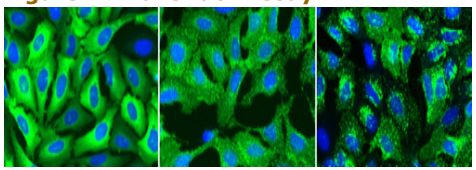


Figure 2a. A universal assay for GPCRs and orphan GPCRs. Left: untreated. Center: Activation of the receptor induces, within seconds, a mass movement of the fluorescence to the cell membrane (pits). Right: In the case of "vesicle former" receptor, within minutes, the fluorescence moves to endocytic vesicles.

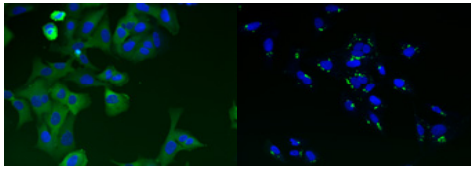


Figure 2b. Transfluor technology requires no prior knowledge of the interacting G-protein. This important feature of Transfluor makes it ideally suited for screening orphan GPCRs (oGPCR). The Ligand Independent Translocation (LITe™) assay (right) is an agonist-independent assay used to verify the translocation of beta-arrestin-GFP in orphan GPCRs. Left: control, right: LITe assay.

Figure 3: Calcium signaling on FLIPR^{TETRA} and FlexStation

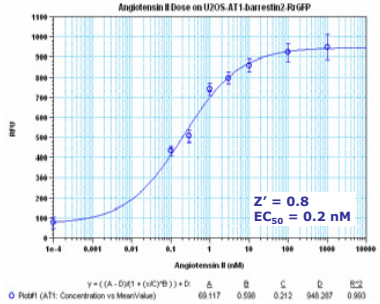


Figure 3a. Calcium Red Dye (MDC's Prototype kit) with FLIPR^{TETRA} (384 wells).

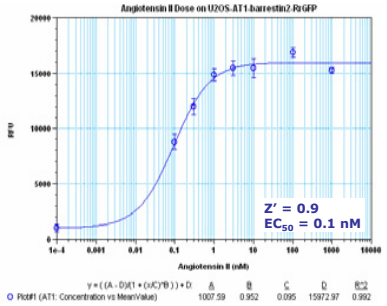
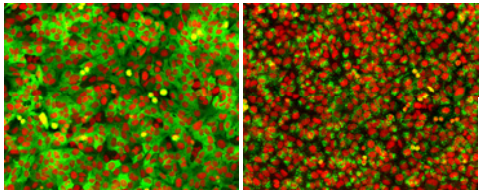
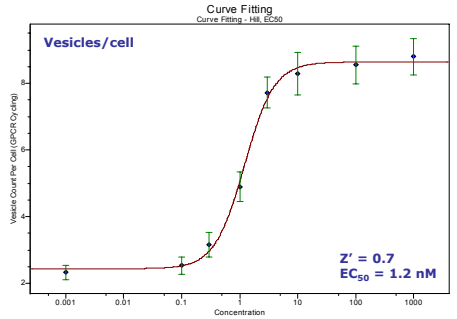


Figure 3b. Calcium Red Dye (MDC's Prototype kit) with FlexStation (96 wells).

Figure 4: Transfluor Assay Imaging

Figure 4a. Left: untreated cells with homogenous distribution of the GFP-beta arrestin fusion protein (in green). The far-red shifted DRAQ5 binds to DNA and allows the identification of the nuclei (in red). The Calcium Red dye does not interfere with this detection. Right: 1 µM Angiotensin II. activation of the receptor induces, within minutes, a mass movement of the fluorescence to endocytic vesicles.





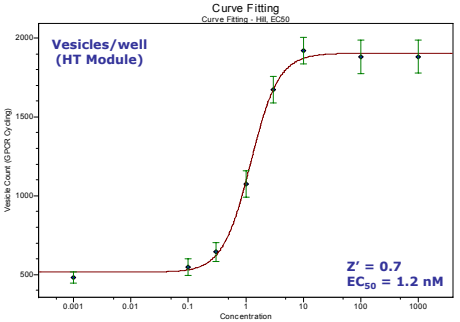


Figure 4b. High data quality is obtained from the Transfluor assay and the pharmacology is comparable to the one obtained with the FLIPR (comparable EC_{50}s). Left: analysis with the Transfluor Module for MetaXpress. Right: analysis with the fast Transfluor HT Module for MetaXpress (< 15 minutes/ 384 well plate). Curve fitting was performed using the AcuityXpress cellular informatics software.

Conclusion

FLIPR reagents are compatible with Transfluor.
- → GFP doesn't significantly interfere with Prototype Calcium Red Kit.

Equivalent performance of Calcium flux and Transfluor assay
- → Transfluor gives excellent results at 8 minutes with the Angiotensin receptor (data not shown).

Software development of Transfluor HT Application Module
- → Less than 15 minutes/ 384 well plate.

FLIPR followed by Transfluor is ideal for pathway mapping
- → Compare cell signaling and beta-arrestin mediated desensitization.
- → Reveal ligands that signal and do not desensitize (NDLs) as well as inhibitors of the desensitization mechanisms (DACs).

Molecular Devices