Cellular dopamine and intracellular calcium signaling using the next generation HTS microplate reader

Mark Koeppel[1] and E.J. Dell[2]
[1] Invitrogen [2] BMG LABTECH Inc.

- **Direct optic bottom reading, dual emission detection and high resolution well scanning are microplate reader features that greatly enhance all cell-based assays**
- **Fluo-4 Direct™ Calcium Assay and Tango™ Dopamine Receptor 1 assay**

Introduction

This application note highlights the direct optic bottom reading performance of the BMG LABTECH microplate readers in two different cell-based fluorescence intensity assays from Invitrogen. First, the intracellular calcium response to histamine was measured in HEK 293 cells using the Fluo-4 Direct™ Calcium Assay. Advanced high resolution cell layer scanning shows a correlation with the calcium signal and cell location in the well. Second, arrestin recruitment through activation of the dopamine D1 receptor with dopamine (D1 agonist) was measured using Tango™ D1-bla U2OS cells and a live cell FRET substrate (LiveBLAzer™). The fluorescent signal generated in the cells by the LiveBLAzer™ substrate greatly benefits from the PHERAstar® *FS´* unique bottom read dual emission detection. The PHERAstar *FS* is the next generation HTS microplate reader because it has many unique features not found on any other instrument. For this application note those unique features include: direct optic bottom reading; dual emission detection; injection at the point of measurement; high resolution well scanning (30x30 matrix); and orbital averaging.

Materials & Methods

- Black, clear bottom 384 well plates (Corning)
- Tango™ D1-bla U2OS DA Assay Kit (Invitrogen)
- Fluo-4 Direct™ Calcium Assay Kit (Invitrogen)
- LiveBLAzer™-FRET B/G Loading Kit (Invitrogen)
- HEK293 cells

Fluo-4 Direct™ Calcium Assay

HEK 293 cells were seeded into 384-well microplates at 15,000 cells/well in 25 ul of assay medium. An equal volume of 2X Fluo-4 Direct™ calcium reagent was then added to the well and the plate was incubated at 37°C in a CO_2 incubator for 60 min. Using the onboard reagent injectors, 5, 10 and 15 µl of histamine (270 nM) were injected into different wells and the intracellular calcium flux was recorded using the fast kinetic (well mode) setting on the microplate reader. Readings were taken every 0.5 sec for 90 sec and the histamine injection occurred at 10 sec.

The fluorescence signal was measured using the direct optic bottom reading and an assay specific Optic Module (EX/EM 485/520). After the calcium response was measured, a bottom reading, high resolution well scan of the cell layer was performed using a 10x10 scan matrix.

Tango™ D1-bla U2OS GPCR Cellular Assay

Tango™ D1-bla U2OS cells were seeded into 384-well microplates at 15,000 cells/well in 32 µl of assay medium. Dopamine, 8 µl of 5X concentration, (columns 1-10, 9 replicates each) was added to the cells to generate a dose response curve then incubated for 5 hr in a CO_2 incubator at 37°C. Following treatment, 8 µl of 6X LiveBLAzer ™ substrate was added for 2 hr at room temperature. Plates were read using direct optic bottom reading and a dual emission Optic Module that excites at 405 nm and emits at both 460 and 530 nm.

Results & Discussion

Fluo-4 Direct™ Calcium Assay

Figure 1 shows the intracellular calcium response measured using Invitrogen's Fluo-4 Direct™ Calcium Assay, in HEK 293 cells upon the injection of three different volumes (5, 10, and 15 µl) of histamine (270 nM). Different ranges can be defined to perform different calculations with those ranges. When the averages were calculated (inset graph Figure 1), some of the replicates were found to vary widely.

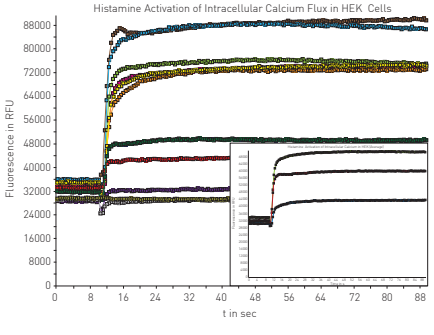

Fig. 1: Fluo-4 Direct™ intracellular calcium response in HEK 293 cells upon injection of histamine (5, 10 and 15 µL of 270 nM) in a 384 well microplate. Inset graph shows the average of four replicates.

After the kinetic measurement, advanced, high resolution cell layer well scanning was performed using direct optic bottom reading and a 10x10 matrix. As can be seen in Figure 2, the number of cells in the middle of each well varied, causing the signal to vary accordingly.

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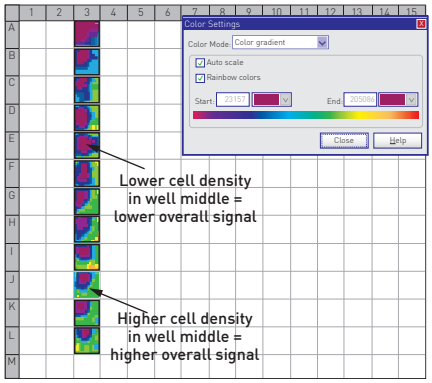

Fig. 2: Bottom reading high resolution cell layer scanning (10x10 matrix) shows the uneven distribution of HEK cells in each well. Samples with higher cell density in the middle of the well gave a more robust calcium response than lower density wells.

Well scanning can thus be used to help correct for cell number. To avoid such uneven cell distribution and results, three things can be done:

1) use pre-coated microplates that allow cells to adhere better;
2) decrease the injection speed so as not to disturb the cell layer; and
3) use the orbital averaging function, which measures and averages readings around the entire well.

Tango™ D1-bla U2OS Cellular Assay

The Tango™ division arrested U2OS cell line was used to measure the activity of the D1 receptor with dopamine through arrestin recruitment. The Tango™ GPCR assay uses beta-lactamase as a reporter to measure receptor activation. GPCR activation in the cell line recruits protease tagged arrestin to the activated GPCR which cleaves a non-native transcription factor tagged to the receptor's C-terminus. The released transcription factor enters the nucleus and stimulates the expression of beta-lactamase. Beta-lactamase is measured with the live cell fluorescent beta lactamase FRET substrate LiveBLAzer™. The substrate contains coumarin and fluorescein linked by the beta-lactamase substrate CCF2. In the non-active state, FRET occurs between the linked coumarin and fluorescein and emits a green signal measured at emission wavelength 530 nm. Upon receptor activation, beta-lactamase expressed in the cells cleaves the substrate resulting in an increased blue signal at 460 nm.

The ratio of blue to green cells is used to assess receptor activation and provides for a robust assay that is convenient for HTS. Figure 3 shows D1 receptor activation by increasing amounts of dopamine. As represented in the inset in figure 3, the cells change in color (green to blue) as increasing amounts of dopamine is added (columns 10 thru 1). A 4-parameter fit of the percent activation versus the log of the dopamine concentration gives an EC_{50} of 380 nM, which corresponds to the published 400 nM. Orbital averaging (2 mm), averaging multiple readings in an orbit in each well, allows for better Z' values (Table 1) and is recommended for cell based assays.

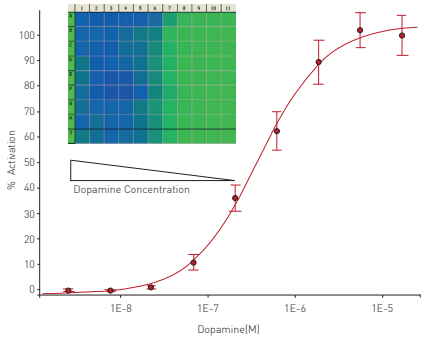

Fig. 3: Decreasing amounts of dopamine, a D1 agonist, (9 replicates) were added to Tango™ D1-bla U2OS cells (inset, columns 1-10) and the percent activation was plotted as a 4-parameter fit (negative control in column 11 was used as 0%).

Table 1: Dopamine Activation of Tango™ D1-bla U2OS Cells

	Z'	R^2	EC_{50}
With 2 mm Orbital Avg	0.76	0.998	380 nM*
Without 2 mm Orbital Avg	0.63	0.997	382 nM

*Corresponds with published results of 400 nM

Conclusion

Two cell-based signaling assays from Invitrogen, Fluo-4 Direct™ Calcium Assay and Tango™-bla U2OS GPCR Assay, were performed on a BMG LABTECH HTS instrument.