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Automated FRET based high-throughput screening with MACSQuant® X flow cytometer

Introduction

Complex networks of interactions between cellular proteins have proven to play a pivotal role in both the regulation of biological systems and the development of disease. Cells utilize and interact with membrane proteins to transduce downstream signals to cells, transport essential ions and molecules, bind the cell to a surface or substrate, and catalyze reactions. Therefore, cellular proteins and their interactions are of major interest to investigate as drug targets. The majority of approved therapeutic targets are proteins, primarily membrane proteins that are able to modify cellular signaling[1].

Technological advancement in high-throughput screening (HTS) assays have evolved to directly monitor protein-protein interactions for compound library screening. In recent years, Förster resonance energy transfer (FRET) assays have found extensive use in HTS due to this being one of the few, non-invasive, radiation-free techniques to study protein interactions within an intact cell in real-time. The FRET principle lies upon the transfer of energy from an excited donor fluorophor to a close-by acceptor fluorophor, resulting in enhanced fluorescence emission of the acceptor[2]. This phenomenon only occurs when the distance between donor and acceptor is less than 10 nm, and the emission spectra of the donor overlaps with the excitation of the acceptor. A major limitation of FRET methodology is achieving a clear FRET signal due to the spectral overlap between donor and acceptor. This requires numerous controls and complicated software calculations to eliminate background signal[3]. A less-artifact prone FRET approach includes fluorescence lifetime imaging (FLIM), which requires specialized equipment and expert knowledge. Fluorescent microscopy on the other hand, has been commonly used to measure FRET. However, this tedious method generates huge amounts of data and essentially precludes the analysis of large cell numbers as well as HTS for protein interactions.

Flow cytometry based assays are non-invasive, sensitive, quantitative and allow the user to measure large numbers of cells and samples rapidly. The flow cytometry based FRET assay has been successfully implemented, standardized and applied to understand complex protein interactions[4,5]. Here, we have utilized our established automated flow cytometry based FRET assay[4] in the MACSQuant® X for HTS of molecules which have a potential inhibitory effect on programed cell death protein-1 (PD-1). PD-1 is an immune checkpoint expressed in T cells and pro B cells. PD-1 signaling results in inhibition of T cell proliferation, survival and effector functions and require binding to its specific ligand receptor PD-L1 or PD-L2. PD-L1 is expressed in several cancers, and therefore drugs targeting the PD-1 signaling pathway are commonly used in cancer immunotherapy[6]. PD-1/PD-L1 complex to exert its effect forms a microcluster in the immunological synapse together with TCR/CD3, and can be detected by assays that measure protein-protein interactions (figure 1).

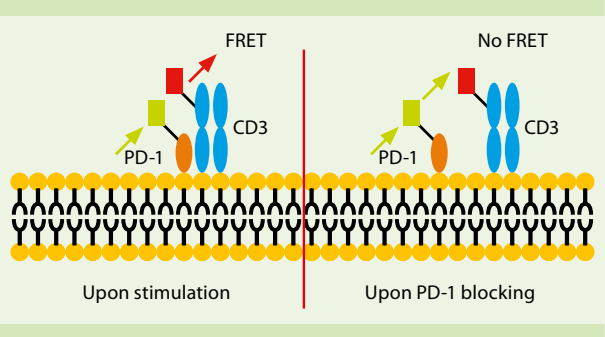

Figure 1: Principle of FRET based detection of PD-1 and CD3 interaction.

The MACSQuant X is a fully automated flow cytometer consisting of fluidics, which are designed to provide rapid sampling to increase sample throughput. A 384 well plate can be processed in less than one hour, with a reliable absolute cell count still being obtained. The orbital shaker attachment ensures proper automatic mixing of samples. Additionally, by using the FRET Express Mode integrated in MACSQuantify™ software, the user is able to automate sample acquisition and analysis, further reducing experimental time and increasing analytical consistency and reproducibility. Here we demonstrate a HTS of 348 compounds to evaluate their potential to inhibit PD-1 signaling at the single cell level using the MACSQuant X flow cytometer.

Method

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor buffy coats. T cell isolation was performed using the Pan T Cell Isolation Kit, human (#130-096-535). 5×10^5 cells were seeded per well in a 384 well plate. For cell activation, which will allow PD-1 and CD3 interaction, Staphylococcus enterotoxin B (SEB) was added. Each of the 348 compounds from the library were added to wells. Duplicate samples of SEB were used as a negative control (displaying successful FRET), and PD-L1 antibody for PD-1 blocking used as a positive control (displaying decreased FRET efficiency). After 3 days of culturing, cells were washed and stained with a mixture of anti-PD-1 acceptor FITC, and donor anti-CD3 VioBlue® antibody conjugates for 10 minutes at 4 °C. After washing and resuspending cells, all samples in the 384 well plate were directly measured using the MACSQuant® X flow cytometer (figure 2).

FRET Express Mode program was set for automated data acquisition and analysis. Control samples: blank, acceptor only and donor only were initially measured to calculate background, spillover and FRET signal for all of the controls and to determine alpha factor and FRET efficiency median which was applied to the rest of the samples for automated FRET efficiency calculation. Finally, FRET efficiency data was normalized and plotted against absolute cell counts for each sample.

*Please consider FRET Express Mode is highly application specific, careful tailoring of experiment is required before applying the technology.

Results

The compound library was screened to monitor their effect on PD-1/CD3 FRET efficiency in T cells collected from healthy donors. After incubation with the assay reagent, samples were acquired automatically in the high-throughput MACSQuant X analyzer. The Express Mode integrated in MACSQuantify software automatically measured and analyzed control samples to exclude background (figure 3A).
The MACSQuant X analyzer was able to successfully measure FRET efficiencies of individual samples treated with compound libraries, in addition to measuring absolute numbers of cells present in each samples. Comparison of cell survival (by cell count) and FRET efficiency (PD-1/CD3 clustering) upon compound library treatment demonstrated clustering of samples.
We monitored 3 dominant clusters (figure 3B):

- **Cluster 1:** Low cell count indicating screened molecule was toxic to the cells.
- **Cluster 2:** High cell count and high FRET efficiency indicating screened molecule was not toxic, however unable to inhibit PD-1/CD3 clustering.
- **Cluster 3:** High cell count and low FRET efficiency indicating screened molecule was not toxic to the cells and able to inhibit PD-1/CD3 clustering. These were considered as potential hits that can go for further detailed characterizations and verifications.

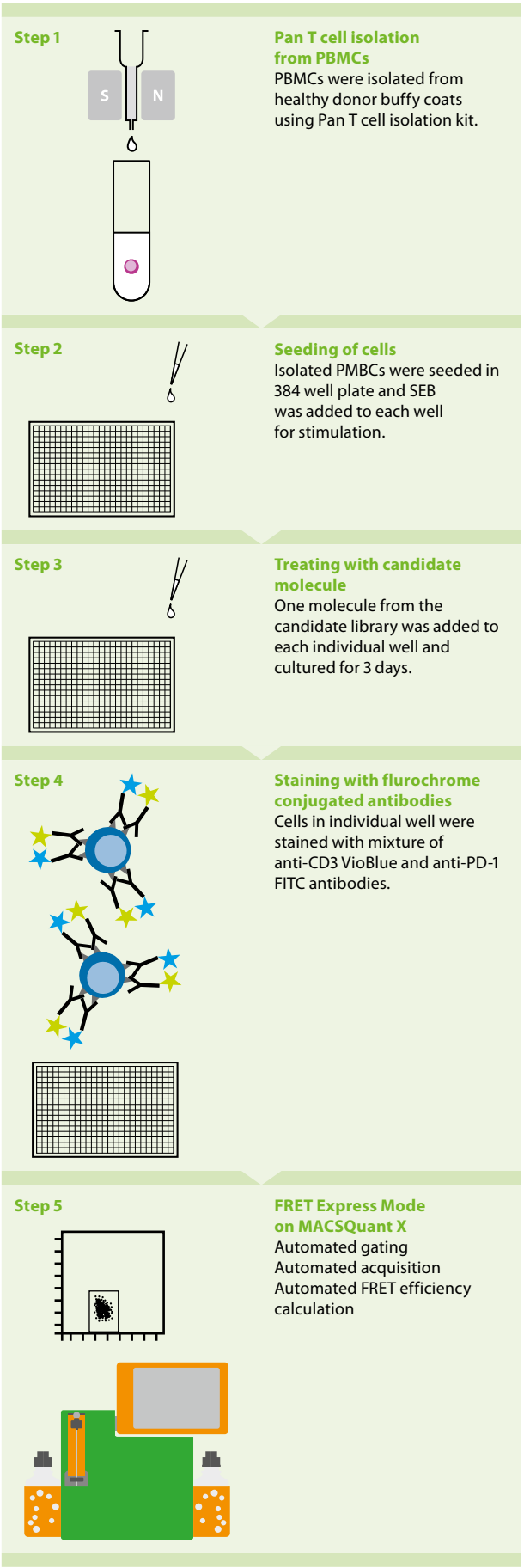

Figure 2: Flow cytometry based automated PD-1/CD3 FRET detection workflow.

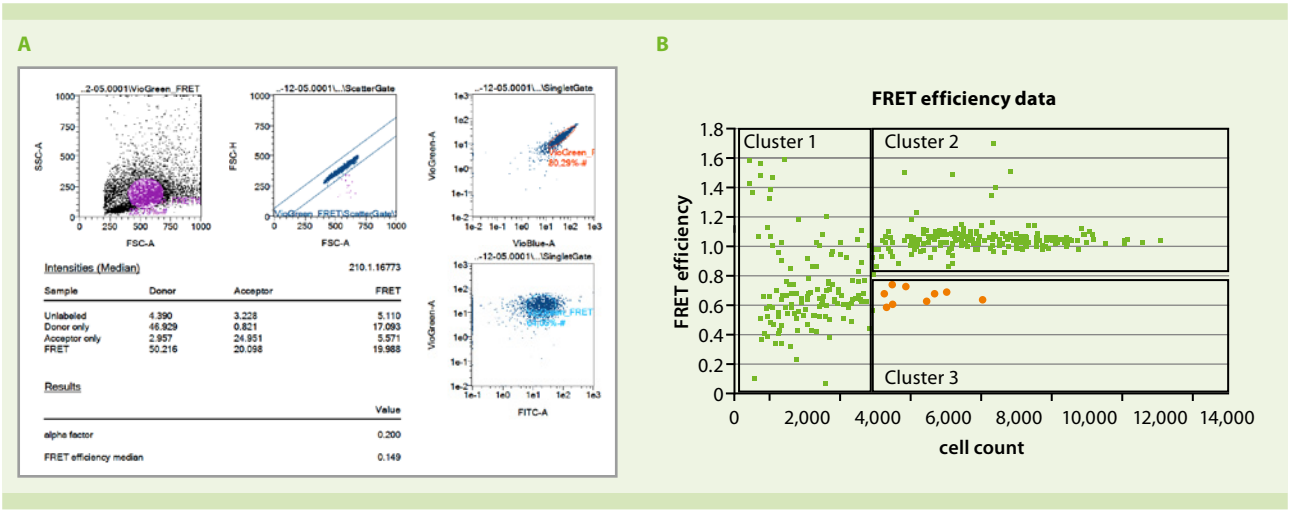

Figure 3: Representative result analysis page from FRET Express mode (A) and analysis of FRET efficiency data comparing normalized FRET efficiency (X-axis) vs. cell count (Y-axis) from individual screened compounds using MACSQuant X Analyzer. Potential hit candidates and negative candidates are displayed as orange dots and green squares respectively (B).

Conclusion

The MACSQuant® X analyzer together with FRET Express Mode, is able to perform fully automated high throughput protein-protein interaction assays, and therefore can be used as a cell-based screening tool in the drug discovery process. Use of such tool can reduce assay time, experimental variability, computational error and confirm reliable data generation. We have shown sophisticated background-free data analysis to measure high-quality FRET efficiency result. In addition to this, analyzing a large number of cells and their absolute count ensures exclusion of potential toxic compounds.

References

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