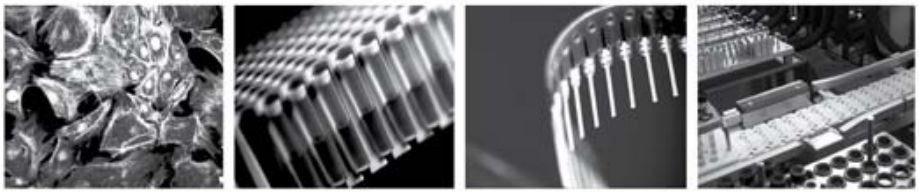

TTP LABTECH

A Mix-and-Read Cell-Based Assay for Antibody Screening Against Epidermal Growth Factor Receptor

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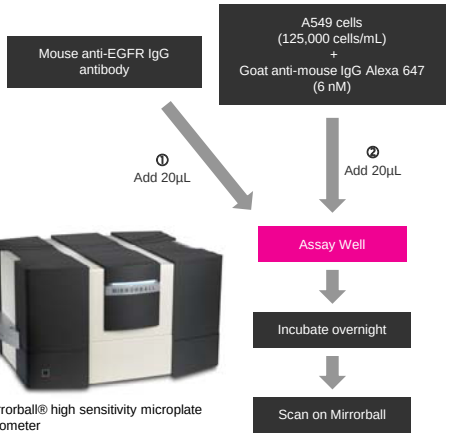
Introduction

Antibodies against a wide range of protein targets have either been approved or are currently under development as therapeutics. The conventional antibody screening assay based on antibody-antigen binding has been the enzyme-linked immunosorbent assay (ELISA). While tedious and consuming, ELISA has proved sufficient for the identification of antibodies directed against secreted antigens. However, cell surface antigens (e.g. GPCRs) provide challenges for ELISA primarily due to the shortage of soluble antigens and high variability resulting from loss of cells during wash procedures. Mix-and-read assays – popularized as FMAT® assays – overcome such problems, while at the same time offering simplified workflows for automation.

We have developed mix-and-read methods for the screening of antibodies against cell surface proteins expressed on live cells. The methods use a high sensitivity microplate cytometer to quantify cellular fluorescence in cultures seeded in microplates. Here, we describe their use for the determination of human epidermal growth factor receptor (EGFR) antibody binding in A549 cells which are known to express high levels of EGFR. A549 cells were incubated with mouse anti-EGFR antibody and fluorescently-labelled anti-mouse IgG antibody. Without washing away unbound antibody or fluorescent tracer, plates were scanned and fluorescence of each cell quantified. Clear concentration-dependent antibody binding was observed with low assay variability. Further improvements were made by dual laser scanning allowing the identification of all cells even in the absence of antibody binding, plus multiplexing of two cell types in the same well.

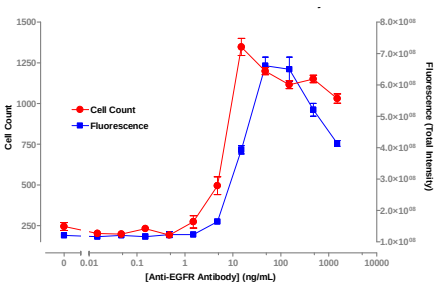
1. Assay Method



Mirrorball® high sensitivity microplate cytometer

Assays were performed using a so-called 'mix-and-read' or homogenous protocol. Briefly, a suspension of A549 cells and goat anti-mouse IgG Alexa 647 conjugate was combined equally with a range of concentrations of anti-EGFR antibody (GR01, Merck Chemicals Ltd) in a 384 microplate. Final concentrations were 2,500 cells per well and 3 nM conjugate. After incubation overnight, the amount of anti-EGFR bound was determined by scanning on a Mirrorball® microplate cytometer (TTP LabTech Ltd, UK).

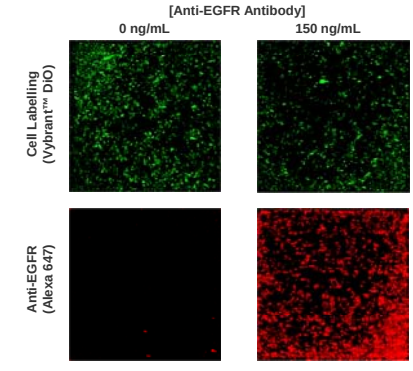
2. Single Laser Scanning

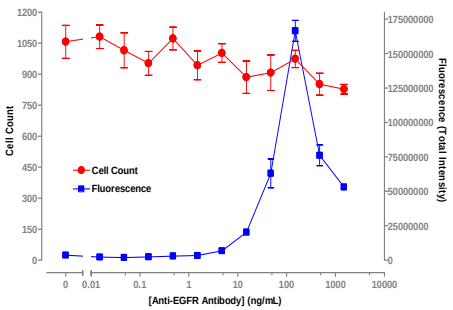


The EGFR assay set-up proved straightforward with minimal development required to run on a Mirrorball. Concentration-dependent increases in cell counts and fluorescence were observed. Cell count appeared to be a more sensitive readout compared to fluorescence with a clear response at 5 ng/mL (30 pM). At concentrations above 100 ng/mL, a reduction in fluorescence total intensity was seen due the well documented 'hook effect'. A similar trend was observed with cell count demonstrating that cell recognition is directly related to the amount of anti-EGFR antibody labelling.

3. Dual Laser Scanning

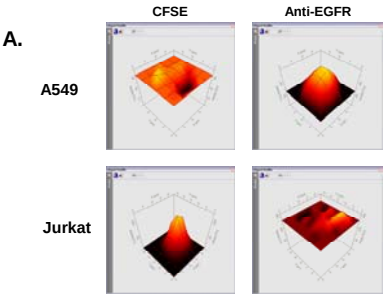
The Mirrorball microplate cytometer offers simultaneous scanning with multiple lasers. This new development has numerous benefits for mix-and-read assays such as improved assay performance from independent cell recognition. To allow detection of all cells, they were labelled with Vybrant DiO (Cat No. V-22886, Invitrogen). DiO is a lipophilic tracer that is highly fluorescent in lipid environments such as cell membranes. Cells were labelled with 30 nM DiO by simply adding the dye to a suspension of cells and anti-mouse IgG Alexa 647. At such low levels of DiO, the cost of addition is low and also the risk of interference with ligand-receptor interaction is minimised. Both control and antibody-positive cells are detectable using their DiO labelling, excited by a 488 nm laser. The Mirrorball simultaneously records the fluorescence emissions for Alexa 647 proving a readout of antibody binding.

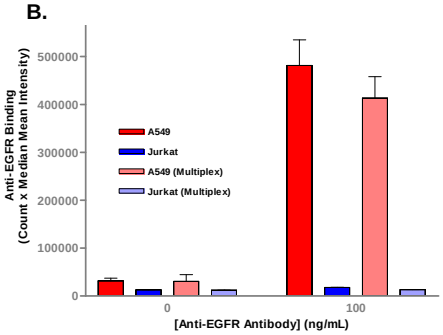




4. Cell Multiplexing

A common approach for antibody discovery is the screening of libraries against parental and transfected cell lines to identify antigen-specific activity. Normally, this requires the running of separate tests for each cell line, however, a Mirrorball allows tests to be performed in a single well.
To illustrate the approach, A549 (EGFR+) and Jurkat (EGFR-) cells were screened for anti-EGFR antibody binding. Jurkat cells were stained with carboxyfluorescein (CFSE; 10 nM) prior to assay. Mirrorball's unique ability to scan simultaneously with multiple lasers allows differentiation of A549 and Jurkat cells (CFSE; 488 nm) based on CFSE staining and quantitation of anti-EGFR binding (Alexa 647; 640 nm).
A. 3D profiles of A549 and Jurkat cells for 100 ng/mL anti-EGFR antibody. Note CFSE staining of Jurkat and positive anti-EGFR binding to A549.
B. Comparison of single and multiplexed assays.





Conclusion

Development of the EGFR binding assay proved straightforward on the Mirrorball. Unlike cell-based ELISA where cells have to be plated 24-48 hours prior to assay, cells for the Mirrorball mix-and-read assay were plated just before the screen. This makes the method suitable for analysis of live cells in adherent and suspension cultures without further manipulations. With a cell-based ELISA, suspension or poorly adherent cells require fixing to the well surface, thereby introducing the risk of detrimental effects such as structural alteration of epitopes in the target antigen.

The Mirrorball microplate cytometer is equipped with 488 nm and 640 nm lasers. Its proprietary optics permit simultaneous scanning with both lasers allowing direct correlation of fluorescence across lasers. When combined with appropriate fluorescent reagents, this unique capability allows independent identification of cells and multiplexing. Both provide improved assay robustness and increased assay throughput. With their simple operation and high sensitivity, Mirrorball and mix-and-read assays are well-suited for high throughput antibody screening against cell surface antigens.

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