

Cell multiplexing in homogeneous antibody binding assays

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introduction

Monoclonal antibody therapy represents one of the most rapidly expanding and exciting segments of the pharmaceutical industry today. This has placed unprecedented demands on the antibody discovery process that has traditionally relied on assay formats such as ELISA and flow cytometry. When screening against antigens expressed in cells, these formats have many disadvantages notably tedious wash steps, detachments of cells from plate surfaces and a requirement for large numbers of cells.

To address these challenges we have employed a mirrorball® high sensitivity microplate cytometer (TTP Labtech) to carry out homogenous cell-based FMAT assays originally developed for the now obsolete ABI 8200 instrument. The combination allows researchers to screen antibody supernatants with minimal reagent additions, no wash steps and achieve excellent sensitivity with only 5 – 10 microlitres of sample.

Using the mirrorball's unique ability to scan simultaneously with up to three lasers, it is also possible to multiplex cell lines, for example to screen against parental and transfected cells in the same well. Cell encoding is achieved by labelling with different fluorophores or a range of fluorophore concentrations. This multiplexing capability results in higher throughput, reduced reagent requirements and more robust data by eliminating interplate variability.

To demonstrate the technique, we have developed a homogeneous assay to quantify binding of human anti-EGFR antibody to receptors expressed in A549 or A431 cells. Jurkat cells served as a negative control for EGFR expression.

1. generic assay method

Assays were performed using a so-called 'mix-and-read' or homogenous protocol.

Briefly, a suspension of cells and anti-mouse IgG conjugate was combined with a range of concentrations of anti-EGFR antibody (GR01, Merck Chemicals Ltd) in a 384-well microplate. Final concentrations were 2,500 cells per well and 3 nM conjugate. After incubation, the amount of anti-EGFR binding was determined by scanning on a mirrorball® microplate cytometer (TTP Labtech Ltd, UK).

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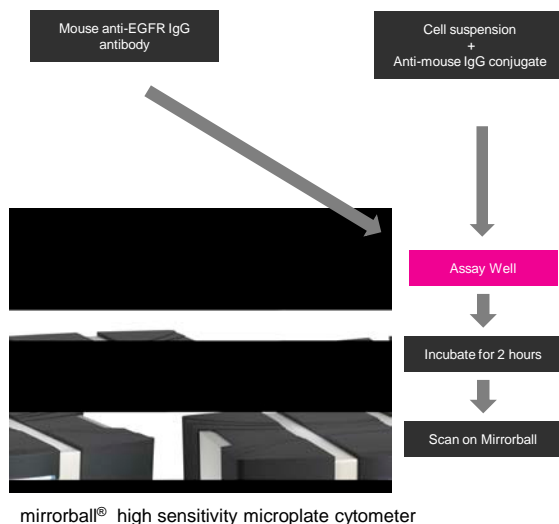
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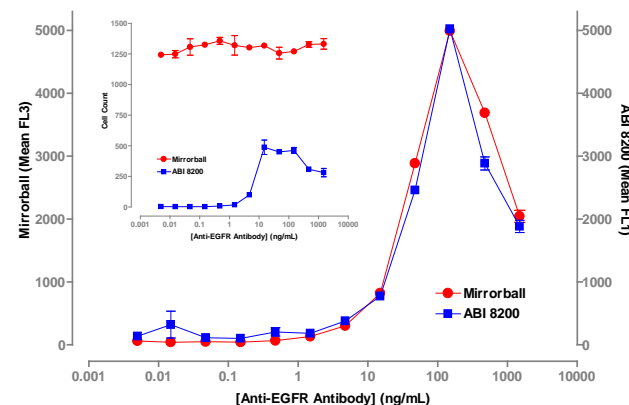
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2. EGFR binding in A431 cells



The EGFR assay set-up proved straightforward with minimal development required to run on a mirrorball.

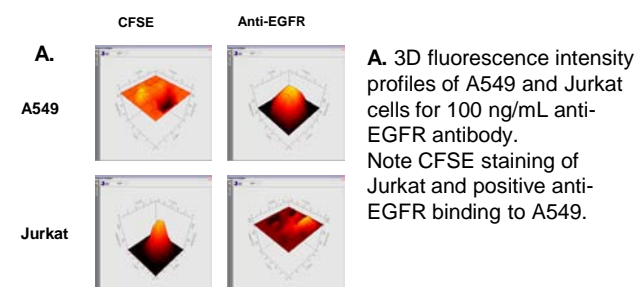
Cells were stained with carboxyfluorescein (CFSE; 10 nM) to allow recognition irrespective of the amount of antibody binding. Concentration-dependent increases in fluorescence were observed with sensitivity of < 5 ng/mL. At antibody concentrations above 100 ng/mL, a reduction in fluorescence total intensity was seen due to the well documented 'hook effect'.

The degree of antibody binding correlated well with similar data from an ABI 8200. However, the stable cell counts reported by the mirrorball contrasted with the inability of the ABI 8200 to reliably identify cells below 15 ng/mL (see inset).

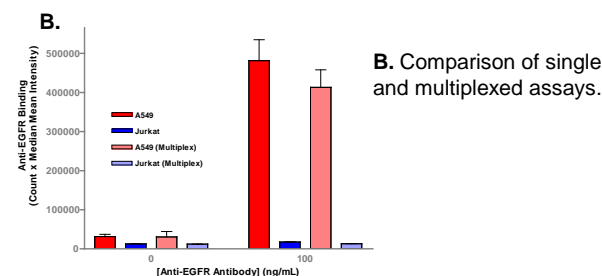
3. multiplexing – single cell staining

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 this approach, A549 (EGFR+) and Jurkat (EGFR-) cells were screened for anti-EGFR antibody binding. Jurkat cells were stained with CFSE (10 nM) prior to assay. mirrorball's unique ability to scan simultaneously with multiple lasers allows differentiation of A549 and Jurkat cells based on CFSE staining (488 nm laser) and quantitation of anti-EGFR binding (Alexa 647; 640 nm laser).



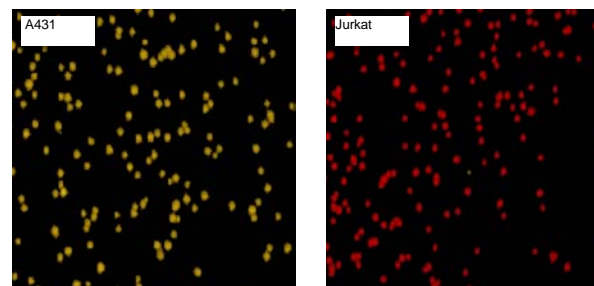
A. 3D fluorescence intensity profiles of A549 and Jurkat cells for 100 ng/mL anti-EGFR antibody. Note CFSE staining of Jurkat and positive anti-EGFR binding to A549.



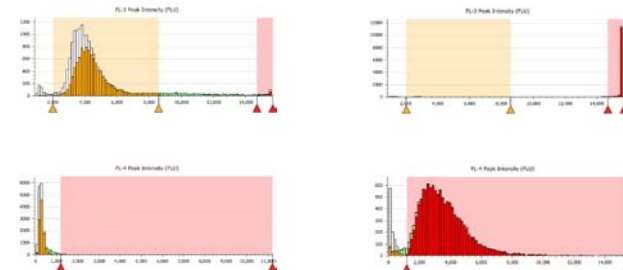
B. Comparison of single and multiplexed assays.

4. multiplexing – dual cell staining

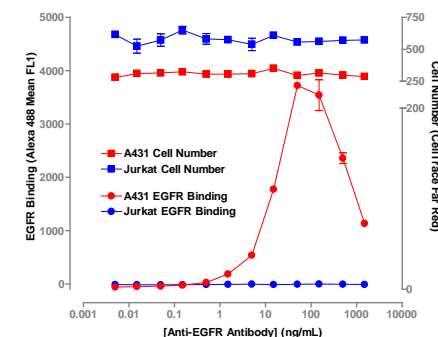
An alternative method for cell multiplexing is to encode cell lines with different concentrations of whole cell stain. The intensity of staining per cell can be recorded and used to define gates for each cell population. The advantage of this method is that both cell lines are counterstained which allows their recognition irrespective of the amount of antibody binding.



Here, we have used CellTrace™ Far Red DDAO-SE (Invitrogen), a far-red cell labelling reagent excitable by the 640 nm laser on mirrorball systems. A431 and Jurkat cells were labelled with 100 nM and 1,000 nM CellTrace™ Far Red, respectively. Representative Well Views (coloured by population) are shown below, together with fluorescence peak intensity histograms for each cell population in channels FL-3 and FL-4.



To demonstrate cell multiplexing, A431 and Jurkat were combined in the EGFR assay. Binding of anti-EGFR antibody was detected using a DyLight 488 labelled anti-mouse conjugate.



Cells were scanned using both 488 nm and 640 nm lasers on mirrorball to differentiate A431 and Jurkat cells, provide a cell count per well and report the amount of anti-EGFR antibody binding. The data show excellent differentiation of the cell lines based on the highly specific binding to A431.

conclusion

The ability to multiplex cell lines adds a new dimension to antibody binding assays. The combination of fluorescent cell stains and TTP Labtech's mirrorball® high sensitivity microplate cytometer enables simple homogeneous protocols to be performed. Decoding of cell lines is straightforward via gating of the fluorescence signals using the multiple detection channels provided by the mirrorball system.

This new approach allows researchers to directly transfer existing protocols being run on ABI 8200 systems without loss of sensitivity, and realise the major benefits of increased throughput and data robustness.

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