A mix-and-read cell-based assay for antibody screening against Epidermal Growth Factor Receptor

Tristan Cope, David Onley, Diana Caracino, Paul Wylie and Wayne P Bowen

TTP LabTech Ltd, Melbourn Science Park, Melbourn, Royston, Hertfordshire, SG8 6EE, UK.

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introduction

Antibodies against a wide range of protein targets have been either approved or are currently under development for therapeutics. The use of flow cytometry to identify antibody-antigen binding has been well characterised.

While flow cytometry has been widely used, it requires the use of suspension cells or adherent cells that are removed from the well, so it is unable to analyse cells *in situ*. In addition, as the cells are passed through a flow tube, low affinity antibodies can dissociate from the antigen as the equilibrium of antibody concentration to antigen is changed. Flow cytometry is typically not a high throughput method for this kind of assay. Though higher-throughput systems are now currently available, they still have to remove cells from a plate which can lead to cross-contamination, and require wash steps which are not ideal for low-affinity antibodies.

We offer an alternative high-throughput method for looking at antibody binding whereby the primary screen can be run through the mirrorball® high sensitivity cytometer (TTP Labtech Ltd) to rapidly identify antibodies of interest.

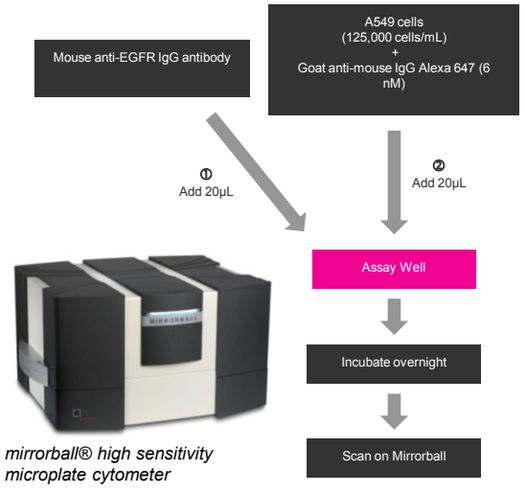
Here we present a sensitive robust, mix-and-read method for the screening of antibodies against cell surface proteins. The mirrorball® high sensitivity cytometer is used to quantify the cellular fluorescence in cultures in 384-well microplates. We describe its use for the determination of human epithelial 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 (Merck Chemicals Ltd) and fluorescently-labelled anti-mouse IgG antibody. Without washing away unbound antibodies, plates were scanned and fluorescence of each cell quantified. Clear concentration-dependent antibody binding was observed with low assay variability. Addition of Vybrant™ DiO, a 488 nm-excitable cell stain, allowed detection of cells irrespective of the amount of anti-EGFR antibody binding for improved assay performance.

This new method provides simple operation, no-wash format, and high sensitivity, and thus is well-suited for high throughput antibody screening

TTP Labtech Ltd
Melbourn Science Park
Melbourn
Hertfordshire SG8 6EE
United Kingdom
tel: +44 1763 262626
fax: +44 1763 261964
sales@ttplabtech.com

TTP Labtech Inc
One Kendall Square
Suite B2303
Cambridge MA 02139
United States
tel: +1(617) 494 9794
fax: +1(617) 494 9795

1. assay method



mirrorball® high sensitivity microplate cytometer

Assays were performed using a 'mix-and-read' or homogenous protocol. Briefly, a suspension of A549 cells and goat anti-mouse IgG Alexa Fluor 647 conjugate was combined equally with a range of concentrations of anti-EGFR antibody (Cat. No. GR01-100UG, 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 high sensitivity cytometer (TTP Labtech Ltd).

2. single laser scanning

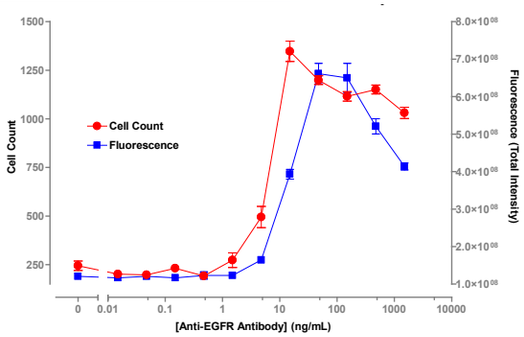


Fig 1: Concentration dependent detection of anti-EGFR antibody. Data shows that with single fluorescent dye, the cell count is directly linked to the level of antibody binding.

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 is equipped with 405nm, 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.

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 Fluor 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 Fluor 647 proving a readout of antibody binding.

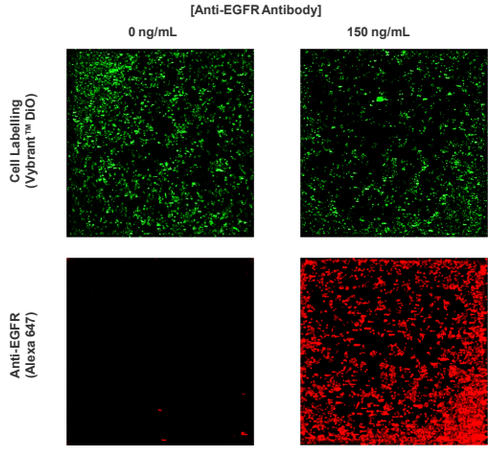


Fig 2: Well images from mirrorball showing total cell count and cells with anti-EGFR antibody binding

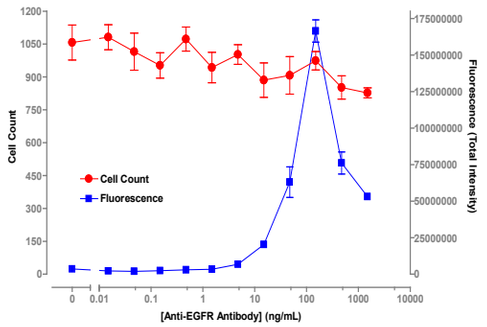


Fig 3: Concentration dependent detection of anti-EGFR antibody using a cell counterstain. Data shows that with a cell counter stain, the cell count obtained is independent of the level of antibody binding to the cell.

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, 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 Fluor 647; 640 nm).

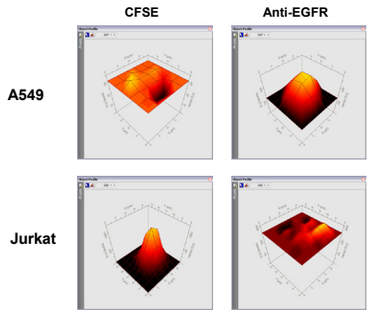


Fig 4: 3D fluorescence profiles of A549 and Jurkat cells with 100 ng/mL anti-EGFR antibody. Note CFSE staining of Jurkat and positive anti-EGFR binding to A549.

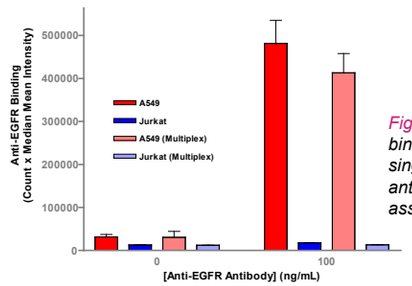


Fig 5: Comparison of binding data from both single and multiplexed antibody binding assays.

conclusion

Development of the EGFR binding assay proved straightforward on TTP Labtech's 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 offers simultaneous scanning with multiple lasers. This has numerous benefits for mix-and-read assays such as improved assay performance from independent cell recognition.

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.