mirrorball®
application note

a multiplexed cell-based assay for antibody screening

introduction

The use of monoclonal antibodies against a wide range of protein targets is an expanding area for the development of therapies and biomarkers of diseases such as cancer and autoimmune disorders. Monoclonal antibodies are produced by cell lines that secrete antibodies against a specific antigen into the growth medium. High-throughput screening is central to antibody discovery programmes due to the large number of clones that need to be assessed.

ELISA versus mix-and-read assays

The use of traditional ELISA techniques for antibody screening of protein targets has many limitations. These assays rely on the immobilisation of antigen to the surface of microplates and are often not suitable for the detection of low solubility antigens such as those found on the cell surface. There have also been concerns about the reliability of ELISAs to identify antibodies that recognize the native conformation of cell surface antigens. Even with soluble antigens it has been suggested that the adsorption of detection antibodies onto microplates may alter protein conformation leading to a failure to identify antigens at target epitopes.

Nowadays “mix-and-read” assay protocols are preferentially used in the biopharmaceutical industry for monoclonal antibody discovery.

“Mix-and-read” assays involve the addition of all the assay constituents to one well followed by an incubation step to allow binding to reach equilibrium. For antibody screening, assays are routinely configured using antigen-coated beads or antigen-expressing cells. Antibody/antigen interaction is reported by the binding of a fluorescently-labelled conjugate which results in an increase in fluorescence associated with the bead or cell. This can be quantified without washing using cytometric analysis to discriminate bound from free fluorescence in test wells. The advantages of cytometric analysis compared to traditional ELISA techniques were highlighted by Lee *et al*. (1) when introducing fluorometric microvolume

assay technology (FMAT). The use of FMAT to analyse “mix-and-read” assays results in high detection sensitivity and throughput compared to ELISA assays, alongside improved identification and detection of antibodies.

high speed multiplexing

TTP Labtech’s mirrorball® is ideally suited to the analysis of “mix-and-read” assays offering high sensitivity for the detection of low expression membrane bound proteins. Not only is it capable of rapid single laser scanning, it can also simultaneously scan with 405, 488 and 640 nm lasers to excite a broad range of fluorophores. This ability allows direct correlation of the resultant fluorescence emissions irrespective of the excitation source and is therefore ideal for the configuration of bead and cell-based multiplex assays in a wide range of antibody screening applications.

This application note describes the use of TTP Labtech’s mirrorball for the determination of antibody binding to human epidermal growth factor receptor (EGFR) using a “mix-and-read” assay as an example of a screen for a cell surface antigen. EGFR ligands are a family of proteins which are involved in the regulation of cell migration, adhesion growth and differentiation.

Over-expression of EGFR is associated with a number of disorders, including breast, lung and colon cancer and the study of inhibition of EGFR expression and activation is of relevance to therapy development.

Analyses of the binding of increasing concentrations of anti-EGFR antibody in two cell lines were tested. An epithelial

key benefits

key benefits versus traditional ELISA

- **improved data quality**
- **greater productivity**
- **cost effective solution**
- **confident decision making faster and within budgets**
- **free up time and precious sample for other value added tasks**

carcinoma cell line, A431, which is known to express high levels of EGFR and Jurkats, a non-EGFR expressing cell line were studied in order to assess the detection sensitivity using mirrorball. A common antibody screen is to profile binding against antigen-expressing and non-expressing cells to identify antigen specific hits. For example, these may be cells transfected with a gene of interest and the corresponding parental cell line.

Using existing screening platforms (e.g. ELISA and FMAT) it would be necessary to set-up duplicate plates to compare the binding activity of each cell line and antigen specificity.

In this application note, we demonstrate that mirrorball is capable of distinguishing antibody binding in more than one cell line in a single well. This ability to multiplex reduces costs, increases throughput and eliminates intraplate variability.

methods

cell lines and cell culture

In this study, Jurkats and A431 cells expressing EGFR were compared. These cell lines, were acquired from Sigma and cultured in the following conditions: For A431 cells, EMEM (EBSS) (Sigma) supplemented with 10% FBS, 2 mM Glutamine (Sigma), 1x Non-essential

ttplabtech

Amino Acids (NEAA) (Sigma) and 100 U/mL; Jurkat cells, RMPI-1640 medium (Sigma), supplemented with 10% FBS and 100 U/mL penicillin, 100 U/mL streptomycin. A431 cells were cultured to 80% confluency and harvested using Trypsin 0.25% with EDTA, washed and resuspended in media. Culture reagents were purchased from Life Technologies unless otherwise stated.

The detection antibody, mouse anti-human epidermal growth factor antibody (∝EGFR mAb), was purchased from Merck Chemicals Ltd (#GR01). AlexaFluor® 488 Goat Anti-Mouse IgG, Fcγ Fragment (AF488∝mouse IgG Fcγ) (115-546-071) from Jackson ImmunoResearch.

CellTrace™ Far Red labelling

Dissolve CellTrace™ Far Red DDAO-SE (Invitrogen) in anhydrous DMSO to 1 mM. All dilutions of stock prior to addition to cells must be made in DMSO. Harvest 1 mL cell suspension in PBS at a density of 1 x 10^6 cells/mL, then prepare for each cell line. Wash cells twice and resuspend in 1 mL of PBS to each tube of cells, add CellTrace™ Far Red DDAO-SE to give final concentrations of: Jurkat cells – 1000 nM, A431 cells – 100 nM. Incubate at 37°C in the dark for 15 minutes. Stop the labelling reaction by addition of 1 mL per tube of 4% FBS in PBS. Incubate the stop mixture at 37°C for 10 minutes. Wash three times with culture medium. Remove supernatant and resuspend in 1 mL per tube of culture medium to give 1 x 10^6 cells/mL.

EGFR assay set up

∝ EGFR mAb was serially diluted in culture medium and 20 µL added to wells of a 384 microplate (Costar #3712). A suspension of cells (either A431 or Jurkats) containing 1.25 x 10^5 cells/mL and 800ng/mL ∝mouse IgG AF488 conjugate was prepared. The assay was started by adding 20 µL of cell suspension to each well (final concentrations were 2500 cells/well and 400 ng/mL detection conjugate). Microplates were incubated for 2 hours at 37°C before the amount of ∝ EGFR bound to the cells was determined by scanning on a mirrorball microplate cytometer.

cell multiplexing

20 µL of ∝ EGFR mAb, serially diluted as described above was added to wells of a 384 microplate. A suspension of equal numbers of (EGFR+) A431 cells and (EGFR-) Jurkat cells containing a total of 1.25 x 10^5 cells/mL and 800ng/mL ∝mouse IgG AF488 conjugate was prepared. The assay was started by adding 20 µL of cell suspension to each well (final concentrations were 2500 cells/well and 400ng/mL detection conjugate). Following a 2 hour incubation at 37°C both staining and antibody binding were quantified by simultaneously scanning the microplates with 488 nm and 640 nm lasers on a mirrorball microplate cytometer.

mirrorball data acquisition and scanning

Samples were scanned using 488 nm and 640 nm lasers as specified. Objects in each well were identified "on-the-fly" using TTP Labtech patented threshold algorithms and both morphological and fluorescence parameters were reported as described previously by Bowen and Wylie (2). Data can be visualised in a variety of ways including histogram or scatter diagrams, 3D fluorescence intensity profiles, or whole well images using mirrorball's Cellista software.

results

detection sensitivity of EGFR mix-and-read assay

In this study, in order to assess the detection limit of EGFR in a cell-based antibody binding assay, A431 and Jurkat cells were incubated with serial dilutions of anti-EGFR mAb and AF488 conjugated goat ∝mouse IgG. Figure 1 shows that the detection level of EGFR in A431 cell line is 5 ng/mL, and there is no binding detected in the Jurkat cell line.

The data shown here using mirrorball demonstrates a similar detection level of EGFR to that observed in a study with the now obsolete ABI 8200 (FMAT).

multiple laser scanning

mirrorball's triple laser capability allows the user to monitor cell count in addition to the detection of multiple fluorescent labelled markers. In this study, cells were labelled with CellTrace Far Red and multiplexed with ∝EGFR mAb using an AF488 conjugate.

Following overnight incubation, cell count and EGFR labelling were analysed using

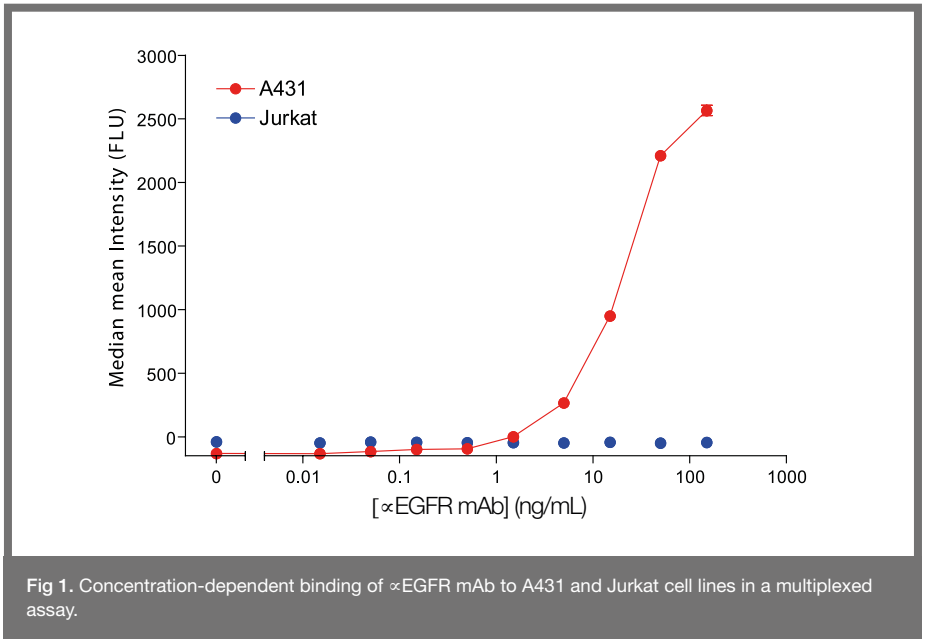

Fig 1. Concentration-dependent binding of ∝EGFR mAb to A431 and Jurkat cell lines in a multiplexed assay.

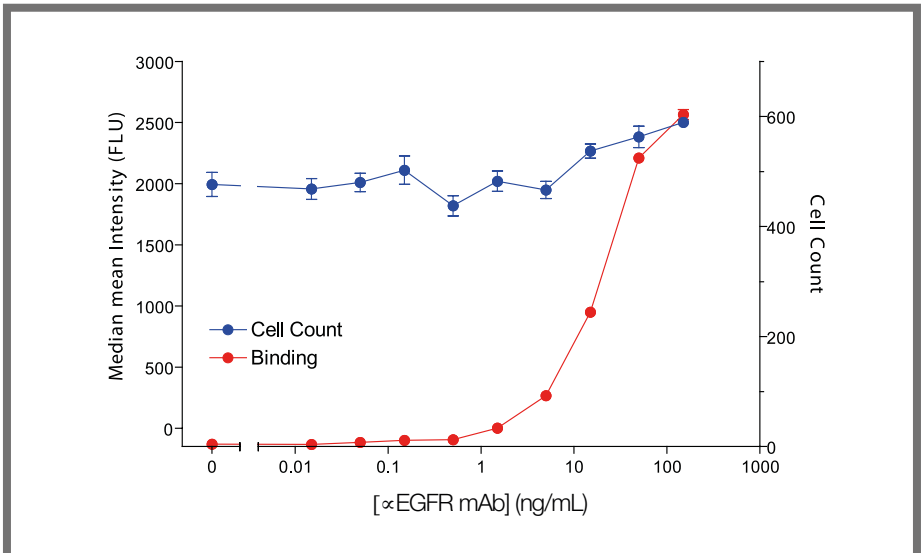

Fig 2. Concentration-dependent ∝EGFR mAb binding to A431 cells. Associate cell counts were derived from independent cell staining with CellTrace Far Red.

dual lasers at 488 nm and 640 nm. Figure 2 shows consistent cell counts alongside an increase in EGFR binding with concentration. Importantly, cell enumeration was possible in the absence of EGFR binding.

cell multiplexing

mirrorball's unique ability to scan simultaneously with multiple lasers allows the differentiation of antigen-expressing and non-expressing cells in a mixed culture of transfected and parental cells. In order to demonstrate this, a mixed suspension of A431 cells (EGFR+) and Jurkat cells (EGFR-) were screened for EGFR binding. Figure 3 demonstrates 3D fluorescence intensity profiles of individual A431 and Jurkat cells for 100 ng/mL E GFR.

discussion

TTP Labtech's mirrorball microplate cytometer provides a robust, "mix-and-read" method for the screening of antibodies against cell surface antigens. It is ideally suited for antibody discovery and its unique optics and software enables highly sensitive detection of binding to proteins that are expressed in low abundance.

The EGFR assay set-up demonstrated in this application note proved straightforward and required minimal development to run on a mirrorball. These "mix-and-read" assays reduce screening times by eliminating the requirement for wash and incubation steps compared to traditional ELISA procedures. In addition, these assays are cost efficient due to decreased sample and reagent usage. mirrorball is the first system in its class to offer simultaneous scanningwith multiple lasers, allowing direct correlation of fluorescence across lasers. When combined with appropriate fluorescent reagents, this unique capability allows independent identification of cells and multiplexing, enabling increased throughput, and producing substantial cost savings and convenience. This was exemplified in the studies using CellTrace Far Red counterstaining where a total cell count could be reported for all wells irrespective of the level of antibody binding. Such data eliminates false positives due to cell seeding variability or cell toxicity.

In addition, the ability to identify the presence of a gene product in wells containing both parental and daughter cell lines using mirrorball has the potential to speed up the screening process significantly. Such an approach was exemplified in this application note by concurrently measuring anti-EGFR binding to A431 and Jurkat cells combined in a single well. Cell multiplexing provides many benefits; it eliminates interplate variability across cell types, doubles assay throughput and minimises sample requirement.

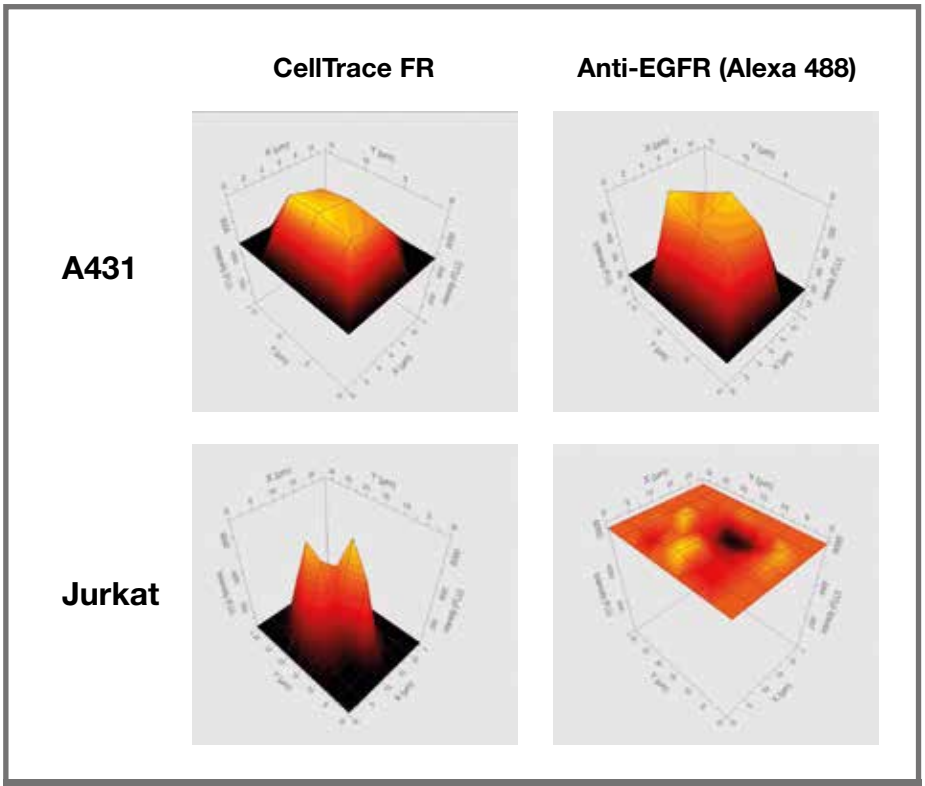

Fig 3. Three dimensional cytometric analysis of single cells. mirrorball's unique ability to scan simultaneously with multiple lasers allows differentiation of A431 and Jurkat cells (based on CellTrace Far Red staining and quantification of anti-EGFR binding (AlexaFluor 488). Data was analysed using mirrorball's Cellista software.

references

1. Lee, R., Tran, M., Nocerini, M. and Liang, M. (2008) A High-Throughput Hybridoma Selection Method Using Fluorometric Microvolume Assay Technology. J. Biomol. Screening 13: pp210-17.

2. Bowen, W. and Wylie, P. (2006) Application of Laser-Scanning Fluorescence Microplate Cytometry in High Content Screening. Assay and Drug Development Technologies. 4: pp209-21.

specifications

TTP Labtech's mirrorball is a high sensitivity microplate cytometer for fluorescence applications. mirrorball enables rapid screening of a broad range of cell and bead-based assays and is equipped with multiple lasers and data collection channels for multiplexed experimental protocols.

Detection technology:	Laser scanning fluorescence cytometry
Laser excitation:	Up to 3 solid state lasers in a single instrument (choice includes 405, 488, 640 nm)
Multicolour detection:	Up to 5 colours simultaneously using photomultiplier tubes
Laser scatter detection:	Single channel (beads only)
Sample format:	96, 384 and 1536 SBS-format microplates
Scan area:	Whole well
Throughput:	Typically 10 - 15 minutes regardless of plate type
Control & analysis software:	Cellista
File export:	Cellista plate files, CSV, FCS and open source TIFF (16-bit)
PC operating system:	Microsoft® Windows® 7 (Professional)
Configurations:	Stand-alone, self-maintained workstation or fully integrated
Laser safety:	Class 1 laser product
Dimensions:	645 mm x 513 mm x 350 mm (25" x 20" x 14") (w x d x h)
Net weight:	Approx 60 kg (132 lbs)
Services:	110/230 V single phase 47/63 Hz 800 W

designed for discovery

discover@ttplabtech.com
www.ttplabtech.com