FluoroSpot-

a tool for detection of multiple protein secretion at the single cell level

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The principle objective of the FluoroSpot assay is the simultaneous measurement of dual cytokine secretion at the single cell level. This is accomplished by using a mixture of monoclonal antibodies (mAbs) with different cytokine specificities in a sandwich assay. Unlike the related ELISpot method, the FluoroSpot assay employs fluorophore-labeled detection reagents, which enable analysis of several cytokines or other secreted analytes in the same well.

The ELISpot assay has proven particularly useful when studying specific immune responses in infections, cancer, allergies and autoimmune diseases. Due to its simplicity and high sensitivity, it has become a standard tool in the development of new vaccines and monitoring of vaccine trials. However, due to the enzyme/substrate based detection, ELISpot is limited with regard to the number of different cytokines/analytes which can be analyzed in each well. Usually only one parameter per well is analyzed and IFN- has been the analyte of choice serving as an immunocorrelate for T-cell responses.

To increase the versatility of the method and in response to the recent interest in antigen specific polyfunctional T cells[1-8], Mabtech has developed FluoroSpot assays that differentiate between cells secreting one or both of two cytokines in the same well[9-11].

The FluoroSpot assay, which is as sensitive and simple to perform as the ELISpot, utilizes biotinylated detection mAb for one analyte and tag-labeled detection mAb for the other analyte (Fig. 1). The detection step is amplified by secondary addition of Streptavidin conjugated to a red fluorophore and an anti-tag mAb labeled with a green fluorophore. Spot analysis is performed with an automated fluorescence reader generating separate images for the two fluorophores. Two-colored spots, derived from cells secreting dual cytokines are identified by an analysis of co-positioned spots in a digital overlay of the single stain images (Fig. 2). This procedure currently allows for analysis of two analytes in the same well, but can principally be extended to detect three or more analytes simultaneously. Experimental systems for three-color FluoroSpot have been described[12] but the method needs further development before commercial reagents are made available.

Example of a FluoroSpot assay procedure for IFN- /IL-2 staining:

1) *A mixture of capture mAbs specific for IFN- and IL-2 is added to an ethanol treated 96-well PVDF plate.*
2) *Cells are added in the presence or absence (control) of specific stimulus.*
3) *Activated cells start to produce and secrete cytokines that bind to the capture mAbs.*
4) *Cells are removed and a mixture of tag-labeled anti-IFN- and biotinylated anti-IL-2 detection mAbs is added.*
5) *To amplify the detection, a mixture of anti-tag-green and Streptavidin-red is added.*
6) *Finally, a fluorescence enhancer solution is added.*

7) *The dried plate is preferably analyzed in an automated fluorescence plate reader fitted with separate filters for the two fluorophores.*

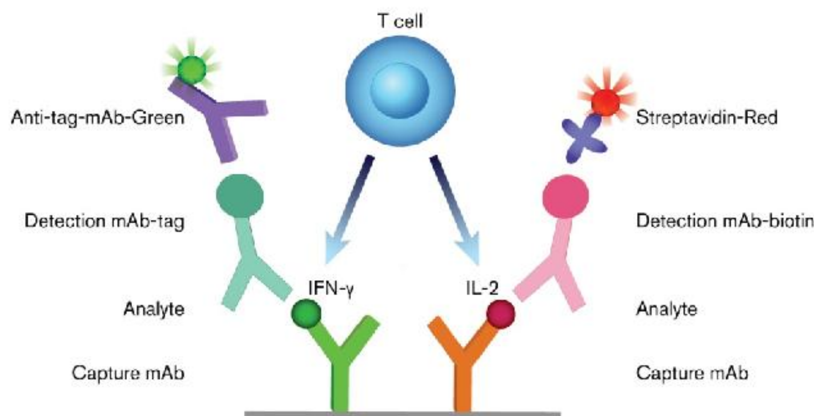


Figure 1. Principle of the FluoroSpot assay

Applications

The FluoroSpot assay is well suited for individual tests as well as for large scale screening. The current method offers several advantages over ELISpot and can be applied to essentially any system where one would like to monitor dual protein secretion at the single cell level. As more than one cytokine can be analyzed in the same sample, a more detailed characterization of T-cell populations can be achieved. Due to its higher sensitivity compared to flow cytometry, the FluoroSpot method is especially attractive in conditions where the producing cells represent only a small fraction of the cell population. The ability to analyze two analytes in the same well is also a great benefit when the supply of cells is limited.

T cells

An interest in many vaccine development studies is to identify antigen-specific polyfunctional T cells. In several studies, cells co-expressing IFN- and IL-2 in particular, but also other cytokines like TNF- and MIP-1 , have been suggested to represent cells more closely related to protective immunity than cells expressing only one of these cytokines[1-8]. Figure 2 shows a FluoroSpot analysis of cells secreting IFN- and/or IL-2 in response to the CEF pool of MHC class-I restricted peptides derived from EBV, CMV and Flu[14]. The CD8+ T-cell mediated response was dominated by IFN- secreting cells of which a portion also secreted IL-2. The majority of IL-2 secreting cells also secreted IFN- .

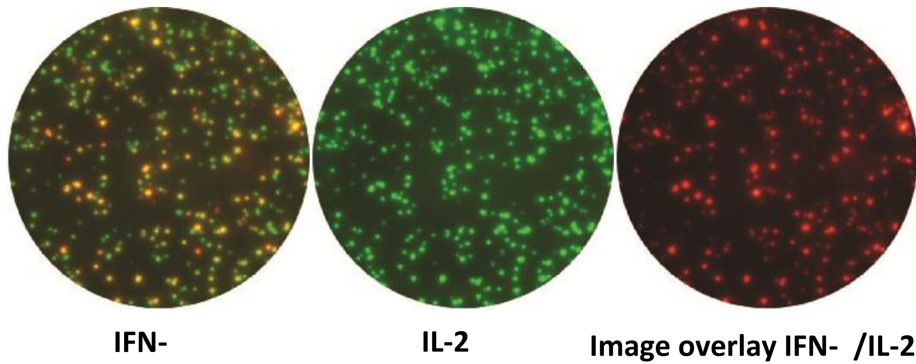


Figure 2. FluoroSpot analysis of human IFN- /IL-2 secreting cells. Peripheral blood mononuclear cells (PBMC; 200 000/well) were stimulated for 16 hours with a pool of MHC class I-restricted peptides14 derived from CMV, EBV and Flu (available from Mabtech as "CEF peptide pool") in the presence of anti-CD28 antibody. Cells secreting both cytokines (about 40% of responding cells) create yellow spots in an overlay of green and red images. The number of double colored spots in control wells with unstimulated cells were <1. Spot analysis was made with an iSpot reader from AID (Autoimmun Diagnostika GmbH).

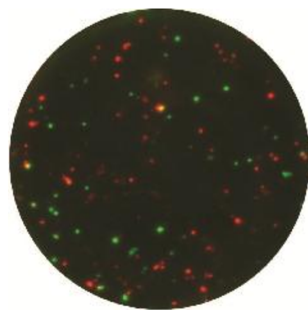


Image overlay IFN- /IL-17A

Figure 3. FluoroSpot analysis of human Th1 and Th17 cells in the same well. PBMC (200 000 cells/well) were stimulated for 16 hours with an antigen extract from Candida albicans. Around 3% of the responding cells were double positive.

One recently published example of the value of the FluoroSpot method to delineate functional T-cell subsets is a study that distinguishes different clinical stages of human tuberculosis infection by using single and dual cytokine profiling[11].

As previously mentioned, an advantage of the FluoroSpot assay is the ability to gain information about different cytokines, which define cellular subsets, in a single well. This will reduce not only the amount of cells needed but also the cost of analysis.

Other examples include the combined analysis of T helper 1 (Th1) versus Th2 or Th1 versus Th17 responses (Fig. 3).

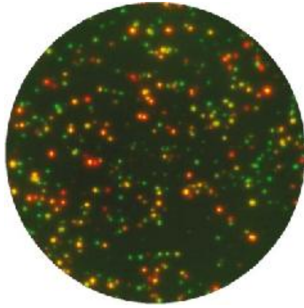
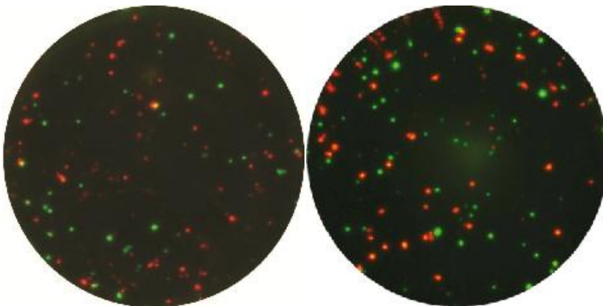


Image overlay IL-6/GM-CSF

Figure 4. FluoroSpot analysis of cytokine secretion by human monocytes. Lipoteichoic acid (LTA) was used to induce Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and IL-6 secretion by purified PBMC. Cells (4000 cells/well) were incubated for 20 hours in the presence or absence of LTA. In the image overlay, yellow spots represent cells secreting both cytokines (about 30% of secreting cells). Wells with cells cultured in medium alone (no stimuli) are not shown but contained <5 spots.

Other immune cells

In addition to T cells, FluoroSpot may also be used to analyze other cells such as monocytes and B cells. Figure 4 shows an analysis of cytokine secretion by monocytes where it may be useful for delineating distinct subpopulations based on their cytokine profile.



(A) Flu-specific IgA (green) and IgG (red) secreting B cells

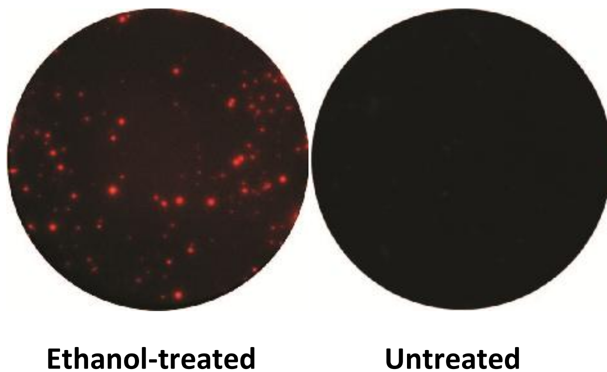
(B) Total IgA (green) and IgG (red) secreting B cells

Figure 5. FluoroSpot analysis of swine flu specific IgA and IgG secretion by in vivo activated human B cells. PBMC were collected before and one week after vaccination with Pandemrix. Wells were coated with 0.75 µg of antigen per well. After overnight culturing of PBMC (200 000 cells/well), spots were detected by adding a mixture of anti-IgA-green and anti-IgG-red. There were no detectable spots before vaccination whereas after vaccination, the frequencies of Ag-specific IgA- or IgG-secreting B cells were approximately one per 5000 PBMC (A).Analysis of B cells secreting IgA and IgG is shown as a reference (B). Wells were coated with a mixture of anti-IgG and anti-IgA capture mAbs. After overnight culturing of PBMC (100 000 cells/well), spots were

detected by adding a mixture of anti-IgA-green and anti-IgG-red. (A) Flu-specific IgA (green) and IgG (red) secreting B cells. (B) Total IgA (green) and IgG (red) secreting B cells.

The FluoroSpot method can identify and enumerate both the total number of antibody-secreting cells in a sample and those secreting antibodies to a specific antigen. Detection based on fluorophores offers the possibility to analyze the secretion of different immunoglobulin isotypes in the same well and thereby saving cell material. Major application areas are detection of B-cell responses in various diseases and those elicited by vaccination.

An example of a vaccine-induced response to swine flu (H1N1) is shown in Figure 5, where the number of antigen-specific IgA- and IgG-secreting cells was investigated before and after vaccination with Pandemrix swine flu vaccine (GlaxoSmithKline).



Ethanol-treated **Untreated**

Figure 6. Impact of ethanol treatment of low autofluorescence PVDF plates prior to the coating step in human IL-2 FluoroSpot analysis. The ethanol treatment will enable more capture antibodies to bind to the membrane which will increase spot number and spot quality.

Technical aspects

Several aspects of the FluoroSpot assay protocol are critical to achieve optimal detection of spots. The choice of highly specific mAbs is the foundation for other aspects of optimization. One important factor that will influence the quality and the number of spots is the concentration of capture antibody. The use of plates with PVDF membrane briefly pre-treated with ethanol prior to coating (Fig. 6), enables efficient binding of capture mAbs. A high density of the capture mAbs is especially critical when monitoring cell populations which secrete low amounts of cytokine. Plates with low autofluorescence PVDF membranes are recommended. After the final washings, a fluorescence enhancer solution is added to the plate to increase the fluorescent spot signals.

Analysis of plates

We recommend the use of an automated reader with filters for FITC and Cy3. The plate should be completely dry before analysis. Although the fluorescence in dry plates has been found to be stable (plates can be saved for months), it is recommended to analyze the plate within one week of development since the PVDF membrane may brighten over time.

Although of low significance, false dual spots may occur, i.e. two adjacent cells secreting either of the two cytokines of interest may erroneously be counted as a dual spot. The number of such spots is primarily dependent on the number of spots of each cytokine in the well and the reader count settings (e.g. maximal distance between spot centers). One way to practically estimate the potential frequency of false dual spots in any given experiment is to analyze overlays of green and red images from different wells[12]. All dual spots in such non-matched overlays will represent false positive spots.

Stimulation of CD28

While antigen-specific T-cell stimulation is critically dependent on the interaction between the T-cell receptor and its cognate antigen, additional co-stimulatory signals from antigen presenting cells and the surrounding cytokine environment are also required. These signals are not always optimally provided under in vitro cell culture conditions, which in turn may lead to suboptimal responses. Furthermore, in the FluoroSpot analysis, the possible biological effect of cytokine capture has to be taken into consideration13.

IL-2 is pivotal in T-cell activation and the presence of IL-2 capture mAbs in a well may result in a reduced availability of free IL-2 in the culture, which in turn may result in reduced activation of IFN- secreting T cells. To compensate for possible suboptimal conditions in the *in vitro* culture and to circumvent the attenuating effect of IL-2 capture, anti-CD28 mAb can be added to the culture. An appropriate concentration of anti- CD28 mAb will not increase the spontaneous secretion of cytokines in the absence of specific stimulus but will elevate the stimulator-specific secretion of IL-2 and thus compensate for the effect of cytokine capture. Alternatively, cells can be pre-activated (about 6h) in vials, prior to their addition to the plate, in which case the “capture effect” will be negligible. Other anti-cytokine capture mAbs may also cause “capture effects” and therefore stimulation of CD28 may be useful in many systems.

Positive controls in FluoroSpot

Relevant controls are crucial in FluoroSpot. The use of a polyclonal T-cell activator, e.g. certain antibodies to CD3, may serve as a positive control both for cell viability and assay functionality. A multitude of T-cell cytokines are elicited by this stimulation and this control can thus be used in a variety of FluoroSpot assays. Other commonly used positive controls include phytohemagglutinin (PHA) and concanavalin A (ConA). Both are potent T-cell activators and can be used in a similar way as anti-CD3 antibodies for activation of CD4+ and CD8+ T cells.

A more specific positive control is the CEF peptide pool14 mentioned earlier, which is based on antigens to which most people have specific T cells. It efficiently induces IFN- production by virus-specific CD8+ T cells in almost 90% of Caucasians. In addition to serving as a positive control, the CEF peptide pool is useful for standardization procedures.

In summary

Mabtech has worked extensively for more than 20 years to optimize the ELISpot protocol. Building on this experience, we have during the last years developed and

optimized the FluoroSpot protocol by determining the best capture/detection mAb pairs, fluorophores, concentration of reagents, choice and treatment of plates, etc. Our goal is to ensure delivery of analytical systems with the most accurate performance.

Mabtech, June 2011

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