Research Techniques Made Simple: Monitoring of T-Cell Subsets using the ELISPOT Assay

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The enzyme-linked immunospot (ELISPOT) assay allows characterization of single-cell immune responses through detection of secreted analytes. Although ELISPOT analysis shares similarities with ELISA, it has some essential differences. In general, the ELISPOT assay uses antibodies to capture and detect analytes of interest released by activated immune cells. Released analytes form specific antibody-antigen complexes and are visualized as spots of enzyme-substrate precipitates. These spots indicate both how many cells secrete the respective analyte and how much analyte is produced per individual cell. Initially developed for the detection of antibody-secreting cells, ELISPOT assays are now frequently performed both in the context of clinical diagnostics and in research on T-cell responses, in particular antigen-specific T-cell subpopulations, as related to allergy, cancer, infections, or autoimmune diseases. The *one spot-one cell* principle allows sensitive detection of specific and rare immune cell subsets. Here we present general principles, applications, and recent modifications of the ELISPOT technique.

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INTRODUCTION

T lymphocytes are a central component of the adaptive immune system that react against pathogens like microbes and viruses. Somatic hypermutation and recombination create a repertoire of T-cell receptors (TCR) to allow selective recognition of pathogenic factors, called *antigen specificity*. T-cell memory is based on specific TCR pools increasing during the entire individual life span. Antigen-specific T cells discriminate between *self* and *nonself* antigens in the adaptive immune response and become activated to in turn stimulate B-cell clones harboring identical antigen specificities. Therefore, the development, activation, and clonal expansion of antigen-specific T cells are critical steps for the adaptive immune response. Antigen-specific T cells are key players in allergy, host defense, and autoimmunity. Therefore, this cell subset is of interest in immunological and clinical research. Antigen-specific T cells and, in particular, their memory subsets occur with very low frequencies. Thus, only very sensitive techniques like the enzyme-linked immunospot (ELISPOT) assay are capable of detecting and analyzing T-cell subsets.

The first ELISPOT assay was described in 1983 for the enumeration of specific antibody-secreting lymphocytes (Czerkinsky et al., 1983; Sedgwick and Holt, 1983) and was further developed in recent decades. Currently, ELISPOT assays are performed for applications ranging from basic research to clinical diagnostics as a highly sensitive and effective method for detection of low-frequency cell subsets on a single-cell level, including T and B lymphocytes, monocytes, dendritic cells, and natural killer cells.

GENERAL PRINCIPLES

Although the ELISPOT assay is based on a detection principle comparable to ELISA, described by Gan and Patel (2013),

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WHAT THE ENZYME-LINKED IMMUNOSPOT (ELISPOT) ASSAY DOES

- The ELISPOT assay enables detection of cytokine or effector molecule secretion on a single-cell level.
- ELISPOT analysis allows for ex vivo characterization of cell function, including antigen-specific T-cell monitoring.
- The ELISPOT assay has a higher sensitivity than the enzyme-linked immunosorbent assay (ELISA) or intracellular staining, facilitating measurement of very low numbers of analyte-producing cells (i.e., as low as 1 cell in 300,000).
- The ELISPOT assay facilitates high-throughput cell screening with low intra- and interassay variability.
- Because cells are not fixed or killed during the ELISPOT procedure, they can be further characterized in subsequent experiments.

LIMITATIONS

- The ELISPOT assay is not applicable for analysis of whole blood but requires isolation of peripheral blood mononuclear cells (PBMCs), tumor-infiltrating lymphocytes, or other cell subsets.
- Detection of rare antigen-specific T cells is highly dependent on an appropriate stimulus.
- Currently, the ELISPOT/FluoroSpot assay only allows simultaneous detection of four analytes.
- The ELISPOT assay measures only the release of soluble analytes and does not allow for further cell phenotyping.
- Spot counts are strictly limited to membrane area per well.

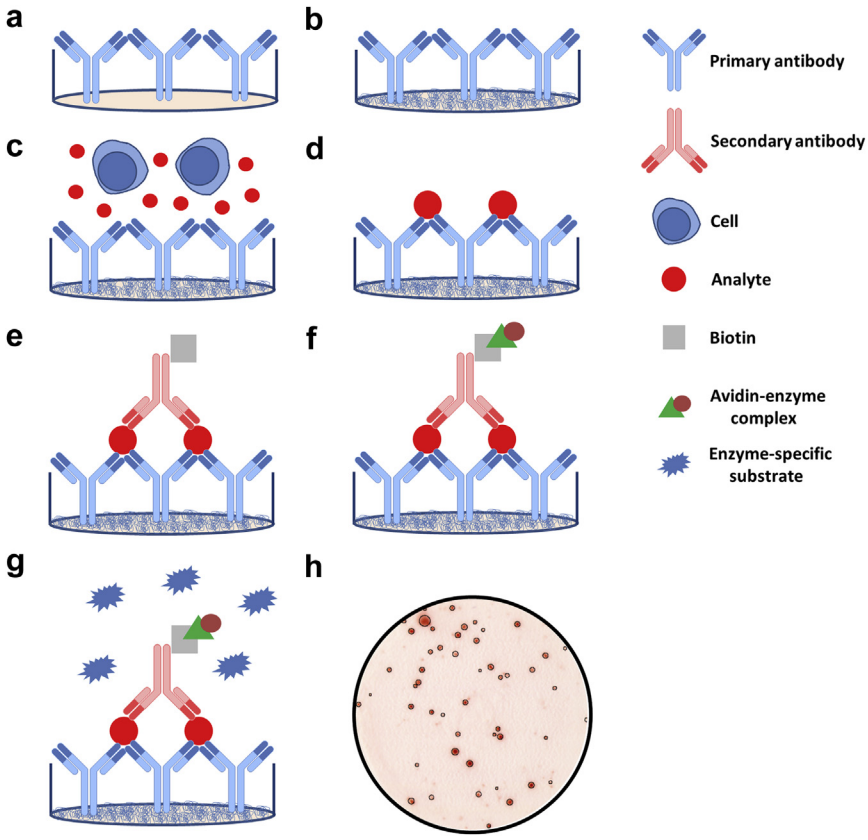

Figure 1. ELISPOT principle. (**a**) Coating of ELISPOT membranes (microwells) with primary (capture) antibody. (**b**) Blocking of unspecific binding sites by saturation of the membrane with protein solution (e.g., phosphate buffered saline + 10% fetal calf serum). (**c**) Addition of a single-cell suspension and activation of cells to secrete analytes (red circles) using an appropriate stimulus. (**d**) Analyte binding to the specific capture antibody followed by removal of cells. (**e**) Addition of a secondary biotinylated (detection) antibody, which binds to the respective analyte-antibody complex. (**f**) Binding of avidin- or streptavidin-conjugated enzyme (e.g., horseradish peroxidase) to the biotinylated detection antibody. (**g**) Addition of chromogenic substrate solution and visualization of spots by enzymatic substrate transformation. (**h**) Representative image of an ELISPOT membrane that can be automatically analyzed by an ELISPOT reader.

it has some essential differences. ELISA measures total analyte concentrations in supernatants removed from cultured cells, whereas ELISPOT analysis defines the frequency (i.e., the number of analyte-secreting cells within a total cell population) and the secretion intensity (i.e., the productivity of an individual cell to secrete the analyte of interest) of cell populations by capturing specific molecules (e.g., cytokines, chemokines, and immunoglobulins) released by activated cells. In general, ELISPOT assays are performed in 96-well plates with nitrocellulose or polyvinylidene difluoride membranes. As a first step, a specific primary antibody against the analyte of choice is coated onto the membrane (Figure 1). After blocking to avoid nonspecific binding of other proteins or biomolecules, single-cell suspensions like peripheral blood mononuclear cells (PBMCs) or tumor-infiltrating lymphocytes in combination with an appropriate stimulus are added to the wells of the ELISPOT plate for a defined incubation time, usually between 2 and 24 hours. Primary antibodies in the immediate vicinity of an activated cell continuously capture the secreted analyte over the entire incubation period. After cell removal, the antibody-antigen complexes are visualized by the stepwise addition of biotinylated secondary detection antibody, enzyme-labeled conjugate, and substrate solution. To detect more than one analyte, enzyme-conjugated and tag-labeled secondary antibodies are used. This will be discussed in more detail in the following sections.

Of note, in contrast to the intracellular detection of analytes by flow cytometry, cells that are carefully removed from the ELISPOT membrane are still viable and can be used for subsequent characterization using other experimental approaches (Meiklejohn et al., 2004). Enzyme-substrate interactions lead to formation of chromogenic precipitates at sites of analyte accumulation, appearing as spots. Visible spots are detected and can be enumerated automatically by special ELISPOT readers, and each individual spot represents a single cell that has released the analyte of interest (Figure 1). Attention should be paid to cell densities and incubation times used in the assay, because high cell numbers and excessive analyte secretion might lead to overlay of individual spots. As mentioned, in addition to cell frequencies, information on the analyte-secreting capacity of a particular cell is given by ELISPOT analysis. The size of a spot directly correlates with the amount of analyte produced by a single cell, providing further information on cell functionality. Thus, the ELISPOT technique is a sensitive method for characterization of analyte release on a single-cell level.

APPLICATIONS

ELISPOT analysis is used in multiple research areas including allergy, infectious diseases, cancer, vaccination studies, and autoimmunity. Because the reproducibility is high and standardization across various laboratories applying validated protocols is quite feasible, the ELISPOT assay represents one of the central methods in clinical diagnostic settings for the investigation and characterization of antigen-specific T cells as, for example, in the context of tracking immune responses

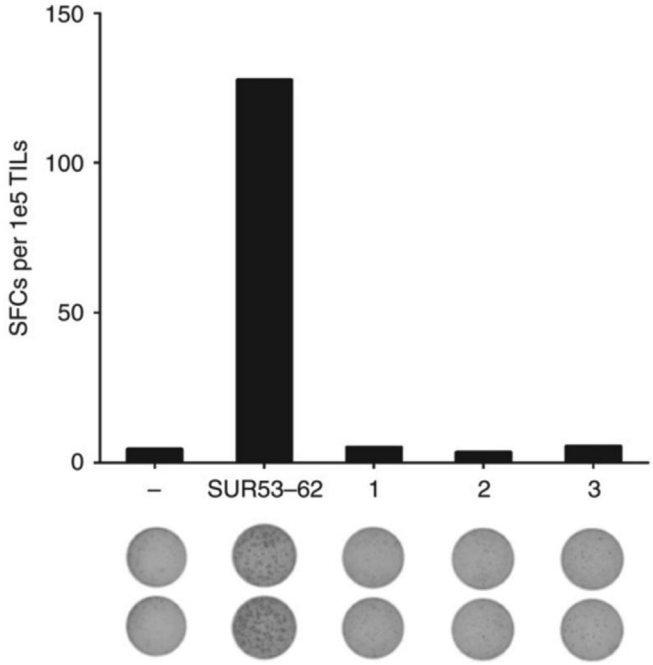

Figure 2. IFN-γ ELISPOT assay from IL-2–expanded TILs after peptide stimulation. Shown are background counts of unstimulated TILs (–) and TILs incubated with "irrelevant peptides," (1) Bcl-X(L) 165–173 (RIAAWMATY), (2) Rho1 (RAGLQVRKNK), and (3) Rho1L2 (RLGLQVRKNK), which did not induce IFN-γ secretion compared with the specific IFN-γ response after stimulation with the survivin-derived epitope SUR53-62 (DLAQCFFCFK). The bars of spot-forming cells represent the number of unstimulated (–) and peptide-stimulated TILs. Respective raw data are depicted by images of ELISPOT membranes in duplicate (reprinted from Junker et al., 2012, with permission from Elsevier). SFC, spot-forming cell; TIL, tumor-infiltrating lymphocyte.

to vaccines (Junker et al., 2012; Slota et al., 2011) (Figure 2), cytomegalovirus (Godard et al., 2004), and tuberculosis (Achkar et al., 2011). Other advantages of the ELISPOT assay lie in the fact that application of both fresh and frozen cell samples show comparable outcomes (McCutcheon et al., 1997) and that high-throughput cell screening is, in contrast to other analyte-detecting approaches (i.e., ELISA, flow cytometry, and real-time PCR), rather simple to perform. For instance, even without automatization, using the standard 96-well ELISPOT assay enables testing of hundreds of samples in parallel under the same conditions. This makes the ELISPOT assay attractive for use in multicenter trials and longitudinal studies, where samples are collected over time, and in field studies where further processing after isolation of single-cell suspensions is impractical. In addition, frozen cell samples can be included as an internal standard to unveil putative interassay variability. Concerning the measurement of released analytes in cell samples, application of the ELISPOT assay is limited only by optimal matching of primary and secondary antibody pairs. A wealth of ELISPOT assays are commercially available for the investigation of common immune cell-associated extracellular proteins like cytokines (e.g., IL-2, IL-10, IFN-γ, tumor necrosis factor-α), chemokines (e.g., CXCL8), proteases (e.g., granzyme B), and growth factors (e.g., granulocyte-macrophage colony-stimulating factor, transforming growth factor-β).

MODIFICATIONS IN THE ELISPOT TECHNIQUE

Stimulus-dependent spot numbers

In general, isolated cell populations can be easily monitored using the ELISPOT assay after in vivo or ex vivo activation. However, the selected stimulus is of particular importance

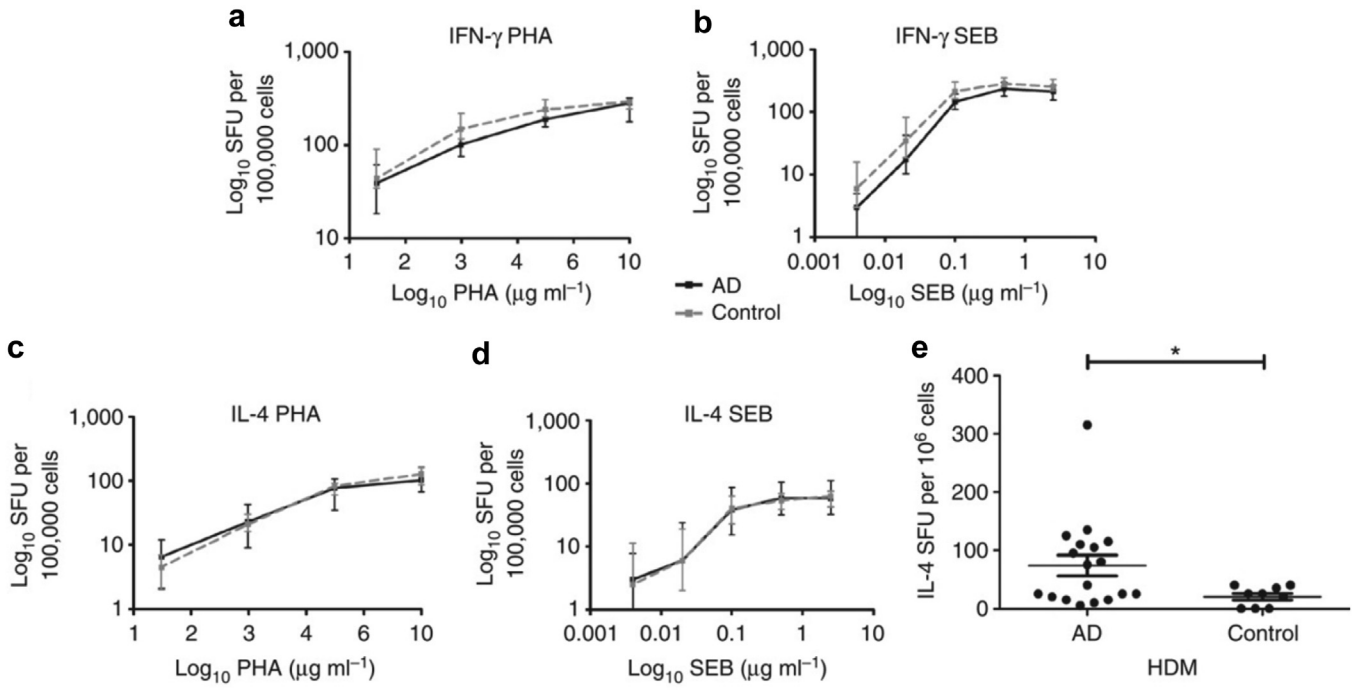

Figure 3. Stimulus-dependent cytokine secretion of PBMCs from patients with AD and healthy control subjects. (**a, b**) IFN-γ and (**c, d**) IL-4 ELISPOT assay of ex vivo PBMCs activated with either (**a, c**) PHA or (**b, d**) SEB in patients with AD (black lines) and in control subjects (gray lines). (**e**) Ex vivo PBMC IL-4 ELISPOT stimulated with house dust mite extract. AD, n = 18; control, n = 10. *$P < 0.05$. (reprinted from Newell et al., 2013, with permission from Elsevier). AD, atopic dermatitis; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; SEB, staphylococcal enterotoxin B; SFU, spot-forming units.

because the nature of antigen (e.g., peptide, whole protein, or mitogen) determines the activation of distinct cell subsets. For example, in vitro T-cell activation by the non-TCR mitogen phytohemagglutinin or by a staphylococcal enterotoxin B-induced direct TCR signal showed no significant differences in T helper (Th) 1 and Th2 cell responses between PBMCs from atopic dermatitis patients and healthy control subjects (Newell et al., 2013). After stimulation of PBMCs with the specific antigen (house dust mite extract), a subset of IL-4–secreting Th2 cells could be detected in patients with atopic dermatitis, which was absent in the control cohort (Figure 3). However, in some instances, that is, for the detection of very infrequent antigen-specific cells, in vitro expansion of the particular cell population might be necessary. By using ELISPOT protocols including both the addition of IL-2 (±IL-7 and/or ±IL-15) followed by restimulation with the respective antigen, an adequate and selective in vitro expansion of antigen-specific T cells can be achieved (McCutcheon et al., 1997; Meiklejohn et al., 2004; Möbs et al., 2010).

Dual color ELISPOT

Important progress in development of the ELISPOT technique was made by introducing the capacity to analyze secretion of more than one analyte from the same cell. In this modified approach, termed *dual color ELISPOT,* two different chromogenic substrates are applied to distinguish between cells expressing two different analytes. For example, after using BCIP/NBT (i.e., 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) as the first substrate solution, blue-colored precipitates appear as spots, indicating high local concentrations of one analyte. In a second incubation step, adding AEC (i.e., 3-amino-9-ethylcarbazole) substrate leads to red spot development where cells secreted the other analyte. Purple-colored spots would then represent single cells secreting both analytes. By application of the dual color ELISPOT technique, up to three distinct cell populations can be distinguished simultaneously in one well. Apart from more information on the individual cell level, this advance in the ELISPOT technique circumvents common difficulties with limited cell numbers, as occurs in clinical trials.

FluoroSpot

A further modification of the ELISPOT method was the implementation of use of fluorescent dyes in the so-called FluoroSpot assay. Simultaneous detection of more than two analytes was limited by the inability of chromogenic detection systems to dissect multicolored spots. In addition to biotinylated detection antibodies, FluoroSpot assays use tag-labeled detection antibodies to enable analysis of two or more analytes in the same well. To amplify analyte detection, different anti-tag- and anti-biotin-fluorophores are added, and after incubation with fluorescence enhancer solution, spots are visualized using an automated fluorescence reader with separate emission filters for each fluorophore. Spot analysis of individual cells secreting more than one analyte is performed by the digital overlay of single fluorescent images. Currently the FluoroSpot method enables parallel detection of up to four analytes using fluorophore-labeled detection reagents. Because of the high number of fluorochromes available, further developments in the FluoroSpot technique, including the detection of even more analytes, can be expected in the near future.

MULTIPLE CHOICE QUESTIONS

1. What does the ELISPOT assay detect?
   A. Total analyte concentrations in culture supernatants
   B. Surface marker expression
   C. Analytes released from cells
   D. Tissue-bound antibodies
2. What source material is needed for ELISPOT analysis?
   A. Whole blood
   B. Tissue
   C. Serum
   D. Single-cell suspensions
3. ELISPOT assays are based on which principle?
   A. One spot-one cell
   B. One spot-one analyte
   C. One spot-one enzyme
   D. One spot-one antibody
4. What might be one reason for the overlay of spots in the ELISPOT assay?
   A. The concentration of stimulus was too low
   B. The detection antibody was absent
   C. The applied cell density was too high
   D. The blocking period was too long
5. Which is not a characteristic feature of the ELISPOT assay?
   A. High sensitivity
   B. High interassay reproducibility
   C. High intra-assay variability
   D. High throughput

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SUMMARY

The ELISPOT assay is a highly sensitive, relatively easy-to-perform, and reproducible method for identification and quantification of even very rare antigen-specific T cells. ELISPOT analysis allows for both high-throughput analysis of T cell-mediated immune responses involved in allergies, cancer, autoimmunity, and inflammatory diseases and long-term monitoring of antigen-specific T cells in clinical trials, emphasizing the substantial role of this assay applied from basic and biomedical research to clinical trials and diagnostics.

CONFLICT OF INTEREST

The authors state no conflict of interest.

CME ACCREDITATION

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

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