Chapter 13

Objective, User-Independent ELISPOT Data Analysis Based on Scientifically Validated Principles

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Abstract

ELISPOT results used to be evaluated visually which, however, is inevitably subjective, inaccurate, and cumbersome. Even when applying automated image analysis to this end, the results are highly variable if the counting parameters are set subjectively. Since objective, accurate, and reproducible measurements are fundamental to science, major efforts have been undertaken over the last decade at CTL to understand the scientific principles behind ELISPOT data and to develop "intelligent" image analysis algorithms based on these principles. Thus, a spot recognition and gating algorithm was developed to automatically recognize the signatures of defined cell populations, such as T cells, discerning them from irrelevant cell types and noise. In this way, the science of ELISPOT data analysis has been introduced, permitting exact frequency measurement against background. As ELISPOT assays become a gold standard for monitoring antigen-specific T-cell immunity in clinical trials, the need has surfaced to make ELISPOT data transparent, reproducible, and tamper-proof, complying with Good Laboratory Practice (GLP) and Code for Federal Regulations (CFR) Part 11 guidelines. Flow cytometry-based and other immune monitoring assay platforms face the same challenge. In this chapter, we provide an overview of how CTL's ImmunoSpot® platform for ELISPOT data analysis, management, and documentation meets these challenges.

Key words: ELISPOT, T cells, Good Laboratory Practice, Code for Federal Regulations, ImmunoSpot®, SmartCount™, SpotMap™, AutoGate™, Data analysis, Data management, Data documentation, Spot morphology, Spot recognition

1. Introduction

For the past few decades, classic T-cell assays, such as proliferation and killer assays, have been a form of art which could be performed successfully only by highly trained personnel (and those who, in addition, were blessed with a "green thumb"), but would not work reliably for most. Moreover, those assays had the reputation of being rather irreproducible, even by such investigators. The introduction of ELISPOT, intracellular cytokine staining (ICS), and

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multimer analysis (e.g., tetramer and pentamer) for T-cell monitoring promised to move the field closer to attaining reliable measurements on T-cell immunity. Recently, studies have been undertaken to investigate the interlaboratory variability of these assay platforms (1). Identical aliquots of cryo-preserved peripheral blood mononuclear cells (PBMCs) had been sent to the participating laboratories for testing, and the results showed up to 35-, 20-, and 100-fold differences, respectively, within data obtained for ELISPOT, ICS, and tetramer. The authors of this study concluded thus: "The high degree in variability makes the comparison between any two laboratories become a game of chance."

This disturbingly high variability of T-cell assay results obtained by the multicenter initiatives might have had many reasons – certainly a major one shared by all assay platforms being data analysis. Indeed, highly discrepant results were obtained even when a single raw ICS file (acquired on a single flow cytometer) was sent to different experienced investigators for analysis (C. Britten, unpublished). In this setting, all assay- or instrument-related variables were removed, the only variable being data analysis. Alerted by the high variability of the results, the international Society of Biological Treatment of cancer (iSBTc) just initiated an "ICS Gating Panel" project when this chapter was written that invites scientists experienced in ICS to develop a gating harmonization strategy.

Another study published concurrently (2) found the opposite to be the case for ELISPOT: ELISPOT data were highly reproducible among different laboratories, with the maximal variation being 0.42-fold (83-fold less than what was reported in ref. 1). Notably, in this latter study, the participants were all ELISPOT novices, and the results of their first ever ELISPOT assays were obtained and reported. However, all participating laboratories followed the same protocol and the data analysis was done by a fully automated platform. Thus, for obtaining highly reproducible ELISPOT data, it is essential to eliminate subjectivity from spot counting and to replace it by fully automated, scientifically validated, and statistics-based principles for spot recognition and gating. In the following, we will outline how this is accomplished by the ImmunoSpot® Software.

1.1. Spot Morphology and Spot Recognition

One key piece of information to be gained from T-cell ELISPOT assays is the frequency of antigen-specific T cells within the entire sample cell population, as measured by the number of T cells engaged in cytokine production following antigen stimulation. This frequency reflects the clonal size of the antigen-specific T cells, and therefore, the magnitude of T cell immunity. Obviously then, one prerequisite for obtaining accurate frequency information is that both the assay and the image acquisition must be optimized for single-cell resolution.

1.1.1. Antigen Presentation

Each spot within an ELISPOT assay reflects on a single cell's secretory activity. In an interferon gamma (IFN-γ) ELISPOT assay, for example,

IFN-γ is captured by the membrane-bound anti-IFN-γ antibody in the area directly surrounding the secreting cell with its size and density reflecting on the amount of cytokine produced by the cell within the assay's entire duration (3) (see also Notes 1 and 2). Spot size and density are thus critical parameters for ELISPOT data analysis (see also Chapters 11 and 12). The kinetics of cytokine production is also reflected by the spot morphology, i.e., its density and general shape. For example, a rapid secretion rate will produce a large, fuzzy spot, whereas the slow but steady release of cytokine will result in a smaller, denser spot (see Note 3). In ELISPOT assays performed with PBMC, the individual antigen-specific T cell will interact by chance with different types of antigen-presenting cells (APC). Each of these APC has different co-stimulatory property. When a T cell becomes activated by an antigen-presenting B cell, it will produce less cytokine, and will do so in a delayed fashion as compared to antigen recognition on a dendritic cell – antigen presentation by a macrophage will provide an intermediate T-cell response (4). For this reason, the spots seen in T-cell assays involving PBMC are invariably heterogeneous in size and density. When it comes to analyzing such results, therefore, it is not the individual spot, but rather the distributions and population kinetics of all spots within an assay that need to be examined. The ImmunoSpot® software that is designed for user-independent analysis of ELISPOT data recognizes first all spots, irrespective of size and density, and then subjects these spots to statistical evaluation to determine spot distributions.

1.1.2. Antigen Dose

In ELISPOT assays, the antigen dose also affects cytokine secretion rates of T cells and, hence, spots morphologies. Stimulation of a T-cell clone with a high dose of antigenic peptide induces stronger cytokine production in the individual T cells (that is, they produce larger and/or more dense spots) than does the stimulation of the same clone with low-dose peptide (3). When stimulated with a single antigen dose, as is frequently the case in ELISPOT assays, high-avidity T cells within the PBMC will produce larger spots than low-avidity T cells. Similarly, increased T-cell co-stimulation was shown to result in increased per-cell productivity (5).

1.1.3. Pathological Variations

In diseases such as HIV, the per-cell cytokine productivity can be significantly reduced, resulting in smaller spots (6). One of the many advantages of ELISPOT over other assays which measure net cytokines in supernatant (ELISA, CBA/Luminex) or mRNA is the ELISPOT assay's ability to determine whether a decreased net cytokine production is caused by a decreased number of cytokine-secreting T cells or by the reduced per-cell productivity of the same number of T cells. In order to account for physiological and pathological variations in per-cell productivity, ELISPOT data analysis software must therefore be highly versatile, with the ability to

recognize and analyze all variants of spots, by automatically fine tuning the counting parameters. Such fine tuning can be done manually (which will be inherently subjective), or by ImmunoSpot® software automatically, and hence in a user-independent fashion.

1.1.4. Assay-Related and Physiological Variations

Spot morphology varies if different antibodies are used for cytokine detections. A capture antibody with low affinity will produce fainter and more diffuse spots than a high-affinity capture antibody. Furthermore, the spot morphology will vary when different concentrations of the same antibody are used for coating. The durations of the assay can also influence spot morphology significantly. Spots grow in size and density when the assay duration is prolonged and the cells secrete continuously, as is the case for T-cell-derived IFN-γ (3). The outcome is different, however, when there is an early burst of production that comes to a halt before the assay is terminated. In such cases, the spot size will continue to grow even after the production of the cytokine has stopped (due to lateral cytokine diffusion caused by the reversibility of its interaction with the membrane antibodies), but spot intensity will fade due to the dilution of the cytokine. The temperature during enzymatic substrate development and the nature of the substrate will also play a role in defining the spot morphology. Red spots developed with horseradish peroxidase/amino-ethyl carbazole (HRP-AEC) differ fundamentally from the blue alkaline phosphatase-nitro-blue tetrazolium chloride/bromo-4-chloro-3′-indolyphosphate *p*-toluidine (ALPH-NBT/BCIP) spots, with the former being more pristine with a fainter background, while the latter more dramatic and fuzzy with a frequently more heavily stained background.

1.1.5. Background Variations

ELISPOT data analysis is further complicated by the fact that the spots occur over a variable background as is inherent to ELISPOT assays. While the analyte is captured around the secreting cells (resulting in the spots), some of it diffuses into the supernatant, and thereafter gets absorbed on the membrane producing a color carpet. This "ELISA effect" is more pronounced in areas of the well where densities of secreting cells are higher, many times at the edge of the well, resulting in a variable background even within a single well. Accurate ELISPOT data analysis therefore not only requires the precise recognition of various spot morphologies, but these must also be recognized over varying backgrounds over different wells or within a single well. Automatic background correction is crucial for accurate analysis of ELISPOT data and is a key feature of the ImmunoSpot® Software (see Notes 4 and 5).

Recognizing spots of different morphologies over various backgrounds is a challenge for automated ELISPOT data analysis. While counting parameters can be manually fine-tuned to accurately analyze spots on a well-to-well basis (in the same cumbersome and subjective way as it is done for flow cytometry), the SmartCount™ module of the ImmunoSpot® software performs these adjustments

fully automatically for walk-away analysis. The ImmunoSpot® software recognizes first all spots, of all sizes and densities, correcting for background variations, and then subjects the spots to statistical evaluation of distributions to automatically set gates (see Fig. 1). Audit trails are produced throughout the process, with overlays of counted spots saved allowing researchers to review the accuracy of the automated process.

1.2. Gating

After the accurate recognition of spots of various size and morphologies over various backgrounds, the next challenge for ELISPOT analysis is accurate gating. As for the analysis of flow cytometry data, also for ELISPOT data analysis, it is inconceivable to meaningfully "count spots" without proper gating (see Fig. 1). In that example provided in Fig. 1, the PBMC were isolated from a subject who was undergoing a cytokine storm. As a consequence, in the media control a high number of cells are seen spontaneously producing IFN-γ – these are primarily NK cells and DC. When antigen is added, the antigen-specific T cells are triggered to secrete IFN-γ – because T cells produce more IFN-γ on a per-cell basis than cells of the innate immune system, a new "juicier" spot category appears over the background. Thus, the spot size and morphology allows researchers to distinguish cytokine production by different cell types within mixed cell populations. In general, T cells produce substantially more cytokine on a per-cell basis, resulting in larger and denser spots than cells of the innate immune system (see Notes 6 and 7). For example, when IL-10 production by PBMC is measured in ELISPOT assays, most of the "antigen-induced" spots are not T-cell derived (as would be expected), but rather are produced by macrophages in response to bacterial lipopolysaccharide (LPS) contamination of the antigen solution. However, such macrophage-derived IL-10 spots are considerably smaller than the IL-10 spots generated by antigen-specific T cells – by gating the latter can be identified (7). While the LPS-induced macrophage-derived spots provide no information on specific immunity, the antigen-induced T-cell-derived IL-10 spots do, since they indicate the presence of regulatory T cells. In order to measure the accurate frequency of the T-cell-derived spots, background spots need to be excluded by setting appropriate "gates." Similarly, small and faint IL-6 spots are produced by macrophages, while antigen-specific T cells produce larger and "juicier" spots that can be identified by gating (8). ELISPOT data analysis software must therefore be capable of distinguishing different populations of spots to determine the gates for the relevant information required for T-cell diagnostics (see Note 8). The gate settings will critically affect the number of spots counted. For this reason, one of the main goals of ELISPOT data analysis has been to establish objective criteria for gating, thereby exorcizing the "ghost of subjectivity" which has haunted ELISPOT data analysis and is haunting flow cytometer data analysis even until today.

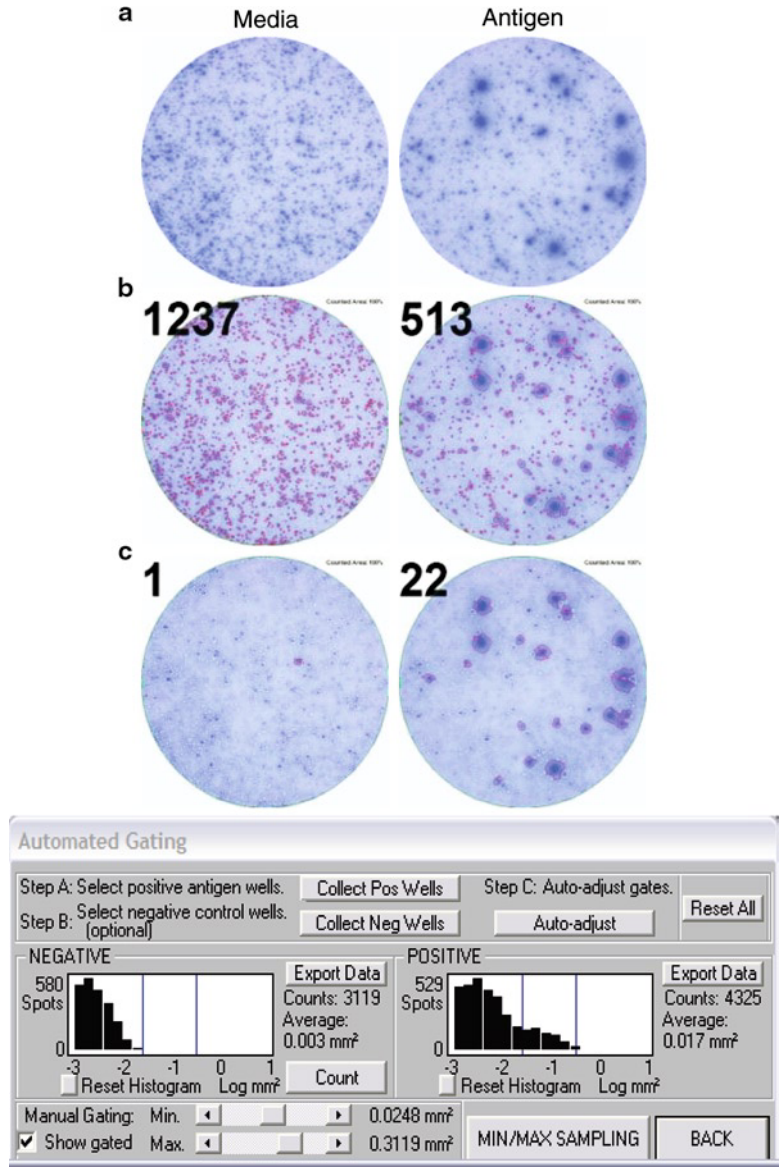

Fig. 1. Statistics-based automated ELISPOT counting. Raw images of a medium control well ("Media") and the corresponding antigen-stimulated well ("Antigen") are shown in the top two *panels* (**a**). Underneath (**b**) are the spot counts obtained without gating, with each spot highlighted. (The ImmunoSpot® software establishes the spot recognition parameters automatically by learning spot morphologies, instead of requiring users to subjectively set a multitude of parameters inevitably leading to variability in counts.) The spots automatically recognized are highlighted by the software, creating complete transparency of the (ungated, intermediate) counting result. While all spots in this medium well are "real" (being produced either by the cells of the innate immune system or by antigen-stimulated T cells), and recognized precisely as spots by the software, for establishing the T-cell count this ungated result is completely wrong. The next step is gating. The ImmunoSpot® software automatically establishes size distributions for the media and the antigen-triggered wells and sets the gates automatically, based on the statistical analysis of the distributions as we established in J. Immunol. 2000 164:1862–72 and J. Immunol. 2001, 167:1353–61, among several other publications. After re-counting with these gates, the counts are now correct and objective (**c**). Thus, there is no human judgment involved in the analysis – anyone in the world analyzing these images with the ImmunoSpot® software would come up with exactly the same scientifically validated count.

The simplest experimental models that were used to establish ELISPOT gating criteria involved the use of T-cell clones that produced IFN-γ. These T cells were activated by the nominal peptide on a clonal population of APCs which cannot express IFN-γ (3). In such experiments, conducted over a wide range of T-cell frequencies, the numbers of T cells plated per well closely matched the numbers of spots detected. Even though the T cells and APC were clonal, the spot sizes varied over a wide range. Closer analysis of the spot size distributions showed that they followed a log-normal distribution. When the peptide dose was lowered, the per-cell productivity decreased, but the size distribution of spots still followed a log-normal pattern. Similarly, when the assay duration was changed, the mean spot size varied, but the log-normal distribution remained. In all subsequent studies of human and murine cells, for clonal and bulk populations, for all cytokines measured (IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, and IFN-γ) and in ELISPOT assays measuring granzyme B, perforin, or TRAIL, this log-normal distribution of spots was observed (2–10). Therefore, by assessing morphologies of a multitude of individual spots, the statistical qualities of the distributions of the spots can be established, allowing the software to automatically set objective criteria for recognizing populations and set the gates, thereby identifying with a 99.7% confidence the spots formed by a specific cell population. In addition, having established these distributional properties, clusters of spots can be recognized and the numbers of spots constituting these clusters can be calculated.

1.3. Hardware Requirements

One limiting factor in the accuracy of ELISPOT data analysis is the hardware used for image acquisition. There is a common misconception that the pixel resolution of the camera is the key factor in determining image quality; however, this is an overly simplistic view. A fine-grain film alone does not provide pristine photographs unless the optics, the illumination, and many other fine details are optimized. Likewise, ELISPOT readers need to be high-end optical instruments to allow for the accurate analysis of ELISPOT data at single-cell resolution. In addition, such readers must feature precise robotic motion control and image centering algorithms, so as to accurately position and capture the membrane surface. Well identity is of regulatory concern and must be verified by slip-proof, encoder-controlled stages, and by faithfully recording the accurate well positions for each well during image acquisition.

The illumination of a well will largely affect the performance of any ELISPOT analyser. An ELISPOT analyser should have a closed architecture to exclude the influence of ambient light. The light source must be optimized to provide even illumination of the well bottom without reflections caused by the wall of the well. A backlight, in addition to the top light, will allow for increased luminescence of the membrane, thus facilitating the separation of adjacent

spots, and the discernment of discrete colors for dual color analysis. The light must be stabilized to permit constant performance over the operation period over a decade of use. Only industry grade illumination will provide constant readings. ImmunoSpot® Series Analyzers have been designed to meet these criteria and are equipped with a user friendly module that permits the user "at the click of a button" to verify the consistent performance of the machine.

A further challenge is harmonizing the performance of different ELISPOT analysers, as desirable for multicenter studies, and as required for the comparability of data generated in different laboratories. No two cameras capture identical images, unless the cameras are fine-tuned to the same calibration standards, along with standardizing lighting conditions and other variables, two ELISPOT readers of the same model from the same manufacturer might provide rather variable counts. Substantial effort has been invested by CTL to create fully harmonized readers that produce consistently identical results; starting with the ImmunoSpot® Series 6 analysers, these have now become available.

2. Materials

1. ImmunoSpot® Series 6 Analyzer (CTL, Shaker Heights, OH).
2. ImmunoSpot® 6.0 Software (CTL).
3. SpotMap™ 6.0 Software (CTL).

3. Methods

3.1. Scanning

In the first step of ELISPOT analysis, an ImmunoSpot® Analyzer scans and saves image files of individual ELISPOT wells of a plate. The machine progresses automatically from well to well, using optical feedback to automatically center on each well, thus compensating for irregularities in the plate geometry. (ELISPOT plates are manufactured using a high-temperature molding process, and are prone to deform as they cool down.) Digital encoders keep track of the precise position of each well, thus helping to confirm well identity and positioning. In addition, the software keeps track of the encoder information, the time stamp, and the identity of the operator for tracking of such information for regulatory purposes.

The end point of the automated scanning process for an ELISPOT plate is a tamper-proof set of 96 image files, each representing a digital photograph of one well from the original 96-well plate. Scanning can also be done for 384-, 12-, 24-, and 6-well formats.

The saved image files allow users to document and analyze ELISPOT assay results long after the original plates have decayed, and to reproduce the analysis results. While "live" analysis of images (i.e., without saving them to a disk file) is also possible, it is not recommended, because this obscures the transparency and reproducibility of the data, and thus violates good scientific and laboratory practice.

For work that demands high-throughput scanning, a robotic arm and stacker can be used to automatically load up to 200 plates a time. The user can instruct the ImmunoSpot® software to automatically count and process all plates instead of tediously working with each plate individually. Grouping of plates together can expedite all phases of the work: counting, quality control, and data export. The Plate Manager module permits one to flexibly group plates from different experiments and different locations for streamlined counting, quality control, viewing, printing, and exporting.

3.2. Analysis

The saved image files can then be processed on the analyzer itself, or on remote workstations equipped with the ImmunoSpot® software. The dissociation of scanning and analysis enables work to proceed more efficiently by permitting an indefinite number of users to analyze images independently, without tying up the core machine.

3.2.1. Automated Analysis

The main steps of automated counting are as follows.

Loading Plate Images

Virtually any number of plates can be loaded at this stage, from a single folder ("Experiment"), individually, or as a group from any number of experiments, due to the flexible software design.

Defining Counting Parameters

Accurate counting requires informing the software about the nature of the spots to be counted. As discussed above, the spot characteristics can vary considerably, depending on the assay conditions and the cytokines under examination. For this reason, the ImmunoSpot® software has been designed to learn and auto-adjust the counting parameters using a simple two-stage process.

Step 1: SmartCount™ for automatic spot recognition (Fig. 2). By clicking on wells that contain characteristic spots for a given assay, the software learns to recognize the cardinal features of the spots of interest. While establishing the appropriate counting parameters for the respective spot type which is fully automated (and therefore objective and reproducible), the parameters can be manually fine-tuned for spot morphology, sensitivity, and a multitude of other criteria. If such adjustments are needed, e.g., for atypical wells that contain artifacts, it is recommended to do these in the quality control step such that the objective counts and the subjective modifications are transparently documented.

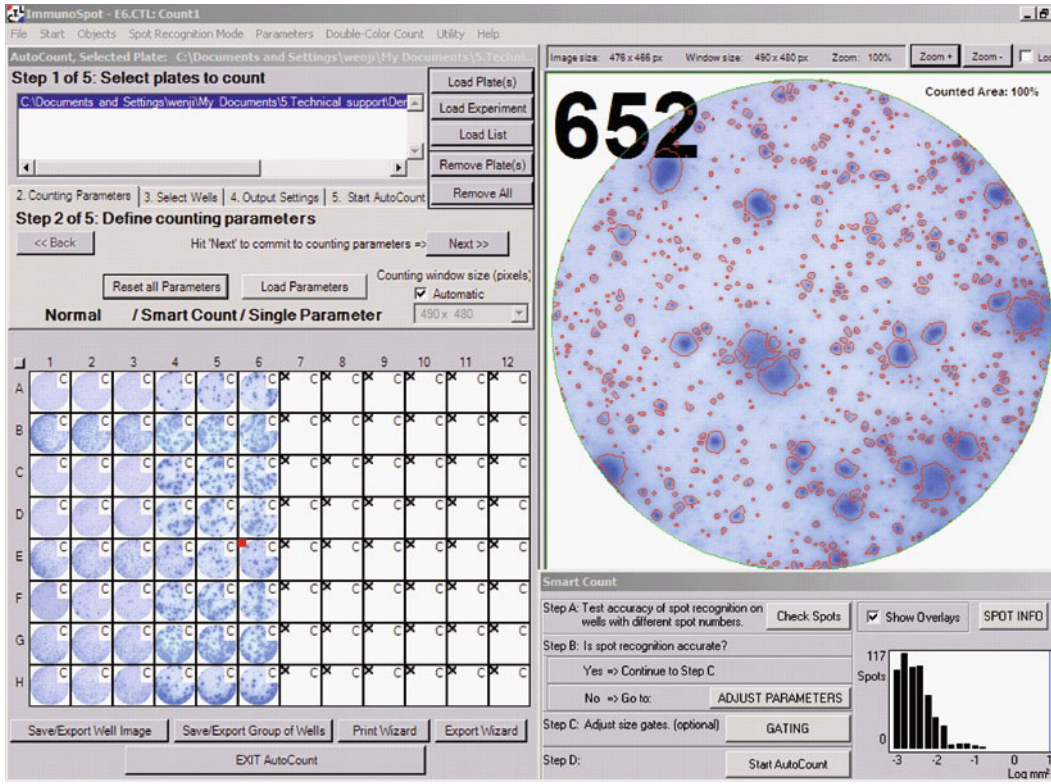

Fig. 2. Recognition of spot morphology. The first step of the analysis process is to "teach" the software the morphology of the spots. This can be done by clicking on wells that contain spots typical of those which are to be analyzed. The software will analyze such spots and highlight those that are being recognized by outlining them. In the subsequent step, the distribution of the spots will be analyzed.

Step 2: AutoGate™ for automatic gating (Fig. 3). After the software is instructed to learn the spot morphology for accurate spot recognition, it needs to understand the distributional properties of the spots. Several wells containing typical spots need to be sampled to accumulate information for an accurate statistical analysis of the spot size distribution. Typically, this requires the sampling of 3–10 wells, a process which takes less than half a minute. At one click of a button, the software will automatically calculate and set the lower and upper gate values based on the spot's distributional properties. The AutoGate™ feature thus allows objective, statistics-based criteria to be used in setting the minimum and maximum gate for spots to be included in the count.

Spots smaller than the lower limit specified by the minimum gate are ignored; i.e., they are excluded from the final spot count. Typically, these are spots secreted by innate immune system and should not be included into the frequency of antigen-specific T cells. Spots larger than the maximum gate value are counted as cell clusters by default: the software automatically estimates the number of cells in the clusters to be included into the count.

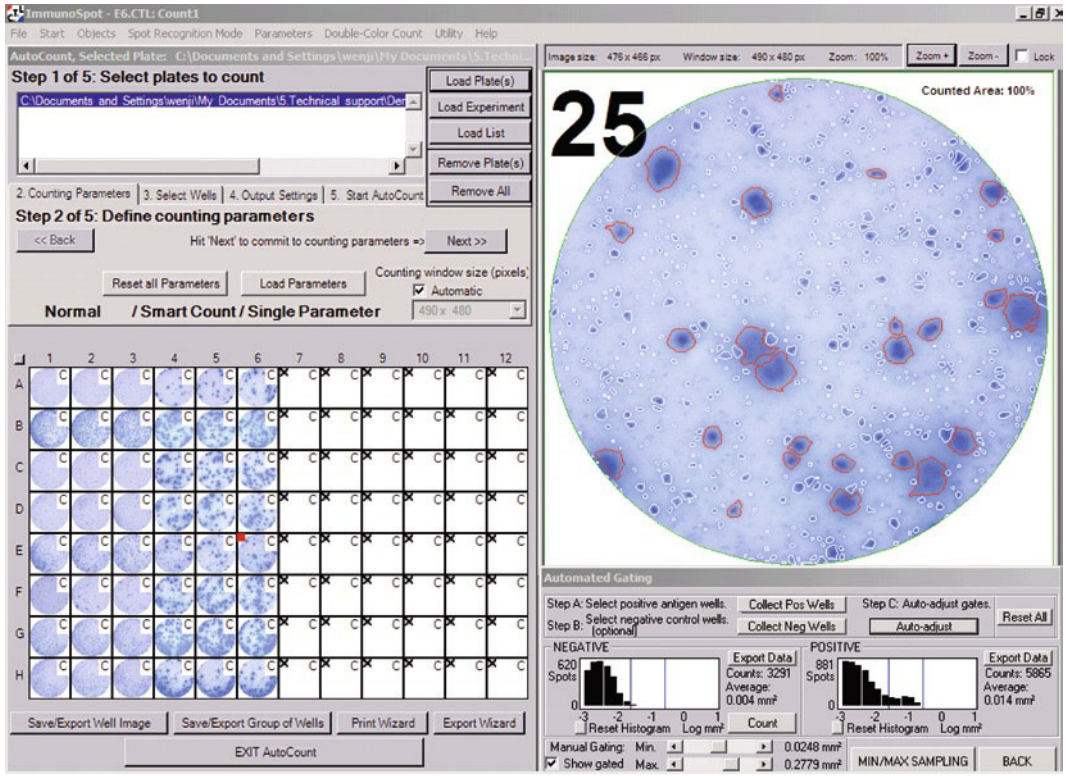

Fig. 3. Establishing the minimal/maximal gates by AutoGate™. The distribution of the spots in the media control wells (columns 1–3 of the plate loaded) is captured in the left histogram (labeled "NEGATIVE"). The distribution of the spots in antigen testing wells (columns 4–6 of the plate loaded) is captured in the right histogram (labeled "POSITIVE"). The "AutoGate" feature uses the distributional properties of this cumulative data to calculate the minimum and maximum limits or "gates" (indicated by the *vertical lines* in the histograms). When the actual well shown is recounted with these limits in place, 627 spots are "gated out," resulting in the spot count of 25 shown at the *top.*

Automated Counting

Once the parameters have been established and assigned to the wells, the software automatically counts spots on any number of plates or sections thereof. Overlays of the raw image files and of the counting results are saved for each well, as are the counting parameters. The results of the counting process thus are transparent, documented, and easily reproduced for subsequent verification in the quality control step.

3.2.2. Quality Control

As ELISPOT assays can be subjected to artifacts, contaminants, damaged or leaking membranes, etc., the ImmunoSpot® software was designed to permit corrections for each of the situations if needed, so that the valuable data could be remedied (see Notes 9–11). For a streamlined review, the ImmunoSpot® software allows the user to view image overlays that indicate which spots have actually been counted and to make corrections if needed (Fig. 4). In the example shown, an artifact that resulted from the clumping of cells by free DNA in a freeze–thawed PBMC sample) has been excised.

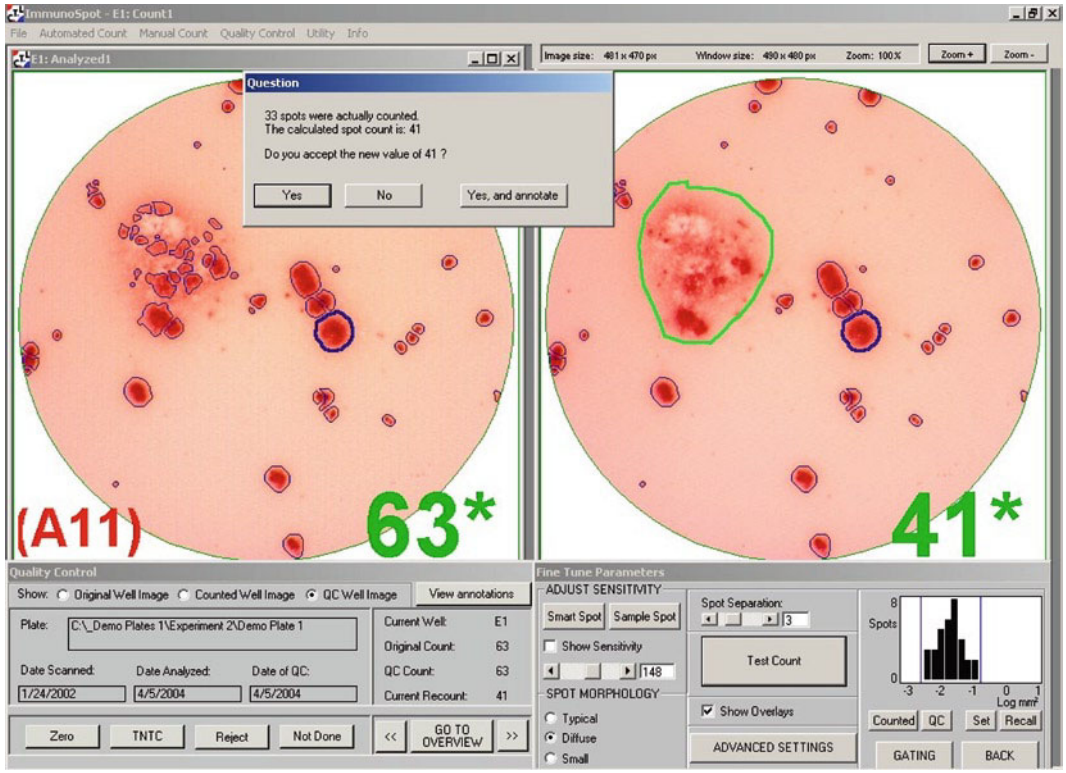

Fig. 4. Quality control in ELISPOT analysis. The image on the left-hand side shows the counting results obtained in automated analysis mode on a well that contains an artifact (in this case, a cell clump caused the spot cluster in the *upper left-hand* quadrant). The cluster was treated as a group of individual spots, resulting in a spot count of 63. In quality control mode, this artifact-containing region can be outlined (in *green*) and excluded from the analysis, as shown on the *right-hand side*. The software then normalizes the spot count by correcting for the size of that region. In the example shown, 33 spots were actually detected, and this count was increased by 8 to compensate for the exclude area, resulting in an adjusted spot count of 41. (The *asterisk* beside the spot count of 41 indicates that this is a re-calculated value, rather than a direct measurement.) In keeping with Good Laboratory Practices, the software saves and annotates all such subjective adjustments made to the objective automated count. This same example also contains a spot near the center of the well that exceeds the upper gate threshold. This spot was automatically outlined in *dark blue* during automated analysis, indicating that it was treated as a cluster. In such cases, the software automatically calculates the number of spots required to generate such a cluster based on the average spot size and density distribution, and re-computes the spot count accordingly (the *asterisk* beside the spot count of 63 likewise indicates that this value was re-calculated, as does the automatically generated A11 annotation code).

The software has calculated how many spots would occupy the excised region if the artifact was not there, assuming an even distribution of spots in the well.

To ensure Good Laboratory Practices (GLPs) compliance, all changes made in QC are recorded and annotated automatically by the ImmunoSpot® software. This allows the principal investigator or regulatory agency to determine at a glance whether the counting results have been changed relative to the automated count, and whether the changes made are accurate and appropriate. As part of this documentation, the software also automatically generates a set

of plates and well image files which can be helpful in preparing presentations, publications, or discussion of the results. Direct PowerPoint Register Mark export function of the software also makes it convenient for the user to arrange groups of wells for direct comparison at desired magnifications.

3.3. ELISPOT Data Management

ELISPOT assays are highly suited for high-throughput work. It is not uncommon for a single well-trained team to test each day hundreds of samples for reactivity to hundreds of antigens. However, even a small assay can contain a flood of information. Just three 96-well plates, for example, require storing 864 image files – raw images, counting overlays, QC images, along with the records of counting parameters, the numbers of spots counted, and the spot size/density statistics for each well. This information needs to be linked to the assay information, i.e., to the source of the cell material tested (e.g., PBMC of donor "X"), to the number of cells per well (so that frequencies can be normalized "per million"), to the antigens tested and their concentrations, and to the cytokines measured. Thus, in even a small three-plate assay, there can be more than 4,320 sets of data which need to be linked together.

The ImmunoSpot® software's SpotMap™ module was specifically designed to manage all data automatically. For each well, and for each plate, the software documents the assay conditions: which cells were plated in which numbers, which antigens were used to challenge the cells and in which concentrations, and which cytokines were measured (Fig 5). Custom plate layouts can be generated in a streamlined fashion for multiplate experiments – the SpotMap™ software will even calculate the amounts of reagents needed for each assay. Once the counting results are available, they can be quickly linked, within the SpotMap™ software, to the other assay parameters: at the click of a button, even the most complex ELISPOT assay can be evaluated, the statistics calculated, and the requested information represented in virtually any desired format.

4. Notes

1. T cells can move around during the assay, causing the spots to develop "tails." This is especially true when T cells have been preactivated in vivo or in vitro, as this makes the cells particularly mobile.
2. Small clusters can result from T cells migrating from one APC to an adjacent APC within the assay's duration while they continue to secrete cytokines. At higher magnification, such clusters are linked by a cytokine trail. Such clusters should be – and are – counted by the ImmunoSpot® software as having

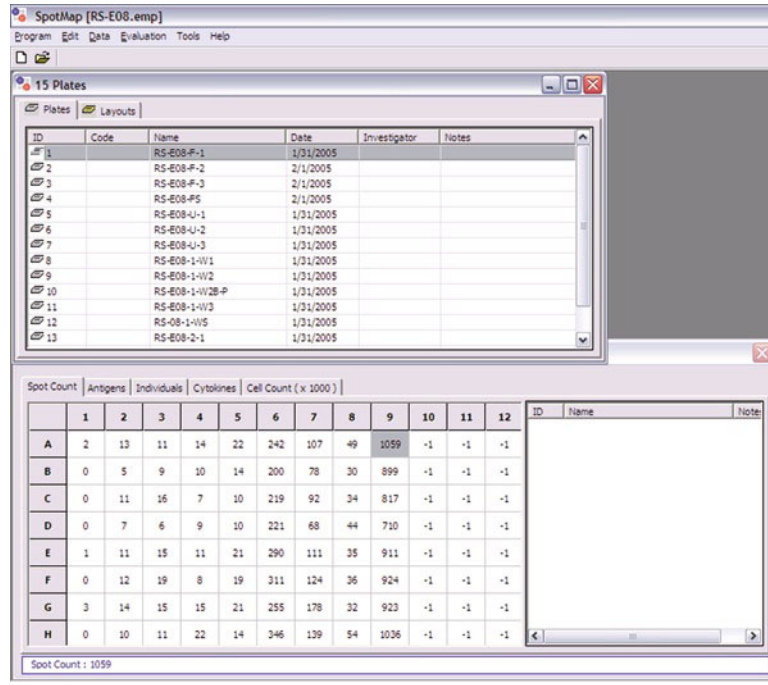

Fig. 5. ELISPOT data management. The spot counts in 96-well format are linked to the plate layout. For each well, the antigen, the test subject, the cytokine, and the number of cells plated are specified. All these data are linked and processed for exporting into a database.

been derived from a single cell. Frequencies can be verified by serial dilution of cells. The number of cells plated and the spot counts are linear in the 100,000–800,000 PBMC per well range (2).

3. Occasionally, white dots can be seen in the middle of the spots. These result from the substrate peeling off, e.g., when the flow rate of the plate washer is too high, or the plates are banged too hard while washing. The "Fill Holes" feature of the ImmunoSpot® software can be used to mask the white dots, allowing for the spots to be counted accurately.
4. In addition to ELISA effects, background coloration can be darker in some parts of the well due to leakage of the membrane or nonspecific protein precipitations. The ImmunoSpot® software will automatically correct for such variations in the background, but if additional adjustments are needed, in the quality control (QC) module, one can compensate by adjusting the "background balance" parameter.
5. The background coloration tends to be elevated in wells with high number of cytokine-producing cells (i.e., the number of

spots) due to the "ELISA effect." The "Auto-light" feature of the ImmunoSpot® Analyser compensates for the increased background coloration. However, for accurate counts in the high-frequency range, it is recommended that one perform serial dilutions of the cells to establish the number of spots below which cell numbers plated and spot counts are linear, and above which spot confluence occurs. That cut-off number will depend on the analyte and on assay conditions used. It can be over 5,000 spots per well, e.g., for an IL-10 assay which produces small spots, and can be as low as 400 spots per well for big fuzzy spots, such as IL-2. Spot counts above the linearity cut-off number are best expressed as too numerous to count (TNTC). The precise value can be established by repeating the experiment with a lower number of cells plated per well.

6. Sometimes, the number of spots in the medium background is high for all samples, due to the stimulatory effects of serum. Even brief exposure to nontested serum, e.g., during washes or during freezing, can drive up the background intensity. The use of serum-free media for cryopreservation, thawing, and testing can eliminate this problem (2).
7. The number of spots in the medium control wells can be high for a single individual out of several tested. This is a common finding for individuals undergoing cytokine storms in vivo, e.g., due to a clinical or subclinical infection or other massive immune stimulation. In such cases, gating cannot distinguish between background and foreground spots (since both are T-cell derived), but statistics can (see also Chapters 14 and 15).
8. Some assays, such as IL-6, IL-10, and TNF, tend to give high-background coloration in general, due to the activation of macrophages on the membrane of the ELISPOT plate. Such background spots are frequently smaller than the antigen-induced spots produced by T cells, and can be gated out automatically by the ImmunoSpot® software.
9. On occasion, the counting parameters established can recognize valid spots for most test subjects, but not for others. For example, spots that are either smaller or larger than usual can be seen with particularly low- (or high-) avidity T-cell responses, or if co-stimulation is decreased (or increased). This is one reason why, as part of any ELISPOT analysis, the researcher should have the option of viewing both the raw images and the counting results in QC mode. This allows the researcher to judge whether the counting parameters established do indeed apply to all subjects under examination. If re-counting of any given subject becomes necessary, the altered parameters are automatically annotated by ImmunoSpot® software, thus drawing attention to the atypical spot morphology or other image characteristics.

10. The well images can contain artifacts due to membrane damage – for example, when the membrane is accidentally scratched with a pipette tip. The affected area can be excised in QC mode, and using the *normalization* algorithm, the spot count is re-computed. This re-normalization is performed by computing the number of spots required to fill the excised area, using the average spot size and distribution density in the rest of the well. The same technique can also be used to correct for cell clumping. For example, if the testing was done in triplicate, and a clump is found in one of the wells, this clump can be excised and the spot count can be normalized. In both cases, these corrections are automatically recorded by the software in the form of annotations added to the well records.
11. Seeing is believing! Never blindly trust ELISPOT counts, whether from your own laboratory or from others! Overlays of both the raw images and the counting results are a simple and transparent way of understanding the assay results and judging the counting accuracy. *Well surveys* containing this information can be printed or exported into graphics files or PowerPoint presentations, allowing assessments of assay results to be performed at a glance. The direct side-by-side display of medium control and antigen wells can speak volumes about the quality of the assay and the appropriateness of spot counts.

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