7

Image Analysis and Data Management of ELISPOT Assay Results

Paul Viktor Lehmann

Summary

The recent renaissance of enzyme-linked immunospot (ELISPOT) assays largely is the result of advances in image analysis. Information on the frequency of antigen-specific T-cells and also on the secretion rate of the individual cells is captured in spots generated using this technique. Although the overall assessment of ELISPOT results can be conducted visually, this is inevitably subjective, inaccurate, and cumbersome. In contrast, objective, and accurate measurements are fundamental to good science. Validated image analysis algorithms and procedures, therefore, have become critical for elevating the quality of ELISPOT assays results. As cytokine and granzyme B ELISPOT assays become the gold standard for monitoring antigen-specific T-cell immunity in clinical trials, the pressure increases to make ELISPOT analysis transparent, reproducible and tamperproof, complying with Good Laboratory Practice and Code for Federal Regulations Part 11 guidelines. In addition, ELISPOT assays in clinical and basic science settings frequently require high degrees of throughput, thus further raising the need for advanced data management and statistical analysis. The ImmunoSpot software portfolio has been specifically designed to meet all these needs, using the techniques described in this chapter.

Key Words: T-cells; ELISPOT; image analysis; cytokine productivity; spot morphology; single cell resolution; spot size gating.

1. Introduction

Each spot within an enzyme-linked immunospot (ELISPOT) assay is not "just a dot," but the footprint of a single cells' secretory activity—one that contains detailed information about the secretory process itself. Understanding the basics of spot formation is critical for performing image analysis, which can withstand rigorous scientific scrutiny.

From: *Methods in Molecular Biology, vol. 302: Handbook of ELISPOT: Methods and Protocols*
Edited by: A. E. Kalyuzhny © Humana Press Inc., Totowa, NJ

1.1. ELISPOT Size and Morphology

In an interferon (IFN)-γ ELISPOT assay, for example, IFN-γ is captured by the plate-bound anti-IFN-γ antibody around the secreting cell. The footprint of the captured IFN-γ will eventually be visualized as an ELISPOT, with its size and density reflecting the amount of cytokine produced by the cell during the assay's entire duration. Spot size and density are thus critical parameters that one using ELISPOT image analysis must take into consideration. The kinetics of the cytokine production also is reflected by the spot morphology, that is, their density and general shape. For example, a rapid secretion rate will produce large, fuzzy spots, whereas the slow-but-steady release of cytokines will result in smaller, denser spots. Evaluating both the size and the morphology of these spots is therefore crucial to performing accurate ELISPOT analysis.

Spot size and morphology frequently allow researchers to distinguish cytokine production by different cell types within mixed cell populations. For example, when interleukin (IL)-10 production by human peripheral blood mononuclear cells (PBMC) is measured in ELISPOT assays, most of the "antigen-induced" spots are not T-cell derived (as would be expected) but are produced by macrophages in response to lipopolysaccharide contamination of the antigen. Such macrophage-derived IL-10 spots are considerably smaller than the IL-10 spots generated by antigen-specific T-cells ***(1)***. Although the lipopolysaccharide-induced macrophage-derived spots provide no information on specific immunity, the antigen-induced T-cell-derived IL-10 spots do because they indicate the presence of T regulatory cells. To measure the latter, the former need to be excluded from the counting results by setting appropriate size thresholds. **Figure 1** illustrates this point on the example of a tumor necrosis factor (TNF)-α assay; the small spots were macrophage-derived, whereas the large spots were generated by T-cells (A. Y. Karulin, and P. V. Lehmann, unpublished results). Similarly, small and faint IL-6 spots are produced by macrophages, whereas antigen-specific T-cells produce larger, "juicier" spots (unpublished data). ELISPOT image analysis must therefore be capable of distinguishing different spot sizes and morphologies to provide information relevant for T-cell diagnostics.

The antigen dose affects the cytokine secretion rate of T-cells. Stimulation of a T-cell clone with a high dose of the nominal antigenic peptide induces stronger cytokine production in the individual T-cells (i.e., it triggers larger and/or denser spots) than does the stimulation of the same clone with low dose peptide ***(2)***. Therefore, when stimulated with a single antigen dose, as is frequently the case in ELIPSOT assays, high-avidity T-cells within the PBMCs produce larger spots than low-avidity clones. Confirming this notion, increased T-cell co-stimulation was shown to result in increased per cell productivity ***(3)***. In diseases such as HIV, the cytokine productivity per cell can be reduced,

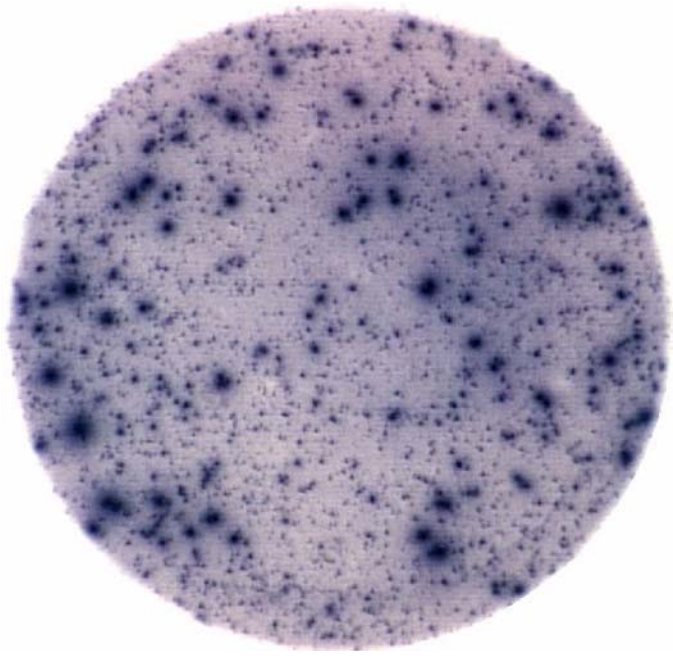


Fig. 1. ELISPOT morphologies as exemplified in a TNF-α assay. Two types of spots are seen, each after log-normal size/density distributions. The large diffuse spots were generated by antigen-induced T-cells, as shown by cell separation experiments; these spots were absent in the medium control wells. The fuzzy morphology of these spots results from a high per cell TNF-α productivity rate. In contrast, cell separation experiments showed that the small spots were generated by macrophages, and these spots were present in medium control wells. The pristine morphology of these spots is caused by the slow but continuous release of the TNF-α. Image analysis can be used to recognize these different morphologies, and thus, to clearly distinguish between T-cell and macrophage-derived spots. (As an aside, the figure also illustrates elevated background intensity in areas of increased secretory activity—the result of an ELISA effect as the cytokine is captured from the supernatant.)

resulting in smaller spots ***(4)***. One advantage of ELISPOT assays is their ability to determine whether decreased net cytokine production in disease states is caused by a decreased number of cytokine-secreting T-cells or from reduced per cell productivity by unchanged frequencies of T-cells. To compensate for physiological and pathological variations in per cell productivity, ELISPOT image analysis tools must therefore be versatile, with the ability to permit fine-tuning of the image processing parameters.

Central to ELISPOT image analysis is the standardization of assay conditions, which can directly impact the spot morphology. Consider the affinity of the capture antibodies: a capture antibody with low affinity will produce fainter, more diffuse spots than a capture antibody of high affinity. Accordingly, the morphology of ELISPOTs can vary considerably when different antibodies (or even different concentrations of the same antibody) are used for coating.

The assay duration also can influence the spot morphology. The 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 *(2)*. 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 (because of lateral cytokine diffusion caused by the reversibility of its interaction with the membrane antibodies) their intensity will fade, however, because of the dilution of the cytokine. The temperature during development, and the nature of the enzymatic reaction will also define the spot morphology. The red spots developed with HRP-AEC differ fundamentally from the blue NBT/BCIP spots, with the former being more pristine (despite ALPH detection being more sensitive than HRP) and having a faint background, whereas the latter is more dramatic and fuzzy with a more heavily stained background.

Once an ELISPOT assay has been standardized, however, the interassay variability of spot morphology becomes negligible. Although spots of different cytokine ELISPOT assays will continue to look different even after standardization, the same counting parameters can be used assay after assay *(4,5)*. Therefore, apart from allowing counting parameters to be fine-tuned, ELISPOT image analysis tools must also allow the user to employ the same parameters for different assays, so as to permit objective comparison of the results of different assays.

1.2. ELISPOT Counts

One key piece of information to be gained from ELISPOT assays is the frequency of antigen-specific T-cells within the entire sample cell pool, as measured by the number of cells that engage in cytokine production after 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 correct frequency information is that both the image acquisition and the assay must be optimized for single-cell resolution.

In all T-cell cytokine ELISPOT assays, a wide spectrum of spot sizes and densities can be seen. Thus, when analyzing ELISPOT results, cut-off values need to be set for the minimum spots sizes and densities to be counted. The maximum spot size must likewise be defined so that clusters of cells can be

identified as such. The minimum and maximum "gates" set will critically affect the number of spots counted. For this reason, one of the main goals of ELISPOT image analysis has been to establish absolute criteria for gating, thereby eliminating the "ghost of subjectivity" that has haunted ELISPOT counts in the pre-image analysis age.

The simplest experimental model that can be used to establish ELISPOT gating criteria involves the use of a T-cell clone that produces IFN-γ. These T-cells were activated by the nominal peptide on a clonal population of antigen-presenting cells (APCs) that cannot express IFN-γ ***(2)***. In such experiments, conducted over a wide range of plated T-cells, the number of T-cells 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 distribution showed that they followed a log normal distribution. When the peptide dose was lowered, the per cell productivity (the mean spot size/density) decreased, but the size distribution still followed a log normal pattern. Similarly, when the assay duration was changed, the mean spot sizes/density varied, but the log normal distribution remained. In all subsequent studies of human and murine cells ***(6)***, 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 granzyme B assays ***(7)***, this log normal distribution of ELISPOTs was noted. Therefore, by measuring the size/density of a multitude of individual spots, the statistical qualities of the spot size distributions can be established, allowing the software to set absolute criteria for the minimum and maximum size gates.

Having established these distributional properties, clusters of cells can be recognized, and the numbers of cells constituting these clusters can be calculated. By rooting ELISPOT image analysis in these objective statistical principles, one can establish absolute criteria for ELISPOT counting, thus eliminating subjectivity and elevating ELISPOT to an exact science.

1.3. Hardware Requirements

One limiting factor in the accuracy of ELISPOT image 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. 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 also are optimized. ELISPOT readers need to be high-end optical instruments to permit accurate analysis of ELISPOT images at single-cell resolution. In addition, such readers must feature precise robotic motion control, so as to accurately position and capture the membrane surface. The identity of the wells is of regulatory concern and must be verified by slip-proof, encoder-controlled stages and by faithfully recording the well position on each plate during image

acquisition. Finally, to ensure consistent performance at single-cell resolution, regular machine calibration is required.

2. Materials

1. ImmunoSpot® Series 3B Analyzer (CTL, Cleveland, OH).
2. ImmunoSpot® 4.0 Software (CTL).
3. SpotMap™ 4.0 Software (CTL).

3. Methods

3.1. Scanning

In the first step of ELISPOT analysis, an ImmunoSpot Analyzer scans and saves image files (basically, digital photographs) of individual ELISPOT wells on 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 identify and positioning. In addition, the software keeps track of the encoder information, the time stamp and the identity of the operator. Systems also can be set up with access limitations for an added measure of security.

The end point of the fully automated scanning process for an ELISPOT plate is a tamperproof set of 96 image files, each representing a digital photograph of one well from the original 96-well plate. Scanning can also be performed using 12- and 24-well formats. The saved files allow users to document and analyze ELISPOT assays long after the original plates have decayed, and to reproduce the analysis results. While "live" analysis of images (that is, without saving them to a disk file) is also possible, it is not recommended because this obscures the transparency and reproducibility of the results, and thus violates good scientific and laboratory practice.

Suggestions for scanning:

- We recommend that plate images that belong together (e.g., plates from the same experiment) be stored in the same folder. The software allows the user to handle all the plates in a folder as a single unit; that is, the user can instruct ImmunoSpot to process all plates within a given folder, instead of tediously loading each one individually. Grouping such plates together can expedite all phases of the work: counting, quality control, and data export.
- We recommend that the scanned images be kept on a read/write storage device, such as the hard drive of the computer on which the counting and quality control will be done.

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

In the first step of analysis, counting parameters are defined, and these parameters are used for analyzing all wells within an assay. This permits the objective comparison of results from different wells or plates of an assay.

The main steps of automated counting are as follows:

1. Loading the plate images. Virtually any number of plates can be loaded at this stage, due to the flexible software design.
2. Defining the counting parameters. The software provides default parameters that have been carefully selected to provide reasonably accurate counts for most ELISPOT assays. If strict, scientific counting precision is not a requirement, one can directly proceed to the spot counting stage.

Accurate counting, however, requires instructing the software about the nature of the spots to be counted (*see* **Note 1**). As discussed above, the spot characteristics can vary considerably, depending on the assay conditions and the cytokines under examination (*see* **Note 2**). For this reason, the ImmunoSpot software has been designed to allow fine-tuning of the counting parameters using a simple two-stage process.

Step 1: Sampling the Spot Morphology Using the "SmartSpot" Feature

By clicking on a spot, the software will analyze and "learn" to recognize the cardinal features of this spot, after which it can proceed to examine all other spots for these features (**Fig. 2**). Although establishing the appropriate counting parameters for the respective spot type is fully automated (and therefore objective and reproducible), the parameters can be manually fine-tuned for morphology, sensitivity, and a multitude of other criteria (*see* **Note 3**).

Step 2 : Gating Using the "Autogate" Feature

After the spot morphology has been defined, the software can be instructed to count all spots that exhibit this morphology (**Fig. 3**). In the process, the spot size distribution parameters can be established. A minimum of 500 spots need to be counted in this way to accumulate enough information for an accurate statistical analysis of the spot size distribution (but *see* **Note 4**). Typically, it takes sampling of approx 10 wells (a process that takes less than a minute) to sample at least 500 spots (*see* **Note 5**). By hitting the "Autogate button," the user can automatically set the lower and upper gate values (that is, spot size thresholds) based on the log-normal distribu-

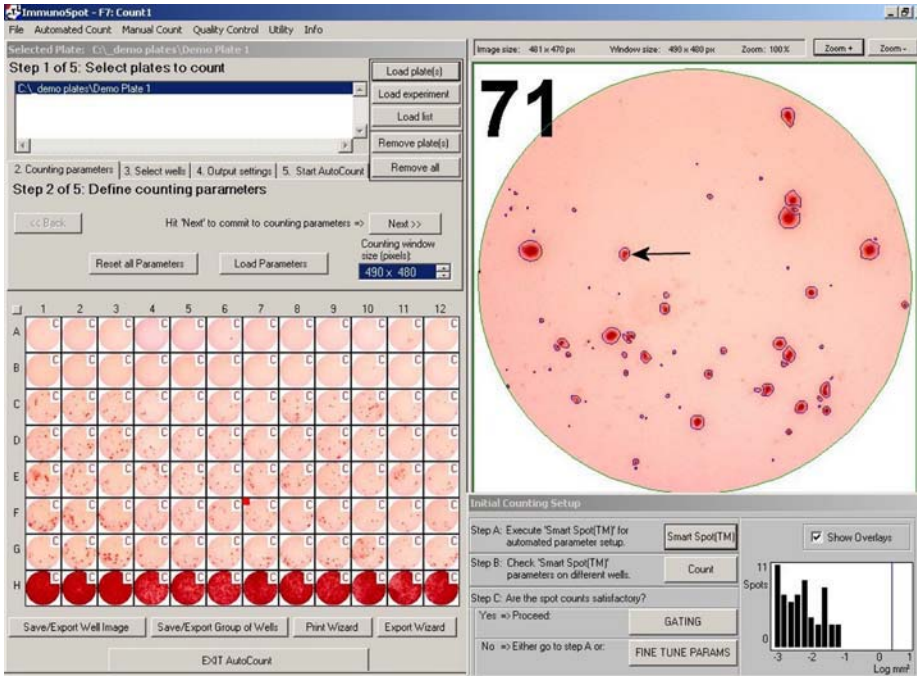
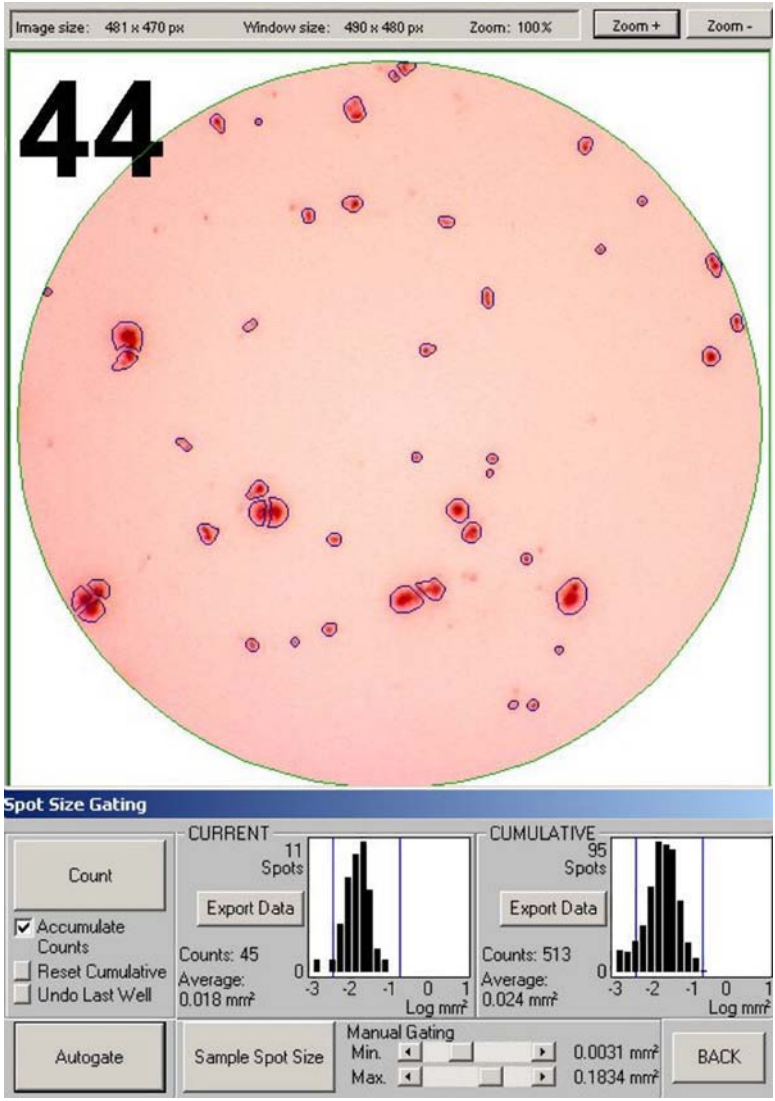


Fig. 2. Recognition of ELISPOT morphology. The first step of the analysis process is to "teach" the software the morphology of the spots of interest. This can be done by sampling characteristic spots (highlighted by arrow). The software then identifies all spots of the same morphology, irrespective of size, and marks each spot recognized as shown. In the subsequent step, the size distribution of the spots will be analyzed.

Fig. 3. Establishing the minimal/maximal spot size/density to be counted by autogating. The size/density distribution of the spots in the current well is captured in the left histogram (labeled "CURRENT"), which shows 45 spots as being recognized. More accurate information on the size/density distribution of ELISPOTS in the assay can be obtained by sampling multiple wells, as captured in the cumulative histogram on the right-hand side. (In this example, the cumulative histogram was generated by sampling 513 spots in 12 wells.) The "Autogate" feature uses the distributional properties of this cumulative data to compute the minimum and maximum spot limits or "gates" (indicated by the vertical lines in the histograms). When the actual well shown is recounted with these limits in place, one small spot is "gated out," resulting in the spot count of 44 shown at the top. Once the morphology and size/density criteria are established, the software applies these very same parameters to the automated counting of any number of wells.



tion properties of the spot-size distribution. The Autogate feature thus allows objective, statistics-based criteria to be used in setting the minimum and maximum spot sizes allowed.

Spots smaller than specified by the minimum gate are ignored, that is, they are excluded from the final spot count. Spots larger than the maximum gate value are either counted as clusters or are excluded altogether. The latter option

permits counting small spots generated by cell type "A," whereas gating out the larger spots generated by cell type "B."

The same counting parameters cannot be applied if results of different assays are to be counted in the same run. For this reason, the software allows the user to define an indefinite number of distinct counting parameter sets (templates) for any array of wells, arranged in any pattern on the loaded plates.

3. 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 (*see* **Note 6**). 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 become transparent, documented, and easily reproduced for subsequent verification in the quality control step.

3.2.2. Quality Control

Because ELISPOT assays can contain artifacts (e.g., contaminants, damaged or leaking membranes; *see* **Note 7**), the results of the automated counting need to be subjected to quality control. A menu in the ImmunoSpot software allows the user to view image overlays that indicate which spots have actually been counted, and to make corrections as needed (**Fig. 4**). In the example shown, a cluster of cells (resulting from the clumping of cells by free DNA in a freeze-thawed PBMC sample) has been excised, and the software has calculated how many spots would occupy that cluster, assuming that these spots have the same average size and density distribution as those within the rest of the well.

To ensure Good Laboratory Practice compliance, all changes made are recorded and annotated. This allows the principal investigator or regulatory agency to determine at a glance whether the counting results are accurate and appropriate. As part of this documentation, the software also generates a set of plate and well image files that can be helpful in preparing presentations, writing publications or discussing the results. Direct PowerPoint export options also make it convenient for the user to arrange groups of wells for presentation or documentation purposes.

3.3. ELISPOT Data Management

ELISPOT assays can require high degrees of throughput. It is not uncommon for a single well-trained team to test hundreds of samples each day for reactivity to hundreds of antigens (*see* **Note 8**). However, even a small assay can contain a veritable flood of information. Just three 96-well plates, for example, require storing 864 image files—raw images, counting overlays, post-quality

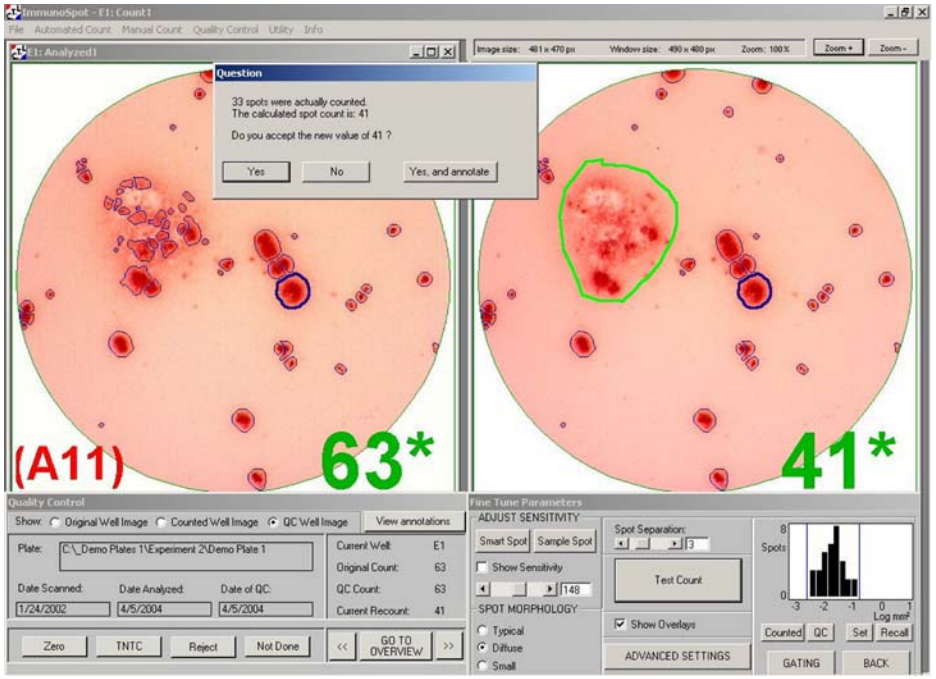


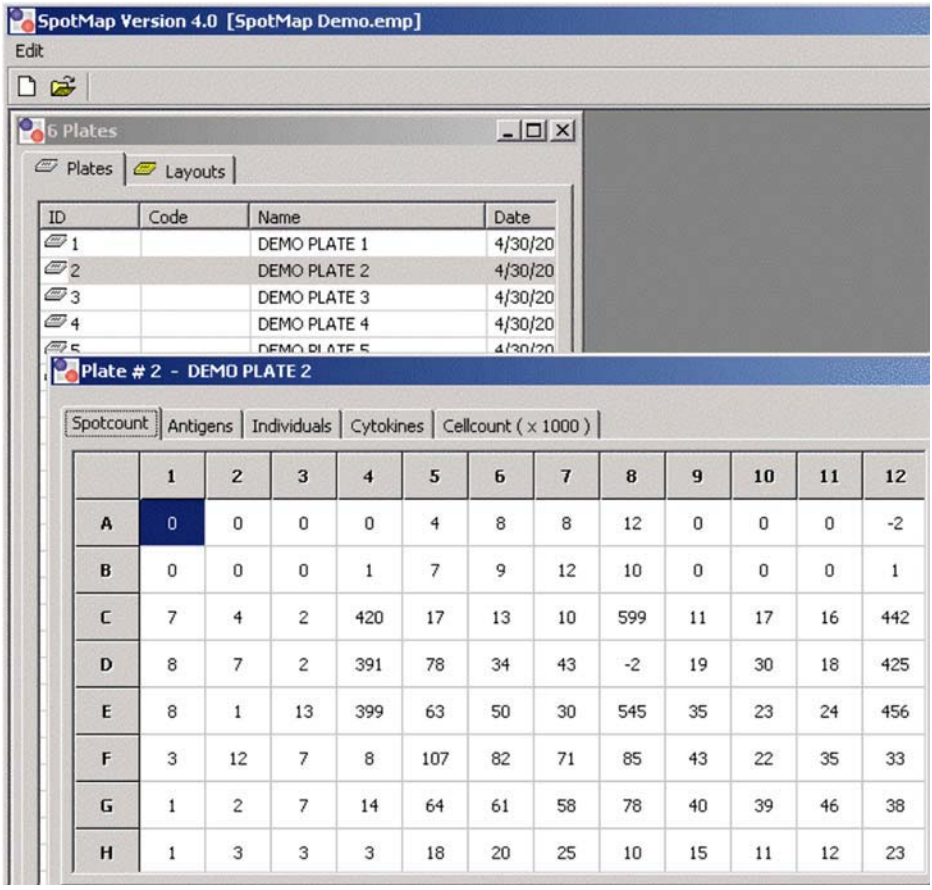
Fig. 4. Quality control step in ELISPOT analysis. The image on the left-hand side shows the counting results obtained in automated analysis mode on a well that contains an artifact (in this case, a cell clump caused the spot cluster in the upper left-hand quadrant). The cluster was treated as a group of individual spots, resulting in a spot count of 63. In control mode, this artifact-containing region can be outlined (on the right) and excluded from the analysis. The software then normalizes the spot count by correcting for the size of the unselected region. In the example shown, 33 spots were actually detected, and this count was increased by eight to compensate for the unselected area, resulting in an adjusted spot count of 41. (The asterisk beside the spot count of 41 indicates that this is a recalculated value, rather than a direct measurement.) In keeping with good laboratory practice, 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 bold during automated analysis (the smaller bold outline in the left and right image), 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 recalculated, as does the automatically generated A11 annotation code.)

control images, along with records of the 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, that is, to the source of the cell material tested (e.g., PBMC of donor 'Z"), to the number of cells per well (so that frequencies can be normalized "per million"), to the antigens tested and their concentration, and to the cytokines measured. Thus, even a small three-plate assay, there can be more than 4320 sets of data that need to be linked together.

The ImmunoSpot software's SpotMap module was specifically designed to manage these data. For each well, and for each plate, the software allows the user to document the assay conditions: which cells were plated in which numbers, which antigens were used to challenge the cells and in which concentrations, and what cytokine was measured (**Fig. 5**). Custom routines help in laying out multi-plate experiments. The software even calculates the amounts of reagent needed for each assay. Once the counting results are available, they can be quickly linked to the other assay parameters. At a click of a button, even the most complex ELISPOT assay can be evaluated, the statistics calculated, and the requested information compiled in an Excel spreadsheet, allowing the results to be represented in virtually any desired format.

4. Notes

1. Occasionally, T-cells move around during the assay, causing the ELISPOTs to develop "tails." This is especially true when T-cells which have been preactivated in vivo or in vitro, as this makes the cells particularly mobile. Such spots can be counted accurately by decreasing the "spot separation tolerance" value.
2. Occasionally, white dots can develop in the middle of the spots. These result from the substrate peeling off, for example, when the flow rate of the plate washer is too high, or the plates are banged too hard while washing. The "Fill Holes" feature can be used mask the white dots away, allowing the spots to be counted accurately.
3. Occasionally, the background coloration can be darker in some parts of the well as the result of leakage in the membrane. This problem can be avoided by decreasing the concentration of Tween used. Additionally, the user can typically compensate by adjusting the "Background Balance" parameter.
4. The background coloration is increased over the entire membrane in a well if the number of cytokine-producing cells (i.e., the number of spots) is high. This is caused by an ELISA effect; that is, cytokine that is not captured around the secreting cell escapes in the supernatant and binds as a "carpet" over the entire well surface. "Autolight" adjustment compensates for the increased background coloration. Some protein antigens can also cause high uniform background by non-specifically binding the detection antibody.
5. Sometimes, the number of spots in the medium background is high for all samples because of the stimulatory effects of serum. Even brief exposure to nontested serum, for example, during washes or during freezing, can drive up the background



	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	4	8	8	12	0	0	0	-2
B	0	0	0	1	7	9	12	10	0	0	0	1
C	7	4	2	420	17	13	10	599	11	17	16	442
D	8	7	2	391	78	34	43	-2	19	30	18	425
E	8	1	13	399	63	50	30	545	35	23	24	456
F	3	12	7	8	107	82	71	85	43	22	35	33
G	1	2	7	14	64	61	58	78	40	39	46	38
H	1	3	3	3	18	20	25	10	15	11	12	23

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 this data is linked and processed for exporting into a database.

intensity. Occasionally, the number of medium background spots is high for a single individual out of several tested. This is a common finding for individuals undergoing a clinical or subclinical infection or other massive immune stimulation in vivo. Some assays, such as IL-6, IL-10, and TNF, tend to give high background coloration in general because of 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.

6. Occasionally, the counting parameters established can produce valid spot 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 (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 quality control mode. This allows the researcher to judge whether the counting parameters established do indeed apply to the all subjects under examination. If recounting of any given subject becomes necessary, the altered parameters are automatically annotated by ImmunoSpot®, thus drawing attention to the atypical spot morphology or other image characteristics.

7. On occasion, the well images contain artifacts caused by membrane damage, for example, when the membrane is accidentally scratched with the pipet. The affected area can be excised in QC mode, and using the *normalization* algorithm, the spot count is recomputed. This renormalization 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 also can be used to correct for cell clustering. For example, if the testing was performed in triplicate and a cluster is found in one of the wells, this cluster 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.
8. 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 assessment 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 spot counting.

Acknowledgments

I would like to thank all those who worked in my Case Western Reserve University-based laboratory on establishing the scientific foundations of cytokine ELISPOT assays. At the postdoctoral level, these are (in alphabetical order): Drs. Don Anthony, Beate Berner, Thomas Forsthuber, Peter Heeger, Alexey Karulin, Damian Kovalovsky, Patrick Ott, Clara Pelfrey, Frauke Rininsland, Stephan Schwander, Oleg Targoni, and Magdalena Tary-Lehmann. Several of my graduate students at Case have made major contributions in our ELISPOT efforts as well: Wolf Bartholomae, Jan Baus, Kamruz Darabi, Marcus Dittrich, Julia Eisenberg, Kristina Feldmann, Judith Gottwein, Robert Guerkov, Thomas Helms, Bernhard Herzog, Maike Hesse, Harald Hofstetter, Thomas Kleen, Christian Kreher, Haydar Kuekrek, Anke Lonsdorf, Kai Loevenbrueck, Stephan Quast, Tarvo Rajasalu, Tobias Schlingman, Britta Stern, and Hualin Yip. At Cellular Technology Limited, I am indebted to the R&D and programming efforts of Johannes Albrecht, Tameem Ansari, Andras Bakos, Ben Matthes, Mark Novak, Jerry Perchinske, Carey Shive, Endre Tary, Norma Sigmund, John Truden, Dean Velasco, and Wenji Zhang.

References

1. Guerkov, R. E., Targoni, O. S., Kreher, C. S., Boehm, B. O., Herrera, M. T., Tary-Lehmann, M., et al. (2003) Detection of low-frequency antigen-specific IL-10-producing CD4+ T-cells via ELISPOT in PBMC: cognate vs. nonspecific production of the cytokine. *J. Immunol. Methods* **279**, 111–121.
2. Hesse, M. D., Karulin, A. Y., Boehm, B. O., Lehmann, P. V., and Tary-Lehmann, M. (2001) A T-cell clone's avidity is a function of its activation state. *J. Immunol*. **167**, 1353–1361.
3. Ott, P. A. Berner, B. R., Herzog, B. A., Guerkov, R., Yonkers, N. L., Boehm, B. O., et al. (2004) CD28 costimulation enhances the sensitivity of the ELISPOT assay for detection of antigen-specific memory effector $CD4^+$ and $CD8^+$ cell populations in human diseases**.** *J. Immunol. Methods* **285**, 223–235.
4. Helms, T., Boehm, B. O., Assad, R. J., Trezza, R. T., Lehmann, P. V., and Tary-Lehmann, M. (2000) Direct visualization of cytokine-producing, recall antigen-specific CD4 memory T-cells in healthy individuals and HIV patients. *J. Immunol*. **164**, 3723–3732.
5. Kreher, C. R., Dittrich, M. T., Guerkov, R., Boehm, B. O., and Tary-Lehmann M, M. (2003). CD4+ and CD8+ cells in cryopreserved human PBMC maintain full functionality in cytokine ELISPOT assays. *J. Immunol. Methods*. **278**, 79–93.
6. Karulin, A. Y., Hesse, M. D., Tary-Lehmann, M., and . Lehmann, P. V. (2000) Single-cytokine-producing CD4 memory cells prevail *in vivo*, in type 1/type 2 immunity. *J. Immunol*. **164**, 1862–1872.
7. Rininsland, F. H., Helms, T., Asaad, R. J., Boehm, B. O., and Tary-Lehmann, M. (2000) Granzyme B ELISPOT assay for *ex vivo* measurement of T-cell immunity. *J. Immunol. Methods* **240**, 143–155.