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High Resolution as a Key Feature to Perform Accurate ELISPOT Measurements Using Zeiss KS ELISPOT Readers

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Summary

The enzyme-linked immunospot (ELISPOT) assay was originally developed for the detection of individual antibody secreting B-cells. Since then, the method has been improved, and ELISPOT is used for the determination of the production of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, or various interleukins (IL)-4, IL-5. ELISPOT measurements are performed in 96-well plates with nitrocellulose membranes either visually or by means of image analysis. Image analysis offers various procedures to overcome variable background intensity problems and separate true from false spots. ELISPOT readers offer a complete solution for precise and automatic evaluation of ELISPOT assays. Number, size, and intensity of each single spot can be determined, printed, or saved for further statistical evaluation. Cytokine spots are always round, but because of floating edges with the background, they have a nonsmooth borderline. Resolution is a key feature for a precise detection of ELISPOT. In standard applications shape and edge steepness are essential parameters in addition to size and color for an accurate spot recognition. These parameters need a minimum spot diameter of 6 pixels. Collecting one single image per well with a standard color camera with 750×560 pixels will result in a resolution much too low to get all of the spots in a specimen. IFN-γ spots may have only 25 µm diameters, and TNF-α spots just 15 µm. A 750×560 pixel image of a 6-mm well has a pixel size of 12 µm, resulting in only 1 or 2 pixel for a spot. Using a precise microscope optic in combination with a high resolution (1300×1030 pixel) integrating digital color camera, and at least 2×2 images per well will result in a pixel size of 2.5 µm and, as a minimum, 6 pixel diameter per spot. New approaches try to detect two cytokines per cell at the same time (i.e., IFN-γ and IL-5). Standard staining procedures produce brownish spots (horseradish peroxidase) and blue spots (alkaline phosphatase). Problems may occur with color overlaps from cells producing both cytokines, resulting in violet spots. The latest experiments therefore try to use fluorescence labels as a marker. Fluorescein isothiocyanate results in green spots and Rhodamine in red spots. Cells producing both cytokines appear yellow. These colors can be separated much easier than the violet, red, and blue, especially using a high resolution.

Key Words: ELISPOT reader; ELISPOT method; spot teacher; camera resolution; pixel size; double labels; fluorescence markers.

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1. Introduction

Despite the pharmaceutical and medical success achieved so far, it is possible to treat only one third of the 30,000 known diseases. In addition, many diseases, such as rheumatism, Alzheimer's disease, AIDS, and cancer, remain incurable ***(1)***. Research throughout the world is focused on cancer. The use of genetic engineering provides scientists with an increasingly better understanding of what malfunctions participate in the generation of malignant tumors on a molecular level. With regard to possible therapies, immunological methods make us particularly optimistic. In this field, the cytokines are of special interest. These proteins are substances created by a variety of cell types contributing to the activation of cells.

Tumor cells have a special characteristic. Like most cells, they have a structure that is specific only for their cell type. This is called a tumor-associated antigen. This structure makes it different from all the other body cells and therefore permits identification ***(2)***. Where identification is possible, we are hopeful a direct remedy will soon be possible.

Cytostasis agents used in standard chemotherapy also affect healthy, proliferating body cells. This results in the known side effects of chemotherapy. The new therapy approach endeavors to use the characteristic structure of tumor cells to specifically attack the tumor itself ***(3)***. Background: The tumor cells produce proteins—so-called antibodies. These antibodies all feature the same specific structure and also are expressed by the tumor. These tumor antibodies can be isolated from the patient's blood with relative ease and are used, so to speak, as the tumor-specific structure for immunization.

A certain cell type in the human immune system, the antigen-presenting cell, commonly known as a "dendritic cell," is able to present an antigen to the so-called T-cells. The T-cells then proliferate and, in the form of cytotoxic "killer cells," specifically attack the structure "shown" to them before by the antigen-presenting cell ***(4)***. To measure the success of therapy, the specific defense cells, which were activated by the therapy, must be detected and recorded in a suitable manner. The T-cells that attack the cells of interest circulate in the patient's blood. The enzyme-linked immunospot (ELISPOT) assay is used to count the number of these specific "killer cells" before and after therapy, providing a measure for the immunologic reaction of the therapy.

In the field of immunology research, more and more molecules secreted in response to immunization are checked whether or not they can be used to develop direct therapy methods. The enzyme-linked immunospot (ELISPOT) technique, meanwhile, became the method to evaluate the effect of these molecules to immune cells on a single cell level. Fields of application are the determinations of therapy successes with regard to immunological responses in cancer

diseases, AIDS, Alzheimer's disease, asthma, and so on. Immunological response does not always mean therapeutical success, but it is a first step in this direction.

Meanwhile, more than 150 cytokines are isolated and detected ***(5)***, and their number is increasing nearly on a daily basis. Because cytokines play an important role during inflammation and diseases, they are the best tool to measure the activation of immune cells. Whereas the previously used enzyme-linked immunosorbent assay (ELISA) method only allows one to measure the concentration of cytokines in the supernatant of a cell culture, the ELISPOT method allows one to detect of cytokine production on a single cell level.

2. The ELISPOT Method

The ELISPOT assay was developed for the detection of individual antibody-secreting B-cells ***(6)***. Since then, the method has been improved in a way that cells can be detected producing only approx 100 molecules of a protein (i.e., cytokine) per second ***(7)***. These high protein concentrations in the cell surrounding will be detected with specific antibodies. When the ELISPOT method is used, the created spots only show an imprint of those cells originating them. The advantage of this method is the fact that the spots are long lasting, and they can be evaluated visually as well as by means of image analysis. Using image analysis, one can automatically determine the number, size, and intensity of spots with an increased objectivity.

For the ELISPOT determination of the production of tumor necrosis factor (TNF)-α or interferon (IFN)-γ or interleukin (IL)-4, IL-5 and more by a single cell, the bottoms of nitrocellulose microtiter plates are covered with antibodies directed against the cytokine of interest. All antibodies not bound to the nitrocellulose are then washed away. The covered wells are now filled with lymphocytes to be tested and negative controls. The T-cells on the membranes secrete the cytokines, which will bind to the antibodies. After a certain incubation time, the cells will be washed out, and the bound cytokines will be marked with a second antibody. This complex will finally be marked with a staining substance. Now single spots can be counted under the microscope ***(8,9)***.

In this way, the spots created by the ELISPOT method are only a reprint of those cells that originally created them. Some advantages are that specimens are not dangerous and that they can be kept for a long time. Furthermore, they can be evaluated, either visually or by means of image analysis, and the evaluation may be repeated for control purposes whenever required ***(7)***.

The ELISPOT method consists of five steps: (1) adding a cytokine specific antibody to the nitrocellulose membranes of a microtiter plate; (2) exclusion

of nonspecific absorption of other proteins; (3) adding cytokine secreting cells in various concentrations; (4) addition of a second anti-cytokine antibody; and (5) detection of the antibody–cytokine complex.

At present two staining techniques are used. The alkaline phosphatase (AP) marker produces blue spots whereas the horseradish peroxidase marker produces brownish ones. The method also is used for the detection of secretions of specific subgroups of lymphocytes or T-cells from peripheral blood, as well as of monocytes and granulocytes ***(7)***.

The immunochemically stained cytokine spots are scanned with a 3-chip CCD color camera on an incident light microscope with motorized stage and auto-focus control. The images are digitized with 24-bit color resolution and evaluated by the KS ELISPOT system. The region for evaluation is determined using the mouse on the system monitor. The definition of the start and end point is sufficient to begin the routine measurement ***(10)***.

All results of the analysis are displayed immediately on the screen. Number, size, and intensity of each single spot can be printed or exported to a file for future statistic evaluation or graphic presentation. All well images can be saved for re-evaluation or documentation purposes.

2.1. Common Problems With ELISPOT Specimens

The ELISPOT assay faces two background problems. First, a variable background intensity of the nitrocellulose may be observed. To overcome this problem, a specific algorithm had been developed, taking into account the varying background conditions in the region when detecting the spots ***(10)***. It is no longer necessary to adjust different threshold values for various positions of a well.

With the aid of a unique spot learning procedure, all system parameters, necessary for a correct spot recognition, are adjusted by simple cursor clicks to desired spots. A further advantage of this new method is the improvement of measurement reproducibility.

The second problem is the occurrence of small and very dark spots that were not generated by secreted cytokines. In the visual evaluation, these spots are differentiated from "true" spots by their sharper edges. True spots always have a dark center with fading color intensity towards the edges. False spots are usually small with a homogeneous intensity. Functions for the differentiation of true spots also have been implemented in the detection algorithm of the KS ELISPOT software ***(10)***. The shape can be tested to decide whether or not a spot exists. Cytokine spots are always round but because of "floating edges" with the background they have a "non-smooth" borderline.

Depending on the magnification used, each experiment can be defined in this way under the tool setup. The spot definition itself is performed by automatic

color segmentation. This algorithm first determines all probable spot positions after the acquisition of a complete well image. Each of these positions will then be checked via edge steepness and form factors for its spot probability.

All these conditions demand certain minimum and maximum magnifications for accurate use of the system. The recognition of differences in edge intensities needs a certain minimum spot diameter regarding the pixel resolution. Acquiring a complete well as one single image often lacks of necessary resolution. This method therefore should only be applied on specimens with mean or large spot diameters. Whenever the acquisition of only one image per well will produce exact results because of only larger spots, the advantage of these systems is the extremely high evaluation speed. An example for this group of instruments is the KS ELISPOT compact, using a stereo microscope Stemi 2000 as input source.

Whenever spots decrease to less than certain minimum diameters, the KS ELISPOT compact may be used in a scanning mode, similar to the KS ELISPOT, that is, using the Axioplan 2 light microscope as input source. This will result in a well image composed from several single images with a higher resolution, which is then evaluated with the correct pixel resolution without any edge problems using a circular measurement frame.

A check for all selected settings can be performed for a complete well. Using the teaching algorithms, all parameters responsible for the determination of a spot (spot diameter, color, saturation, contrast, shape, slope) can be trained by simply clicking to spots. In a similar way false spots can be eliminated from the parameter set. The finally accepted settings can be saved for the routine usage in different experiments under separate names.

2.2. Important Requirements of an Automatic ELISPOT Evaluation System

1. Easy handling: The system should be developed for use in routine laboratories and easily adaptable to new preparation methods. In the KS ELISPOT, this is achieved by the unique spot learning procedure.
2. Complete package: All system parts, such as the microscope, scanning stage, control unit, computer, and software, should be available from one source to guarantee a maximum performance.
3. Minimum adjustments: Especially for the routine usage, only a few adjustments should be necessary to start the evaluation.
4. Overview or full-resolution image: During evaluation, the permanent display of the overview well image under evaluation is essential for control purposes. The access to the full resolution image, including the display of results, also is very important.
5. Storage of measurement results: Spot results (number, single areas, and single intensities) have to be stored in a simple file format like text files for external processing and graphic presentation.

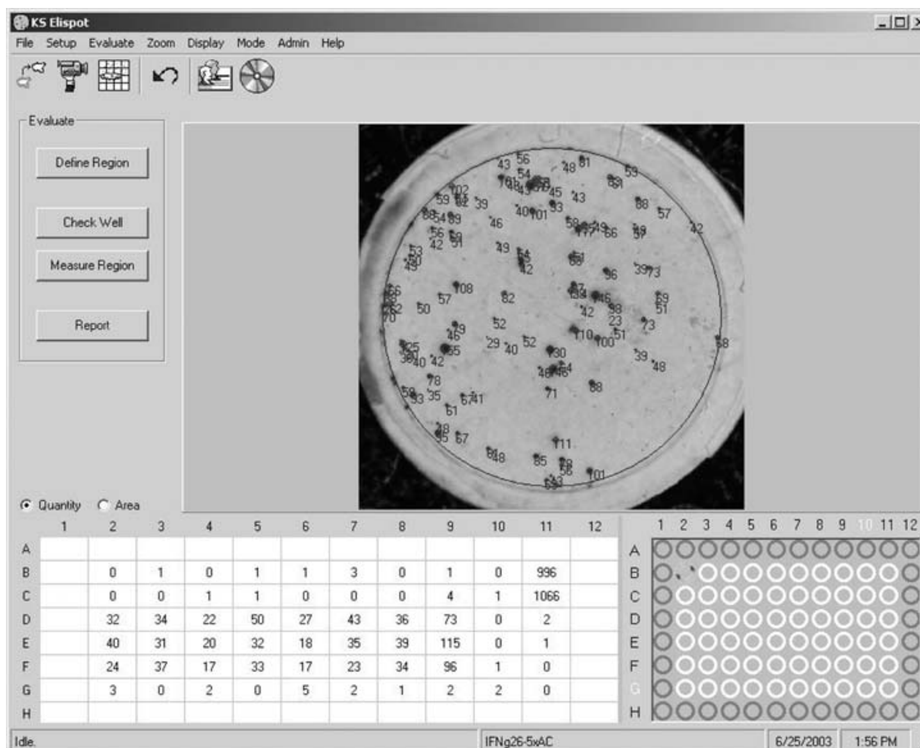


Fig. 1. Example of an easy-to-use interface: only four buttons for routine measurements, a window for the display of well images during the measurement, a plate field for the definition of the region of interest, and a result table for the immediate display of count results.

6. Data output: All measurement results should possibly be stored in an internal format to be saved and externally processed. As an alternative reports like a Word protocol may be produced showing experiment description, measurement data, and parameter settings, as well as a plate image of all measured wells and a plate image with all detected spots in each measured well.
7. Storage of images: Images of all measured wells should be saved for re-evaluation and documentation purposes, if needed.

An example of the tool buttons to be used for routine evaluations are listed below (*see also* **Figs. 1** and **2**):

1. **Define Region:** Defines the plate region for evaluation, resulting in the number of wells to be measured. At the same time the well positions and the stage co-ordinates are synchronized.

Fig. 2. Only four buttons are used for routine applications.

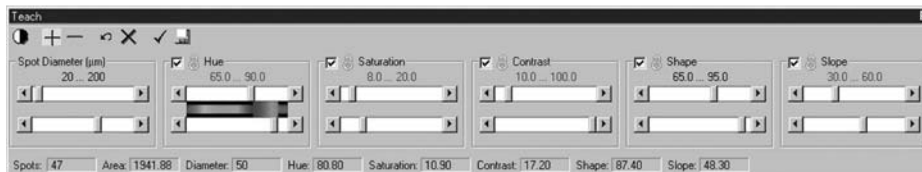


Fig. 3. Example for a spot teacher. Spots in the well image will be selected by simple mouse clicks to adapt the sliders to their values.

2. **Check Well**: Performs a test evaluation of one selected well to check the system settings and to perform the learn procedure.
3. **Measure Region:** Evaluates all selected wells of a microtiter plate.
4. **Report:** Displays results page with the option to print and save the data.

2.3. Learning Method for Spot Recognition

At least when setting up an ELISPOT reader for the first time, it will be necessary to train the software in the appearance of the spots inside the specimen. A tool like a spot teacher is extremely helpful because it uses a specifically developed learning method to recognize the spots. This learning algorithm extends all its parameters for spots that were selected from clicks on not-yet-recognized spots using the cursor and the left mouse key (*see* **Fig. 3**). In this way all spot parameters are adapted automatically. Spot definitions created in

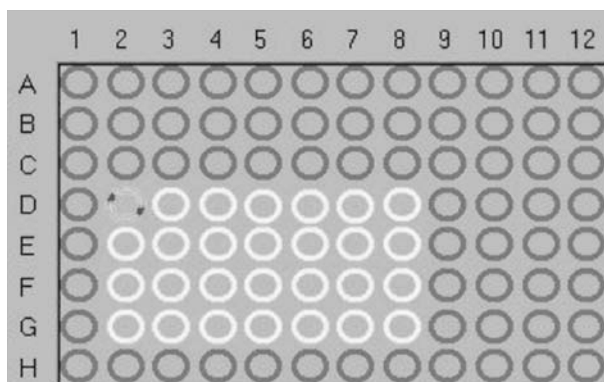


Fig. 4. The region of interest per plate can be defined by simply marking the wells inside that region with the mouse.

this way can be stored for future use. For a better control all recognized spots have to be displayed graphically together with their diameters.

2.4. Routine Use

An easy way to define the region for evaluation may be performed in a plate table on the screen using the mouse (*see* **Fig. 4**). To start a measurement the definition of only the start and end well should be necessary. It should be possible to eliminate not required positions from the measurement by mouse clicks. The possibility to define various regions per plate with different configurations for the evaluation can be very helpful.

For longer-lasting evaluations, the immediate presentation per well of the results of an analysis (spots per well) is absolutely necessary for the control. During the evaluation period, the indication of the likely time for the complete evaluation of the entire plate is advantageous when permanently updated, together with the estimated time for the completion of the evaluation—as a value and as a progress bar.

A general background correction is useful with specimens having extremely high background staining and at the same time high and dense spot numbers (this is often the case with positive controls). This helps to recognize the spots to an acceptable amount (*see* **Fig. 5**). Variable local spot background settings offer alternatives for a better spot recognition. Variable internal image resolutions allow the best compromise between evaluation accuracy and system speed.

2.5. Performance

After the definition of the overall region to be measured, one single configuration file should define all necessary settings for the system (*see* **Fig. 6**). The

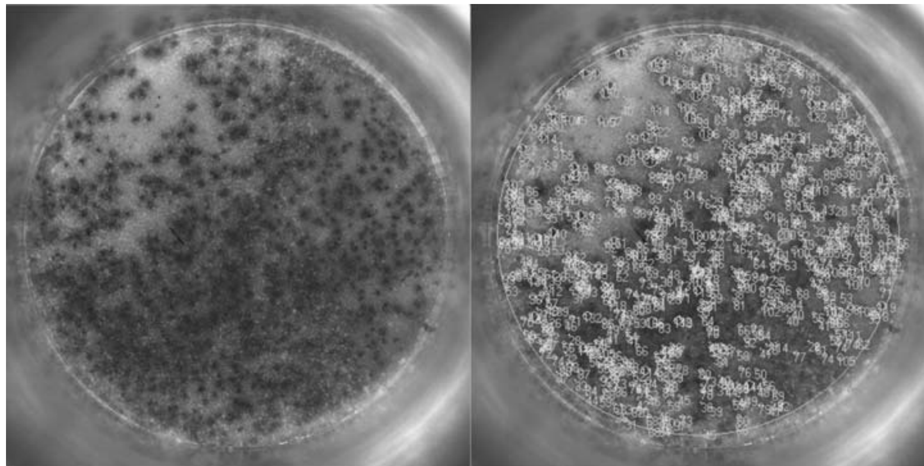


Fig. 5. Typical well image from a positive control. Spots are very dark on an intense background. Partially, the spots and the background are confluent. Using special correction functions the spots still can be recognized in an acceptable amount.

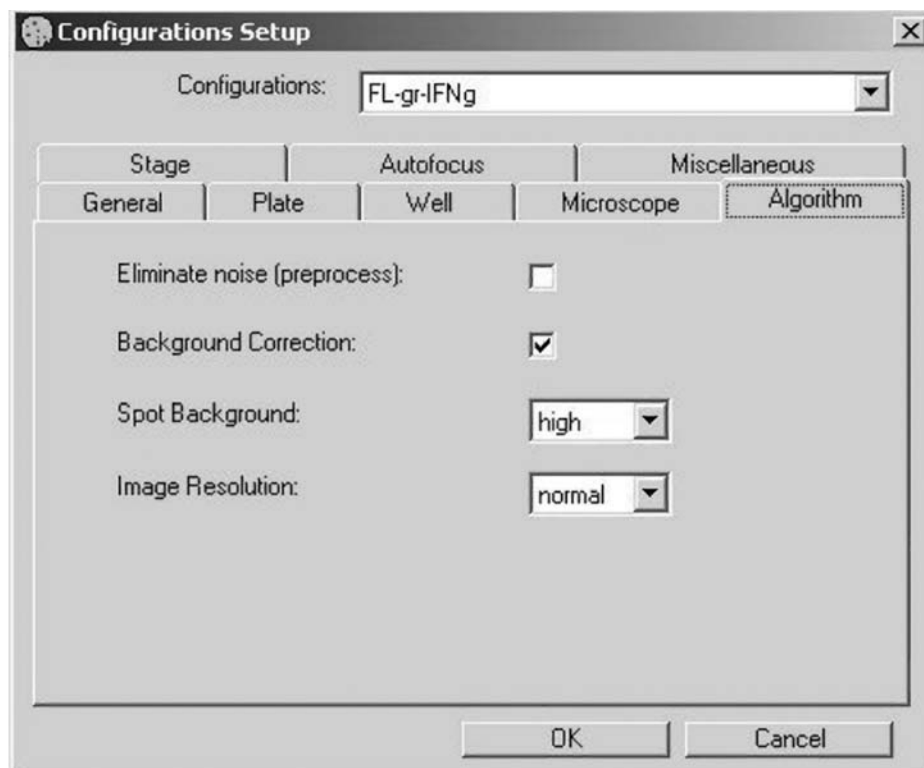


Fig. 6. All parameters for the evaluation of an ELISPOT experiment will be defined in a configuration window on several tab-sheets. Each configuration can be saved for a future usage.

possibility of multiple definitions within one plate to evaluate various regions with differently defined setups is a nice feature that helps facilitate evaluations.

Before the measurement of a whole plate or even of a series of plates, it is advisable to first test the system settings of the used configurations on some selected positive and negative wells for their correct function. In case modifications become necessary, a teach function that allows one to adapt all relevant spot parameters by simple mouse-clicks to the wrongly recognized spots in the well is extremely helpful.

Usually by means of image analysis, there are six parameters that will describe best a real spot (*see* **Fig. 3**) and separate it from other particles in the specimen, like dirt, debris, scratches, and others. They are listed below:

1. The **Spot Diameter** may be preset to a minimum and maximum value. The smallest expected spot size is about 25 μm for human IFN-γ cytokines. TNF-α and usually mouse IFN-γ cytokines produce much smaller spots, as small as 15 μm in diameter.
2. **Hue** is the definition of color values with lower and upper limit. Values range continuously from red (90–15) via yellow (15–40) and green (40–65) to blue (65–90) on a scale from 0 to 100.
3. **Saturation** defines the color saturation with lower and upper limit.
4. **Contrast**: defines the overall contrast range for spots. This should be a position independent relative contrast, taking into account also the local surroundings of the spot.
5. The **Shape** is the definition of the form factor. High values represent an ideal circle and lower values describe shapes more and more differing from circles.
6. The **Slope** defines the edge steepness of spots. Ideal spots usually have values in the mid range, and higher values mean steeper edges.

For the control of the settings, access to the raw data is essential. The defined limit values should also be printed later with the measurement results so that one is able to repeat the measurements at a later time or allow the re-evaluation of them on a different machine.

It is advisable to already have all measurement results available immediately after the scanning of the plate. Sometimes it may be helpful to have all well images recorded for repeated evaluations in the phase of finding the best settings for the evaluations of an experiment series of plates. This also is of advantage when a new evaluation of the identical wells in a plate is necessary, as with double stains, where IFN and IL are labeled at the same time, and red, blue, and violet spots have to be measured in each well. The re-evaluation for the different colors on stored images will be much faster, when no new scanning of the specimen is necessary.

This will be even of more importance when fluorescence labels for the different cytokines are used because fluorescence markers are extremely sensitive to exposure time, especially with ultraviolet light. In this case, a first run to record the

images will allow multiple evaluation runs for adapting the settings and measurement variables and finally perform all evaluation steps in the different channels.

2.6. Specimens

Suitable specimens are original 96-well microtiter plates, filtration plates with nitrocellulose membranes, or nitrocellulose membranes transferred to sticky foils. Some users prefer to remove the membranes from the plates and stick them on a plastic foil before the measurement to avoid reflections from the well side-walls during image acquisition. In this case, the specimen should be mounted very flush with the plate of the stage (best applied with spray adhesive and a roller squeeze).

Special motor stage plates with markers for the driving ranges are necessary to use with peeled-off nitrocellulose membranes; for the use with complete nitrocellulose plates, suitable specimen holders needs to be available as well.

3. A Short Introduction to the KS ELISPOT Software

KS ELISPOT compact is a system with a complete solution for precise and automatic evaluation of ELISPOT assays. It has been developed especially for use in routine laboratories with regard to a high-speed throughput of specimens. Using the KS ELISPOT system (**Fig. 7**) with a light microscope with incident illumination, motor stage, and automatic focusing unit, the aim is high resolution for highest measurement precision and accuracy. The images of the cytokine spots are taken in both cases with a true color camera.

After the digitization, the KS ELISPOT software proceeds with automatic data processing. For routine usage, easy handling is essential. Special importance was attached to the design of the user interface to reduce the user elements to a minimum. Finally, only four buttons are used (*see* **Fig. 2**), which encourages minimal training times to learn the system. Special types of specimen holders guarantee the usage of either whole plates or removed nitrocellulose membranes on a sticky foil. Finally, all data may be transferred directly into a spreadsheet program for further evaluation or graphical display.

First, the region for evaluation in the microtiter plate field on the screen of the system must be marked with the cursor. Next the stage position has to be synchronized with the program coordinates. This will be performed with the **Define Region** button. The system is now ready for the evaluation of a plate.

Using the **Check Well** function, the system settings can be tested for their functioning before the evaluation of a complete plate . Using the Check Well function is always a prerequisite to use the **Teach** algorithm to adapt the spot recognition or to define a new configuration setting for multiple evaluation patterns.

The test well can be selected and addressed by a single right mouse click in the plate overview. The stage will move to the center position of the selected

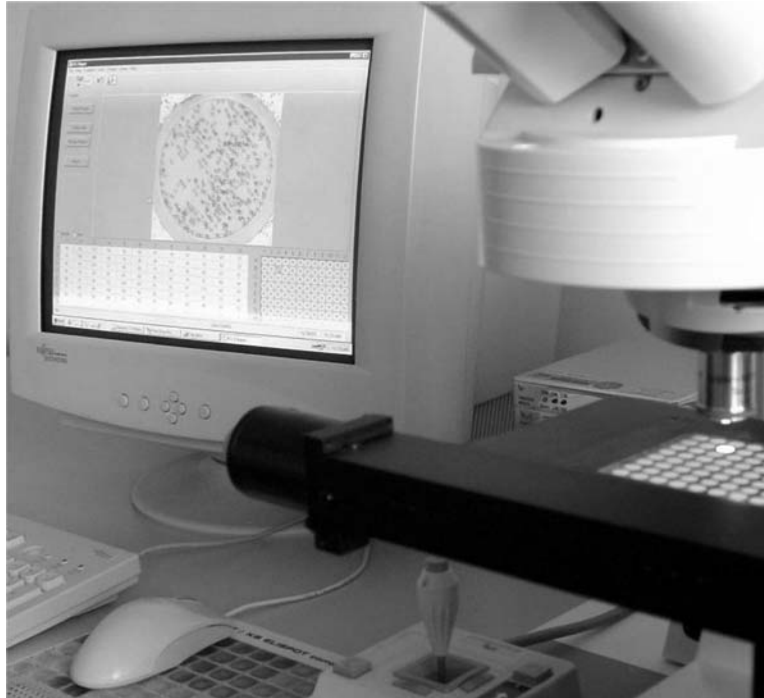
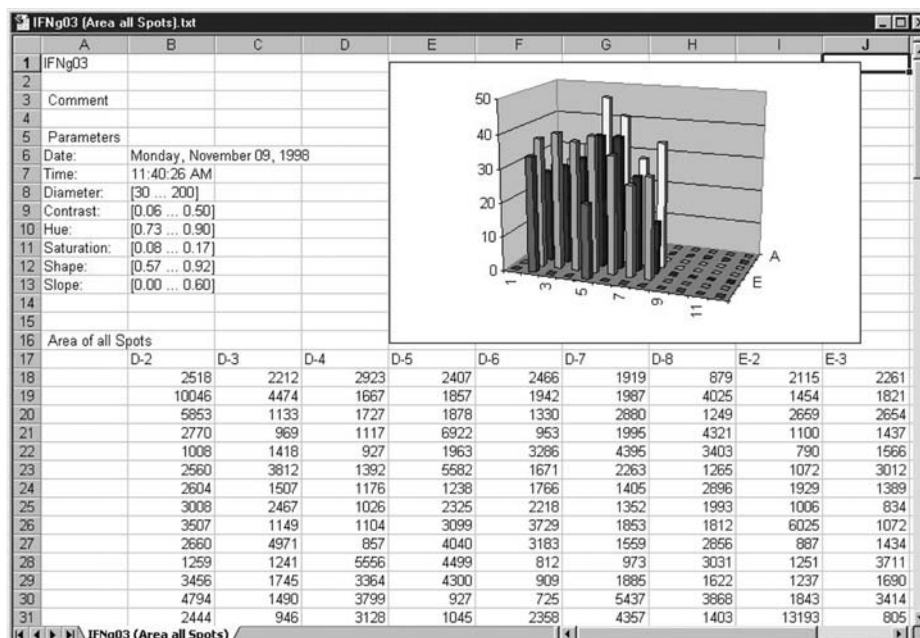


Fig. 7. KS ELISPOT reader.

well and perform a meander scan. From all images, one complete well image will be generated, displayed in a reduced resolution, and evaluated in full resolution. The evaluation result will be displayed in the overlay plane of the image. On the resulting image a check of the limit settings with the **Teach** algorithm is possible.

The **Measure Region** function will start the evaluation of the complete plate. The stage will move to the center of the first well position and initiate the autofocus. Then the well position will be scanned in a meander mode. From all fields, a complete well image will be generated and displayed in a reduced mode in the image field of the user interface.

After a short time, the result will be displayed in the overlay (spot indication and spot diameter). The stage will now move to the next well position and the sequence is repeated until the last well is evaluated. All rejected positions will be skipped. With each stage movement, an estimation is performed for the duration of the complete evaluation procedure and shown in a separate window. After the evaluation of the last well position, the stage will automatically return to the start position.



	A	B	C	D	E	F	G	H	I	J
1	IFNg03									
2										
3	Comment									
4										
5	Parameters									
6	Date:	Monday, November 09, 1998								
7	Time:	11:40:26 AM								
8	Diameter:	[30 ... 200]								
9	Contrast:	[0.06 ... 0.50]								
10	Hue:	[0.73 ... 0.90]								
11	Saturation:	[0.08 ... 0.17]								
12	Shape:	[0.57 ... 0.92]								
13	Slope:	[0.00 ... 0.60]								
14										
15										
16	Area of all Spots									
17		D-2	D-3	D-4	D-5	D-6	D-7	D-8	E-2	E-3
18		2518	2212	2923	2407	2466	1919	879	2115	2261
19		10046	4474	1667	1857	1942	1987	4025	1454	1821
20		5853	1133	1727	1878	1330	2880	1249	2659	2654
21		2770	969	1117	6922	953	1995	4321	1100	1437
22		1008	1418	927	1963	3286	4395	3403	790	1566
23		2560	3812	1392	5582	1671	2263	1265	1072	3012
24		2604	1507	1176	1238	1766	1405	2896	1929	1389
25		3008	2467	1026	2325	2218	1352	1993	1006	834
26		3507	1149	1104	3099	3729	1853	1812	6025	1072
27		2660	4971	857	4040	3183	1559	2856	887	1434
28		1259	1241	5556	4499	812	973	3031	1251	3711
29		3456	1745	3364	4300	909	1885	1622	1237	1690
30		4794	1490	3799	927	725	5437	3868	1843	3414
31		2444	946	3128	1045	2358	4357	1403	13193	805

Fig. 8. Example for exported raw data into an Excel spreadsheet with a graphic presentation of the spot size distribution.

A mouse click on the **Report** button will open a separate result window. These results may be printed or saved in different data files. In case single well images are needed in higher resolution, all well images may be kept temporarily as ".JPG" files with display resolution. As an alternative the images may be stored with full resolution.

3.1. Results

Number, size, and intensity of each single spot can be printed via the results page, or stored in data files for further statistical evaluations or graphic displays (*see* **Fig. 8**). Images of single wells can be stored for documentation purposes. Apart from title, date, and spot size limits, all single spot values are stored in well number order. For each well the number of spots, the areas and the intensities are available. In addition to the measurement data all adjustment parameters will be stored.

As an alternative to the internal data format, a result protocol may be automatically generated in Microsoft Word. This protocol exists of a title page with the general experiment description, the results of spot counting and area measurements, as well as the parameter settings for the spot definition. Furthermore,

a color overview image of all evaluated wells in a plate is created and additionally an overview image showing all recognized spots in each measured well.

4. Why Is Resolution so Important?

Usually a standard camera in combination with a framegrabber will result in a single image size of 750×560 pixel. KS ELISPOT is collecting several images per well that are combined to one common well image for the evaluation.

A digital integrating camera like the AxioCam MRc has a resolution of 1300×1030 pixels and offers excellent options for the correction of colors and background directly in the camera itself. Using this camera resolution, fewer images per well are necessary for identical overall resolution. More than three standard camera images are necessary for the same resolution as one single Axiocam MRc image.

All reader adjustments end in a certain possible minimum as well as maximum magnification for the use of the system. To recognize a difference in edge intensities, a minimum diameter of the spots is necessary with regard to the pixel resolution. Using just one camera image per well will result in insufficient resolution. Evaluating a series of single images with higher magnification will cause problems with those spots that cut the image edges as well as with those spots located close to the well edge. Therefore, KS ELISPOT first generates one high-resolution image of a complete well from multiple camera images. This image will then be evaluated with correct pixel resolution, without any edge problems, using a circular measurement frame.

The decisive advantage of the Zeiss KS ELISPOT system in comparison with other evaluation methods is the direct increase of the value of scientific data. Results are clearly more reproducible than from manual evaluations. This was shown in a recent investigation at the Sloan Kettering Memorial Hospital in New York (S. Janetzki et al., submitted), comparing the results from different scientist among each other, counting manually and using an ELISPOT reader. Finally, using a reader, in addition to the counting, area and intensity values of each single spot are available.

Compared with other systems, another advantage of the KS ELISPOT system is its resolution. The images are taken with a Zeiss microscope and, as a minimum, 12 images are collected for each well. Collecting only one single image per well will result in a resolution that is too low to include all spots in a specimen (*see* **Fig. 9**). In experiments with IFN-γ, the spot size be as small as 25 μ diameter, and in TNF-α, applications, 15 μ. A 750 ± 560 pixel image of a well can only resolve a pixel size of approx 12 μ. This is absolutely not sufficient to measure most of the smaller spots, especially in the case of TNF-α. This is only guaranteed using a precise microscope optic with a good resolu-

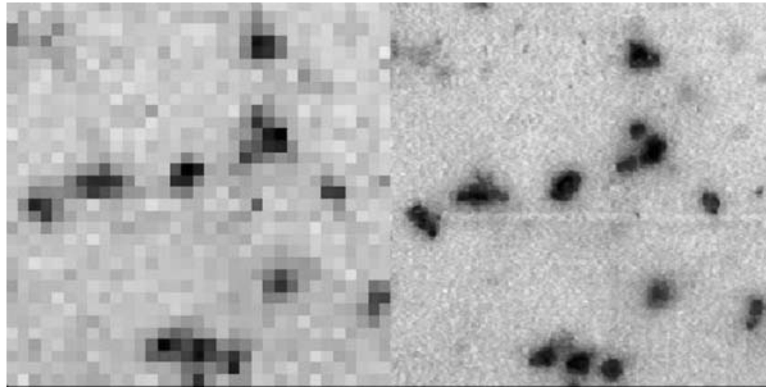


Fig. 9. Resolution comparison in an example of a TNF-α ELISPOT specimen using a standard camera. Left: resolution of one image per well. Right: resolution of the identical area with 12 images per well.

tion, resulting in pixel sizes between 2.0 and 2.5 μ, giving at least a 6 pixel diameter even of the smallest spots *(2)*.

In a recently performed comparison of different readers at the University Clinics in Würzburg (S. Schaed et al., unpublished data), using 1, 4, or 12 images per well, it was shown that one image per well resolution cannot produce accurate results for spots smaller than 50 μ diameter. For experiments with IFN-γ at least four images with a standard camera are necessary to get accurate and reproducible results from spots with 30 μ diameter. With 12 images per well, all possible spot sizes are always recognized correctly, even below a diameter of 20 μ. Using a high-resolution digital camera like the Axiocam MRc, the number of images per well that are needed to obtain accurate results for the smaller spots can be reduced to one from four images per well.

5. Future Developments in the Field of ELISPOT Evaluations

5.1. Measurement of Double Cytokine Production by Standard Staining Methods

To detect more than one cytokine produced by a cell at the same time, different approaches have been tried. Currently two different staining methods are used, as mentioned earlier. The horseradish peroxidase method will produce brownish spots, and the alkaline phosphatase method blue ones. If a cell is producing both cytokines, the result will be violet spots.

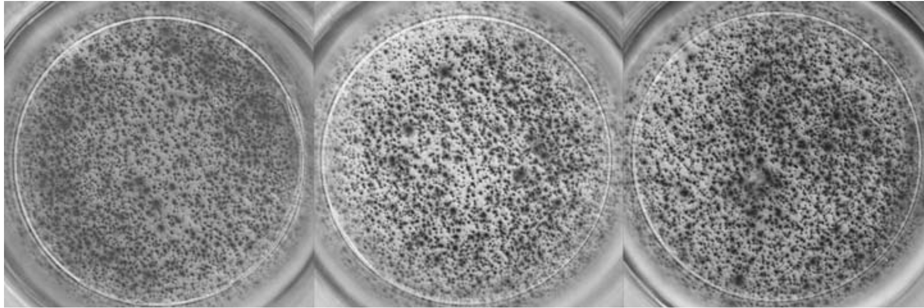


Fig. 10. ELISPOT specimen with similar number of spots per well. Some wells are labeled with a red marker (left), others with a blue marker (middle), and some with both markers resulting in violet spots (right). Result of counts per well with teaching on each color and cross check: Setting 1: color 88–97 (red), setting 2: color 57–62 (blue), setting 3: color 82–95 (violet). *See* **Color Plate 3** following page 50.

Well	Setting	Count	Setting	Count
A2 (red)	1	597	2	0
A4 (blue)	2	672	1	0
A6 (violet)	3	660	1	462
A6 (violet)	3	660	2	0

In the test setups parameters could be defined to clearly separate the blue from the red spots, and the blue from the violet spots. But there was always an overlap of more than 60% between the red and the violet spots. This overlap is too big to accept any result as a clear separation between cells only producing one cytokine from those producing both cytokines.

These tests have been performed on special specimen prepared by MabTech to test the usability of standard staining procedures for the detection of multiple labeled cells (*see* **Fig. 10**). Without better new staining procedures these methods will not result in good and valid data, as long as no clear separation between the colors can be guaranteed.

5.2. Measurement of Double Cytokine Production by Fluorescence Methods

To measure fluorescent specimen an appropriate filter is necessary in the microscope, and the camera must be capable to integrate signals. Using a Sony DXC 950 or a Hitachi HV-C20A the shutter time must be set to an adequate integration time. For the trigger signal, a special cable may be necessary

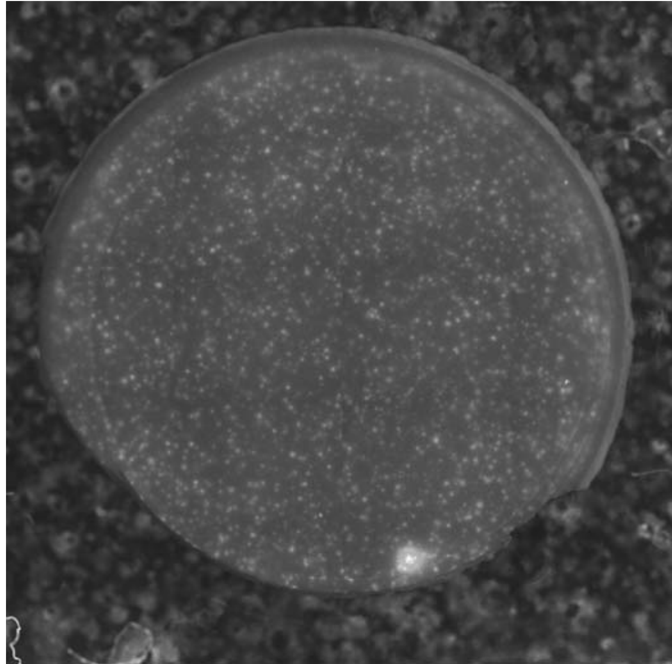


Fig. 11. ELISPOT specimen labeled with fluorescence markers for IFN-γ (fluorescein isothiocyanate, green spots) and IL-5 (rhodamine, red spots). Spots from cells expressing both cytokines appear yellow. Count results: red spots, 229; green spots, 291; yellow spots, 64. *See* **Color Plate 4** following page 50.

between camera and frame-grabber. The integration time for these cameras is only adjustable at the cameras themselves and cannot be controlled by the evaluation software. This solution therefore is not really user-friendly.

The AxioCam MRc as an alternative is an integrating digital camera that can be adjusted to an optimum fluorescence exposure time directly from the evaluation software. Various settings for different experiments may be saved in their own configuration files, and easily recalled when necessary. The handling of this system is much easier and can easily be used in a routine environment.

Fluorescent signals usually result in bright spots on dark background (*see* **Fig. 11**). This is different to the usually expected dark spots on a bright background from standard specimen. A special functionality is therefore necessary to enable the ELISPOT software to perform the measurement. Fluorescence images in addition are not as bright as standard images and require a special illumination for the different wavelengths to produce the requested signals.

But once the system is configured in the correct way, the separation between the signals is much easier and more accurate as with the mixed conventional staining method.

IFN-γ will be labeled with a green fluorescence dye (fluorescein isothiocyanate), and IL-5 with a red dye (rhodamine). Those spots from cells producing both cytokines will appear yellow. These colors can be separated much easier than the violet between red and blue resulting with the conventional stainings. The method is currently under development at the European Hospital Georges Pompidou in Paris ***(11)***, but the first results presented at the first French ELISPOT workshop in September 2003 are more than promising.

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