Application Note

Immunology

Stimulation of IL-2 Secretion in Human Lymphocytes

Using the Agilent BioTek Lionheart LX automated microscope to image and analyze silver-stained ELISpot assays

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Abstract

Lymphocytes are used in several fields of research including autoimmune disorders, infectious diseases, vaccine development, and hematological malignancies. The ELISpot assay monitors *ex vivo* cellular immune responses to antigenic stimuli. While the original ELISpot assays used PVDF membrane plates, recently the assay format has developed to use a silver-staining process that allows clear-bottom plates to be used. This application note uses the Agilent BioTek Lionheart LX automated microscope in conjunction with Agilent BioTek Gen5 microplate reader and imager software to quantitate changes in IL-2 secretion in Jurkat cells using a silver-stain ELISpot assay format.

Introduction

Cytokines are small molecular weight proteins or peptides secreted by many cell types (particularly immune system cells) that regulate the duration and intensity of the immune response. The cytokine interleukin 2 (IL-2) is a pleiotropic cellular regulatory molecule that is produced by lymphoid cells in response to several stimuli. It plays a role in preventing autoimmune diseases by promoting differentiation of immature T cells into regulatory T cells.[1] In addition, it causes the differentiation of T cells into effector T cells and memory T cells when the original T cell was stimulated by an antigen.[2]

T cell activation is normally initiated by the interaction of a cell surface receptor to its specific ligand molecule along with a co-stimulatory molecule.[3] This binding event triggers the rapid hydrolysis of inositol phospholipids to diacylglycerol and inositol phosphates by phospholipase C (PLC).

Diacylglycerol is an allosteric activator of protein kinase C (PKC) activation and inositol phosphates, which trigger Ca^{2+} release and mobilization, resulting in a cascade of additional cellular responses mediating T cell activation. One of these cellular responses is the production and secretion of IL-2. Triptolide is a diterpene triepoxide, that is a potent immunosuppressant and anti-inflammatory agent. Triptolide has been shown to inhibit the expression of IL-2 in activated T cells at the level of purine-box/nuclear factor and NF-κB mediated transcription activation. (Figure 1).[4]

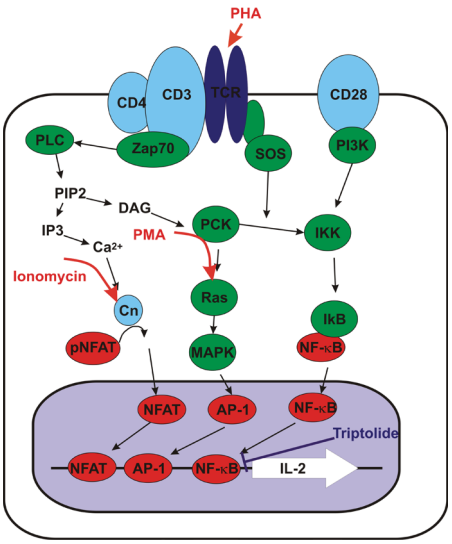

Figure 1. Schematic of signal cascade for stimulation of IL-2 secretion.

While Jurkat cells are a leukemic T cell line known to produce IL-2, under normal growth conditions little IL-2 is produced. Only after stimulation will substantial amounts of the cytokine be expressed.[5] Phytohemagglutinin (PHA) is a lectin that binds to the sugars on glycosylated surface proteins, including the T cell receptor (TCR), and nonspecifically binds them. The result is the low level stimulation of the signal cascade required for IL-2 secretion.[6] Likewise, Phorbol myristate acetate (PMA) is a small organic compound that has a structure analogous to diacylglycerol and diffuses through the cell membrane into the cytoplasm where it directly activates PKC, used in combination with Ionomycin, a calcium ionophore that triggers calcium release also resulting in a low-level signal. However, when PMA and a co-stimulator such as PHA stimulate Jurkat cells IL-2 concurently, production is strongly enhanced.[7]

The silver spot ELISpot assay procedure is similar to that of a conventional ELISpot. The plates are first coated with the appropriate capture antibody. Cultured secreting cells are added to the wells along with any experimental mitogen or antigen. Cells are maintained for a period of time after which they are removed. The analyte remains bound to the capture antibodies in close proximity to the location on the plate where the cell that secreted the analyte was situated. After removal of the cells and any unbound materials, a biotinylated detection antibody is added followed by an enzyme conjugate with incubation to allow binding and wash to remove unbound materials after each. Spots are formed with a substrate that deposits silver on the plate bottom at the locations of the original analyte capture. The resultant spots are then analyzed and counted by image analysis (Figure 2).

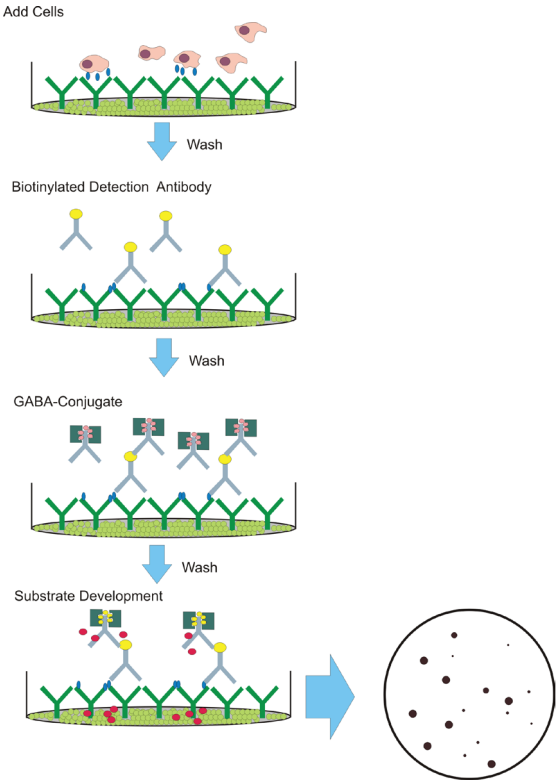

Figure 2. ELISpot silver stain procedure.

The main difference between the ELISpot assays used in this study is that the microplates use a transparent membrane, allowing inverted geometry microscopes to be used to quantify the silver spots. This allowed the use of the Lionheart LX automated microscope.

Materials and methods

Human IL-2 ELISpot silver stain kit was obtained from U-CyTech Biosciences (Utrecht, The Netherlands). Phorbol 12-myristate (PMA)(part number P8139), phytohemagglutin-L (PHA)(part number 11249738001), and triptolide (part number XXXX) were purchased from Millipore-Sigma (Burlington, MA). Ionomycin (part number 407952) was from EMD-Millipore (Burlington, MA). Jurkat cells (human lymphoma) were from ATCC (Manassas, VA).

Cell culture

Jurkat cells were grown in RPMI-1640 plus 10% FBS supplemented with 2 mM glutamine, penicillin, and streptomycin. The suspension cells were split 1:5 with fresh media every 2 to 3 days. For experiments, unless otherwise indicated, cells were plated at a density of 1×10^5/well in 96-well plates, previously coated with IL-2 antibody. Jurkat cells were stimulated to secrete IL-2 with a combination of PMA (50 ng/mL), Ionomycin (1 µg/mL), and PHA (1 µg/mL).

Triptolide inhibition

Jurkat cells were plated at 1×10^5/well in 50 µL of complete RPMI media. After allowing cells to recover for 1 hour at 37 °C in a humidified 5 % CO_2 environment, triptolide treatment was added in complete RPMI media at 4x of final concentration to each well in 50 µL. After treating with triptolide for 1 hour, IL-2 stimuli mixture (2x) was added in 100 µL for a final volume of 200 µL. After stimulating for 24 hours, plates were assayed using an ELISpot kit.

ELISpot assay

A human IL-2 ELISpot kit from U-CyTech Biosciences was used for these experiments. The assays were performed according to the kit instructions. The plates are first coated with the appropriate concentration of capture antibody and allowed to absorb overnight at 4 °C. The unbound antibody is aspirated and the plate is manually washed 3x with PBS. The wells are then filled with a blocking solution (200 µL) and allowed to incubate for at least 1 hour at room temperature. Cultured Jurkat cells are added to the wells along with any experimental mitogen or inhibitor in a total volume of 200 µL. Cells are maintained for a period of 24 hours after which they are removed by washing six times with 250 µL PBS-Tween 0.05% using an Agilent BioTek MultiFlo FX multimode dispenser. A biotinylated detection antibody (100 µL) is added to the well and allowed to incubate for 60 minutes at 37 °C, after which unbound detection antibody is removed by washing. A streptavidin-GABA conjugate is then added (100 µL) and incubated at 37 °C for 60 minutes. Again, unbound conjugate is removed by washing and two-part substrate is then added that deposits silver grains onto the well bottom. Reactions are halted after 60 minutes at room temperature by washing with deionized water (250 µL) three times and allowed to dry. Entire wells are then imaged.

Plate washing

Plates were washed according to the assay kit instructions using a MultiFlo FX multimode dispenser. Wash buffer consisted of PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 7.4 mM) supplemented with 0.05% Tween 20. Unless specifically indicated, plates were washed six times with 250 µL buffer per well.

Plate imaging

Cultures were imaged using a Lionheart LX automated microscope configured with enhanced brightfield. The imager uses a combination of LED light sources in conjunction with band pass filters and dichroic mirrors to provide appropriate wavelength light. A series of images were taken with a 4x objective to create a montage (4 × 5), that covered the entire well. Images were focused automatically using brightfield dual peak autofocus routine in Gen5.

Analysis

Montage tiles (20 per well) were first stitched into a single image file before background subtraction. After background subtraction, objects were identified based on a threshold value and size criteria (Table 1).

Table 1. Image processing and image analysis parameters for ELISpot quantitation.

Parameter	Value
Channel	Brightfield
Size	4,528 × 4,158 (35.91 Mb)
Crop Edges	Yes
Reduce Image	50%
Preprocessing	
Background	Light
Flattening	Auto
Rolling Ball Diameter	1,430 µm
	434 pixels
Image Smoothing	0
Spot Counting	
Channel	Tsf[Stitched[Bright Field]]
Threshold	Value: 15,000 Background: Light Split touching object: Yes Fill holes in masks: Yes
Advanced Options	
Smoothing	0
Background	5% lowest pixels
Minimum Object Selection	50 µm
Maximum Object Selection	500 µm
Include Edge Object	Yes
Entire Image	No (disc plug)

Results and discussion

Initial experiments demonstrate the specificity of the ELISpot reaction. As shown in Figure 3, Jurkat cells that have been stimulated with a combination of PMA/Ionomycin and PHA exhibit numerous spots, while unstimulated cells produce few if any silver spots. Treatment alone without Jurkat cells also does not produce any spots.

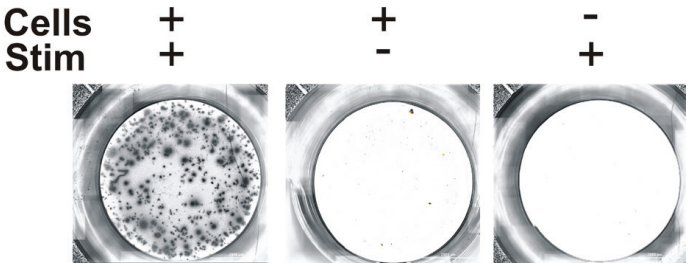

Figure 3. Specificity of the IL-2 ELISpot reaction. Images of ELISpot wells that have Jurkat cells that have been treated with or without PMA (1 ng/mL), Ionomycin (1 µg/mL) and PHA (1 µg/mL). Negative control that lacks cells, but received stimulant.

The deposited silver from the conversion of the substrate by the conjugate enzyme results in very dark spots against a light background. It was noted that in wells with high numbers of spots the intensity of the spot was diminished. The cause of this is most likely depletion of the reagents within the well. To capture all of the spots under these circumstances, a threshold value of 15,000 was used for all experiments. The relationship to the threshold value and positive objects from several wells is depicted in Figure 4.

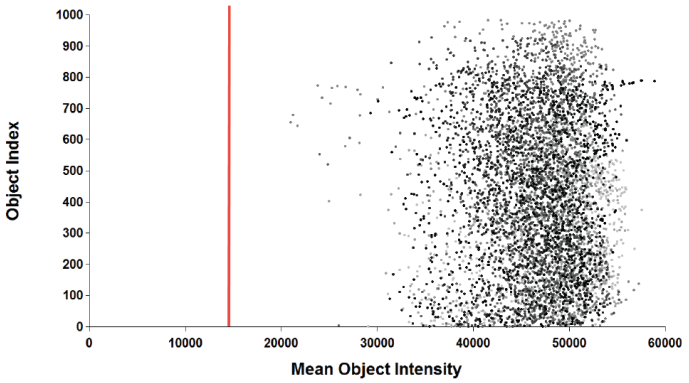

Figure 4. Scatterplot of the object mean intensity versus object number. The object mean intensity for all positive objects from seven separate wells in a typical experiment were plotted against the object designation number. The positive threshold criteria of 15,000 is depicted as the red line.

Correct sizing of the identified objects is critical for accurate determinations. The intent of the ELISpot assay is to identify and quantitate the number of cells responding to specific stimuli. The antibody-coated plate captures its specific target rather than the actual secretory cell. While most of the secreted analyte will be captured in the area immediately surrounding the position of the cell, some of the analyte will diffuse into the media and be captured elsewhere. The high concentration of analyte near the cell will result in a spot as large or larger than the physical size of the cell, while dispersed analyte will result in very small silver deposits. Because the density of the deposited silver grains is very high, it is critical that the selected spots are sized appropriately. Figure 5 demonstrates the number of dense silver grains 1 µm or greater present in a typical ELISpot well. Only those spots exceeding 50 µm in size are designated as true spots.

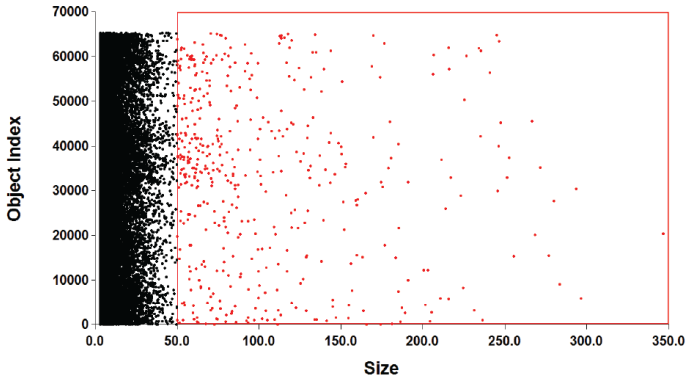

Figure 5. Scatterplot of object size versus object number. All spots achieving a threshold of 15,000 greater than 1 µm in size were plotted against their designation number. Spots designated as positive (size 50 µm) are indicated in red.

The number of recorded spots produced from stimulated cells is proportional to the number of secreting cells. When a titration of Jurkat cells are exposed to a fixed concentration of stimulant, the number of counted spots is proportional to the cell number. As demonstrated in Figure 6, increasing numbers of cells in a well results in an increase in the number of spots counted. Cell counts above 20,000 per well resulted in the spots coalescing together. Subsequent experiments used 10,000 cells per well.

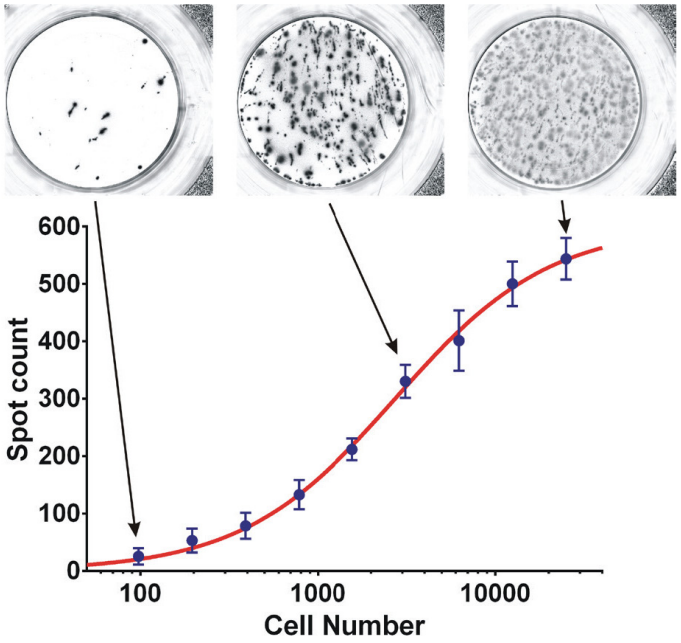

Figure 6. Jurkat cell titration. Jurkat cells were seeded at various concentration into an ELISpot plate and stimulated with 50 ng/mL PMA, 1 µg/mL Ionomycin, and 30 µg/mL PHA for 24 hours. The ELISpot plate was then assayed for IL-2 secretion. Data points represent the mean of eight determinations.

Secretion of IL-2 following stimulation of Jurkat cells is time-dependent. Virtually no spots were observed in the first 2 hours after stimulation. Maximum secretion, as determined by spot number, was observed after approximately 8 to 10 hours in the presence of the stimulant. Further exposure did not result in an increase in the number of observed spots (Figure 7).

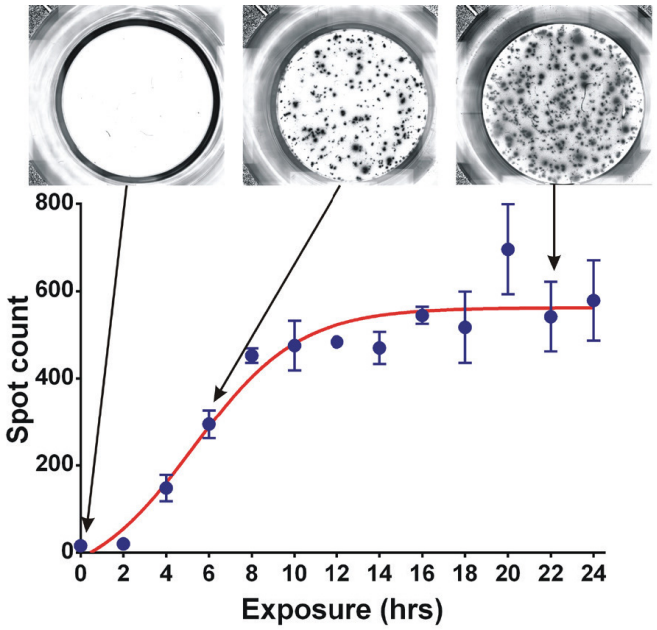

Figure 7. The effect of stimulation exposure time on IL-2 secretion. Jurkat cells were exposed to 50 ng/mL PMA, 1 µg/mL Ionomycin, and 30 µg/mL PHA for various periods of time before being assayed for IL-2 secretion using a silver-staining ELISpot assay. Data points represent the mean of six determinations.

Stimulation of IL-2 secretion by a mixture of PMA, Ionomycin, and PHA is dose-dependent. As observed in Figure 8, when a serial dilution of the compound mixture is used to stimulate IL-2 secretion, with increasing dilution (lower concentration) fewer spots are observed with the ELISpot assay for IL-2.

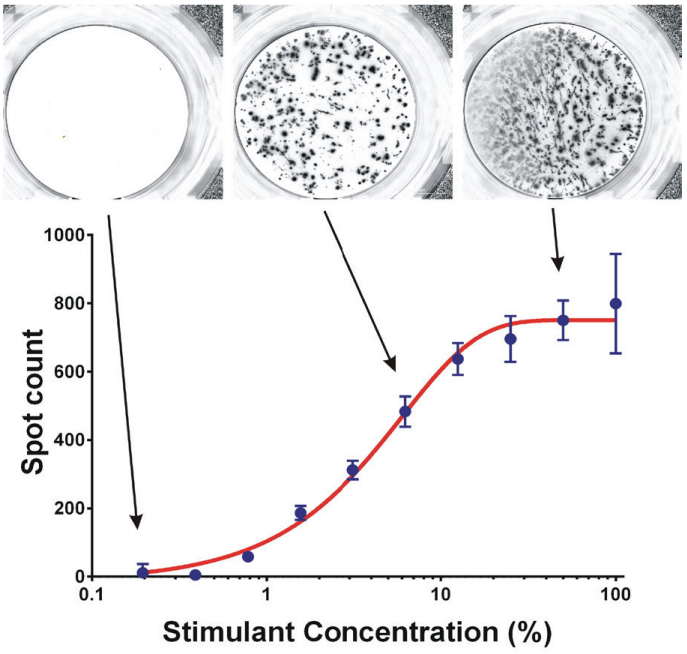

Figure 8. Titration of stimulate mixture. Jurkat cells were stimulated with various dilutions of PMA/Ionomycin and PHA mixture for 24 hours in an ELISpot plate coated with IL-2 antibody. Concentration is expressed as a percentage of the highest concentration (50 ng/mL PMA, 1 µg/mL Ionomycin, 30 µg/mL PHA). After stimulation, IL-2 secretion was assessed and spots counted. Data points represent the mean of six determinations.

Pretreating Jurkat cells with triptolide for 1 hour before stimulation reduces IL-2 secretion in a dose-dependent manner. As demonstrated in Figure 9, increasing concentrations of triptolide results in fewer spots indicative of an IL-2 secreting cell. In these experiments, a stimulatory dose that was 80% of maximum was used. The IC_{50} under these conditions was determined to be 40 nM, which is similar to reports in the literature.[8]

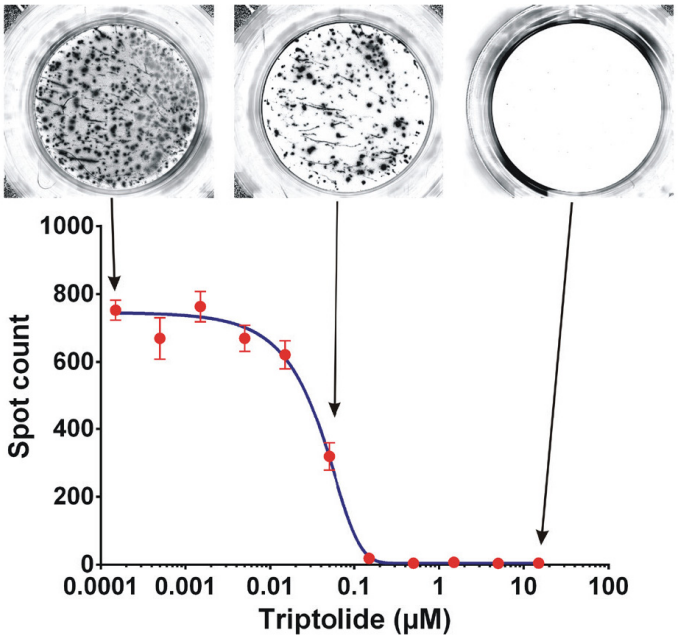

Figure 9. Inhibition of IL-2 secretion by triptolide. Jurkat cells preincubated for 60 minutes with various concentrations of triptolide were stimulated with 6 ng/mL PMA, 0.125 µg/mL Ionomycin, and 3.7 µg/mL PHA to secrete IL-2. After 24 hours, the ELISpot plate was assayed for IL-2 secretion. Data represent the mean of seven data points.

Conclusion

These data demonstrate the use of the Agilent BioTek Lionheart LX automated microscope in conjunction with Agilent BioTek Gen5 microplate reader and imager software to image and analyze silver-stained ELISpot assay plates. The combination of a PHA/Ionomycin and PHA have been shown to markedly stimulate IL-2 secretion in cultured Jurkat cells. Without stimulation, IL-2 is virtually absent. The stimulation of IL-2 secretion is both dose- and time-dependent. The ability of triptolide, a known transcription inhibitor, to prevent IL-2 secretion suggests that new protein synthesis is required after stimulation.

ELISpot is a sensitive assay to monitor the *ex vivo* cellular immune response at the single cell level by detecting secreted proteins released by cells. This technique has been derived from the sandwich enzyme-linked immunosorbent assay (ELISA) to accommodate the use of whole cells to identify the frequency of the secreting cells. As such, there are a number of critical parameters that need to be optimized for experiments to be successful. Depending on the degree of cellular secretion, developed spots can be quite large. The expected number of positive cells is of greater importance than the total number of cells used initially. The presence of too many secreting cells results in individual spots coalescing making a numerical determination difficult. For example, an investigation of a relatively rare secreting event would require a greater number of cells to be seeded compared to a more common event. Timing of the response relative to the stimulation or the inhibition is important. Receptor-mediated events often will take longer to elicit a response than a stimulatory molecule that can interact within the cell directly. It is important that an appropriate interval between stimulation and measurement be used. The testing of inhibitors still requires a stimulating agent to be present. In these experiments, it is important that a less than maximal concentration of the stimulatory agent be used to avoid masking any inhibitory effects.

The Lionheart LX is an ideal platform to interpret silver-stained ELISpot assays. The imager supports microscopic imaging with objectives from 1.25x to 100x that can be installed in a 6-position objective turret. While this research only used brightfield illumination, the imager supports fluorescence-based microscopy with LED and filter cubes. Gen5 microplate reader and imager software, besides controlling reader function, can be used to automatically perform stitching of separate montage image tiles, perform background subtraction, and mask off regions outside the well before analysis.

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