

Stimulation of Human Peripheral Blood Mononuclear Cells

Using the Agilent BioTek Cytation 7 Cell Imaging Multimode Reader to Image and Analyze ELISpot Assays

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Abstract

Human peripheral blood mononuclear cells (PBMCs) are routinely isolated from blood samples and then used in several fields of research including autoimmune disorders, infectious diseases, vaccine development and cancers. The ELISpot Assay monitors *ex vivo* cellular immune responses to antigenic stimuli. Here we use the Agilent BioTek Cytation 7 cell imaging multimode reader in conjunction with Agilent BioTek Gen5 microplate reader and imager software to quantitate changes in cytokine secretion in PBMCs using the colorimetric ELISpot assay format.

Introduction

Human peripheral blood mononuclear cells (PBMCs) are differentially stimulated to secrete a number of cytokines as a result of a receptor mediated cascade based on the cell type and the stimuli. The response of this diverse group of cells to different stimuli offers insights into their role in disease and the development of treatment modalities.

PBMCs are peripheral blood cells that have a round nucleus.¹ These cells consist of lymphocytes (T-, B-, and NK-cells) as well as monocytes. Other peripheral blood cells either have no nuclei (erythrocytes and platelets) or have multi-lobed nuclei (neutrophils, basophils, and eosinophils). In humans, lymphocytes make up the majority of the PBMC population, followed by monocytes, and only a small percentage of dendritic cells.²

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.³ In addition, IL-2 causes the differentiation of T cells into effector T cells and memory T cells when the original T cell was stimulated by an antigen.⁴ Interferon gamma (IFN- γ), is a cytokine critical for innate and adaptive immunity against infections. IFN- γ is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops.⁵ The importance of IFN- γ in the immune system stems in part from its ability to inhibit viral replication directly, and from its immune-stimulatory and immunomodulatory effects. Aberrant IFN- γ expression is associated with a number of auto-inflammatory and autoimmune diseases.

T-cell activation is normally initiated by the interaction of a cell surface receptor to its specific ligand molecule along with a costimulatory molecule.⁶ 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). PKC activation and inositol phosphates, which trigger Ca^{2+} release and mobilization, result in a cascade of additional cellular responses mediating T cell activation (Figure 1). Two

of these cellular responses are the production and secretion of IL-2 and IFN- γ . Triptolide is a diterpene triepoxide that is a potent immunosuppressant and anti-inflammatory (Figure 2). 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- κ B mediated transcription activation.⁷

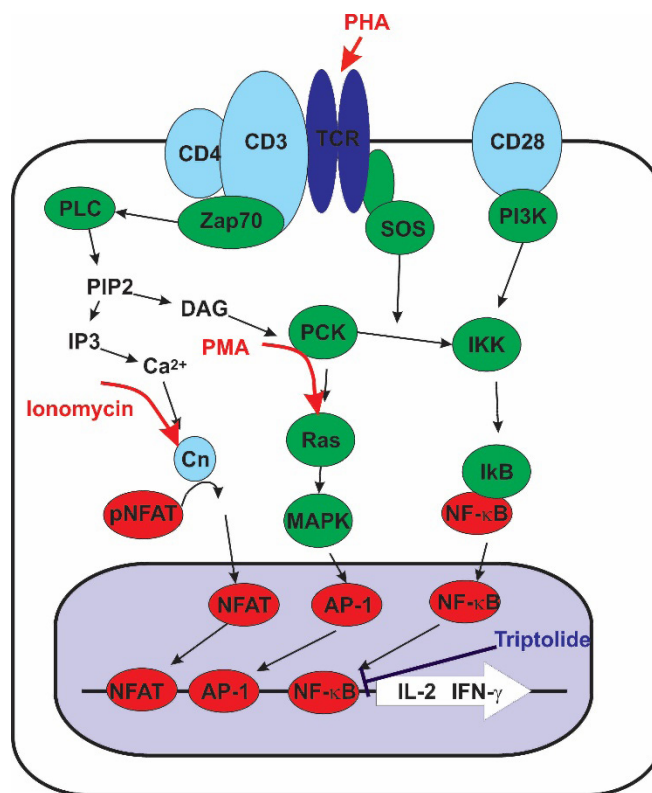


Figure 1. Schematic of signal cascade for stimulation of IL-2 and IFN- γ secretion.

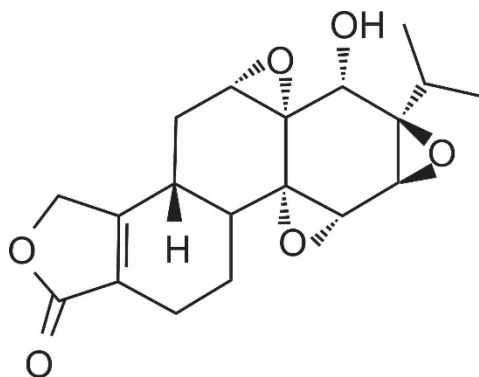


Figure 2. Structure of triptolide.

While some PBMCs are known to produce IL-2 and INF- γ , under normal growth conditions little is produced. Only after stimulation will substantial amounts of the cytokines be expressed.⁸ 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 or INF- γ secretion.⁹ Likewise, Phorbol myristate acetate (PMA) is a small organic compound, which has a structure analogous to diacylglycerol, that diffuses through the cell membrane into the cytoplasm where it directly activates Protein Kinase C (PKC). When used in combination with ionomycin, a calcium ionophore, which triggers calcium release, it results in a moderate level of cytokine release. However, when PMA and a costimulator, such as PHA, stimulate PBMC cells concurrently, cytokine production is strongly enhanced.¹⁰

The ELISpot assay procedure is very similar to that of a conventional ELISA. The plates are first coated with the appropriate capture antibody. Cultured secreting cells are added to the wells along with any interested experimental mitogen or antigen. Cells are maintained for a period of time after which they are removed. The secreted analyte remains bound to the capture antibodies in close proximity to the location on the plate where the cell that produced the analyte was situated. After removal of the cells and any unbound materials, a detection antibody (usually biotinylated) is added followed by an enzyme conjugate with an incubation to allow binding and a wash to remove unbound materials after each step. As the substrate is converted by the conjugate enzyme to colored compounds, spots on the plate membrane bottom at the locations of the original analyte capture are formed. The resultant spots are then analyzed/counted using image analysis. (Figure 3).

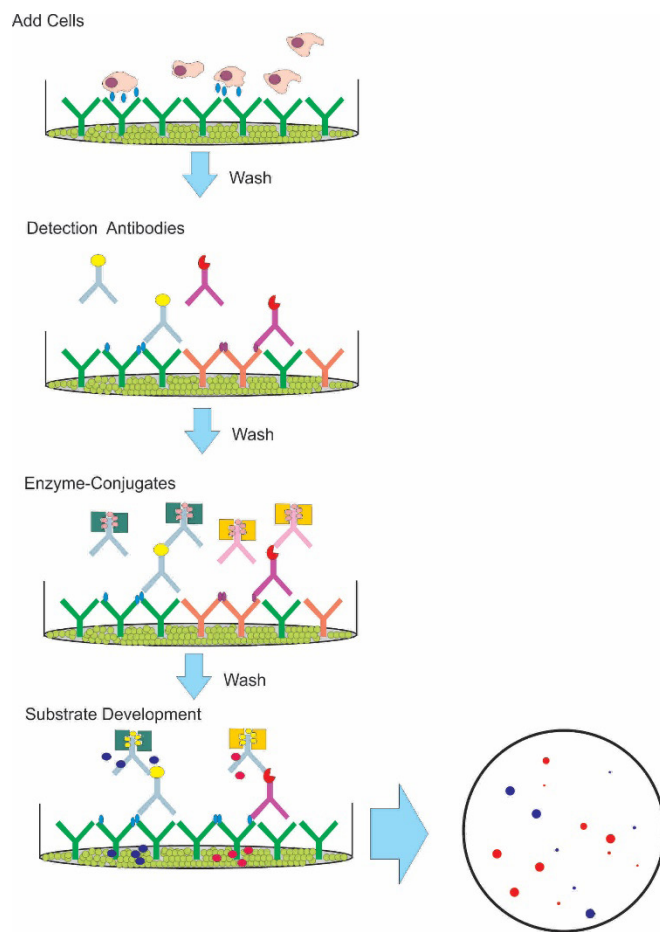


Figure 3. ELISpot stain procedure.

Experimental

Materials and methods

Human IL-2 ELISpot colorimetric kit was obtained from U-CyTech biosciences (Utrecht, The Netherlands) and a two color human IFN- γ /IL-2 ELISpot kit was from Cellular Technology Limited (Cleveland, OH). Phorbol 12-myristate (PMA), and triptolide (part number T3652) were purchased from Millipore-Sigma. Ionomycin (part number 407952) was from EMD-Millipore. Human PBMCs were obtained from Astarte Biologicals (Bothell, WA). White PVDP membrane 96-well (part number MSIP4W10) were from Millipore-Sigma.

Cell culture: Purified human PBMCs were received and maintained frozen until needed. After rapid thawing cells were immediately diluted 1:10 in RPMI-1640 plus 10% FBS supplemented with 2 mM glutamine, penicillin and streptomycin. Cells were centrifuged at 300 g for 10 minutes and the supernatant removed. Cells were resuspended in 10 mL of fresh RPMI media, counted and diluted as needed to provide a density of 5×10^4 cells/well.

Plate coating: Either a human IL-2 ELISpot kit from U-CyTech Biosciences or a 2-color human INF- γ /IL-2 kit from CTL were used for these experiments. PVDF membrane plates are first coated with the appropriate concentration of capture antibody (anti-IL-2 or anti-FTN- γ) 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 μ L) and allowed to incubate for at least 1 hour at room temperature. Blocking buffer is aspirated without washing immediately before the addition of cells.

Cell seeding: Unless otherwise indicated, cells were plated in 96-well membrane plates previously coated with antibody at a density of 5×10^4 /well. PBMCs were stimulated to secrete IL-2 with a PMA (50 ng/mL), ionomycin (1 μ g/mL) mixture. Typical experiments used a volume of 100 μ L for cells followed by the addition of 100 μ L of stimulant mixture at a 2x concentration.

Triptolide inhibition: PBMCs were plated at 5×10^4 /well in 50 μ L volume of complete RPMI media. After allowing cells to recover for 1 hour at 37 °C, in a humidified 5% CO₂ environment, triptolide treatment was added in complete RPMI media at 4x of final concentration to each well in 50 μ L. IL-2 stimuli mixture (2x) was then added in 100 μ L for a final volume of 200 μ L.

One-color ELISpot assay: The assays were performed according to the U-Cytech BioSciences kit instructions. After seeding, cells were incubated for 24 hours, at 37 °C in a humidified 5% CO₂ environment plates and then assayed using an ELISpot kit. Briefly, cells were removed by washing 5x with 250 μ L PBS-Tween 0.05% using an Agilent BioTek MultiFlo FX multimode dispenser. A biotinylated detection antibody (100 μ L) is added to the well and allowed to incubate for 60 minutes at 37 °C or overnight at 5 °C, after which unbound detection antibody was removed by washing. A streptavidin-HRP conjugate was then added (100 μ L) and incubated at 37 °C for 60 minutes. Again, unbound conjugate is removed by washing. Next a two-part AEC substrate was added that deposits dye onto the well membrane bottom. Reactions were halted after 30 minutes at RT by washing with deionized water (250 μ L) 3x using the MultiFlo FX and allowed to dry in the dark. Entire wells were then imaged.

Two-color ELISpot development: The assays were performed according to the C.T. L. Immunospot 2-color ELISpot kit instructions. After seeding, cells were incubated for 24 hours, at 37 °C in a humidified 5% CO₂ environment plates were then assayed using an ELISpot kit. Briefly, cells removed by washing 5x with 250 μ L PBS-Tween 0.05% using a MultiFlo FX multimode dispenser. A detection antibody solution (80 μ L/well) was added to the well and allowed to incubate at room temperature (RT) for 120 minutes, after which unbound detection antibody is removed by washing. Tertiary solution (80 μ L/well) was added and allowed to incubate for 60 minutes at RT. Unreacted reagents were removed by washing 2x with PBS-Tween, followed by 2 washes with dH₂O and then allowed to air dry in the dark. Blue developer solution was then added (80 μ L/well) and incubated for 15 minutes at RT. Reaction was stopped by washing 3x with dH₂O. Red developer solution was then added (80 μ L/well) and incubated at RT for 7 minutes. Plate was the washed 3x with dH₂O. Plate is air dried in the dark for at least 2 hours prior to imaging.

Plate washing: Plates were washed according to the assay kit instructions using a MultiFlo FX. Wash buffer consisted of PBS (NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM, KH₂PO₄ 7.4 mM) supplemented with 0.05% Tween 20. Unless specifically indicated, plates were washed five times with 250 μ L buffer per well.

Plate imaging: Prepared microplates were imaged using a Cytation cell imaging multimode reader configured with an upright color camera. The imager uses a white LED light source in conjunction with a color digital camera. A series of images were taken with the 2x lens in order to image the entire well in a single frame. Once the focal plane and camera exposure were determined manually, images were captured automatically using a fixed focal height routine using reflected light in Gen5.

Table 1. Image capture and preprocessing parameters.

Imaging	
Parameter	Value
Channel	Upright camera
Light Source	Reflected light
Objectives	2x
Focus	Fixed focus
Crop Image	Yes
Preprocessing	
Parameter	Value
Image Set	Red + green + blue
Background	Light
Background Flattening	Rolling ball: 470 µm 136 pixels Priority: fast speed Image smoothing: 0

Analysis

Table 2. Image analysis parameters.

Object Selection	
Parameter	Value
Channel	TSF[Green]
Threshold	Value: 7000 Background: Light Split touching obj.: Yes Fill holes in mask: Yes
Advanced Options	Smoothing: 0 Background: 20
Object Size Selection	Min: 25 Max: 500 Include edge objects: Yes Entire image: Yes
Object Analysis	
Parameter	Value
Cell Count	Count
Calculated Metrics	
Parameter	Value
Channel	Blue: Mean[TSF[Blue]] Red: Mean[TSF[Red]]
Red-Blue Ratio Custom Metric	
Metric	Name
M1	Mean[TSF[Blue]]
M2	Mean[TSF[Red]]
Equation	M1/M2
Subpopulation Analysis	
Subpopulation	Metric Value
Red Spot	Blue-red ratio <0.85
Blue Spot	Blue-red ratio >1.00
Red and Blue Spot	0.85> Red-blue ratio <1.00

Results and discussion

Initial experiments demonstrate the specificity of the ELISpot reaction. PBMCs that have been stimulated with a combination of PMA/ionomycin produce numerous spots, while unstimulated cells produce few if any. Treatment alone without PBMCs does not produce any spots.

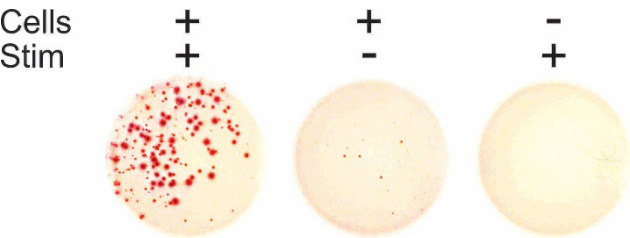


Figure 4. Specificity of IL-2 ELISpot reaction. Images of ELISpot wells containing PBMCs treated with or without PMA (1 ng/mL), Ionomycin (1 µg/mL). Negative control that lacks cells, but received stimulant.

Correct sizing of the identified objects is necessary 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 intense deposits. Figure 5 demonstrates the number of spots present in a typical ELISpot well. Only those spots exceeding 25 µm are designated as true spots.

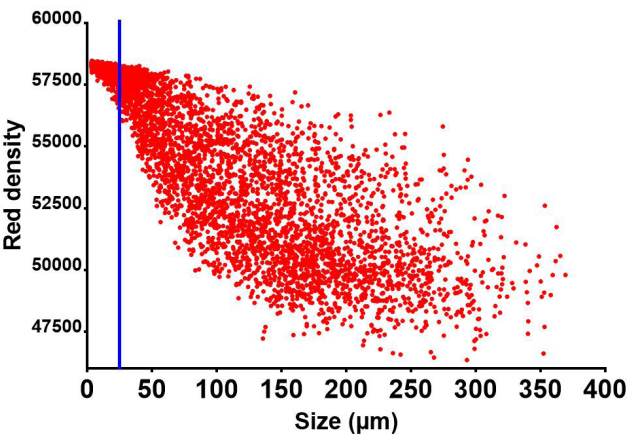


Figure 5. Scatterplot of object size vs red density. All spots achieving a green threshold of 7000 greater than were plotted against their designation number. The size threshold of 25 µm is indicated with a blue vertical line.

The number of recorded spots produced from stimulated cells is proportional to the number of secreting cells. When a titration of PBMCs 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 cell number in a well results in an increase in spots counted. Cell counts above 50,000 per well resulted in the spots coalescing together. Subsequent experiments used 5,000 cells per well.

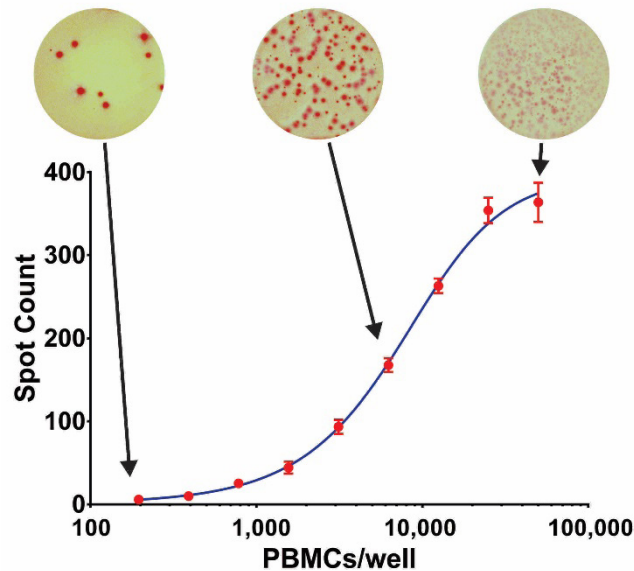


Figure 6. Cell titration. PBMC were seeded at various concentration into an ELISpot plate and stimulated with 50 ng/mL PMA, 1 µg/mL Ionomycin for 24 hours. The ELISpot plate was then assayed for IL-2 secretion. Data points represent the mean of 8 determinations.

Stimulation of IL-2 secretion by a mixture of PMA and Ionomycin is dose dependent. As observed in Figure 7, increasing concentration of PMA produces more spots.

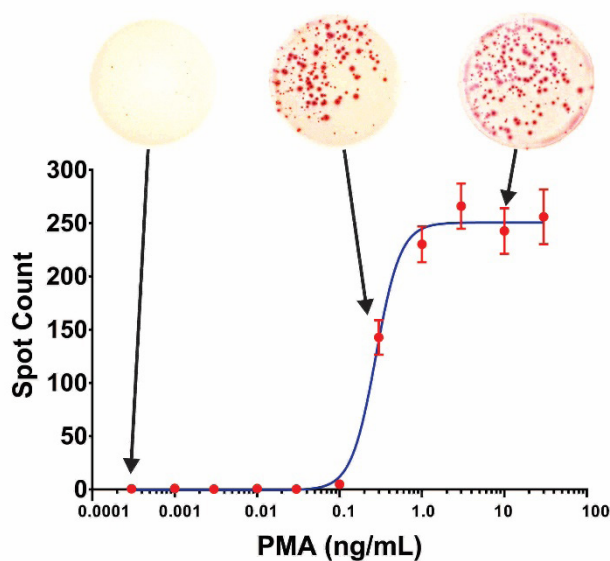


Figure 7. Titration of PMA stimulate. PBMCs (5000 cells/well) were stimulated with various dilutions of PMA and 1 μ g/mL Ionomycin for 24 hours in an ELISpot plate coated with IL-2 antibody. After stimulation IL-2 secretion was assessed and spots counted. Data points represent the mean of 7 determinations.

Pretreating PBMCs with triptolide for 1 hour prior to stimulation reduces IL-2 secretion in a dose-dependent manner. Increasing concentrations of triptolide result in fewer spots indicative of an IL-2 secreting cell (Figure 8). In these experiments, a stimulatory dose that was 80% of maximal was employed. The IC_{50} under these conditions was determined to be 40 nM, which is similar to reports in the literature.⁸

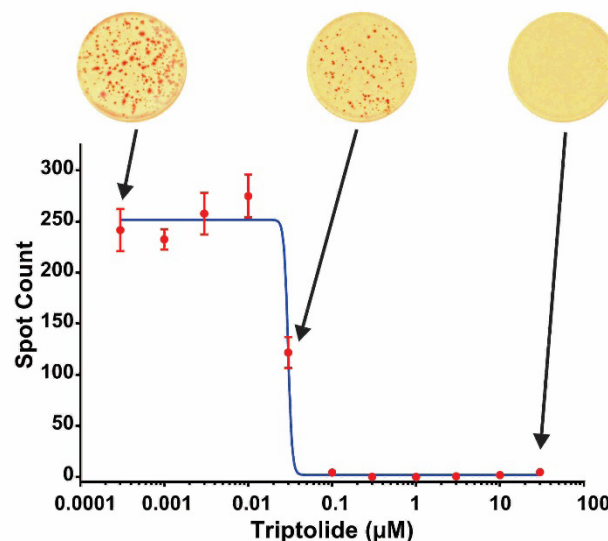


Figure 8. Inhibition of IL-2 secretion by triptolide. PBMCs (5000 cells/well) were pre-incubated for 60 minutes with various concentrations of triptolide were stimulated with 6 ng/mL PMA, 1 μ g/mL Ionomycin, to secrete IL-2. After 24-hours ELISpot plate was assayed for IL-2 secretion. Data represents the mean of 7 data points.

Multiplex ELISpot assays are available to quantitate a number of different analytes simultaneously. While there are several fluorescence-based assays that provide information for up to 4 analytes in a single well, colorimetric ELISpot assays are limited to two analytes per well. Initial experiments using a two-color ELISpot specific to human IL-2 and IFN- γ demonstrated the specificity of the assay to specifically identify IL-2 or IFN- γ secreting cells. In this assay, cells secreting IL-2 can be visualized by the formation of blue spots, while those secreting IFN- γ form red spots. As observed in the control experiment (Figure 9), wells coated with anti-IL-2 antibodies only form blue spots, while wells coated with only anti-IFN- γ antibodies only form red spots. Wells receiving both coating antibodies formed both red and blue spots, while cells lacking PBMCs or PMA stimulation failed to form any spots.

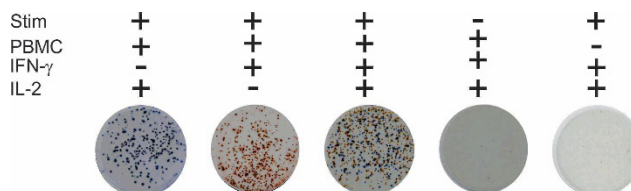


Figure 9. Specificity of two-analyte ELISpot detection. Images of ELISpot wells that have PBMC that have been treated with or without PMA (10 ng/mL). Well membranes were coated with both IL-2 and IFN- γ specific antibodies and color developed for either IL-2 or IFN- γ or both. Negative control that lacks cells, but received stimulant.

Discrimination between red and blue colored spots can be achieved using the differences in red and blue densities of the spots. The histogram plots in Figure 10 demonstrate differences in the calculated red/blue density ratio between red only and blue only control wells. The mean of the red/blue ratio plus two times its standard deviation can be used as the upper limit for red-only spots. Likewise, the mean minus two standard deviations of the blue spot controls defines the lower limit of the red/blue ratio for blue spots. Spots with ratio values between these two thresholds are considered to be both blue and red.

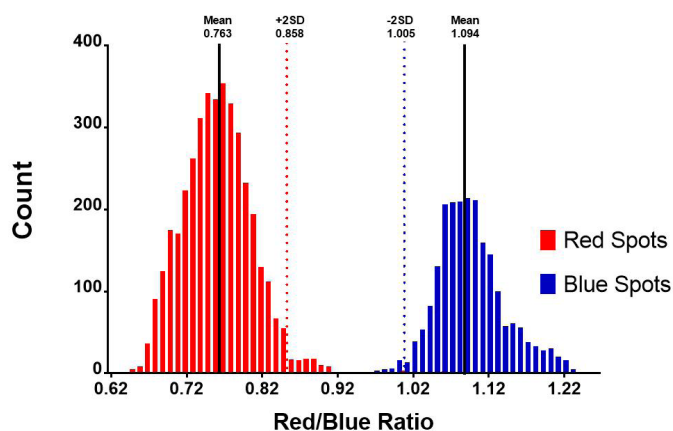


Figure 10. Frequency histogram analysis of red/blue ELISpot intensity ratio values. The frequency of red/blue ratio values from 8 red only and blue only control wells. The mean and the mean plus or minus 2 times the standard deviation of the population are indicated.

These threshold values can be used to quantitate single color reactions where only IL-2 or IFN- γ reactions are developed. As shown in Figure 11, both IL-2 and IFN- γ cytokines are secreted when PBMCs are stimulated with PMA. The stimulation occurs in a concentration dependent fashion with the EC_{50} values being very similar ($EC_{50}=0.05$ ng/mL). Interestingly, twice as many PBMCs, as measured by the spot count, are likely to secrete IFN- γ as compared to IL-2.

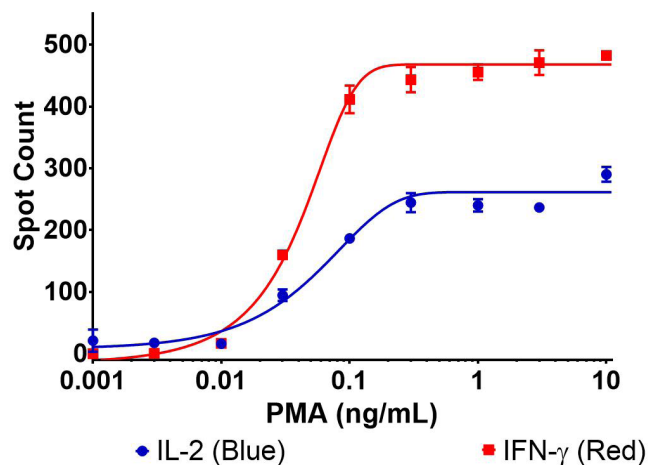


Figure 11. Comparison of IL-2 and IFN- γ secretion by PBMCs after stimulation with PMA. PBMCs were stimulated with PMA in a PVDF membrane ELISpot plate coated with both IL-2 and IFN- γ capture antibodies. After 24 hours plates were processed and colors were developed in parallel wells. Spots (red and blue) were quantitated and plotted as a function of PMA concentration. Data represents the mean and standard deviation of duplicate wells.

Multiplex, 2-color analysis in the same well can be performed when both colors are developed using the same criteria. A frequency histogram of the data from several separate wells where both colors were developed in the wells is depicted in Figure 12. When the images are examined by eye, most wells have spots that are visibly either red or blue, with a smaller percentage that appear as a mixture. These observations are corroborated by the frequency histogram depicted in Figure 12 that demonstrates that the identified spots have a spectrum of red/blue ratio values. There are two obvious peaks based on the red/blue ratio that correspond to the red and blue spots observed when only one color has been developed. Between their respective cutoff values is a significant number of spots that have an intermediate red/blue ratio.

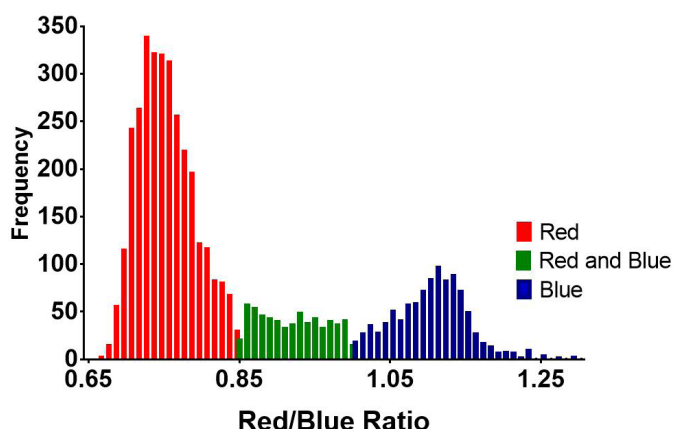


Figure 12. Frequency histogram of ELISpot red/blue ratio values. The red/blue ratio of ELISpot spots from 8 wells of a two-color ELISpot assay plate were plotted as a function of frequency. Subpopulations analysis based on cut off values for red, blue or red and blue spots are indicated by color.

These visibly correspond to spots that appear purple (i.e. a mixture of red and blue). The relative number of spots identified as red or blue is similar to the numbers identified when a single color was developed. When one analyzes the data with a scatter plot that compared the red/blue ratio to spot size, two loose clusters of spots that correspond to red or blue spots are observed along with a number of intermediate ratio spots (Figure 13). While all three subpopulations of spots have the same range in size, red spots tend to be more numerous and smaller in size than spots identified as blue.

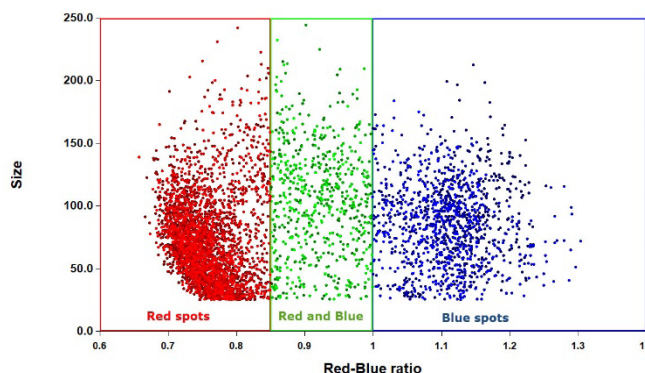


Figure 13. Scatter plot of ELISpot red/blue ratio values. The red/blue ratio of ELISpot spots from 8 wells of a two-color ELISpot assay plate were plotted as a function of size. Subpopulations analysis based on cut off values for red, blue or red and blue spots are indicated.

This multiplex analysis can be used on individual wells with different experimental conditions. Figure 14 demonstrates the response of PBMCs to PMA stimulation where both blue (IL-2) and red (IFN- γ) colors are developed in the same well. As with separate color development, PMA stimulated cytokine secretion on PBMCs in a concentration dependent manner. Also, more PBMCs secreted IFN- γ than IL-2, with equivalent EC_{50} values. If one compares the total number of cells that secrete IFN- γ (red only spots plus red and blue spots) or the total number of cells that secrete IL-2 (blue only spots plus red and blue spots) the numbers are consistent with wells where only one color was developed.

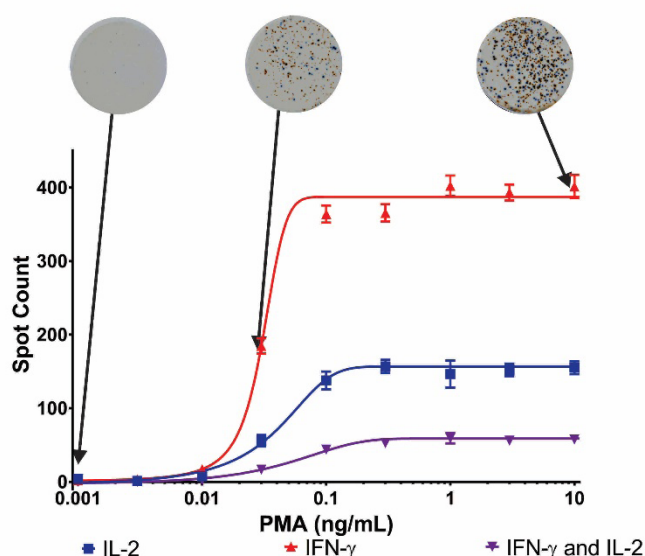


Figure 14. Comparison of IL-2 and IFN- γ secretion in stimulated PBMCs. PBMCs were stimulated with various concentrations of PMA using a PVDF membrane 96-well plate that was pre-coated with both anti-IL-2 and anti-IFN- γ antibodies. After ELISpot processing, the plate wells were imaged and the images analyzed using Gen5. Subpopulation analysis defined spots that were either red, blue or a mix of red and blue. The number of each spot subpopulation was plotted against PMA concentration. Data represents the mean and standard deviation of 4 determinations.

Results and discussion

These data demonstrate the utility of the Agilent BioTek Cytation 7 cell imaging multimode reader in conjunction with Agilent BioTek Gen5 microplate reader and imager software to image and analyze colorimetric PVDF ELISpot assay plates. The combination of a PMA/ionomycin has been shown to markedly stimulate IL-2 secretion in PBMCs. Without stimulation, IL-2 is virtually absent. The ability of triptolide, a known transcription inhibitor, to prevent IL-2 secretion suggests that new protein synthesis is required after stimulation.¹¹

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 enzymelinked 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 in order 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 the 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 as compared to a more common event. Timing of the response relative to the stimulation and/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 appropriate interval between stimulation and measurement be utilized. 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, lest it mask any inhibitory affects.

The Cytation 7 is an ideal platform to interpret colorimetric PVDF membrane ELISpot assays. The imager supports digital top-down color imaging with 2x, 4x and 8x microscope objectives that are factory installed. The 2x objective can capture the entire well in a single image, making it ideal for 96-well ELISpot determination. If desired, higher resolution can be obtained by using a higher magnification objective and a montage of the well. Using this camera both reflected or transmitted light can be used for optimal imaging. While this research only used the upright top-down camera with PVDF membrane plates, the imager also supports bright field imaging using an inverted camera for silver stain ELISpot assays. In addition, the inverted microscope 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 stitch of separate montage image tiles, perform background subtraction and mask off regions outside the well prior to analysis.

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