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Application Note

Development of a FluoroSpot duplexing assay to measure immune response in cryopreserved PBMCs

Introduction

The ELISpot (Enzyme Linked Immuno-Spot) assay provides an effective method of measuring protein secretion of immune cells at the single cell level.[1,2] This assay has seen a resurgence in recent years as researchers attempt to gain a better understanding of immune responses in a variety of applications from cancer research to immunogenicity testing and vaccine development.[3] We developed a multiplexed fluorescent ELISpot (FluoroSpot) assay for quantifying the number of pre-existing, antigen-reactive T cells from cryopreserved disease state peripheral blood mononuclear cells (PBMCs). T cell interferon-gamma (IFN-γ) immune response was compared between commercially available disease state (CMV-seropositive) and normal (CMV-seronegative) cryopreserved PBMC cells from different donors. We show that cryopreserved PBMCs from donors with previous exposure to CMV exhibited significant presence of virus-specific T cells when compared to PBMCs from non-exposed donors based on IFN-γ and IL-2 secretion.

Since microporous membranes offer vastly improved binding characteristics over standard polystyrene surfaces, most ELISpots assays are performed on polyvinylidene fluoride (PVDF) membrane filter plates. Low auto-fluorescence PVDF filter plates such as MultiScreen®$_{HTS}$-IPFL filter plates enable fluorescent detection and multiplexing of FluoroSpot assays.

In the standard FluoroSpot assay, cytokine-specific antibodies are immobilized onto 96-well membrane filter plates. Cells are then seeded in the presence or absence of stimulating agents. Activated cells secrete cytokines that bind capture antibodies in the immediate vicinity of the expressing cell. Cells are then washed away. Spot detection can be accomplished using fluorescently labeled secondary antibodies or detection antibodies with appropriate fluorescent conjugates. Spot numbers are then tallied using image-based spot readers with accompanying analysis software.

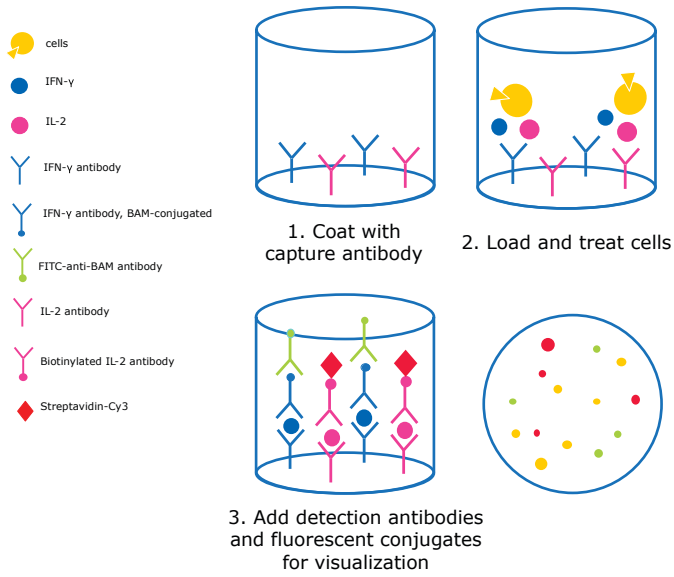


Figure 1. FluoroSpot workflow.

Materials

- Disease state and normal cryopreserved peripheral blood mononuclear cells (cryopreserved PBMCs from BioIVT)
- MultiScreen®$_{HTS}$-IPFL filter plates (Millipore® #MSIFS4510)
- Anti-human IFN-γ (MabTech #3420-3-250)
- Anti-human IFN-γ, biotinylated (MabTech #3420-6-250)
- Anti-human IL-2 (MabTech #3445-3-250)
- CMV peptide pool for human CD4 and CD8 T cells (Mabtech #3619-1)
- Anti-CD3 antibody (CD3-2) (MabTech #3605-1)

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- Anti-IFN-γ (7-B6-1), BAM-conjugated
- Anti-BAM antibody, FITC-labeled
- Biotinylated anti-human IL-2 (MT8G10)
- Streptavidin-Cy3
- FluoroSpot Enhancer (MabTech #3641-F10)
- RPMI 1640 + 10% FBS
- PMA (Millipore® #5005820001)
- Ionomycin (Millipore® #I3909)
- 35% ethanol
- Sterile water
- 1X dPBS

Methods

Plate preparation (under sterile conditions)

1. Wash plate 3X with sterile dPBS (200 µL per well).
2. Add 200 µL/well of cell incubation medium containing 10% fetal calf serum and incubate for 30 minutes at room temperature to block.

Cell incubation (under sterile conditions)

3. Thaw frozen PBMCs and allow to rest at 37 °C for 1 hour before plating.
4. Remove blocking/conditioning medium and add cell suspension with stimuli to plate (100 µL/well).
5. Incubate plate at 37 °C, 5% CO_2 incubator overnight.

Assay (Detection)

6. Remove cells and wash the plate 5X with dPBS (200 µL per well).
7. Dilute detection antibodies in dPBS containing 0.1% BSA and add 100 µL per well; incubate wells for 2 hrs at room temperature
8. Wash 5x with PBS (200 µL per well).
9. Add 100 µL per well of fluorophore conjugates diluted in dPBS containing 0.1% BSA and incubate for 1 hr at room temperature. Protect from light.
10. Wash 5x with dPBS (200 µL per well).
11. Add 50 µL per well of fluorescence enhancer for 10 minutes at room temperature.
12. Empty plate by flicking to remove the fluorescence enhancer. Do NOT wash or tap against paper towels.
13. Remove the underdrain.
14. Leave the plate to dry overnight. Protect from light.
15. Dried plates were used to obtain well images using CTL ImmunoSpot® analyzer, equipped with filters for the fluorophores.

Results

Cryopreserved disease state PBMCs from CMV-seropositive donors (N=4) and normal cryopreserved PBMCs from CMV-seronegative donors (N=3) were used for this study. The assay was initially developed as a single-plex assay measuring IFN-γ response after stimulation with an antigenic CMV peptide pool. IFN-γ response was clearly observed when using the cryopreserved PBMCs from CMV-seropositive donors **(Figure 2)**. Titration of PBMC cell number resulted in a corresponding decrease in the number of spots visualized. The MultiScreen® filter plates with low auto-fluorescent PVDF membranes exhibited excellent resolution of fluorescent spots.

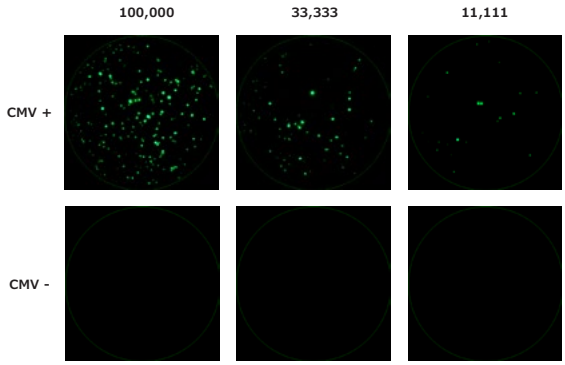


Figure 2. Detection of rare immune responders against CMV-derived peptides using a FluoroSpot assay. Cryopreserved PBMCs from CMV seropositive and CMV seronegative donors were tested for the presence of CMV antigen specific T cells capable of secreting IFN-γ upon CMV peptide stimulation. Seven different donors (4 CMV-positive and 3 CMV-negative) were tested for IFN-γ response. Clear antigenic responses were seen with all CMV-positive donors ranging from 0.09 to 0.76% of input PBMCs. Representative images from CMV seropositive and negative donors are shown.

Tabulated results are shown in **Figure 3.** Overall, a negligible number of spots were counted from normal (non-disease state) cryopreserved PBMCs. Frozen disease state PBMCs from the four CMV-seropositive donors exhibited varied but robust response in the optimized assay.

			Average IFNg Spot count 100,00/Well	
Donor #	**CMV Status**	**Lot Number**	**Activated with CMV peptide Pool**	**CMV pep, %**
1	Negative	**LS1161393A**	3	0.003
3		**172009009-04**	1	0.001
6		**172009443-04**	3	0.003
2	Positive	**BRH1515656**	295	0.30
4		**LS1167106A**	760	0.76
5		**172009743-04**	85	0.09
7		**172003256-07**	134	0.13

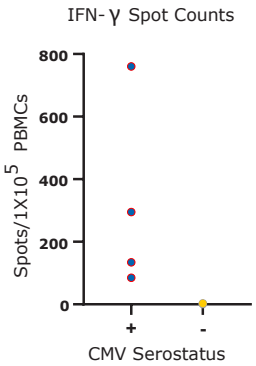


Figure 3. A) Quantitation of FluoroSpot results using cryopreserved PBMCs from seven donors. **B)** Direct comparison of spot counts from wells seeded with 10,000 PBMCs. While donor-to-donor variability was observed, cryopreserved PBMCs from CMV-seropositive donors exhibited significant increases ($p < 0.01$) in the number of spots observed upon CMV peptide stimulation when compared to cryopreserved PBMCs from CMV-seronegative donors.

Next, we performed a direct comparison of the overall sensitivity of the single-plex FluoroSpot assay to that of conventional ELISA and flow cytometry. While the ELISA exhibited excellent sensitivity (limit of detection ranging in low pg/mL), its ability to detect rare responders was severely limited in comparison to FluoroSpot. For example, IFN-γ was only detected in one of the CMV+ donor samples by ELISA, whereas both CMV+ samples showed a significant increase in spot counts over CMV- samples in the FluoroSpot assay. Similarly, flow cytometry showed a high level of sensitivity where small populations (<1%) of antigenic responders could be detected. However, when comparing two CMV+ donors, only one of the donors could be seen as showing statistical significance over corresponding non-activated controls. Taken together, these results clearly demonstrate ability of FluoroSpot to quantify low level antigenic responders in comparison to ELISA and flow cytometry.

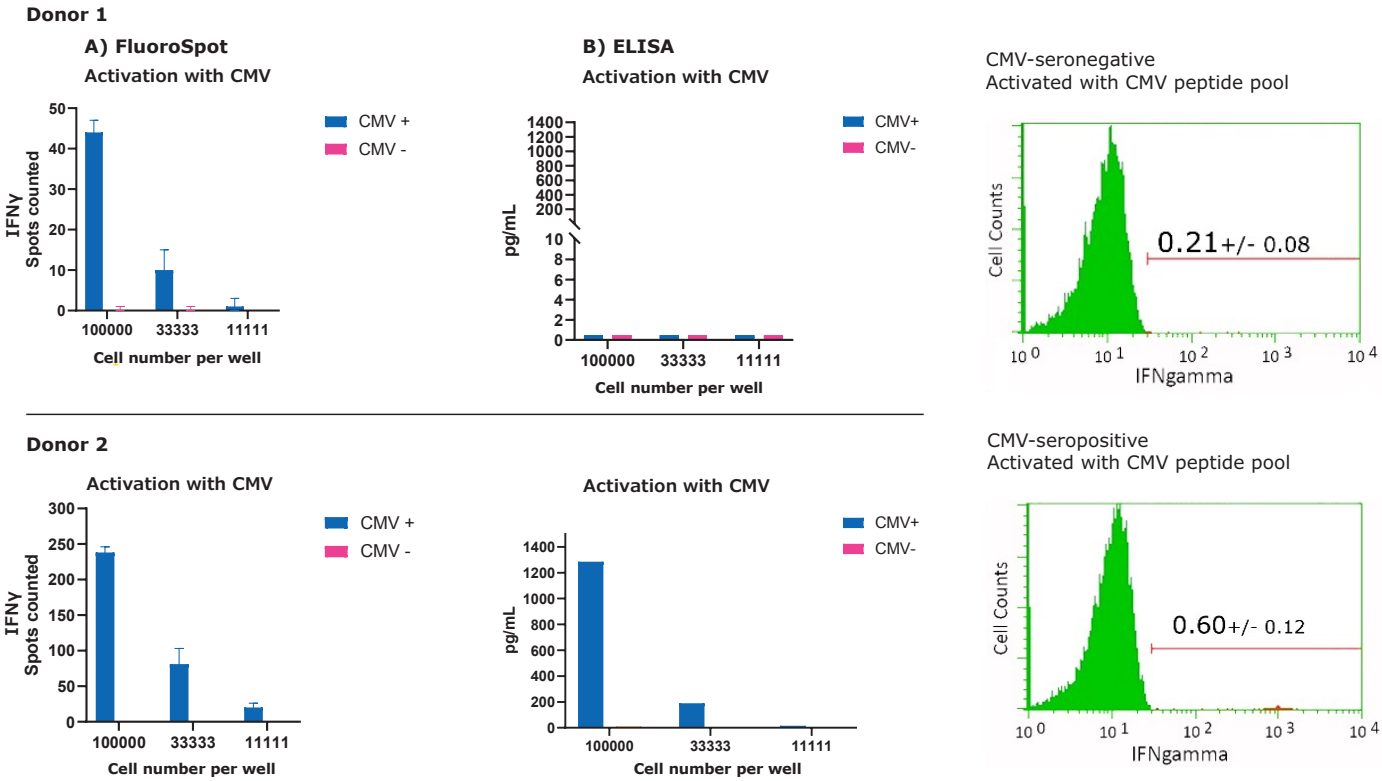


Figure 4. Direct comparison of FluoroSpot with ELISA and flow cytometry. **A)** Quantification of FluoroSpot results showing number of IFN-γ secreting cells upon antigenic stimulation. **B)** Corresponding ELISA results where total IFN-γ secreted from antigen responding cells are quantified. **C)** Corresponding flow cytometry results. Shown are percent IFN-γ positive cells from CD45 gated cells. Donor 1 (upper panel) showed 0.21+/-0.08 percent positive upon antigenic (CMV peptide pool) stimulation compared to 0.09+/-0.05 percent positive for non-stimulated cells ($p>0.05$, Student's t-test). Only donor 2 (lower panel) showed a significant level of antigenic responders 0.6+/-0.12 compared to 0.1 +/-0.03 for activated and non-activated cells, respectively ($p<0.05$, Student's t-test). Notice how only one of the CMV+ donors show detectable level of antigenic IFN-γ responses by ELISA and flow cytometry, whereas both CMV+ donors show detectable level of IFN-γ secretion by FluoroSpot.

In addition to its ability to detect rare antigenic responders, FluoroSpot assays can offer the ability to detect multiple types of antigenic responders simultaneously. We optimized a multiplexed FluoroSpot assay to detect both IL-2 and IFN-γ responses using normal and disease state PBMCs upon anti-CD3 stimulation. In addition to its sensitivity, FluoroSpot can also be used to detect multiple analytes simultaneously in each well.

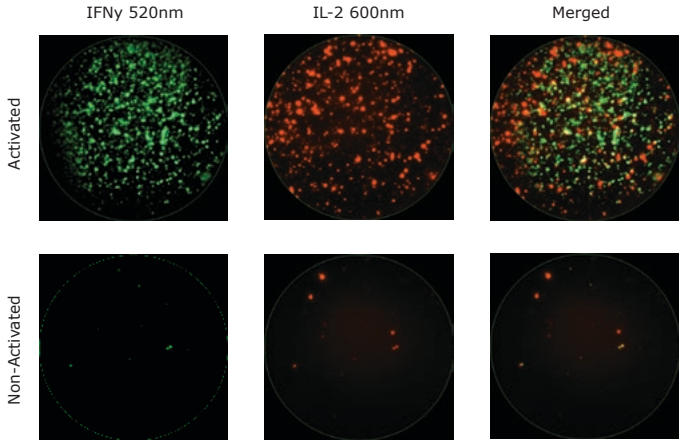


Figure 5. Dual FluoroSpot assay. Cryopreserved PBMCs were treated with (upper panel) or without (lower panel) anti-CD3 antibody overnight. IFN-γ (green spots) and IL-2 (red spots) were detected simultaneously using 520 nm and 600 nm cut off filters (limited band-width filters), respectively.

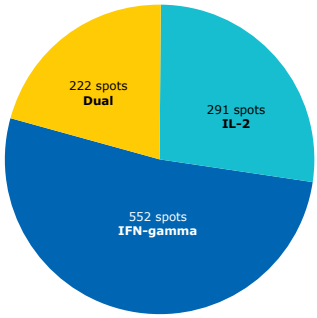


Figure 6. Tabulated results from duplexing assay.
Pie chart shows the distribution of single and dual expressors.

Conclusions

A FluoroSpot assay was used to evaluate cytokine production from normal (CMV-seronegative) and disease state (CMV-seropositive) cryopreserved PBMCs stimulated with a CMV peptide pool at the single cell level. Significant differences in IFN-γ spot formation were observed between the two populations, where previous donor exposure to cytomegalovirus correlated to an increased number in CMV antigen-specific T cells. The sensitivity of the FluoroSpot assay was compared to ELISA and flow cytometry. Results suggested the FluoroSpot assay exhibits greater sensitivity, with the ability to detect rare responders with better fidelity. A duplexing FluoroSpot assay was then developed, enabling simultaneous detection of IL-2 and IFN-γ from the same well. These assays used a MultiScreen®$_{HTS}$-IP-FL filter plate containing a low auto-fluorescent PVDF membrane for cellular discrimination and visualization. The FluoroSpot assays exhibited well-defined spots and were able to discriminate single and dual responders, suggesting highly sensitive and robust assay performance. FluoroSpot provides highly sensitive assays for detection of rare responders in single-plex and multiplexed formats.

References

1. Czerkinsky, C., Nilsson, L., Nygren, H., Ouchterlony, O., and Tarkowski, A. (1983). A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. J Immunol Methods 65 (1–2): 109–121.
2. Sedgewick, J. and Holt, P. (1983) A solid-phase immunoenzymatic technique for the enumeration of specific antibody secreting cells. J. Immunol. Methods. 57:301-309.
3. Sahin, U., Muik, A., Derhovanessian, E. et al. (2020) COVID-19 vaccine BNT162b1 elicits human antibody and TH1 T cell responses. Nature. 586:594–599.

Ordering Information

Description	Qty/Pk	Sterile	Cat. No.
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