Application Note

Novel Imaging and Data Analysis Techniques for Quantifying and Reporting Immunological Synapses:

Pairing Amnis® FlowSight® technology and IDEAS® Software with FCS Express™ Image Cytometry

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Abstract:

Interaction between antigen-specific T cells and antigen presenting cells (APC) cognate ligand involves reorganization of the cytoskeleton and recruitment of adhesion and signaling molecules to the site of intercellular contact. Sustained adhesion of T cells to APCs and formation of the immunological synapse after T cell receptor stimulation are required for the antigen-specific response. One way to measure an immunological synapse is by fluorescently labeling the molecules that have been recruited to the synapse and imaging via confocal or conventional fluorescence microscopy. However, immunological synapses are often rare and therefore difficult to analyze objectively and statistically by traditional microscopy methods. In this study, the Amnis® FlowSight® imaging flow cytometry platform was employed to collect imagery of large numbers of T cells and antigen presenting cells (APC) incubated with varying doses of antigen to assess the percentage of T cells involved in an organized immunological synapse. FCS Express™ Image Cytometry software was used to report and graph donor variation and dose dependent results with real time gating and statistics for quick and robust analysis across all samples. Advanced data analysis techniques in FCS Express combined with imaging flow cytometry technology allows researchers to more rapidly assess the results of experiments with visual cues amongst a large sample size and with “real time” access to statistical results as populations and samples change.

Materials and Methods:

The Amnis® brand FlowSight® imaging flow cytometer equipped with the 405 nm, 488 nm and 642 nm lasers was use for this study. For each sample 30,000 events were acquired. Single color controls were used to calculate a spectral crosstalk matrix that was applied to the data for spectral compensation among the detection channels. The resulting compensated data file was initially analyzed using image-based algorithms available in the IDEAS® 6.2 image analysis software package to generate the Features and Masks. All cell based images in the application note are high resolution exports from IDEAS 6.2 software.

After segmentation and initial data analysis in IDEAS, data files were saved in the .daf / .cif file format which can be loaded directly in FCS Express 6 image cytometry analysis software (De Novo Software). FCS Express enables users to utilize advanced data handling and reporting tools to analyze multiple data files simultaneously for regression analysis and group comparisons while maintaining real time linking between gates, images, statistics and advanced charting tools. The suite of analysis tools provided by FCS Express further eliminate the need for additional statistical, charting, and reporting software by utilizing integrated regression charts for calculation of half maximal inhibitory/effective concentrations, bar charts for samples and sample group comparisons, significance testing, as well as high resolution scatter plots and histograms with overlays. All charts and data visualizations presented in this application note were generated directly in FCS Express and exported in high resolution format for publication.

Quantifying the frequency of organized immunological synapse in a dose dependent manner

Staphylococcal enterotoxin B (SEB) is an enterotoxin produced by the bacteria *Staphylococcus aureus*. SEB, also is known as a 'superantigen' and a member of a class of antigens that cause non-specific activation of T-cells. It has been reported that superantigens are capable of activating up to 20% of the body's T-cells.[1] In this experiment, Raji B cells were loaded with SEB to make APCs in a dose dependent manner. The SEB-loaded APCs were incubated with human T cells purified from human peripheral blood. After incubation the cells were fixed, permeabilized and labeled with CD3-PE TexasRed (T cells, orange), CD19-AF488 (Raji Bcells, green), phalloidin-AF647 (actin, red) and DAPI (nuclear stain, purple). We used the FlowSight imaging flow cytometer to assess the frequency of conjugates with an organized immunological synapse. Image analysis is done using image-based algorithms available in the IDEAS 6.2 image analysis software package. Dose response results were calculated and analyzed using FCS Express Image Cytometry software and verified via real time gating on plots and images. The dose response curves were used to identify appropriate treatments for studies on donor variation and the effects of cytochalasin D (Cyto D), an actin polymerization inhibitor.

Polymerization and concentration of actin at the immunological synapse results in a high local pixel intensity. A "Valley" mask operation on the DAPI image defined the region of contact between cell conjugates. The actin intensity was then quantified within the Valley mask, Figure 1. In addition, the overlap between the cells was measured by using the Co-localization wizard in the IDEAS 6.2 software. The Co-localization wizard measures the Bright Detail Similarity between two images (the cross correlation of the smaller fluorescence features), in this case CD3 and CD19.

Higher Bright Detail Similarity scores are associated with greater overlap between the cells and an organized immunological synapse. By plotting the Bright Detail Intensity of the actin in the Valley mask vs the Bright Detail Similarity of the CD3 and CD19 events, the number of cells with an organized immunological synapse may be resolved for quantitative analysis. Figure 2 shows representative images of conjugates without an organized immunological synapse (Figure 2A) and conjugates with an organized immunological synapse (Figure 2B).

Figure 1A

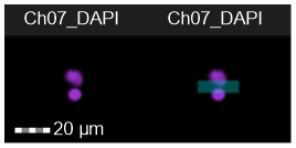


Figure 1B

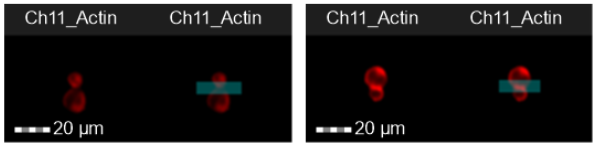
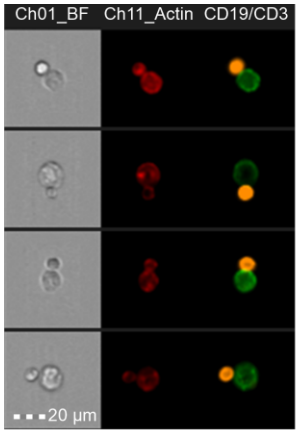
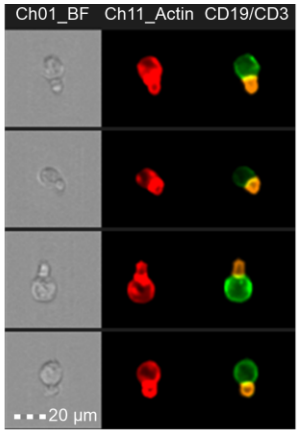


Figure 1: A - DAPI Images of Cell Conjugate (purple) with the Valley mask (blue) identifying the region of contact between the cells. B – Actin images (red) of Cell Conjugates without (left) and with (right) an organized immunological synapse. The Valley mask (blue) is used to quantify the actin intensity between the two cells where a synapse would form.

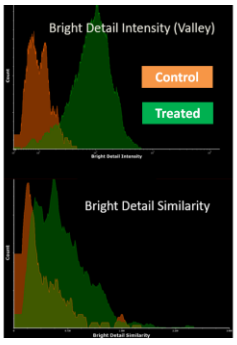
A. No Organized Immunological Synapse



B. Organized Immunological Synapse



C. Histogram Overlays



D. Gating Strategy

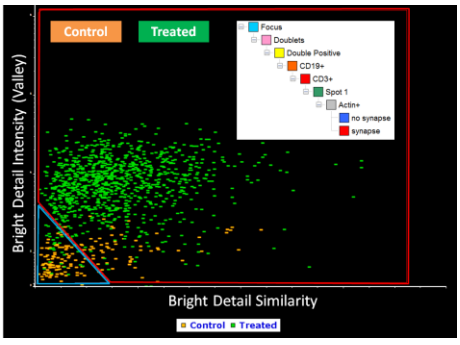


Figure 2: A) Representative images from the No Synapse gate, B) and from the Synapse gate, C) Histogram overlays for the Bright Detail Intensity (Valley) and Bright Detail Similarity channels were used to for initial assessment and review of the data sets in FCS Express (orange overlay and green overlay represent the control and treated samples respectively). Note that only by collecting both parameters via the valley mask and co-localization wizard can the synapse population be resolved. D) Bright Detail Intensity vs Bright Detail Similarity were plotted in FCS Express to define gates on the Synapse (Red gate) vs No Synapse (Blue gate). Events collected from the control (Orange dots) and treated sample (Green dots) were overlaid on a two dimensional plot to help define the optimal gating position and strategy for downstream analysis. Additional gates used to arrive at the final gates are shown (D-inset).

Parameters for Bright Detail Intensity (Valley) and Bright Detail Similarity were first assessed with histogram overlays for the control and treated populations (Figure 2C). It is important to note that assessment of a single parameter on its own would not yield sufficient information to resolve the population of interest as seen in histogram overlays of the control and treated populations for each individual channel in figure 2C. However, when combining the results from the co-localization wizard and the valley mask operation one can easily resolve cells in synapse or no synapse as seen in figure 2D. The additional gates used to arrive at the synapse and non-synapse populations on the bivariate plot are outlined in figure 2D (inset).

Assessing drug treatment concentrations and donor variation

To assess donor variation (Figure 4) and minimal dosage for a Cyto D inhibition measurements (Figure 6) two donor samples were treated with increasing amount of SEB (Figure 3). Donor samples were treated with 0 to 10 ug/mL SEB and EC50 values were calculated in FCS Express Image Cytometry based on the percentage of cells observed in the synapse gate (Figure 2D). A large variation is observed between the two donors as expected (see Figure 4) resulting in an inflection point averaging 5.49 ug/mL. Based on these results, we selected a dose of 5 ug/mL SEB for the donor variation study as it provides significant T-cell activation without inducing an overwhelming response. A lower dose was chosen for the Cyto D study since T-cells only needed to be activated but not to the extent that the inhibitor would be ineffective. Samples exhibited a response at doses as low as 0.01 ug/mL (Figure 3 top). A final dosage of 0.5ug/mL SEB exhibits the desired response for the Cyto D study.
For these studies it is important to note that T cells from healthy donors are used and that individuals can exhibit large differences in the levels of T cell activation in response to SEB. A variation study using 5 donors was conducted to demonstrate the degree of difference between donors. Each donor's T cells were incubated with 0 (control) or 5ug/mL SEB loaded Raji cells. The average percentage of T cells in an immune synapse gate for the 5 donors is shown (figure 4) where each bar represents the mean with error bars describing the range of donor variation. Control (2.9 ± 1.1) and SEB Treated (11.8 ± 4.1) with a p value of 0.01.

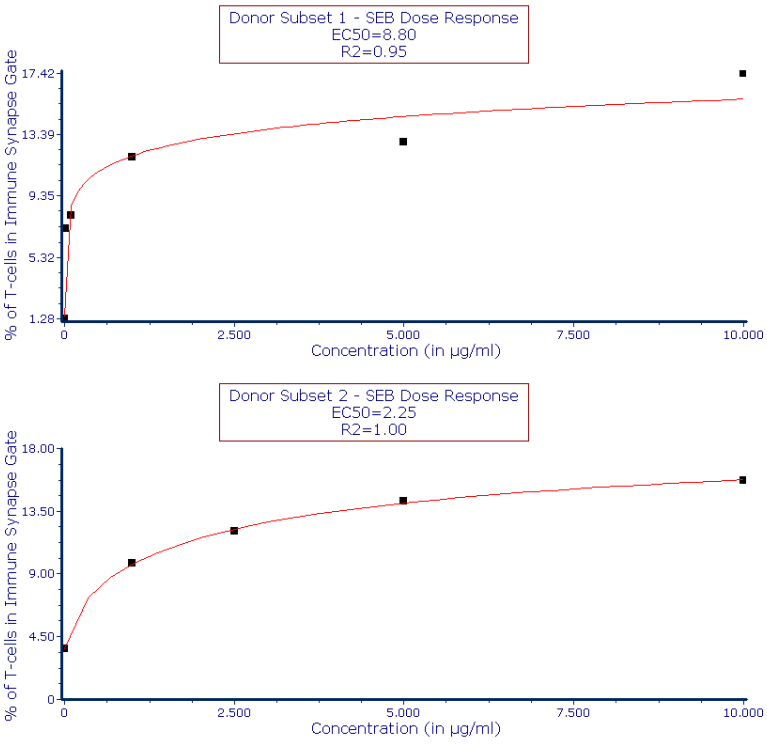


Figure 3: Two donor samples were treated with increasing doses of SEB to determine the minimum dosage required for an inhibitory response of Cytochalisin D as well as an appropriate dosage for a donor variation study. A response was seen at a dosage as low as 0.01 ug/mL (top) while the average half maximal concentration was determined to be 5.49 ug/mL for the two donors.

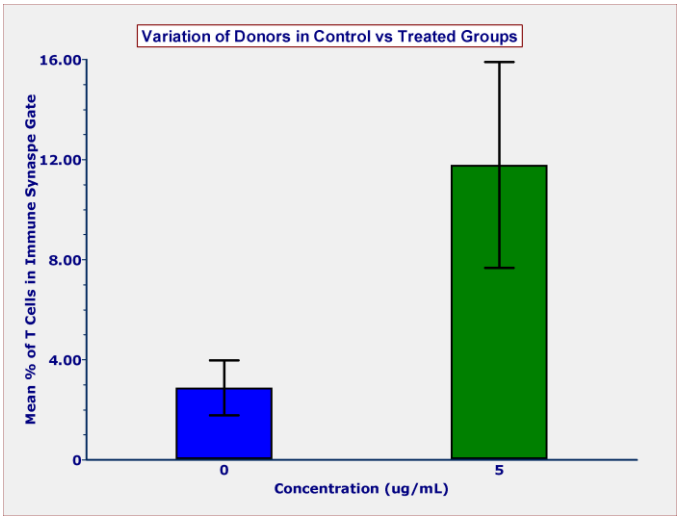


Figure 4: The variation of T cell activation amongst five separate donors was assessed by calculating the mean % of T Cells in the Synapse Gate across all donors for both untreated (0) and treated (5 ug/mL) groups. The control group exhibited a mean of 2.9 +/- 1.1 percent gated events while the treated showed 11.8 +/- 4.1 percent with a p value of 0.01.

Cytochalasin D affects immune synapse formation in a dose dependent manner

Cyto D is a cell permeable fungal toxin and potent inhibitor of actin polymerization. Cyto D is known to induce actin aggregation resulting in irregular spots of F-actin [2]. Figure 5 confirms and demonstrates the effect on actin when cells are treated with Cyto D. An increase in actin accumulation may be observed between the control (Figure 5A and 5C) and increasing doses of Cyto D (Figure 5B and 5C) as the cells become brighter and aggregates are formed. In this experiment, Raji cells are treated with 0.5 ug/mL SEB to form Antigen Presenting Cells (APCs). These APCs are then mixed with T cells previously incubated with increasing concentrations of Cyto D. Due to the actin aggregation that occurs when cells are treated, typical gating strategies as presented in figure 2D become difficult as populations shift due to the increasing amount of actin aggregation that is unrelated to the formation of an immune synapse. However, the percentage of T cell in conjugates is another easily measurable metric that we used in lieu of percentage T cell in an organized immunological synapse.

The percentage of T cell in conjugates is a function of the total number of events present in the no synapse and synapse gate (Figure 2D) divided by the total number of T Cells. FCS Express was used to quickly recalculate the percentage of T cells in conjugates metric and plot the results at each dosage point. The resulting curve shows that Cyto D effects the number of T Cell Conjugates formed in a dose dependent manner with a half maximal inhibitory concentration of 2.47 ug/mL (Figure 6).

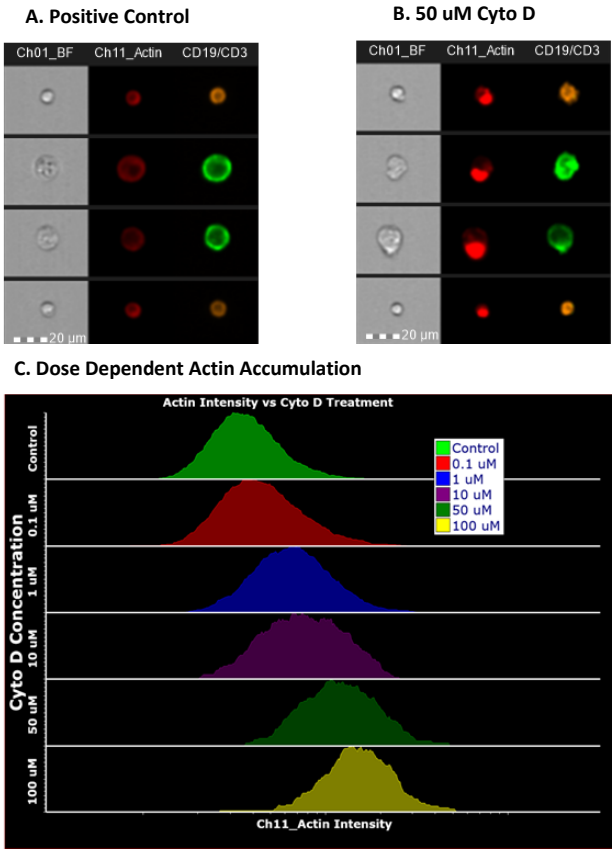


Figure 5: T cell and APC cell images of cell treated with A) 0.5ug/mL SEB (Positive control) and cells treated with B) 0.5ug/mL SEB and 50uM Cyto D. Bright Field, actin (red) and composite image CD19/CD3 (green-APC, orange-T cell) are shown. The 50 uM Cyto D treated sample images show clear actin accumulation and aggregation of actin. C) Histogram overlays of the control sample and samples treated with increasing doses of Cyto D demonstrates dose dependent accumulation of actin when assessing the intensity of Actin staining.

Conclusions

Imaging flow cytometry combines the quantitative power of large sample sizes common to flow cytometry with the information content of microscopy. This study utilized the FlowSight and its companion IDEAS software to demonstrate the utility and function of the "Valley" mask algorithm and the Bright Detail Similarity feature in relation to Immune Synapse detection. The results were calculated and reported in FCS Express Image Cytometry allowing for real time review of data with direct linkage to the imagery to confirm gating strategies and phenotypes of interest. The combination of image collection, advanced morphology analysis and masking algorithms from the FlowSight/IDEAS technology and real time analysis from FCS Express Image Cytometry allowed researchers to quickly and easily quantify and report the complexities of Immune Synapse formation for multiple study formats.

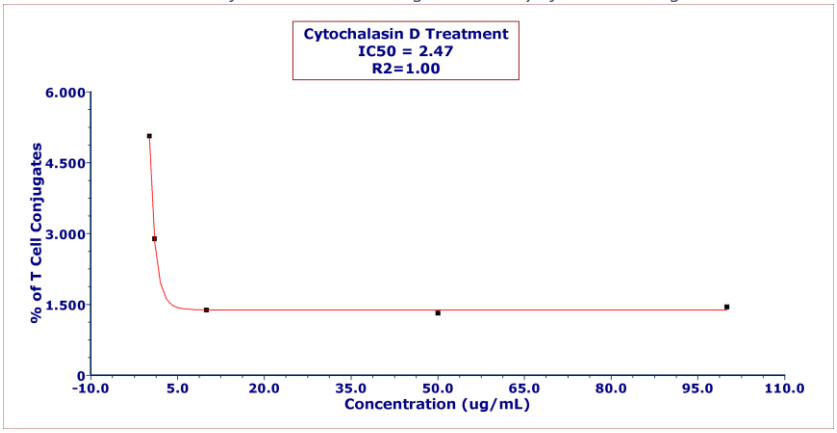


Figure 6: The percentage of T Cell Conjugates decreases in a dose dependent manner in response to increasing concentration of Cytochalasin D. A half maximum effect is observed at 2.47 ug/mL in the sample tested.

[1] Li H., Llera A., Malchiodi E.L., Mariuzza R.A. The structural basis of T cell activation by superantigens. Annu. Rev. Immunol. 1999;17:435–466. doi: 10.1146/annurev.immunol.17.1.435.

[2] Mortensen K., Larsson LI. Effects of cytochalasin D on the actin cytoskeleton: association of neoformed actin aggregates with proteins involved in signaling and endocytosis. Cell Mol Life Sci. 2003 May;60(5):1007-12. doi:10.1007/s00018-003-3022-x