Application Note

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Illuminating T Cell–APC Interactions Using Multispectral Imaging Flow Cytometry

Abstract

Interactions between antigen-specific T cells and the cognate ligand of antigen presenting cells (APCs) involve cytoskeleton reorganization, and recruitment of adhesive and signaling molecules to the site of intercellular contact. Sustained adhesion of T cells to APCs and formation of an immunological synapse are required for T cell receptor (TCR) stimulation and the antigen-specific response.

Immunological synapse formation may be measured 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.

To overcome these problems, we employed the Cytek® Amnis® ImageStream®X Mk II imaging flow cytometer to collect imagery of a large number of cells to:

- Identify cell conjugates and assess the percentage of T cells involved in an organized immunological synapse
- Investigate the recruitment of the LFA-1 adhesion molecule and Lymphocyte-specific protein tyrosine kinase (Lck) signaling molecule to the synaptic complex
- Assess the degree of T cell activation by measuring the translocation of NF-kB from the cytoplasm to the nucleus in the T cell in an objective and statistically significant manner

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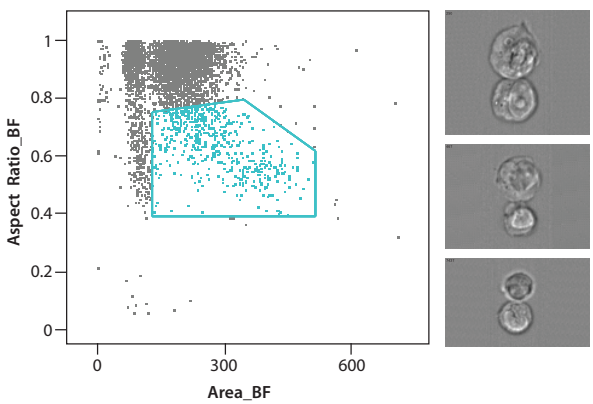
Identifying conjugates

The following method was used to identify T cell–APCs conjugates. The antibodies used may change based on the specific experiment.

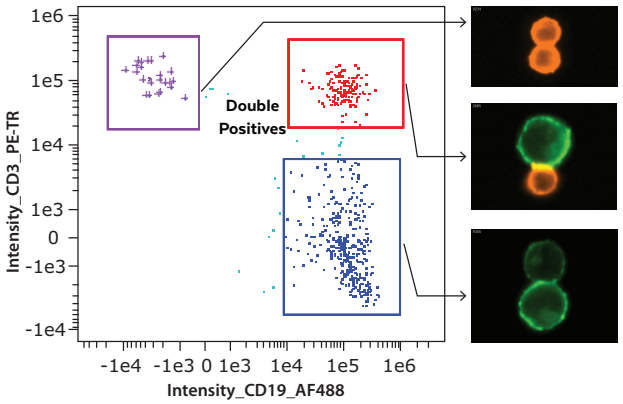
In these experiments, Raji B cells were loaded with *Staphylococcal enterotoxin B* (SEB) to create APCs. The SEB-loaded APCs were incubated with human T cells purified from peripheral blood. After incubation, the cells were fixed, permeabilized, and labeled with CD3-PE-TexasRed (T cells, orange) and CD19-AF488 (Raji B cells, green).

An ImageStream^X Mk II imaging flow cytometer with a 60X objective was used to assess the frequency of conjugates with an organized immunological synapse. Image analysis was performed using the Cytek® IDEAS® image analysis software package. Figure 1 demonstrates how to isolate cell conjugates using the IDEAS software.

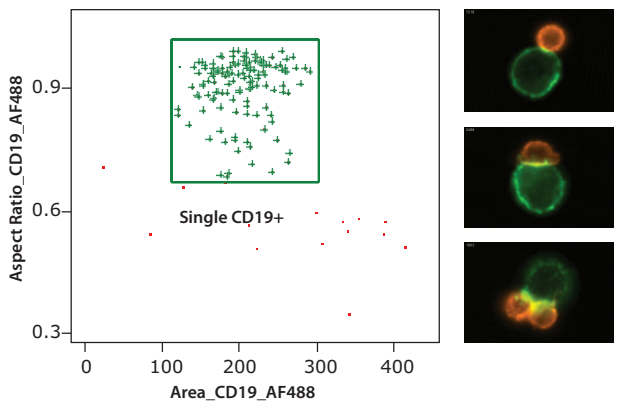
Figure 1. Gating strategy to isolate conjugates for immunology synapse analysis



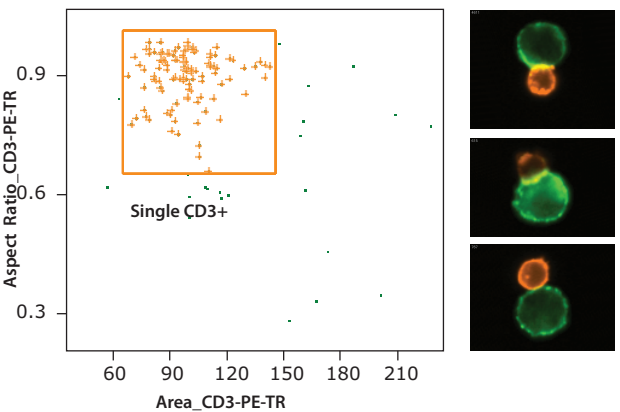
Step 1. Plot the Aspect Ratio vs. Area of the brightfield (BF) image to identify doublets.



Step 2. Plot the Intensity CD3 vs. CD19 to identify doublets with both CD3 (orange) and CD19 (green).



Step 3. Plot the Aspect Ratio vs. Area of the CD19 signal from the double positive population to identify doublets with only one CD19+ cell.



Step 4. Plot the Aspect Ratio vs. Area of the CD3 signal from the doublets to identify those with only one CD3+ cell.

This final population consists of doublets with only one CD19+ Raji B cell and one CD3+ T cell. In each study shown, this method was used to identify the T cell–APC conjugates. These T cell–APC conjugates were then used for further analysis.

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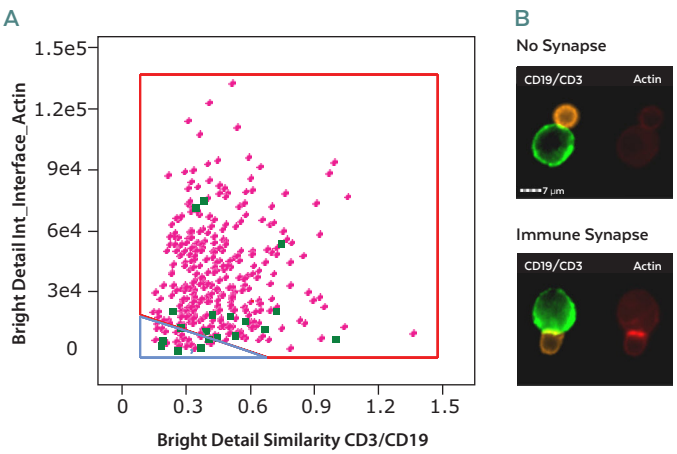
Identifying organized immunological synapses in the conjugates

By plotting the Bright Detail Intensity of the actin at the interface vs. the Bright Detail Similarity of CD3 and CD19, we are able to quantify the percent of T cells in an organized immunological synapse (**Figure 2**). Polymerization and concentration of actin at the immunological synapse results in high local pixel intensity, which is quantified by masking the cell contact area and using the Bright Detail Intensity feature of the IDEAS Software. The co-localization of CD3 and CD19 was measured by using the co-localization wizard within IDEAS, which measures the cross-correlation of the small fluorescence details (Bright Detail Similarity) between the CD3 and CD19 images. The percent of T cells in the immune synapse gate was 6.9% for the SEB-treated sample vs. 0.49% for the control (no SEB) sample.

Figure 2. (**A**) Scatter plot of Bright Detail Intensity in the interface* of the actin signal vs. the Bright Detail Similarity of the CD3+ and CD19+ signals. (**B**) Representative images from no synapse and immune synapse gates. CD3 (T cells, orange); CD19 (APC, green); Actin (red).



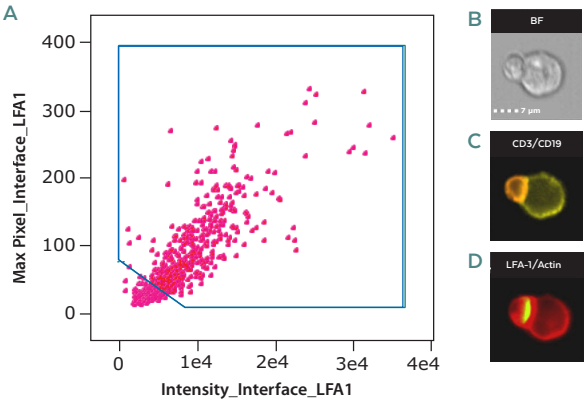
Green squares = No SEB control
Pink diamonds = SEB-treated
Red box = Immune synapse gate
Blue box = No synapse gate

*Interface is defined as a mask that covers the region where the two cells connect. This mask varies by experiment.

LFA-1 migration to the immunological synapse

Lymphocyte function-associated antigen 1 (LFA-1) is found on all T cells and functions as an adhesion molecule by binding to ICAM-1 on APCs. During an organized immunological synapse, there is an accumulation of LFA-1 and actin at the interface. In this study, we visualized LFA-1 (CD11a-FITC) and actin (phalloidin-AF647) accumulation within the immune synapse (**Figure 3**).

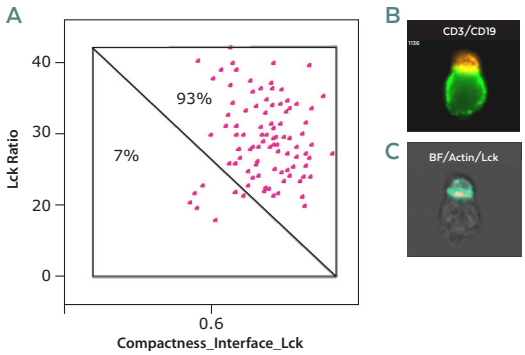
Figure 3. (**A**) Scatter plot of Max Pixel Intensity vs. Intensity of LFA-1 at the interface. The blue region represents cells with a high LFA-1 signal. 68% of the cells in the immune synapse exhibited high LFA-1. Representative image from the blue region. (**B**) BF, brightfield; (**C**) CD3 (orange); CD19 (yellow); (**D**) LFA-1 (green); Actin (red).



Lck migration to the immunological synapse

Lck phosphorylates ITAMs of the TCR complex, which in turn recruit ZAP-70 to the TCR. We measured the localization of the signaling molecule Lck within an organized immunological synapse. Plotting the Lck intensity ratio vs. Compactness of Lck we were able to identify events with Lck at the immune synapse (**Figure 4**).

Figure 4. (**A**) Scatter plot of the ratio of Lck intensity in the synapse to the total Lck intensity (Lck ratio) vs. the Compactness of Lck at the interface. Representative images of a SEB-treated cell. CD3 (orange); (**B**) CD19 (green); (**C**) actin (phalloidin-Texas Red, light blue); Lck (LcK-AF647, red).



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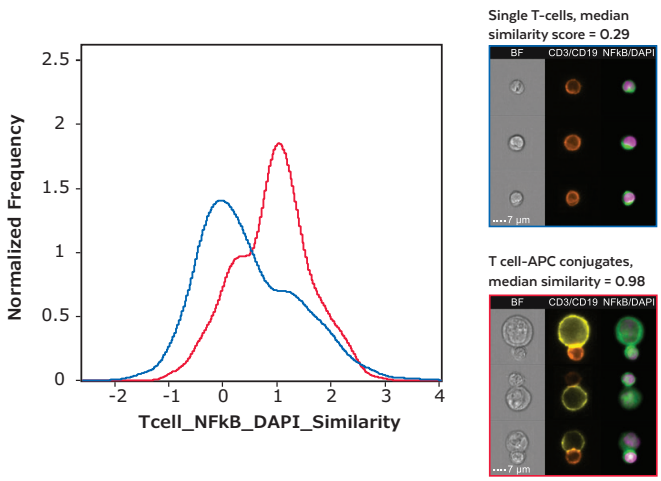
NF-kB translocation in T cell conjugated to APCs

The Nuclear Factor kappa B (NF-kB) transcription factor plays a central role in regulating many key processes in mammalian cells, including immune and inflammatory responses, proliferation, apoptosis, and cell survival.

Presentation of antigens to T cells in cognate ligand/MHC complexes results in stimulation and T cell activation with subsequent NF-kB nuclear translocation. In this study, we measured NF-kB translocation in T cells which were in contact with SEB-loaded APCs (T cell-APC conjugates) and compared it to NF-kB translocation in single T-Cells. IDEAS Software was used to calculate the Similarity score of NF-kB to the DAPI nuclear images in just the T-Cells. The median Similarity score for the T cells in T cell-APC conjugates was 0.98; compared to the median Similarity score of 0.29 for the single T cells not in conjugates. This indicates T-Cell activation from APC binding (**Figure 5**).

Figure 5. Similarity score histogram of single T cell (blue) and T cells in T cell-APC conjugates (red). Representative images of cells with median Similarity scores for each are shown: BF, brightfield; CD3-PE-TexasRed (orange); CD19-PE (yellow); NF-kB-FITC (green); DAPI (pink).



The scatter plot shows the Similarity score of single T cells (blue) and T cells in T cell - APC conjugates (red).

Conclusions

Imaging flow cytometry combines the quantitative power of large sample sizes common to flow cytometry with the information content of microscopy. This study used the ImageStream^X Mk II imaging flow cytometer and its companion IDEAS data analysis software to demonstrate in an objective and statistically robust manner that there is a significant increase in the number of cell conjugates with an organized immunological synapse when the APCs have been loaded with SEB. In addition, there is an increase in NF-kB translocation when T-cells are in contact with APCs.

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