Application Note

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The Kinetics of CpG Internalization and Sub-cellular Organelle Co-localization Within Circulating Human Plasmacytoid Dendritic Cells

Abstract

Plasmacytoid dendritic cells (pDCs) are key players in the innate immune response to viruses. Although rare—occuring in <0.2% of circulating peripheral blood cells—pDCs secrete the potent antiviral cytokine IFN-α in response to HSV, mainly via TLR-mediated signaling upon recognition of viral DNA motifs. As ligand-bound TLR9 enters the endosome, MyD88-dependent IRF-7 and NF-κB signaling is promoted, resulting in the sustained anti-viral responses mediated by the pDCs. The lack of available cell lines and the poor adaptability of pDCs to culture has rendered the study of TLR-mediated internalization and sub-cellular trafficking of viral particles, as well as TLR-mediated signaling within pDCs, difficult by traditional biochemical or microscopic techniques.

We have shown that nuclear translocation events within non-adherent primary cells can be quantified by correlating a transcription factor with nuclear images collected using the Cytek® Amnis® ImageStream®X imaging flow cytometer. We have also demonstrated the ability to quantify sub-cellular trafficking of internalized molecules in tumor cells using the ImageStream^X system.

The ImageStream^X system acquires up to 12 images per cell—including brightfield, darkfield, and fluorescence—at rates exceeding 5,000 cells per second. The digital images obtained are analyzed using Cytek® IDEAS® software, a powerful statistical image analysis program which includes tools for objective numerical scoring as well as cell population characterization. The ability to score large volumes of automatically acquired images streamlines the processes of investigating internalization, sub-cellular trafficking, and nuclear translocation within rare primary immune system cells such as pDCs.

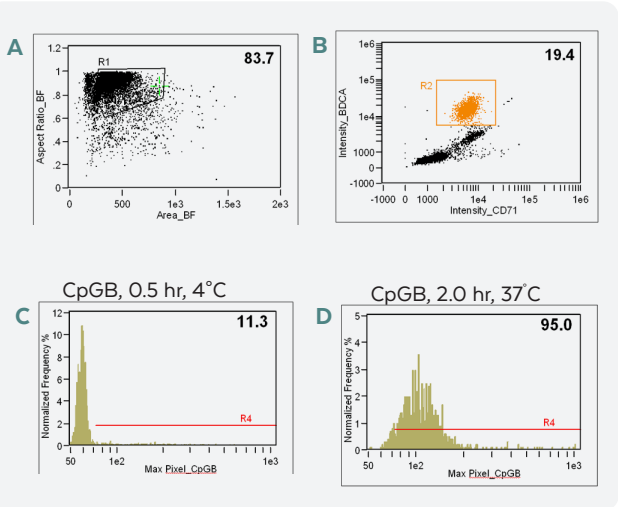
In this study, we used the ImageStream^X imaging flow cytometer to measure CpGB-induced nuclear translocation of NF-κB, as well as the internalization and intracellular trafficking of CpGB to endosomes and lysosomes within pDCs, using image-based quantitative metrics.

Results

Identification of pDCs Associated With CpGB

To limit analysis to an intact, single pDC, we gated events with an intermediate BF area and a high aspect ratio, which distinguished single cells (R1) from debris (low BF area) and multi-cellular (high DRAQ5 intensity and low aspect ratio) events (**Figure 1A**). Figure 1B shows gating for BDCA+ cells. The percentages of single cells and single cells that are pDCs are indicated in the upper right corner of each plot. Because the CpGB stain is concentrated in small areas, we used the CpGB Max Pixel feature to identify pDCs that were associated with CpGB (**Figure 1C**).

Figure 1. Gating strategy to identify single pDCs positive for CpGB at 4˚C and 37˚C



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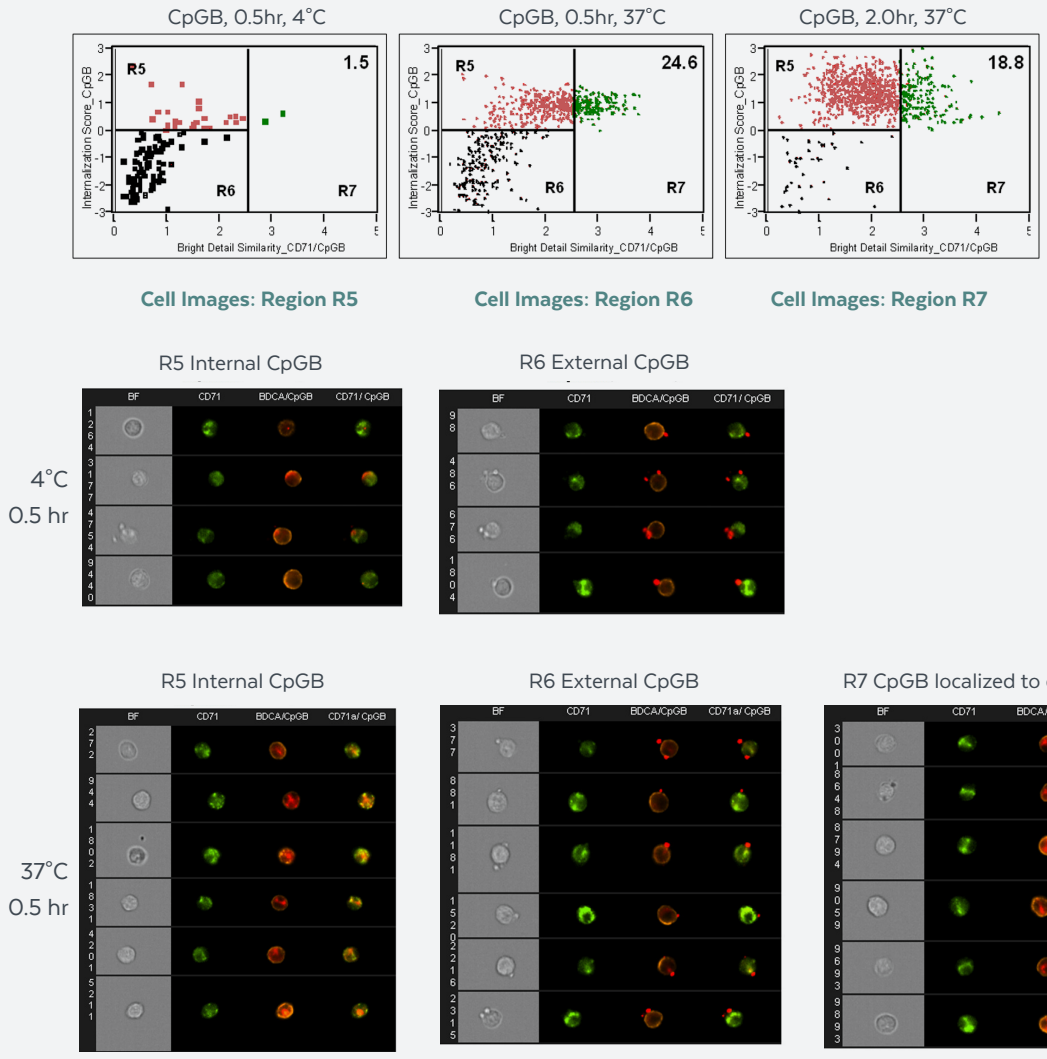
Correlation Between CpGB and CD71

To measure the relative co-localization of pDC-associated CpGB to the early endosomes, we calculated the correlation between the bright details of the CpGB and CD71 image pair for each pDC using the Bright Detail Similarity feature (x-axis). This feature correlates only the portion of each image in the optimal plane of focus and ignores data contributed by background pixels within the cells. As CpGB molecules internalize and co-localize to the endosome, the similarity between the CpGB and CD71 images increases. Region R7 was drawn to include those cells with clear visual evidence of co-localization. The Internalization feature (y-axis) is derived from the ratio of CpGB intensity inside the cell to the total CpGB intensity. The internalization score is conditioned such that a value greater than zero indicates that more CpGB is found inside the cell compared to outside the cell (**Figure 2**).

Figure 2. Scatter plots showing the internalization and location of CpGB at 4°C, and 37°C for 0.5 and 2.0 hours and representative images from region 5 (internal), region 6 (external), and region 7 (internal and localized to endosomes).



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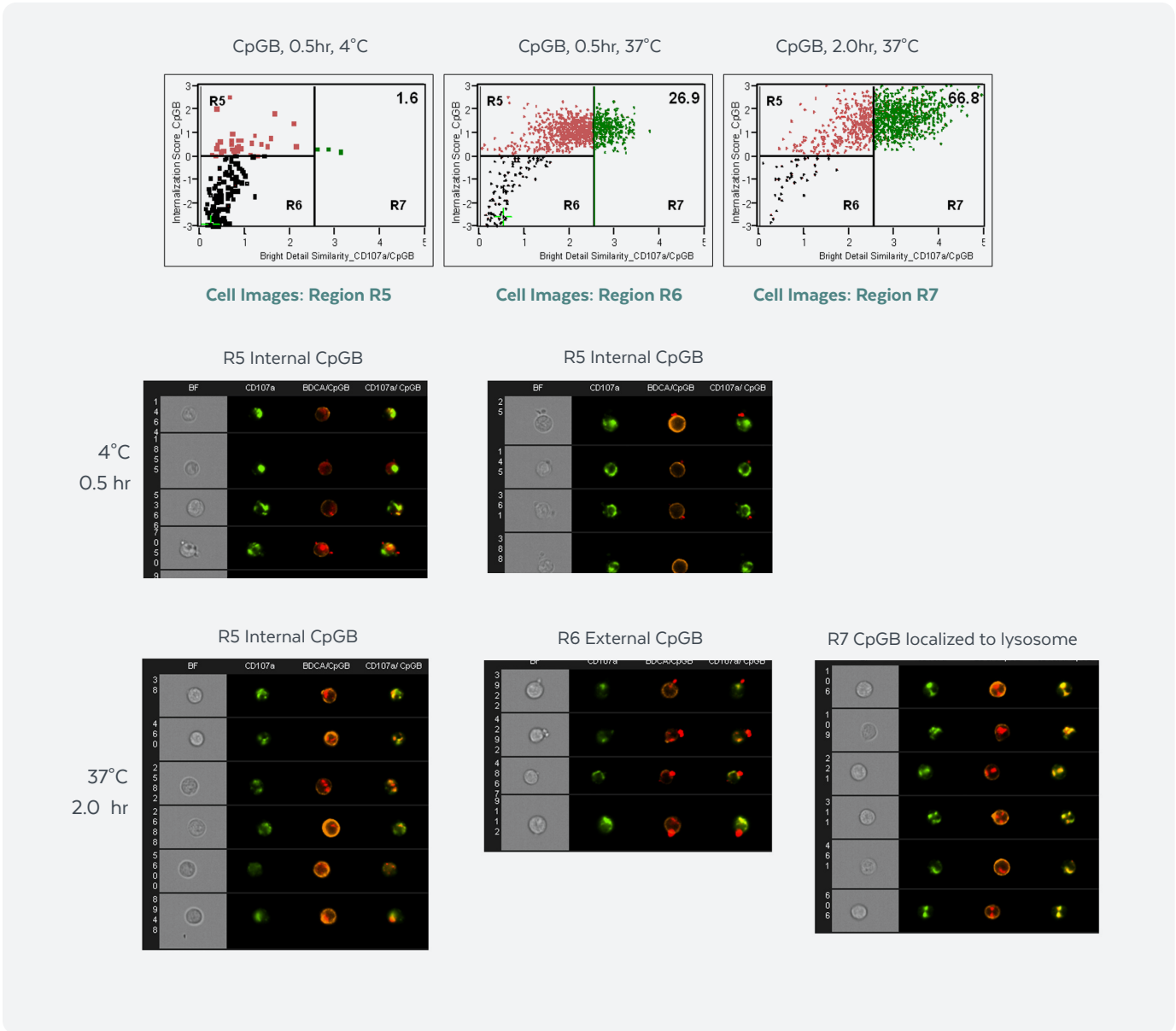
Correlation Between CpGB and CD107a

The relative co-localization of pDC-associated CpGB to the lysosomes was measured using a calculated correlation between the bright details of the CpGB/organelle (CD107a) image pair for each pDC. As CpGB molecules co-localize to the lysosome, the similarity between the CpGB and CD107a images increases. Region R7 was drawn to include cells with clear visual evidence of co-localization. The Internalization feature (y-axis) is derived from the ratio of CpGB intensity inside of the cell to the total CpGB intensity. The internalization score is conditioned such that a value greater than zero indicates that more CpGB is found inside the cell compared to outside the cell (**Figure 3**).

Figure 3. Scatter plots showing the internalization and location of CpGB at 4˚C, and 37˚C for 0.5 and 2.0 hours and representative images from region 5 (internal), region 6 (external), and region 7 (internal and localized to lysosomes).



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To measure nuclear translocation of NF-κB, we measured the Similarity of the NF-κB and DRAQ5-labeled nuclear image pair for each pDC in the file (**Figure 4**). The more NF-κB that is in the nucleus, the more similar the image pair will appear and the larger the Similarity value will be. The R6 gate identifies the region of positive correlation for the score, and the percentage of pDCs that fall within R6 is indicated in the upper right corner of the plots (**Figure 5**).

Figure 4. Histograms of Similarity scores showing the translocation of NF-κB in the pDCs from a control (Mock), 30, 60 and 120 minutes.

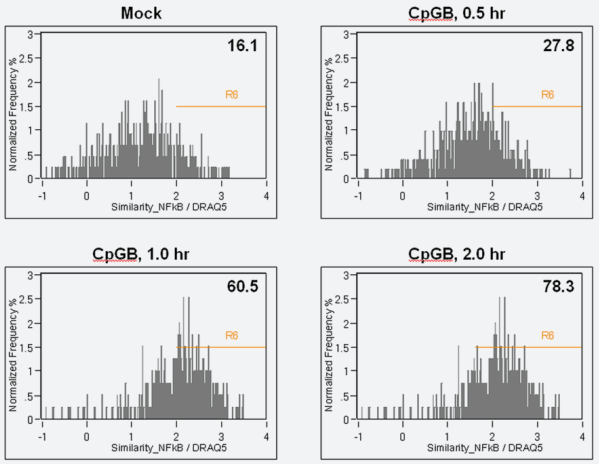
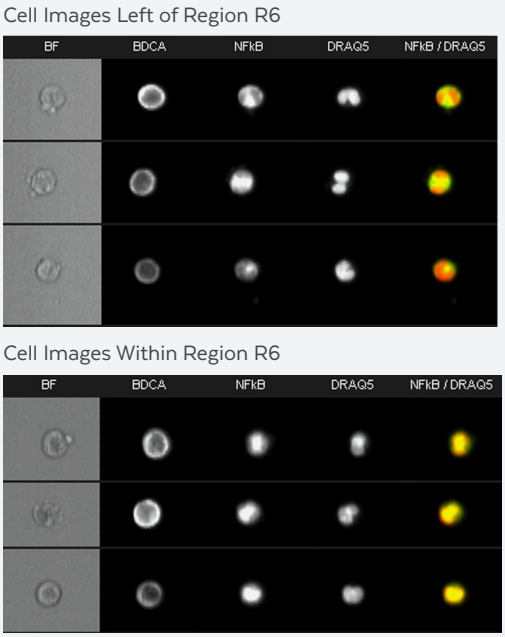


Figure 5. Representative images of cells from Figure 4.



Conclusion

This study demonstrates how the ImageStream^X imaging flow cytometer can be utilized to measure CpGB-induced nuclear translocation of NF-κB, as well as the internalization and intracellular trafficking of CpGB to endosomes and lysosomes within pDCs using image-based quantitative metrics. By combining the power of digital fluorescence microscopy with the speed and sensitivity of flow cytometry, the ImageStream^X system performs applications with ease that would be impossible using either technique alone.

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