The Effects of Cytoskeletal Perturbation on ZAP70 Clustering During T-Cell Activation

Will Heffernan

Spring 2022

Abstract

ZAP-70 is one of many molecules involved in signal transduction during the activation of a T-cell. This experiment was conducted to investigate how perturbation of the cytoskeleton affects ZAP-70 clustering at T-cell antigen receptors. Activated T-cells were observed through both interference reflection microscopy and total internal reflection fluorescence microscopy, with post processing conducted using various softwares. From results including cell areas and mean fluorescence intensities, it was indicated that various molecules do have different effects on the clustering of ZAP-70, potentially due to their effects on the cytoskeleton. Going forward, not only should more molecules be used for cytoskeletal perturbation, but more subject cells should be used for a more expansive and complete understanding of signal transduction and other intracellular processes.

Introduction

T-cells, a crucial component of the adaptive immune system, are known for their roles in protecting the body by destroying potentially harmful cells, such as bacteria, or those that are a viral vector. While research has come a long way in understanding the mechanisms of T-cells, there is still much to grasp on how external factors could affect their inner workings.

T-cells are activated through signal transduction, made possible through the mediation done by T-cell antigen receptors, or TCRs (Smith-Garvin, J., Koretzky, G., & Jordan, M., 2009). Through something of a signal cascade, a plethora of molecules interact with various sections of the TCRs during activation. Following the phosphorylation of immunoreceptor-based tyrosine activation motifs (ITAMs), a protein tyrosine kinase (TPK) by referred to as the zeta-chain-associated protein kinase-70, or ZAP70 is recruited and activated, binding to TCRs and allowing for the continuation of signal transduction within the T-cell (Yi, J., Balagopalan, L., Nguyen, T., McIntire, K., & Samelson, L., 2019).

This ZAP70 molecule plays an ostensibly role in the activation of T-cells, as the recruitment of ZAP70 is critical to the proper signal transduction within the cell, just as the the recruitment of plenty of other molecules involved in the process are.

The goal of this experiment was to investigate the effects of perturbation of the T-cell cytoskeleton on the clustering of ZAP70 molecules during the activation of the T-cell. The drugs used to induce this perturbation were 0.05% dimethyl sulfoxide (DMSO), 1 μM Jasplakinolide, 2 μg/ml of vascular cell adhesion molecule-1 (VCAM-1), and 100 μM Y-27632, as well as an L-15 medium as a control environment.

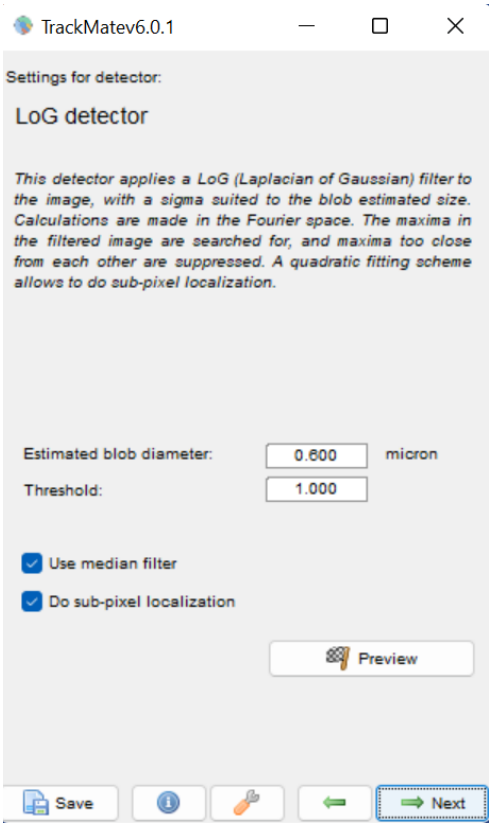
Methodology

Setup

This experimental setup was designed by multiple members of the Cellular Dynamics lab to analyze intracellular components and their processes. A line of Jurkat T-cells with GFP-marked ZAP70 were used for this experiment. To image the cells, both interference reflection microscopy (IRM) and total internal reflection fluorescence microscopy (TIRF) were used. IRM was used in order to isolate a region of interest from each movie and create boundaries which would be used in analysis of the ZAP70 molecule. TIRF, on the other hand, was used to look specifically at the clusters of ZAP70 molecules within the cells during activation.

Analysis

Using ImageJ, the regions of interest were defined from the IRM movies, while intensities of each of the pixels in each frame were recorded using the TIRF movies. Utilizing the region of interest threshold and data from the ZAP70 movies, a number of scripts were devised in MATLAB in order to extract mean fluorescence intensities (MFIs) as well as area for each cell over their interval of frames. This data was used to create individual as well as some comparative plots of the MFIs.



Trackmate interface using the LoG detector.

In preparation for the segmentation of ZAP70 clusters in the ImageJ plug-in, Trackmate, signal to noise

ratios (SNRs) were estimated using line scans of the movies within ImageJ. The SNR estimate was made by locating the peak intensity of a single cluster within the cell and dividing it by the surrounding intensity, or noise, where another cluster was not present. Continuing with this preparation, it was found that the ideal threshold for segmentation, as defined in the Trackmate interface, was between 1.0 and 2.0. While a threshold below 1.0 included far too many spurious ZAP70 clusters to manually remove later, any threshold above 2.0 risked excluding clusters of interest. With about 10 pixels being the diameter of the clusters that are of interest, it was determined from the dimensions of the movies that about 0.6 microns would be appropriate for the estimated blob diameter for segmentation.

After completing segmentation, it was possible to import data on the detected clusters into excel spreadsheets to characterize the spatial distribution of the clusters.

Results

Representative images from IRM (fig. 1a-1b) and TIRF (fig. 1c-1d) movies are shown at two time intervals. Generally, the region of interest in the IRM movies slowly grows in diameter, while the TIRF movies exhibit a significant spike in fluorescence within the first few minutes, tapering off as time passes.

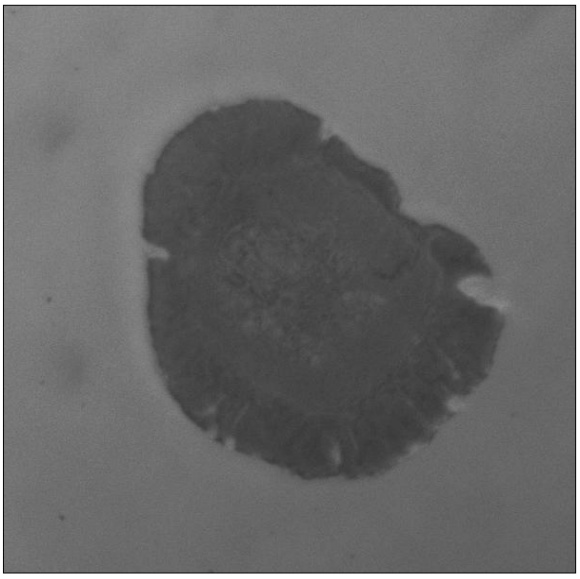


Figure 1a. IRM region of interest of an L15 cell at 1 minute.

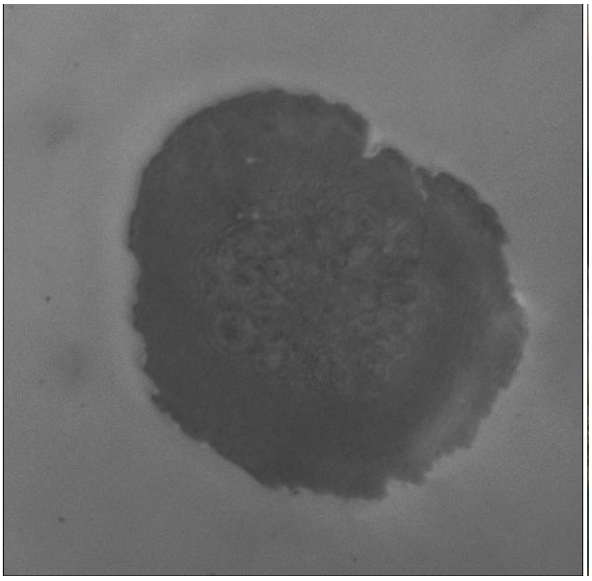


Figure 1b. IRM region of interest of an L15 cell at 5 minutes.

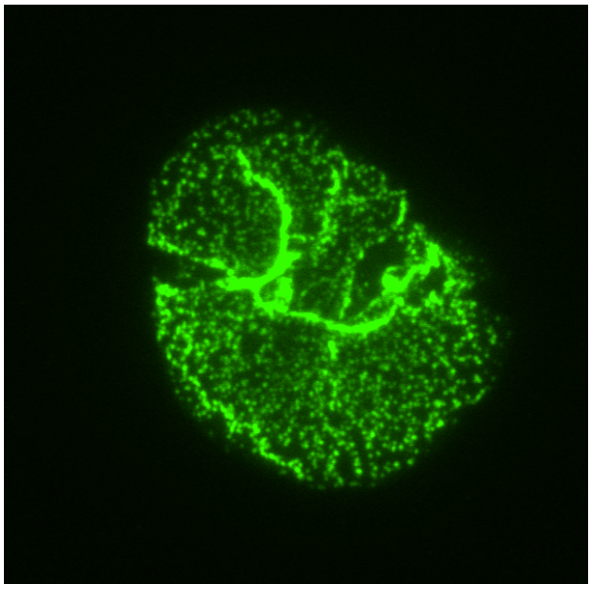


Figure 1c. TIRF image of ZAP70 clusters in an L15 cell at 1 minute.

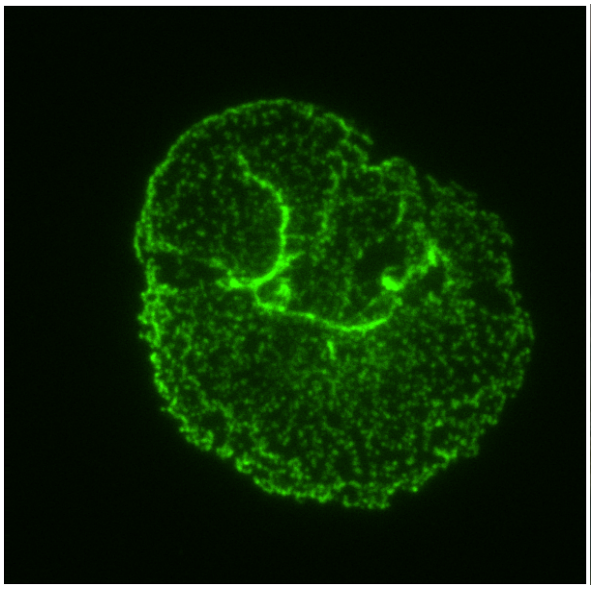


Figure 1d. TIRF image of ZAP70 clusters in an L15 cell at 5 minutes.

Plots of cell areas over time seem to confirm these observations with all but one cell exhibiting a clear growth in area over the 10 minute time interval (fig. 2a). As seen from the

average MFI of all L15 cells over time (fig. 2b), the intensity also seems to quickly peak between 80-100 seconds, with a significant decline thereafter.

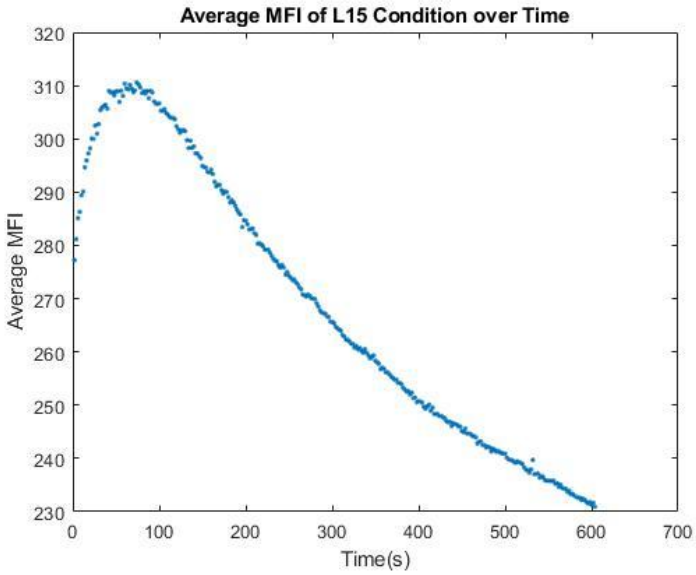


Figure 2a. Average MFI of cells treated with L-15 over time.

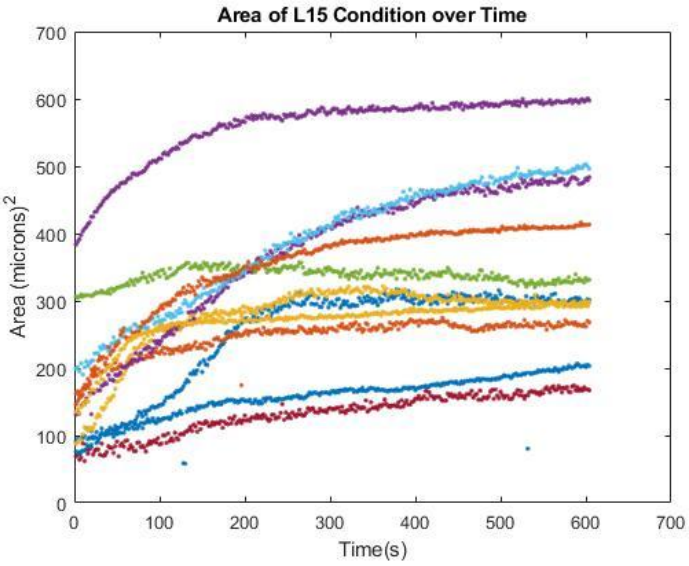


Figure 2b. Areas of cells treated with L-15 over time.

Plots of the cell areas and average MFI for the DMSO control condition are shown. The cell areas stick to the trend of gradual growth over time (fig. 3a), while the average MFI (fig. 3b) begins at a higher intensity, around 350, and reaches a higher maximum average MFI than the cells treated with L15.

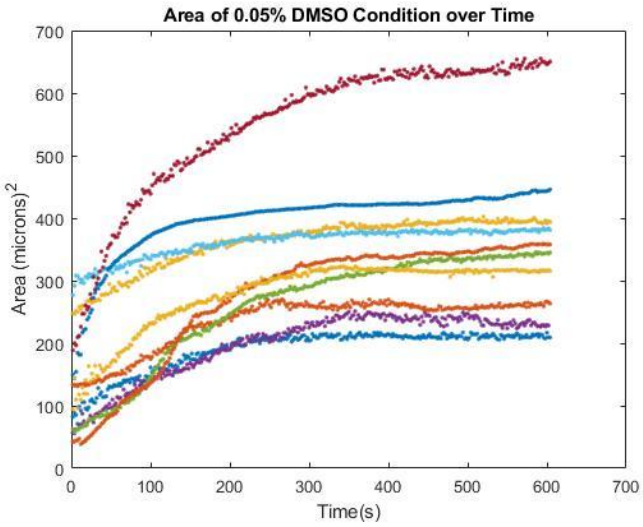


Figure 3a. Areas of cells treated with DMSO over time.

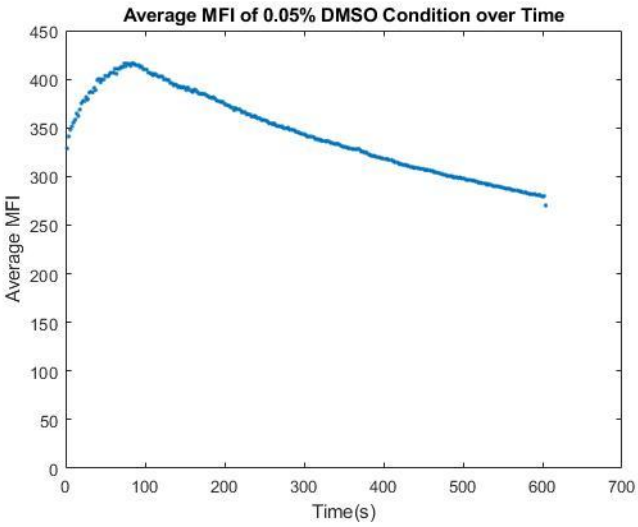


Figure 3b. Average MFI of cells treated with DMSO over time.

Plots of the cell areas as well as average MFI over time are shown for cells treated with 1 μM Jasplakinolide, an inducer of actin polymerization (NCBI, 2022). The cell areas remain consistent with the trend of growth over time (fig. 4a) while the average MFI of cells treated with Jasplakinolide reaches a maximum of about 335 (fig. 4b), higher than cells treated with L15 and lower than those treated with the DMSO.

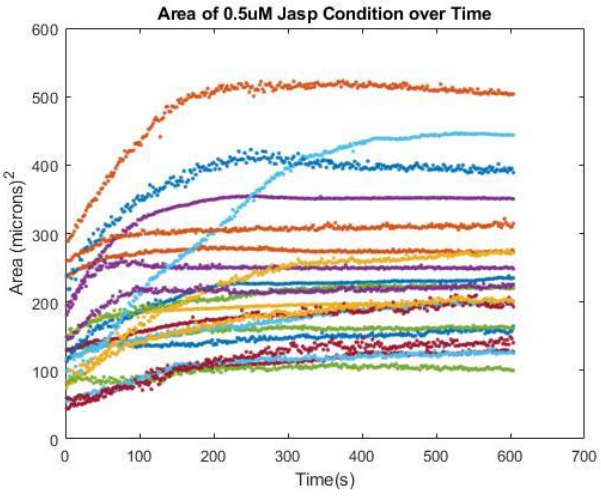


Figure 4a. Areas of cells treated with Jasplakinolide.

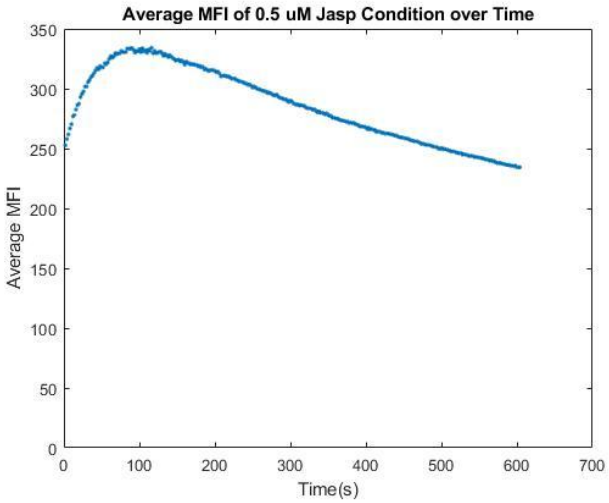


Figure 4a. Average MFI of cells treated with Jasplakinolide.

Shown are plots of cell areas and average MFI of the cells treated with 100 μM Y-27632. Again, cells exhibit a gradual growth in area over the 10 minute time frame (fig. 5a) but the average MFI of the cells over time begin at 250 and reach an intensity of around 330 (fig. 5b), most closely resembling the intensity profile of the cells treated with Jasplakinolide.

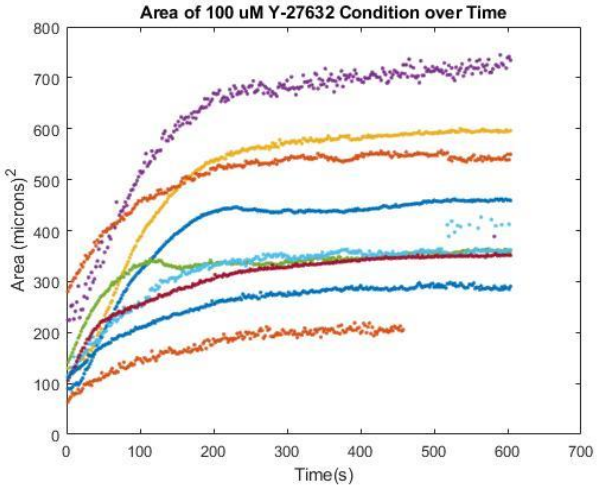


Figure 5a. Areas of cells treated with Y-27632 over time.

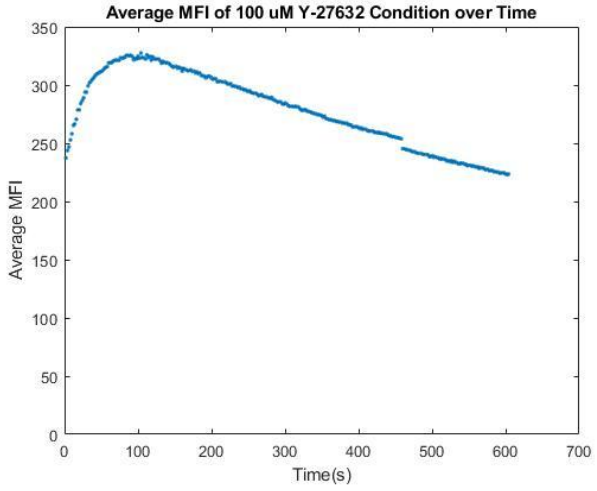


Figure 5b. Average MFI of cells treated with Y-27632 over time.

The last condition included in this analysis was the 2 μg/ml VCAM-1. The areas over time for cells treated with the VCAM-1, unlike those under other conditions, exhibit a significant growth within the first 40-110 seconds then decline in the following frames (fig. 6a). The average MFI plot for these cells, however, follows the general trend of an early maximum followed by decline, with these cells reaching a maximum average MFI of about 280 (fig. 6b).

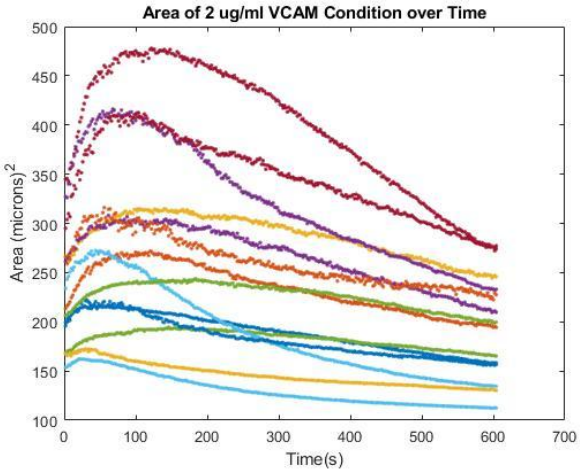


Figure 6a. Areas of cells treated with VCAM-1 over time.

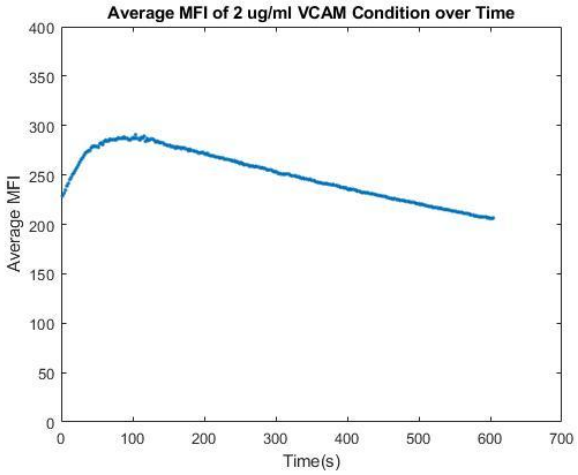


Figure 6b. Average MFI of cells treated with VCAM-1 over time.

A line scan used to find the SNR of a cell treated with L15 is shown (fig. 7a). The peak value of the line scan would be divided by the minimum value to estimate the SNR. Using the SNR and observations from using various quality threshold values, segmentation of the fluorescently marked clusters would be completed (fig. 7b-7d).

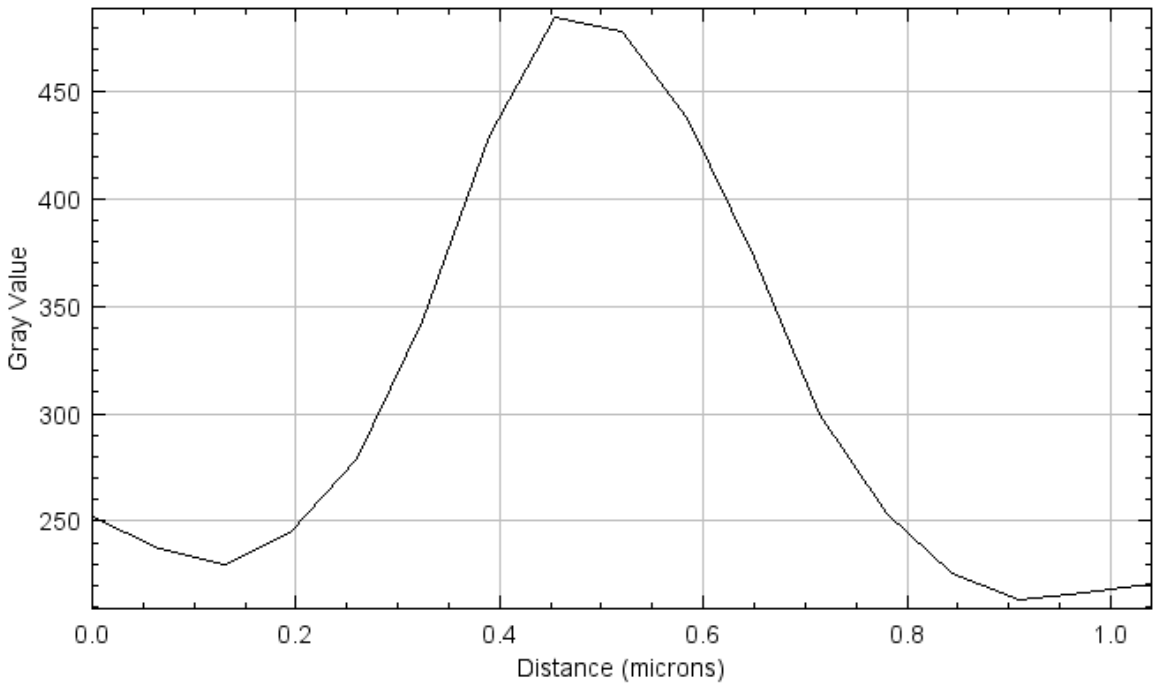


Figure 7a. Representative line scan across just over 1 micron of a cell treated with L15.

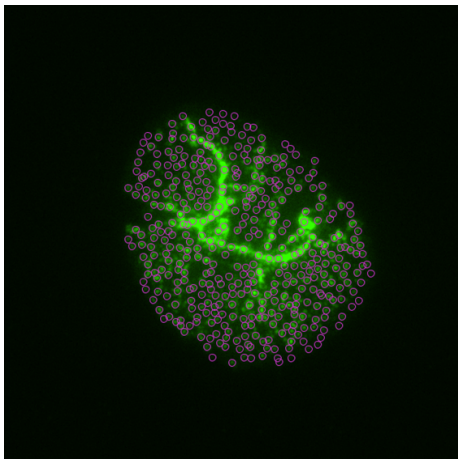


Figure 7b. Segmentation of a cell treated with L-15. Estimated SNR: 3.3. Quality threshold: 1.0.

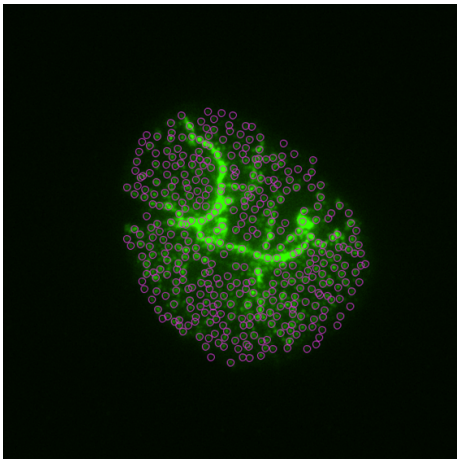


Figure 7c. Segmentation of a cell treated with L-15. Estimated SNR: 3.3. Quality threshold: 1.5.

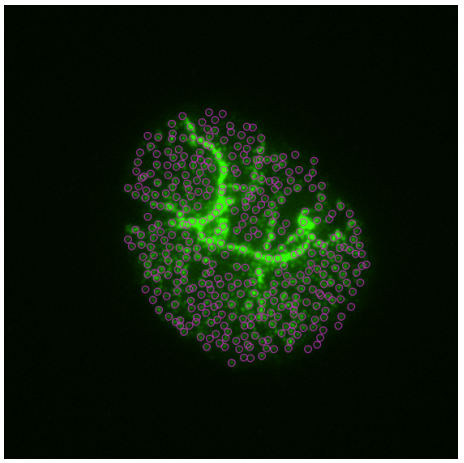


Figure 7d. Segmentation of a cell treated with L-15. Estimated SNR: 3.3. Quality threshold: 2.0.

Discussion

Between numerical as well as observed results, it would seem that the substance with the greatest effect on ZAP-70 clustering is the VCAM-1. While T-cells receiving the other treatments did exhibit some slight changes from the L-15 control, those treated with VCAM-1 showed the most profound differences in the MFI over time of cells and especially the area of the cells over time. This notable decrease in cell area over time may be due to the VCAM-1's stimulation of actin stress fibers (Kong, D., Kim, Y., Kim, M., Jang, J., & Lee, S., 2018). The stimulation of actin stress fibers may lead to significant contraction of the cytoskeleton, and thus, the notable decrease in area. This contraction may also explain the lower MFI exhibited by the cells treated with VCAM-1, as the clusters begin to stack on each other and less intensity found at the TCRs with ZAP-70 may be observed.

All of the cells treated with the 0.05% DMSO actually grew in area and unexpectedly so, as DMSO treatment has previously been observed to lead to development of thick stress fibers in the cytoskeleton, resulting in increased adhesion to substrates and inhibition of cell growth (Lampugnani, M., Pedenovi, M., Niewiarowski, A., Casali, B., Donati, M., Corbascio, G., &

Marchisio, B., 1987). Additionally, cells treated with DMSO had a significantly higher average MFI over time than those in the L-15 control medium, which leads to the conclusion that there is likely some overlooked interaction between DMSO and the T-cell that enhances the expression of ZAP-70 or its fellow TPKs.

Cells treated with both the Y-27632 and Jasplakinolide exhibited slightly higher average MFIs over time than those in the L-15 medium. More notable is how much longer this higher intensity is sustained than in the control-treated cells. Jasplakinolide functions as an actin polymerizer, while Y-27632 has been observed to inhibit contractions in some smooth muscle cells (Rees, R., Ralph, D., Royle, M., Moncada, S., & Cellek, S., 2001). From these mechanisms of action, it can be concluded that some interaction of the T-cells with Jasplakinolide or Y-27632, through inhibition of cell contraction or actin polymerization, respectively, increases the amount or intensity of clustering of ZAP-70 during T-cell activation.

Going forward, this experiment should, if possible, be replicated on as many molecules involved in signal transduction as possible, in T-cells and beyond. This may lead to a better understanding of T-cell activation and the signaling pathways of cells as a whole. Additionally there are more mediums which could be used to test other cytoskeletal perturbations on the recruitment of signaling molecules in cell activation.

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