

# Media Cybernetics Application Note

## Visualizing the Accumulation of CD3 at the Immune Synapse in Activated T-cells Using 3D Constructor®

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### Introduction

T lymphocytes (T cells) play an important role in orchestrating an adaptive immune response to foreign pathogens. A key event for T cell activation is an appropriate interaction between the T cell antigen receptor (TCR) and a limited number of foreign major histocompatibility complexes (MHCp) displayed on the surface of the antigen-presenting cell (APC) junction (1, 2). Other co-stimulatory and adhesion molecules are also bound in the cell-cell junction. Much attention has been devoted to these extracellular binding events (3, 4), as well as to the intracellular steps involved in signal transduction pathways implicated in T cell activation (5). Most of these individual events are compared to the time scale over which full T cell commitment is achieved. An important recent discovery is that T cell antigen recognition is accompanied by the formation of a specialized cell-cell junction that has been labeled the immunological synapse (6-9). The mature synapse is characterized by a specific pattern of segregated cell surface molecules in the T cell-APC junction (7, 8). In the immune synapse, CD3 is a protein localized at the center of the T cell/APC junction, and is important for T cell activation. The G3BP protein is a novel regulator of T cell activation. Striking images obtained with video microscopy demonstrate that the mature synapse is several microns in diameter, evolves over many minutes (~30 min), and is sustained for well over an hour (7, 8). Investigating these slow processes may provide mechanistic insights that have not been easily accessible by studying the fast events noted earlier.

Although data on synapse formation and the underlying molecular events are emerging at a rapid rate, several questions concerning the genesis of the synapse and its biological function remain unresolved. These questions are motivating a plethora of experiments. The resulting wealth of data must be understood in terms of a mechanistic framework if it is

to be useful for controlling the immune response. Scanning confocal microscopy has been a valuable tool in viewing the cell to cell junction of the immunological synapse in two dimensions. To gain a better perspective of the immunological synapse, 3D Constructor, a plug-in module for Image-Pro Plus, was used to view the cell to cell interaction in three dimensions. Investigating these slow processes may provide mechanistic insights that have not been easily accessible by studying the fast events noted earlier.

### Methods

A Multi-photon laser scanning confocal microscope (Bio-Rad MRC 1024 SE) was used to acquire image stacks of T-Cell extracellular binding events leading to the immunological synapse. Image stacks were taken at different time points, and confocal control settings were kept constant between images. Time points were 5/10/20/60 minutes following the APC/T cell contact. The cells were stained with anti-rat Alexa 594 (Red) for the zeta chain of the CD3 protein, which makes a complex with the T cell receptor. The cells were also stained with anti-mouse Alexa 488 (Green) for the G3BP protein, which is a novel regulator of T cell activation.

To view apparent CD3/G3BP protein co-localization, a surface value was determined using a thresholding method found in 3D Constructor (Media Cybernetics, Silver Spring, MD). The threshold was used to determine the extent of co-localization along the immunological synapse. Threshold values were selected that represented the degree of red/green co-localization within the image volume, and kept constant for all images analyzed.

### Results

Three dimensional projections indicate that expression of both the G3BP (green) and CD3 (red) proteins are localized mainly at the immunological synapse in activated T cells for all time points (Figure 1). The

control cell shows no significant accumulation of red and green signal.

Three dimensional visualization of the immune synapse indicated that the degree of G3BP and CD3 protein signal expression is affected by T-cell activation (Figure 3). Signal accumulation tended to be the most abundant at 5 minutes following activation, and then declined with time. Three dimensional rendering of the images revealed that endogenous G3BP forms a partially organized structure resembling a cluster.

## Discussion

A novel aspect of our work is the identification of the spatial organization of TCR signaling proteins, not only in the plane of the membrane, but also perpendicular to it. The anchoring of molecular components inside the cell at a distance from the plasma membrane, upon formation of an immune synapse, is referred to as a “parasynaptic” location. Signal accumulation of CD3 and G3BP was most abundant directly following T-cell activation as expected, since these proteins are important for T-cell activation. The staining for endogenous G3BP clearly revealed a partly organized structure resembling a cluster of budies or vesicles that a 2 dimensional structure couldn’t show. Much of the G3BP protein is poorly soluble in detergent-containing buffers (biochemical data not shown). Therefore, it seems that the G3BP structure may be associated with cytoskeletal elements and perhaps endocytic or lysosomal vesicles involved in down-modulation or recycling of surface molecules, such as those of the CD3/TCR complex. Alternatively, since the T-cell becomes polarized towards the APC, G3BP may be directed from the Golgi apparatus and/or the trans-Golgi network. These possibilities will need to be addressed experimentally. In either case, our findings indicate that the TCR-associated signaling molecules in the immune synapse are organized into a three-dimensional machinery of higher complexity than previously demonstrated by two dimensional visualization.

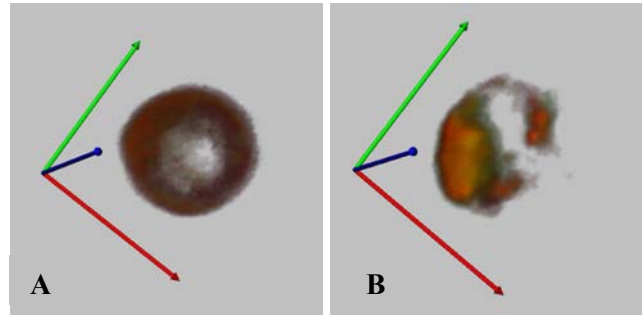


Figure 1. Three dimensional projections of Control (A) and Activated (B) T cells. The amount of co-localization can be observed by the change in relative color intensities in the two volumes. Areas with brighter intensities indicate a higher level of apparent co-localization. Axes, indicated by the colored bars (X=Red Y=Green Z=Blue), represent the spatial orientation of the T cells.

The CD3 and G3BP proteins appear to be forming a cluster of “budies” or vesicles at the T-cell/APC junction upon activation of the immune synapse (Figure 2).

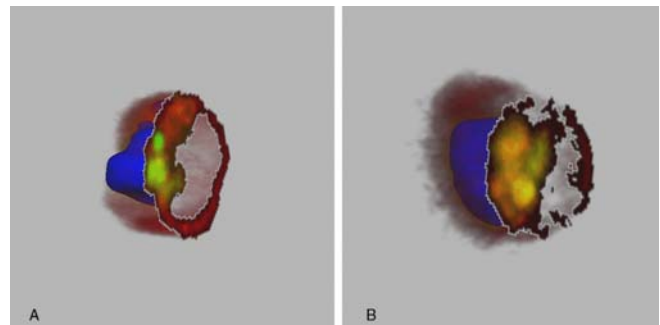


Figure 2. Three dimensional projections at 20 min (A) and 10 min (B) displaying a cluster pattern of CD3 (red) and G3BP (green) proteins. Co-localization and protein clustering can be viewed by creating an isosurface (blue) mask, and by viewing a defined orthogonal slice through the co-localized volume.

A thresholding tool was used to display the degree of CD3/G3BP protein co-localization at the immune synapse. The control T-cell shows a very small co-localized area, indicating that CD3 and G3BP are in low abundance prior to T-cell activation. The activated T-cell at 5 minutes has a large area of red/green co-localization at the immune synapse (Figure 3).

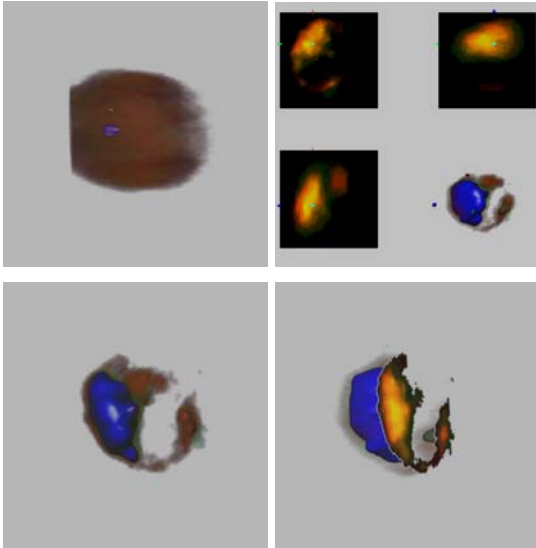


Figure 3. The control T cell (A) shown in X/Y orientation with apparent co-localization displayed as solid objects within the volume. Projections for the experimental T cell at 5 minutes are shown in views B-D. To facilitate observation of the co-localized areas, numerous visualization methods may be used. Co-localization can be viewed by orthogonal projection (B), creating an isosurface over the apparent co-localization (C), and by viewing a cut-away (orthogonal slice) through the co-localized volume (D).

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