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Qualitative and quantitative analysis of the immune synapse in the human system using imaging flow cytometry

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Abstract:	The immune synapse is the area of communication between T-cells and antigen-presenting cells (APCs). T-cells polarize surface receptors and proteins towards the immune synapse to assure a stable binding and signal exchange. Classical confocal, TIRF or super-resolution microscopy have been used to study the immune synapse. Since these methods require manual image acquisition and time-consuming quantification it is challenging to quantify rare events. Here we describe a workflow that enables morphological analysis of tens of thousands of cells. Immune synapses are induced between primary human T-cells in pan-leukocyte preparations and Staphylococcus aureus enterotoxin B (SEB)-loaded Raji cells as APCs. Image acquisition is performed employing imaging flow cytometry, also named InFlow microscopy, which combines features of a flow cytometer and a fluorescence microscope. A complete gating strategy for identifying T-cell/APC couples and the analysis of immune synapses is provided. As this workflow allows the analysis of immune synapses in unpurified pan-leukocyte preparations and hence requires only a small volume of blood (i.e. 1 mL) it can be applied to samples from patients. Importantly, several samples can be prepared, measured and analyzed in parallel.
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Cover letter

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Dear Dr. Metha,
Dear Dr. Jaydev Upponi

Please find our manuscript entitled: "*Qualitative and quantitative analysis of the immune synapse in the human system using imaging flow cytometry*".

We made all modifications that were suggested by the editor and the reviewers (see point-by-point reply).

I look forward to hearing from you and to produce the movie together with you.

Yours sincerely

Guido Wabnitz

TITLE:
Qualitative and quantitative analysis of the immune synapse in the human system using imaging flow cytometry

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KEYWORDS:
Immune synapse, flow cytometer, Iimaging flow cytometry, T cells, actin cytoskeleton, human, adaptive immune system

SHORT ABSTRACT:
Here, we describe a complete workflow for the qualitative and quantitative analysis of immune synapses between primary human T cells and antigen-presenting cells. The method is based on imaging flow cytometry, which allows the acquisition and evaluation of several thousand cell images within a relatively short period of time.

LONG ABSTRACT:
The immune synapse is the area of communication between T cells and antigen-presenting cells

(APCs). T cells polarize surface receptors and proteins towards the immune synapse to assure a stable binding and signal exchange. Classical confocal, TIRF, or super-resolution microscopy have been used to study the immune synapse. Since these methods require manual image acquisition and time-consuming quantification, the imaging of rare events is challenging. Here, we describe a workflow that enables the morphological analysis of tens of thousands of cells. Immune synapses are induced between primary human T cells in pan-leukocyte preparations and *Staphylococcus aureus* enterotoxin B (SEB)-loaded Raji cells as APCs. Image acquisition is performed with imaging flow cytometry, also called In-Flow microscopy, which combines features of a flow cytometer and a fluorescence microscope. A complete gating strategy for identifying T cell/APC couples and analyzing the immune synapses is provided. As this workflow allows the analysis of immune synapses in unpurified pan-leukocyte preparations and hence requires only a small volume of blood (*i.e.,* 1 mL), it can be applied to samples from patients. Importantly, several samples can be prepared, measured, and analyzed in parallel.

INTRODUCTION:

T cells are major regulators of the adaptive immune system and are activated through antigenic peptides that are presented in the context of major histocompatibility complexes (MHC). Full T-cell activation requires two signals, the competence signal via the antigen-specific T-cell receptor (TCR)/CD3 complex and the costimulatory signal via accessory receptors. Both signals are generated through the direct interaction of T cells with antigen-presenting cells (APCs). Mature APCs provide the competence signal for T-cell activation through MHC-peptide complexes, and they express costimulatory ligands (*e.g.,* CD80 or CD86) to assure the progression of T-cell activation[1]. One important function of costimulation is the rearrangement of the actin cytoskeleton[2-4]. The cortical F-actin is relatively static in resting T cells. T-cell stimulation through antigen-bearing APCs leads to a profound rearrangement of the actin cytoskeleton. Actin dynamics (*i.e.,* fast actin polymerization/depolymerization circles) enable the T cells to create forces that are used to transport proteins or organelles, for example. Moreover, the actin cytoskeleton is important for developing a special contact zone between T cells and APCs, called the immune synapse. Due to the importance of the actin cytoskeleton to the immune synapse, it has become essential to develop methods to quantify changes in the actin cytoskeleton of T cells[5-9].

By means of actin cytoskeletal aid, surface receptors and signaling proteins are segregated in supramolecular activation clusters (SMACs) within the immune synapse. The stability of the immune synapse is assured by the binding of receptors to F-actin bundles that increase the elasticity of the actin cytoskeleton. Immune synapse formation has been shown to be critical for the generation of the adaptive immune responses. The detrimental effects of a defective immune synapse formation *in vivo* were first realized in patients suffering from Wiskott Aldrich Syndrome (WAS), a disease in which actin polymerization and, concomitantly, immune synapse formation are disturbed[10]. WAS patients can suffer from eczema, severe recurrent infections, autoimmune diseases, and melanomas. Despite this finding, it is currently not known whether immune synapse formation differs in the T cells of healthy individuals and patients suffering from immune defects or autoimmune diseases.

Fluorescence microscopy, including confocal, TIRF, and super-resolution microscopy, were used to uncover the architecture of the immune synapse[11-14]. The high resolution of these systems and the possibility of performing live-cell imaging enables the collection of exact, spatio-temporal information about the actin cytoskeleton and surface or intracellular proteins in the immune synapse. Many results, however, are based on the analysis of only a few tens of T cells. Moreover, T cells must be purified for these types of fluorescence microscopy. However, for many research questions, the use of unpurified cells rather than the highest-possible resolution is of the utmost importance. This is relevant if T cells from patients are analyzed, since the amount of donated blood is limited and there might be the need to process many samples in parallel.

We established microscopic methods that allow the analysis of the actin cytoskeleton in the immune synapse in the human system[15-17]. These methods are based on imaging flow cytometry, also called In-Flow microscopy[18]. As a hybrid between multispectral flow cytometry and fluorescence microscopy, imaging flow cytometry has its strengths in analyzing morphological parameters and protein localization in heterogeneous cell populations, such as pan-leukocytes from the peripheral blood. We introduced a methodology that enables us to quantify F-actin in T-cell/APC conjugates of human T cells from whole-blood samples, without the need of time-consuming and costly purification steps[17]. The technique presented here comprises the whole workflow, from getting the blood sample to the quantification of F-actin in the immune synapse.

PROTOCOL:

1. Preparation of pan-leukocytes

1.1) Draw 1 mL of peripheral blood from a healthy donor (or patient) in a heparinized syringe. Make sure to have approval by the responsible ethics committee for the blood donation.

1.2) Mix 1 mL of human peripheral blood with 30 mL of ACK lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.0) in a 50-mL tube and incubate for 8 min at room temperature.

1.3) Fill the tubes with PBS and centrifuge at 300 x g for 6 min. Aspirate the supernatant and resuspend the pellet in 30 mL of ACK lysis buffer.

1.4) Repeat steps 1.2 and 1.3 until the supernatant is clear. Finally, wash the cells in PBS, centrifuge at 300 x g for 6 min at room temperature, and resuspend the cell pellet in 2 mL of culture medium (RPMI1640 + 10% FCS). Incubate the cells at 37 °C for 60 min.

2. Loading of Raji cells with SEB

2.1) Prepare two 15-mL Falcon tubes with $1.5x10^6$ Raji cells per tube. Spin down the cells (300 x g for 6 min at room temperature) and discard the supernatant.

2.2) Resuspend the cells in residual medium (about 50-100 µL), add 1.9 µL (1.9 µG) SEB, and incubate at room temperature for 15 min. Add 5 mL of culture medium, spin down the cells

(300 x g for 6 min at room temperature), and resuspend the pellet in culture medium (RPMI1640 + 10% FCS) at a density of $1x10^6$ cells/mL.

3. Induction of immune synapses and staining protocol

3.1) Pipette 500 µL of the preparation of SEB-loaded Raji cells into a FACS tube and 500 µL of the preparation of unloaded Raji cells into another FACS tube. Add 650 µL of pan-leukocytes to each tube and spin down the cells (300 x g for 10 min at room temperature). Discard the supernatant and resuspend the pellet in 150 µL of culture medium (RPMI1640 + 10% FCS). Incubate at 37 °C (typically for 45 min).

3.2) Gently vortex the cells (10 s at 1,000 rpm) and add 1.5 mL of paraformaldehyde (1.5%) during the vortex to fix the cells. Stop the fixation by adding 1 mL of PBS + 1% BSA. Pellet the cells (300 x g for 10 min at room temperature and resuspension the cell pellet in 1 mL of PBS + 1% BSA after incubating at room temperature for 10 min.

3.3) Pellet (300 x g for 10 min at room temperature) and resuspend the cells in 100 µL of PBS + 1% BSA + 0.1% saponin for 15 min at room temperature to permeabilize the cells (96-well plate, U-shaped).

3.4) Wash the cells in PBS + 1% BSA + 0.1% saponin with centrifugation (300 x g for 10min at room temperature) and resuspend the cell pellet in 50 µL of PBS + 1% BSA + 0.1% saponin containing fluorophore-labelled antibodies or compounds (CD3-PE-TxRed (1:30), Phalloidin-AF647 (1:150), and DAPI (1:3,000)).

3.5) Incubate the cells at room temperature in the dark for 30 min. Wash the cells 3 times by adding 1 mL of PBS + 1% BSA + 0.1% saponin. Centrifuge at 300 x g for 10 min at room temperature. Re-resuspend the cells in 60 µL of PBS for imaging flow cytometry.

4. Image acquisition using a flow cytometer

NOTE: The following image acquisition procedure and data analysis are based on imaging flow cytometry using software such as imagestream (IS100), INSPIRE, and IDEAS. However, other flow cytometers and analysis software can also be used.

4.1) Open the analysis software on the computer connected to the imaging flow cytometer and click on Initialize Fluidics of the Instrument menu. Apply the beads on the right port when prompted to do so.

4.2) Load the default template from the File menu and click on Run/Setup. Choose Beads from the View dropdown menu.

4.3) Adjust the bright-field illuminator by clicking on Set Intensity if the indicated value is below 200.

4.4) Run the calibration and test routine in the Assist tab by clicking on Start All.

4.5) Click on Flush/Lock/Load and apply the samples in the left port when prompted to do so. After loading the cells in the flow cytometer, open the Cell Classifier and adjust the values as follows: peak intensity upper limit at 1,022 for each channel, peak intensity lower limit at 50 for channel 2 (DAPI) and channel 5 (CD3-Pe-TxR), area lower limit at 50 for channel 1 (side scatter), and upper limit at 1,500.

4.6) Change the excitation laser power to 405 nm (15 mW), 488 nm (200 mW), and 647 nm (90 mW) in the Setup tab.

4.7) Switch the View dropdown menu between Cells and Beads to evaluate the cell classifier and laser power adjustments.
NOTE: Make sure that all cells and cell couples are found the Cell View and that cell clumps, debris, and images with saturated pixels are found in the Debris View by changing the cell classifiers and/or the excitation laser powers.

4.8) Define the sample name and the amount of images to acquire (15,000-25,000 for samples and 500 for compensation controls) in the Setup tab. Click on Run/Setup to start the acquisition.

5. Data analysis
5.1) Transfer the raw image files (.rif) to the data analysis computer and open the analysis software.

5.2) Produce a compensation matrix following the instructions of the Compensation dropdown. Save the compensation matrix as comp_Date.ctm.

5.3) Open a sample raw image file (.rif) and apply the comp_Date.ctm in the window that appears to produce the compensated image files (.cif) and the default data analysis file (.daf).

5.4) Open the compensated image file. Convert the images to color mode and adjust the lookup tables to obtain optimal visible colors in the Image Gallery Properties toolbar. Obtain an RGB-merged image using the Composite tab of the Image Gallery Properties toolbar.

5.5) Open the Mask Manager from the Analysis dropdown. Create masks to define the T cells and the immune synapse, as follows:

5.5.1) Select the T-cell mask: "(Fill(Threshold_Ch05, 60)." Select the valley mask: "Valley(Ch02,3)." Select the T-cell synapse mask: "T-cell mask AND Valley(M02,Ch02, 3)."

5.6) Open the Feature Manager from the Analysis dropdown. Calculate the following features:

5.6.1) For the total CD3 expression in T cells, select "Intensity_T-cells_Ch5." For the total amount of F-actin in the T cells, select "Intensity_T-cells_Ch6." For CD3 expression in the

immune synapse, select "Intensity_T-cell synapse_Ch5." For the amount of F-actin in the immune synapse, select "Intensity_T-cell synapse_Ch6."

5.6.2) To calculate the T-cell area, select "Area_T-cells." To calculate the T-cell immune synapse area, select "Area_T-cell synapse."

5.7) Determine the F-actin and CD3 enrichment in the immune synapse by using the equation in the Feature Manager:

$$F\text{-}actin\ enrichment\ (\%) = \frac{\text{Intensity}_{\text{T}}\text{-cell synapse}_{\text{Ch6}}}{\text{Intensity}_{\text{T}}\text{-cells}_{\text{Ch6}}} x100$$

$$CD3\ enrichment\ (\%) = \frac{\text{Intensity}_{\text{T}}\text{-cell synapse}_{\text{Ch5}}}{\text{Intensity}_{\text{T}}\text{-cells}_{\text{Ch5}}} x100$$

5.8) Apply the following gating strategy by using histograms and dot plots from the Analysis area (for further details, see References 17 and 19):

5.8.1) Discard out-of-focus cells by plotting the "Gradient RMS_M2_Ch2" in a histogram; set the threshold at 15.

5.8.2) Plot the SSC versus CD3 intensity in a dot plot. Set a gate on CD3-positive events.

5.8.3) Plot the "Aspect ratio" of M02 (DAPI stain) versus the area of M02 (Dapi stain). Gate on T-cell singlets and cell couples accordingly. Correct for true cell couples using the area of the synapse mask, as described previously[17,19].

5.8.4) Determine the amount of F-actin in T-cell singlets and T cells of T-cell/APC couples and the percent of F-actin in the immune synapse.

REPRESENTATIVE RESULTS:

A major goal of the method described here is the quantification of protein enrichment (*e.g.,* F-actin) in the immune synapse between surrogate APCs (Raji cells) and T cells in unpurified pan-leukocytes taken from low-volume (1 mL) human blood samples. The screenshot in Figure 1 gives an overview of the critical gating strategy of this method. It shows the image gallery on the left and the analysis area on the right (Figure 1). The image gallery shows the "In Focus" gate. The depicted images are mainly granulocytes and two T-cell/APC couples. F-actin and CD3 are enriched in cell couple number 30272. The gating strategy to quantify the amount of cell couples with such enrichment is shown in the analysis area and is described in the protocol (see step 5.8) or elsewhere[17,19]. The last dot plot in the analysis area displays the amount (percent protein) of CD3 (x-axis) and F-actin (y-axis) in the immune synapse. If more than 30% of the protein was located within the immune synapse mask, it was considered as protein enrichment in the immune synapse[20]. Dot plots containing enrichment data were used to produce a typical final result (Figure 2A). The imagery on the left shows sample images of T-cell/APC conjugates,

with low amounts of CD3 and F-actin in the immune synapse, whereas on the right, T-cell/APC conjugates are depicted with a strong enrichment of CD3 and F-actin. The amount of CD3 at the immune synapse (percent protein) is plotted on the x-axis, and the amount of F-actin at the immune synapse is plotted on the y-axis. The percentage in the gate represents the amount of T cells that have an enrichment of CD3 and F-actin at the immune synapse in the presence of a superantigen. In the absence of superantigen, 15% percent of the T cells showed an enrichment at the immune synapse of both CD3 and F-actin. The amount of cells increased to 29% in the presence of superantigen. A single quantification of CD3 enrichment (Figure 2B) or F-actin enrichment (Figure 2C) is shown in the presence and absence of superantigen. These results show that, in the absence of superantigen, 18.3 ± 3.5% and, in the presence of superantigen, 34.3 ± 4.0% of the total F-actin amount in the cells were accumulated at the immune synapse. Interestingly, there was already CD3 accumulation in the absence of superantigen (16.6 ± 2.1% of the total CD3 amount), which was significantly increased by the addition of superantigen (24.6 ± 3.0% of the total CD3 amount). This method allows the quantification of how much protein is accumulated at the immune synapse between T cells and APCs.

FIGURE LEGENDS:

Figure 1: Gating strategy for the identification of immune synapses in pan-leukocyte preparations.
The figure shows a screenshot of the software and contains a complete analysis workflow for the evaluation of CD3 and F-actin enrichment in the immune synapse of T cells conjugated to APCs in the presence of SEB. Images are displayed in the image gallery on the left (Ch2 = DAPI; Ch5 = CD3-PETxR; CH6 = Phalloidin AF647; and Merge = Combined image containing Ch2, Ch5, and Ch6). The software provides an image display toolbar to adjust for lookup tables, mask display, and color/grayscale mode. The right portion of the screenshot shows the analysis area and analysis toolbar. The analysis area contains histograms and dot plots as follows: 1) Histogram to find cells in focus according to the gradient RMS feature of the DAPI staining. 2) Gating on T cells according to the expression of CD3 (Intensity_MC_Ch05, x-axis) and the side scatter profile (Intensity_MC_Ch01, y-axis). 3) Gating on potential T-cell/APC couples according to the aspect ratio of the DAPI stain (Aspect ratio_M02, y-axis) and the side scatter profile (Intensity_MC_Ch01, x-axis). 4) Gating on true T-cell/APC couples according to the area synapse mask (x-axis) and the area of the CD3 stain (y-axis). 5) Gating on mature immune synapses defined by enrichment (>30% protein content in the interface) of CD3 (x-axis) and F-actin (y-axis) in the immune synapse.

Figure 2: Imaging flow cytometry data on CD3 and F-actin enrichment in the immune synapse.
A) The dot plots in the center show the percentage of CD3 (x-axis) and F-actin (y-axis) in the immune synapse. T-cell/APC conjugates with a mature immune synapse in the absence (upper part) or presence (lower part) of SEB are depicted. The images on the left show T-cell/APC couples with a low degree of CD3 and F-actin enrichment, whereas the images on the right display T-cell/APC couple with a high degree of CD3 and F-actin enrichment (mature immune synapse). The scale bar on the lower left represents 10 µm. The result is representative of three independent experiments.

B-C) Mean CD3 (B) or F-actin (C) enrichment in the immune synapse in the presence or absence of SEB is shown as percent protein (n = 3; SE).

DISCUSSION:

The workflow presented here enables the quantification of immune synapses between human T cells (*ex vivo*) and APCs. Notably, erythrocyte-lysed pan-leukocytes were used as T-cell sources, making T-cell purification steps dispensable. The B-cell lymphoma cell line Raji served as surrogate APCs. This bears significant advantages, since it allows comparisons between blood donors of the T-cell side of the immune synapse. Furthermore, autologous DCs are hardly available directly from peripheral human blood. The production of monocyte-derived dendritic cells (moDCs) takes several days, making the application to clinical studies challenging. However, imaging flow cytometry and the analysis strategy presented here can be applied to other APCs (*e.g.,* transdifferentiated neutrophils)[20]. Moreover, it was demonstrated that the immune synapse between CD4 T cells and *ex vivo* DCs[9] or B cells[21] can be assessed for mice using imaging flow cytometry. Thus, this method can be broadened to assess APC function in addition to the T-cell side of the immune synapse.

The most critical steps of this methodology are mixing the pan-leukocytes and APCs in a small volume and performing the correct gating strategy to exclude false-positive events while at the same time minimizing the loss of true immune synapses. While we describe the measurement of CD3 and F-actin accumulation, this method is not restricted to these proteins and can be expanded to other surface receptors, such as LFA-1[17,19]. Moreover, the addition of fluorophore-labelled antibodies to identify T-cell subgroups (*e.g.,* CD4, CD8, CD45RA, or chemokine receptors) would allow for the exploration of the nature of T cells displaying a certain immune synapse phenotype. Importantly, the amount of blood that is necessary for such an analysis is low (maximum: 1 mL). While the first generation of imaging flow cytometers, as used here (IS100), have an image acquisition rate of about 100 cells/s, the third generation of imaging flow cytometers (IsX^{MKII}) allows much higher image acquisition rates (up to 2,000 cells/s using a 40x objective). Thus, the actual workflow, from drawing the blood to data analysis, requires a considerably short time period (*i.e.,* one day). Notably, while the presented image analysis is based on imaging flow cytometry, cell preparation and the induction of immune synapses (*i.e.,* the first part of the workflow) can be applied to other imaging techniques[22].

In contrast to flow cytometry, imaging flow cytometry-obtained dot plots contain information about protein localization in addition to protein expression data. One strength of imaging flow cytometry is that cells (or cell couples) of any selected gate can be evaluated by eye; the gate boundaries can be adjusted accordingly, simply by clicking on the dots and inspecting the corresponding image. In our experiments, we found that 30% protein enrichment is a reliable value to consider the respective cell couple as having the protein of interest enriched. Once the gates are finally set, the features and gating strategy are saved in a separate file (.ast) and can be applied to each following sample to assure an unbiased evaluation.

The weaknesses of imaging flow cytometry are the limitation in resolution (0.5 µm using a 40x

objective) and the fact that only one focus plane can be analyzed. Therefore, this method is not suitable to analyze the fine structure of the immune synapse and the occurrence of microclusters[14]. To analyze such architecture-related aspects of the immune synapse, alternate techniques, such as confocal, TIRF, or super-resolution microscopy, are needed. The strength of imaging flow cytometry is the amount of images that can be acquired per sample (up to 25,000). Notably, each image is segmented, which means that the background is knocked out, and there is usually only one solitary cell or cell couple per image. These characteristics of imaging flow cytometry form the basis for automated analysis at the single-cell level. Moreover, the high amount of cells that are analyzed per sample allow for the reliable identification of rare events (*i.e.,* subpopulations or cell couples with a frequency below 1%). Importantly, the workflow presented here can be applied to the study of immune synapse formation in *ex vivo* T-cells in pan-leukocytes from patients suffering from immune-related diseases (*e.g.,* primary immunodeficiency disorders).

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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Fig. 1

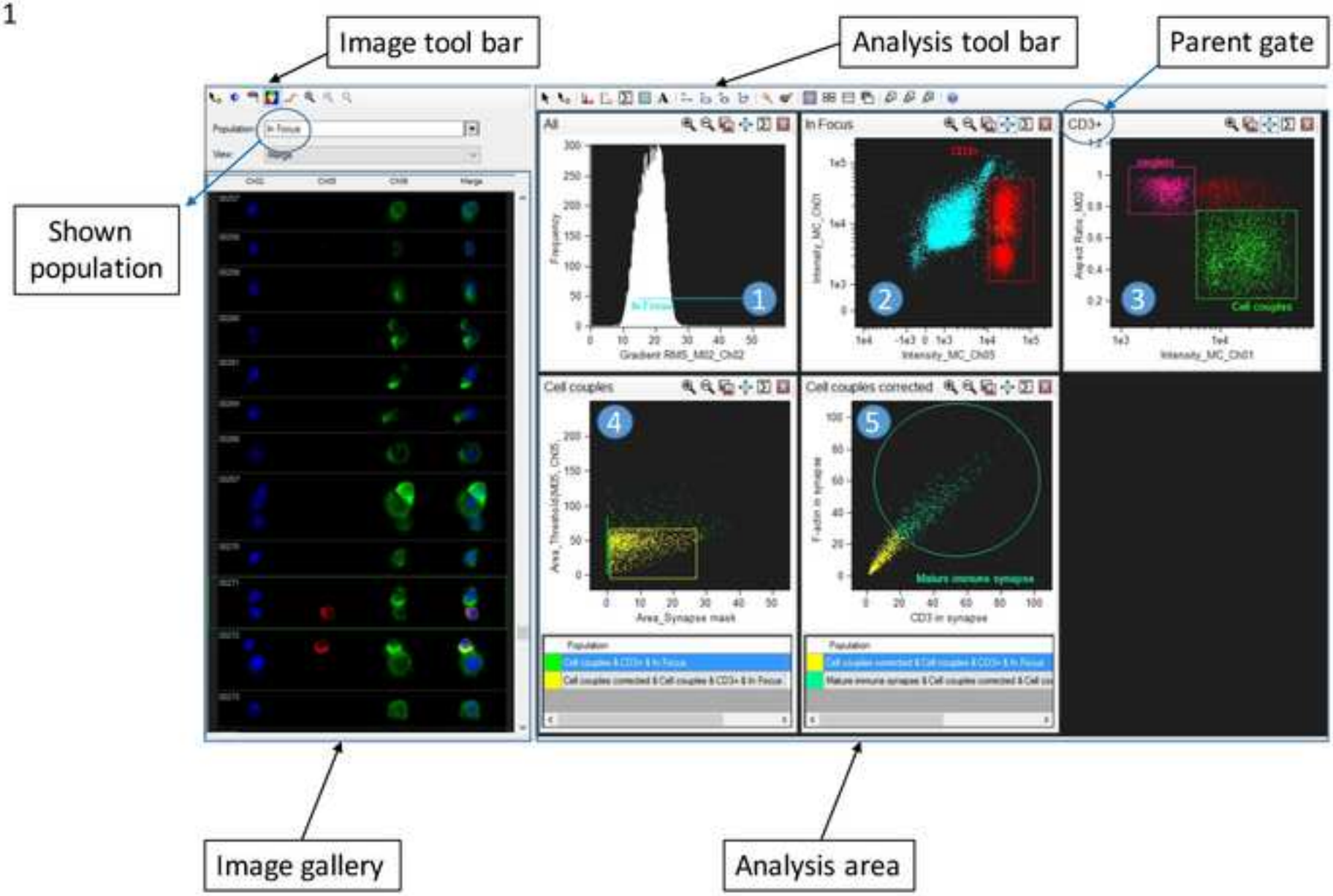

Figure 2

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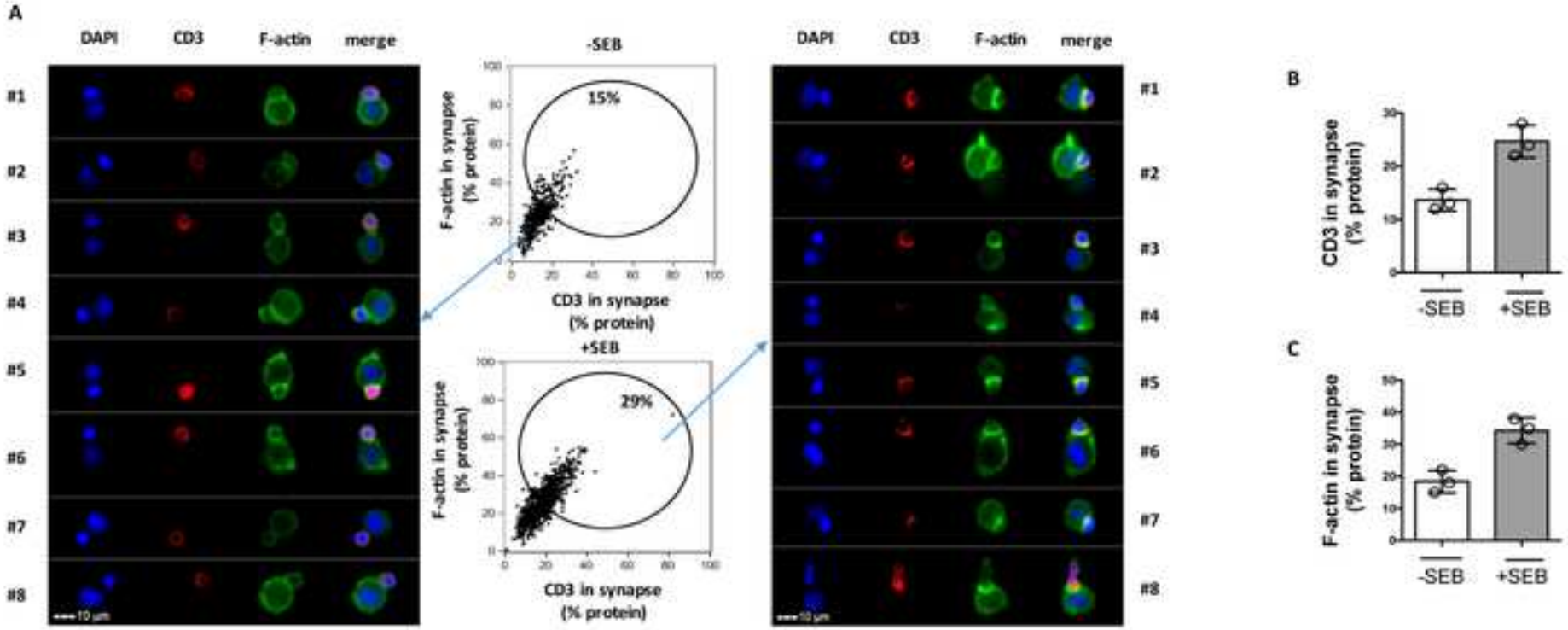
Fig. 2



Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Multifuge 3 SR	Heraeus		
RPMI 1640	LifeTechnologies	#11875085	500ml
FCS	Pan Biotech	#3302-P101102	
Polystyrene Round Bottom Tube	Falcon	#352054	5ml
Kulturflasche	Thermo Scientific	#178883	
Dulbecco's Phosphate Buffered Saline	Sigma	D8662	
Bovine Serum Albumin	Roth	#8076.3	
Saponin	Sigma	S7900	
Paraformaldehyd	Sigma Aldrich	#16005	
FACS Wash Saponin			PBS 1%BSA 0,1% Saponin
Reaktionsgefäß	Sarstedt	72.699.002	0,5ml
Speed Bead	Amnis	#400041	
Minishaker MS1	IKA Works	MS1	
Mikrotiterplatte	Greiner Bio One	#650101	96U
Enterotoxin SEB	Sigma Aldrich	S4881	
DAPI	Sigma-Aldrich	D9542	1:3000
CD3-PeTxR	Invitrogen	MHCD0317	1:30
Phalloidin-AF647	Molecular Probes	A22287	1:150
IS100	Amnis		Imaging flow cytometer
IDEAS	Amnis		Software
INSPIRE	Amnis		Software

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Author(s): Guido Wabnitz, Henning Kirchgessner, Yvonne Samstag

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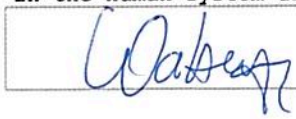
Signature:	
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Qualitative and quantitative analysis of the immune synapse in the human system using imaging flow cytometry

Guido H. Wabnitz, Henning Kirchgessner, Yvonne Samstag

Point-by-point reply

Editors comment
Please abbreviate all relevant journal titles. See, for example, refs 12 and 15.

Authors reply
The journal titles are now abbreviated.

Editors comment
•There are many minor grammatical errors and incidences of odd phrasing in the manuscript. We recommend proofreading by a native English speaker.
These include, but are not limited to:
-Line 48: analysis is restricted to a limited number of cells and does not allow to evaluate rare events
-Take care not to crash units, and please use uppercase L for liter. E.g. 1ml should be 1 mL.
-Additionally, 37°C should be 37 °C -60min should be 60 min
-300g should be 300 g, etc.
-Do not use decimal point in place of comma in units. For example, 1.200 rpm should be 1,200 rpm or 1200 rpm.
-3.5: “temperature in the dark for 30 min..” two periods. -5.4: “coloured”; “colours” – please use American English throughout.
-Line 257: “The imagery on the left shows sample images of…”
-Line 291: “…can be evaluated by eye in an imagery…” -Line 289: “In opposite to flow cytometry…”

Authors reply
We changed the manuscript accordingly.

Editors comment
•Additional detail is required:
-3.2, 3.3, 3.4 and others “pellet and resuspend” – is this always at 300g / 10 min at room temperature? Please include all centrifuge conditions for each step.
-3.4 “Then, wash cells by centrifugation” – was them with what wash medium?
Unnecessary branding should be removed from the Figure 1 legend: IDEASTM does not need to be named here.

Authors reply
We added all missing information and removed the brand in the figure legend.

Editors comment
Results:
-“The number of T-cells that form a couple with APCs and have simultaneously CD3 and F-actin enriched is of prime importance to compare different samples.” Please edit for grammar and also expand on this. Why is it of “prime importance”?

Authors reply
This paragraph has been revised. See also comments of reviewer #3.

Editors comment
-Please describe the individual elements of the screenshot and how they are related in more detail in the legend for Figure 1.

Authors reply
The figure and figure legend are revised.

Editors comment
•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

Authors reply
The are no original figures from any other publication. However, the exact gating strategy is based on Wabnitz et al. 2015 (doi:10.1016/j.jim.2015.03.003). Please indicate if a re-print permission is needed.

Editors comment
•JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

Authors reply
The DOIs are now provided.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The article describes a fast method to analyze the immune synapse directly ex vivo with limited amount of blood.

Major Concerns: None.

Minor Concerns:

Reviewers comment
The protocol 1 does only describe the lysis of the erythrocytes, but not the purification of the CD3+ T cells by MACS as mentioned in the Table for Materials/Equipment.

Authors reply
All experiments were performed with pan-leukocytes, without purification of CD3+ T-cells. Information about MACS reagents and equipment are now removed.

Reviewers comment
Moreover, the concentrations and distributers of Phalloidin, anti-CD3 Ab and DAPI are missing. Please add this information.

Authors reply

We now included that details in the material list. Moreover, a description of distributors and concentrations of Phalloidin, CD3 and DAPI are provided in the protocol section 3.3 and the materials template.

Additional Comments to Authors: N/A

Reviewer #2:

Manuscript Summary:

The authors describe in detail their method for using imaging flow cytometry to quantify f actin accumulation at the immunological synapse.

Major Concerns:

I have no major concerns.

Minor Concerns:

Reviewers comment

1) The authors could strengthen the work by highlighting that others have done this using ex vivo APC rather than loaded cell lines (eg Markey et al, already referenced in the manuscript), thus broadening the applicability to assessing APC function rather than simply T cell response capacity (as is the case in the experiments the way they are performed here using a loaded cell line as the APC). Experiments of this type can now be done on a range of instruments, and while the cell preparation may be the same the specific work flow, instrument settings etc will not apply, and addressing this would help the work have broader applicability.

Authors reply

We agree with the reviewer that a broader description of potential applications of the workflow would strengthen the manuscript. We revised the first paragraph of the discussion and included this information.

Reviewers comment

2) The method is clearly written for use with the Amnis/Millipore Imagestream instrument and IDEAS software, but nowhere do the authors state this clearly (it is mentioned as an aside to a point re: acquisition speed in the discussion).

Authors reply

The instrument and software are now clearly described in the first paragraph of chapter 4 and the material template.

Additional Comments to Authors: N/A

Reviewer #3:

Manuscript Summary:

This study analyzes the immune synapsis (IS) by flow cytometry. It describes a method to identify cell conjugates with CD3 and F-actin at T-Raji cell junctions. It is difficult to determine the validity of this methodology to quantify formation of IS, as well as to calculate the percentage of a given protein at the IS. The figures are poorly explained. I did not find this method useful to analyze IS formation. A useful procedure to study IS with T-cells and SEB-loaded Raji cells should provide two types of parameters:

Reviewers comment

1.- The percentage of cells forming IS in the presence and absence of SEB (negative control). It is important to quantify formation of conjugates with and without SEB, as well as

the number of conjugates with CD3 at the T cell region that contact the Raji cells, which identifies IS formation.

Authors reply
A quantification of cells with a mature immune synapse is now included in Fig. 2A.

Reviewers comment
2.- The percentage of a given protein (CD3, F-actin…) at the IS region. Many studies on IS formation study translocation of a protein to the IS. Therefore, it is necessary to quantify how much protein (CD3, F-actin….) accumulates at the T-Raji contact area. A method that determines the amount (%) of a protein at the IS in T cells would be relevant. These parameters should be determined and presented in a revised manuscript, which must describe properly the results and the figures.

Authors reply
The authors are thankful for this suggestion. Quantification of the amount of protein at the immune synapse is exactly what the presented workflow was developed for. This point was not clearly enough stated in the manuscript. Thus, a quantification of CD3 and F-actin enrichment in the T-cell/APC interface is now included in Fig. 2 B and C.

Reviewers comment
Perhaps the authors should consider the use of transfected T cells (Jurkat cell line).

Authors reply
One major strength of the here presented method is that the immune synapse can be analyzed between APCs and primary human T-cells without purification of T-cells, but rather using pan-leukocytes. Thus, Jurkat cells would be out of the scope of the here presented workflow.

Major Concerns: N/A *Minor Concerns:* N/A *Additional Comments to Authors:* N/A