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TITLE:

Imaging the Human Immunological Synapse

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SUMMARY:

This protocol images both the immunological synapse formation and the subsequent polarized secretory traffic towards the immunological synapse. Cellular conjugates were formed between a superantigen-pulsed Raji cell (acting as an antigen-presenting cell) and a Jurkat clone (acting as an effector helper T lymphocyte).

ABSTRACT:

The purpose of the method is to generate an immunological synapse (IS), an example of cell-to-cell conjugation formed by an antigen-presenting cell (APC) and an effector helper T lymphocyte (Th) cell, and to record the images corresponding to the first stages of the IS formation and the subsequent trafficking events (occurring both in the APC and in the Th cell). These events will eventually lead to polarized secretion at the IS. In this protocol, Jurkat cells challenged with Staphylococcus enterotoxin E (SEE)-pulsed Raji cells as a cell synapse model

was used, because of the closeness of this experimental system to the biological reality (Th cell-APC synaptic conjugates). The approach presented here involves cell-to-cell conjugation, time-lapse acquisition, wide-field fluorescence microscopy (WFFM) followed by image processing (post-acquisition deconvolution). This improves the signal-to-noise ratio (SNR) of the images, enhances the temporal resolution, allows the synchronized acquisition of several fluorochromes in emerging synaptic conjugates and decreases fluorescence bleaching. In addition, the protocol is well matched with the end point cell fixation protocols (paraformaldehyde, acetone or methanol), which would allow further immunofluorescence staining and analyses. This protocol is also compatible with laser scanning confocal microscopy (LSCM) and other state-of-the-art microscopy techniques. As a main caveat, only those T cell-APC boundaries (called IS interfaces) that were at the right 90° angle to the focus plane along the Z-axis could be properly imaged and analyzed. Other experimental models exist that simplify imaging in the Z dimension and the following image analyses, but these approaches do not emulate the complex, irregular surface of an APC, and may promote non-physiological interactions in the IS. Thus, the experimental approach used here is suitable to reproduce and to confront some biological complexities occurring at the IS.

INTRODUCTION:

The main goal of the method is to generate immunological synapse (IS) cell-to-cell conjugates formed by an antigen-presenting cell (APC) pulsed with SEE superantigens and an effector Th cell, and to register the images corresponding to the first stages of immunological synapse formation and the subsequent trafficking events (occurring both in the APC and the Th cell), that eventually will lead to polarized secretion at the IS. The establishment of the IS by T lymphocytes upon binding of their cell receptor (TCR) to antigens bound to MHC-II on the APC organizes an extremely dynamic, malleable and critical instance involved in antigen-specific, humoral and cellular immune responses^{1,2}. The IS is defined by the formation of a special supramolecular activation complex (SMAC) pattern characterized by an actin reorganization process³. Upon IS construction by T lymphocytes with an APC, the polarization of the secretory vesicles towards the IS appears to be implicated in polarized secretion at the synaptic gap. This focused machinery appears to unambiguously supply the immune system with a superbly regulated stratagem to enhance the efficacy of critical secretory effector roles of T lymphocytes, while reducing nonspecific, cytokine-arbitrated stimulation of bystander cells, killing of irrelevant target cells and apoptotic suicide via activation-induced cell death (AICD)⁴.

The consequence of the IS varies on the nature of both the T lymphocyte and the APC. The synaptic contact of Th cells (typically CD4+ cells) with the APC displaying antigen-associated to MHC-II produces the activation of the T cell (cytokine secretion, proliferation, etc.) and, in some instances, apoptosis via AICD⁴. For cytotoxic T lymphocytes (CTLs) (principally CD8+ cells) interacting with APC presenting antigen associated to MHC-I, the outcome differs on the pre-stimulation or not of the CTLs with antigen. Thus, naïve CTLs identifying antigen-MHC-I complexes on the APC are “primed” to destroy target cells and divide. Primed CTLs also establish synapses with target cells (i.e., cells infected by viruses or tumor cells) producing antigen-specific cell extermination^{5,6}.

The polarized secretion of exosomes at the immunological synapse is a developing and challenging area of research involved in relevant immune responses⁷. It has been demonstrated that multivesicular bodies (MVB) carrying intraluminal vesicles (ILVs) experience polarized transportation towards the IS^{8,9} (**Video 1**) upon TCR stimulation with antigen. The fusion of these MVB at the synaptic membrane induces their degranulation and the release of the ILVs as exosomes to the synaptic cleft^{8,10}. This occurs in IS formed by Th-type Jurkat cells that were challenged with SEE superantigen-coated Raji cells acting as APC¹¹, TCR-stimulated CD4+ lymphoblasts, and primed CTLs. Thus, synapses made by Jurkat cells constitute a valuable model to study polarized secretory traffic of exosomes. In addition, several decades of investigation have shown that many fundamental insights into TCR signaling came from studies with transformed T-cell lines, and indeed the best known of these model systems is the Jurkat leukemic T-cell line¹².

The formation of a fully developed IS produces several crucial biological outcomes, including activation of Th cells, activation of naïve CTLs or target cell killing by primed CTLs, apart from anergy or AICD⁵. Therefore, there are two major types of secretory IS established by T lymphocytes that result in very diverse, but similarly critical, immune effector functions^{1,6,13}. On one hand, the IS from primed cytotoxic T lymphocytes (CTLs) induces rapid polarization (ranging from seconds to few minutes) of lytic granules (called “secretory lysosomes”) towards the IS. The degranulation of the lytic granules induces the secretion perforin and granzymes to the synaptic cleft¹⁴, which are pro-apoptotic molecules. The secreted perforin and granzymes subsequently induce killing of the target cells^{15,16}. CTLs develop temporary synapses, lasting only few minutes, as the target cells are murdered^{3,17}. This is probably due to the circumstance that the optimal CTL task requires a rapid and temporary contact in order to distribute as many lethal strikes as possible to numerous target cells^{3,17}. In contrast, Th lymphocytes such as Jurkat cells generate stable, long-standing IS (from 10-30 min up to hours), since this appears to be necessary for both directional and incessant secretion of stimulating cytokines^{3,17}. The cytokines are also enclosed in secretory vesicles and some of them (i.e., IL-2, IFN- γ) experience polarized transport to the IS¹⁷ and secretion. One essential characteristic of the IS is the formation of exploratory, weak and transient contacts between the T cell and the APC (**Video 1**) that may produce a stronger interaction and the establishment of a mature IS, providing that the TCR identifies the cognate antigen-MHC complexes and that appropriate co-stimulatory connections are established⁵. Both the beginning of the initial contacts and the founding of a mature, totally productive IS, are inherently stochastic, fast and asynchronous processes^{5,18}. Furthermore, there is a scarce frequency in the creation of cell-to-cell conjugates¹⁹, which may constitute a challenge for imaging techniques (please refer to Results and Discussion sections).

Another main challenge in examining polarization of the microtubule-organizing center (MTOC) and secretory granules in T lymphocytes is that the entire process is fast (seconds to few minutes), particularly in CTLs. Considering these facts, most early approaches confronted an end point strategy in which APC/target cells and T lymphocytes are mixed jointly and converged by low speed centrifugation, to favor cell-to-cell conjugate creation, incubated for several minutes, fixed and subsequently evaluated for the relocation of MTOC and/or secretory vesicles towards the IS²⁰. This approach has two significant limitations: no lively trafficking data was

achieved and high levels of background MTOC/secretory granules polarization were obtained, probably due to the stochastic character of IS establishment¹⁸. Furthermore, any correlation between TCR-stimulated, initial signaling events (i.e., intracellular calcium rises, actin reorganization) and secretory vesicle polarization is problematic to investigate. Thus, imperative provisions for appropriate imaging of the IS in living cells combine to enhance the cell-to-cell conjugate materialization, to synchronize the generation of the IS and, when possible, to guarantee the establishment of cellular conjugates at defined microscope XY fields and Z positions. Several strategies have been developed in order to avoid all these problems. It is out of the scope of this paper to explain these methods, their benefits and weaknesses. Please refer to the previously published reviews tackling these important points^{1,4,5,21}.

The fact that IS made by Th lymphocytes are long-lived, and the circumstance that in Th lymphocytes the MTOC, lymphokine-containing secretory granules and MVB take from several minutes up to hours to move and dock to the IS²² makes the Th-APC IS an ideal candidate for imaging using the protocol described here.

PROTOCOL:

1. Preparation of slides to adhere Raji cells

1.1. Add 150 μ L of fibronectin (100 μ g/mL) per well to an 8 microwell chamber slide (plastic-bottom chamber slide) and incubate it for 30 min to 1 h at 37 °C. This adhesion substrate will allow the binding of Raji cells to the well bottom (Step 2), the formation of living conjugates with Jurkat cells (Step 4) cells, and time-lapse microscopy capture (step 6), and is also compatible afterwards with optional paraformaldehyde (PFA) fixation (step 7).

NOTE: For acetone fixation, use glass bottom chamber slides and poly-L-lysine (20 μ g/mL) instead of fibronectin, since acetone dissolve plastic. The 8 microwell chamber slide (1 cm² well, 300 μ L maximum volume) or equivalent is a flexible and appropriate format.

1.2. Aspirate fibronectin using a 200 μ L automatic pipette and wash each well with 200 μ L of PBS for 2 min with gentle shaking. Repeat this wash one more time. The chamber slide can be stored at this stage with PBS for 1-2 weeks at 4 °C.

2. Adhesion of Raji cells to the chamber slides and 7-amino-4-chloromethylcoumarin (CMAC) labeling

2.1. Transfer 10 mL of a confluent (1-2 x 10⁶ cells/mL) pre-culture of Raji cells to a 15 mL, V-bottom tube. Mix well and use 10 μ L to count the cells on Neubauer chamber or equivalent.

2.2. Centrifuge the remaining cells at 300 x g for 5 min at room temperature. Aspirate and discard the supernatant.

2.3. Gently resuspend the cell pellet in warm complete culture medium (RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 10 mM HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin) at a concentration of 10^6 cells/mL. Use the equation given below:

$([]_{\text{initial}} \times V_{\text{initial}} = []_{\text{final}} \times V_{\text{final}})$, where $[]_{\text{initial}}$ represents initial cell concentration, V_{initial} = initial volume of the cell suspension, $[]_{\text{final}}$ = final cell concentration, V_{final} = final volume of the cell suspension.

NOTE: Depending on the cell concentration of the starting culture, it is possible to collect more cells than needed but it is important to maintain the remaining cells in culture (37 °C) till the end of the experiment in order to prevent potential problems (see Step 2.6).

2.4. Label Raji cells to allow for their identification during the synaptic conjugate formation.

In this experiment, 7-amino-4-chloromethylcoumarin (CMAC) labeling is performed in step 2.4.2.

2.4.1. Transfer the required number of Raji cells in the culture medium to a 2 mL tube. For the 8 microwell chamber slide, a total of 1.6 mL of the cell suspension is needed (200 µL per well).

2.4.2. Add CMAC to a final concentration of 10 µM. Keep the cells in the dark by covering the tube with aluminum foil since CMAC is light-sensitive. 200 µL containing 2×10^5 Raji cells are needed per 1 cm² well. Thus, if 8 wells need to be prepared, 1.6×10^6 Raji cells are required.

NOTE: Labeling Raji cells with cell tracker blue (CMAC, UV excitation and blue emission) distinguishes them from Th cells when the synaptic conjugates are formed. This dye is compatible with PFA and acetone fixatives and allows further immunofluorescence procedures. Try to avoid light exposition. The labeling of Raji cells in a pool with CMAC followed by resuspension before the adhesion of the Raji cells to the fibronectin-coated chamber slides ensures the homogeneous labeling of Raji cells with CMAC among different wells.

2.5. Resuspend CMAC-stained cells and transfer 200 µL of the cell suspension to each well of the fibronectin-coated chamber slides prepared in step 1.1-1.2. Incubate the chamber slide at 37 °C, 5% CO₂ for 30 min-1 h.

NOTE: The adhesion and CMAC labeling will simultaneously occur at this step and this saves time. Please be aware that Raji cells will sediment quickly and caution needs to be taken to maintain a homogeneous concentration in the cell suspension before seeding. It is not necessary to wash CMAC at this stage by centrifugation, since CMAC washing will be more easily done at step 2.7 (when the labeled Raji cells are already adhered to the chamber slides). Since CMAC is present in the cell suspension in large excess, the blue fluorescence background is too high to distinguish the blue-stained cells. Check cell CMAC fluorescence in step 2.7 after CMAC washing.

219 2.6. Ensure that Raji cells are adhered to the bottom of the wells by gentle shaking of the
220 chamber slides on the microscope. Ensure that the cells display gaps among each other and are
221 not confluent (Figure 1, middle panel). 50-60% of cell confluence is appropriate.

222
223 2.6.1. If most of the cells efficiently adhere to the chamber slide and no cell gaps are observed,
224 wash each well with warm complete medium and resuspend the medium with a 200 μ L
225 automatic pipet to detach cell excess. Check confluence after each resuspension step.

226
227 2.6.2. If the cells do not adhere, repeat the adhesion step again and increase adhesion time
228 and/or cell number.

229
230 NOTE: It is possible to stop here, incubate the chamber slide at 37 °C, 5% CO₂ overnight (O/N),
231 and continue with the protocol the next day. Please confirm the next day that Raji cells remain
232 adhered and CMAC-labeled by using fluorescence microscopy.

233
234 2.7. Wash each well again carefully with warm supplemented RPMI to eliminate excess
235 CMAC and check for blue emission with the fluorescence microscope (Figure 1).

236
237 NOTE: To avoid the use of immersion oil (sticky and viscous) and high numerical aperture oil
238 objectives, extra-long distance (i.e., 20x or 40x) objectives can be used to quickly check CMAC
239 labeling using the fluorescence microscope.

240 241 3. Pulse of CMAC-labeled Raji cells with Streptococcus Enterotoxin E

242
243 3.1. Add Streptococcus Enterotoxin E (SEE, 1 μ g/mL) to each well. SEE can be conveniently
244 diluted in cell culture medium (working solution at 100 μ g/mL) from the SEE frozen stocks (1
245 mg/mL in PBS). Use 2 μ L of the 100x working solution per 200 μ L microwell.

246
247 CAUTION: Use gloves for this step and dispose of the used tip into the biohazard box.

248
249 3.2. Incubate the chamber slide at 37 °C, 5% CO₂ for at least 30 min. The SEE effect lasts for
250 at least 3-4 h.

251
252 NOTE: SEE can be added to the wells at different time points when required, if distinct time-
253 lapse setups are planned (step 5) and/or depending of the start time point for the end point
254 experiments (step 6).

255 256 4. Preparation of Jurkat cells

257
258 4.1. Use a previously growing culture of Jurkat cells ($1-2 \times 10^6$ cells/mL) for this experiment.
259 Use cells from a standard culture flask or from a previous transfection following standard
260 electroporation protocols, as previously described²³. The transfection of Jurkat cells will allow
261 time lapse visualization of the traffic of secretory granules in living cells. For instance, when

GFP-CD63 (a marker of MVB) is expressed the movement of GFP-CD63-decorated vesicles can be recorded (**Video 1**).

4.2. **Observe the cells under a phase contrast microscope.** If an excess of dead cells (>20-30%) are observed, perform Ficoll density gradient centrifugation using standard protocols²⁴, to eliminate the excess of dead cells (dead cells exhibit higher density than living cells) prior to use (see Discussion).

4.3. **Transfer the cells to a 15 mL tube, V-bottom tube,** and use 10 μ L for counting using hemocytometer.

4.4. **Centrifuge the remaining cells as described in step 2.2.** Discard the supernatant and resuspend the cells at the same concentration as Raji cells (1×10^6 /mL) using fresh, warm culture medium. Follow steps 2.2-2.3.

4.5. **Maintain the Jurkat cells in culture (37 °C, 5% CO₂) while waiting for the Step 4.**

NOTE: In the second option (transfection), the number of living cells is going to be much lower than in the first one. Thus, consider using a higher starting cell culture volume in order to have enough cells for the experiment. From 10×10^6 Jurkat cells per electroporation cuvette and transfection, only $2-4 \times 10^6$ Jurkat cells will survive after 48 h of transfection and some of these cells will be lost during the Ficoll step. Thus, one electroporation cuvette is generally sufficient to challenge the adhered SEE-pulsed Raji cells from 8 micro wells (1.6×10^6 transfected Jurkat cells needed).

5. Co-seeding of Raji and Jurkat cells

5.1. **Take the chamber slides containing the CMAC-labeled, SEE-pulsed, adhered Raji cells out of the incubator from step 3.2.** It is not necessary to wash the CMAC at this stage since this was previously done in Step 2.7.

5.2. **Aspirate carefully the culture medium of each well, one by one, from one corner of the well using an automatic 200 μ L pipette. Do not let the medium in the well dry out completely.**

5.3. **Immediately replace the medium with 200 μ L of resuspended Jurkat cells in cell culture medium (1×10^6 /mL) prepared in step 4.5.** If time lapse imaging is performed, go to step 6 immediately after this step, since Jurkat cells tend to sediment and form synaptic conjugates very quickly. For convenience, the microwells containing SEE-pulsed, adhered Raji cells that do not receive seeding with Jurkat cells at this stage should be maintained with cell culture medium until subsequent challenge with Jurkat cells. This will flexibly allow subsequent challenge with Jurkat cells for additional time lapse or reverse kinetic, end point experimental approaches.

5.4. If a time lapse is going to be performed, quickly proceed to step 6. This involves the co-culture for 1-2 h on the microscope stage incubator or equivalent at 37 °C, 5% CO₂ to allow the synaptic conjugate formation and simultaneous image acquisition. If end point analysis, but no time lapse, is foreseen check conjugate formation after the co-culture period using the microscope (as in **Figure 1**) before fixing the cells (step 7).

6. Time lapse imaging of emerging synaptic conjugates

6.1. Prepare the microscope and incubation chamber prior to imaging. For the example shown in **Video 1**, the detailed microscopy settings are shown in **Figure 2**.

NOTE: If a time lapse experiment is planned, all the microscope settings and complements (ambient cell culture chamber, etc.) should be prepared **before** adding the Jurkat suspension to the chamber slide with adhered Raji cells. The following steps are described for a commercial microscope (**Table of Materials**). However, any inverted fluorescence microscope equipped with a cell culture incubator can be used.

6.1.1. Use a microscope with a 60x oil-immersion, high numerical aperture when imaging polarized traffic.

6.1.2. Ensure that the automatic focus system is switched on and adjust the offset to focus the Raji cells bound to the bottom surface. Please refer to **Figure 1**, **Video 1** and **Video 2**.

6.2. After Jurkat addition to each well containing the adhered Raji cells in step 5.3, quickly locate the microwell chamber slide on the pre-heated (1-2 hours) microscope stage incubator (i.e., OKOlabs) and select some XY positions with the microscope, fields in which it is likely to record an emerging IS formation made by, for instance, a Jurkat-transfected cell falling into the microscope focus.

6.3. Use a pre-heated microscope stage incubator since it was observed that a temperature-stabilized stage maintains stable X,Y,Z positions. Criteria for a convenient XY field are: well-focused and non-confluent Raji cells (i.e., displaying gaps among cells) and the presence of transfected Jurkat cells (this can be checked by combining transmittance and UV or GFP channels). Jurkat cells will sediment very quickly (few minutes) on the chamber slide, and the chances to image emerging synapses will decrease with time (**Figure 1**). It is possible either to finish the experiment after the defined time lapse or proceed to the Step 7 and fix the conjugates for subsequent immunofluorescence and analyses.

NOTE: It is possible to select up to 16 different microscope fields from up to 4 different microwells for simultaneous, multi-well time-lapse acquisition with the proper temporal resolution (1-2 min per frame). The limitation relies on both the number and intensity (affecting camera exposition) of the diverse fluorochromes to be imaged (dependent on the number of expressed fluorescent proteins, apart from CMAC). One way to increase the frame rate is recording for the CMAC channel in only one out of each “n” time frames for GFP (i.e., n=8, as

shown in **Figure 2**), since Raji cells are adhered to the well bottom and do not easily move as Jurkat cells. In addition, this benefits the cell viability, since frequent UV light exposure may damage the cells. Try to adjust the time frame rate to 1 frame every 1 minute or less (i.e., 20 seconds per frame in **Video 1, Figure 2**) since the polarization of MVB takes a few min to hours to complete. A microscope equipped with a motorized epi-fluorescence turret and appropriate band-pass fluorescence filters or equivalents are necessary to perform this multichannel capture.

7. End point formation of synaptic conjugates and fixation

7.1. If only an endpoint experiment is planned (1-2 hours incubation to allow synaptic conjugate formation is appropriate), incubate the chamber slide at 37 °C, 5% CO₂ for 1-2 h. Check conjugate formation after the culture period (as in **Figure 1**) and, subsequently, fix the conjugates with acetone or PFA (fixation will depend on the antigens and antibodies to be used in the subsequent immunofluorescence). In this case the incubation does not require a microscope stage incubator. Please refer to **Video 3** for an example.

7.2. To fix the cells, wash the well by gentle shaking with warm RPMI (37 °C) medium without FCS (albumin from serum may precipitate with acetone fixation). Aspirate and add 200 µL of PFA or pre-chilled acetone to each well. Incubate the chamber slide at room temperature (RT) or on ice, respectively, for 20 min.

NOTE: For the acetone fixation, pre-chill the acetone at -20 °C and pre-chill the chamber slide at 4 °C. Remove plastic lids when acetone is used to fix the cells cultured in the 8 well glass-bottom chamber slides.

7.3. Wash each well twice with PBS and add 200 µL of quenching solution (PBS, 50 mM NH₄Cl). Incubate the chamber slide at 4 °C.

NOTE: At this stage the chamber slide can stay for at least one month at 4 °C before performing the immunofluorescence protocol, as described⁸. The lid prevents evaporation.

8. Image processing

8.1. Perform post-acquisition image deconvolution (i.e. Huygens deconvolution or equivalent, **Table of Materials**) of time-lapse series and/or still photos of fixed cells. Deconvolute by employing an appropriate software (i.e., using the “wide field” optical option in Huygens) and the correct optical parameters. Deconvolution necessitates for the image processing of the measured point spread function (PSF) of the microscope⁴.

8.2. Alternatively, use the software to calculate the idealized PSF by automatic loading of the optical parameters included among the metadata from the microscope files. These optical parameters comprise fluorochrome wavelength, refraction index, numerical aperture of the objective, and the imaging technique (confocal, wide-field, etc.)⁴.

8.3. Subsequently, the imaging software uses the PSF and diverse deconvolution algorithms (i.e., QMLE and CMLE in Huygens software) in a step-by-step accumulative, calculation process, whose results than can be continuously visualized and stopped (or resumed) when required by the user. At this stage the user can change the number of convolutions and/or the signal to noise ratio and resume deconvolution. The deconvolution software works well with time lapse series (X,Y,T) (**Video 2**) and Z-stacks (X,Y,Z) (**Video 3**). The deconvoluted channels were subsequently merged to the CMAC, raw channel, since cytosolic, diffuse fluorochromes do not improve by deconvolution^{4,9}.

REPRESENTATIVE RESULTS:

We have followed the described protocol in order to generate Jurkat-Raji immune synapse conjugates and to properly image the early stages of IS formation. Our aim was to improve the early approaches²⁰ previously followed to study the polarization of the MTOC and the secretory machinery towards the IS. These approaches were based on an end point strategy that did not allow imaging of the IS formation or the early synaptic events, since in these strategies the IS formation obligatory occurred in the pellet of mixed, centrifuged cells, but not on a microscope. Our protocol was designed to avoid this main caveat, since the approach was based on the use of an appropriate cell concentration (Steps 2 and 3), to favor the formation of the cell-to-cell IS conjugates on the own microscope chamber slides (8 micro well chamber slide), mounted on the pre-heated microscope stage incubator (in Step 4). This strategy induces the IS formation, simultaneously to the time-lapse imaging capture (**Video 1**).

Figure 1 represents synaptic Jurkat-Raji conjugates that were obtained following the protocol (step 5.2). The image represents the first frame from a representative, time-lapse experiment. 2×10^5 Raji cells and 2×10^5 Jurkat cells were added to a 1 cm^2 well. The upper panel shows transmittance channel, the middle panel consists of CMAC (blue) channel (Raji cells), and the lower panel shows both transmittance plus CMAC merged channels. Yellow arrows label some synaptic conjugates, as a reference, whereas green arrows indicate synaptic conjugates made by one Jurkat cell and several Raji cells (complex conjugates). Decreasing cell concentrations (10^5 or less cells in the 1 cm^2 well) will circumvent the formation of complex cellular conjugates, but there may not be enough cell conjugates for subsequent analysis of polarized traffic (see below), that in turn will decrease also the chances to find and to image emerging synaptic conjugates.

Figure 2 represents screenshots corresponding to the imaging parameters used for the simultaneous capture of the two different fluorochromes (CMAC and GFP-CD63) by using appropriate software (i.e., NIKON NIS_AR) in a representative time lapse experiment corresponding to **Video 1**.

Video 1 (Immunological synapses, raw) represents a Jurkat T lymphocyte expressing GFP-CD63 (a marker of multivesicular bodies-MVB, green vesicles) and the formation of a double synapse between 1 Jurkat cell and 2 Raji cells labelled with CMAC, in blue (one of them undergoes engulfment). The simultaneous movement of MVB inside the Jurkat cell towards synapse areas

was recorded (capture frame rate = 1 frame every 20 seconds for GFP-CD63; video reproduction speed= 2 frames per second). The synaptic conjugates were obtained and imaged following the protocol described above (Step 6.2), which allowed imaging of emerging synapses (**Video 1**). The simultaneous capture of both GFP-CD63 and CMAC fluorescence channels was performed by using an appropriate software (i.e., NIS-AR, **Table of Materials**). The video represents the raw data from a representative, time-lapse experiment. The automatic focus system was defined with an appropriate offset with respect to the Raji cells bound to the glass bottom, to assure a stable focus along the experiment.

Video 2 (immunological synapses, after deconvolution) corresponds to **Video 1**, but the deconvolution of the GFP-CD63 fluorescence channel images was performed by employing an appropriate deconvolution software (i.e., Huygens), using the “wide field” optical option and the proper optical parameters (step 8). This deconvoluted channel was subsequently merged to the CMAC, raw channel. The improvement of both signal-to-noise ratio and sharpness of the image is evident. The deconvolution was performed post-acquisition, as described above. For specific details regarding the deconvolution software please refer to⁴.

Video 3 (immunological synapse, after fixing and immunofluorescence staining) represents a Z-stack (z-step size = 0.8 μ m, 5 frames) of a fixed IS conjugate after the step 6 of the protocol (acetone fixation). After fixation, immunofluorescence was performed following standard protocols²⁵ by using Phalloidin to visualize the F-actin (green), anti-CD63 to visualize MVB (magenta), anti- γ -tubulin to visualize MTOC (red). CMAC (blue) labels the Raji cell. Subsequently the conjugate was imaged by epifluorescence and several channels deconvoluted using the “wide field” optical option and the proper optical parameters (step 7). The deconvoluted channels were subsequently merged to the CMAC, raw channel, as indicated in the different panels (CMAC plus Transmittance-TRANS, CMAC plus Phalloidin, CMAC plus anti-CD63 and CMAC plus anti- γ -tubulin, respectively). White arrow labels the synapse, whereas green arrow labels the MVB and the yellow arrow labels the MTOC. The detailed quantification of MVB and MTOC polarization has been explained elsewhere²⁵.

The approach presented here involves the formation of cell-to-cell conjugates and, simultaneously, the time-lapse acquisition by wide-field fluorescence microscopy (WFFM) followed by image processing (post-acquisition deconvolution). This tactic improved the signal-to-noise ratio (SNR) of the images, enhanced their temporal resolution, and allowed the synchronized acquisition of several fluorochromes in emerging synaptic conjugates⁴. In addition, the protocol is well matched with subsequent end point cell fixation methods (paraformaldehyde, acetone or methanol), which would allow further immunofluorescence staining and analyses²⁵ (**Video 3**). This protocol is also compatible with laser scanning confocal microscopy and other state-of-the-art microscopy techniques.

FIGURE AND TABLE LEGENDS:

Figure 1. Representative double-size microscopy field of synaptic conjugates. The image represents the first frame from a representative, time-lapse experiment following the protocol.

The upper panel shows the transmittance channel, the middle panel CMAC channel (Raji cells) and the lower panel both merged channels. Yellow arrows label some synaptic conjugates, as a reference. Green arrows indicate complex synaptic conjugates (i.e., one Jurkat cell establishing synapses with more than one Raji cell). Captured with a 40x EWD (0.6 NA) objective.

Figure 2. Time lapse microscope settings. The image corresponds to several screenshots corresponding to the imaging parameters used for the simultaneous capture of the two different fluorochromes (CMAC and GFP-CD63) using appropriate software (i.e., NIKON NIS_AR) in a representative time lapse experiment corresponding to **Video 1**. Each frame for GFP-CD63 channel was captured every 20 seconds. Only one frame for a UV channel out of eight frames for GFP channel was captured in order to maintain cell viability along the experiment.

Video 1. Immunological synapses made by a Jurkat cell expressing GFP-CD63, raw data. Raji B cells labelled with cell tracker blue (CMAC, blue) were pulsed with SEE for 30 min and synapses with Jurkat cells expressing GFP-CD63⁸ were formed. Time-lapses corresponding to GFP-CD63 and CMAC channels were captured (20 seconds per frame; video reproduction speed = 2 frames per second) and a representative example is shown. Captured with a 60x PLAN APO (1.4 NA) objective.

Video 2. Immunological synapses made by a Jurkat cell expressing GFP-CD63, after deconvolution. Same as **Video 1**, but images were deconvoluted using a deconvolution software. The improved signal-to noise ratio and the enhanced sharpness, due to the elimination of contaminant out of focus fluorescence, is evident.

Video 3. Fixed and immunostained immunological synapse. The image shows a representative fixed synaptic conjugate after Step 7 of the Protocol and subsequent immunofluorescence. CMAC (blue) labels raji cells, transmittance (TRANS) to show the synaptic conjugate (white arrow), Phalloidin labels F-actin (green), anti-CD63 labels MVB (magenta, green arrow) and anti- γ -tubulin labels MTOC (red, yellow arrow), respectively. These channels were imaged by epifluorescence, deconvoluted and merged as indicated. The video includes a Z-stack (z-step size = 0.8 μ m, 5 frames). White arrow labels the synaptic contact area, whereas the green arrow labels the MVB and the yellow arrow labels the MTOC.

DISCUSSION:

A limitation of this protocol is that not all synapses will be ideally oriented perpendicular to the optical axis. Using this technique, there is no way to predict and/or to influence the ideal orientation for immune synapse imaging. To fix this problem, we exclude from the subsequent analyses all the randomly captured synapses that finally do not fulfill the ideal criteria. These synapses, opportunely enough, are not very frequent. However, it is possible to circumvent this limitation using several experimental approaches⁴.

The polarized CD63 release (degranulation) can be quantified by other complementary techniques such as cell surface staining of CD63 (CD63 relocation to the cell surface) in living cells (not fixed and not permeabilized), after step 6, and subsequent washing and fixation, as

previously shown⁸. In addition, CD63 release on exosomes^{8,9} and exosome quantification by nanoparticle tracking analyses^{9,25} can be performed. These approaches are certainly compatible with our protocol, provided fixation is being performed **after** the cell surface immunofluorescence of living cells.

We have found the ideal number of cells (for a 1 cm² well in the 8-well chamber slide) is 4 x 10⁵ cells (2 x 10⁵ Raji cells and 2 x 10⁵ Jurkat cells) since they adhere efficiently to the bottom of the well. Binding efficiency to plastic (using fibronectin), or glass bottom (using poly-L-lysine) microslides is not usually a problem. Higher cell numbers may produce no gaps among adhered cells and, subsequently, complex synaptic conjugates (**Figure 1**); both situations are not desirable when single cell-to-cell conjugates need to be imaged in, for instance, MTOC or secretory granule polarization experiments. Lower number of cells may decrease the chances to find conjugates, especially when transfected Jurkat cells are challenged with APC to generate synapses. Please note that we have observed that a temperature-stabilized stage incubator in place on the microscope X/Y stage **before** the onset of the protocol (i.e., 1-2 h in advance to stabilize the stage/microscope setup) maintains stable X,Y,Z parameters, which is crucial for proper imaging. Automatic focus system will eventually compensate small Z variations.

When certain gene transduction techniques such as electroporation are used to express fluorescent chimeric proteins (i.e., GFP-CD63) in the Jurkat clones (**Video 1**), a considerable fraction of cells may die after electroporation. This can be a problem since although dead cells do not form synapses, when they are in excess over the living, transfected cells, they may interfere with the formation of conjugates made by living Th cells. We have found that careful elimination of dead cells from transfected cultures by using a density gradient medium following standard protocols before the conjugate formation step can indeed increase the chances to properly image conjugates. In addition, low transfection efficiencies (<20%) can be an important caveat since this, combined with a moderate conjugate formation efficiency (around 60%)²⁵, will decrease the probability to find conjugates made by transfected cells. This is not a problem when non-transfected cells are used to obtain conjugates in end point experiments and subsequent fixation. The 8 microwell chamber slides are compatible with conventional immunofluorescence protocols. This increases the flexibility of the above protocol with different purposes. Fixation with acetone can be a problem to consider when using chamber slides with plastic-bottom wells. However, there are commercially available 8 microwell microscope chamber slides containing glass bottoms, which are compatible with acetone fixation. Remove plastic lids when acetone is used to fix the cells cultured in 8 microwell glass-bottom chamber slides.

It is recommended that the microscope is equipped with a motorized XY stage, motorized epifluorescence turret and automatic focus system (e.g., Perfect Focus System) or equivalent supplements. When multi-well acquisition is required²⁵, the automatic focus system will ensure a stable focus all along the experiment. Previous experience indicates that by establishing an appropriate focus offset on the Raji cells, both the movement of T cells (**Video 1**) and the microscope stage/chamber slide movements in XY multi-point experiments, may be compensated. This is indeed convenient for multiwell time-lapse capture.

A black and white, panchromatic and cooled charged coupled device (CDD) camera was used but higher-sensitivity, fluorescence scientific complementary metal-oxide semiconductor (sCMOS) camera is desirable, since this will decrease camera exposure times and will enhance temporal resolution. The short camera exposure times we have used (ranging from 100 ms to 500 ms) combined with the automatic fluorescence shutter allows prolonged time lapse capture (up to 24 h) with an adequate time resolution (1 frame per minute or less, for up to 16 XY positions) without significant fluorescence bleaching and/or loss in cell viability. The motorized stage allows multi-point (XY) capture and increases the chance to find and image the emerging and developing synapses in the ideal orientation, but also permits image acquisition in multiwell chamber slides when different Jurkat clones need to be simultaneously conjugated²⁵. High numerical aperture of the objective (i.e. 60x, 1.4) is necessary in order to obtain the best results when analyzing the traffic of secretory granules.

The RAJI-SEE-Jurkat constitutes a well-established immunological synapse model that has been used by a myriad of researchers since it was originally described¹¹. We have adapted our protocol to this model in order to properly image the early stages of IS formation. Our aim was to improve the early approaches²⁰ previously followed to study the polarization of the MTOC and the secretory machinery towards the IS. It is remarkable that the conjugates made with this protocol produce F-actin reorganization at the synapse, configuring a canonical SMAC, concomitantly to the MVB polarized traffic²⁵. These crucial events have been also analyzed and validated by confocal microscopy²⁵.

Kinetic differences in polarized traffic exist among different types of IS. For example, the polarized transport of lytic granules from CTLs takes place in seconds or very few minutes, whereas several cytokine-containing vesicles from Th lymphocytes take from minutes up to several hours to finish. These temporal dissimilarities must be taken into account in advance, in order to design the best strategy and to select the most appropriate experimental and imaging approach, since for some imaging stratagems (i.e., laser scanning confocal microscopy (LSCM)), time can be a limiting factor since capture time is much higher than the appropriate time resolution (1 min or less)⁴. This is not a limitation when wide-field fluorescence microscopy (WFFM) is used as described in the protocol above. Since in CTLs, the polarization of MTOC towards the synapse lasts only a few minutes^{3,6,17}, diverse specific state-of-the-art microscopy approaches different to that described here (but harboring higher spatial and temporal resolution) are necessary in order to properly image these synapses^{26,27}, mainly when several microscope fields (multi-point capture) are imaged. These high-resolution, new approaches can be also utilized for imaging the synapses made by Th lymphocytes, although economical and/or logistic reasons (i.e., the core equipment required for some of these imaging techniques costs 6-7 times more than the one described here) could certainly constitute a limitation for these state-of-the-art imaging methods⁴. The fact that IS made by Th lymphocytes are long-lived, and the circumstance that in Th lymphocytes the MTOC, the lymphokine-containing secretory vesicles and MVB take from several minutes up to hours to transport and dock to the IS²², makes this protocol an ideal, affordable approach for imaging the Th-APC IS.

WFFM in combination with post-acquisition image deconvolution constitutes an interesting approach and not only economic reasons support this strategy. The intrinsic poor resolution in the Z axis (the most important caveat of the technique) can be improved by using post-acquisition image deconvolution⁴ (compare **Video 1** with **Video 2**). Deconvolution uses a calculation-based, image processing approach that can improve signal to noise ratio and image resolution and contrast²⁷ up to 2 times, down to 150-100 nm in XY axis and 500 nm in the Z axis⁴.

The use of higher-sensitivity, high readout speed and wide dynamic range, new fluorescence sCMOS cameras will improve the quality of the images and will reduce fluorescence bleaching. The flexibility offered by the cell-to-cell conjugation protocol described here allows the combination of the described cellular approach with several state of the art microscopy techniques, both in living cells but also in fixed cells, and the expected outcome will indeed improve our knowledge of the immunological synapse.

Although we have implemented and validated the protocol by using easy-to-handle, well established cell lines, the potential the approach may allow visualization of more physiological interactions when primary T cells and different types of antigen-presenting cells (such as dendritic cells of macrophages) are used⁵. In this context, this protocol has been also extended and validated by using superantigen (SEB)-pulsed mouse EL-4 cell line used as an APC, to challenge primary mouse T lymphoblasts⁹. Indeed primary T lymphocytes, CTLs in particular, rendered more short-lived and dynamic synaptic contacts (see Suppl. Video 8 in reference⁹) to those seen with the SEE-Raji and Jurkat model. The variability of synaptic contact modes can best be seen for primary T-cell interactions with dendritic cells or B cells in two-dimensional in vitro tissue equivalents that can be also recorded and analyzed by using this protocol. In addition, apart from superantigens, the technique can be used to image other types of synapses. For instance, it could be used in a TCR transgenic, antigen-specific T cell model, for instance using the ovalbumin specific murine OT1/OT2 system or by transfection of T cells with antigen-specific T cell receptors. This opens a myriad of experimental possibilities for the immediate future.

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DISCLOSURES

The authors declare no conflict of interest.

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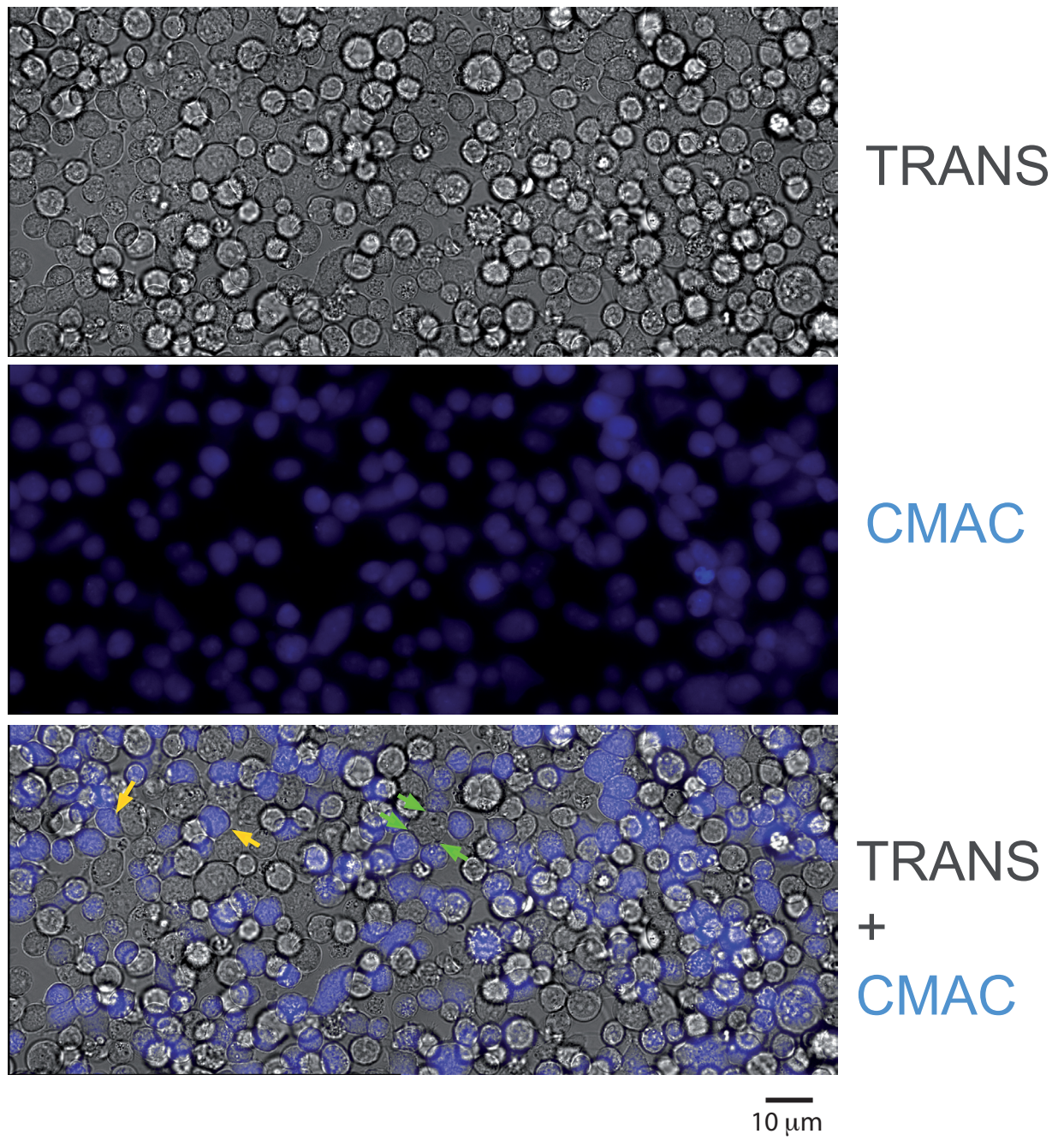


Fig. 1

Microscope settings (time lapse)

Time
settings:
20 sec

CMAC (UV
filter)
camera
settings:
100 msec
(1 frame out
of 8)

GFP-CD63
(GFP filter)
camera
settings:
426 msec

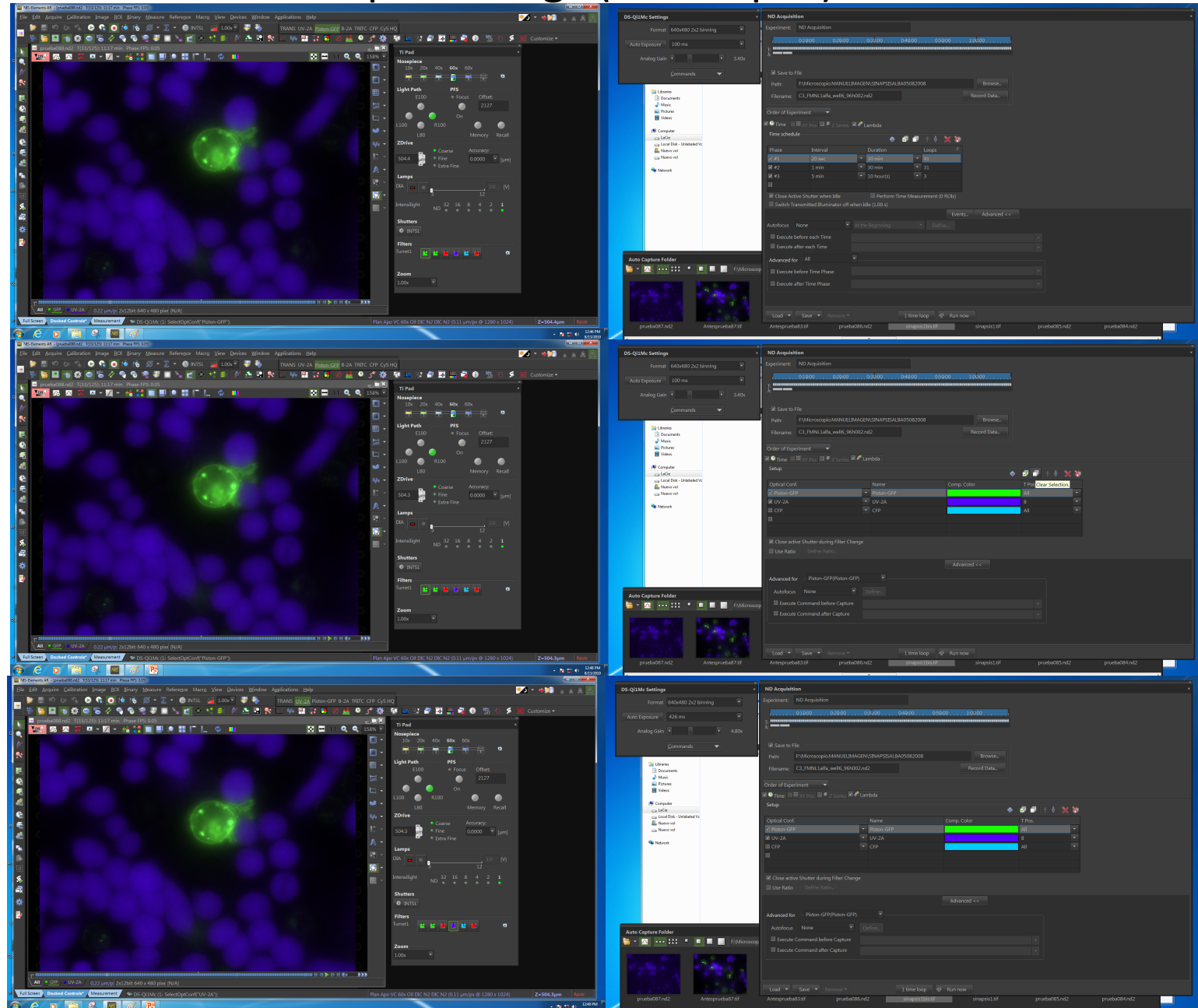
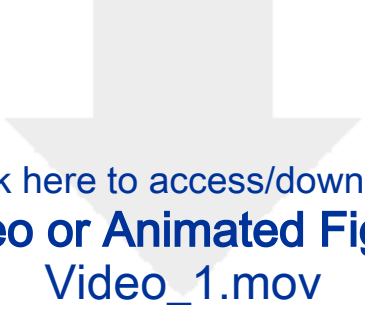
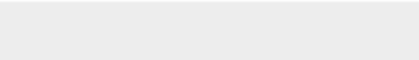
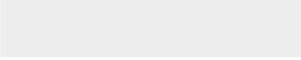
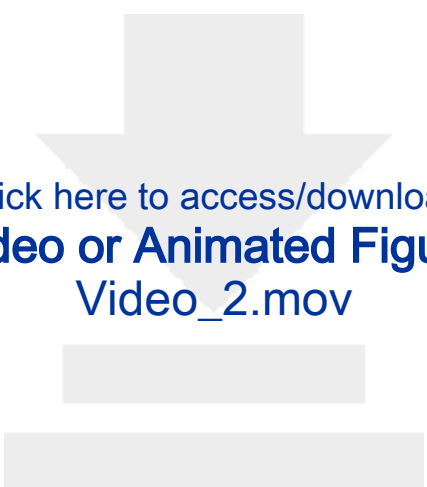


Fig. 2

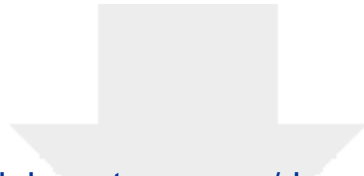


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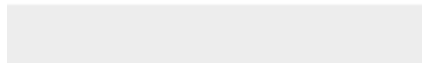
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Camera Nikon DS-QI1MC	Nikon	MQA11550	Cooled Camera Head
CMAC	ThermoFisher Scientific	C2110	Cell tracker blue
JURKAT cells	ATCC	ATCC TIB-152	Effector T lymphocytes
μ-Slide 8 well ibiTreat, μ-Slide 8 well Glass-Bottom	IBIDI	Cat.No: 80826, 80827	Cell culture and cell imaging supports
Microscope NIKON Eclipse Ti-E	Nikon	NIKON Eclipse Ti-E	Wide-field fluorescence, fully-motorized
Microscope Stage Incubator with 3-channel manual gas mixer and gas bubbler/ humidity module	OKOLAB	H201-NIKON-TI-S-ER	Cell culture atmosphere
Raji Cells	ATCC	ATCC CCL-86	APC
RPMI medium GIBCO	ThermoFisher Scientific	21875034	Culture medium
Streptococcus Enterotoxin E (SEE)	Toxin Technology, Inc	EP404	Bacterial Toxin
Software Huygens Essential	SVI	Huygens Essential	Image Deconvolution software
Software Image J	NIH	Image J	Image software
Software Nikon NIS-AR	Nikon	NIS-Elements AR	Image capture and analysis software

microscope equipped with Perfect Focus System (PFS) option



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Madrid 2 September 2019

Dr. Vineeta Bajaj
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Dear Editor,

Thank you very much to invite us to contribute to JoVe and for the useful comments regarding our 3rd revision. We acknowledge all the raised editorial points. Consequently, we have amended our manuscript to satisfy Dr. Vineeta's points. Enclosed you will find our revised manuscript entitled "**Imaging the human immunological synapse**" by Gamboa et al. Changes within the manuscript have been tracked to identify all of the edits.

All the authors concur with the submission. The material is original research, that has not been previously published and is not currently being considered for publication elsewhere. The authors declare there are no competing financial interests in relation to the work described.

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