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## Chapter 9

### Imaging vesicular traffic at the immune synapse

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### Summary

Immunological synapse formation is the result of a profound T cell polarization process that involves the coordinated action of the actin and microtubule cytoskeleton, as well as intracellular vesicle traffic. Endosomal vesicle traffic ensures the targeting of the T cell receptor (TCR) and various signaling molecules to the synapse, being necessary for the generation of signaling complexes downstream of the TCR. Here we describe the microscopy imaging methods that we currently use to unveil how TCR and signaling molecules are associated to endosomal compartments and deliver their cargo to the immunological synapse.

### 1. Introduction

Immunological synapses are characterized by the accumulation and clustering of TCRs, cosignaling receptors like CD4 and CD28, inhibitory receptors like CTLA4 and PD1, adhesion molecules like LFA-1, and a number of signaling molecules of the TCR signaling cascade, including the tyrosine kinases Lck and ZAP70, the adapter molecules LAT and SLP76, and the serine-threonine kinases PKC0 and Erk1/2. Dynamic clustering of these various molecules ensures TCR signal regulation, long term T cell activation and effector functions, like cytokine production or the expression of surface ligands by T helper cells, or the release of cytotoxic granule content by cytotoxic T cells (1, 2). In order to accumulate and cluster at the immunological synapse, those molecules need to be transported to and/or retained at the T cell-antigen presenting cell contact site. Two main mechanisms have been proposed to transport proteins to the synapse: cytoskeleton-based movement at the plasma membrane (3) and transport via intracellular vesicles (4). While the former mechanism was shown to transport the LFA-1 integrin, the latter was shown to transport the TCR-CD3 complex subunits (5-7), the tyrosine kinase Lck (8, 9), the adapter LAT (10, 11) and the inhibitory receptor CTLA4 (12). Different endosomal compartments and regulatory transport proteins are involved. These different endosomal compartments appear intermingled in the T cell cytoplasm, but they are distinct with regard to endosomal markers and are differentially regulated when targeting TCR and signaling molecules to the synapse (4, 13). Thus, the TCR-CD3 complex was shown to be transported via transferrin receptor positive recycling endosomes (5), while the TCR $\zeta$  subunit is associated to Rab8 and intraflagellar transport (IFTs) protein-regulated compartments (7, 13, 14). Moreover, Lck traffic is regulated by MAL (15, 16), Unc119 (17), and Rab11 and its effector FIP3 (13) (Bouchet et al, submitted). Finally, LAT was found associated to Rab7, Rab27 and Rab37 endosomes and its targeting to the synapse regulated by the SNARE VAMP7, the calcium sensor synaptotagmin-7 and IFT20 (11, 13, 18, 19). Intracellular vesicle traffic and actin and microtubule cytoskeleton rearrangements are tightly interconnected processes that ensure immunological synapse architecture, molecular clustering, signaling complex dynamics and ultimately the regulation of T cell activation (4, 20, 21). Interestingly, the subcellular localization and activity of the GTPase Rac1, a master regulator of the actin cytoskeleton crucial for immunological synapse formation and function, is regulated by Rab11 and its effector FIP3 (21).

Here we describe the methods we have been developing to localize TCR and signaling molecules together with endosomal regulatory proteins at the immunological synapse and to decipher the functional importance of this endosomal transport.

### 2. Materials

#### 2.1. Cells

1. Jurkat T cell leukemia cells, J77 Clone 20 cells (J77cl20) and Raji B cell lymphoma cells have been previously described (5, 9, 22). Cells are cultured in RPMI 1640 + GlutaMAX<sup>™</sup> + Phenol Red medium (Gibco<sup>®</sup>, Life Technologies<sup>™</sup>, No. 61870-010) supplemented with 10% fetal calf serum and 10 mM Hepes. Jurkat and Raji cells are cultured at a density average of 0.5-1 x 10<sup>6</sup> cells/mL, splitting the cultures every 2-3 days.

2. Peripheral blood T cells from healthy donors are isolated by centrifugation through Ficoll-Hypaque using Unisep Maxi tubes (Eurobio, No. U-10) (*see* **Note 1**).  $CD4^+$  T cells are further purified using the  $CD4^+$  T cell isolation kit (Miltenyi Biotech, 130-096-533) (*see* **Note 2**). After isolation they are cultured at 2 x  $10^6$  cells/mL in RPMI 1614 medium supplemented with 10% FCS, 1 mM sodium pyruvate and 1% MEM Non-essential aminoacids.

### 2.2. Microscopy materials

1. Confocal microscope: LSM 700 confocal microscope (Zeiss) equipped with a Plan-Apochromat 63× objective and ZEN software (Zeiss).

2. Square glass coverslips 20 x 20 mm. Coverslips are coated with 500  $\mu$ L poly-L-Lysine (0.002% w/v in water) during 20 minutes at room temperature, washed with water and air dried before use (*see* **Note 3**).

3. Round glass coverslips 12 mm diameter. Coating as in 2, using 150  $\mu$ L per round coverslip.

3. Glass Slides 76 x 26 mm

4. ProLong<sup>®</sup> Gold Antifade mounting medium with DAPI (Molecular Probes<sup>®</sup>, Life Technologies<sup>™</sup>, No.
P36935).

### 2.3. Chemicals and biological products

1. Poly-L-lysine MW: 150-300 kDa, 0.1% (w/v) (Sigma-Aldrich<sup>®</sup>, No. P8920). Coating solution 0.002% in water.

Paraformaldehyde: (Electron Microscopy Sciences, No 15714, aqueous stock solution 32%).
 Paraformaldehyde solution 8% (w/v) in water (*see* Note 4).

3. Triton X-100 0.1 % (v/v) in phosphate buffer pH 7.5, 150 mM NaCl (PBS).

4. Methanol.

5. Bovine serum albumin, 1% (w/v) in PBS (PBS-BSA). Store solution at 4°C.

6. *Staphylococcus* enterotoxin E superantigen (SEE, Toxin Technology Inc), 10  $\mu$ g/mL in PBS. Biohazard (*see* **Note 5**).

7. FITC-coupled Phalloidin probe from Molecular Probes (1/100 dilution).

8. Primary antibodies for immunofluorescence: mouse monoclonal IgG2a anti-Rab11, clone 47, and IgG2b anti-Rac1, clone 102 (Becton Dickinson) are used at 25  $\mu$ g/mL and 1  $\mu$ g/mL, respectively. Mouse monoclonal IgG2b anti-Lck, clone 3A5 (Santa Cruz Biotechnology) is used at 2  $\mu$ g/mL. Mouse monoclonal IgG1 anti-CD3 $\epsilon$ , clone UCHT1, (BioLegend Inc) is used at 10  $\mu$ g/mL. Rabbit anti centrin-3,

gift of M. Bornens (Institut Curie, France) is used at 1/400 dilution. Mouse IgG2b anti- $\beta$ -tubulin, clone KMX1 (Millipore) is used at 10 µg/mL. Rabbit anti-phospho ZAP70 (Y319) (Cell Signaling) is used at 1/100 dilution and mouse monoclonal IgG2a anti-phosphoTCR $\zeta$  (Y142), clone K25-407.69 (Becton Dickinson), are used at 5 µg/mL.

9. Secondary antibodies for immunofluorescence: highly cross-adsorbed Cy3-coupled goat antimouse IgG2a, anti-mouse IgG2b and anti-rabbit IgG (Jackson Immuno-Research Laboratories) are used at 1/100 dilution. FITC-coupled goat anti-mouse IgG1 (Southern Biotech) is used at 0.7 µg/mL. Alexa Fluor 488–coupled goat anti-fluorescein (Molecular Probes) is used at 1 µg/mL.

10. Antibody used for pseudo-synapses: mouse IgG1 anti-CD3ε, clone UCHT1 (Bio Legend) is used at 500 ng/mL.

11. Expression vectors: pEGFP-C1/FIP3 and FIP3-I738E mutant expression vectors (23). pEGFPN1/Rab11 WT, Rab11-Q70L and Rab11-S25N mutants provided by Dr. A. Echard (Institut Pasteur, Paris). pEF-BOS/Rac1-cMyc WT, Rac1-G12V and Rac1-T17N mutants (Komuro et al, 1996).

12. siRNA oligonucleotides: We provide here an example of our recent work (21). siRNA duplexes based on the human RAB11-FIP3 sequence had been described previously by others: (5'-AAGGGATCACAGCCATCAGAA-3') (24) and (5'-AAGGCAGTGAGGCGGAGCTGTT-3') (25). Stock solutions are stored in aliquots at -20°C and used at 1 nM per transfection (21) (*see* **Note 6**).

### 2.4. Transfection systems

1. Neon<sup>™</sup> Transfection System and Neon<sup>™</sup> Transfection 100 μL Kit, containing electrolytic buffer E2, resuspension buffer R, 100 μL Neon<sup>™</sup> tips and Neon<sup>™</sup> Electroporation tubes (Invitrogen<sup>™</sup>, Life Technologies<sup>™</sup>).

2. Nucleofector<sup>™</sup> 2b Device and Primary Cell Nucleofector<sup>™</sup> Kit, containing Nucleofector<sup>™</sup> Solution, single use pipette and Amaxa<sup>™</sup> 100 µl aluminum electrode cuvettes (Lonza).

### 3. Methods:

#### 3.1. T cell transfection by electroporation

For expression vectors transfection, cell synapses assays are done 24 h after T cell transfection. For siRNA treatment of T cells, two sequential transfections at 24 h interval are performed, and cell synapses are performed 72 h after the first siRNA transfection. In case of DNA plasmid transfection on siRNA-transfected cells, two sequential transfections at 24 h interval are performed for the siRNA, followed by DNA plasmid transfection 48 h after the first siRNA transfection. Synapses in this case are performed 24 h after transfection of expression vectors (see **Note 7**).

### 3.1.1. Transfection of T cell lines using the Neon™ Transfection System

Transfection is done with 5-10 x  $10^6$  cells. Use 10 µg of DNA plasmid or 1 nM of siRNA for 10 x  $10^6$  cells. Complete with Resuspension Buffer R to equilibrate different volumes for the same amount of DNA or siRNA when preparing Eppendorf tubes with plasmid DNA or siRNA.

1. Pre-incubate at 37°C one flask with 10 mL T cell culture medium (RPMI 1640 supplemented with 10% FCS and 10 mM Hepes) for each transfection. Prepare Eppendorf tubes with DNA plasmid or siRNA.

2. Harvest the amount of cells required, centrifuge at 290 xg, at 20°C for 4 min. Wash twice in PBS and aspirate supernatant.

3. Insert a new Neon<sup>™</sup> tube into the Neon<sup>™</sup> Pipette Station and fill it with 3 mL Electrolytic buffer E2. Turn on Neon<sup>™</sup> device, and select electroporation protocol (voltage: 1400 V, width: 10 ms, pulses: 3)

4. Resuspend the cell pellet in 100  $\mu$ L Resuspension buffer R per required transfection.

5. Add 100  $\mu$ L resuspended cells in each Eppendorf tube with DNA plasmid or siRNA, and mix gently.

6. Take 100  $\mu$ L of the cells-plasmid mix using the Neon<sup>M</sup> pipette and a 100  $\mu$ L tip, avoiding air bubbles. Change Neon<sup>M</sup> tip for every transfection with different DNA or siRNA. Insert the Neon<sup>M</sup> pipette with the sample vertically into the Neon<sup>®</sup> tube and press start.

7. Once electroporation is complete, remove Neon<sup>™</sup> pipette and transfer the sample into the prewarmed culture flask and incubate at 37°C, 5% CO<sub>2</sub> for 24-48 h (*see* **Note 8**).

### 3.1.2. Transfection of purified primary CD4<sup>+</sup> T cells using Nucleofector™ 2b Device

Transfection is done with 5-10 x  $10^6$  cells. Use 5 µg of DNA plasmid or 30-300 nM of siRNA for 10 x  $10^6$  cells. Complete with Resuspension Buffer R to equilibrate different volumes for the same amount of DNA or siRNA when preparing Eppendorf tubes with plasmid DNA or siRNA.

1. Pre-incubate at 37°C one flask with 1 mL Primary CD4<sup>+</sup> T cell culture medium (RPMI 1614 medium supplemented with 10% FCS, 1 mM sodium pyruvate and 1% MEM Non-essential aminoacids) per 10x10<sup>6</sup> cells transfected. Prepare 1.5 mL Eppendorf tubes with DNA plasmid or siRNA.

2. Harvest the amount of primary CD4<sup>+</sup> T cells required, centrifuge at 453 xg at 20°C for 10 min. Wash twice in PBS.

3. Turn on Nucleofector<sup>™</sup> 2b Device and select electroporation protocol (U014).

4. Add corresponding volume of DNA plasmid or siRNA to the Amaxa cuvette per transfection required.

5. Resuspend the cell pellet in 100  $\mu$ L Amaxa buffer per 10 x 10<sup>6</sup> CD4<sup>+</sup> cells.

6. Take 100  $\mu$ L of resuspended cells and add them to the cuvette with DNA plasmid or siRNA. Mix gently, avoiding air bubbles.

7. Insert cuvette in the Nucleofector<sup>™</sup> 2b Device and electroporate the cells using protocol U014.

8. Use single-use pipettes to recover cells and transfer to the pre-warmed flask with medium. Incubate 10 min at 37°C.

9. Count cells and resuspend to a final concentration of 2 x  $10^6$  cells/mL in RPMI 1614 medium supplemented with 10% FCS, 1 mM sodium pyruvate and 1% MEM Non-essential aminoacids at 37°C, 5% CO<sub>2</sub> for 24-48 h (see Note 8).

### 3.2. Immunological synapse formation between T cells and antigen presenting cells

1. Pulse antigen-presenting cells with superantigen: Harvest Raji cells (5 x  $10^6$  cells/mL) by centrifuging at 290 xg at 20°C for 4 min. Resuspend in RPMI 1640 (without serum) supplemented with 10 µg/mL Staphylococcus enterotoxin E superantigen (SEE) and incubate for 30 min at 37°C.

2. Immunological synapse formation: Harvest Jurkat J77 Clone 20 cells by centrifuging at 290 xg at, 20°C for 4 min and resuspend at 5 x  $10^6$  cells/mL RPMI 1640 (without serum). Jurkat J77 Clone 20 cells are incubated with pulsed-Raji cells at 1:1 ratio, at 37°C during 5, 15 or 30 min in RPMI 1640 medium (without serum) in 1.5 mL Eppendorf tubes at 37°C in a water bath.

3. Plating cells on coverslips: 160  $\mu$ L of conjugated cells are plated onto poly-L-lysine-coated square coverslips and kept for 3 min at room temperature (*see* **Note 9**).

4. Fixation: 160  $\mu$ L of 8% paraformaldehyde are mildly dropped onto the coverslips containing cell suspensions (final concentration of paraformaldehyde 4%), and incubated for 20 min at room temperature. After incubation, paraformaldehyde is removed and coverslips washed with PBS, 1 % (w/v) bovine serum albumin (PBS-BSA) (*see* **Notes 4 and 10**).

5. Saturation: Nonspecific binding is prevented by 15 min incubation, in PBS-BSA.

6. First antibody preparation. Primary antibody (or mix of primary antibodies in case of multiple staining), at the recommended dilution, is suspended in PBS-BSA, 0.1% Triton X-100.

7. Cell immuno-staining: 80 μL drops of primary antibody solution are deposited on Parafilm<sup>®</sup> and coverslips with fixed cells are turned over on drops (*see* **Note 11**). Incubate for 1 h at room temperature, protected from light.

8. Washes: After primary antibody incubation, coverslips are washed twice in PBS-BSA by submerging them several times in a beaker using forceps to handle the coverslips.

9. Second antibody preparation: Secondary antibody (or mix of secondary antibodies in case of multiple staining) is resuspended in PBS-BSA.

10. Staining of coverslips: 80  $\mu$ L drops of secondary antibody solution are deposited on Parafilm<sup>®</sup> and coverslips are turned over on drops, as in step 7. Incubate for 45 min – 1 h at room temperature, protected from light.

11. Washes: After secondary antibody incubation, coverslips are washed twice in PBS-BSA by submerging in a beaker using forceps to handle the coverslips. Drain the washing solution by setting the edge of the coverslip on absorbent paper, in order to remove excess PBS-BSA before mounting.

12. Mounting: Drops of 20  $\mu$ L of ProLong Gold Antifade mounting medium with DAPI are deposited on 76 x 26 mm slides and coverslips are turned over on the drops. Let dry mounted slides overnight at room temperature, protected from light. Once dried, slides can be stored at 4°C and used during 2-4 weeks (*see* **Note 12**).

13. Microscopy analysis: Cells are observed under a LSM 700 confocal microscope (Zeiss) equipped with an oil-immersion Plan-Apochromat  $63 \times$  objective. Z-stack optical sections are acquired at different increments depending on image analysis performed afterwards: 0.2 µm depth increments for deconvolution and colocalization analysis and 1 µm depth increments for fluorescence intensity analysis. Green and red laser excitations are intercalated to minimize fluorescence spill over different channels. Image acquisition is done with ZEN software (Zeiss).

### 3.3. Pseudosynapse formation on anti-CD3-coated coverslips.

1. Coating coverslips with anti-CD3 antibodies. Prepare 10  $\mu$ g/mL anti-CD3 (UCHT-1) in PBS. Round poly-L-Lysine coated coverslips are turned over 50  $\mu$ L drops of anti-CD3 solution and incubated 2 h at 37°C in an humidified chamber or overnight at 4°C (*see* **Note 13**). Coverslips are washed once in PBS and saturated with 200  $\mu$ L RPMI 1640 with serum to prevent nonspecific binding to poly-L-Lysine.

2. Cell resuspension. Jurkat J77 Clone 20 cells or  $CD4^+$  primary T cells are washed twice by centrifugation at 290 xg at 20°C for 4 min and resuspended at 2 x 10<sup>6</sup> cells/mL in RPMI 1640 medium without serum.

3. Plating of cells on coverslips. 100  $\mu$ L of cells are plated onto anti-CD3-coated coverslips during 3, 5 or 15 minutes 37°C in a humidified chamber kept at 37°C during the assay.

4. Fixation. 100  $\mu$ L of 8% paraformaldehyde are mildly dropped onto the coverslips containing cell suspensions (final concentration of paraformaldehyde 4%) and incubated for 20 min at room temperature. After incubation PFA is removed and coverslips washed with PBS-BSA (*see* **Notes 4 and 10**).

5. Saturation. Nonspecific binding is prevented by 15 min incubation in PBS-BSA.

6. First antibody preparation. Primary antibody (or mix of primary antibodies in case of multiple staining) at the recommended dilution is suspended in PBS-BSA, 0.1% Triton X-100.

7. Staining of coverslips. 30  $\mu$ L drops of primary antibody solution are deposited on Parafilm<sup>®</sup> and coverslips with fixed cells are turned over on drops (*see* **Note 11**). Incubate for 1 h at room temperature, protected from light.

8. Washes. After primary antibody incubation, coverslips are washed twice in PBS-BSA by submerging several times in a beaker using forceps to handle the coverslips.

9. Second antibody preparation. Secondary antibody (or mix of secondary antibodies in case of multiple staining) is resuspended in PBS-BSA.

10. Staining of coverslips. 30 µL drops of secondary antibody solution are deposited on Parafilm<sup>®</sup> and coverslips are turned over on drops, as done in step 7. Incubate for 45 min-1 h, at room temperature, protected from light.

11. Washes. After secondary antibody incubation, coverslips are washed twice in PBS-BSA by submerging in a beaker using forceps to handle the coverslips. Drain the washing solution by setting the edge of the coverslip on absorbent paper, in order to remove excess PBS-BSA before mounting.

12. Mounting. Drops of 20  $\mu$ L of ProLong Gold Antifade mounting medium with DAPI are deposited on 76 x 26 mm slides and coverslips are turned on the drops. Let mounted coverslips harden overnight at room temperature, protected from light. Slides are then stored at 4°C and may be used during 2-4 weeks (*see* **Note 12**).

13. Microscopy analysis. Cells are observed under a LSM 700 confocal microscope (Zeiss) equipped with an oil-immersion Plan-Apochromat  $63 \times$  objective). Z-stack optical sections are acquired at different increments depending on desired image analysis: 0.2 µm depth increments for deconvolution and colocalization analysis and 1 µm depth increments for fluorescence intensity analysis. Green and red laser excitation are intercalated to minimize fluorescence spill over different channels. Images acquisition is done with ZEN software (Zeiss).

### 3.4. Staining of microtubules

1. Immunological synapses or pseudo-synapses are performed as in 3.2 and 3.3 until the fixation step.

2. For microtubules staining, 8% paraformaldehyde is mildly dropped onto the coverslips with cells and incubated for only 10 min at room temperature. Directly after incubation, coverslips are placed

in 6- or 24-well plates with ice-cold methanol and stored at -20°C for at least 1 h for further fixation and permeabilization. Before staining coverslips are washed once with PBS-BSA.

3. The remaining steps are performed as in 3.2 for immunological synapses with antigen presenting cells, or 3.3 for pseudo-synapses (Figure 1).

### 3.5. Quantitative image analysis

1. Deconvolution of confocal images is used to improve image rendering, especially for 3D reconstruction and prior to colocalization analysis. Deconvolution is performed on Z-stacks of confocal optical sections obtained at 0.2  $\mu$ m depth increments, using Huygens Professional software (Scientific Volume Imaging).

2. Colocalization analysis is performed with FiJi software (open platform for scientific image analysis). JaCoP plugin or Colocalization Threshold option of Fiji are used.

Pearson's correlation coefficient corresponds to the linear relationship between intensity of pixels in the two analyzed channels. Mander's coefficient is defined as the ratio of the summed intensities of pixels from one image for which the intensity in the second channel is above the threshold (26).

Costes Automatic Threshold allows us to consider only the pixels in each channel showing statistical correlation, as explained in Costes et al (27) (Figure 2).

3. Fluorescence intensity analysis. Using FiJi, in a chosen channel, create a Z-projection of the image (whole cell, or a number of optical sections corresponding to an intracellular compartment) and select a region of interest (i.e. immunological synapse, or an intracellular compartment), depending on experimental requirements. Then, in the other channel, measure fluorescence intensity of the selected area of interest (Figure 3).

### 4. Notes:

1. Density-Gradient centrifugation to isolate peripheral blood mononuclear cells (PBMC). Ficoll separation: spin Ficoll tubes 5 min at 453 xg at room temperature. Lay 28 mL of blood per tube of 15 mL Ficoll-Hypaque-Lymphoprep. Centrifuge 30 min at 805 xg at room temperature with no brake. Aspirate plasma layer containing PBMCs and add it to new empty falcon tubes (15 mL recovered PBMCs per 50 mL falcon tube). Add 35 mL RPMI no FCS per 15 mL of recovered PBMCs per tube. Spin 10 min at 453 xg at room temperature. Discard supernatants and pull pellets together to one tube. Wash twice with 50 mL RPMI no FCS (10 min at 453 xg). Resuspend pellet in 50 mL of RPMI no FCS and count cells using cell counter or Trypan blue vital staining and Malassez chambers (Figure 4).

2. Purification of CD4<sup>+</sup> T cells using MACS CD4 Cell Isolation Kit II: follow manufacturer's instructions.

3. Coverslip coating with poly-L-lysine: prepare a 1:50 dilution of 0.01% poly-L-Lysine solution in water. Cover a flat surface with Parafilm, and place round or square coverslips over it. Add 500  $\mu$ L for square coverslips or 150  $\mu$ L for round coverslips of the 1:50 poly-L-Lysine dilution over the coverslips, covering the surface completely. Incubate 20 min at room temperature and remove excess. Wash once with water and dry the coverslips before use.

4. Paraformaldehyde. We recommend purchasing a stock commercial solution, avoiding manipulation of paraformaldehyde powder, in order to prevent toxicity. Paraformaldehyde solution manipulation should be carried out under a chemical hood.

5. *Staphylococcus* enterotoxins are a biohazard and should be manipulated as such. Inactivation of all solutions and materials in contact with the toxin should be performed at the end of experiments using an excess of 0.5% (w/v) sodium hypochlorite for at least 1 h. Dispose as biohazard.

6. siRNA efficacy to inhibit protein expression depends on the messenger RNA stability and the turnover of each protein. A dose response assay is useful to set the appropriate experimental conditions for different proteins.

7. Schematic representation of the transfection time line (Figure 5).

8. Different plasmids might need different expression times in order to reach enough protein levels. Expression may be different in Jurkat and in primary T cells.

9. Cover with Parafilm a flat surface and place the poly-L-Lysine coated coverslips over it (poly-L-Lysine side of the coverslips facing up). Add cell suspension over the coverslip, trying to distribute it evenly over the surface.

10. Once fixed and washed, round or square coverslips containing cells may be stored in 6- or 24-well plates, respectively, in an excess of PBS-BSA, for 1-2 weeks at 4°C. Staining does not require to be performed immediately after fixation, except for protein phosphorylation staining, which requires freshly prepared cells for optimum results.

11. Cover with parafilm a flat surface and label areas for different staining solutions planned. Deposit 80 µL drops of primary antibody mix on the parafilm. Take the coverslips with fixed cells and drain the excess of PBS-BSA by setting the edge of the coverslip on absorbent paper. This prevents dilution of antibody staining solutions. Place coverslips over primary antibody drops on Parafilm, cells facing the drops. Create a humidified chamber for the incubation by placing wet pieces of paper around the parafilm, and cover everything with a lid or opaque surface, in order to protect from light.

12. Once slides have been analyzed, it is recommended to store them at -20°C, to prevent loss of fluorescence. While flourochrome fluorescence tends to be stable, GFP fluorescence is lost much faster.

13. Cover with Parafilm a flat surface and deposit 50  $\mu$ L drops of anti-CD3 antibody mix on the parafilm. Create a humidified chamber for the incubation by placing wet pieces of paper around the parafilm, and cover everything with a lid.

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### **Figure legends**

### Figure 1 : Microtubule detection at T cell « pseudosynapses »

A: Schematic representation of T cells spreading on anti-CD3-coated coverslips. B: Jurkat cells spread 3 min on an anti-CD3-coated coverslip.  $\beta$ -tubulin staining. Confocal image treated by deconvolution. A 4  $\mu$ m Z- projection close to the coverslip is shown. Scale bar = 5  $\mu$ m

### Figure 2 : Colocalization analyses of Rac1, Rab11 and Rab11-FIP3.

A, B: Immunostaining of Jurkat T cells for endogenous Rab11 and Rac1 GTPases. A Z-stack of confocal images were treated by deconvolution. A 4  $\mu$ m Z- projection of the area where the intracellular pericentrosomal compartment is located is shown. Pearson's correlation coefficient (R) in the region framed was measured and the graph is shown on the right panel. Examples of different experimental conditions. A: control cells. Colocalization between Rab11 and Rac1. B: Cells silenced for the expression of the Rab11 effector FIP3. Colocalization between Rab11 and Rac1. C: Effect of GFP-FIP3 (green) overexpression in endogenous Rac1 localization (red). Colocalization between FIP3 and Rac1. Scale bar = 5  $\mu$ m

### Figure 3 : Quantification of fluorescence accumulation at the immunological synapse

Immunological synapse of a Jurkat (J77cl20) lymphocyte with a Raji B lymphocyte pulsed with SEE superantigen (antigen presenting cell, APC). Confocal image was treated by deconvolution. A 4  $\mu$ m Z-projection is shown. From left to right: Lck (green), CD3 surface staining (red), merge of fluorescence images and phase contrast image. Rectangles show the region of interest in which quantification of the intensity of fluorescence is performed. It can be then referred to the total fluorescence of the cell or to a region at the opposite side of the synapse. Scale bar = 5  $\mu$ m.

Figure 4: Scheme of a Ficoll gradient centrifugation tube after centrifugation.

Figure 5. Schematic representation of the transfection time line.



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