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Summary

In T lymphocytes, the immune synapse is an active zone of vesicular traffic. Directional transport of vesicular receptors and signaling molecules from or to the immune synapse has been shown to play an important role in T cell receptor (TCR) signal transduction. However, how vesicular trafficking is regulating the activation of T cells is still a burning question and the characterization of these intracellular compartments remains the first step to understand this process. We describe herein a protocol, which combined a separation of membranes on flotation gradient with an affinity purification of *Strep*-tagged fusion transmembrane proteins with *Strep*-Tactin® resins, allowing the purification of membranes containing the *Strep*-tagged molecule of interest. By keeping the membranes intact this protocol leads to the purification of molecules physically associated with the *Strep*-Tagged protein as well as of molecules present in the same membrane compartment: transmembrane proteins, proteins strongly associated with the membranes, luminal proteins. The example shown herein is the purification of membrane compartment prepared from T lymphocytes expressing LAT fused to a *Strep*-Tag.

Key words: T lymphocyte, Immune synapse, flotation gradient, *Strep*-Tag-*Strep*-Tactin affinity purification, LAT.

1. Introduction

How the activation of T cells is regulated remains a fundamental question for understanding the adaptive immune response. It has been 17 years now that the immune synapse has been described by both A. Kupfer's and ML. Dustin's groups [1,2]. Since then a growing number of papers have investigated the spatio-temporal organization of this structure as well as the mechanisms involved in its formation and its role in T cell activation. The application of microscopy techniques, revealed that different receptors expressed by T lymphocytes and involved in T-cell activation localized to defined supramolecular activation clusters (SMACs) in the contact zone between the T lymphocyte and the antigen-presenting cell (APC), with antigen receptors distributing to the center of this region (cSMAC), and adhesion molecules accumulating at the periphery (pSMAC) [1,2]. Development of high resolution microscopy then allowed for a better description of the dynamic of these contact regions, showing that receptors and signaling molecules aggregate in dynamic microclusters, which form at the periphery of the immune synapse and migrate toward its center [3,4]. These microscopy based analysis also allowed the characterization of the polarized trafficking and secretion of vesicular components at the immune synapse [5-7].

Since these early studies, it has become evident that the immune synapse is an active zone for directional exocytosis, endocytosis, receptor recycling and more generally vesicular traffic [8-11]. Yet, how vesicular trafficking is involved in T cell activation is still only partially understood.

Several groups including ours have shown that intracellular vesicles containing signaling molecules are actively involved in signal transduction processes in T lymphocytes, providing unique platforms for specific signaling complexes to assemble or be activated [12-15]. These intracellular vesicles, which localize close to or interact with the membrane at the immune

synapse, may regulate T-cell activation by transporting cargo to or from specific sites of the immune synapse. They may also directly control T lymphocyte signaling process by acting as mobile platforms that compartmentalize and organize signaling inside the cell. Along this line, it is worth noting that location and intracellular trafficking regulate the signaling induced by many receptors such as EGFR [16], TGFβR [17], TLR [18] and BCR [19]. Intracellular location of signaling also matters in T lymphocytes. Indeed, differential intracellular locations of signaling molecules have been shown to translate to different functional outcomes [20-22].

Finally, there is emerging evidence that different pools of sub-synaptic vesicles containing signaling molecules exist [14,23,24]. Yet, their relative role and content are unknown. The mechanisms controlling the traffic of these different vesicular compartments and their interaction with the immune synapse are still a matter of debate [25,13,15,26,14].

To better understand T cell signaling at the immune synapse, it thus seems important to develop tools that allow the purification of these different intracellular compartments and to analyze their content.

We herein describe a protocol of purification of membrane compartments containing the adaptor molecule LAT. This molecule, which was cloned at the same time as the first characterization of the immune synapse [27,28], plays a key role in T cell activation. It is present both at the plasma membrane and in vesicles [29]. The traffic of the vesicular pool of LAT to the immune synapse requires the SNARE protein VAMP7 [15]. Our protocol is based on a first step of separation of different intracellular organelles by flotation gradient and a second step of affinity purification of *Strep*-tagged fusion proteins (*Strep*-tag® is a nine amino acid peptide with intrinsic streptavidin-binding activity) on resins coated with *Strep*-Tactin® (an engineered streptavidin that binds *Strep*-tag®) [30]. Because the purification is done in the absence of detergent, it not only allows the purification of molecules that are

physically associated with the tagged-bait, as usually done for interactome studies [31,32] but also the purification of molecules that are present in the same membranes/vesicles being integral or lumenal proteins of the compartments (see Fig19.1). We think that this protocol, presented here with the example of LAT, can be used to purify membrane compartments containing other tagged transmembrane proteins.

2. Materials

Prepare all solutions using ultrapure water. Solutions are stored at room temperature unless stated otherwise.

2.1. Solutions and material for cell disruption and gradient preparation.

1. Cells: LAT deficient JCAM2.5 Jurkat T cells [33] expressing the mouse LAT- *Strep*-tag® protein [31].
2. Homogenization buffer: 0.25 M sucrose, 10 mM Tris-HCl pH 7.4, 1 mM EDTA
3. Iodixanol dilution buffer: 0.25 M sucrose, 60 mM Tris-HCl pH 7.4, 6mM EDTA.
4. cOmplete ^{TM}, EDTA-free protease inhibitor cocktail from Roche Life Science. Dissolve 1 tablet in 2 mL of water according to the manufacturer instructions to obtain a 25x concentrated stock solution (store at -20°C).
5. Halt^{TM} Phosphatase Inhibitor cocktail from ThermoFisher Scientific. Solution is diluted 100x in appropriate solution. (store at 4°C)
6. OptiPrep^{TM} (Axis-Shield) density gradient medium is a solution of 60% iodixanol in water with a density of 1.32 g/mL
7. Phosphate-buffered saline (PBS)
8. RPMI-1640
9. 2 mL glass Dounce homogenizer
10. Needles (Terumo) 25Gx5/8" and 2 mL Syringes
11. SW 55 Ti Rotor, Swinging Bucket (Beckman-Coulter)
12. Ultra-clear centrifuge tubes 5 mL (Beckman-Coulter)
13. RIPA lysis buffer: 25 mM Tris-HCl pH 7.4 , 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl.

2.2. Solutions for the immunoprecipitation and elution steps.

1. *Strep*-Tactin® Sepharose® resin (IBA) 50% suspension (50% suspension in 100mM Tris-HCl pH 8.0 ; 1 mM EDTA; 150 mM NaCl) (Fig19.1B)
2. Sepharose® 50% suspension (IBA) (Fig19.1C)
3. IBA washing buffer: 100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA.
4. Biotin elution buffer: IBA washing buffer supplemented with 2 mM of D-biotin (IBA). Store at 4°C

2.3. Western blot

5. SDS PAGE running buffer: 25 mM Tris-HCl pH 8.3, 0.192 M Glycine, 0.1% SDS
6. Western blot transfer buffer: 25 mM Tris, 0.192 M glycine and 20% methanol
7. Tris buffered saline (TBS; 10x) containing 0.05% Tween-20 (TBST)
8. Blocking solution: 5% BSA in TBST. Filter on 0.45µm and store at 4°C.
9. BIORAD Mini-PROTEAN® TGX Stain-free™ Precast gels 4%-15%
10. 4x Laemmli sample buffer (277.8 mM Tris-HCl pH 6.8, 44.4% (v/v) glycerol, 0.02% bromophenol blue, 5% SDS, BIORAD)
11. Reducing sample buffer 10x: 500 mM Dithiothreitol (Novex™)
12. PVDF transfer membrane (Immuno-Blot®, BIORAD)
13. ECL Blotting substrate (Pierce)

2.4. Antibodies

See Table 19.1

3. Methods

3.1. Cells stimulation.

It is very important to avoid any difference of temperature. Place the centrifuge at room temperature and pre-warm medium at 37°C.

1. Harvest and count the cells expressing the transmembrane *Strep*-tag® protein of interest. (*see* **Note 1**)
2. Wash the cells twice in RPMI medium to remove the FCS contained in culture medium: fill a 50 mL tube with RPMI pre-warmed at 37°C and spin for 5 min at 300x*g*. Remove the supernatant, resuspend the pellet and spin one more time.
3. For the stimulation, resuspend the cells in RPMI at 100 10^6/mL and then transfer 1 mL of this cell suspension in an Eppendorf tube (2 mL). Do as many tubes as needed. Leave the cells at 37°C for 5 min (in a water bath) without any stimulation. Meanwhile, prepare the appropriate dilutions of anti-CD3 and anti-CD28 antibodies (in RPMI). Anti-CD3 Ab is used at 12.5µg/mL and anti-CD28 Ab at 25 µg/mL. (*see* **Note 2**)
4. Add antibodies to the cells directly in Eppendorf tubes and mix by gently pipetting up and down without vortexing the cells. Incubate at 37°C (in water bath) for the requested period of time.
5. To stop the activation, add 1 mL of ice-cold PBS to the cells and spin immediately for 5 min at 250x*g* at 4°C.

3.1. Cells disruption

It is important to keep samples on ice as much as possible. To avoid any contamination between samples, rinse extensively the Dounce homogenizer with sterile water and do the last rinse with homogenization buffer.

1. Resuspend the cells pellet obtained in 3.1.5 in 1.5 mL of ice-cold homogenization buffer supplemented with both protease and phosphatase inhibitors. (*see* **Note 3**)

2. At this step, take an aliquot of the cell suspension (100µl) and resuspend into RIPA lysis buffer containing protease and phosphatase inhibitors. This is your total lysate control. (*see* **Note 4**)
3. Transfer the cell suspension into the Dounce homogenizer and apply 25 strokes with the pestel to induce cells breakage.
4. Transfer the suspension into a new Eppendorf tube (2 mL)
5. Homogenize by 15 passages through a 25GA needle fitted onto a 2 mL syringe. (*see* **Note 5**)
6. Centrifuge 3 min at 900x*g* at 4°C; discard the pellet containing nuclei and unbroken cells and keep the supernatant which contains cell membranes.

3.2. Membranes centrifugation

1. Transfer the supernatant in a 5 mL ultracentrifuge tube and centrifuge at 65000x*g* for 1 h in a SW55Ti rotor. (*see* **Note 6**)
2. Transfer the supernatant in a new Eppendorf tube, it contains the cytosolic proteins.
3. Resuspend the pellet into 1.2 mL of homogenization buffer supplemented with both protease and phosphatase inhibitors.
4. Homogenize by 5 passages through a 25GA needle fitted onto a 2 mL syringe.

3.3. Flotation gradient

1. For each gradient, prepare extemporaneously 1.3 mL of a 20% Iodixanol solution (mix 1vol of Optiprep™ with 2 volumes of Iodixanol dilution buffer) and 1.2 mL of a 10% iodixanol solution (mix 1 volume of Optiprep™ with 5 volumes of Iodixanol dilution buffer). (*see* **Note 7**)

2. Mix the volume of membranes suspension obtained in 3.2.4 with the same volume of Optiprep^{TM} to reach a final concentration of 30% Iodixanol (dilution 1:2 from the 60% original Iodixanol solution). Place this sample at the bottom of an ultracentrifuge tube.
3. Slowly overlay on the top of the 30% Iodixanol solution 1.3 mL of 20% Iodixanol solution and then overlay 1.2 mL of the 10% Iodixanol solution. It is important to avoid mixing the gradient layers. (*see* **Note 8**)
4. Centrifuge the gradient at 350000x*g* for 3 h at 4°C (SW55Ti rotor) with low acceleration (4) and no brake. (*see* **Note 9**)
5. After centrifugation, collect carefully 10 fractions of 490 µL from the top using a smooth 1 mL pipet. (*see* **Note 10**)

3.4. Western blot analysis of the different fractions

1. For each fraction transfer 49 µL in a new Eppendorf tube and mix with 4.9µl of 10x reducing sample and 17 µL of 4x Laemmli buffer.
2. Heat the samples at 95°C for 5 min. Make sure to briefly spin if condensation was formed in the tube.
3. Load 15 µL of each sample on a 4-15% SDS-PAGE gel and proceed with electrophoresis till the dye front has reached the bottom of the gel. (*see* **Note 11**)
4. Transfer on PVDF membrane.
5. Incubate the membrane with TBST-5%BSA for 1 h at RT under gentle agitation.
6. Incubate the membrane with primary antibodies OV/N at 4°C under gentle agitation. For references see 2.4.
7. Wash 3 times 15 min with TBST

8. Incubate with the appropriate HRP-conjugated secondary antibodies for 1h under gentle agitation.
9. Wash 4 times 15 min with TBST.
10. Proceed with chemiluminescence detection of proteins. (*see* Fig**19.2A**)

3.5. Precipitation and Elution of the membranes containing the *Strep*-tag® protein

1. Vortex carefully the *Strep*-Tactin® Sepharose® IBA resin (hereafter called resin). Transfer the required quantity in an Eppendorf tube. (*See* **Note 12 and 13**)
2. Wash the resin 3 times with IBA washing buffer: add 1 mL of IBA washing buffer to the resin. Mix by inverting the tube several times. Centrifuge at 2000xg for 1 min. Remove the supernatant. Repeat the centrifugation and washing steps two more times. (*See* **Note 14**)
3. Resuspend the resin in IBA washing buffer supplemented with protease and phosphatase inhibitors. The volume of resuspension is equal to the *Strep*-tag® protein sample volume. (*See* **Note 15**)
4. Add the sample containing membranes with *Strep*-tag® protein (prepared in 3.3) to the resin and mix by inverting the tube. (*See* **Note 16**)
5. Incubate for 1h30 at 4°C on a rotating wheel.
6. Centrifuge at 2000xg for 1 min. Carefully transfer the supernatant to a new Eppendorf tube. This corresponds to the "unbound membranes" (see Fig19.1.E).
7. Wash the resin/membranes pellet 5 times as in 3.5.2. but add phosphatase and protease inhibitors to the IBA washing buffer.

8. After the last wash, carefully remove the supernatant and add 120 µL of elution buffer containing 2 mM D-biotin to the pellet containing membranes bound *Strep*-tag® protein coupled to the resin (Fig19.1F). Incubate for 8 min on ice.
9. Centrifuge at 2000xg for 1 min. Remove the supernatant: it corresponds to eluted membranes containing the *Strep*-tag® proteins (Fig19.1G). (*See* **Note 17**)
10. For each elution sample, transfer 20 µL in an Eppendorf tube and mix with 2 µL of 10x reducing sample buffer and 10 µL of 4x Laemmli buffer. Proceed to immunoblotting as in 3.4.
11. Following the last elution step, resuspend the resin in 500 µL of IBA washing buffer and transfer 50 µL in an Eppendorf tube, mix with 5 µL of reducing sample buffer and 18 µL of 4x Laemmli buffer. This will be the control of the efficacy of elution (material staying on *Strep*-tactin® resin after elution). Proceed to immunoblotting as in 3.4. (*See* **Note 18**)

4. Notes

1. This protocol was optimized to purify membranes containing LAT from JCAM2.5 cells (LAT deficient Jurkat cells) expressing the mouse chimeric LAT coupled to a *Strep*-tag® [30,31,33].
2. For each gradient, use between 100-400 10^6 cells. If more than 500 10^6 cells are used, the mechanical lysis will be less efficient and will reduce both the gradient separation and the protein yield.
3. Use 150-200 10^6 cells/1.5 mL. Increase the volume of homogenization buffer in proportion.

4. The total lysate can be immunoblotted for specific phospho-proteins in order to ensure that the stimulation was successful. A proper activation of the cells can also be detected in the different fractions following the flotation gradient. (*see* Fig**19.3**)
5. These 2 sequential steps of homogenization should break between 60% and 80% of the cells without damaging nuclei. Cells breakage can be checked by Trypan blue staining under a microscope.
6. Ultracentrifuge settings: acceleration maximum, brake maximum
7. Carefully mix the content of the Optiprep™ bottle before use. Keep the Optiprep™ solution at room temperature, sterile and protected from light.
8. Carefully equilibrate the tubes between layers to ensure that the centrifuge will be balanced during centrifugation.
9. Without brake, it will take about 1 h for the rotor to stop.
10. The density of each fraction can be measured using a refractometer (Carl Zeiss). After centrifugation, the gradient results in a linear increase in density and can be assessed to ensure the reproducibility of the experiments.
11. Each lane contains 1/40 of total fraction proteins. (*see* Fig**19.2A**)
12. The Sepharose suspension has a tendency to stick to plastic pipettes and tips. Moreover 200 µL tips are too tight to let the resin go through so cut off the end of the tip to ensure reproducible pipetting.
13. Resin binding capacity (from IBA data sheet): 1 mL of sedimented resin (corresponding to 2 mL of a 50 % suspension) is able to purify 50 to 100 nmol recombinant *Strep*-tag® protein (up to 3 mg in the case of a 30 kDa protein)
14. You can make a mark on the Eppendorf tube to visualize the top of the pellet formed by the resin. Indeed, the resin is whitish and the pellet can be difficult to see.

15. In our experiments, the volume of fraction sample used for purification is 400 µL, so we resuspend the resin in 400 µL of IBA washing buffer supplemented with proteases and phosphatases inhibitors.
16. In the example described herein, fraction 3 was precipitated because it contains LAT and the vesicular SNARE protein VAMP7 that was shown to control LAT trafficking [15] (see figure**19.2A**) and thus constitute an enriched pool of the LAT containing vesicles transported to the immune synapse.
17. Elution steps can be repeated to improve the yield of protein bait recovery (*see* Fig**19.4**). Each application will require preliminary settings to determine recovery yields of precipitation and of elution.
18. To control for the efficacy of the precipitation and elution steps, run side by side on a gel samples from fraction 3 (before purification, 3.4.1), unbound membranes (3.5.6), elution (membranes containing the *Strep*-tag® proteins, 3.5.10) and resin after elution (non-eluted proteins, 3.5.11). Use an antibody corresponding to the *Strep*-tag® protein and quantify its amount in each sample. The comparison of the amount of *Strep*-tag® protein found in fraction 3 and in "unbound membranes" will give the efficiency of purification, whereas the comparison between the eluted material and material still bound to resin after elution will give the efficiency of elution.
19. As a control for the specificity of the purification, you can run the experiment in the exact same conditions but using a "nude" Sepharose, which does not contain the *Strep*-Tactin® (*see* Fig**19.1.C**). Proteins that will bind in a non-specific manner on the resin will constitute the background of the experiment.
20. Also, negative control can come from non-activated conditions or using cells that do not express the *Strep*-tag® protein.

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Figure legends

Fig19.1: Experimental approach followed to purify membranes containing a chimeric LAT protein fused to a *Strep*-tag®. (A) Membranes containing the chimeric LAT are recovered by flotation gradient (in the example presented herein fraction 3 is used, see Fig19.2). (B-D) Specific purification of membranes containing chimeric LAT are obtained with Sepharose coated with *Strep*-Tactin. Non specific binding is obtained with Sepharose alone (C: "Nude" Sepharose). (E) Membranes that do not contain the chimeric LAT are not retained on Sepharose-StrepTactin (unbound membranes). (F) Membranes containing the chimeric LAT are eluted with an excess of biotin. Molecules recovered in this fraction contain: protein interacting with LAT, membrane associated or transmembrane proteins present in the same membranes as LAT, lumenal proteins from LAT bearing vesicles.

Fig19.2: A. Western blot analysis of the different fractions collected after flotation gradient. Presence of the different intracellular organelles is followed using antibodies for specific markers: mitofilin for mitochondria, LAMP2 for lysosomes, GM130 for cis-Golgi, TGN46 for trans-Golgi, gp 96 for endoplasmic reticulum, VAMP7 for vesicular compartment and CD45, TfR, CD3ζ, LAT for plasma membrane and endocytic compartments. B: Electron-microscopy images showing immunogold labeling for LAT on the vesicles present in fraction 3. The size of the vesicles is between 50 and 300nm.

Fig19.3: Western blot analysis of the phosphorylated signaling molecules in the different fractions. JCAM2.5 T cells expressing a chimeric LAT fused to *Strep*-Tactin® were either left unstimulated or stimulated for 15 min with anti-CD3 and anti-CD28 antibodies and the 10 fractions obtained after flotation gradient were immunoblotted and probed with phospho-specific antibodies: anti-phospho-PLCγ1 (P-PLCγ1), anti-phospho-CD3ζ (P-CD3ζ), anti-phospho-LAT (P-LAT) or probed with a total anti-LAT antibody.

Fig19.4: Western blot analysis of eluted proteins. Fractions 3 obtained from unstimulated or anti-CD3/CD28 stimulated JCAM2.5 T cells expressing a chimeric LAT fused to *Strep*-Tactin® (see Fig19.3) were subjected to purification with either "nude" Sepharose (non specific binding) or Strep-Tactin® Sepharose (specific binding). Membranes were then eluted with D-biotin 3 times (Elution n° 1,2,3) and probed with anti-LAT and anti-CD3ζ antibodies. Two bands are revealed with the anti-CD3ζ, the upper band corresponding to the phosphorylated form of the protein.

Table **19.1**: List of antibodies used in the protocol.

Company	Antigen Recognized	Catalog Number	Clone	Host	Final Concentration or Dilution*
Western blot antibodies					
Santa Cruz	CD3ζ	SC-1239	6B10.2	Mouse	0.2 µg/mL
In Vitrogen	Transferrin receptor	136800	H68.4	Mouse	0.5 µg/mL
Genetex	Lamp2a	63319		Rabbit	1/1000
E-Biosciences	CD45	14-0459-82	H130	Mouse	0.5 µg/mL
ABCAM	GM130	Ab52649	EP892Y	Rabbit	0.11 µg/mL
ABCAM	TGN46	Ab16052		Rabbit	0.5 µg/mL
ThermoFisher Scientific	Mitofilin	PA5-30419		Rabbit	2 µg/mL
Upstate Technology-Millipore	LAT	06-807		Rabbit	1 µg/mL
Enzo	GP96	ADI-SPA-850	9G-10	Rat	1 µg/mL
T.Galli's lab	VAMP7			Rabbit	1/200
Cell Signaling	P-PLCγ1(Tyr783)	CS#2821		Rabbit	1/1000
BD Biosciences	P-CD3ζ (Tyr142)	BD #558402	K25-407.69	Mouse	0.5 µg/mL
Upstate Technology-Millipore	P-LAT (Tyr191)	07-278		Rabbit	1 µg/mL
Stimulating antibodies					
Biolegend	CD28	302923	CD28.2	Mouse	12.5 µg/mL
E-Biosciences	CD3ε	16-0037-85	OKT3	Mouse	25 µg/mL
HRP-conjugated antibodies					
Jackson Immunoresearch	HRP-anti-mouse Ig	115-035-146		Goat	0.08 µg/mL
Jackson Immunoresearch	HRP-anti-rat Ig	112-035-143		Goat	0.08 µg/mL
Jackson Immunoresearch	HRP-anti-rabbit Ig	111-035-144		Goat	0.08 µg/mL

*Dilution is given when antibody concentration is not known.

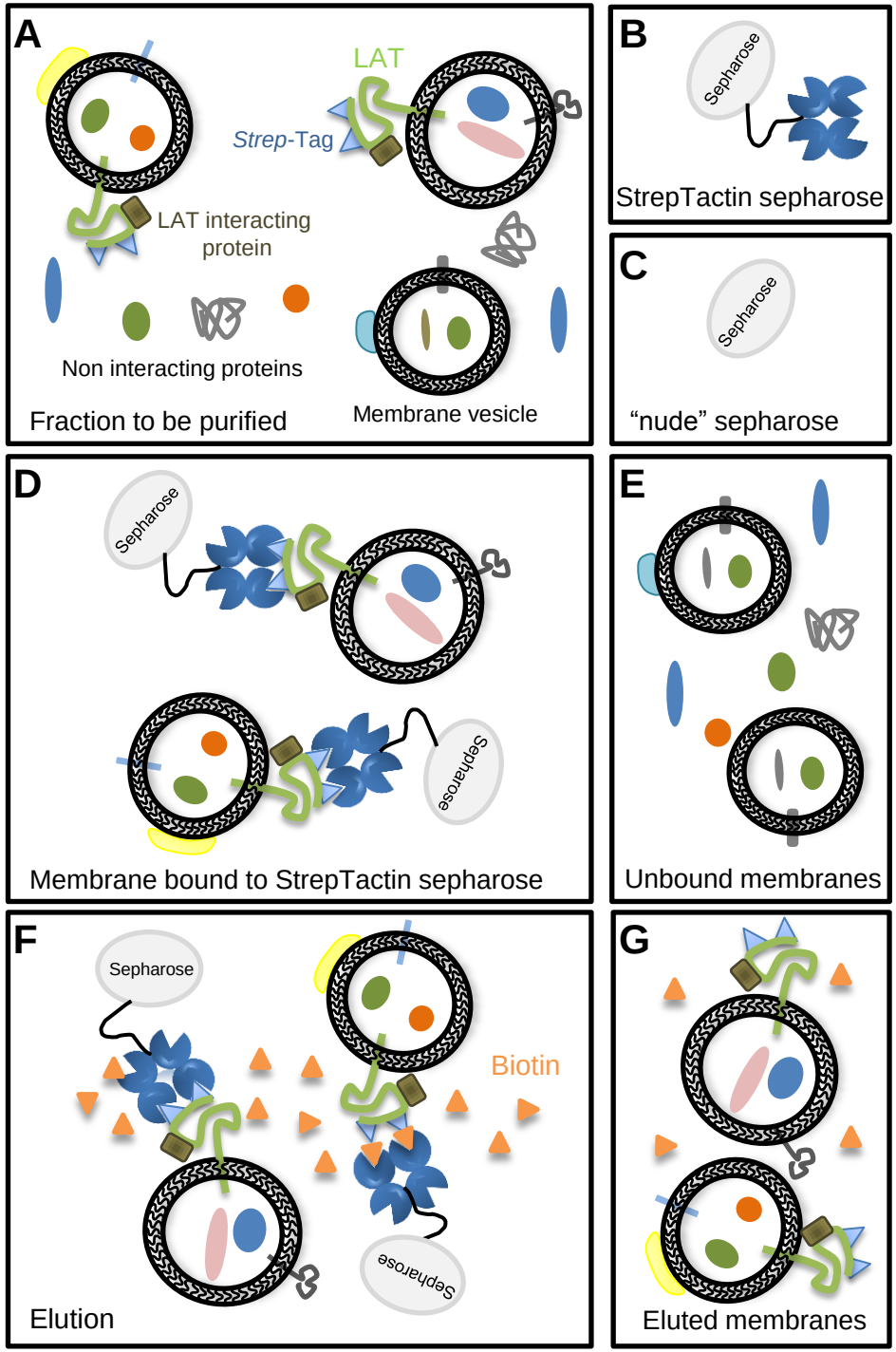
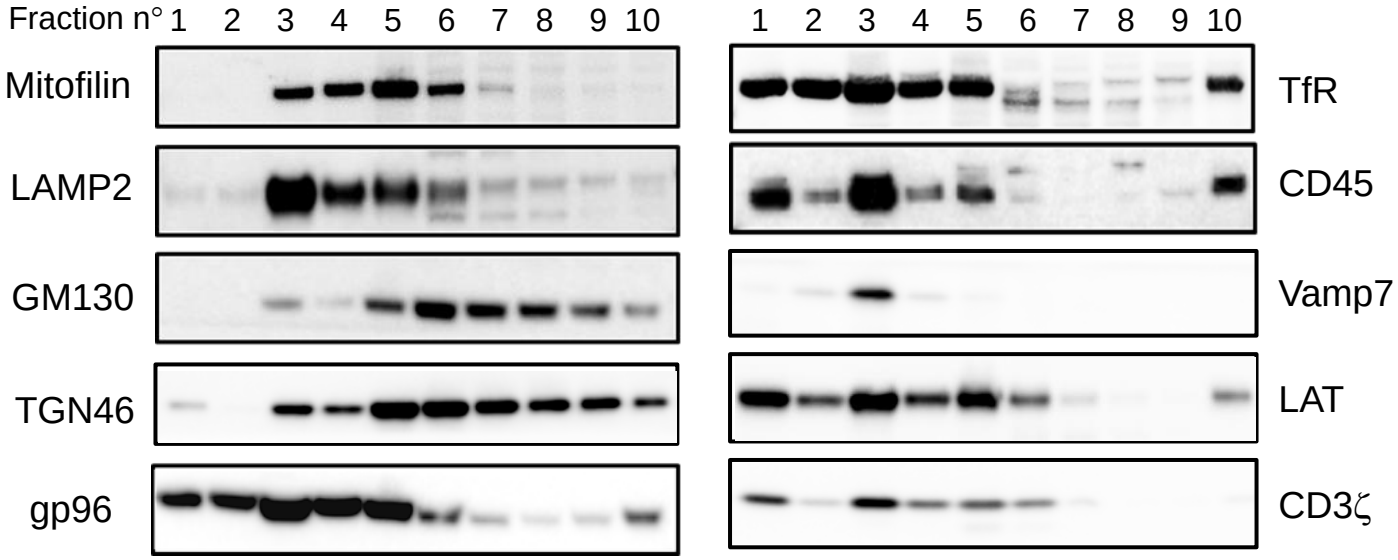

A
LAT
Strep-Tag
LAT interacting protein
Non interacting proteins
Fraction to be purified
Membrane vesicle
B
Sepharose
StrepTactin sepharose
C
Sepharose
“nude” sepharose
D
Sepharose
Sepharose
Membrane bound to StrepTactin sepharose
E
Unbound membranes
F
Sepharose
Biotin
Sepharose
Elution
G
Eluted membranes

Fig19.1

Fig19.2

A


Fraction n° 1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
Mitofilin
LAMP2
GM130
TGN46
gp96
TfR
CD45
Vamp7
LAT
CD3ζ

B

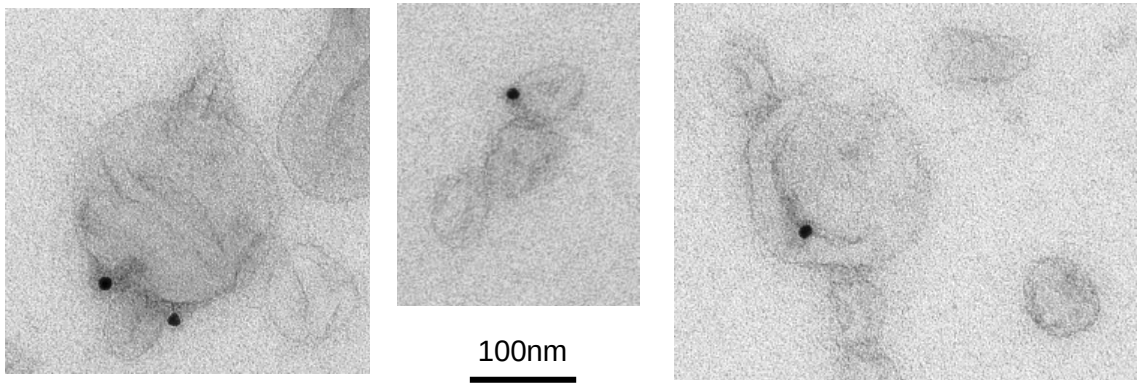

100nm

Fig19.3

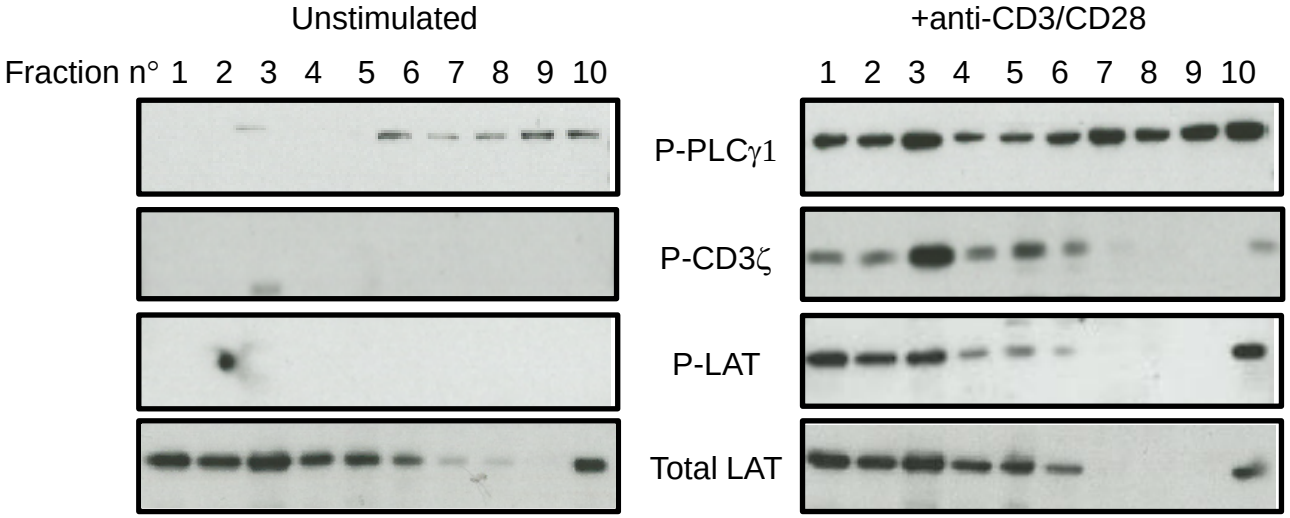
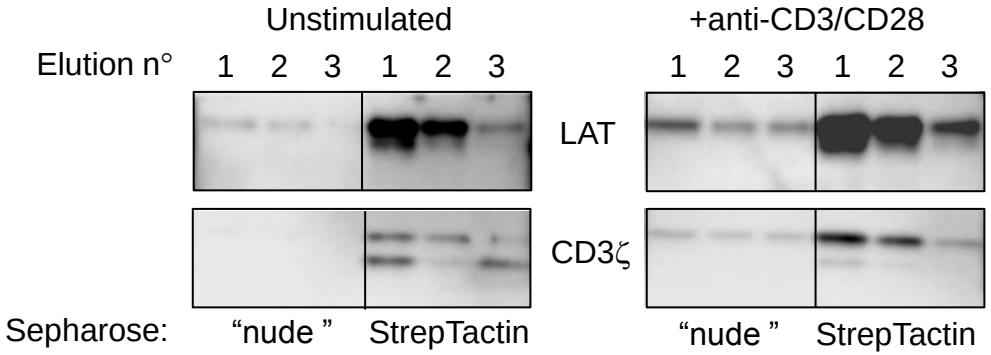

Unstimulated
+anti-CD3/CD28
Fraction n° 1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
P-PLCγ1
P-CD3ζ
P-LAT
Total LAT

Fig19.4


Unstimulated
+anti-CD3/CD28
Elution n°
1 2 3 1 2 3
1 2 3 1 2 3
LAT
CD3ζ
Sepharose:
“nude ”
StrepTactin
“nude ”
StrepTactin