Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE63130R2
Full Title:	Preparation of Bead-Supported Lipid Bilayers to study the particulate output of T cell Immune Synapses
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Additional Information:	
Question	Response
Please specify the section of the submitted manuscript.	Immunology and Infection
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Oxford/Oxfordshire/UK
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TITLE:

Preparation of Bead-supported Lipid Bilayers to Study the Particulate Output of T Cell Immune Synapses

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SUMMARY:

Here we present the protocol for the stepwise reconstitution of synthetic antigen-presenting cells using Bead-Supported Lipid Bilayers and their use to interrogate the synaptic output from activated T cells.

ABSTRACT:

Antigen-presenting cells (APCs) present three activating signals to T cells engaged in physical contact: 1) antigen, 2) costimulation/corepression, and 3) soluble cytokines. T cells release two kinds of effector particles in response to activation: trans-synaptic vesicles (tSVs) and supramolecular attack particles, which transfer intercellular messengers and mediate cytotoxicity, respectively. These entities are quickly internalized by APCs engaged in physical contact with T cells, making their characterization daunting. This paper presents the protocol to fabricate and use Bead-Supported Lipid Bilayers (BSLBs) as antigen-presenting cell (APC) mimetics to capture and analyze these trans-synaptic particles. Also described are the protocols for the absolute measurements of protein densities on cell surfaces, the reconstitution of BSLBs with such physiological levels, and the flow cytometry procedure for tracking synaptic particle release by T cells. This protocol can be adapted to study the effects of individual proteins, complex ligand mixtures, pathogen virulence determinants, and drugs on the effector output of T cells, including helper T cells, cytotoxic T lymphocytes, regulatory T cells, and chimeric antigen receptor-expressing T cells (CART).

INTRODUCTION:

The immunological synapse (IS) is a pivotal molecular structure formed at the interface of cells engaged in physical contact that facilitates the regulated exchange of juxtracrine information. Different ISs have been described in the literature, and a growing body of evidence suggests these molecular hubs are a conserved feature of cellular networks. Various immune cells, including B cells, natural killer cells, dendritic cells, macrophages, and T cells, exchange information via the assembly of short-lived contacts[1]. Multiomic studies are advancing the understanding of novel subsets of leukocytes and stromal cells driving pathogenic cellular networks and expressing surface proteins with unknown functions. As synthetic APCs, BSLBs allow the direct investigation of the functional role of individual proteins in the integration of activating signals, namely antigens and

47 costimulation/corepression, by T cells and the resulting release of effector particles referred
48 to as signal four.
49
50 This paper describes the protocols and critical technical points to consider while using BSLBs
51 to mimic the surface composition of model APCs. The protocols for the quantitative
52 measurement of immune receptors and other surface proteins on APCs are presented along
53 with the protocol for the reconstitution of synthetic APCs containing these measured
54 quantities. Then, the steps required for coculturing T cells and BSLB are presented along with
55 the protocol for the quantitative measurement of trans-synaptic particle transfer using flow
56 cytometry. Most remarkably, BSLBs facilitate studying a plasma membrane-derived
57 population of tSVs termed synaptic ectosomes (SEs). T-cell antigen receptor-enriched (TCR+)
58 SEs are shed in response to TCR triggering[2] and efficiently captured by BSLBs[3], representing
59 an excellent readout to assess the agonistic properties of antigens and the modelled
60 membrane composition. CD63+ exosomes and supramolecular attack particles (SMAPs) are
61 also released by stimulated T cells and captured by BSLBs. They can be used as additional
62 readouts of activation and the resulting exocytic and lytic granule secretion by T cells. The
63 mobilization of exocytic vesicles to the interacting pole of the T cell also facilitates the
64 directional release of cytokines, such as IL-2, IFN-γ, and IL-10 in response to activation[4-8].
65 Although T-cell released cytokines can also be detected on BSLBs, a more dedicated study is
66 currently under development to validate the quantitative analysis of cytokine release at the
67 immunological synapse.
68
69 To interrogate how specific membrane compositions influence T cells' synaptic output
70 requires defining the physiological density of the target membrane component. Flow
71 cytometry-based quantifications of cell surface proteins are an essential step in this protocol
72 and require: 1) the use of antibodies with known numbers of fluorochromes per antibody
73 (F/P), and 2) benchmark beads providing a standard reference for interpolating fluorochrome
74 molecules from measured mean fluorescence intensities (MFIs).
75
76 These benchmark standards consist of five bead populations, each containing an increasing
77 number of equivalent soluble fluorochromes (MESFs), which span the dynamic range of
78 arbitrary fluorescence detection. These standard populations yield discrete fluorescence
79 peaks, facilitating the conversion of arbitrary fluorescence units into MESFs by simple linear
80 regression. The resulting MESFs are then used alongside antibody F/P values to calculate the
81 average number of bound molecules per cell (or BSLB in later steps). The application of
82 estimated cell surface areas to the average number of detected molecules then enables the
83 calculation of physiological densities as molecules/μm^2. This quantification protocol can also
84 be adapted to the measurement of protein densities on T cells and the biochemical
85 reconstitution of membrane compositions mediating the formation of homotypic T cell
86 synapses (i.e., T-T synapses[9]). If needed, the valency of antibody binding can be further
87 estimated by using recombinant targets labeled with known numbers of fluorochromes per
88 molecule. Then, the antibody-binding valency can be calculated for the same BSLB population
89 by simultaneously comparing the number of bound fluorescent proteins and quantification
90 antibodies (using two different quantification fluorochromes and MESF standards).
91
92 The reconstitution of APC membranes requires the assembly of supported lipid bilayers (SLBs)
93 on silica beads[1]. Liposome stocks containing different phospholipid species can be harnessed

94 to form a versatile lipid-bilayer matrix, enabling the anchoring of recombinant proteins with
95 different binding chemistry (the preparation of liposomes is detailed in [10]). Once the
96 physiological density (or densities) of the relevant ligand "on cells" is defined, the same flow
97 cytometry protocol is adapted to estimate the concentration of recombinant protein needed
98 to coat BSLBs with the target physiological density. Two different anchoring systems can be
99 used either in combination or separately.
100
101 First, SLB containing a final 12.5 mol% of Ni^{2+}-containing phospholipids is sufficient to provide
102 approximately 10,000 His-tag binding sites per square micron[10] and works well to decorate
103 BSLBs with most commercially available proteins whose physiological densities do not exceed
104 this maximum loading capacity. The second loading system harnesses biotin-containing
105 phospholipids (as mol%) to load biotinylated anti-CD3e Fab (or HLA/MHC monomers) via
106 streptavidin bridges. The combination of these two BSLB decoration methods then enables
107 the flexible tailoring of BSLBs as synthetic APCs. For highly complex APC surface compositions,
108 the mol% of phospholipids and proteins can be increased to load as many proteins as the
109 question at hand requires. Once the working concentrations of proteins and mol% of
110 biotinylated phospholipids are defined, BSLBs can be assembled to interrogate the synaptic
111 output of T cells with multiparametric flow cytometry.
112
113 **PROTOCOL:**
114
115 **1.   Measurement of cell surface protein densities with quantitative flow cytometry**
116
117 1.1.   Prepare 0.22 μm-filtered human flow cytometry buffer (hFCB) by adding EDTA (to a
118 final 2 mM concentration) and human AB serum (to a final 10%) to sterile phosphate-buffered
119 saline (PBS), pH 7.4 (see **Table 1**). Filter the solution using a 0.22 μm pore filter unit to remove
120 serum impurities and store at 4 °C.
121
122 1.2.   Recover the cells and sediment them by centrifugation at 300 × *g* for 5 min at room
123 temperature (RT).
124
125 1.3.   Wash the cells twice with PBS. In each washing step, resuspend the cells in PBS to the
126 original volume (before centrifugation) and spin down at 300 × *g* for 5 min at RT.
127
128 1.4.   Count trypan blue-stained cell suspensions in a hemocytometer[11]. Alternatively, count
129 cells using electric current exclusion. For the latter, follow the CASY-TT manufacturer's
130 instructions (see the **Table of Materials**).
131
132 NOTE: The CASY-TT cell counter allows the determination of percent viable cells, cell size, and
133 cell volume.
134
135 1.5.   Calculate the volume of PBS containing 1:1,000 dilution of Fixable Viability Dye eFluor
136 780 or similar (see the **Table of Materials**) required to resuspend the cells to a staining
137 concentration of 10^7 cells/mL.
138
139 1.6.   Resuspend the cells using the viability dye–PBS solution and incubate on ice for 30
140 min.

141

142   1.7.   Remove the viability dye by adding one volume of ice-cold hFCB. Spin down at 300 ×
143   *g* for 5 min at 4 °C.

144

145   NOTE: From now onwards, keep the cold chain unbroken.

146

147   1.8.   Wash the cells with hFCB containing 1:50 dilution of Fc Receptor Blocking Solution (see
148   the **Table of Materials**). Bring the volume to a final concentration of 10^7 cells/mL and incubate
149   for an additional 15 min to achieve efficient FcgR blocking.

150

151   1.9.   Distribute 100 µL of the cell suspension (i.e., 10^6 cells) per well of a U-bottom or V-
152   bottom 96-well plate. Keep the cells on ice and protected from light (cover with aluminum
153   Foil).

154

155   1.10.   Prepare an antibody master mix in hFCB by defining the optimal antibody
156   concentrations for each fluorochrome-conjugated antibody, and most importantly, for those
157   antibodies used to determine surface protein densities. For example, for quantification of
158   ICAM-1 expression on tonsillar cell populations, as shown in **Figure 1**, prepare a mix
159   containing 1:200 dilutions of anti-CD4, anti-CD19, and anti-CXCR5 together with saturating
160   concentrations of an anti-ICAM-1 antibody with known AF647 fluorochromes per antibody
161   (i.e., 10 µg/mL, which was defined by independent antibody titration experiments).

162

163   1.11.   As additional controls, prepare an antibody master mix containing the relevant
164   **antibody isotype controls (at the same effective concentrations as their counterparts**
165   **conjugated with the same fluorochromes** for background subtraction), i.e., use 10 µg/mL of
166   a relevant AF647 isotype control.

167

168   NOTE: In the example above, such an isotype control is used to subtract background
169   fluorescence from the true ICAM-1 signal on cells.

170

171   1.12.   When quantifying protein densities on cell subsets present at low frequencies within
172   tissues, prepare fluorescence minus one (FMO) controls containing all staining antibodies
173   except for the markers of interest (see further details in [12]).

174

175   1.13.   Spin down the 96-well plate containing the cells at 300 × *g* for 5 min at 4 °C, discard
176   the supernatant, and resuspend the cells in 50 µL of either the quantification antibody master
177   mix or the isotype antibody master mix.

178

179   1.14.   Incubate the cells for at least 30 min at 4 °C and 400 rpm using a plate shaker. Protect
180   the plates from light (cover with aluminum foil).

181

182   1.15.   Wash the cells three times using hFCB and centrifuge at 300 × *g* for 5 min at 4 °C.

183

184   1.16.   Resuspend the cell pellet using 200 µL of PBS (i.e., to a final concentration of $5 × 10^6$
185   cells/mL).

186

187   1.17.   For acquisition:

188

189   1.17.1. If using a standard BD FACS Loader, transfer the samples to 5 mL polystyrene round-
190   bottom tubes (see the **Table of Materials**).

191

192   1.17.2. If using High-throughput Samplers (HTS, also referred to as plate readers), proceed
193   immediately to step 1.19.

194

195   1.18.   Before proceeding with the data acquisition for the MESF standards, check the
196   fluorescence intensity linearity maximum and minimum limits for the quantification channels.

197

198   1.19.   **Before compensation**, acquire data for the MESF standards, ensuring both the
199   dimmest and brightest populations fall in the linear range of measurement.

200

201   1.20.   Acquire the compensation samples. Keep the photomultiplier tube (PMT) voltage
202   values for the quantification channels unchanged to preserve the dynamic range of detection.
203   Perform slight adjustments in the PMT voltage of other channels before calculating the
204   compensation matrixes.

205

206   1.21.   Calculate and apply compensation.

207

208   1.22.   Acquire and save a minimum of 2×10^4 total MESF beads for each of the quantification
209   channels.

210

211   1.23.   Select the population of single cells based on their side and forward light scattering
212   areas (SSC-A and FSC-A, respectively, as shown in **Figure 1A** (i)), followed by the selection of
213   events inside the time continuum (**Figure 1A** (ii)) and low for time of flight (W) in both FSC
214   and SSC as compared to their heights (i.e., FSC-W/FSC-H, followed by SSC-W/SSC-H gating as
215   shown in **Figure 1A** (iii) and (iv), respectively). Define a final single-event gate containing
216   events with proportional FSC-A versus FSC-H distribution (**Figure 1A** (v)).

217

218   1.24.   Acquire control samples (Isotype-labeled and FMO controls).

219

220   1.25.   Acquire samples and record until a minimum of 10,000 target cells have been
221   acquired.

222

223   NOTE: the robust determination of average molecular densities requires the analysis of
224   several donors across independent experiments. This is crucial when analyzing either cell
225   subsets found in reduced frequencies or derived from scarce biological material (e.g., from
226   human tissue biopsies).

227

228   1.26.   Wash the cytometer running for 5 min with a FACS cleaning solution followed by 5 min
229   of ultrapure water before shutting down the instrument. If using the HTS, follow the options
230   under the tab **HTS and Clean Plate program**.

231

232   NOTE: To reduce sample-to-sample carryover, activate **sit (sampler) flush** or **high-throughput**
233   **sampler wash** options, which will automatically wash the cytometer between tubes or wells.

234 Before starting the acquisition, run ultrapure water for 10 min at a high flow rate to remove
235 any unwashed biological contaminants from the cytometer sample line.
236
237 1.27. Export the Flow Cytometry Standard (FCS) files.
238
239 **2.   MESF overcorrected mean (or median) fluorescence intensity (MFI) regression**
240 **analyses**
241
242 2.1.   Open the flow cytometry analysis software and load the experiment FCS files. Select
243 the population of beads based on their side and forward light scattering areas (SSC-A versus
244 FSC-A), as shown in **Figure 1A** (i).
245
246 2.2.   Control data quality by checking the distribution of single events over time, as
247 indicated in step 1.24.
248
249 NOTE: Bubbles create gaps in the distribution of events over time; this typically appears at
250 the beginning of acquisition using the HTS. Avoid selecting events flanking gaps in the
251 acquisition time as these add measurement errors due to optical aberrations.
252
253 2.3.   Focus on the single events.
254
255 2.3.1. Cells
256
257 2.3.1.1.   Identify single cells first based on their SSC-A and FSC-A distribution (**Figure 1A**
258 (i)), followed by events low for W in the sequential gates FSC-W/FSC-H (singlets-1, **Figure 1A**
259 panel (iii)) and SSC-W/SSC-H (singlets-2; **Figure 1A** panel (iv)). Define an additional singlets-3
260 gate by selecting events with proportional FSC-A and FSC-H (**Figure 1A**, panel (v)). Finally,
261 identify live cells as those negative for the fixable viability dye.
262
263 2.3.2. MESF beads
264
265 2.3.2.1.   Follow the same singlets-1 to singlets-3 discrimination as in step 2.3.1.1 (**Figure**
266 **1B**, panels (i) to (v)). Identify each of the MESF populations (blank, 1, 2, 3, and 4) based on
267 their fluorescence intensity levels (see **Figure 1B**, panel (vi)).
268
269 2.4.   Extract the MFI of MESF fractions blank and 1 to 4.
270
271 2.5.   Generate corrected MFI (cMFI) values for MESF fractions 1 to 4 by subtracting the
272 MFIs of the blank bead population from each fraction.
273
274 2.6.   Calculate the line of best fit for the relationship between cMFIs and the MESF values
275 provided by the vendor (independent variable, being fraction blank equal to zero).
276
277 2.7.   Extract the slope (**b** in the equation below) of the linear regression of MESF over cMFI
278 (**Figure 1B** panel (viii) shows a regression in which y = a + bx; with a = 0).
279

280 2.8.   Extract the median (for bimodal fluorescence distributions) or mean (for normal or
281 log-normal fluorescence distributions) fluorescence intensities from the populations of
282 interest (TFH and B cells in **Figure 1A** panel (vii).
283
284 2.9.   As in steps 2.5–2.7, extract the MFI values from isotype-labeled control cells.
285
286 2.10.   If the F/P of isotype controls is the same as the quantification antibodies, correct the
287 MFIs of stained cells by subtracting the MFIs from Isotype-labeled cells.
288
289 2.11.   If the F/P of the isotypes differs from those of quantification antibodies, extract the
290 MESF from the isotypes by dividing the MFIs of isotype-labeled cells with the slope calculated
291 in step 2.7. Subtract Isotype MESF from quantification MESF before estimating bound
292 quantification antibodies.
293
294 2.12.   Divide the MESF by the F/P of the quantification antibodies to estimate the number
295 of bound molecules per cell (Molec.$_{cell}$ as shown in the flow diagram of **Figure 1C**).
296
297 2.13.   Divide the number of bound molecules with the estimated cell surface area (CSA
298 (μm^2)) to extract the density of proteins as molec./μm^2 (**Figure 1C**). Refer to the
299 representative results section for more details.
300
301 **3.   Functional phospholipid species to use in the calibration of proteins coating BSLBs**
302
303 3.1.   Use 0.4 mM **DOPC** (1,2-dioleoyl-sn-glycero-3-phosphocholine) as the major
304 component of the lipid matrix forming the supported lipid bilayer. Dilute all other lipids
305 species in this DOPC solution.
306
307 3.2.   Use between 0.2 and 1 mol% of 0.4 mM **DOPE** (1,2-Dioleoyl-sn-glycero-3-
308 phosphoethanolamine) conjugated to dyes, including ATTO 390, 488, or 565 (see the **Table of
309 Materials**), to generate BSLBs with intrinsic fluorescence.
310
311 NOTE: The intrinsic fluorescence of BSLBs facilitates the identification of single BSLBs and
312 single cells in synaptic transfer experiments.
313
314 3.3.   Use 12.5 mol% of 0.4 mM **DGS-NTA(Ni) (**1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-
315 carboxypentyl) iminodiacetic acid) succinyl]) to anchor His-tagged proteins. Keep the mol% of
316 DGS-NTA(Ni) constant and perform 2-fold titrations of the His-tagged proteins starting with
317 100 nM as the highest concentration. Leave one condition with no protein as a negative
318 control for absolute quantifications.
319
320 NOTE: Accessory signals and adhesion molecules, such as ICAM-1, are designed with a 12-His
321 tag to increase the affinity of the protein for DGS-NTA(Ni).
322
323 3.4.   Use **Biotinyl Cap PE** (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap
324 biotinyl)) to anchor biotinylated proteins via biotin-streptavidin-biotin bridges. Use a 5-fold
325 serial titration of 0.4 mM Biotinyl Cap PE covering between 10 mol% and 0 mol% (as negative
326 control). Keep the concentration of streptavidin and biotinylated proteins constant (200 nM)

in both calibration experiments and the reconstitution of synthetic APCs for synaptic transfer experiments.

NOTE: The titration of Biotinyl Cap PE sets the mol% required for loading physiological monobiotinylated HLA peptide or anti-CD3ε Fab to be used as monomeric antigens or antigen surrogates, respectively. In the reconstitution of synthetic APCs experiments, a fixed mol% of Biotinyl Cap PE is used to produce a target density of antigen or anti-CD3ε Fab.

4.   Preparation of supplemented HEPES buffered saline containing 1% serum albumin

NOTE: Supplemented HEPES-buffered saline containing 1% human serum albumin (HBS/HSA) or 1% bovine serum albumin (HBS/BSA) is required in the washing and protein loading steps of BSLBs (**Table 1**). Prepare a 10x HBS stock solution and the working buffer as fresh as possible; keep refrigerated and use within one month. While BSA is a cheaper alternative to HSA, it provides efficient blockage of Ni-chelating lipids[13] and is recommended for high-throughput experiments.

4.1.   Prepare a 10x stock buffer solution of supplemented HBS containing 200 mM HEPES, 7 mM Na_2HPO_4, 1400 mM NaCl, 50 mM KCl, 60 mM glucose, 10 mM $CaCl_2$, and 20 mM $MgCl_2$.

NOTE: Dissolving $MgCl_2$ is highly exothermic and a burn hazard. Add this salt slowly and on a large volume of solvent. Avoid preparing concentrated solutions (i.e., 1 M) of these salts as they tend to precipitate over time, making their precise addition to the working buffer difficult.

4.2.   Filter the 10x solution using a 0.22 µm filter unit and keep it sterile at 4 °C.

4.3.   For 500 mL of HBS/has, take 50 mL of the supplemented 10x HBS solution, adjust the pH to 7.4 if needed, and make up the volume to 483.4 mL with ultrapure water.

4.4.   Add 16.6 mL of 30% HSA solution to the 483.4 mL of HBS, pH 7.4.

4.5.   For 500 mL of HBS/BSA, dissolve 5 g of BSA in HBS and incubate at 37 °C for 30 min. Then, mix gently at RT by inverting the bottle periodically until there are no visible protein crystals or clumps.

4.6.   Filter the resulting solution from step 4.5 using a 0.22 µm filter unit (see the **Table of Materials**) and store it at 4 °C.

5.   Protein density calibrations on BSLBs

5.1.   Before taking the 5.00 ±0.05 µm diameter non-functionalized silica beads, mix the stock solution well and resuspend any big clumps of beads sedimented on the bottom of the flasks.

NOTE: Silica beads tend to sediment quickly, which might lead to counting errors. Mix vigorously by pipetting up and down half of the maximum volume of a P1000 micropipette.

374

375   5.2.   Dilute 1 µL of bead solution in 1,000 µL of PBS, count the beads using a
376   hemocytometer chamber, and calculate their concentration per mL.

377

378   NOTE: Trypan blue staining is not needed for visualizing silica beads.

379

380   5.3.   Calculate the volume of silica beads needed for 5×10^5 final BSLBs per point of the
381   titration.

382

383   5.4.   Transfer the required volume of silica beads to a sterile 1.5 mL microcentrifuge tube.

384

385   5.5.   Wash the silica beads three times with 1 mL of sterile PBS, centrifuge the beads for 15
386   s on a benchtop microcentrifuge at RT (at fixed rpm).

387

388   NOTE: When removing the washing solution, avoid disturbing the bead pellet. A small buffer
389   column will not affect the spreading of the liposomes composing the liposome master mix as
390   these are also in PBS.

391

392   5.6.   Prepare three volumes of the liposome master mix to assemble the BSLB on the
393   washed silica beads (e.g., if the initial total volume of silica beads is 20 µL, prepare a minimum
394   of 60 µL of the liposome master mix).

395

396   **5.7. For Biotinyl Cap PE mol% titrations**

397

398   5.7.1.   Prepare the lipid master mixes containing 5-fold dilutions of Biotinyl Cap PE.

399

400   5.7.1.1.   Dilute the 0.4 mM Biotinyl Cap PE mol% in a 100% DOPC matrix.

401

402   5.7.1.2.   Mix each Biotinyl Cap PE mol% titration point at a 1:1 (vol:vol) ratio with a
403   solution of 0.4 mM 25% DGS-NTA(Ni) such that a final 12.5 mol% of Ni-containing lipids is
404   present in all titrations.

405

406   NOTE: The 12.5 mol% (vol:vol%) of Ni-containing lipids represent the mixed lipid composition
407   of BSLBs on which His-tagged proteins can also be tested in parallel calibrations. For example,
408   since all liposome stocks are prepared at the same molar concentration, to reach the target
409   mol% mixture in 200 µL of final liposome mix, simply mix 100 µL of 25 mol% of Ni-containing
410   DGS-NTA with 100 µL of 100 mol% DOPC.

411

412   5.7.2.   Transfer 5×10^5 washed silica beads to 1.5 mL microcentrifuge tubes, such that one
413   Biotinyl Cap PE mol% titration point is assembled per tube.

414

415   5.7.3.   Add the Biotinyl Cap PE mol% titration master mixes to the washed silica beads and
416   gently mix by pipetting up and down half of the total volume. Avoid forming bubbles, which
417   in excess destroy the lipid bilayer.

418

419   5.7.4.   Add Argon (or Nitrogen) gas on the tube containing the now-forming BSLBs to displace
420   air and protect the lipids from oxidation during mixing.

421

422 5.7.5. Add Argon to the 0.4 mM lipid stocks before storage and manipulate using a sterile
423 technique.

424

425 NOTE: Connect a small tubing to the Argon/Nitrogen gas cylinder. Before adding gas to the
426 tubes, adjust the gas cylinder regulator so that the pressure is set no higher than 5 psi.
427 Connect a sterile pipette tip to the outlet tubing to direct the gas stream inside the liposome
428 stock for 5 s and quickly close the lid. In the case of lipid stocks, seal the tube's lid with paraffin
429 film before storing it at 4 °C.

430

431 5.7.6. Move the BSLBs to a vertical, variable-angle laboratory mixer (see the **Table of**
432 **Materials**) and mix for 30 min at RT using an orbital mixing of 10 rpm.

433

434 NOTE: This step will prevent the sedimentation of beads during the formation of the
435 supported lipid bilayer.

436

437 5.7.7. Spin down the beads by centrifuging for 15 s at RT on a benchtop minicentrifuge, and
438 then wash three times with 1 mL of HBS/HSA (BSA) to remove excess liposomes.

439

440 5.7.8. Block the formed BSLBs by adding 1 mL of 5% casein or 5% BSA containing 100 µM of
441 NiSO$_4$ to saturate NTA sites and 200 nM streptavidin to coat all biotin-anchoring sites on the
442 BSLBs uniformly. Mix gently by pipetting up and down half of the total volume and incubate
443 in the vertical mixer for **no longer than 20 min at RT** and 10 rpm.

444

445 5.7.9. Spin down the BSLBs by centrifuging for 15 s at RT on a benchtop minicentrifuge, and
446 then wash three times with 1 mL of HBS/HSA (BSA) buffer.

447

448 NOTE: Keep washed beads vertically with a small volume of wash buffer covering the BSLBs.
449 Avoid the dehydration of the BSLBs as air will destroy the lipid bilayer.

450

451 5.8. **For the titration of His-tagged proteins on 12.5 mol% of DGS-(Ni) NTA-containing**
452 **BSLBs**

453

454 5.8.1. Prepare three volumes of liposome master mix containing a final 12.5 mol% of DGS-
455 NTA(Ni).

456

457 5.8.2. Use the liposome master mix to resuspend the washed silica beads and gently mix by
458 pipetting up and down half of the total volume. Avoid forming bubbles, which in excess
459 damage the lipid bilayer.

460

461 5.8.3. Add Argon (or Nitrogen) gas on the tube containing the now-forming BSLBs to displace
462 air and protect the lipids from oxidation during mixing.

463

464 5.8.4. Add Argon to the 0.4 mM lipid stocks before storage and manipulate using a sterile
465 technique.

466

5.8.5. Move the BSLBs to the vertical mixer and mix for 30 min at RT using orbital mixing at 10 rpm.

5.8.6. Spin down the beads by centrifuging for 15 s at RT on a benchtop minicentrifuge, and then wash three times with 1 mL of HBS/HSA (BSA) to remove excess liposomes.

5.8.7. Block the formed BSLBs by adding 1 mL of 5% casein (or 5% BSA) containing 100 μM of NiSO₄ to saturate NTA sites on the BSLBs. Mix gently and incubate in the vertical mixer for **no longer than 20 min at RT** and 10 rpm.

5.8.8. Wash three times using HBS/HSA (BSA) to remove the excess blocking solution.

5.8.9. In a new U-bottom 96-well plate prepare 2-fold serial dilutions of the proteins.

5.8.9.1.   Prepare a starting concentration of 100 nM for the protein of interest in a total volume of 200 μL of HBS/HSA (BSA) buffer, distribute 100 μL of this solution in the first column, and the remaining 100 μL on top of column #2 containing 100 μL of HBS/HSA (BSA) buffer.

5.8.9.2.   Continue by serially transferring 100 μL from column #2 to column #3 and repeat as necessary to cover all titration points. Leave the last column of the series with no protein, as this will be used as the blank reference for quantification.

5.8.10. Resuspend the prepared BSLBs in a volume such that 5×10^5 BSLB are contained in 100 μL of HBS/HSA (BSA) buffer.

5.8.11. Transfer 100 μL of the BSLB suspension to wells of a second U-bottom 96-well plate, such that each well receives 5×10^5 BSLBs.

5.8.12. Spin down the second plate containing BSLBs for 2 min at $300 \times g$ and RT and discard the supernatant.

5.8.13. Transfer the 100 μL volumes from the protein titration plate to the plate containing the sedimented BSLBs. Mix gently, avoid excess bubble generation while pipetting, and incubate for 30 min at RT and 1,000 rpm using a plate shaker. Protect from light with aluminum foil.

5.8.14. Wash the plate three times with HBS/HSA (BSA) buffer using sedimentation steps of $300 \times g$ for 2 min at RT.

5.8.15. If the recombinant protein used in the calibration is directly conjugated to fluorochromes and has known F/P values, proceed to step 5.8.20.

5.8.16. If the recombinant protein used in the calibration is unlabeled or conjugated to fluorochromes or no MESF bead standard is available, use Alexa Fluor 488 or 647-conjugated antibodies with known F/P values to stain the protein-coated BSLB.

514 5.8.17. Stain with saturating concentrations of quantification antibodies.
515
516 NOTE: Depending on the target expression level, these range between 5 and 10 µg/mL.
517
518 5.8.18. Stain for 30 min at RT and 1,000 rpm using a plate shaker. Protect from light using
519 aluminum foil.
520
521 5.8.19. Wash twice with HBS/HSA (BSA) buffer and once with PBS using sedimentation steps
522 of 300 × g for 2 min at RT.
523
524 NOTE: Use PBS, pH 7.4 to resuspend the washed BSLBs before acquisition. Do not use buffers
525 containing protein, as this leads to the formation of bubbles during the automatic mixing of
526 samples with high-throughput samplers.
527
528 5.8.20. Acquire the MESF standards, making sure the brightest peaks remain in the linear
529 detection range for the quantification detector (channel), as shown in **Figure 1B** (vii).
530
531 5.8.21. Acquire the samples manually or using HTS. If using the latter, resuspend BSLBs in 200
532 µL of PBS and acquire 100 µL using a flow rate between 2.5 and 3.0 µL/s, a mixing volume of
533 100 µL (or 50% of the total volume if the resuspension volume is less), a mixing speed of 150
534 µL/s, and five mixes to ensure BSLBs are monodispersed.
535
536 5.8.22. Export the FCS files.
537
538 5.8.23. Focus on single events for the analyses (**Figure 2A**), as doublets or triplets will
539 introduce error in the determination. Use nested identification of single events as indicated
540 in protocol step 1.2.3.
541
542 5.8.24. Measure the MFI of each MESF fraction (1–4) and subtract the MFI of blank beads to
543 extract corrected MFIs (cMFI).
544
545 5.8.25. Perform a linear regression analysis to extract the slope (**b**) of MESF over the cMFI
546 calculated for MESF standards, which will be used in step 5.33.
547
548 5.8.26. Extract the MFI of each titration point and subtract the MFI of beads without protein
549 to obtain cMFIs.
550
551 5.8.27. Divide the cMFIs with the slope calculated in step 5.31 to extract the MESF bound to
552 BSLBs for each titration point.
553
554 5.8.28. Divide MESF bound to BSLBs by the F/P value of the quantification antibody to extract
555 the average number of molecules bound per BSLB.
556
557 5.8.29. Using the diameter of the BSLBs (5.00 ± 0.05 µm), extract the bead surface area (SA =
558 $4pr^2$) to calculate the final densities of protein (molec./µm^2) per titration point (protein
559 concentration).
560

561 5.8.30. Perform a new regression analysis of **protein concentration** over **protein density** to
562 calculate the slope (b) of the line of best fit.
563
564 NOTE: The concentration of 12.5 mol% of DGS-NTA(Ni) confers a maximum anchoring
565 capacity of approximately 10,000 molec./μm^2 [10] without inducing the nonspecific activation
566 of T cells or affecting the lateral mobility of the SLBs.
567
568 6. **Performing synaptic transfer experiments between T cells and BSLBs**
569
570 **6.1.** Before running the synaptic transfer experiment
571
572 6.1.1. Acquire non-fluorescent BSLBs, BSLBs with fluorescent lipids, unstained cells (or
573 compensation beads; see the **Table of Materials**), and single-color-stained cells (or
574 compensation beads) to identify the instrument's fluorescence spectrum interactions. Focus
575 on those detectors with high spillover spreading to redesign the polychromatic panel,
576 increase sensitivity, and reduce the measurement error on critical detectors (see [14]).
577
578 6.1.2. Titrate the detection antibodies to find the optimal concentration, enabling positive
579 events detection without compromising the detection of negatives.
580
581 NOTE: Repeat this step whenever there is an antibody lot change as the F/P values and
582 brightness vary from batch to batch.
583
584 6.1.3. Optional: Optimize the PMT voltages by acquiring the sample at different voltage
585 ranges (i.e., a voltage walks) to find the PMTs leading to optimal signal over noise (i.e.,
586 separation of negatives and positives while ensuring the signal of the brightest population
587 remains in the linear range).
588
589 **6.2.** Measurement of T-cell output transfer to BSLBs
590
591 6.2.1. Prepare supplemented RPMI 1640 (herein R10 medium) containing 10% of heat-
592 inactivated fetal bovine serum (FBS), 100 μM non-essential amino acids, 10 mM HEPES, 2 mM
593 L-glutamine, 1 mM sodium pyruvate, 100 U/ml of penicillin, and 100 μg/mL of streptomycin.
594 Use R10 medium to culture and expand T cells.
595
596 6.2.2. On **day 1**, isolate T cells from peripheral blood or leukoreduction system (LRS)
597 chambers. Use immunodensity cell isolation and separation kits (see the **Table of Materials**)
598 for enrichment of human CD4$^+$ and CD8$^+$ T cells.
599
600 6.2.3. Seed the cells at a final concentration ranging between 1.5 and 2.0 × 10^6 cells/mL,
601 using 6-well plates with no more than 5 mL total per well.
602
603 6.2.4. Activate T cells using a 1:1 ratio of human T cell activation (anti-CD3/anti-CD28)
604 magnetic beads (see the **Table of Materials**) and add 100 IU of recombinant human IL-2 to
605 support cell proliferation and survival.
606

607 6.2.5. On **day 3**, remove the activating magnetic beads using magnetic columns (see the **Table**
608 **of Materials**). Ensure that magnetic beads remain attached to the sides of the tube before
609 recovering the cells for additional washing steps.

611 6.2.6. Wash the magnetic beads once more with 5 mL of fresh R10 medium, mix well, and
612 put them back in the magnet**.** Recover this volume and mix with the cells recovered in step
613 6.2.5.

615 6.2.7. Resuspend the cells to 1.5–2 × 10^6 cells/mL in fresh R10 containing 100 U/mL of IL-2.
616 Replenish medium after 48 h, making sure that the last addition of IL-2 is 48 h before the day
617 of experimentation (days 7 to 14 of culture).

619 6.2.8. **On the day of the synaptic transfer experiment**, prepare the **Synaptic Transfer Assay**
620 **medium** (see **Table 1**) by supplementing Phenol Red-free RPMI 1640 medium with 10% FBS,
621 100 µM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml of
622 penicillin, and 100 µg/mL of streptomycin. Do not include recombinant human IL-2.

624 6.2.9. Count cells using either trypan blue staining or electric current exclusion and
625 resuspend them to a final concentration of 2.5 × 10^6 cells/mL in medium. If excessive cell
626 death is observed (>10%), remove dead/dying cells using a mixture of polysaccharide and
627 sodium diatrizoate (see the **Table of Materials**) as follows:

629 6.2.9.1.   Layer 15 mL of cell culture on top of 13 mL of the polysaccharide–sodium
630 diatrizoate solution.

632 6.2.9.2.   Centrifuge for 1,250 × *g* for 20 min at RT with minimum acceleration and
633 deacceleration. Collect the cell layer (cloud) in the interface between the medium and
634 the polysaccharide–sodium diatrizoate solution.

636 6.2.9.3.   Wash the cells at least twice with prewarmed Synaptic Transfer Assay medium
637 (prepared in step 6.2.7).

639 6.2.9.4.   Count and resuspend the cells to a final concentration of 2.5 × 10^6/mL using
640 Synaptic Transfer Assay medium. Coculture 100 µL of this cell suspension with BSLBs (see step
641 6.2.15).

643 6.2.10. Calculate the number of BSLBs needed for the experiment; consider all the different
644 protein master mixes and antigen titrations to be tested (either biotinylated HLA/MHC-
645 peptide monomers or monobiotinylated anti-CD3ε Fab).

647 6.2.11. Assemble the BSLBs by following the same steps from protocol section 5 but this time,
648 combine all the titrations of proteins and lipids required to reconstitute a complex APC
649 membrane (**Figure 3A**). Keep the same vol: vol relationships between the initial silica beads
650 volume and the volume of protein mix used during calibrations, as well as times and
651 temperatures used in the loading of BSLBs. For example, if 0.5 µL of silica beads/well and 100
652 µL/well of protein mix were used for the initial calibration, maintain 5:1,000 vol:vol ratios to
653 prepare the BSLBs to be cocultured with T cells.

6.2.12. Once BSLBs have been loaded with the protein mix of interest, wash the BSLBs twice with HBS/HSA (BSA) to remove excess unbound proteins. Use sedimentation speeds of 300 × g for 2 min at RT in each washing step and discard the supernatants.

6.2.13. Resuspend the $5 × 10^5$ BSLBs per well in 200 µL.

6.2.14. Transfer 100 µL per well to a new U-bottom 96-well plate to make a duplicate, such that the final amount of BSLB per well is $2.5 × 10^5$.

6.2.15. Spin down the BSLBs at 300 × g for 2 min and RT and discard the supernatant.

6.2.16. Resuspend the BSLBs using 100 µL of T cell suspension; mix gently to prevent the formation of bubbles.

6.2.17. Incubate the cocultures for 90 min at 37 °C.

NOTE: Alternatively, cells and beads can be resuspended in HBS/HSA (BSA) buffer instead of Phenol-Red free RPMI for the coculture. In this case, the incubation must be performed in a non-CO₂ incubator as this gas will rapidly acidify the buffer in the absence of bicarbonate.

6.2.18. Cool down the BSLB–T cell cocultures by first incubating the cells at RT for a minimum of 15 min. Protect them from light.

6.2.19. Centrifuge the cells for 5 min at 500 × g and RT; discard the supernatant.

6.2.19. Resuspend the cells in RT 2% BSA–PBS (Ca^{2+} and Mg^{2+}-free) for blocking. Place the cells on ice for 45 min. Protect from light.

6.2.20. While incubating the cells, prepare the antibody master mix using ice-cold 0.22 µm-filtered 2% BSA in PBS as a staining buffer, which will provide extra blocking.

NOTE: Some batches of antibodies conjugated to Brilliant Violet dyes tend to bind to BSLBs nonspecifically. Blocking with 5% BSA–PBS helps to reduce this noise. **From now on, make sure to keep the cold chain unbroken.**

6.2.21. Spin down the cocultures at 500 × g for 5 min and 4 °C. Before discarding the supernatants, briefly make sure the pellet is present by inspecting the bottom of the 96-well plate using a backlight.

6.2.22. Using a multichannel pipette, resuspend the cells in the staining master mix containing optimized antibody concentrations.

NOTE: Use pipette tips with no filter to prevent the generation of bubbles and errors in the distribution of staining volumes.

6.2.23. Include isotype-labeled cells and BSLBs, fluorescent and non-fluorescent BSLBs, and cells and BSLBs stained alone. Respect the total number of events per well for all controls (i.e., only BSLBs containing 5×10^5 BSLB/well, and only cell controls containing 5×10^5 cells/well to avoid a relative increase of antibodies per stained event).

6.2.24. Mix gently by pipetting up and down half of the volume and incubate for 30 min on ice. Protect from light.

6.2.25. Wash the cells and BSLBs twice using ice-cold 2% BSA–PBS, pH 7.4, and sedimentation steps of $500 \times g$ for 5 min at 4 °C. Resuspend the washed cocultures in 100 µL of PBS and acquire immediately.

6.2.26. **If fixation is needed,** fix using 0.5% w/v of PFA in PBS for 10 min, wash once, and keep in PBS until acquisition. Protect from light.

6.2.27. **Before compensation**, acquire MESF standards, ensuring both the dimmest and brightest populations fall in the linear range of measurement.

6.2.28. **Acquire compensation samples, calculate compensation, and apply the compensation matrix (link) to the experiment.**

6.2.29. Acquire and save a minimum of 2×10^4 total MESF standards for each of the quantification channels.

6.2.30. **For acquisition using high-throughput samplers,** set instrument acquisition to standard, set sample acquisition to 80 µL (or 80% of total volume), sample flow rate between 2.0 and 3.0 µL/s, sample mixing volume of 50 µL (or 50% of the total volume to avoid bubble formation during mixing), sample mixing of 150 µL/s, and mixing per well between 3 and 5.

6.2.31. Acquire a minimum of 1×10^4 single BSLBs per sample (refer to **Figure 3B** panels (i)-(vi) for the reference gating strategy).

6.2.32. Wash the cytometer running for 5 min a cleaning solution followed by 5 min of ultrapure water before shutting down the instrument. If using the HTS, follow the options under the tab **HTS and the Clean** option.

6.2.33. Export FCS files.

7.   Measuring the synaptic transfer of particles to BSLB

7.1.   Open the experiment FCS files. Select the population of cells and BSLBs based on their side and forward light scattering areas (SSC-A versus FSC-A), as shown in **Figure 3B** (i).

7.2.   Select the events within the continuous acquisition window (**Figure 3B** (ii)).

7.3.   Focus on the single events of both **cells and BSLB;** identify single cells first based on low W in the sequential gates FSC-W/FSC-H (singlets-1, **Figure 3B** panel (iii)) and SSC-W/SSC-

747   H (singlets-2; **Figure 3B** panel (iv)). Define an additional singlets-3 gate by selecting events
748   with proportional FSC-A and FSC-H (**Figure 3B**, panel (v)).
749

750   7.4.   Extract the MFI of MESF fractions blank and 1 to 4 and from single cells and MESF for
751   each experiment sample.
752

753   7.5.   Generate corrected MFI (cMFI) values for MESF fractions 1 to 4 by subtracting the MFI
754   of the blank bead population from each fraction.
755

756   7.6.   Generate cMFIs for single BSLBs and cells (refer to **Figure 3C** panel (i)).
757

758   7.7.   Use the signal from BSLB stained with isotype control antibodies to correct the MFI of
759   BSLB stained with antibodies against the relevant T cell markers. Use cMFI to calculate the
760   normalized synaptic transfer percent (NST%) by using the equation shown in **Figure 3C** panel
761   (ii).
762

763   7.8.   If interested instead in the particles specifically transferred in response to TCR
764   triggering, subtract the signal from null BSLBs from the MFI of agonistic BSLBs. Use this cMFI
765   to calculate the **TCR-driven NST%** by using the equation shown in **Figure 3C** panel (ii).
766

767   7.9.   **If interested in determining the total number of molecules transferred** as particle
768   cargo across the T cell-BSLB interface, acquire MESF benchmark beads using the same
769   instrument settings and acquisition session for T cell-BSLB cocultures.
770

771   7.10.   Analyze MESF bead populations and extract their cMFIs as indicated in protocol steps
772   5.8.15 to 5.8.17. Calculate the slope of the line of best fit for the regression analysis of MESF
773   over cMFI.
774

775   7.11.   Use the calculated slope to extract the number of MESF deposited on BSLBs. Use cMFIs
776   calculated using either isotype controls or null BSLB as blanks to extract the number of MESF
777   transferred specifically to stimulating BSLBs.
778

779   7.12.   Calculate the number of molecules of markers transferred to BSLBs by dividing the
780   calculated (average) MESFs per BSLB by the F/P value of the quantification antibody.
781

782   **REPRESENTATIVE RESULTS:**
783

784   **FCM for absolute protein quantification on the cell surface**
785   The reconstitution of BSLBs presenting physiological densities of ligands requires the
786   estimation of total protein densities on the modeled cell subset. To reconstitute BSLBs,
787   include any relevant ligand expected to play a role in the signaling axis of interest alongside
788   proteins supporting the adhesion and functional interaction between BSLB and cells, such as
789   ICAM-1 and costimulatory molecules, e.g., CD40, CD58, and B7 receptors (CD80 and CD86).
790   Additional proteins can be added depending on the question at hand, including costimulatory
791   molecules such as ICOSL[3], PD-L1, and PD-L2[15]. For any other molecules, reconstitute BSLBs
792   using molecular densities determined by directly analyzing cells with quantitative flow
793   cytometry. For directly conjugated antibodies, BioLegend provides F/P values for each

794 antibody lot number. Antibodies can also be labeled in-house and the F/P ratios determined
795 by spectrophotometry, providing an alternative when there are no commercial antibodies
796 conjugated to the desired fluorochrome. Since we use the same antibodies to calibrate the
797 number of recombinant proteins on the surface of cells and BSLBs, there is no need to correct
798 the antibody-binding valency as this remains constant. If the antibody-binding valency is
799 required, use both recombinant proteins and antibodies with known F/P to decorate BSLBs
800 and compare molecules of loaded recombinant proteins with the number of bound antibodies
801 following staining under saturating conditions.
802
803 The bound antibody molecules per cell can be estimated using a dedicated monochromatic
804 flow cytometric measurement or a polychromatic panel of antibodies intended to estimate
805 absolute protein densities on a relatively infrequent subset of cells within a tissue of interest,
806 such as palatine tonsils. **Figure 1** shows representative measurements of densities of ICAM-1
807 in CXCR5+ B cells and follicular T cells (TFH) as an example. The same staining protocol and
808 flow cytometry analysis principles shown in **Figure 1A** can be used to measure protein
809 densities on epithelial cells, stromal cells, monocytes, monocyte-derived dendritic cells
810 (moDCs), or on B and T lymphocytes in other human and mouse tissues. For tissues different
811 from blood and tonsils, caution must be taken when isolating cells using protease cocktails,
812 as the prolonged exposure of cells to digesting enzymes reduces cell surface expression levels.
813
814 Focus the acquisition and analyses on single, live cells within the continuous acquisition
815 window (**Figure 1A** ii), as events outside the time continuum aberrantly scatter light,
816 compromising quantification. To increase the accuracy of determinations, reduce the
817 nonspecific staining of APCs by efficient blocking of FcγRs. The human serum and EDTA
818 present in hFCB enable efficient FcγR blocking while chelating free Ca^{2+} to reduce the
819 spontaneous aggregation of cells during their manipulation and staining (black arrows in
820 **Figure 1A** (iii) and (iv) show remaining doublets in suspensions of tonsillar cells).
821
822 Keeping track of the instrument performance by using the setup and tracking beads and the
823 software (or similar; see the **Table of Materials**) is critical for the reproducibility of
824 quantifications over time, especially in later steps when the synaptic transfer of particles to
825 BSLB is measured using only MFIs (i.e., for fluorochromes for which there are no MESF
826 standards). Similarly, check the linear range (i.e., **linearity minimum and linearity maximum**)
827 of arbitrary fluorescence units for the quantification detector to be used alongside the MESF
828 standards such that each calibration point keeps the linear relationship between fluorescence
829 and the number of fluorochromes.
830
831 The preparation of samples "blank" for fluorescence, or samples providing an idea of the
832 background staining noise, are essential to subtract the nonspecific fluorescent signal. Isotype
833 controls and/or biologically null samples (e.g., knockouts) are essential to correct for the
834 background signal of cells and extract the true signal derived from the quantification
835 antibodies (**Figure 1A** panel (viii)). Similarly, standard MESF beads use a dedicated blank
836 population to subtract the background signal from each truly positive bead population (**Figure
837 1B** panels (vii) and (viii)). Once the regression analyses are performed and the slope defining
838 the relationship between MESF and corrected MFIs is extracted, the conversion to absolute
839 molecular densities follows simple mathematical operations (**Figure 1C**).
840

841 To estimate CSA in **Figure 1C**, electrical current exclusion (CASY-TT) was used to extract
842 measurements of cell volume and diameter from thousands of cells. The resulting CSA
843 estimated from the calculation of surface area for spheres ($4\pi r^2$) varies with the activation
844 state of the cells, with observed values of 170.37 ± 4.91 μm^2 for nonactivated B cells and
845 234.52 ± 1.53 μm^2 and 318 ± 24.45 μm^2 for nonactivated and activated T cells, respectively.
846 These CSAs are comparable to those estimated by imaging techniques such as three-
847 dimensional refractive index tomography of nonactivated lymphocytes[16].
848
849 Once the range of physiological densities has been defined (e.g., by comparing surface
850 densities on cells undergoing different activation programs), BSLBs can be used to model
851 those surfaces. A titration of biotinylated antigenic HLA-peptide monomers provided by the
852 NIH tetramer facility (or monobiotinylated monomeric anti-CD3ε-Fab) can be used alongside
853 ICAM-1 12-His to reconstitute a canonical APC membrane. Commercial proteins tagged with
854 6, 9, 12, and 14 His can be used to decorate the surface of BSLB (see **Figure 2A** for examples
855 with ICAM-1 12-His). Protein titrations together with quantitative FCM analyses provide a
856 robust methodology to reconstitute physiological APC surfaces and test their effect on the
857 synaptic output of different T cell subsets.
858
859 For studying the output of T cell synapses, use a 1:1 BSLB-to-T cell ratio to ensure that, on
860 average, one cell will interact with one BSLB over the studied period. We have observed that
861 the material transfer is proportional to the incubation time, providing a versatile platform for
862 detecting molecules transferred in low quantities across the cell:BSLB interface. An
863 appropriate panel design is thus critical to increasing the sensitivity and reliability of the
864 detection of trans-synaptic material, as in the case of tSVs, the output varies between 25 and
865 36 vesicles/cell/20 min[2,3]. Test first the spectral spillover of each fluorochrome-labeled
866 antibody and lipid. When high spillover is observed, we recommend the titration of staining
867 antibodies and the percent of fluorescent lipids composing the BSLB, as well as PMT voltage
868 walks to reduce the spreading error on compensated samples and enhance the signal over
869 noise ratio, respectively (refer to[12,14,17] for a dedicated introduction to the subject).
870
871 The use of quantification controls, including null BSLBs (lacking antigen or anti-CD3ε Fab) and
872 either knockout cells or isotype-labeled samples[18], is essential to accurately measure the
873 transfer of effector tSV, such as SEs, as well as supramolecular attack particles released within
874 the synaptic cleft. Use highly abundant cell-surface proteins such as CD4, CD2, or CD45
875 alongside synthetic fluorescent lipids (DOPE Atto conjugates) to identify the population of
876 single cells and BSLB upon cold-based dissociation of conjugates. Focus the analyses on the
877 geometric mean or median of fluorescence intensities in single BSLB and cells (CD4 is used in
878 **Figure 3B**). SEs are a specialized type of tSV derived from the PM, and their transfer to BSLB
879 is evidenced by the gain of marker signal on BSLBs with the consistent loss of signal on the
880 surface of the interacting cells (refer to TCR on BSLBs as shown in **Figure 2B**, violet arrows).
881 Null BSLBs lacking either antigen or anti-CD3ε are an excellent reference to keep track of the
882 specific gaining of TCR (and other T cell markers) on BSLB resulting from stimulating
883 interacting cells via their TCR complex.
884
885 **FIGURES AND TABLE LEGENDS**
886 **Figure 1: Absolute quantification of proteins on the surface of APCs.** (**A**) Example of
887 quantitative flow cytometry measurements of ICAM-1 on the surface of tonsillar B cells (Foll.

888 Bc) and helper T cells (TFH). (**i–vii**) Gating strategy for analyzing single CXCR5$^+$ Bc and TFH
889 isolated from human palatine tonsils. Shown is the sequential gating strategy for identifying
890 single, live events contained within the continuous window of acquisition. **(iii–iv)** black arrows
891 indicate doublets. (**viii**) overlaid histograms showing the cell surface expression of ICAM-1
892 (teal histograms) compared to FMO controls (grey histograms) and FMO controls labeled with
893 relevant isotypes (black histograms, which overlap with the grey histograms) of the
894 populations shown in (vii). Arrows indicate the direction for the nested gating strategy used
895 to identify CXCR5$^+$ B cells (Bc; CD19$^+$) and TFH (CD4$^+$). (**B**) Extraction of absolute molecules on
896 the surface of tonsillar cells from MFI requires regression analyses of MESF benchmark beads
897 acquired using the same instrument setting as the cells shown in **A**. (**i–v**) Shown is the
898 sequential gating strategy for identifying single, live events contained within the continuous
899 window of acquisition. (**vi**) Gating and measurement of MFIs from different standard MESF
900 populations. (**vii**) shown are overlaid histograms of the MESF populations identified in (**vi**).
901 The values displayed on the top right represent the MFIs for each of the 5 MESF populations
902 (blank, 1, 2, 3, and 4). (**viii**) G Linear regression of MESF over cMFI for the MESF populations
903 shown in (**vii**). Shown is the slope (**b**) for extracting MESF bound to cells from data in **A**. (**C**) In
904 the extraction of the number of molecules, follow simple mathematical operations starting
905 with the application of the slope calculated in (**viii**) from measured MESF cMFI (cMFI$_M$) and
906 reference MESF values (MESF$_R$). To extract the MESF bound to cells (MESF$_{cells}$), divide the
907 corrected MFI of cells (cMFI$_{cells}$) by the calculated slope. Then, to calculate the number of
908 molecules bound to cells (Molec.$_{cells}$) MESF$_{cells}$, divide by the F/P of the detection
909 (quantification) antibody. Finally, to calculate the molecular density on the surface of cells
910 (D$_{cells}$), divide Molec.$_{cells}$ by the estimated cell surface area (CSA$_E$). Abbreviations: X =
911 independent variable; Y = dependent variable (measured fluorescence), cMFI$_M$ = MESF
912 corrected MFI; MESF$_R$ = reference MESF values; MESF$_{cells}$ = estimated MESF per cell; cMFI$_{cells}$
913 = corrected MFI cells; Molec.$_{cells}$ = estimated molecules per cell. D$_{cells}$ = estimated density on
914 cells; CSA$_E$ = estimated Cell Surface Area.
915
916 **Figure 2: Reconstitution of BSLB with recombinant ICAM-1 and the measurement of**
917 **particulate transfer to BSLBs.** (**A, i–vi**) Flow cytometry analysis of BSLBs reconstituted with
918 increased densities of recombinant monomeric ICAM-1 12-His (rICAM-1). **(i–v)** As in **Figure 1**,
919 focus the gating strategy on single BSLBs within the continuous window of acquisition. Note
920 the gap immediately before the *time continuum* gate, which was excluded to prevent errors
921 of measurement. **(vi)** Good protein quality often results in the homogeneous coating of BSLB
922 at high concentrations, with the observation of narrow fluorescence distributions (low
923 Coefficient of Variation, see histograms in **vi**). (**B**) Regression analyses of ICAM-1 reference
924 concentration (C$_R$) over measured density (D$_M$). Use the slope to calculate target
925 concentrations of protein (C$_T$) to achieve the density of cells (D$_{cells}$) measured in the
926 experiments in **Figure 1**. Abbreviations: 12-His = 12-histidines tag; D$_M$ = measured molecular
927 densities; C$_R$ = reference concentrations of the rICAM-1; C$_T$ = target concentration (to be
928 interpolated); D$_{cells}$ = densities measured in cells (see also Fig. 1C).
929
930 **Figure 3: Measurement of T cell synaptic particles transferred to BSLBs.** (**A; i–v**) Flow diagram
931 showing the critical steps for the co-culturing of T cells with BSLBs reconstituting model
932 membranes and the subsequent measurement of particle transfer with flow cytometry. **(iv)**
933 Blue and dark yellow diagrams show the relative distribution and location of cells and BSLBs
934 in biparametric flow cytometry plots. **(v)** Fluorescence distribution histograms displaying the

935 relative gain of fluorescence of agonistic BSLBs (dark yellow) compared to null BSLBs (grey).
936 (**B**) Exemplary synaptic transfer experiment. (**i–vi**) Shown is the gating strategy to identify
937 single BSLBs and cells within the continuous acquisition window. Violet arrows indicate the
938 direction of analysis, which continues in C. (**C**) (**i**) Focus the analyses on the MFI of single cells
939 (blue) and single BSLBs (yellow). (**ii**) Equations to calculate the normalized synaptic transfer
940 (NST%, top) and T_{max}% (bottom) from the cMFI calculated for BSLB and cells. (**iii–vi**) Overlaid
941 histograms showing the change of fluorescence intensity distributions for cells (blue shades)
942 and BSLBs (yellow shades) across different densities of the T-cell activating anti-CD3ε-Fab,
943 including non-activating (grey) and activating with either 250 (soft color value) or 1,000 (high
944 color value) molec./μm^2. Numbers in different color values represent the NST% measured for
945 the BSLB histograms shown in yellow. The overlaid histograms show the overarching
946 hierarchy in the synaptic transfer of T cell vesicles positive for different markers. For this
947 composition of BSLBs (200 molec./μm^2 of ICAM-1 and increasing densities of anti-CD3ε-Fab),
948 tSVs are transferred to BSLB with TCR$^+$(**iii**) > CD81$^+$(**iv**) > CD4$^+$(**v**) > CD28$^+$(**vi**). As demonstrated
949 in previous articles, TCR and CD81 are components of SEs and are transferred with
950 comparatively higher efficiencies to CD4, despite the latter being expressed at comparatively
951 higher surface levels. SE shedding results in the loss of cell surface CD81 and TCR and the gain
952 of these signals on BSLBs (open purple arrows for 250 molec./μm^2, and closed purple arrows
953 for 1,000 molec./μm^2 in yellow histograms). (**D**) Improper cooling down of conjugates leads
954 to cells ripping off the SLB from silica beads as seen from comparing input beads (left
955 biparametric plot) and conjugates subjected to rapid cooling down from 37 °C to 4 °C.
956 Compare also with **Figure 3B** panel (**vi**). Abbreviations: PRF1 = perforin 1; NST% = normalized
957 synaptic transfer; Tmax% = percent of maximum observed transfer (for control or reference
958 condition); tSVs: trans-synaptic vesicles; SEs: synaptic ectosomes.
959
960 **Table 1: Buffers used in this protocol.**
961
962 **DISCUSSION:**
963 BSLBs are versatile tools for studying the particulate effector output of T cells stimulated with
964 model APC membranes. The flexibility of the method allows the reconstitution of complex
965 and reductionist membrane compositions to study the effects of ligands and their signals on
966 the secretion of tSVs and supramolecular attack particles and their components. We have
967 tested this technology on various T cells, including preactivated TH, CTL, Tregs, and CART[1].
968 This protocol also works for the measurement of synaptic particle release of freshly isolated
969 and quiescent T cells. One limitation of using freshly isolated T cells is that these quiescent
970 populations produce a different profile of trans-synaptic particles, which correlates with their
971 cell surface composition (see [15] for more details).
972
973 As a simple flow cytometry panel, we recommend the use of anti-TCR clone IP26, anti-CD81
974 clone 5A6, anti-perforin (PRF1) clone B-D48 or anti-CD40L clone 24-31, and ATTO 390 or ATTO
975 565-containing lipids (to confer BSLBs an intrinsic fluorescence). To help discriminate single
976 cells from single BSLBs and conjugates, we recommend the use of anti-CD4 clone OKT4 and/or
977 anti-CD45 clone HI30, which are transferred to BSLBs at limited levels despite being expressed
978 at very high levels at the cell surface (see **Table of Materials** for further details on
979 fluorochromes and other validated antibodies). Panels with a higher number of
980 fluorochromes can be designed but require a systematic evaluation of the fluorescence
981 spectrum spillover of each fluorochrome analyzed. To increase sensitivity, try different

982 titrations of quantification antibodies ranging from 0.5 µg to 20 µg/mL final, and repeat
983 whenever new stocks of antibodies are used. To ensure reproducibility of the absolute and
984 relative measurements of particle transfer, titrate and calculate the binding capacity of each
985 new lot of biotinylated and Ni-chelating phospholipids, as they might differ significantly from
986 lot to lot. The gradual cool-down of T cell-BSLB conjugates is critical to increasing the
987 sensitivity of detection of particles with low abundance. The rapid cool-down of cocultures
988 leads to the destruction of BSLBs, as evidenced by the significant loss of lipid fluorescence
989 (**Figure 3D**).
990
991 Different metrics can be used to measure the particulate output of T cell immune synapses
992 depending on the experimental question at hand. For instance, when minor differences are
993 expected in the baseline expression levels of surface proteins sorted into budding SEs, a
994 normalized synaptic transfer (NST%) metric can be used (**Figure 3C** panel (ii) top equation).
995 The latter quantifies the percent MFI signal on BSLBs as a function of the total, combined MFI
996 of cells and beads. One caveat from this approach is the analysis of transferred markers not
997 expressed on the PM, such as components of supramolecular attack particles[19]. As these
998 elements reach BSLBs by pathways independent of PM transit, such as intracellular store
999 exocytosis, the calculation of NST% is not recommended as the result will be inflated because
1000 the numerator will be divided by a comparatively small denominator (cell surface expression
1001 level). Instead, use corrected MFIs to compare the deposition of perforin and granzymes
1002 between null BSLBs and BSLBs presenting increasing densities of antigen or anti-CD3ϵ Fab.
1003 Alternatively, to track intracellular elements transferred to BSLBs, use for comparison either
1004 the estimated absolute molecules transferred or the percent of signal with regard to the
1005 maximum signal transferred to BSLBs (T_{max}%) (**Figure 3C** panel (ii) bottom equation). For
1006 T_{max}%, use either cMFI or molecules measured on the BSLB sample (or condition) presenting
1007 the highest antigen density as the reference Tmax. When analyzing different donors, use
1008 donor-specific Tmax for comparisons. When studying the material transferred in response to
1009 the specific triggering of the TCR, MFIs can also be corrected to the level of background
1010 transfer observed on antigen-negative (null) BSLBs.
1011
1012 Estimating the absolute number of molecules transferred to BSLBs is a more robust method,
1013 as this involves MESF calibration with the measurement of molecules as the endpoint and a
1014 better comparison of independent experiments. Tmax% offers a similar normalization across
1015 independent experiments and is particularly useful when using polychromatic FCM for
1016 intracellular markers, such as perforin and granzymes, or for markers for which no MESF
1017 standard is available. Tmax% is simply the percentage of signal in any given condition to the
1018 condition with the highest transfer in the control condition (e.g., vehicle/untreated controls
1019 for drug studies, control guide RNAs for CRISPR/Cas9 libraries). Further, Tmax% can be used
1020 for both cMFIs and an absolute number of molecules and has been particularly useful for the
1021 side-by-side comparisons of the effects of gene deletions on the synaptic output of T cells.
1022 The latter is evident when gene-editing leads to high variability in the dynamic range of
1023 immune receptor expression among independent donors and experiments, which might
1024 impact absolute and NST% measurements.
1025
1026 BSLBs have facilitated the capture and characterization of the synaptic output of different T
1027 cell types, which otherwise are difficult to isolate due to their rapid internalization by APCs[2].
1028 The physical stability of BSLBs also provides a platform for the fluorescence-activated cell

1029 sorting of BSLBs that have been surveyed by T cells, thus enabling the biochemical
1030 characterization of highly pure particle preparations by mass spectrometry and nucleic acid
1031 sequencing technologies. The latter facilitates the detailed characterization of intercellular
1032 messengers shed by T cells under a broad range of experimental conditions. Different
1033 questions can be addressed, including how these particulate messengers change among
1034 different T cell types and activation states, how canonical and noncanonical antigen receptors
1035 (i.e., chimeric antigen receptors), and how agonistic and antagonistic membrane signals
1036 influence particle composition. We are currently developing this technology further for the
1037 quantitative characterization of cytokines secreted at the immune synapse of stimulated T
1038 cells. The latter requires the careful study of combinations of recombinant cytokine receptors
1039 and antibodies, providing efficient capture of interleukins, interferons, and chemokines
1040 deposited on BSLBs following T cell activation. These synthetic APCs can be adapted to model
1041 the surface composition of cell types suspected to produce tSV-like intercellular messengers,
1042 such as stromal and innate immune cells. BSLBs can also be adapted to screen new
1043 pharmacological compounds seeking the positive and negative modulation of the exocytic
1044 and tSV secretion machinery for the treatment of cancer and other pathologies. Finally, BSLBs
1045 can also be used to discover virulence determinants modulating T cell function in infectious
1046 diseases[20].
1047

1057

1058 **DISCLOSURES:**
1059 The authors have no conflict of interest to declare.
1060

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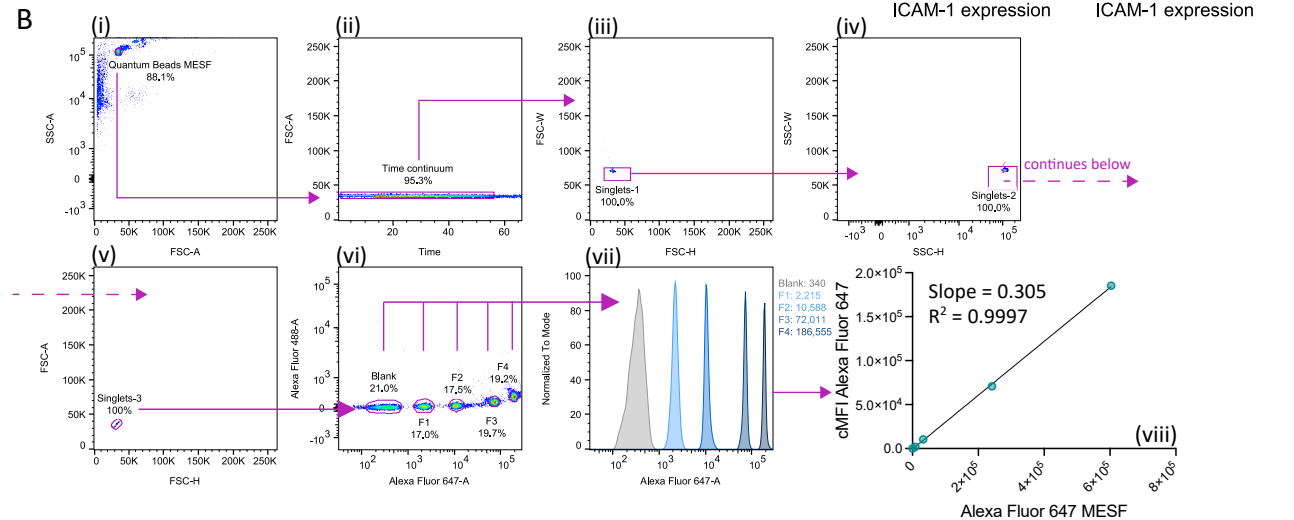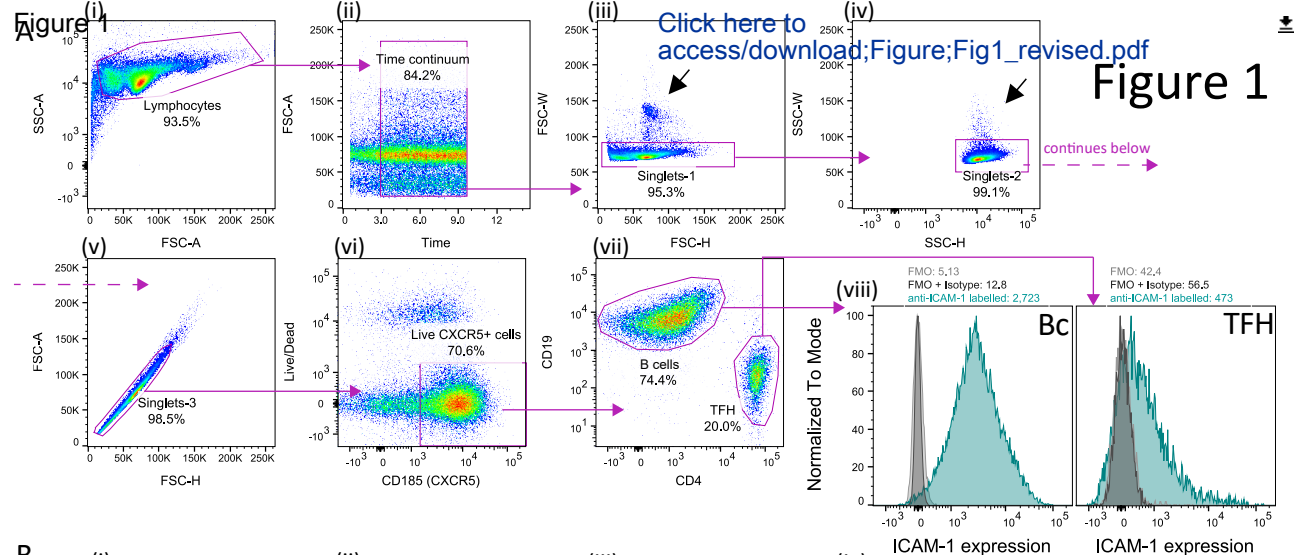
Figure 1

C

$$cMFI_M = 0.305 * MESF_R \longrightarrow MESF_{cells} = \frac{cMFI_{cells}}{0.305} \longrightarrow Molec._{cells} = \frac{MESF_{cells}}{F/P\ Ab} \longrightarrow D_{cells} = \frac{Molec._{cells}}{CSA_E}$$
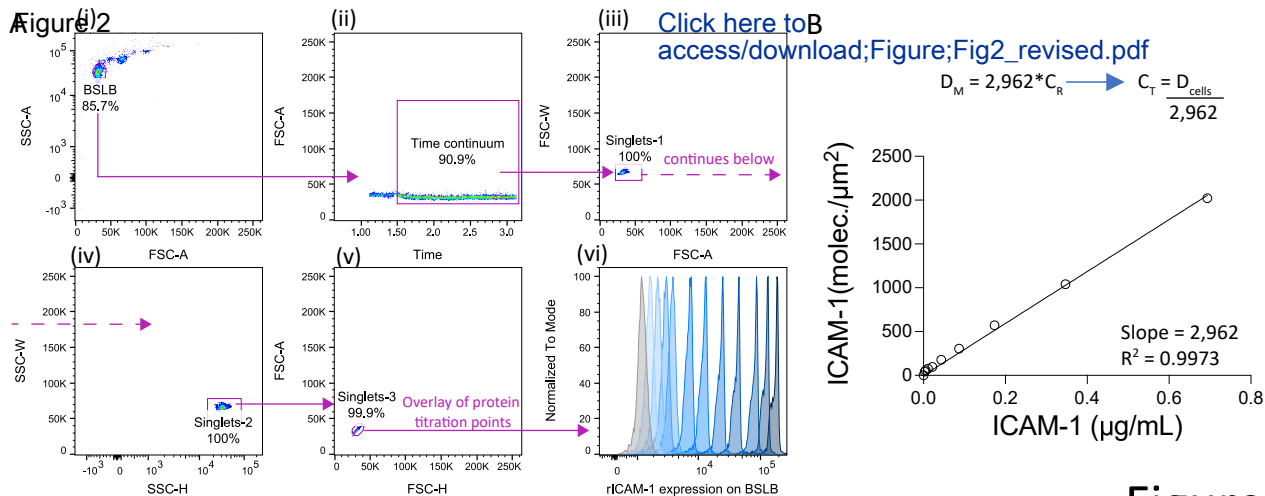
A Figure 2

(i) (ii) (iii)

B

$$D_M = 2{,}962 * C_R \longrightarrow C_T = \dfrac{D_{cells}}{2{,}962}$$

(iv) (v) (vi)

Slope = 2,962
$R^2 = 0.9973$

Figure 2

Figure 3

SVG version of Figure 1

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SVG version of Figure 3

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Table 1

Human Flow cytometry buffer	Component		[mM required]	mL for 500 mL of buffer
	PBS pH 7.4 (no Mg^{2+}, no Ca^{2+})			450
	Human AB serum, heat inactivated			50
	EDTA 0.5 M		2	2

HBS 10x (1 L)	Component	g/mol	[mM required]	g for 1 L of buffer
	HEPES	238.3	200	47.66
	NaCl	58.44	1370	80.0628
	KCl	74.55	50	3.7275
	Na_2HPO_4	141.96	7	0.99372
	D-Glucose	180.16	60	10.8096
	$CaCl_2$	110.98	10	1.1098
	$MgCl_2$ **(highly exothermic)**	95.21	20	1.9042

HBS/HSA buffer (500 mL)	Component	Volume	
	HBS 10x	50	mL
	30% HSA solution	16.6	mL
	* Adjust pH to 7.4		
	MiliQ H_2O	433.4	mL

HBS/BSA buffer (500 mL)	Component	Volume	
	HBS 10x	50	mL
	MiliQ H_2O	450	mL
	* Adjust pH to 7.4		
	BSA	5	g

BSLB blocking; casein solution	Component	Volume	
	0.22 µm-filtered 5% casein solution (store stocks at -20 °C)	1	mL
	10 mM $NiSO_4$ (in water; store 0.22 µm-filtered stock at 4 °C)	10	µL

BSLB blocking; BSA solution	Component	Volume	
	0.22 µm-filtered 5% BSA solution in PBS (store stocks at -20 °C)	1	mL
	10 mM $NiSO_4$ (in water; store 0.22 µm-filtered stock at 4 °C)	10	µL

10% FBS-supplemented RPMI 1640	Component	Volume	
	RPMI 1640 (no glutamine)	500	mL
	Fetal Bovine Serum, heat-inactivated	50	mL
	HEPES 1 M	5.5	mL
	Non-essential aminoacids 10 mM	5.5	mL
	L-glutamine 200 mM	5.5	mL
	Sodium Pyruvate 100 mM	5.5	mL
	Penicillin/Streptomycin (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin)	5.5	mL

Synaptic Transfer Assay Media	Component	Volume	
	RPMI 1640 (Phenol-red free, no glutamine)	500	mL
	Fetal Bovine Serum, heat-inactivated	50	mL
	HEPES 1M	5.5	mL
	Non-essential aminoacids 10 mM	5.5	mL
	L-glutamine 200 mM	5.5	mL
	Sodium Pyruvate 100 mM	5.5	mL
	Penicillin/Streptomycin (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin)	5.5	mL

Table of Materials

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Table of Materials
Table of Materials_R2_63130.xls

KENNEDY
INSTITUTE OF RHEUMATOLOGY I NDORMS

UNIVERSITY OF OXFORD

November 8th, 2021

Dear Vidhya Iyer, Ph.D.
Review Editor
The Journal of Visual Experimentation
625 Massachusetts Ave.,
2nd Floor, Cambridge, MA

Dear Vidhya,

Thank you very much for submitting your letter indicating that our protocol has passed the second round of review and is considered for publication in the JOVE contingent upon revising the reviewers' points.

Once more, we are very grateful to the reviewers and editors. Their comments have continued improving the overall quality of our work, which we feel will help others adopt this novel and quickly evolving methodology. Please, find attached the point-by-point letter addressing each of the referees' comments in the pages continuing below.

We have indicated the pages and lines of the most critical points raised by the reviewers and kept "track changes" to facilitate the revision of the manuscript. We hope the current version of the manuscript is acceptable for publication in the Journal of Visual Experimentation.

We look forward to hearing from you.

Yours sincerely,

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Point by point address to reviewers' comments:

Reviewer #2:
Manuscript summary:
The authors have sufficiently addressed my comments in the revised manuscript and I recommend acceptance.

Answer: Once more, we would like to thank the reviewer for all the suggestions to our work.

Reviewer #3:
Manuscript Summary:
The present JoVE manuscript by Cespedes-Donoso and Dustin illustrates different protocols for including relevant proteins regulating cell-cell interaction in bead supported lipid bilayers, for measuring the density of proteins on the surface of beads and cells and for estimating the proteins released by cells that are conjugated with these functionalized beads. These authors have previously used these methodologies in relevant articles describing the transfer from T cells to APCs of different effectors and mediators thanks to the T cell ability to release vesicles at this cell-cell contact. These are very useful and interesting protocols for researchers in the field and abroad. The manuscript is clearly organized; however, some points may be clarified by authors.

Major Concerns:
A major issue is that authors do not include the possibility of other kind of vesicles, apart from the ectosomes or the attack particles, which can be delivered by T cells or other cells to contacting cells. As an example, some cytokines are known to be secreted in a directional form from T cells to APCs, as well as intraluminal vesicles from multivesicular bodies or lytic granules. All these elements are transported by vesicles or organelles and delivered to the APCs, and could be potentially detected at the surface of the beads. This is an important concept that should be clearly included in the introduction and discussion sections.

Answer: We thank the reviewer by pointing out to this very important concept in the biology of T cell communication. We have mentioned the biology of cytokine secretion at the immune synapse in the Introduction. In the discussion section, we have also mentioned that this is a non-validated application of the method, which we are currently developing. Please refer to page 2, lines 65 to 70; and page 22, lines 1076 to 1080 of the revised manuscript.

Other Concerns:
Line 46: BSLBs instead of BSLB? Can authors revise the text to fix singular and plural forms?

Answer: We thank the reviewer for pointing to this error. We have corrected the manuscript accordingly.

Line 51: do these BSBLs allow the study of other kind of synaptic-delivered vesicles, apart from ectosomes? Are BSBLs useful to distinguish between these vesicle populations? Please, include this information.

Answer: We thank the reviewer for suggesting this change. We have amended the introduction to include other types of trans synaptic particles including CD63+ exosomes, and supramolecular attack particles, which derive from intracellular stores. Please refer to page 2, lines 63 to 65 of the revised manuscript.

Line 59: 1) the use of antibodies with known numbers of fluorochromes per antibody (F/P): Can authors secure the number of antigens (1-2) bound by each antibody, and is it included in their calculations for their estimations of physiological density of molecules? A comment on this may be useful to readers here, even if lines 554-557 state that: "Since we use the same antibodies to calibrate the number of recombinant proteins on the surface of cells and BSLBs, there is no need to correct the antibody binding valency as this remains constant…."

Answer: We thank the reviewer for suggesting this change. We have included a statement in the abovementioned paragraph to clarify that correcting for the antibody avidity is possible by using fluorescent recombinant proteins with known F/P ratios. Please refer to page 2, lines 89 to 94.

Line 158: "Acquire relevant samples and a minimum of 104 target cells for quantification". Is this minimum number of cells enough to assess differences in the density of molecules? Please, include an estimation/comment on this issue.

Answer: We thank the reviewer for pointing to this key point. The number of target cells to analyse will depend on the abundance of the cell subset and the abundance of starting tissue material. In some cases, recording 10,000 events might require half an hour of acquisition per sample, or ultimately be limited by the total number of cells collected from a given human tissue. We have added a note to indicate that a more robust analysis requires the collection of data from multiple donors and across independent experiments. Please refer to page 5 and lines 276-279 of the revised manuscript.

Line 159: do authors find relevant to wash the cytometer between samples to facilitate acquisition? If the answer is yes, please, include a note on this.

Answer: We thank the reviewer for suggesting this note. We have added a note recommending activating either the sit flush option or the washing option in high throughput samplers to reduce the noise from sample to sample carry over. Please refer to lines 285-291 in pages 5 and 6 of the revised manuscript.

Line 257: point 5.2 should not be there.

Answer: We thank the reviewer for pointing to this error. We have amended the manuscript accordingly.

Line 258: How do authors avoid sedimentation of silica beads usually?

Answer: We have included a comment in the manuscript to clarify this point. Please refer to new page 8, lines 428 to 429, and page 10, lines 488-489.

Line 287: Can authors explain how they add the argon/nitrogen gas to the tube containing the BSLBs during mixing?
Answer: We have included a comment in the manuscript to clarify this point. Please refer to current page 10, lines 481 to 485.

Line 427: include the medium to resuspend the cells (some information is missing).

Answer: We have included the step in which the synaptic transfer assay medium was prepared and clarified its composition. Please refer to current page 14, lines 673 to 676.

Line 459: Does "in absence of CO_2" mean "in an non-CO_2 incubator"? I recommend using this later term.

Answer: We thank the reviewer for suggesting this correction. We have amended the manuscript accordingly.

Line 503 and others: please use a unique term when referring to the BD HTS cytometer.

Answer: We thank the reviewer for suggesting this correction. Since Hight Throughput Samplers are sell by several vendors and for a variety of commercially available instruments, we have used a more general term instead.

Reviewer #4:

Manuscript Summary:

This protocol provides both a wealth of details into the practical, in-the-lab aspects of this assay and a high-level overview of the underlying foundations. The overall concept of replacing antigen-presenting cells with bead-supported lipid bilayers is powerful, and does deserve a discussion as accomplished in this manuscript. The revisions are responsive to comments in the original review, and improve the manuscript. I recommend accepting this manuscript for the next stage of production with minor revisions.

Minor Concerns:

There are organizational issues. While additional copy-editing will be done, I would like to illustrate specific problems:

1) Line 256, there appears to be an extra line return, which breaks the numbering system. Fixing this could have a cascading effect on sections, so should be carefully addressed.

Answer: We thank the reviewer for pointing to this error. We (and the reviewing editor) have modified the text accordingly to correct for any structural and spelling errors while keeping the unique format of JOVE.

2) Line 427, something is missing at the end of the highlighted text.

Answer: We thank the reviewer for pointing to this error. We have modified the manuscript to improve its readability.

3) Line 42, "BSLB" is used in the introduction before definition. There is a definition in the Abstract, but if the Abstract is meant to stand alone, the acronym should be defined again in the Introduction.

Answer: We thank the reviewer for pointing to this error. We have modified the revised manuscript accordingly.

4) The Comments/Description section (starting page 47 of the review document) is missing indicators from the main text.

Answer: We thank the reviewer for pointing the unclarity of this section. We (and the reviewing editor) have modified the text and associated tables accordingly to correct for any structural and spelling errors while keeping the unique format of JOVE.