UNIVERSITY OF TURKU

This is a self-archived – parallel published version of an original article. This version may differ from the original in pagination and typographic details. When using please cite the original.

This is a post-peer-review, pre-copyedit version of an article published in

BOOK The Immune Synapse. Methods in Molecular Biology, vol 2654.

The final authenticated version is available online at https://doi.org/10.1007/978-1-0716-3135-5_25

DOI 10.1007/978-1-0716-3135-5_25

CITATION Cunha, D.M., Hernández-Pérez, S., Mattila, P.K. (2023). Isolation of the B Cell Immune Synapse for Proteomic Analysis. In: Baldari, C.T., Dustin, M.L. (eds) The Immune Synapse. Methods in Molecular Biology, vol 2654. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-3135-5_25

Diogo M. Cunha[1,2,3], Sara Hernández-Pérez[1,2,3], and Pieta K. Mattila[1,2,3#]

[1] Institute of Biomedicine, and MediCity Research Laboratories, University of Turku, Finland

[2] Turku Bioscience, University of Turku and Åbo Akademi University, Turku, Finland

[3] InFLAMES Research Flagship, University of Turku

[#] Corresponding author

Corresponding author:

Pieta K. Mattila

E-mail: pieta.mattila@utu.fi

Isolation of the B cell immune synapse for proteomic analysis

Diogo M. Cunha, Sara Hernández-Pérez, and Pieta K. Mattila

Running head: Immune synapse proteomics

Abstract

Recent technical developments have fueled increasing utilization of proteomics to gain new insights into various aspects of cellular behavior. In this chapter, we describe a method to specifically isolate immune synapses from mouse primary B cells. The method utilizes antibody-coated magnetic beads to induce the formation of the immune synapses and describes a protocol for the extraction of the cell-bead adhesions for mass spectrometry analysis. Finally, this method enables unveiling the large-scale protein content of the immune synapse.

Keywords: Immunological Synapse Immune synapse, B cells, cell adhesion, proteomics, mass spectrometry

1. Introduction

As an important part of our adaptive immune system, B cells carry vital functions by mounting high-specificity antibody responses and orchestrating other branches of the immune response. These functions largely rely on the recognition of specific antigens via the B cell receptor (BCR) [1, 2]. Even though B cells strongly respond to soluble antigens, the dominant form of antigen recognition *in vivo* is mediated by antigen-presenting cells (APCs), such as dendritic cells, follicular dendritic cells, and macrophages [3–5]. These cells can display antigens on their surface, and notably, it has been demonstrated that surface attachment of antigen lowers the threshold for BCR activation [6].

The interaction between an antigen displaying cell and a B cell triggers the formation of an activation platform called the immune synapse (IS) [5]. Antigen recognition by the BCR prompts robust downstream phosphorylation events as well as recruitment of key players regulating B cell activation and differentiation, such as Vav1, Btk, and Syk, to the synapse. This platform is a central assembly point for BCR signalosomes, in which downstream signaling is initiated and antigen is internalized for further processing [7, 8]. The formation and coordination of the IS relies on the plasticity of the cytoskeleton and polarization of the microtubule network [9, 10]. Not only does the cytoskeleton provide structural force and support to the morphological changes involved in IS formation, but it also regulates signaling microcluster formation and gathering, antigen internalization, vesicle trafficking, and even controls the diffusion of the BCR with direct consequences to BCR activation [11–15].

BCR signaling, together with the morphological rewiring that enables the formation of the IS, exerts such an influence in the cellular dynamics and processes that capturing this complexity presents a serious experimental and technical challenge. In the last decade, we have witnessed a steep

development of advanced imaging techniques [16], next-generation sequencing (NGS) technologies [17, 18] and mass spectrometry (MS)-based proteomics [19, 20] to address B cell activation. These techniques have allowed identification of several novel proteins and mechanisms regulating BCR signaling and B cell activation. However, most studies largely neglect the particular responses provoked by surface-bound antigen, the predominant *in vivo* form of antigen encounter. As such, a technique to comprehensively study the proteins recruited to the IS following recognition of surface-bound antigen is set to provide a fuller picture of the plethora of different molecular players located at the IS as well as to facilitate a more detailed understanding of the mechanisms of B cell activation.

Here, we describe a novel pull-down approach to extract proteins recruited to the IS during B cell activation. The method was developed based on previous protocols described for integrin isolation using adherent cells [21] and IS of NK cells (Meyer et al., *submitted*). In our protocol, IS formation is triggered using magnetic beads coated with surrogate antigen to mimic BCR activation by surface-tethered antigens. Beads coated with anti-transferrin receptor antibodies are used as control. The extracted samples are prepared for mass spectrometry analysis to reveal the protein content of the IS.

2. Materials

2.1 Preparation of the beads coated with activatory and non-activatory ligands

1. Tosyl-activated magnetic beads, such as Dynabeads™ M-450 Tosyl-activated (#14013, Thermo Fisher Scientific)
2. Magnetic separation rack, such as MagnaRack™ (#CS15000, Thermo Fisher Scientific)

3. Fragment AffiniPure F(ab')$_2$ Fragment Goat Anti-Mouse IgM, μ chain specific (#115006020, Jackson ImmunoResearch or equivalent)
4. Purified rat anti-mouse CD71, clone C2 (#553264, BD Bioscience or equivalent)
5. Thermo-shaker (TS-100, Biosan, or equivalent)
6. Washing Buffer 1: 0.1 M sodium phosphate buffer, pH 7.4-8 (*see* **Note 1**)
7. Washing Buffer 1 + BSA: 0.1 M sodium phosphate buffer + 1% BSA, pH 7.4-8 (*see* **Note 1**)
8. Washing Buffer 2: Phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and 2 mM Ethylenediaminetetraacetic acid (EDTA), pH 7.4 (*see* **Note 1**)
9. Washing Buffer 3: 0.2 M Tris 0.1% BSA (w/v), pH 8.5 (*see* **Note 1**)
10. 20 mM HEPES
11. 25 mM bis(sulfosuccinimidyl)suberate (BS3) (#21580, Thermo Fisher Scientific or equivalent)

2.2 Primary mouse B cell isolation

1. Wild-type mice, strain C57BL/6NCrl or similar (*see* **Note 2**)
2. 70 μm cell strainer
3. Sterilized surgical scissors and forceps
4. B cell isolation buffer: PBS, 2% fetal calf serum (FCS), 1 mM EDTA
5. Negative B cell isolation kit (EasySepTM Mouse B Cell Isolation Kit, #19854, StemCell Technologies or equivalent)
6. Complete RPMI (cRPMI): RPMI 1640 with 2.05 mM L-glutamine supplemented with 10% FCS, 50 μM β-mercaptoethanol, 4 mM L-glutamine, 10 mM HEPES and 100 U/ml penicillin/streptomycin
7. Sterile conical Falcon tubes of 50 and 15 mL

2.3 Conjugate formation

1. Imaging Medium: RPMI 1640 without glutamine and phenol red supplemented with 1% FCS and 20 mM HEPES
2. Di-tert-butyl peroxide (DTBP) crosslinker (#20665, Thermo Fisher Scientific or equivalent)
3. Quenching Buffer: Tris-HCL 1 M, pH 8.5
4. Inverted microscope EVOS fl (illumination system based on LED cubes,40x AMG PlanFluor objective), or similar, enabling fast sample evaluation.
5. Sterile 96-well plates

2.4 Synapse Isolation

1. Cytoskeletal Buffer (CSK buffer); 300 mM sucrose, 100 mM sodium chloride, 10 mM PIPES, pH 6.8, 3 mM magnesium chloride (*see* **Note 3**)
2. CSK buffer + 0.5% Triton X-100 (*see* **Note 3**)
3. Protease inhibitors, such as Pierce™ Protease Inhibitor Mini Tablets, EDTA-free (#A32955, Thermo Fisher Scientific)
4. Bioruptor Sonicator (Diagenode), or equivalent
5. 2x Laemmli buffer with 5% β-mercaptoethanol: (45 µL of 4x Laemmli Sample with 5 µL of beta-mercaptoethanol and 50 µL of deionized H_2O)

2.5 In-gel digestion

1. Eppendorf® Protein LoBind tubes (#EP0030108116-100EA, Merck, or equivalent) (*see* **Note 4**)
2. Milli-Q H_2O
3. Gradient protein electrophoresis polyacrylamide gel, such as Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gels (#4569034, Bio-Rad).
4. Pierce™ Zinc Reversible Stain Kit (#24582, Thermo Fisher Scientific, or equivalent)
5. Tris-glycine pH 8: 25 mM Tris, 192 mM glycine

6. Acetonitrile (ACN), LC-MS Grade (#51101, Thermo Fisher Scientific or equivalent)
7. Dithiothreitol (DTT), preferentially in no-weigh format for accuracy
8. Iodoacetamide (IAA)
9. Ammonium bicarbonate (NH_4HCO_3), LC-MS Grade
10. Sequencing grade modified Trypsin (v511A, Promega or equivalent)
11. Formic acid (HCOOH), LC-MS Grade
12. Sterilized Scalpers and Tweezers
13. Vacuum concentrator system

3. Methods

The following protocol describes the necessary steps for the successful conjugation of 20 million mouse primary B cells with 20 million antibody-coated magnetic beads to mimic IS formation, and extraction of the formed IS and control adhesions for mass spectrometric analysis. The full protocol takes a couple of days mainly due to incubation times during the bead preparation (Figure 1).

3.1 Coating of magnetic beads

1. Vortex the stock bottle of beads for a minimum of 30 s at maximum speed.
2. Prepare 40 million magnetic beads per condition by adding 20 million magnetic beads (50 µl) per tube into two autoclaved Eppendorf tubes (*see* **Note 5 and 6**).
3. Add 1 mL of Washing Buffer 1 and resuspend.
4. Place the Eppendorf in a magnetic separation rack for 1 min and discard the supernatant using vacuum suction.
5. Repeat washing (steps 3 and 4).
6. Remove the Eppendorf from the magnet and resuspend the beads in 960 µl of Washing Buffer 1.

7. Add 200 µg of ligand antibodies per 1 ml of stock beads (= 10 µg of protein for 50 µl of stock beads). As ligands, we added 20 µl of purified rat anti-mouse CD71 per 20 million magnetic beads and 7.7 µl of Affinipure $F(ab')_2$ fragments of goat anti-mouse IgM per 20 million magnetic beads.
8. Incubate for 1-2 hours at RT with shaking (1000 rpm).
9. Add 200 µl of Buffer 1 + BSA (final concentration of BSA in the Eppendorf = 0.167%) (*see* **Note 7)**.
10. Incubate overnight (16-24 h) at 37°C with shaking (1000 rpm) (*see* **Note 8)**.
11. Meanwhile, prepare 25 mM BS3 crosslinker solution by resuspending 4.32 mg of BS3 in 600 µl of 20 mM HEPES (*see* **Note 9)**.
12. Place the tube with beads in a magnetic separation rack for 1 min and discard the supernatant.
13. Add 1 ml of 20 mM HEPES, mix and incubate for 5 min with gentle tilting and rotation.
14. Place the tube with beads back in the magnetic separation rack for 1 minute and discard the supernatant.
15. Repeat the washing steps 13 and 14.
16. To crosslink the antibodies to the beads, resuspend beads in 275 µl BS3 solution and incubate for 2h at RT with shaking (1000 rpm) (*see* **Note 10**).
17. Place the tube with beads back in the magnetic separation rack for 1 min and discard the supernatant.
18. Add 1 ml of Washing Buffer 3, mix and incubate for 5 min with gentle tilting and rotation (*see* **Note 11**).
19. Place the tube with beads back in the magnetic separation rack for 1 minute and remove the supernatant.
20. Repeat the washing steps 18 and 19.

21. Resuspend the beads in 1 ml of Washing Buffer 3. Incubate overnight (16-24 h) at RT with shaking (1000 rpm) to ensure quenching of the B3 cross-linker and any remaining tosyl groups on the beads (*see* **Note 11**).
22. Place the tube in a magnet for 1 min and discard the supernatant.
23. Add 1 ml of Washing Buffer 2 and incubate for 5 min with gentle tilting and rotation.
24. Place the tube in the magnetic separation rack for 1 minute and remove the supernatant.
25. Repeat washing as in steps 23 and 24 of this section.
26. Resuspend the coated beads in 1 ml of Washing Buffer 2 (*see* **Note 12**).
27. Count the beads using an Automated Counter (*see* **Note 13**).
28. Coated beads are stored for up to 1 month at +4ºC in 1 mL of Washing Buffer 2.

3.2 Primary mouse B cell isolation (sterile work)

1. Sacrifice 2 WT mice (i.e. C57BL/6NCrl genetic background, 2-3 months old) by an appropriate method.
2. Spray the dead bodies with 70% ethanol and harvest the spleens.
3. Get rid of fat tissue and other unrelated remaining tissues and place the spleens in an Eppendorf tube on ice.
4. Fill 2 wells of a 6-well plate with 2 ml of isolation buffer each.
5. Place a 70 µm cell strainer in the wells and mash the spleens.
6. Collect the splenic lymphocytes into a conical 50 mL falcon tube and wash the strainers with an additional 1 mL of isolation buffer.
7. Centrifuge to pellet the splenocytes for 5 min at +4°C (450g), continue to B cell isolation using a negative B cell isolation kit following the manufacturer's recommendations and count the isolated cells (*see* **Note 14**).

3.3 Conjugate Formation

1. Thaw the CSK buffer (stored at -20°C) and equilibrate the DTBP crosslinker to room temperature.
2. Turn on the thermo-shaker and heat up to 37°C.
3. Take 25 x 10^6 cells per condition and pellet them by centrifugation for 5 minutes at 4ºC (450g).
4. Resuspend the cells in 2 mL of Imaging Medium per condition and split them into two Eppendorf tubes (1 mL per tube) (*see* **Note 15**).
5. Keep the cells at 37°C while preparing the beads for conjugate formation.
6. Vortex the ligand-coated magnetic beads for a minimum of 30 s at maximum speed to resuspend thoroughly.
7. Take 25 million beads and mix in a total volume of 500 µL of Imaging Medium per condition.
8. Mix the cells (1 mL, 12.5 million cells per Eppendorf tube) with the beads (250 µL, 12.5 million per Eppendorf tube) to a final volume of 1250 µL per tube (two tubes per condition), to initiate the conjugate formation (*see* **Note 16**).
9. Incubate at 37°C for 15 min in the thermo-shaker (1000 rpm) (*see* **Note 17**).
10. Add 32 µL of 100 mM DTBP solution (final concentration 2.5 mM) to each sample to crosslink the proteins and stabilize the adhesive structures for isolation (*see* **Note 18**).
11. Incubate for 5 minutes at 37ºC with rotation (*see* **Note 18**).
12. Quench the DTBP crosslinker by adding 200 µL of Quenching buffer to each tube (final concentration 150 mM).
13. Incubate for 5 minutes at RT.
14. Keep the tubes on ice.
15. Put aside a sample of 5 µL of the suspension into a 96 well plate containing 95 µL of PBS. To evaluate the success of the conjugate formation, image the sample in the 96 well plate using an inverted brightfield microscope (such as EVOS) (Figure 2A) (*see* **Note 19**).

3.4 Synapse Isolation

1. Place the magnetic separation rack on ice. All reagents should be ice cold.
2. Place the cell-bead conjugate samples in the magnet for >1 minute and collect the supernatant (*see* **Note 20**).
3. Resuspend conjugates in 1 mL of CSK buffer and agitate gently up and down (*see* **Note 21**).
4. Place the tubes back in the magnetic separation rack, remove supernatant and repeat the wash as in steps 2 and 3 above.
5. Resuspend the samples in 1 mL of CSK buffer + 0.5% Triton X-100 supplemented with protease inhibitor tablets according to the manufacturer's recommendation to permeabilize cell membrane and help preserving the cross-linked proteins.
6. Put aside a sample of 5 µL of the suspension into a 96 well plate containing 95 µL of PBS. When there is a suitable break in the protocol, to visualize conjugate formation in washed samples, image the sample in the 96 well plate using an inverted brightfield microscope (such as EVOS) (Figure 2B).
7. In order to remove the cell bodies from the beads, sonicate the samples in a sonicator using a program optimized beforehand to the samples (*see* **Note 22**).
8. After sonication keep samples on ice.
9. Put aside a sample of 5 µL of the suspension into a 96 well plate containing 95 µL of PBS. When there is a suitable break in the protocol, to visualize cell body detachment from beads, image the sample in the 96 well plate using an inverted brightfield microscope (such as EVOS) (Figure 2C).
10. Place the tubes in the magnetic separation rack for >1 minute and remove the supernatant (*see* **Note 23**).

11. Add 1 mL of CSK buffer + 0.5% Triton X-100 supplemented with protease inhibitor tablets and agitate gently up and down.
12. Repeat the steps 10 and 11 to wash 5 more times.
13. Put aside a sample of 5 µL of the suspension into a 96 well plate containing 95 µL of PBS. When there is a suitable break in the protocol, to visualize successful washing of the beads and better evaluate the success of sonication, and image the sample in the 96 well plate using an inverted brightfield microscope (such as EVOS) (Figure 2D).
14. Remove the supernatant using the magnet.
15. Add 20 µL of 2x Laemmli buffer with 5% β-mercaptoethanol and incubate the sample tubes for 30 minutes at 70°C thermo-shaker (1000 rpm) to denature the proteins and elute the from the beads.
16. Cleave the DTBP crosslinker by incubating the sample further for 10 minutes at 95°C on a thermo-shaker (1000 rpm) (*see* **Note 24**).
17. Separate the beads using the magnet and gently transfer the supernatants, containing the extracted immune synapse or control adhesion proteins, into new Eppendorf tubes. These eluted samples can be stored at -20ºC for several days (*see* **Note 25**).

3.5 In-gel digestion

1. Run the eluted samples on a gradient polyacrylamide gel suitable for a wide range of polypeptides for 1 cm (approximately 20 minutes).
2. Stain the gel with reversible zinc staining, according to the manufacturer's indications, for protein quantification (*see* **Note 26**).
3. Excise the zinc-stained gel bands with a clean scalpel, split in 3 pieces and place them in three tubes, Eppendorf® Protein LoBind, or equivalent.
4. Chop each gel piece further into smaller pieces inside the Eppendorf. (*see* **Note 27**).

5. Destain the gel pieces with 1 mL of Tris-glycine pH 8 until Zinc Reversible Stain disappears (*see* **Note 26**).
6. Wash the gel pieces with 1 mL of Milli-Q water for 10 minutes with gentle rotation and discard supernatant.
7. Repeat the wash two more times as described in step 6.
8. Shrink and dehydrate the gel pieces by covering the gel pieces with 150 µL of ACN.
9. Wait until the gel pieces become white (about 5-10 min) and remove all ACN.
10. To reduce disulphide bonds, rehydrate the gel pieces in 150 µL of 20 mM DTT for 30 min at 56°C. Remove the excess liquid if needed (*see* **Note 28**).
11. Repeat shrinking and dehydration of the gel pieces as described in step 8 of this section.
12. Rehydrate the gel pieces by adding 150 µL of 55 mM IAA for 20 min in the dark RT to block reoxidation of disulphide bonds. Remove the excess liquid if needed (*see* **Note 28**).
13. Wash the gel pieces two times with 100 µL of 100 mM ammonium bicarbonate.
14. Repeat shrinking and dehydration of the gel pieces as described in step 8 of this section.
15. To start enzymatic digestion, add 75 µl of 0.02 µg/µL trypsin solution and allow it to absorb to the gel pieces for 20 min on ice (*see* **Note 29**).
16. After 20 min, add about 150 µL solution containing 40 mM NH_4HCO_3/10 % ACN to completely cover the gel pieces and keep them wet during enzymatic cleavage (*see* **Note 30**).
17. Incubate the samples for up to 18 hours at 37 °C. The incubation with trypsin generates the peptides for mass spectrometry analysis.
18. Add about 225 µL ACN (equal volume with the digestion mixture used in steps 15 and 16), vortex and incubate 15 min at 37°C.
19. Collect the supernatant to another Eppendorf tube and repeat the extraction with 150 µL of solution containing 50 % ACN / 5% Formic Acid.

20. Combine the supernatants containing the eluted peptides and dry the samples in a vacuum concentrator (*see* **Note 31**).
21. Store the dried peptides at -20ºC.
22. Immediately prior to mass spectrometry analysis, dissolve the peptides in 10 µL 2% Formic Acid by vortexing, incubate at 37ºC for 15 min and vortex again (*see* **Note 32**).

4. Notes

1. Ideally, the buffers should be prepared fresh, but can also be stored at +4 ºC for up to 1 month.
2. We use mice at age of 2-3 months.
3. CSK buffer can be stored at -20ºC for a few months. Store CSK without Triton and add Triton every time before use.
4. To avoid plasticizer contamination, which can be detrimental to mass spectrometry, tubes made of high-quality polypropylene must be used.
5. For good coating efficiency, we have determined that a maximum of 20 million magnetic beads should be coated per one 1.5 mL Eppendorf tube.
6. During the coating of the beads, there are many washing steps where some beads are lost. To account for that, in our experimental setup, 40 million beads were initially prepared per condition, in two different Eppendorf tubes. This ensures the recovery of a minimum of 25 million coated beads for conjugation with cells.
7. BSA is added to aid the orientation and presentation of ligands.
8. Based on the manual provided for tosyl-activated Dynabeads by the manufacturer, maximal chemical binding is achieved after 16–24 hours at 37℃. Coupling at 18℃ to 25℃ (room temperature) would require an incubation time period longer than 48 hours to obtain the same degree of chemical binding. Consider if your ligand is temperature-sensitive in order to determine the right temperature and time.

9. BS3 is moisture sensitive. Equilibrate at RT for 30 minutes before opening the vial to avoid condensation.
10. BS3 is an amine-to-amine crosslinker. The addition of BS3 helps to avoid leakage of the bead-bound antibodies to the final adhesion protein elutes, subject to mass spectrometry.
11. Tris in the Washing Buffer 3 de-activates remaining free tosyl groups, as well as quenches the BS3 crosslinker.
12. Antibody-coated beads may typically be stored in Washing Buffer 2 (optionally containing 0.02% sodium azide) at 2-8°C for months. The stability of coated beads with other ligands should be determined individually. For example, beads coated with fibronectin do not store for long.
13. During the coating steps, beads may be lost by suction or other errors, so an accurate count of the number of final beads is recommended.
14. The purity (for example, surface staining for B220 and IgM) and viability (for example, Zombie Violet) of the isolated B cells should be checked by flow cytometry routinely after each isolation.
15. The volume and amount of cells were optimized to ensure the optimal formation of single conjugates (single cell:bead contacts). It may vary depending on the experiment setup, cells, coating ligand and magnetic beads used.
16. The ratio of cells to beads used is 1:1. This ratio provided a balanced output of single conjugate formation compared to other ratios tested, but again, this may vary depending on the experiment setup, cells, coating ligand and magnetic beads used.
17. For the experimental procedure described, the activation timepoint used was 15 minutes. This can be changed depending on the experimental setup.
18. DTBP crosslinker is a membrane-permeable reversible crosslinker. DTBP has been considered a powerful tool to stabilize protein-protein interactions in different biological settings. The chosen

DTBP conditions (2.5 mM, 5min) were determined by a minimal concentration and time that yielded sufficient protein retrieval. Depending on the given experimental application, incubation time may be adjusted to fit the reader's criteria.

19. Conjugation with activatory ligand was determined to be more effective than conjugation with non-activatory ligand.
20. The supernatant collected is stored in a new refrigerated Eppendorf. This one contains the cells not bound to any beads and can be used to give a rough estimate of the number of conjugates formed. In our experiments, the activatory ligand beads have approximately 70% conjugation rate, while the non-activatory ligand beads have approximately a 50% conjugation rate.
21. The magnetic separation rack used has two components: a magnetic band and a rack for test tubes. When agitating gently to homogenize conjugates in the CSK buffer, the tube rack is separated from the magnet and rotated slowly up and down. Pipetting conjugates to homogenize was deemed too harmful for the samples.
22. Sonication is required to remove the cell bodies from the beads, leaving only the crosslinked proteins at the adhesion structures for elution. This is a critical step that is sensitive to various parameters including the concentration of conjugates and efficiency of the cross-linking. Different sonication programs (time and intensity) bring different results and the best settings for each experimental procedure should be optimized beforehand. Our example protocol with our sonicator had settings of HIGH, 30" on/30" off, for 5 cycles.
23. Washing the beads should be performed with extra care to avoid any loss of weakly crosslinked proteins from the crosslinked immune synapse complexes.
24. For effective DTBP crosslinker cleavage, an incubation with 2x Laemmli buffer with 5% β-mercaptoethanol for 10 minutes with a temperature higher than 90ºC is required.
25. When preparing solutions and handling your samples keep in mind that the most common keratin contaminations, causing problems in mass spectrometry, arise from dust, hair or skin.

26. Zinc staining is a sensitive and fast method for protein detection in polyacrylamide gels immediately after electrophoresis. Here, it is used also to quantify the loaded amount of eluted proteins from the different ligand-coated beads using whole cell lysates (WCL) with known protein concentration as standards (in our experimental setup, 1 and 3 µg of WCL was used). Ideally, matching amounts of protein should be submitted for mass spectrometry analysis for each sample. Additionally, the dye can be removed easily for subsequent sample preparation and is compatible with mass spectrometry analysis.
27. The volumes detailed here are calculated for our experimental setup. Gel lanes were run for a length of 1 cm and cut into 3 pieces. Each gel piece was then placed in a different Eppendorf and further chopped into smaller pieces. Too big gel pieces lead into reduced peptide recovery, while smaller pieces can be discarded during pipetting steps, thus, the user should consider the aimed final size of the gel pieces beforehand and perform chopping similarly in all the samples.
28. On one hand, DTT acts as a reducing agent able to convert cysteine disulphide bonds into free sulfhydryl groups. On the other hand, IAA is an alkylating agent that reacts with free sulfhydryl groups of cysteine to form S-carboxyamidomethyl-cysteine, which cannot be reoxidized to form disulphide bonds. In this conformation, trypsin has maximum access to the cleavage sites within the proteins.
29. Gel pieces should be completely saturated with trypsin solution (when saturated, the gel pieces will be transparent). More trypsin solution should be added if gel pieces are not initially covered. Trypsin solution diffuses into the gel and cleaves the proteins into peptides. After 20 minutes on ice, gel pieces should be transparent. If not, more trypsin solution should be added.
30. The presence of low amounts of ACN (10% v/v) enhances trypsin activity by accelerating and improving the cleavage of the proteins during tryptic digestion.

31. We have used Medium Capacity Integrated Vacuum Concentrator System (#SPD1030A-115, Thermo Fisher Scientific) with 45ºC heating for 20 minutes and maximum vacuum pressure of 5.1, using manual run. Other vacuum centrifuges with comparable settings can be used.
32. The complexity of the protocol correlates with the finetuning of the conjugation (cells + beads). Conjugation efficiency with the non-activatory controls was found significantly lower than the conjugation with activatory ligand coated beads, which generated almost 50% more conjugates. To enable a direct comparison between conditions, the same amount of protein should be analyzed by mass spectrometry. Inherently, we recommend to assess the amount of eluted protein obtained from the tested conditions and compensate for that difference in future conjugation assays. Moreover, sonication settings have to be actively tested for efficient sonication without much protein loss, which presents a glaring challenge since only limited quantities of protein were retrieved for the mass spectrometry analysis (in the range of 2-4 µg per sample). Hence, if higher protein quantities are warranted, optimization of the protocol for higher numbers of conjugates should be performed.

Acknowledgements

We thank Dr. Alexandre Carisey (St Jude's Research Hospital, Memphis, USA) for his generous help with setting up the protocol. We also acknowledge Prof. Jordan Orange and Dr. Anna Meyer for their pioneering efforts in the immune synapse isolation in NK cells (Department of Pediatrics, Vagelos College of Physicians and Surgeons, Columbia University Irving Medical Center, New York, New York, USA). Turku Proteomics Facility of Turku Bioscience and Biocenter Finland are acknowledged for providing the research infrastructures. This work was supported by the Academy of Finland (decision number: 339810 to PKM), Sigrid Juselius foundation (to PKM), Erasmus+ program (to DMC), the Finnish Cultural Foundation (Suomen Kulttuurirahasto; to SHP), and by InFLAMES Flagship Programme of the Academy of Finland (decision number: 337530).

5. References

1. Marshall JS, Warrington R, Watson W, Kim HL (2018) An introduction to immunology and immunopathology. Allergy Asthma Clin Immunol 14:49. https://doi.org/10.1186/s13223-018-0278-1

2. Nicholson LB (2016) The immune system. Essays Biochem 60:275–301. https://doi.org/10.1042/EBC20160017

3. Batista FD, Iber D, Neuberger MS (2001) B cells acquire antigen from target cells after synapse formation. Nature 411:489–494. https://doi.org/10.1038/35078099

4. Kuokkanen E, Šuštar V, Mattila PK (2015) Molecular control of B cell activation and immunological synapse formation. Traffic 16:311–326. https://doi.org/10.1111/tra.12257

5. Batista FD, Harwood NE (2009) The who, how and where of antigen presentation to B cells. Nat. Rev. Immunol. 9:15–27

6. Carrasco YR, Batista FD (2007) B Cells Acquire Particulate Antigen in a Macrophage-Rich Area at the Boundary between the Follicle and the Subcapsular Sinus of the Lymph Node. Immunity 27:160–171. https://doi.org/10.1016/j.immuni.2007.06.007

7. Avalos AM, Ploegh H (2014) Early BCR Events and Antigen Capture, Processing, and Loading on MHC Class II on B Cells. Front Immunol 5:92. https://doi.org/10.3389/FIMMU.2014.00092

8. Hou P, Araujo E, Zhao T, Zhang M, Massenburg D, Veselits M, Doyle C, Dinner AR, Clark MR (2006) B cell antigen receptor signaling and internalization are mutually exclusive events. PLoS Biol 4:1147–1158. https://doi.org/10.1371/journal.pbio.0040200

9. Yuseff MI, Reversat A, Lankar D, Diaz J, Fanget I, Pierobon P, Randrian V, Larochette N, Vascotto F, Desdouets C, Jauffred B, Bellaiche Y, Gasman S, Darchen F, Desnos C, Lennon-

Duménil AM (2011) Polarized Secretion of Lysosomes at the B Cell Synapse Couples Antigen Extraction to Processing and Presentation. Immunity 35:361–374. https://doi.org/10.1016/J.IMMUNI.2011.07.008

10. del Valle Batalla F, Lennon-Dumenil AM, Yuseff MI (2018) Tuning B cell responses to antigens by cell polarity and membrane trafficking. Mol Immunol 101:140–145. https://doi.org/10.1016/j.molimm.2018.06.013

11. Tolar P (2017) Cytoskeletal control of B cell responses to antigens. Nat Rev Immunol 2017 1710 17:621–634. https://doi.org/10.1038/nri.2017.67

12. Mattila PK, Batista FD, Treanor B (2016) Dynamics of the actin cytoskeleton mediates receptor cross talk: An emerging concept in tuning receptor signaling. J. Cell Biol. 212:267–280

13. Porat-Shliom N, Milberg O, Masedunskas A, Weigert R (2013) Multiple roles for the actin cytoskeleton during regulated exocytosis. Cell Mol Life Sci 70:2099. https://doi.org/10.1007/S00018-012-1156-5

14. Mattila PK, Feest C, Depoil D, Treanor B, Montaner B, Otipoby KL, Carter R, Justement LB, Bruckbauer A, Batista FD (2013) The Actin and Tetraspanin Networks Organize Receptor Nanoclusters to Regulate B Cell Receptor-Mediated Signaling. Immunity 38:461–474. https://doi.org/10.1016/j.immuni.2012.11.019

15. Liu C (2018) B Cell Receptor Signaling. 1707:. https://doi.org/10.1007/978-1-4939-7474-0

16. Wang JC, Bolger-Munro M, Gold MR (2018) Imaging the interactions between B cells and antigen-presenting cells. Methods Mol Biol 1707:131–161. https://doi.org/10.1007/978-1-4939-7474-0_10/COVER/

17. Raybould MIJ, Rees AR, Deane CM (2021) Current strategies for detecting functional

convergence across B-cell receptor repertoires. https://doi.org/101080/1942086220211996732 13:. https://doi.org/10.1080/19420862.2021.1996732

18. Breden F, Luning Prak ET, Peters B, Rubelt F, Schramm CA, Busse CE, Vander Heiden JA, Christley S, Bukhari SAC, Thorogood A, Matsen FA, Wine Y, Laserson U, Klatzmann D, Douek DC, Lefranc MP, Collins AM, Bubela T, Kleinstein SH, Watson CT, Cowell LG, Scott JK, Kepler TB (2017) Reproducibility and reuse of adaptive immune receptor repertoire data. Front Immunol 8:1418. https://doi.org/10.3389/FIMMU.2017.01418/BIBTEX

19. Awoniyi LO, Šuštar V, Hernández-Pérez S, Vainio M, Sarapulov A V., Petrov P, Mattila PK APEX2 proximity biotinylation reveals protein dynamics triggered by B cell receptor activation. bioRxiv 2020.09.29.318766

20. Satpathy S, Wagner SA, Beli P, Gupta R, Kristiansen TA, Malinova D, Francavilla C, Tolar P, Bishop GA, Hostager BS, Choudhary C (2015) Systems-wide analysis of BCR signalosomes and downstream phosphorylation and ubiquitylation . Mol Syst Biol 11:810. https://doi.org/10.15252/msb.20145880

21. Jones MC, Humphries JD, Byron A, Millon-Frémillon A, Robertson J, Paul NR, Ng DHJ, Askari JA, Humphries MJ (2015) Isolation of integrin-based adhesion complexes. Curr Protoc Cell Biol 2015:9.8.1-9.8.15. https://doi.org/10.1002/0471143030.cb0908s66

Figure legends

Figure 1. Overview of the immune synapse isolation protocol. Primary B cells are isolated from mouse spleen and then conjugated with magnetic beads coated with surrogate antigen anti-IgM $F(ab')_2$, or anti-CD71 antibodies as control. Conjugation with activatory beads leads to IS formation. The samples are crosslinked to stabilize the adhesions on the beads. Sonication coupled with washing removes the cell bodies, debris and other contaminants. IS proteins bound on the beads are then eluted and prepared for mass spectrometry analysis.

Figure 2. Monitoring of the conjugate formation and sonication efficiency. Conjugation using 25 million mouse primary B cells and 25 million magnetic beads coated with activatory or non-activatory antibodies was performed as described. Inverted brightfield microscopy was used to image **(A)** conjugates after adding the DTBP crosslinker at 15 minutes after the start of the conjugation, **(B)** after washing away the unbound cells, **(C)** after sonication, and **(D)** after washing off the cell bodies and debris. The results are representative of a set of 12 experiments.