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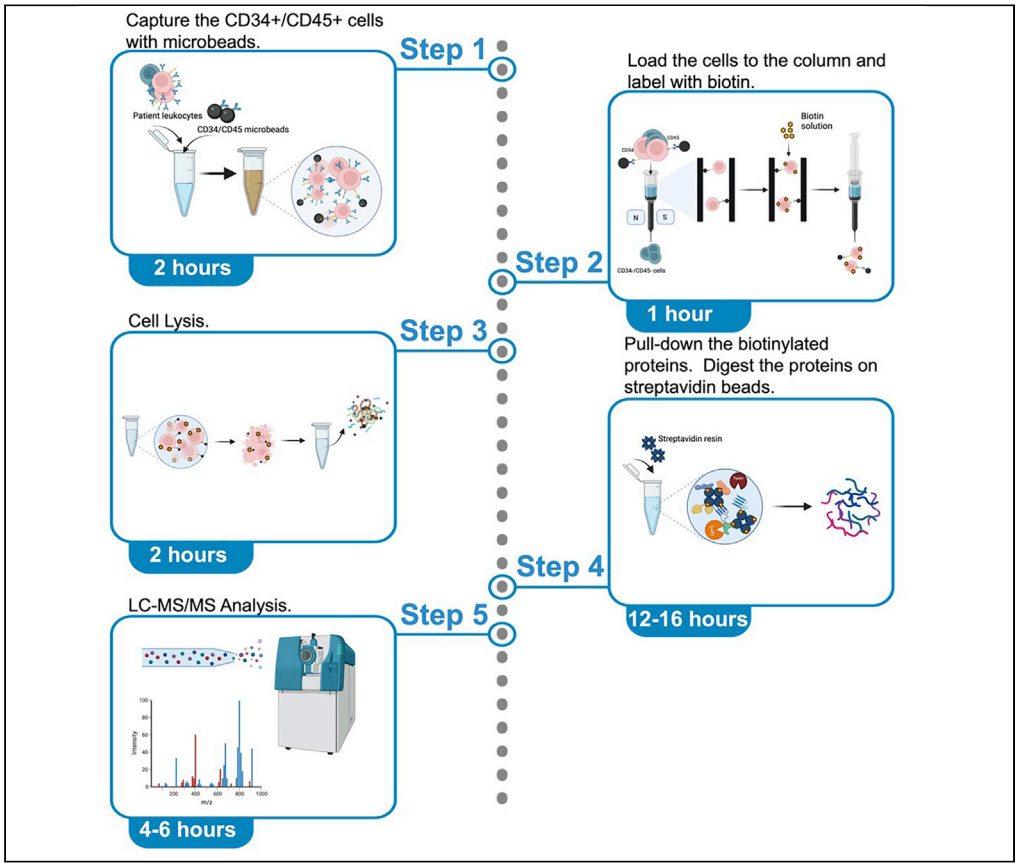
Protocol for cell surface biotinylation of magnetic labeled and captured human peripheral blood mononuclear cells



Analysis of the surfaceome of a blood cell subset requires cell sorting, followed by surface protein enrichment. Here, we present a protocol combining magnetically activated cell sorting (MACS) and surface biotinylation of the target cell subset from human peripheral blood mononuclear cells (PBMCs). We describe the steps for isolating target cells and their in-column surface biotinylation, followed by isolation and mass spectrometry analysis of biotinylated proteins. The protocol enables in-column surface biotinylation of specific cell subsets with minimal membrane disruption.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Serife Ayaz-Guner, Mustafa Burak Acar, Dudu Boyvat, ..., Fatma Fındık, Musa Karakükçü, Servet Özcan

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Highlights

The protocol combines MACS and cell surface biotinylation in MACS column

The protocol enables the capturing and biotinylation of human blood cell subsets

This protocol can be adapted to any cell type with available surface markers

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Protocol for cell surface biotinylation of magnetic labeled and captured human peripheral blood mononuclear cells

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SUMMARY

Analysis of the surfaceome of a blood cell subset requires cell sorting, followed by surface protein enrichment. Here, we present a protocol combining magnetically activated cell sorting (MACS) and surface biotinylation of the target cell subset from human peripheral blood mononuclear cells (PBMCs). We describe the steps for isolating target cells and their in-column surface biotinylation, followed by isolation and mass spectrometry analysis of biotinylated proteins. The protocol enables in-column surface biotinylation of specific cell subsets with minimal membrane disruption.

BEFORE YOU BEGIN

Cell surface proteins constituted one-third of the cell proteome and are essential for many critical cellular processes.[1] Since these proteins are readily accessible on the surface, they can account for a sizable portion of drug targets and are used as diagnostic biomarkers for many diseases.[2] Surface biotinylation, a commonly applied technique for the enrichment of surface proteins, uses impermeable biotin molecules to label surface proteins specifically.[3] Nonetheless, the traditional biotinylation technique consists of several washes and centrifugations. These steps disrupt the membrane integrity and cause the labeling of intracellular proteins. In addition, the conventional biotinylation technique is not specific to any cell subset. The cell surface biotinylation technique, detailed below, explains how to identify the surface proteome of the leukocytes obtained from donors. Incorporating magnetically activated cell sorting $CD34^+$ and $CD45^+$ cells from the patient sample and surface protein biotinylation enables us to isolate leukocytes from PBMCs and directly biotinylate the cell surface on the beads. It should be mentioned that in-column surface biotinylation works for positive sorting strategies. Magnetic column selection and biotinylation technique combination could also be applied to different cell subset selection with specific surface markers such as $CD10^+$ and $CD19^+$ leukocytes and any circulating cell

type with available sorting microbeads. The efficiency of the approach depends on the interaction of sorting antibodies and surface antigens.

Ethical approval

With the approval of Erciyes University's Clinical Research Ethics Committee (Decision No: 2019/176), the patients participating in the study were informed and their consent was obtained.

Buffers and reagents

1. Prepare 500 mL Phosphate-buffered Saline (0.1 M Sodium Phosphate Dibasic, 0.0027 M Potassium Chloride, 0.15 M NaCl, 0.0018 M Potassium phosphate monobasic) adjust the pH to 7.2 by adding 1M HCl.
2. Prepare 100 mL MACS labeling buffer (PBS, 2 mM EDTA, 0.5% BSA).
3. Prepare 100 mM Sulfo-NHS-SS-Biotin stock solution in 100 µL DMSO. Use 50 µL of Biotin stock solution and bring up to 4 mL final volume with PBS.
4. Prepare 500 mL Tris-buffered Saline (0.025 M Tris, 0.15 M NaCl) and adjust the pH to 7.2.
5. Prepare 200 mM stock Tris(2-carboxyethyl) phosphine (TCEP) in 500 µL ultrapure water.
6. Prepare 50 mM Triethylammonium bicarbonate (TEAB) from 1 M stock.
7. 1 M Iodoacetamide (IAA) in 54 µL 50 mM TEAB.
8. Dissolve 1 mg RapiGest in 500 µL 50 mM TEAB to prepare a 0.2% RapiGest-TEAB solution.
9. Prepare In-Stage conditioning buffer (0.1% TFA and 50% ACN).
10. Prepare In-Stage washing buffer (0.1% TFA).
11. Prepare In-Stage elution buffer (0.1% TFA and 80% ACN).

Note: Prepare the biotin solution and In-Stage buffers just before use. For TCEP and IAA, it is better to use fresh buffers.

Note: When dissolving biotin, dissolve it in a nitrogen (N_2) filled chamber to provide a desiccating and inert environment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Peripheral blood sample (25-year-old, male human subjects)	Erciyes University Hospitals	NA
Chemicals, peptides, and recombinant proteins		
Potassium phosphate monobasic	Sigma-Aldrich	P0662-500G
Potassium phosphate dibasic	Sigma-Aldrich	P3786-500G
Potassium chloride	Sigma-Aldrich	P9541
Iodoacetamide (IAA)	Aldrich Chemistry	C0267-500G
Trypsin Gold	Promega	V5280
BSA	Sigma	A-4503 10G
Tris	Sigma-Aldrich	T1503-500G
EDTA 0.5M pH:8.0 UltraPure	Invitrogen	15575-038
NaCl	Sigma Life Science	S5886-500G
RIPA	Sigma Life Science	R0278-50ML
Protease Inhibitor Cocktail Tablet	Roche	11 873 580 001
RapiGest	Waters	186001861-1MG
TCEP	Aldrich Chemistry	C4706-2G
TEAB	Sigma-Aldrich	T7408-100ML
DMSO	Sigma-Aldrich	D2650

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Streptavidin-FITC	Bio-Rad	STAR2B 10223
Lys-C	Promega	V5073
Trypsin Gold	Promega	V5280
Trifluoroacetic acid (TFA)	Sigma-Aldrich	302031-100ML
B-Galactosidase	SCIEX	4465867
7-AAD	BD	344563
Ficoll®-Paque	Sigma-Aldrich	GE17-5442-02
SepMate™-50 (IVD)	STEMCELL Technologies	85450
Methanol LC-MS/MS Grade	Sigma-Aldrich	1.15333.2500
Acetonitrile (ACN) LC-MS/MS grade	Fisher Chemical	A955-1 1L
Formic acid (FA)	Fisher Chemical	A117-50 50ML
Water LC-MS/MS grade	Sigma-Aldrich	1.1
Critical commercial assays		
CD34 microbead	Miltenyi Biotech	130-050-301 2ML
CD45 microbead	Miltenyi Biotec	130-045-801
EZ-LinkTM Sulfo-NHS-SS-biotin	Thermo Fisher Scientific	21331
Software and algorithms		
FACS Diva 8.0.1	BD	NA
Analyst® TF v 1.6	SCIEX	NA
Peak View (1.2)	SCIEX	NA
ProteinPilot 4.5 Beta	SCIEX	NA
CytoScape 3.9.1, ClueGo 2.5.9	CytoScape Team	NA
RStudio	RStudio, PBC	NA
Other		
T75 Cell Culture flask	BD	353118
LS MACS column	Miltenyi Biotec	130-042-401
Cell strainer	BD Falcon	352350
FACS tube	BD Falcon	352235
C18 Disk	Supelco	66883-U
Magnetic separator	Miltenyi Biotec	130-098-308
MACSmix Tube Rotator	Miltenyi Biotec	130-090-753
MACS Column LS	Miltenyi Biotec	130-042-401
Vacuum concentrator	Eppendorf	NA
Ultrasonic water bath	ISOLAB	62105001
Thin glasses	VWR	548-0020
Thin glasses cap	VWR	548-0834
MS tubes	VWR	548-0018
C18 High Resolution 2000	GL Sciences	5020-20E0111522
C18 Trap Column	GL Sciences	5020-10028
Hemocytometer	Thermo	NA
Flow cytometry (FACSAria III)	BD	NA
Eksigent expert nanoLC 425 integrated with ABSciex TripleTOF® 5600+	ABSciex	NA

MATERIALS AND EQUIPMENT

Phosphate-Buffered Saline (PBS) (Store at + 4°C for one month)		
Reagent	Final concentration	Amount
Sodium Phosphate Dibasic	0.1 M	8.19 g
Sodium Chloride	0.15 M	4.38 g
Potassium Chloride	0.0027 M	0.1 g
Potassium phosphate monobasic	0.0018 M	0.122 g
dH_2O		Adjust to volume to 500 mL

MACS Labelling Buffer (Store at +4°C for one week)		
Reagent	Final concentration	Amount
EDTA (0.5 M)	2 mM	80 μL
BSA (10%)	0.05%	1 mL
PBS		Adjust to volume to 20 mL

Biotin Stock Solution (Store at −20°C for one week)		
Reagent	Final concentration	Amount
Sulfo-NHS-SS Biotin	100 mM	6 mg
DMSO		Adjust to volume to 100 μL

Biotin Labeling Solution (Prepare Fresh)		
Reagent	Final concentration	Amount
Biotin Stock Solution (100 mM)	1.25 mM	50 μL
PBS		Adjust to volume to 4 mL

Tris-Buffered Saline (TBS) (Store at + 4°C for six months)		
Reagent	Final concentration	Amount
Tris	0.025 M	1.51 g
NaCl	0.15 M	4.38 g
dH_2O		Adjust to volume to 500 mL

TCEP Solution (Prepare Fresh)		
Reagent	Final concentration	Amount
TCEP	200 mM	28.6 mg
dH_2O		Adjust the volume to 500 μL

TEAB Solution (Prepare Fresh)		
Reagent	Final concentration	Amount
TEAB	50 mM	25 μL
dH_2O		Adjust the volume to 500 μL

IAA Solution (Prepare Fresh)		
Reagent	Final concentration	Amount
IAA	1 M	10 mg
TEAB		54 μL

RapiGest SF Preparation (Prepare Fresh)		
Reagent	Final concentration	Amount
RapiGest	0.2%	1 mg
TEAB		Adjust the volume to 500 μL

Conditioning Buffer (Prepare Fresh)		
Reagent	Final concentration	Amount
TFA	0.1%	1 μL
ACN	50%	500 μL
MS Grade Water		Adjust to volume to 1mL

Wash Buffer (Prepare Fresh)		
Reagent	Final concentration	Amount
TFA	0.1%	2 μL
MS Grade Water		Adjust to volume to 2 mL

Elution Buffer (Prepare Fresh)		
Reagent	Final concentration	Amount
TFA	0.1%	2 μL
ACN	80%	1.6 mL
MS Grade Water		Adjust to volume to 2 mL

MS Sample Solution (Prepare Fresh)		
Reagent	Final concentration	Amount
ACN	5%	50 μL
Formic acid	0.1%	1 μL
MS Grade Water		Adjust the volume to 1 mL

STEP-BY-STEP METHOD DETAILS

PBMC isolation

Timing: 30 min

This section describes density gradient centrifugation to isolate peripheral blood mononuclear cells (PBMS) from whole blood using Ficoll-Paque.

1. Dilute 10 mL of blood with DPBS supplemented with 2% FBS at a 1:1 ratio.
2. Add 15 mL of lymphocyte separation medium (Ficoll-Paque) into the SepMate (commercial ficoll-based blood cell separation tubes).
3. Carefully layer the diluted blood sample (20 mL) onto the Ficoll-Paque media solution by slowly adding with tubes tilted (30°–45°) horizontally.
4. Centrifuge at 1200 × *g* for 10 min.
5. Take the above part of SepMate to a new falcon tube.
6. Adjust the volume with DPBS to 50 mL then centrifuge at 300 × *g* for 10 min.

Note: Optionally, cells might be frozen with a cryoprotectant.

Note: Do not mix the Ficoll-Paque media solution with the diluted blood sample when layering the sample.

Magnetic-activated cell sorting of target cells

Timing: 90 min

This section describes positive selection of $CD34^+$ and $CD45^+$ monocytes by magnetic activated cell sorting (MACS) from Miltenyi Biotec (Figure 1). The method could be applied for isolation of different cell types with a similar positive selection approach.

Note: We suggest using swing-bucket centrifugation to reduce the loss of cells.

7. Collect the cells into a conical tube and centrifuge at 125 × *g* for 10 min.

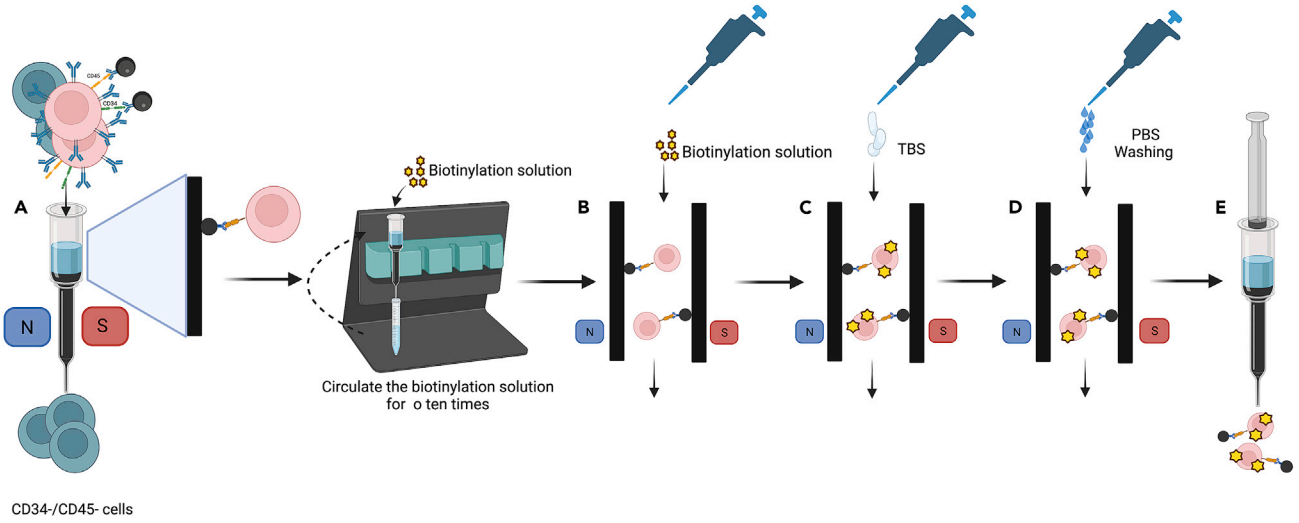


Figure 1. Schematic representation of the biotin labeling of the CD34$^+$ and CD45$^+$ cells

(A) Labelling of target cells by magnetic bead. (B) Biotinylation in MACS column. (C) Termination of biotinilation with TBS. (D) PBS washing step (optional). (E) Elution.

8. Dissolve the cell pellet in 10 mL PBS.
9. Count the cells with the help of a hemocytometer.
10. Continue with 2 × 10^7 cells.
11. Centrifuge at 125 × *g* for 10 min. Repeat the PBS washing step 2 twice.
12. Dissolve the cell pellet in 80 µL MACS buffer.

Note: Be gentle when dissolving the cells. Try not to use small pipette tips; narrow tips may increase cell damage.

13. Add 20 µL CD34 and CD45 microbeads to the cell suspension.
14. Mix gently and incubate at +4°C for 15 min; rotate slowly.
15. Add 2 mL labeling buffer and centrifuge the cells at 125 × *g* for 10 min to wash the cells.
16. Place the LS (largescale) MACS column on the magnetic separator.
17. Rinse the column by adding 2 mL MACS buffer.
18. Add the labeled cells into the MACS column. Unlabeled cells will flow through the column.
19. Wash the cells with 3 mL MACS buffer once then wash with PBS 3 times.

Note: If cells do not attach to the column in magnetic field, see troubleshooting problem 1 for workable solutions. In case of slow or no flow through on the column, see troubleshooting problem 2 for possible solutions.

⚠ CRITICAL: Ensure that the MACS buffer is thoroughly washed/removed from the column, as it can interfere with the biotinylation process. Additional washing steps with PBS can be performed/are recommended.

Surface biotinylation of the cells in the MACS column

Timing: 40 min

This section describes an efficient way of surface biotinylation of the cells in the MACS column (Figure 1).

20. Place a 15 mL Falcon tube to collect the flow-through solution and add the biotinylation solution into the column.
21. Take the flow-through (biotinylation solution) and add it to the column again. Repeat this process at least 10 times.

Note: The labeling process should be limited to a maximum of 30 min at room temperature.

Note: The flow rate depends on the scale of the column. Therefore, the process needs to be repeated for at least 15 min, and the repeat process needs to be adjusted accordingly.

22. Apply 2 mL TBS buffer into the column to quench the biotinylation reaction. Repeat the TBS wash 3 times.
23. Add 2 mL MACS buffer to the column.
24. Remove the column from the magnetic separator.
25. Plunge the column into the collection tube to elute the surface biotinylated cells.

Validation of surface biotin labeling

Timing: 20 min + Flow cytometry analysis

This section describes a flow cytometry-based method to validate biotinylation of surface proteins and plasma membrane integrity.

26. Take 75 μL (it should be approximately 10^6 cells) from the eluted cells obtained from step C6 to validate biotinylation.
27. Take 10^6 cells from the non-biotinylated $CD34^+$ $CD45^+$ PBMCs into a separate tube as a control to measure the non-specific streptavidin bindings.
28. Take 10^6 cells from the non-biotinylated $CD34^+$ $CD45^+$ PBMCs into a separate tube as an autofluorescent to adjust the fluorescent intensity threshold. (Do not add any fluorescence-emitting substance).
29. Adjust the volumes to 100 μL with PBS.
30. Add 3 μL streptavidin-FITC onto the 100 μL of cells (steps 26 and 27) and incubate at RT for 15 min in a dark place.
31. Add 2 μL 7-AAD to the samples and incubate at RT for 2–3 min.
32. Complete the volumes to 2 mL with PBS.
33. Analyze the cells with appropriate settings in a Flow cytometry instrument (Figure 3).

Note: After flow cytometry analysis, over 80% of the population should be FITC positive and 7-AAD negative. If the percentage of FITC positive cells is below 80%, do not continue the downstream workflow. This situation may result from low biotinylation efficiency (troubleshooting problem 3). The presence of a high number of 7-AAD-positive cells could indicate cell death. We recommend an additional wash during the MACS step.

Optional: If there is a need to confirm the MACS step, researchers may add properly selected antibodies to validate the purity of the cell population. One should remember that the fluorochrome chosen should not be in the spectrum of Streptavidin conjugated FITC and 7-AAD.

Isolation of biotinylated surface proteins

Timing: 60 min

This section describes cell lysis and isolation of biotinylated proteins using streptavidin resin from the lysate (Figure 2).

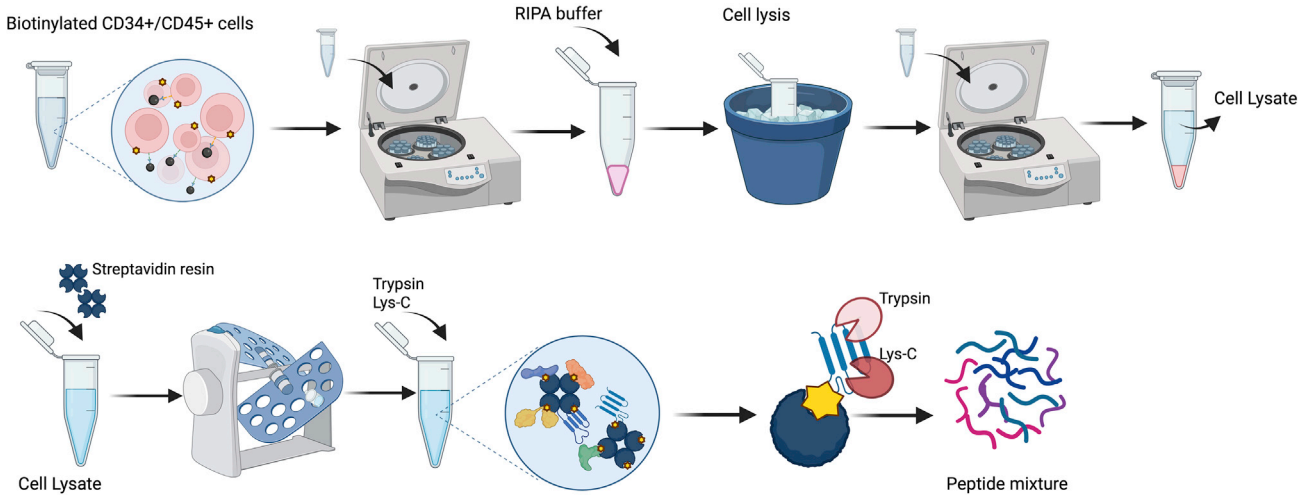


Figure 2. Schematic representation of On-Bead digestion of biotin-labeled cells

34. Centrifuge the cells obtained from step 25 at 125 × *g* for 10 min.
35. Add protease inhibitor (2% v/v) to the 500 µL RIPA lysis buffer and dissolve the cell pellet.

Note: Pipetting will improve the efficiency of cell lysis; repeat 20 times at least.

36. Incubate the cells on ice for 30 min and apply a 5-s vortex every 10 min through dispersion and disruption of the cell pellet.

Note: DNase (0.5 µL/mL Benzonase) could be applied to reduce the viscosity of the lysate.

Note: If it is available, Bioruptor sonication can be applied.

37. Centrifuge the cell lysate at 15000 × *g* for 10 min at +4°C.
38. Transfer the supernatant to a clean collection tube.

⚠ CRITICAL: Use protein low-bind tubes after this step.

39. Wash the 75 µL streptavidin resin with 500 µL PBS and centrifuge at 1000 × *g* for 1 min to clean the storage solution.
40. Repeat the washing step with 100 µL lysis buffer to equilibrate the resin with the cell lysate.
41. Combine the streptavidin resin with cell lysate and incubate at RT for an hour on a rotator mixer.
42. Centrifuge the resin at 1000 × *g* for 1 min; the unbound proteins will remain in the supernatant.

Note: Unbound proteins can be discarded or stored for further analysis.

43. Wash the resin with 100 µL lysis buffer and centrifuge at 1000 × *g* for 1 min.
44. Wash the beads with 1 mL PBS and centrifuge at 1000 × *g* for 1 min; repeat the washing step 3 times.

⚠ CRITICAL: Additional extensive washing steps (step 44) are strongly recommended to prevent detergent contamination for the mass spectrometry.

Pause point: Keep the samples −80°C, proteins could be stored on beads for couple of months.

On-bead digestion

Timing: 30 min + overnight incubation. 4 h + 15 min

This section describes enzymatic digestion of enriched biotinylated proteins on streptavidin agarose bead for mass spectrometry analysis (Figure 2).

45. Suspend the resin in 300 µL 0.2% (w/v) RapiGest solution containing TEAB.
46. Add TCEP and IAA to the solution at a final concentration of 20 and 40 mM, respectively.
47. Incubate the resin at 60°C for 30 min and cool on ice.
48. Add 200 ng Lys-C to the resin mixture and incubate overnight at 37°C.
49. After Lys-C incubation, add 800 ng Trypsin-Gold to the resin and incubate for 4 h at 37°C.
50. Centrifuge the resin at 10000 *g* for 1 min; transfer the supernatant into a low-bind collection tube.
51. Acidify the sample with TFA (1% final concentration) before loading it into Stage Tips.
52. Activate the Stage Tips with the addition of 250 µL In-Stage conditioning buffer.
53. Add 250 µL In-Stage washing buffer to equilibrate the Stage Tips.
54. Load the acidified samples into the Stage Tips and wash the peptide-bounded tips with washing buffer. Repeat the washing step 3 times.
55. Add 50 µL In-Stage elution buffer and elute the peptides with a syringe; apply pressure on the In-Stage tips to facilitate the elution process.

Note: Additional extensive washing steps (step 54) are strongly recommended to prevent detergent contamination for the mass spectrometry.

Note: In our experience, repeating the elution step is increases the peptide yield. If a high volume of elution is not preferred 25 µL elution can be repeated 2 twice.

56. Dry the eluate with a vacuum concentrator and store at −20°C.

Note: On-bead Digestion protocol adapted from Bonn and Otto (2018).[4]

Pause point: Keep the samples −80°C, lyophilized peptides could be stored for couple of years.

LC-MS/MS analysis

Timing: 30 min + 180 min LC-MS/MS + 4–6 h Protein Identification

This section illustrates parameters of mass spectrometry analysis of enriched membrane proteins. The parameters depend on the available device.

Note: Proteomic analysis may be conducted with any high-resolution LC-MS configuration if compatible or equivalent to the ABSciex ToF 5600+ System.

57. Dissolve the dried samples in 12 µL of the sample solution (5% ACN, 0.1% Formic Acid),
    a. Sonicate the samples in the ultrasonic water bath for 1 min.
    b. Centrifuge the samples at 15000 × *g* for 20 min at 4°C.
    c. Transfer the supernatant into insert-vials.
58. Inject 4 µL of peptide mixture in a trap elute mode and separate in a reverse phase analytical column.

Note: LC separation method and parameters depend on the available instrument and columns. We applied 4 µL of peptide mixture to Monocap Column (1.8 µm HSS T3 75 µm × 250 mm) with

Table 1. Nano-LC gradient

Time (min)	A%	B%
0	85	15
157	45	55
162	5	95
170	5	95
177	90	10
180	90	10

trap elute mode. A 180-min gradient (Table 1) with a 350 nL/min flow rate for the peptide elution was used.

59. Identify peptides using a high-resolution mass spectrometry instrument. The mass spectrometry data acquisition and analysis will vary based on the mass spectrometry to be employed.

Note: We employed Eksigent expert nanoLC 425 integrated with ABSciex TripleTOF® 5600+. The Data Dependent Acquisition (DDA) approach was applied for peptide analysis. Positive TOF MS scanning type (Accumulation time: 0.250 s, Collision Energy (CE): 10, Declustering Potential (DP): 100, TOF Masses 350.000–1250.000 Da) was used to do the precursor ion (Precursor-Ion) analysis. Positive Product Ion scanning type was used to analyze product ions created by fragmented precursor peptides. Collision Energy (CE): rolling (Accumulation Time: 0.0500 s. TOF Masses 230.000–1500.000 Da, Declustering Potential (DP) 100, Collision Energy Spread (CES) 10. The top 35 candidate molecules (Top35) for each study cycle were selected based on their mass and charge (Switch criteria: >350.000 m/z, 1250.000 m/z, Ion tolerance 150.000 mDA).

60. Collect and analyze mass spectrometry raw data with accompanying software.
61. Identify proteins via proteomics search engine.
    a. Use organism specific reference proteome database.
    b. Apply Carbamidomethyl as a fixed modification, and Thioacyl modification as a variable modification during data analysis.

Note: The mass spectrometry raw data was collected and analyzed using Analyst® TF v 1.6 (ABSciex). Peak View (1.2) Mass fingerprint ProteinPilot 4.5 Beta (ABSciex) generated by the reporting of the device) were used to evaluate the peptides and product ions. The proteins, their modifications and isoforms were identified using the current protein session retrieved from UniProtKB (UniProtKB: UP000005640, Organism ID:9606, 06_2022). We used global false discovery rate (FDR) 1%. Every identified protein must be represented with at least 2 unique peptides.

Note: Perform the Gene Ontology Cellular Component Analysis with the list of identified proteins. In case of high cytoplasmic ontology annotation in the list, see troubleshooting problem 4 for potential solutions. For covering a small number of identified proteins via a protein search engine, see troubleshooting problem 5 for potential solutions.

EXPECTED OUTCOMES

Our protocol allows efficient isolation and surface biotinylation of $CD34^+$ and $CD45^+$ leukocytes from PBMCs. Once we applied traditional surface biotinylation in a suspension, we observed that one-third of the biotinylated population come from dead cells (Figure 3). We did surface biotinylation of magnetically labeled cells in the MACS column to discard dead cells and prevent cell death during the extended surface biotinylation process. In the fourth row of Figure 3, we have successfully biotinylated 90.6% of the population and a four-fold reduction in the death cell population.

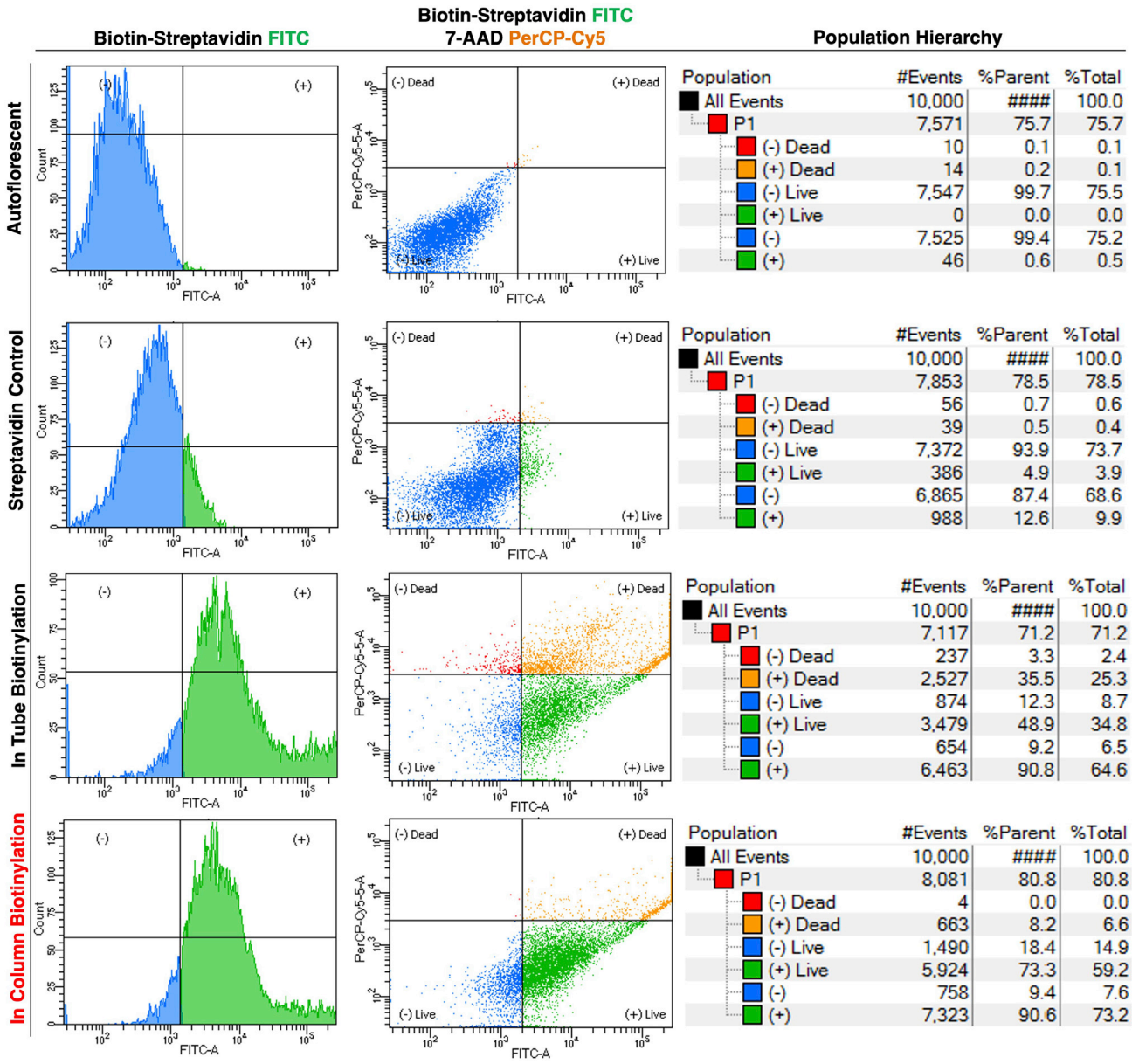
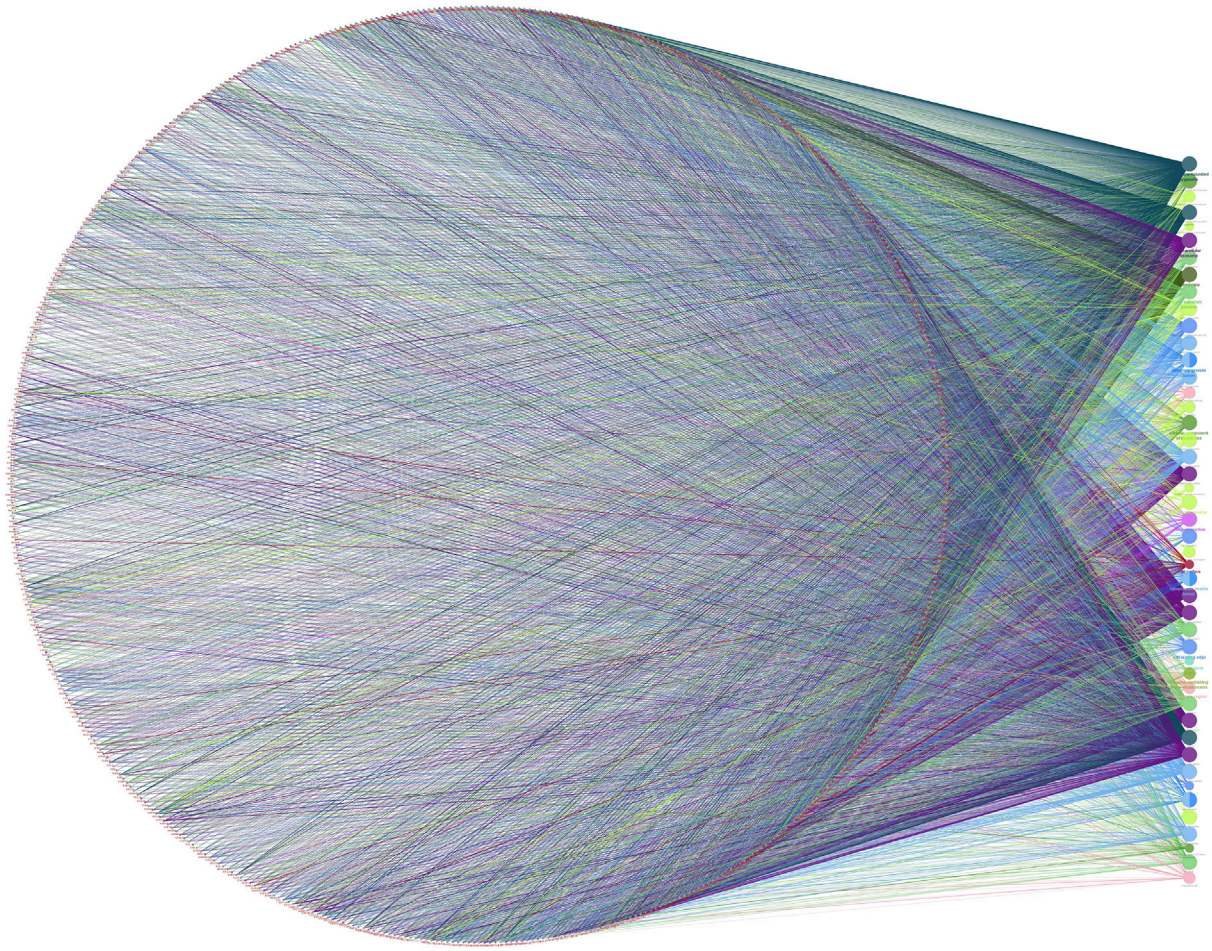


Figure 3. Flow cytometry comparison analysis of In-Tube and On-Column biotinylation

Our protocol identified 887 proteins (1% FDR) from LC-MS/MS analysis. With this method, we isolated 679 proteins (1% FDR). Gene Ontology Cellular Compartment analysis showed that most of these isolated proteins are annotated with "Membrane," "Plasma membrane," "Cell surface," and "Extracellular Space" (Figure 4).

LIMITATIONS

Our method could be applied to various cell populations as long as commercially available antibodies against surface markers are present. For a particular type of cell, there may not be specific surface markers for selection. One of this protocol's significant limitations is the paramagnetic columns' scale. The scale of the column determines the initial and maximum number of labeled cells. This limitation directly depends on the vendors and available column sizes. The labeled cell number

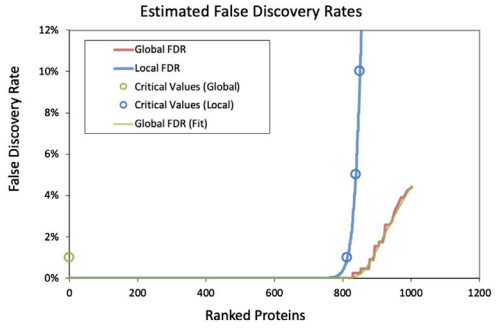


Protein Level FDR Analysis

Proteins Identified at Critical False Discovery Rates

	Number of Proteins Detected		
Critical FDR	**Local FDR**	*Global FDR*	**Global FDR from Fit**
1,0%	*813*	*889*	**887**
5,0%	**839**		
10,0%	**851**		

** It is recommended you use numbers in bold and avoid using numbers in italics.*



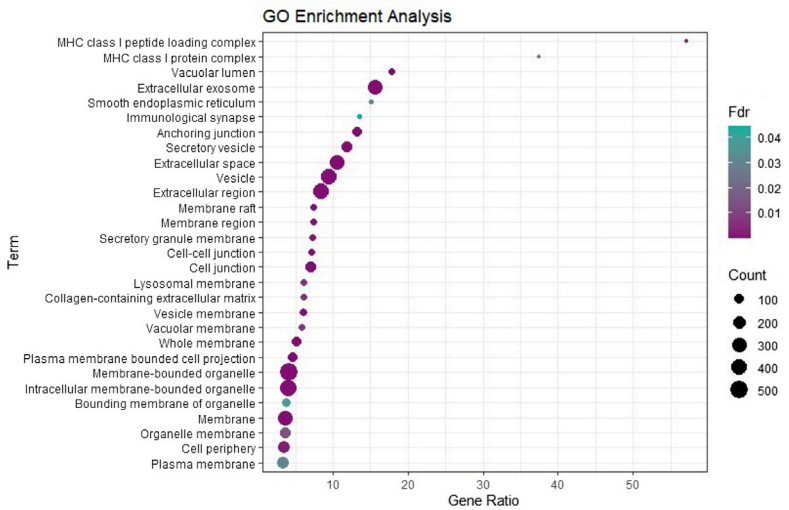


Figure 4. FDR and GO Cellular Component analysis of the biotinylated proteins

should be around 5 × 10^6 for blood cells to identify biotinylated proteins correctly. Starting and labeled cell numbers could vary according to the type of cell, their percentage in the population, and the instrument's sensitivity. The consistency and efficiency of the methodology may be subject to the interaction of sorting antibodies and surface antigens. The expression level of sorting antigens should be high enough for efficient capturing. Notably, we applied our method to a cultured B-lymphoblast cell line (CCRF-SB) and obtained similar biotinylation efficiency using CD19 antibodies.

TROUBLESHOOTING

Problem 1

Cells do not attach to the column (Step: magnetic-activated cell sorting of target cells).

Potential solution

Cells might not be expressing the targeted surface marker; expression of surface markers should be validated using flow cytometry analysis.

Problem 2

Cells could clog the columns (Step: magnetic-activated cell sorting of target cells).

Potential solution

Cell strainer (with desired mesh) should be used before applying the column.

Problem 3

Decreased biotinylation yield (Step: validation of surface biotin labeling).

Potential solution

- Prepare the biotin solution just before use, after dissolving try to use it within a couple of minutes.
- Avoid a moisturized/humid environment when dissolving the biotinylation reagent with anhydrous DMSO for long-term storage.
- Prepare your experiment set-up to use all dissolved biotin or try not to freeze the biotin solution in high volume.
- Primary amine-containing buffer usage may cause a decrease in the biotinylation yield. Before starting the biotinylation reaction ensure cells are washed with PBS extensively.

Problem 4

The high amount of intracellular protein contamination (Step: LC-MS/MS analysis).

Potential solution

- Biotin will be dissolved in DMSO, affecting the cell membrane's integrity. Try to dilute DMSO within the proper range to keep the membrane intact. A concentration of less than 5% DMSO is recommended.
- The column washing step, especially before biotin addition, can be repeated 3–5 times to eliminate dead cells.
- After streptavidin resin binding, additional washings can be done to prevent potential non-biotinylated protein contamination.
- Performing the biotinylation at a lower (2°C–8°C) temperature might be tried.

Problem 5

Identification of the low number of biotinylated proteins via LC-MS/MS analysis (Step: LC-MS/MS analysis).

Potential solution

- Detergents coming from the lysis buffer could suppress the ionization efficiency of peptides. Additional washing steps of streptavidin agarose beads and in-stage tip purification should be considered.
- A small number of labeled cells might decrease the identified protein. We recommend an increase in the starting number of cells.
- Since membrane proteins are highly glycoslylated, glycans could be removed by glycosylases.
- The type and amount of digestion enzyme should be optimized according to need.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Servet Ozcan, ozcan@erciyes.edu.tr.

Materials availability

This study did not generate new materials.

Data and code availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[5] partner repository with the dataset identifier PXD038027

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AUTHOR CONTRIBUTIONS

Conceptualization, S.A.-G., M.B.A., S.O.; Methodology, S.A.-G., M.B.A., D.B., H.G., H.B., M.G., S.K.Y., N.A., F.F., M.K., S.O.; Writing and editing, S.A.-G., M.B.A., D.B., M.G., S.O.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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