SUPPLEMENTARY INFORMATION

Nanowire array chips for molecular typing of rare trafficking leukocytes with application to neurodegenerative pathology

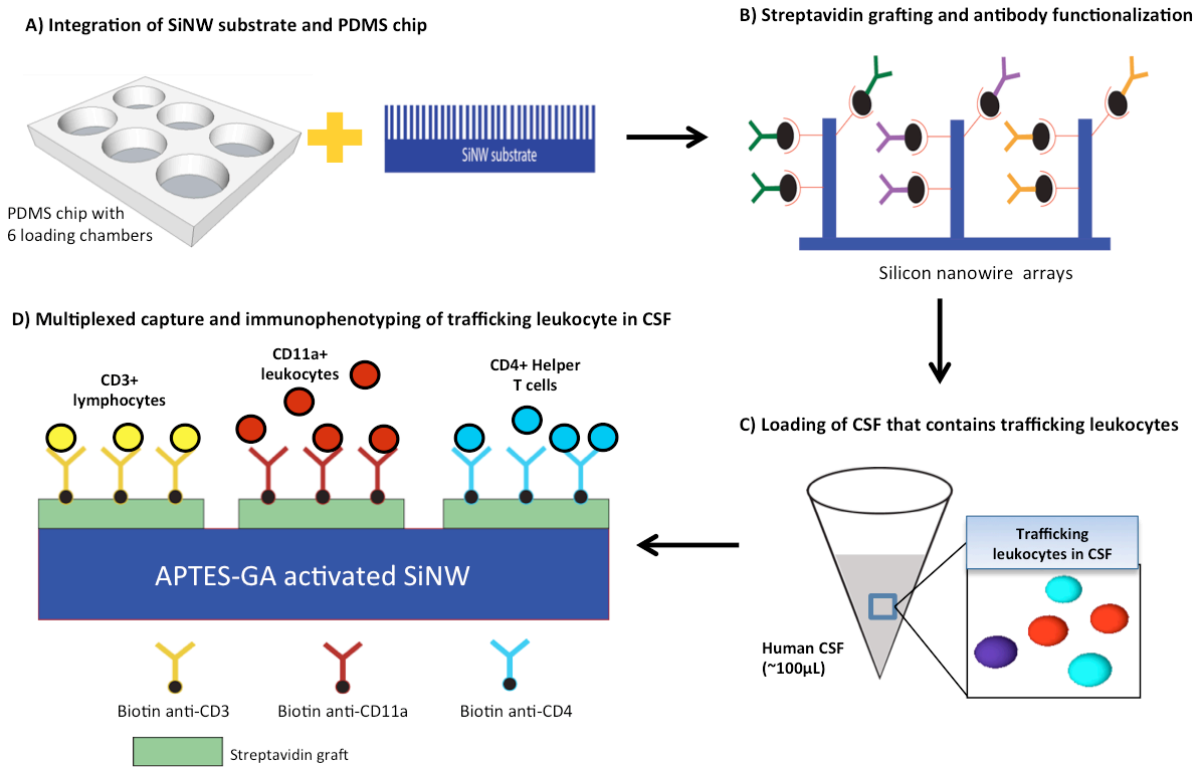
Minsuk Kwak[a], Dong-Joo Kim[b], Mi-Ri Lee[b], Yu Wu[a], Lin Han[a], Sang-Kwon Lee[bc] and Rong Fan*[ad]

[a] *Department of Biomedical Engineering*, Yale University, New Haven, CT 06520, USA.
[b] *Department of Semiconductor Science and Technology, Chonbuk National University, Jeonju 561-756, Korea*
[c] *Department of Physics, Chung-Ang University, Seoul 156-756, Korea.*
[d] *Yale Comprehensive Cancer Center, New Haven, CT 06520, USA.*

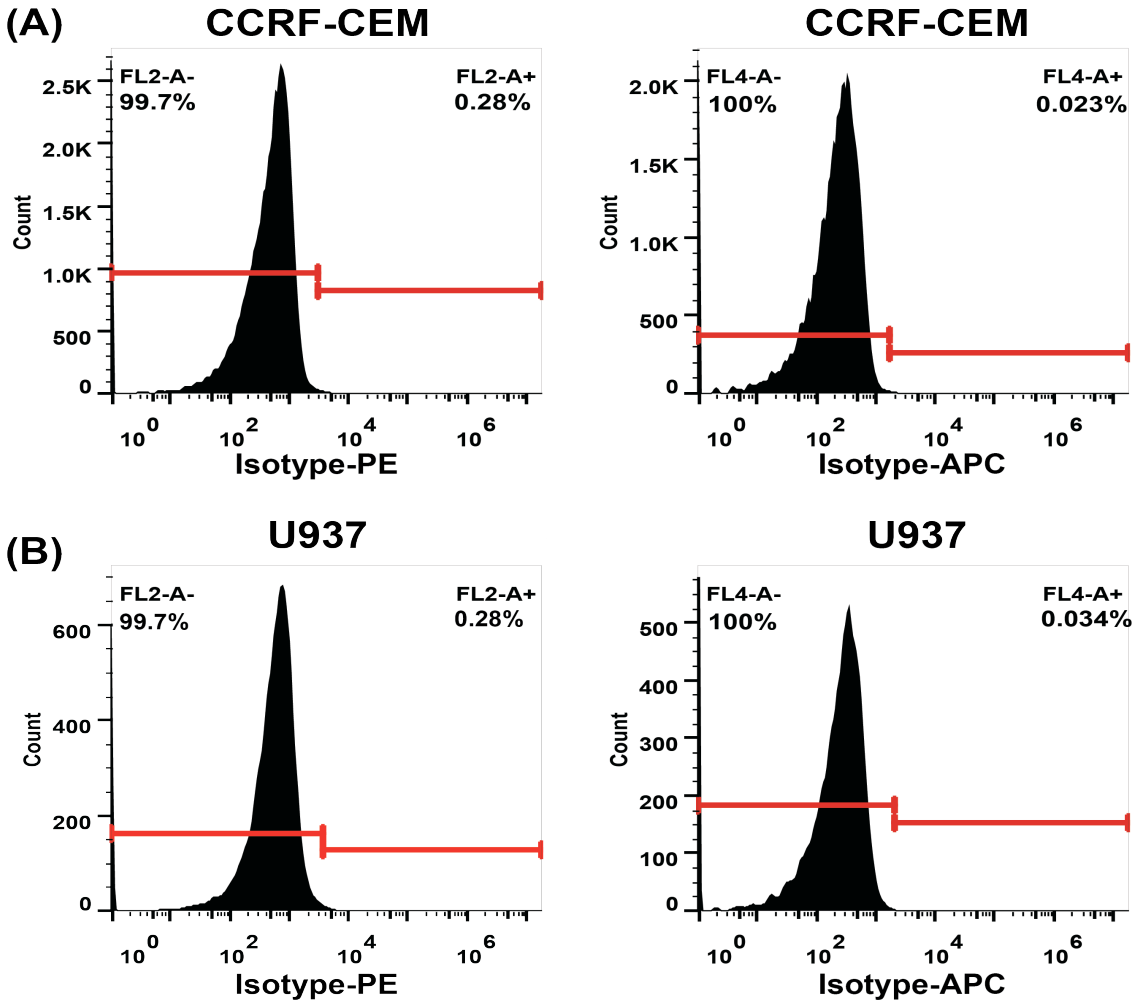
* Address correspondence to: Rong Fan, Department of Biomedical Engineering, Yale University, 55 Prospect St., MEC 213, New Haven, Connecticut 06520, USA. Phone: 203.432.9905; Fax: 203.432.1106; E-mail: rong.fan@yale.edu.

Supplementary Figure S1	The schematic illustration of biochemical functionalization of silicon nanowire arrays and multiplexed capture of rare trafficking leukocytes in CSF.
Supplementary Figure S2	Gating of two-color flow cytometry of CCRF-CEM and U937 based on isotype control.
Supplementary Figure S3	Validation of multiplexing and immunophenotyping using U937 cells.
Supplementary Figure S4	The capture purities and yields of CD4+ and CD8+ T cell mixture on antibody-functionalized silicon nanowire substrates.
Supplementary Figure S5	The number of leukocytes from Alzheimer's disease patients' cerebrospinal fluid (CSF) captured on the SiNW substrates not functionalized with antibody.
Supplementary Figure S6	Enlarged views of fluorescent images showing capture specificity of CD4+ and CD8+ T lymphocytes from rare trafficking leukocytes in CSF samples.
Supplementary table S7	Multiplexed immunophenotyping of trafficking leukocytes when volume of loaded CSF varies from 200μL to 50μL.
Supplementary table S1	Quantification and multiplexed immunophenotyping result of CCRF-CEM cells using the silicon nanowire-based rare cell analysis platform.
Supplementary table S2	Quantification and multiplexed immunophenotyping result of U937 cells using the silicon nanowire rare cell analysis platform. U937 cells were loaded with the cell number of 700 cells per loading chamber.
Supplementary table S3	Highly efficient capture and multiplexed quantification of rare trafficking leukocytes in cerebrospinal fluid (CSF) of Alzheimer's disease patients.
Supplementary results	Results from additional validation experiments.

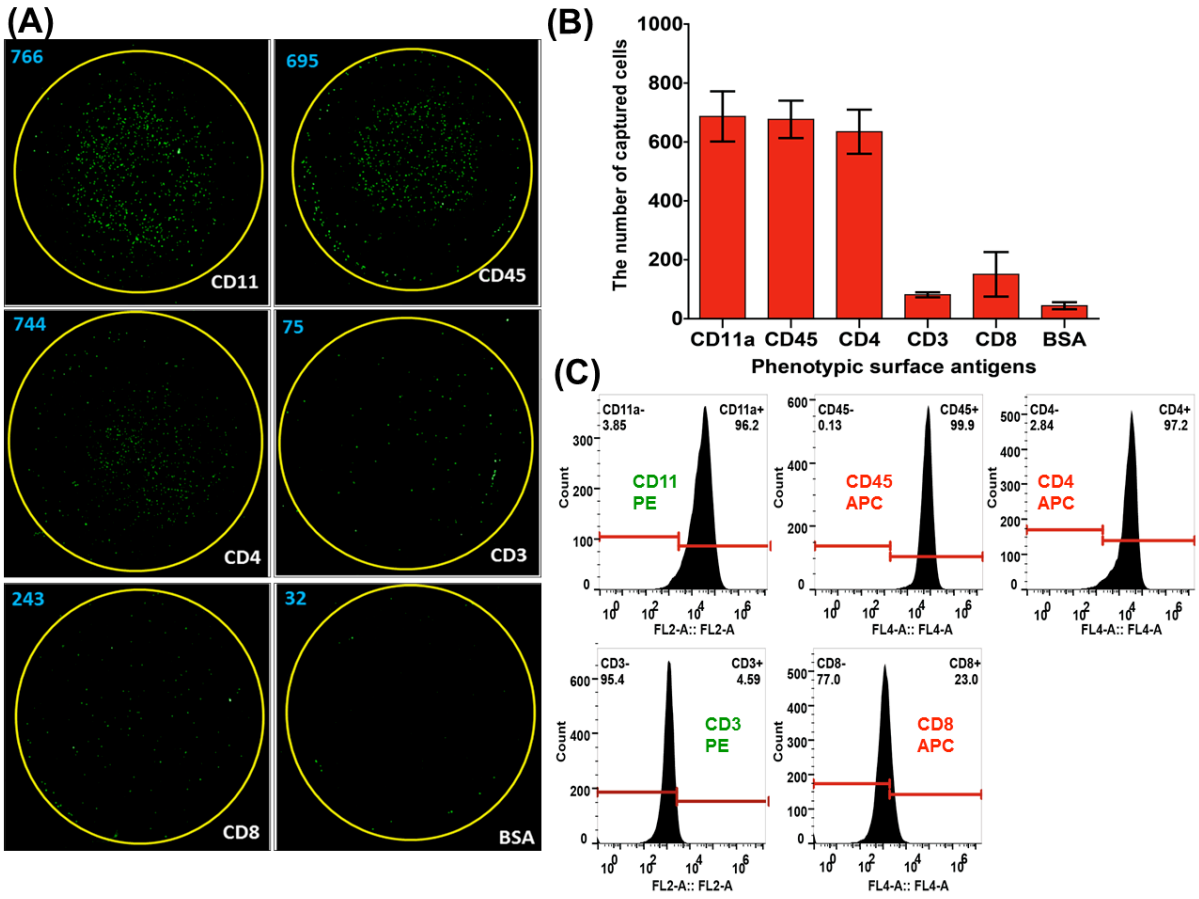
Supplementary Figures 1-7



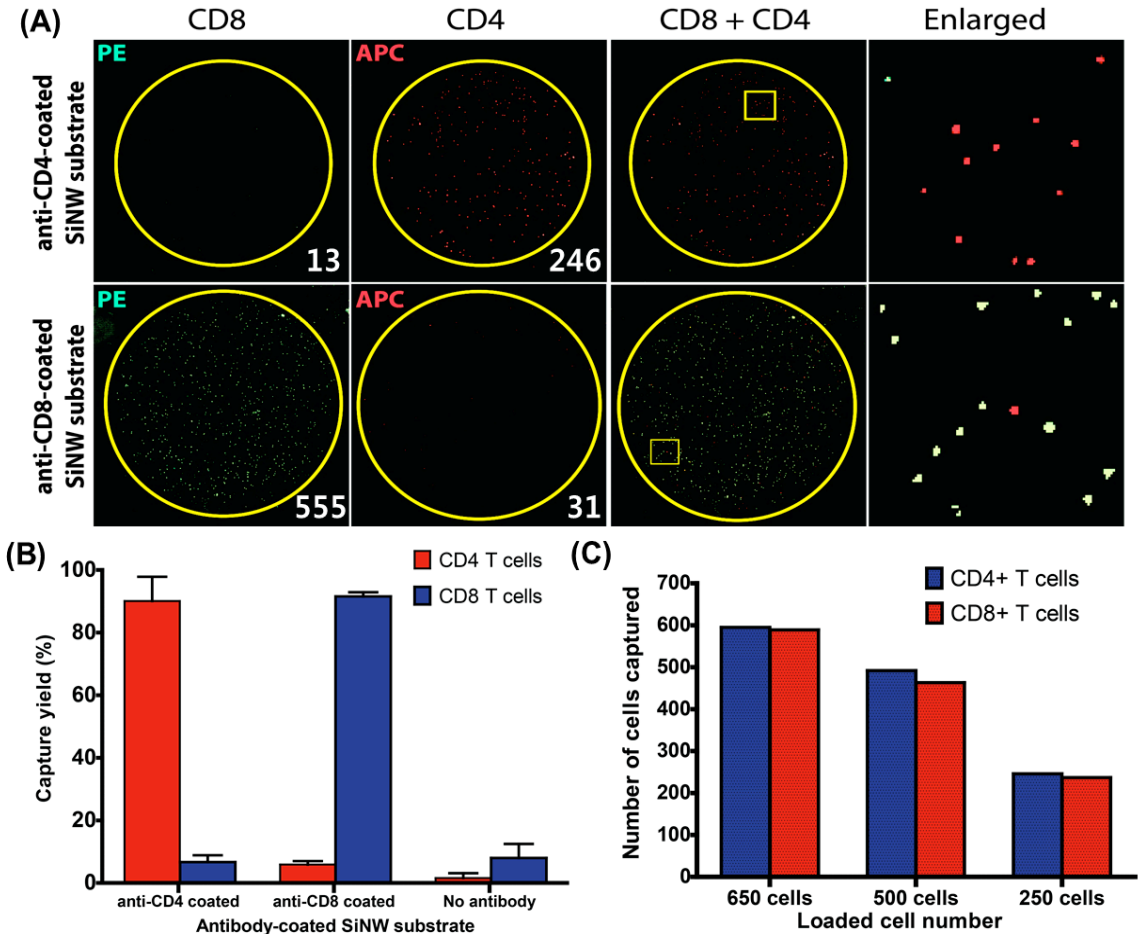
Supplementary Figure S1. The schematic illustration of biochemical functionalization of silicon nanowire arrays and multiplexed capture of rare trafficking leukocytes in CSF. **(A)** After its fabrication, a silicon nanowire (SiNW) substrate was chemically modified through a series of chemical reaction. The chemically activated SiNW substrate is integrated with a PDMS mold that contains multiple loading chambers. The loading reservoir-containing PDMS slab was thermally bonded for 30 minutes at 80 °C onto the SiNW substrate. **(B)** The thermal integration with a PDMS mold creates multiple cylindrical chambers into which streptavidin and biotinylated capture antibodies are introduced to generate multiple distinct capture regions. Each capture region is functionalized with biotin-conjugated antibody (K_d = $1x10^{-15}$) against the surface antigen of a specific leukocyte phenotype. High-affinity interaction between streptavidin and biotin allows the functionalization of SiNW with biotinylated monoclonal antibodies. Functionalized with the antibodies, each capture region can attract and capture specific leukocyte subsets that express the corresponding surface antigen. **(C)** For each loading well, the CSF sample of 50 µL was introduced. The device loaded with CSF was incubated on ice for 45 minutes to allow for the selective capture of specific trafficking leukocyte subsets. **(D)** The graphical illustration of multiplexed capture and phenotyping of trafficking leukocytes onto the functionalized SiNW substrate. As shown in the schematic, the silicon nanowire array contains multiple, distinct capture regions. Each capture region can selectively attract and separate the phenotypically distinct leukocyte subsets. The number of the capture regions is same as the number of loading chambers created within the PDMS slab.



Supplementary Figure S2. Gating of two-color flow cytometry of CCRF-CEM and U937 based on isotype control. (A) Histograms represent the data collected from CCRF-CEM cells labeled with isotype control antibodies that serve as negative controls. (B) Histograms represent the data collected from U937 cells labeled with isotype control antibodies that serve as negative controls. The left figure represents the data collected from FL2 laser that excites PE. The right figure represents the data collected from FL4 laser that excites APC. The gates were located right to the negative peaks of isotype controls.

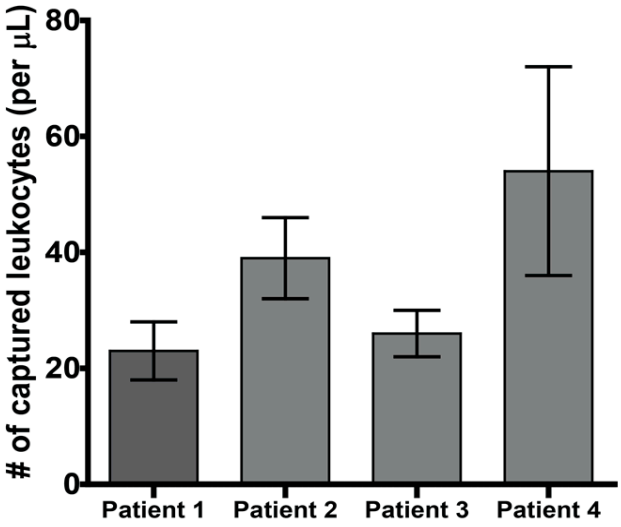


Supplementary Figure S3 Validation of multiplexing and immunophenotyping using U937 cells. A) Fluorescence images of U937 cells captured on different capture regions of an antibody-functionalized nanowire substrate. After immobilization and removal of non-specific cells, the captured cells were stained with fluorophore-conjugated antibodies against immune cell antigens (PE-anti CD11). Excitation wavelength: 532nm. B) Quantification of U937 cells detected on distinct capture regions. Each capture well is coated with one capture antibody against the surface antigen of specific leukocyte phenotype so that only the cells expressing the specific antigen will be captured. Each chip can feature multiple capture regions, allowg multiplexed immunophenotyping. For each well, 700 cells were introduced. The bar height represents the mean number of captured cells from 5 repeated experiments in 5 separate devices. C) Flow cytometric analysis of U937 leukocyte population. Flow cytometry, the conventional method for immunophenotyping, determined the cellular distribution of phenotypically diverse subsets composing U937 cells. The cells were labeled with fluorescence-conjugated (APC or PE) antibodies against the same leukocyte antigens used to functionalize a nanostructure-based cell analysis chip. The number on the top right corner of each panel represents the proportion of the cells that are positive for antigen expression.



Supplementary Figure S4. The multiplexed phenotyping of CD4+ and CD8+ T cell mixture on antibody-functionalized silicon nanowire substrates to validate capture efficiency and capture specificity for primary immune cells. (A) Fluorescence micrographs of SiNW substrates which were functionalized with either CD4 or CD8 antibodies. Primary human CD4+ and CD8+ T lymphocytes (Astarte Biologics) were mixed with the ratio of 1:1. The top row shows the images of the cells captured on the anti-CD4 functionalized substrate, and the bottom row shows the images of the cells captured on the anti-CD8 functionalized substrate. The first column shows the images of captured CD8+ T cells, which were immune-stained with PE-conjugated CD8 antibody (green – 532nm), and the second column shows the images of captured CD4+ T cells, which were immune-stained with APC-conjugated CD4 antibody (red – 635nm). The number highlighted on bottom right corners of each image indicates the number of cells detected and enumerated. The third column shows the overlapped images of CD8+ T cells detected by 532nm channel and CD4+ T cells detected by 635nm channel. The fourth column shows the enlarged images of the confined regions highlighted in yellow rectangles in the overlapped images. (B) The capture efficiency of CD4+ T cells and CD8+ T cells on the SiNW substrates coated with CD4 antibody, CD8 antibody, or BSA (no antibody). Each result and error bar represents an average with standard deviation (n=4). The antibody-coated substrates displayed vastly improved capture efficiency for the target cells expressing corresponding surface antigens. The substrates not coated with any antibody showed significantly lower capture efficiency for both leukocyte types. (C) The number of captured CD4+ and CD8+ T lymphocytes on the bio-functionalized SiNW arrays. The SiNW substrate was conjugated with streptavidin and functionalized with either biotin-CD4 antibody or biotin-CD8 antibody. Varying numbers of CD4 and CD8 T cells (250, 500, and 650 cells) were mixed in a 1:1 ratio, and loaded onto the SiNW arrays. The blue bars represent the numbers of CD4+ T cells captured and detected on the platforms when the T cell mixtures contained 650, 500, and 250 CD4+ T cells, respectively. The red bars represent the number of CD8+ T cells captured and detected on the platforms when the T cell mixtures contained 650, 500, and 250 CD8+ T cells.

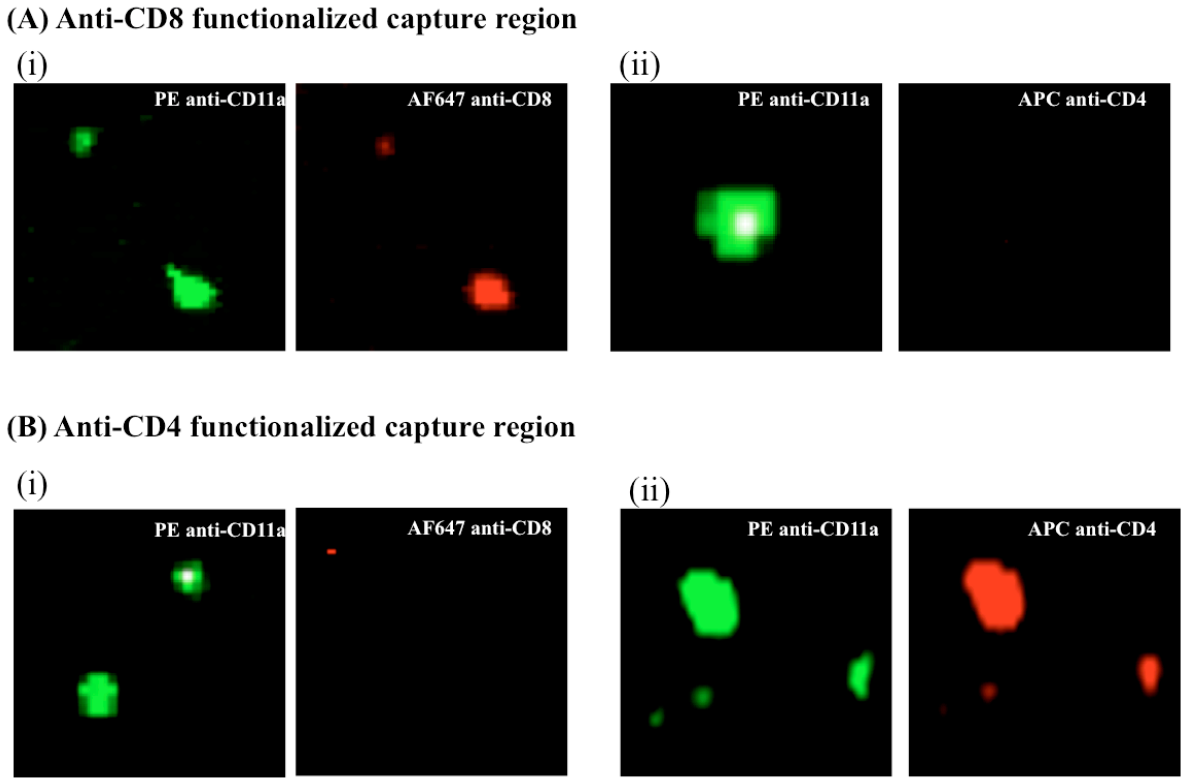
(A)



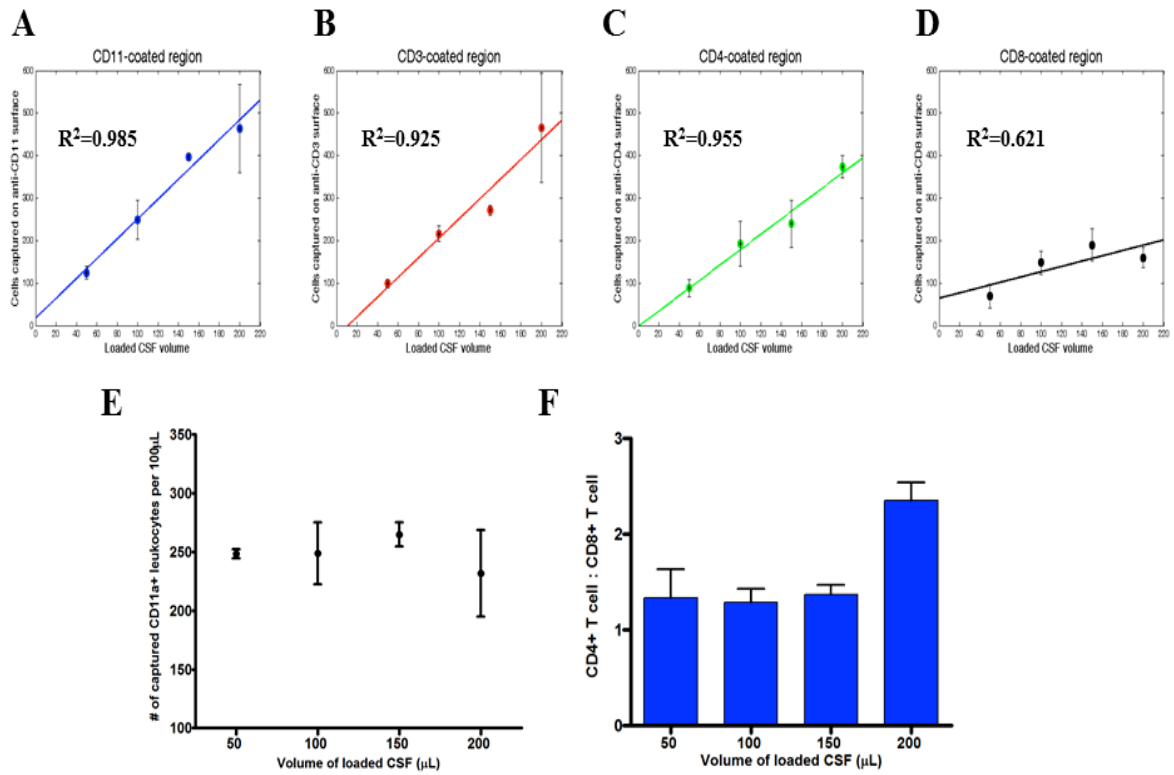
(B)

	Average (Standard Deviation)
Patient 1	23.0 (7.07)
Patient 2	39.2 (9.89)
Patient 3	26.5 (5.66)
Patient 4	54 (25.5)

Supplementary Figure S5. The number of leukocytes from Alzheimer's disease patients' cerebrospinal fluid (CSF) captured on the SiNW substrates not functionalized with antibody. The CSF samples from four different patients were used, and the experiment was repeated three times (n=3). (A) The graphical representation of the number of CSF leukocytes captured and detected. (B) The table shows the average and standard deviation values from each patient. All numbers represents the number of cells/μL.



Supplementary Figure S6. Enlarged views of fluorescent images showing capture specificity of CD4+ and CD8+ cells. **(A)** Fluorescent images of U937 mononuclear leukocytes bound on the capture region coated with biotin anti-CD8. (i) *Left panel:* The cells are stained positive for PE-conjugated CD11a antibody (excitation wavelength: 532nm, green). This confirms that the captured cells are leukocytes. *Right panel:* The cells are stained positive for AlexaFluor647 (AF647)-conjugated CD8 antibody (excitation wavelength: 635nm, red). The result suggests that the captured leukocytes are CD8+ cytotoxic T lymphocytes. (ii) *Left panel*: The cells are stained positive for PE-conjugated CD11a antibody (excitation wavelength: 532nm, green). *Right panel:* The cells are stained negative for APC-conjugated CD4 antibody (excitation wavelength length: 635nm, red). This implies that the captured cells do not express CD4 surface antigens. **(B)** Fluorescent images of U937 cells bound on the capture region coated with biotin anti-CD4. (i) *Left panel*: The cells are stained positive for PE-conjugated anti-CD11, confirming their identity as leukocytes. *Right panel*: The cells are stained negative for AF647-conjugated anti-CD8, indicating the captured cells do not express CD8 molecules. (ii) *Left panel*: The cells are stained positive for PE-conjugated anti-CD11a. Right panel: The cells are stained positive for APC-conjugated anti-CD4. These results confirm that a capture region functionalized with antibody against CD4 and CD8 leukocyte antigens can selectively capture CD4-expressing leukocytes and CD8-expressing leukocytes, respectively.



Supplementary Figure 7. Multiplexed immunophenotyping of trafficking leukocytes when volume of loaded CSF varies from 200μL to 50μL. (A) Correlation of the number of leukocytes captured on CD11a antibody-functionalized arrays as a function of volume of CSF loaded into each capture interface. The solid blue line represents a linear regression, indicating a strong linear relationship (R^2=0.985). (B) Correlation of the number of leukocytes captured on CD3 antibody-functionalized arrays as a function of volume of CSF loaded into each loading chamber. The solid red line represents a linear regression, indicating a strong linear relationship (R^2=0.925). (C) Correlation of the number of leukocytes captured on CD4 antibody-functionalized arrays as a function of volume of CSF loaded into each loading chamber. The linear regression indicates a strong linear relationship between the number of captured CD4+ T cells and the loaded CSF volume (R^2=0.955). (D) Correlation of the number of leukocytes captured on CD8 antibody-functionalized arrays as a function of volume of CSF loaded into each loading chamber. The regression shows a good linear relationship (R^2=0.621). (E) The normalized densities of CSF trafficking leukocytes (per 100 μL CSF) for different volumes of CSF loaded (50, 100, 150, and 200μL). The density of trafficking leukocytes remains relatively consistent over varying CSF volumes. (F) The CD4 T cells/CD8 T cells ratio based on immunophenotyping of individual AD patient's CSF when different volumes of CSF (50, 100, 150, and 200μL) were loaded into each chamber. The CD4/CD8 ratio remains relatively constant when quantities of loaded CSF varied. Each result and error bar represents an average with standard deviation from three repeats.

Supplementary Tables 1-3

Supplementary table S1. Quantification and multiplexed immunophenotyping result of CCRF-CEM cells using the silicon nanowire-based rare cell analysis platform. CCRF-CEM cell suspensions were loaded with varying cell numbers ranging from 400 to 1000 per loading chamber. The experiments were repeated three times with the different cell numbers using different silicon nanowire substrates.

CCRF-CEM	Exp.1		Exp.2		Exp.3		Proportion Average (%)	Standard Deviation
	Cell count	Proportion (%)	Cell count	Proportion (%)	Cell count	Proportion (%)		
Total loaded	400		800		1000			
CD11/CD45	397	99.25	536	67	982	99.2	88.4833	18.6051
CD3	182	45.5	164	20.5	400	40	35.3333	13.1371
CD4	332	83	721	90.125	854	85.4	86.175	3.6252
CD8	188	47	240	30	438	43.8	40.2667	9.034
(-) control	75	18.75	199	24.875	230	23	22.2083	3.1383

Supplementary table S2. Quantification and multiplexed immunophenotyping result of U937 cells using the silicon nanowire rare cell analysis platform. U937 cells were loaded with the cell number of 700 cells per loading chamber. The experiments were repeated four times using different silicon nanowire substrates.

U937	Cell count						
	Exp.1	Exp.2	Exp.3	Exp.4	Average (cell count)	Average (Proportion, %)	Standard Deviation (%)
CD11	766	568	723	689	686.5	98.07	12.15
CD45	702	578	748	659	671.75	95.96	10.33
CD3	75	82	87	N/A	81.33	11.62	0.86
CD4	744	578	596	621	634.75	90.68	10.71
CD8	243	70	122	218	163.25	23.32	11.59
(-) control	32	35	53	55	43.75	6.25	1.70

Supplementary table S3. Highly efficient capture and multiplexed quantification of rare trafficking leukocytes in cerebrospinal fluid (CSF) of Alzheimer's disease patients. Trafficking leukocytes from the CSF samples collected from 5 different individual Alzheimer's disease patients (see supplementary table 4 for more information) and one mixed pool CSF sample were captured and characterized using streptavidin-capture antibody functionalized silicon nanowire substrates. The numbers in the each cell of the table represents the number of captured and detected leukocytes at each capture region. CSF of 50-100 μL was loaded into each loading/capture chamber.

All the numbers of the captured leukocytes were normalized to # cells/100 μL.

		CD11	CD3	CD4	CD8	(-) cont.
Patient 1	Exp.1	135	57	103	80	28
	Exp.2	123	144	179	98	56
	Exp.3	101	142	126	82	27
	Average	119.7	114.3	136	86.67	37
	(Stdv)	(17.24)	(49.66)	(38.97)	(9.87)	(16.46)
Patient 2	Exp.1	329	285	347	205	55
	Exp.2	194	160	154	158	83
	Exp.3	245	-	167	52	12
	Average	243	222.5	212.33	127.7	44.33
	(Stdv)	(87.02)	(88.4	(107.9)	(78.31)	(35.53)
Patient 3	Exp.1	190	-	146.25	165	70
	Exp.2	370.0	243.8	210.0	153.8	58.8
	Exp.3	196.3	202.5	233.8	197.5	45.0
	Exp.4	197.5		220	122.5	47.5
	Average	238.4	223.1	202.5	159.7	55.3
	(Stdv)	(87.77)	(29.16)	(38.74)	(30.96)	(11.47)
Patient 4	Exp.1	245	256.25	217.5	165	46.25
	Exp.2	451.25	323.75	405	288.75	97.5
	Exp.3	432.5	341.25	285	227.5	60
	Exp.4	320	252.5	211.25	138.75	90
	Average	362.2	293.4	279.7	205	73.44
	(Stdv)	(97.28)	(45.69)	(89.97)	(67.10)	(24.31)
Patient 5	Exp.1	312.5	322.5	240	132.5	55
	Exp.2	244	190	200	202	62
	Exp.3	272.5	206.3	151.3	132.5	75.0
	Exp.4	336.7	326.7	313.3	171.7	76.7
	Average	291.4	261.4	226.1	159.7	67.17
	(Stdv)	(41.22)	(73.33)	(68.52)	(33.72)	(10.43)

		CD11	CD3	CD4	CD8	(-) cont.
Mixed pool	Exp.1	277.5	237.5	251.25	178.75	47.5
	Exp.2	362	282	226	157	41
	Exp.3	215	210	160	107.5	47.5
	Exp.4	-	355	196.25	100	65
	Average	284.8	271.1	208.4	135.8	50.25
	(Stdv)	(62.22)	(59.20)	(52.84)	(52.56)	(10.30)

Supplementary Results

1. Determination of distinct immunophenotypes of human monocytes (U937) using biofunctionalized-nanowire enabled multiplexed molecular phenotyping

To further verify the platform's capability to efficiently capture low-abundance immune cells and accurately determine their phenotypic expression profiles, we conducted multiplexed phenotyping of U937 cells and compared the results with flow cytometry phenotyping. Quantification of cells captured in other biofunctionalized wells revealed that anti-CD45 coated wells captured almost all cells (~96.0%), indicating U937 is predominantly composed of leukocytes. In contrast, significantly fewer cells were detected in anti-CD8 coated region (~22%) and very few were captured in the well (11%) coated with antibody against the T lymphocyte surface marker CD3 (**Supplementary Figure S4, Supplementary Table 2**). A significant reduction in U937 binding to the regions grafted with antibodies specific for CD3 and CD8 is consistent with the previous work that prominent T lymphocyte surface markers such as CD3 or CD8 are not expressed at the detectable levels in U937 cells[49]. The side-by-side comparison between biofunctionalized nanowire-enabled and flow cytometry immunophenotyping revealed that the results from our nanowire-enabled platform were in good agreement with that from flow cytometry. The differences in the proportions of specific phenotypic subsets determined by two independent technologies were not statistically significant for all five surface antigens (p-value>0.05).

2. The capture purity of target primary leukocytes when mixed with non-target cells on the silicon nanowire substrates

Additional experiments were conducted to investigate the capture purity and capture efficiency of target and non-target cells when they were mixed together. The purpose of these experiments was to illustrate that our platform's capture regions functionalized with the antibodies against specific phenotypic antigens can capture the target cells expressing the specific antigens with high selectivity while inhibiting non-specific capture of non-target cells to the insignificant level. The SiNW substrates were coated with either CD4 or CD8 antibodies. Primary human CD4+ and CD8+ T lymphocytes were mixed in 1:1 ratio at the varying cell numbers. We then introduced the mixture composed of approximately 250 CD4+ and CD8+ T cells, 500 CD4+ and CD8+ T cells, or 600 CD4+ and 600 CD8+ T cells into anti-CD4 coated, anti-CD8 coated, or BSA-treated substrate capture regions. The captured cells were immune-stained with PE-CD8 and APC-CD4 antibodies for detection so that the captured CD4+ T cells, which illuminate red fluorescence, could be easily distinguished from CD8+ T cells, which illuminate green fluorescence (**Supplementary Fig. S5A**). The fluorescent images of the substrates and captured cells clearly demonstrated that On the substrates coated with CD4 antibodies, most of the captured cells (~90%) were CD4+ T cells (target cells) while very few were CD8+ T cells (non-target cells), resulting in the capture purity greater than 90% (93.1±1.92%) and the capture impurity less than 10% (6.86±1.92%) (**Supplementary Fig. S5A-C**). In this case, the capture purity is the proportion of captured target cells (CD8+ T cells) captured on anti-CD4 coated substrates. Similarly, on the anti-CD8 functionalized nanowire substrates, most of the captured cells were CD8+ T cells (target cells) while the fraction of CD4+ T cells (non-target cells) among all captured cells was small, again resulting in the capture purity greater than 90% (93.9±1.10%) and the capture impurity less than 10% (6.07±1.10%) (Supplementary Fig. 5A, C). When the mixture was loaded into anti-CD4 coated substrates, the capture yield for CD4+ T cells (90.1±7.74%) was significantly higher than that of non-target cells (6.67±2.16%) (**Supplementary Fig. S5B**). Moreover, in anti-CD8 coated substrates, the capture yield for target CD8+ T cells (91.6±1.30%) was significantly higher than that of non-target CD4+ T cells (5.92±1.06%) (**Supplementary Fig. S5B**).