SUPPORTING INFORMATION

High-throughput secretomic analysis of single cells to assess functional cellular heterogeneity

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Supporting Information includes Supporting Methods, Supporting Figures 1-15, and Supporting Tables 1-2

SUPPORTING METHODS

Fabrication of antibody barcodes. The mold for PDMS replica is a silicon master etched with deep reactive-ion etching (DRIE) method. It was pre-treated with chlorotrimethylsilane (Aldrich) vapor overnight to facilitate PDMS release. PDMS prepolymer and curing agent (RTV615, Momentive) was mixed completely (parts A and B in 10:1 ratio) and poured onto the silicon master. Air bubbles were removed via vacuum desiccator for 1 h, and the PDMS was cured in the oven at 80 ^{o}C for 2 hrs. After curing, the PDMS layer was peeled off the mold and holes for inlet and outlet ports were punched. The device was cleaned via sonication in ethanol and 2-propanol before bonding with a poly-L-lysine microarray slide (Erie Scientific). The assembly was then baked in the oven at 80 ^{o}C for 2 hrs to strengthen the bonding. The PDMS microchip for antibody flow patterning contains 20 separate microchannels which can pattern up to 20 different antibodies respectively. The typical width and pitch of antibody barcode is 20 μm , 40 μm respectively in the PDMS flow patterning microchip.

For the flow patterning of the antibody barcodes, 1.5 μL of different antibodies (**Supplementary Table 1**) were injected into microchannels separately and flowed through the microfluidic channels until dry. All the antibodies used in experiments are summarized in supplementary table 1. Antibodies are immobilized on the poly-L-lysine glass slide to form the antibody barcode. After flow patterning, the glass slide can be stored in the refrigerator at 4 ^{o}C, and the PDMS layer will be released before use.

Fabrication of sub-nanoliter microchamber array chips. The mold for the sub-nanoliter microchamber array is a silicon master etched with DRIE method. It was also pre-treated with chlorotrimethylsilane (Aldrich) vapor overnight to facilitate PDMS release. The sub-nanoliter microfluidic chamber array chips for single cell capture were fabricated out of PDMS (RTV615, Momentive, parts A and B in 10:1 ratio) using soft lithography techniques. Air bubbles were removed via vacuum desiccator for 1 h, and the PDMS was cured in the oven at 80 ^{o}C for 2 hrs. The sub-nanoliter microchamber array chips contain 5044 cell capture chambers.

Cell culture and stimulation. Human A549 cell line was cultured in F12/K medium supplemented with 10% fetal bovine serum (FBS, ATCC). Human U937 cell line was purchased from ATCC (American Type Culture Collection, ATCC) and cultured in RPMI 1640 medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS, ATCC). Every 100 uL of U937 cell suspension was differentiated with 1 μL 20 μg/mL phorbol 12-myristate 13-acetate (Fisher) and challenged by 1 μL 1 mg/mL lipopolysacchride (Calbiochem) to activate Toll-like receptor 4 (TLR4) signaling before its suspension was pipetted onto PDMS microwell array.

Human tissue specimens. Human samples were obtained from individuals with meningioma from Yale Pathology. The use of human samples was approved by the appropriate human use committees (Yale University YPTS HIC protocol # 25173). Patient primary tissue was first minced with a scalpel and then placed in 1x TrypLE Select (Invitrogen). Tissue was triturated with fire-polished glass pipettes and allowed to incubate for 5 mins at 37 ^{o}C. The cell suspension was triturated again with fire-polished glass pipettes and allowed to incubate for another 5 mins at 37 ^{o}C. The cell suspension was then strained through a 40 um cell strainer (BD Falcon) and washed with DMEM-F12 (Gibco, Invitrogen). The suspension was then centrifuged at 300 RCF for 5 min. The pellet was resuspended in DMEM-F12. Red blood cell lysis solution (Miltenyi Biotec) was then used to remove erythrocytes and centrifuged at 300 RCF for 5 min. The cells were then resuspended in DMEM-F12 at 10^6 cells/mL.

Single cell trapping with PDMS microwell array. Before performing the single cell trapping experiment, the PDMS microwell array and antibody barcode glass slide was blocked with 3% BSA solution (Sigma) respectively for 2 hrs and then rinsed with fresh cell medium. Cells were suspended in fresh medium just before cell capture. The PDMS microwell array was placed facing upward and cell culture medium solution was removed until a thin layer was remained on the PDMS microwell array surface. Cell suspension was pipetted (50-200 uL) onto the microwell array and allowed to setlle for 10 mins so that cells would fall into the microwells. The antibody glass slide was put on the top of PDMS microwell array with antibody barcode resting on the cell capture chambers. Then the PDMS microwell array and glass slide were clamped tightly with screws and pressure was distributed by springs. Single cells will be trapped in the microwell array and the assembly was allowed to incubate for 24 hours to allow for cell secretion. After the trapped cells were incubated for 24 hours, the screws were released to remove the antibody barcode glass slide, and ELISA immunoassay procedures were performed and the results were detected and analyzed with Genepix scanner and software.

Population micro array. Cell population assay was performed on custom printed antibody microarray, which was spotted with a Spotbot 3 microarrayer (Arrayit) on poly-L-lysine glass slides. 12 identical subgroups which had the same antibody pattern were printed on each glass slide. After printing, the antibody glass side was kept in a wet box (containing saturated NaCl solution at 75% relative humidity) for 5 hours. Before cell population assay, the glass side was bonded with a PDMS microwell slab and blocked with 3% BSA solution for 2 hours. Then cell culture supernatant was added into different microwells and allowed to incubate for 1 hour. Following incubation, ELISA immunoassay procedures are performed, and the results were detected and analyzed with Genepix scanner and software.

Immunoassay procedures. ELISA procedures were followed to translate secreted cytokines by single cells into detectable signals. A mixture of biotinylated detection antibodies (**Supplementary Table 1**) were pipeted onto the glass slide and incubated for 45 min at room temperature to complete the sandwich immunoassay followed by washing with 3% BSA solution. APC dye-labeled streptavidin (eBioscience, 200μL, 5 μg/mL) was added onto glass slide and incubated for another 45 min. Following, the glass slide was washed with 3% BSA again and blocked with 3% BSA for 0.5 hr. Following the BSA blocking, the glass slide was dipped in DPBS, DPBS, DI water, DI water sequentially and finally blown dry.

Fluorescence detection and analysis. Genepix 4000B and 4200A scanners (Molecular Devices) were used to obtain scanned fluorescent images for FITC and APC channels. Two color channels 488 (blue) and 635 (red) were used to collect fluorescence signals. The image was analyzed with GenePix Pro software (Molecular Devices) by loading and aligning the microwells array template followed by extraction of fluorescence

intensity values. Fluorescence results were extracted with the image analysis tool in GenePix Pro. The fluorescence results were then matched to each of the chambers of the sub-nanoliter microchamber array chips analyzed via optical imaging.

Automated optical image analysis and cell counting. The assembly was imaged on an automatic microscope stage (Prior) to acquire optical images recording the number and location of cells in each microwell. Automated whole chip optical images were taken with a Paxcam (Paxit!) attached to a Nikon Diaphot SA optical microscope (Nikon), and stitched together using Paxit software (Paxit!). After imaging, the assembly was put into an incubator at 37 ^{o}C for 24 hrs to allow for cell secretion. After 24 hours of incubation, cells are imaged again to observe cell function. A home developed software using OpenCV (Intel) was written to automate cell counting, and the cell counts were matched with the extracted fluorescent data to their respective cell chambers.

Data and Statistical analyses. All fluorescent scanned barcodes were processed with Genepix software to extract background subtracted average fluorescent signal for all bars in each barcode set. A home developed Matlab (MathWorks) code was created for automated extraction of fluorescent data and generation of scatterplots. Excel (Microsoft) and OriginPro 8 (OriginLab) was used to compile extracted data. Heatmaps and unsupervised clustering were generated from the extracted data using the software Cluster/ Treeview (Eisen Laboratory). Statistical analysis was conducted in Excel, OriginPro 8, and R (R Development Core Team).

SUPPORTING FIGURES

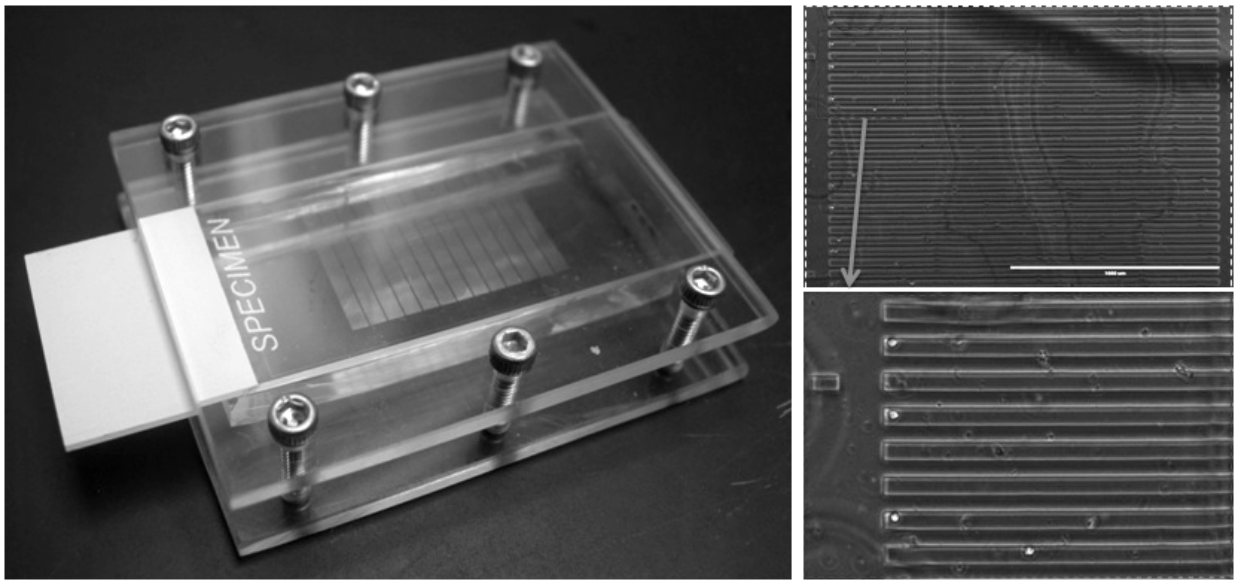

Figure S1. Assembly of a complete single cell secretomic analysis device. A high-density antibody array glass slide and a 5440-microchamber PDMS slab were clamped together with two transparent plates using springs and screws.

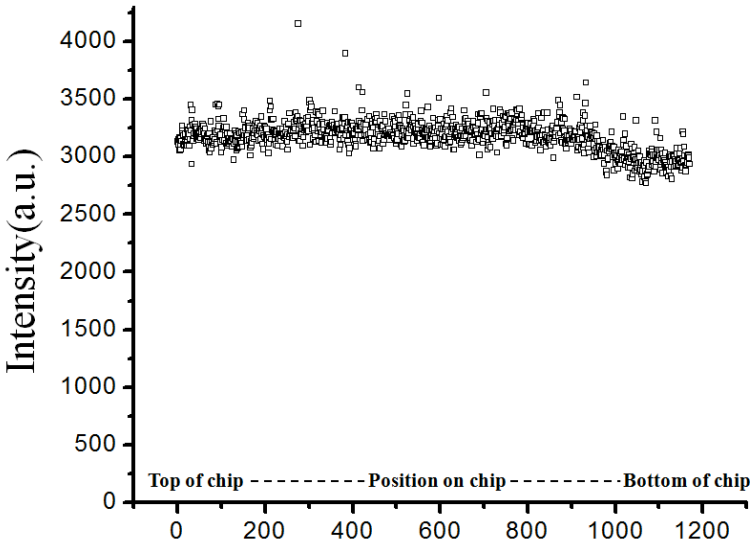

Figure S2. Evaluation of the whole chip uniformity of the protein barcode array. Quantification of fluorescence intensity across the flow patterned poly-L-lysine slide (3cmx2cm) reveal excellent uniformity of the immobilized proteins (FITC-BSA), which ensures the validity of using this high-density barcode array technology to assess single cell heterogeneity.

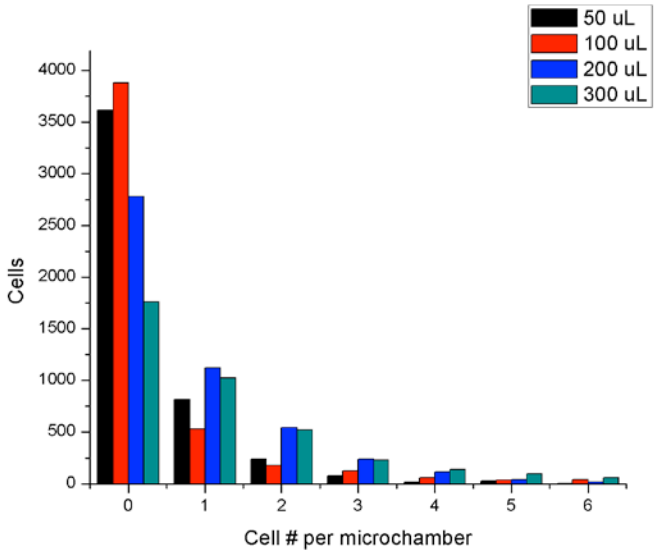

Figure S3. Distribution of the number of cells across a whole microchip. Four experiments were performed with different quantities of cell suspensions (cell density: 10^6 cells/mL).

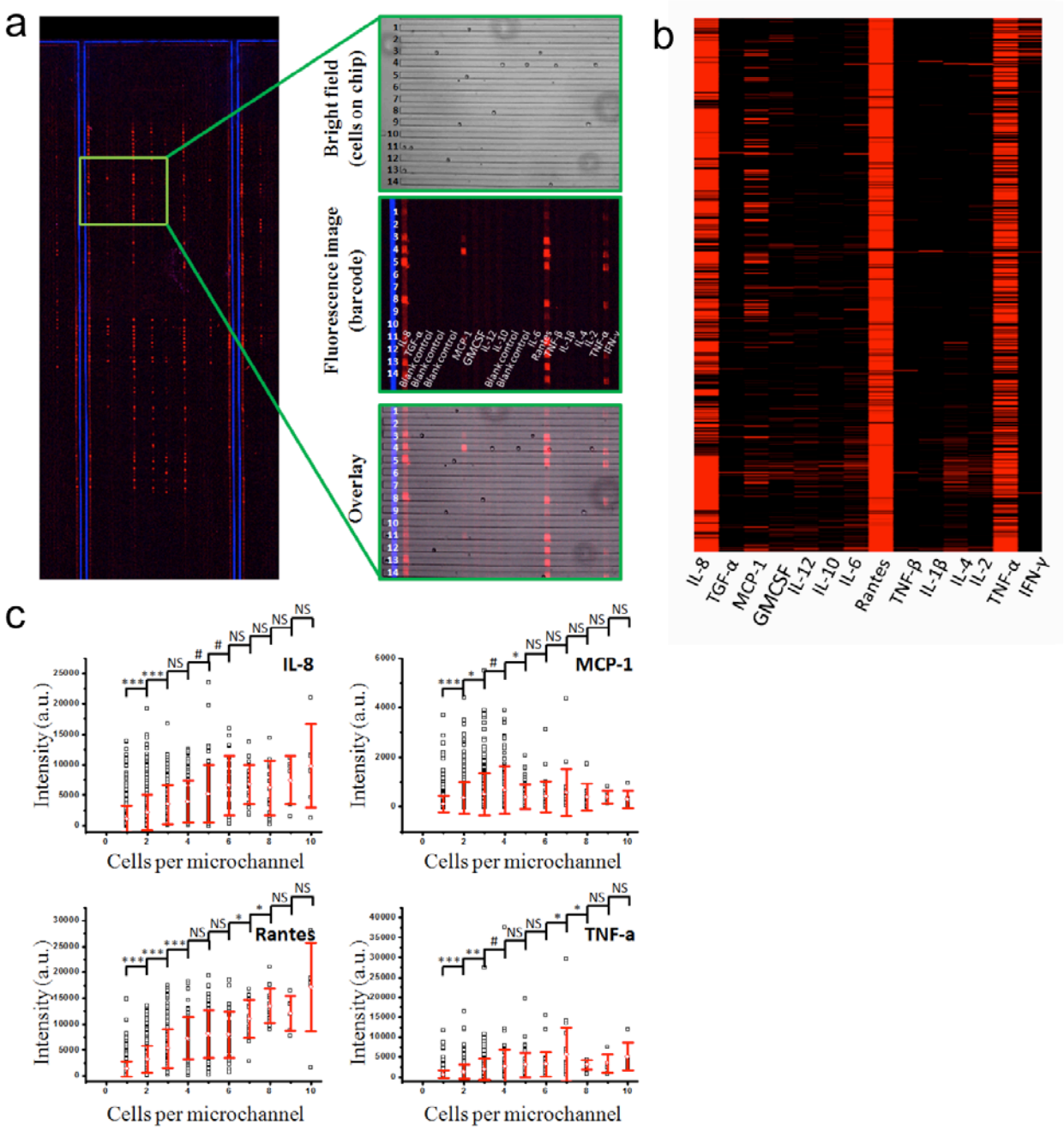

Figure S4. Single-cell secretomic analysis on U937 cell lines. (**a**) Representative region of the scanned image showing the raw data of single cell secretomic measurement. Three subpanels on the right are optical micrograph, fluorescence image and overlay for 14 microchambers. (**b**) Heat map that shows the profile of 14 proteins secreted from 551 single cells (U937). Each row is a single cell and each column corresponds to a protein of interest. (**c**) Scatter plots showing fluorescence intensity measured for four selected proteins (IL8, MCP-1, RANTES and TNFa) versus the number of cells in a microchamber. The cells were stimulated with 20 μg/mL of PMA to differentiate to macrophage and then challenged by 1 mg/mL of LPS to become activated right before they were loaded onto the microchip to secretion analysis.

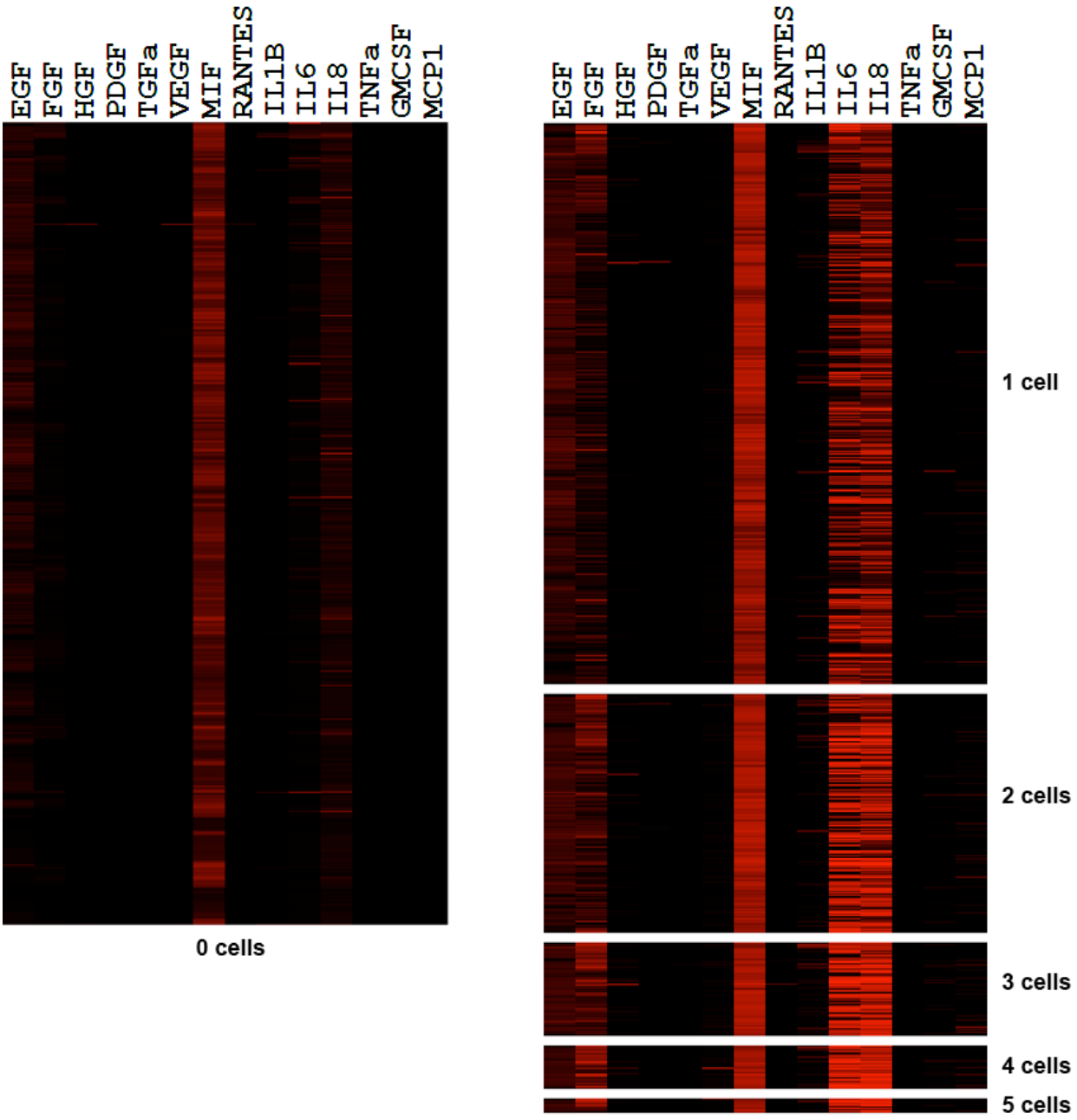

Figure S5. Heat map that shows the profile of 14 proteins secreted from chambers with U87 cell line. Each row is a cell chamber and each column corresponds to a protein of interest. Zero cells (n=1821), single cells (n=1278), two cells (n=544), three cells (n=214), four cells (n=100), and five cells (n=35).

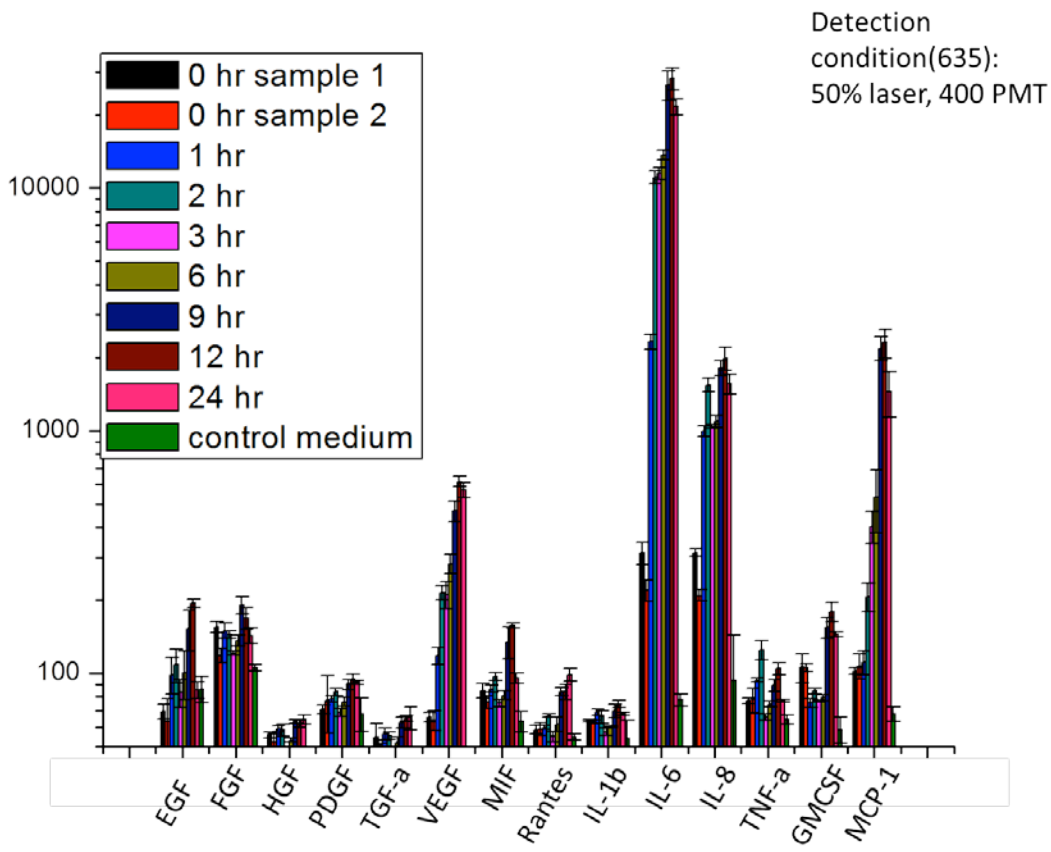

Figure S6. Population kinetics for U87 cell line. Control (MEM medium), secretion supernatant from population at different time points (0 hr, 1 hr, 2 hr, 3 hr, 6 hr, 9 hr, 12 hr, 24 hr)

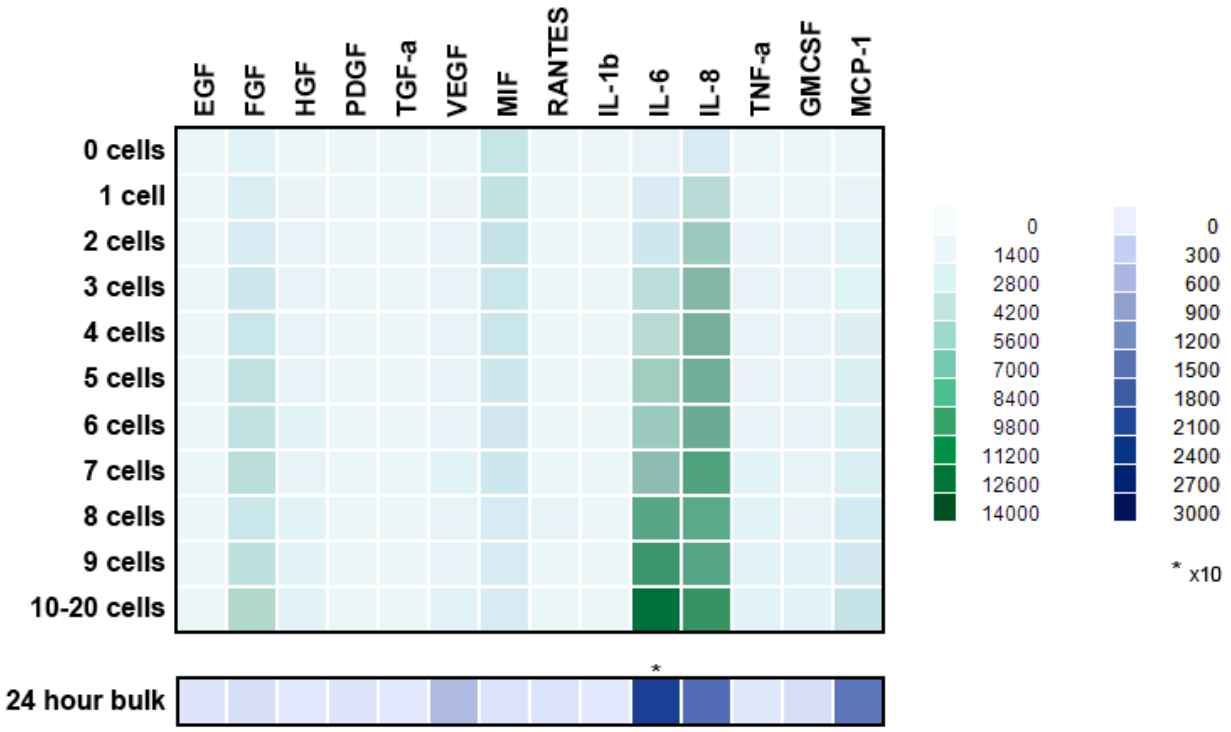

Figure S7. Average signal of U87 cells within the single cell platform approaching bulk patterns (green) and the 24 hour bulk secretion profile of U87 cells (blue)

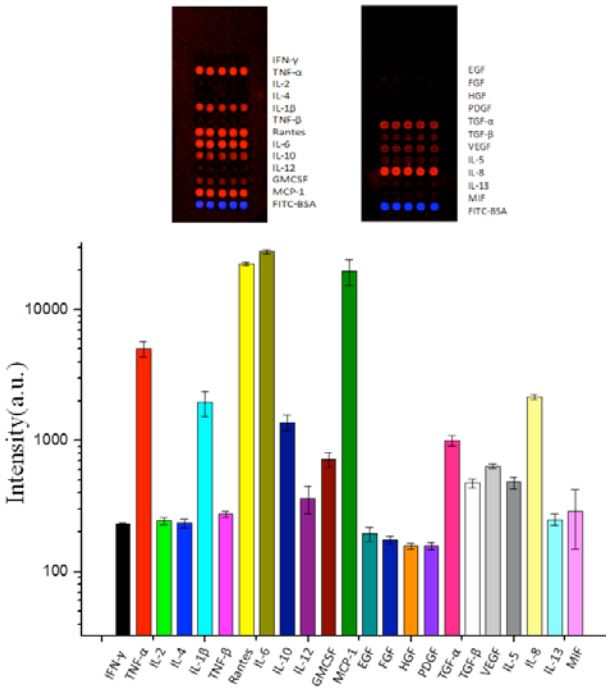

Figure S8. Control experiment: U937 population secretion measurement. Proteins secreted from a large population of U937 cells were measured by a conventional antibody microarray (upper panels). Quantification of all 23 proteins are shown in the lower panel.

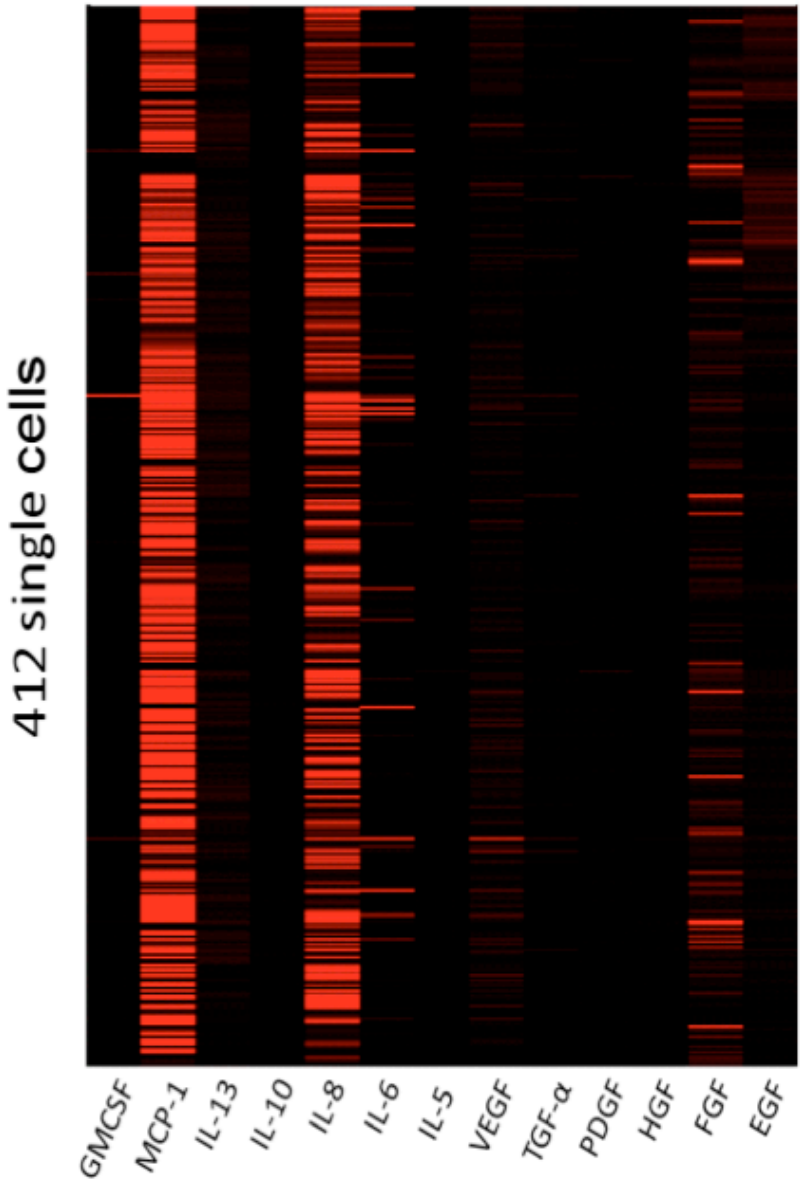

Figure S9. Heat map showing protein secretion profiles of single A549 cells at their basal level without stimulations.

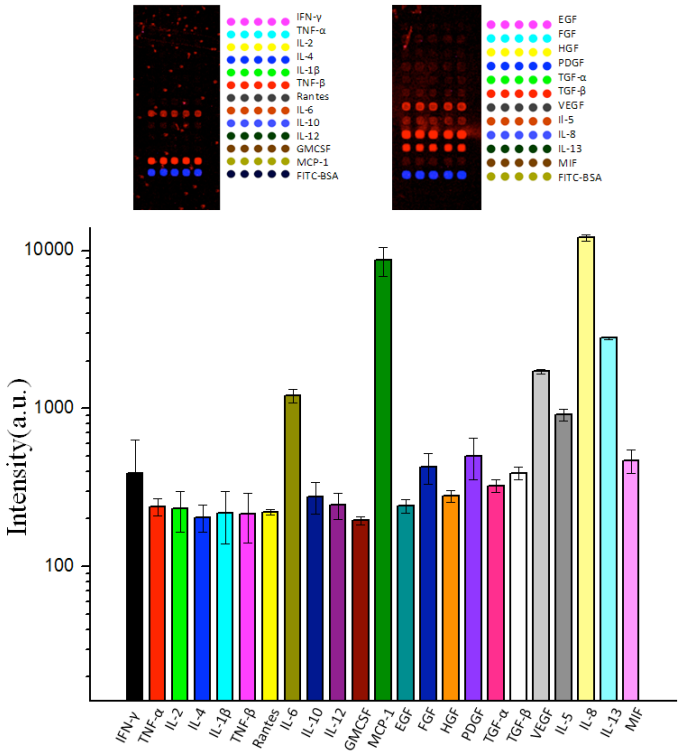

Figure S10. Control experiment: A549 population secretion measurement. Proteins secreted from a large population of A549 cells were measured by a conventional antibody microarray (upper panels). Quantification results of all 23 proteins are shown in the lower panel.

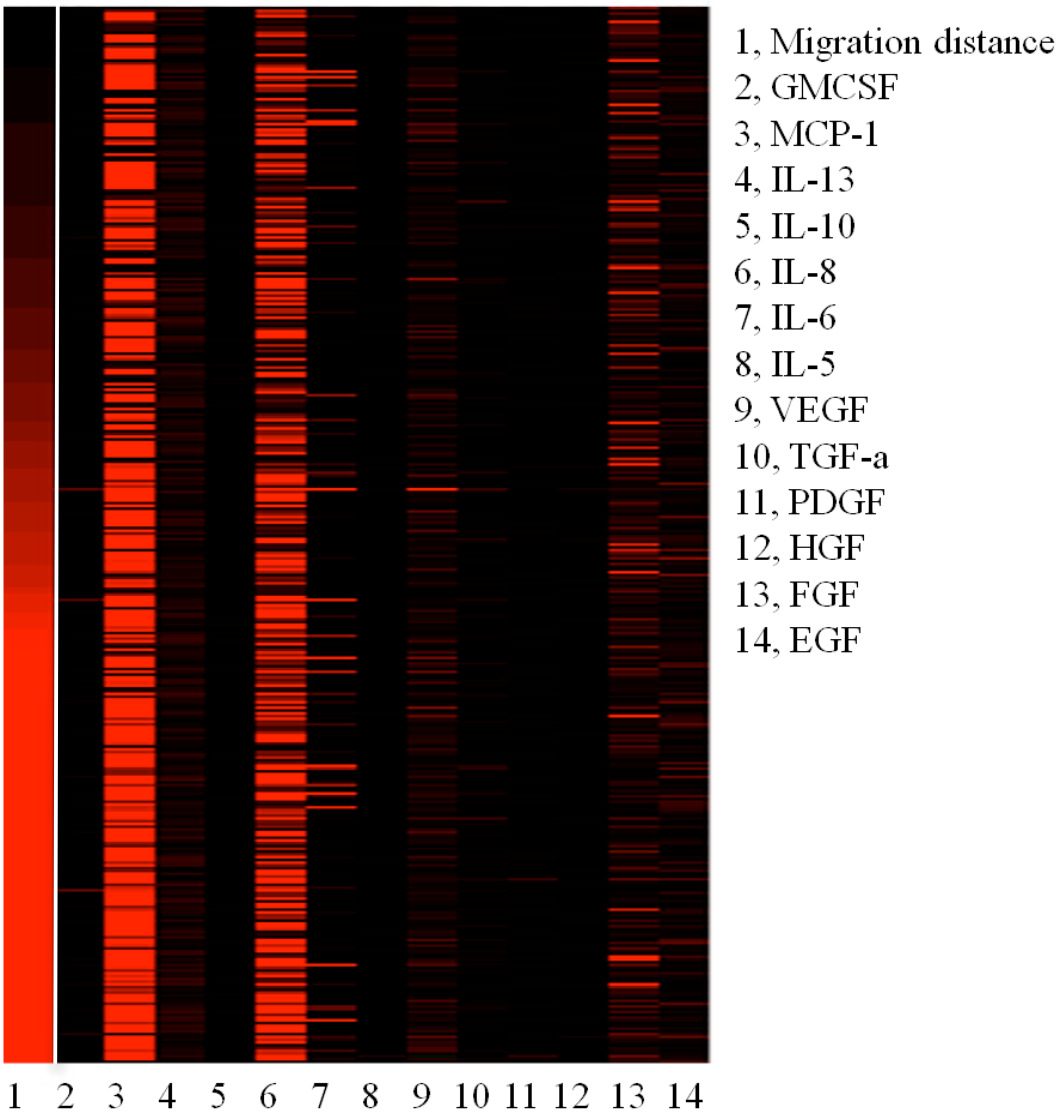

Figure S11. Heat map showing the correlation between A549 single cell migration distance and its corresponding protein secretion signals.

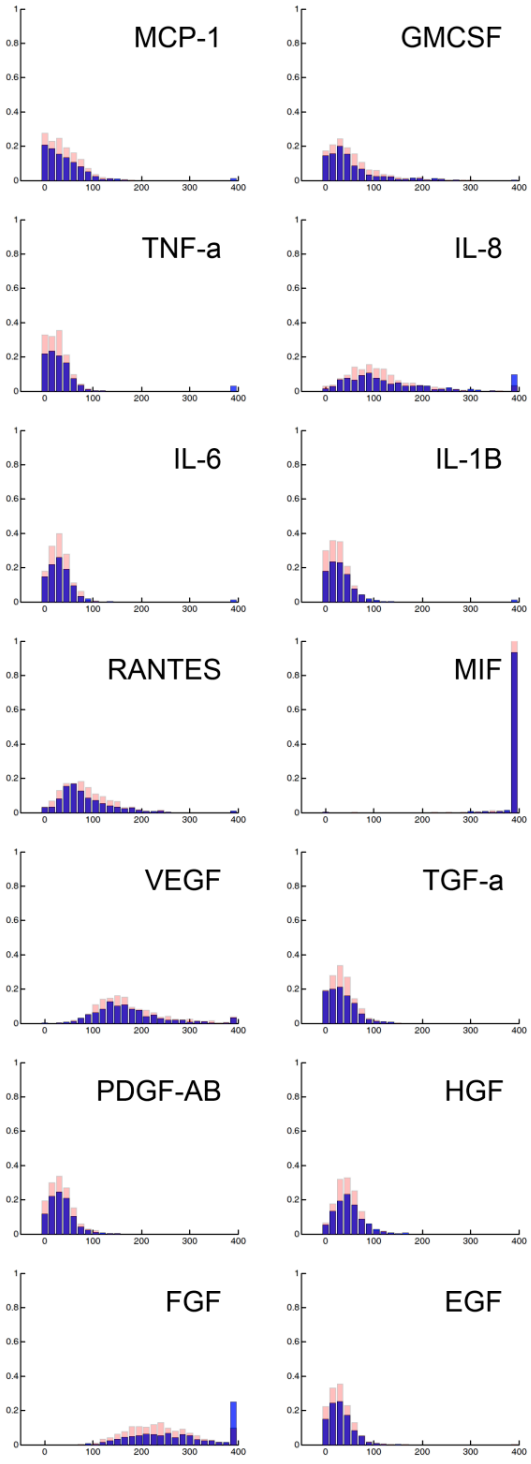

Figure S12. Histogram plots of individual proteins measured on the sample from Patient 1. *(red = zero cells, blue = single cells)*

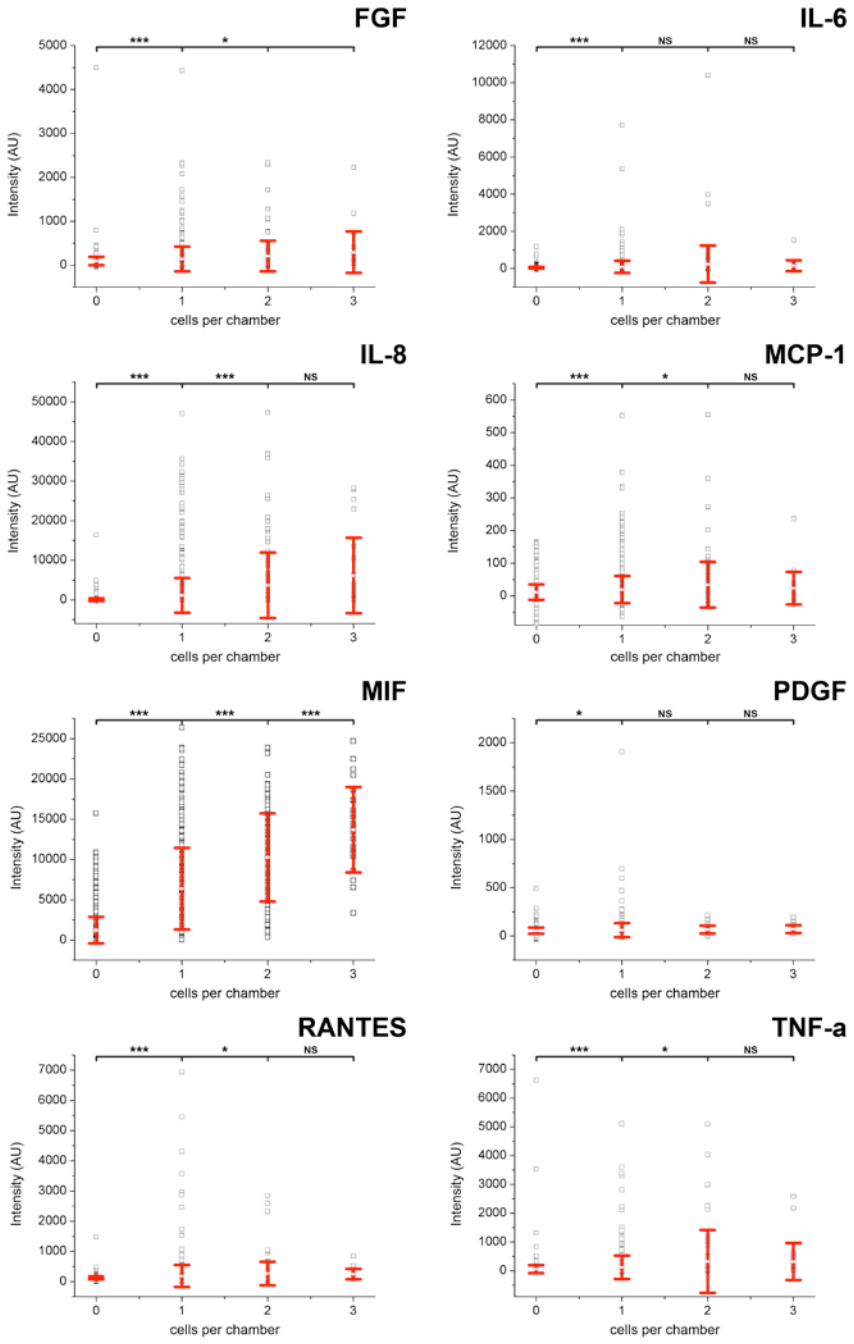

Figure S13. Scatter plots showing fluorescence intensity measured for eight selected proteins from Patient 2. (FGF, IL-6, IL-8, MCP-1, MIF, PDGF, RANTES, TNF-a)

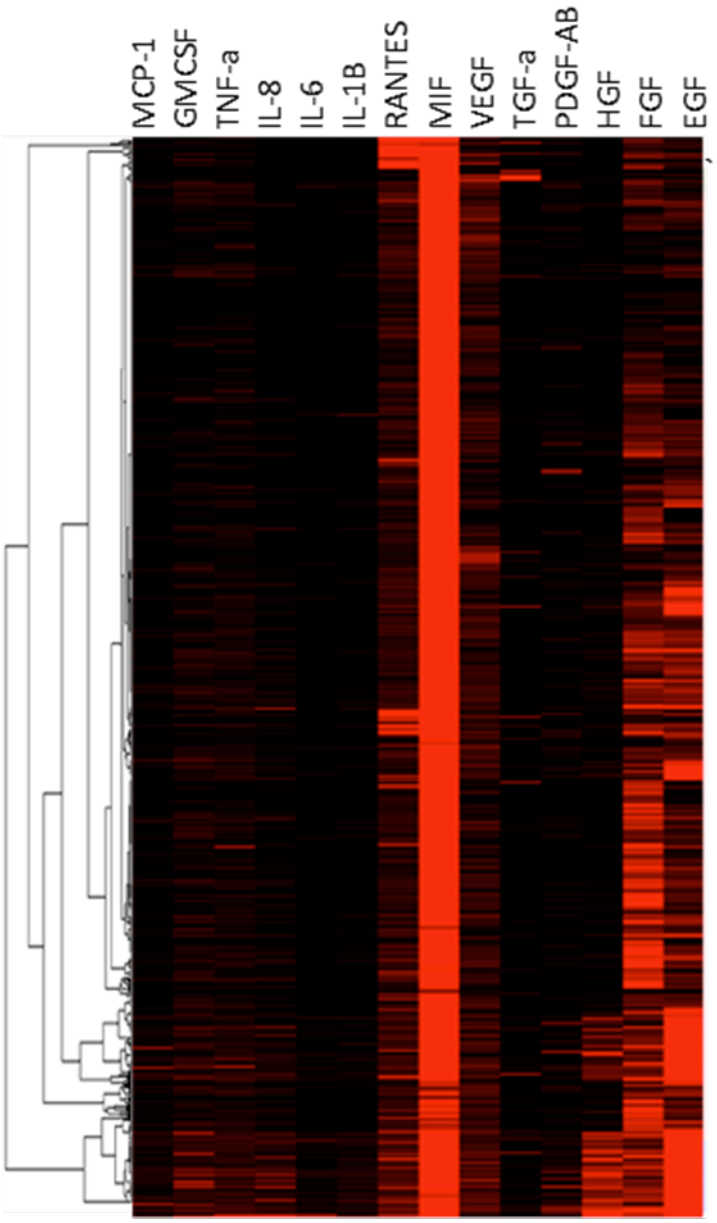

Figure S14. Single cell protein secretion profiling on the sample from Patient #3, a transitional meningioma patient.

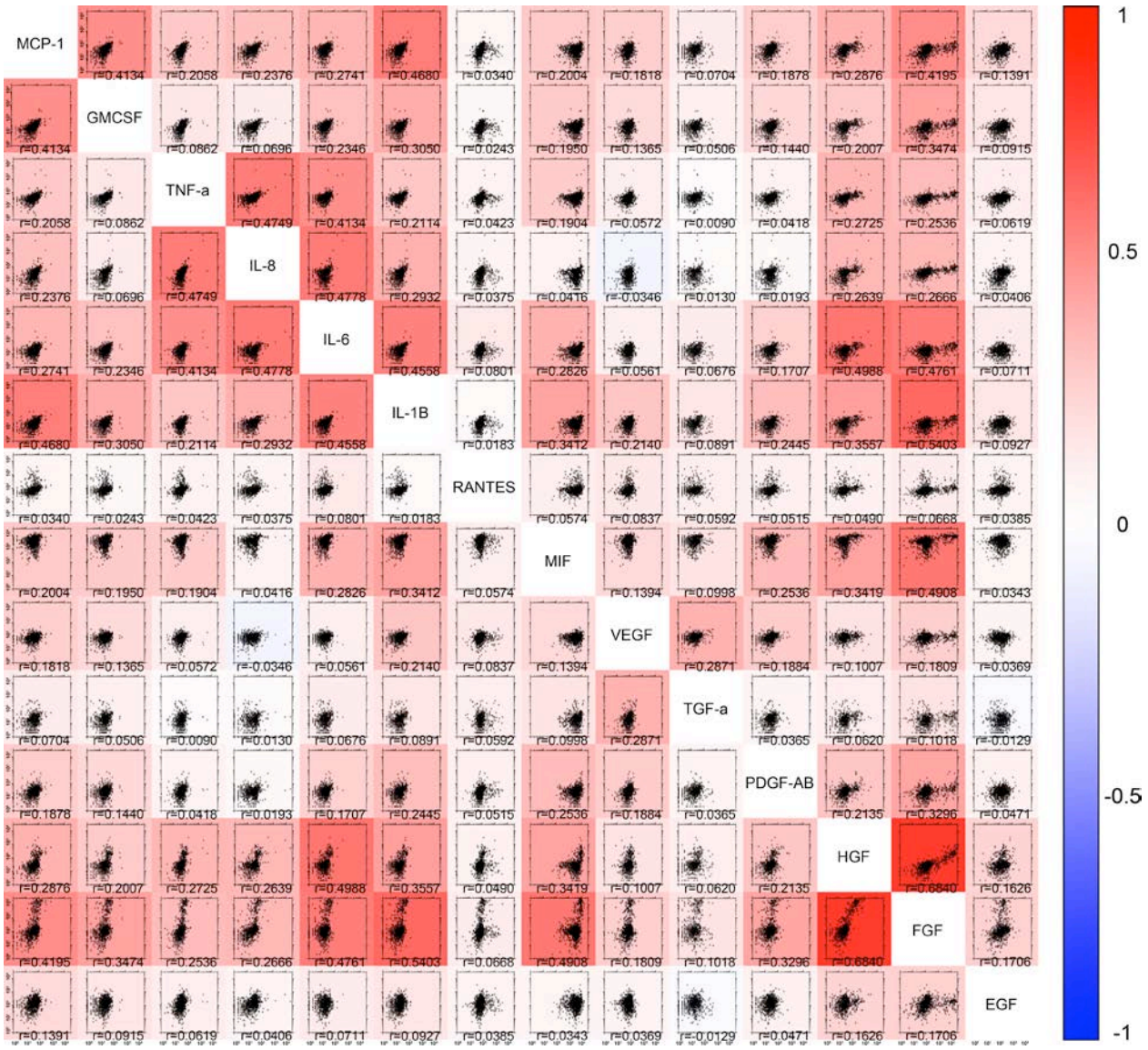

Figure S15. Scatter plots and protein correlation analysis of the single cell secretion data obtained from Patient 3.

SUPPORTING TABLES

Table S1. List of all antibodies used in our study

Primary antibody (vendor: clone)(catalog No.)	Secondary antibody (vendor: clone) (catalog No.)
Mouse Anti-Human IFN gamma (ebio: NIB42)(14-7318)	Anti-Human IFN gamma Biotin(ebio: 4S.B3)(13-7319)
Anti-Human TNF alpha Purified(ebio: MAb1)(14-7348)	Anti-Human TNF alpha Biotin(ebio: MAb11) (13-7349)
Rat Anti-Human IL-2(ebio: MQ1-17H12)(14-7029)	Rabbit Anti-Human IL-2 Biotin(ebio: Polyclonal) (13-7028)
Mouse Anti-Human IL-4(ebio: 8D4-8) (14-7049)	Mouse Anti-Human IL-4 biotin(ebio: MP4-25D2)(13-7048)
Anti-human IL-1b(ebio: CRM56)(16-7018)	Mouse Anti-Human IL-1 beta Biotin(ebio: CRM57)(13-7016)
Mouse Anti-Human TNF beta(ebio: 359-238-8)(14-7329)	Mouse Anti-Human TNF beta Biotin(ebio: 359-81-11) (13-7327)
Mouse anti-human RANTES(R&D) (DY278)	Goat anti-human RANTES(R&D) (DY278)
Rat Anti-Human IL-6(ebio: MQ2-13A5) (14-7068)	Rat Anti-Human IL-6 biotin(ebio: MQ2-39C3)(13-7068)
Rat Anti-Human IL-10(ebio: JES3-9D7)(14-7108)	Rat Anti-Human IL-10 biotin(ebio: JES3-12G8)(13-7109)
Mouse Anti-Human IL-12(ebio: B-T21 (BT21))(14-7128)	Mouse Anti-Human IL-12 biotin(ebio: C8.6)(13-7129)
Anti-Human GMCSF(BD)(555126)	Anti-Human GMCSF biotin(BD)(555126)
Mouse Anti-CCL2 (MCP-1)(ebio: 5D3-F7)(14-7099)	Armenian Hamster Anti-CCL2 (MCP-1) Biotin(ebio: 2H5)(13-7096)
Mouse Anti human EGF(R&D) (DY236)	Goat Anti human EGF Biotin (R&D) (DY236)
Mouse Anti human FGF basic(R&D) (DY233)	Mouse Anti human FGF basic Biotin (R&D) (DY233)
Mouse Anti human HGF (R&D) (DY294)	Goat Anti human HGF Biotin (R&D) (DY294)
Mouse Anti human PDGF-AB (R&D) (DY222)	Goat Anti human PDGF-AB Biotin (R&D) (DY222)
Goat Anti human TGF-a Biotin (R&D) (DY239)	Goat Anti human TGF-a Biotin (R&D) (DY239)
Mouse Anti human VEGF (R&D) (DY293B)	Goat Anti human VEGF Biotin (R&D) (DY293B)
Mouse Anti human MIF (R&D) (DY289)	Goat Anti human MIF Biotin (R&D) (DY289)
Rat Anti-Human IL-5(ebio: TRFK5)(14-7052)	Rat Anti-Human IL-5 biotin(ebio: JES1-5A10)(13-7059)
Mouse Anti-Human IL-13(ebio: PVM13-1)(14-7139)	Rabbit Anti-Human IL-13 biotin(ebio: Polyclonal)(13-7138)

Table S2. Summary of the patient medical records.

Patient	Sample code	Gender	Age	Tumor type	Grade	Location
1	RFa 10-28-11	Female	64	Glioblastoma	4	Left frontal
2	RFa 06-11-12	Female	66	Glioblastoma	4	Right side not specified
3	RFa 04-02-12	Female	47	Transitional Meningioma	1	Right side