NOMA: a high-throughput microchip for robust sequential measurements of secretions from the same single-cells

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Abstract

Despite advances in single-cell secretion analysis technologies, lacking simple methods to reliably keep the live single-cells traceable for longitudinal detection poses a significant obstacle. Here we developed the high-density NOMA (narrow-opening microwell array) microchip that realized the retention of ≥97% of trapped single cells during repetitive detection procedures, regardless of adherent or suspension cells. We demonstrated its use to decode the correlation of protein abundance between secreted extracellular vesicles (EVs) and its donor cells at the same single-cell level, in which we found that these two were poorly correlated with each other. We further applied it in monitoring single-cell protein secretions sequentially from the same single cells. Notably, we observed the digital protein secretion patterns dominate the protein secretion. We also applied the microchip for longitudinally tracking of the single-cell integrative secretions over days, which revealed the presence of "super secretors" within the cell population that could be more persistent to secrete protein or extracellular vesicle for an extended period. The NOMA platform reported here is simple, robust, and easy to operate for realizing sequential measurements from the same single cells, representing a novel and informative tool to inspire new observations in biomedical research.

Introduction

Cell heterogeneity plays a critical role in shaping cell physiology and fates in most biological processes[1, 2]. Different single-cell analysis technologies, including genomics[3], transcriptomics[4-6], and proteomics[7-9], etc.[10], are rapidly developing to decode cell heterogeneity at different levels. As a critical player in mediating intercellular communications, cell secretion also exhibits high heterogeneity[11]. For example, polyfunctional immune cells, which could secrete multiple cytokines simultaneously, are highly predictive of protective immunity or patient responses in immunotherapy clinical trials[11-13]. Discriminating the secretory phenotypes at the single-cell resolution provides a direct way to evaluate the heterogeneity in cell states and activation under different physiological and diseased conditions, which could be of critical importance in basic cell biology, biomarker discovery, and disease diagnosis [14-20]. Many efforts have been made to improve the magnitude of multiplexity in secretion profiling at a single-cell resolution[18, 21-23]. For example, Wang et al. introduced a multiplexed in situ tagging (MIST) strategy that combines ssDNA encoded microbead arrays with a DNA encoded antibody library to realize tens of protein profiling in single cells through multicolor and multicycle successive imaging[22]. By combining spatial and spectral encoding, Lu et al. realized the 42 multiplexed protein secretion profiling from individual macrophage cells, representing the highest multiplexed capability to date for secretomic detection in single cells[18].

Besides high multiplexity, dynamic change is another inherent nature of cell secretion [14, 24-26], which brings more complexity in detection. Resolving the secretion information from the same single-cells at different time points could provide a new dimension of information for an in-depth understanding of how individual cells would evolve upon external stimulation, which is attracting increasing attention in recent years[24, 27-30]. Imaging-based methods, including SPR[31, 32], scattered light[29], photonic crystal resonant imaging[28], optofluidic[33], etc., have been developed to visualize the secretion information continuously. Due to the limited

binding sites and detection regions, these methods usually suffer from low throughput (several), low multiplexity, or a short detection time (within hours). To obtain multiplexed secretion information from more single-cells, high-throughput single-cell analysis platforms need to be employed. Currently used high throughput single-cell secretion analysis tools, including ELISpot (Fluorospot)[34], intracellular cytokine staining (ICS) flow cytometry with either fluorescence or mass spectrometry (CyTOF) detection[7], can only be used in snapshot measurements at an endpoint due to that they can not retain live cells after the detection. Microchip-based platforms, such as microengraving[14, 30, 35, 36], single-cell barcode microchip[16, 23, 37], can track single-cell secretions at different time points due to the cell adherence on the PDMS microchips. However, these methods suffered from significant cell loss during repetitive detection steps. It is reported that around 50% of single cells would be lost during sequential measurements, even for adherent cells, which brings not only the cell loss problem but also the potential cell bias in the process. Junkin et al. reported a microvalve-based integrated microfluidic device to profile single-cell secretion dynamics [24]. However, due to the complexity of microvalve control, the number of single cells profiled simultaneously is relatively low (only dozens), and the operation procedures are very complicated, making it difficult for widespread use. DropMap employed the high throughput droplets to compartmentalize single cells into stationary two-dimensional droplets array, enabling sensitive kinetic secretion analysis[25]. But the limited oxygen, nutrient supply in the pL-droplets limited the cell culture within 12 h. Besides, the closed design makes it difficult to introduce perturbation during the process. Despite these recent advances, it remains an unmet need to reliably keep the live single-cells traceable for longitudinal detection in a simple way. Minimizing cell loss during sequential readout with the same single-cells is the most critical and technically challenging.

Herein, we developed the high-density NOMA microchip to realize the retaining of ≥97% of trapped single cells to makes single-cells traceable in dedicated spatial position during repetitive secretion detection procedures. Notably, the high retention

efficiency was verified with both adherent and suspension cells by two researchers independently, demonstrating the high fidelity of the microchip. The platform makes it possible to track the information from the same live single-cells sequentially. We demonstrated the potential of the NOMA microchip in evaluating secretion functions from the same single-cells by three applications: decoding the correlation of protein abundance between secreted EV and its donor cell from the same single cells, measuring the protein secretion sequentially from the same single cells, and longitudinally tracking of the single-cell integrative secretions over days.

Results and discussion

High-density NOMA microchip realizes high-efficiency retention of single-cells

The NOMA microchip is a two-layered polydimethylsiloxane (PDMS) microwell array (**Figure 1A**), in which the bottom layer is a 200 μm diameter microwell array, and the top layer is a corresponding 30 μm diameter perforated microwell array. The total number of microwells is 4000, ensuring high throughput analysis. The top PDMS layer, fabricated with spin coating, was characterized with SEM (**Figure 1B & Figure S1**) to ensure that microwells were fully perforated. The top and bottom layers were cured with PDMS at different crosslinking ratios (10:1 and 5:1) and bonded together irreversibly after alignment (**Figure 1C**). The resulting volume of each narrow-opening microwell is ~3 nL (corresponds to 3.3×10^5 cells/mL cell density for single-cell occupancy), which is sufficient to accommodate single cells inside and keep them in the viable state over days (**Figure 1D**).

To trap single cells inside the microwells, cell suspension can be pipetted directly on the top of a NOMA microchip, which was pretreated with oxygen plasma to be hydrophilic to facilitate cell loading and minimize nonspecific adsorption. The cells would fall into microwells, and the cell distribution follows Poisson statistics, in which ~25% of microwells could be occupied by individual cells when the PDMS microwells were enclosed by putting a glass slide on the top for detection (**Figure S2**). To profile the information from the single cells retained in the microwells, different types of detection methods can be employed. Here we demonstrated the detection

with an antibody-coated glass slide to capture the secretion information (including proteins and extracellular vesicles) from these single cells. Briefly, the antibody patterned glass slide was sealed with the NOMA microchip for incubation, during which the target secretions would be captured on it. The glass slide would then be removed to finish one round of capture&detection by immuno-sandwich assay. To enable the sequential measurements from the same single cells, a new antibody-coated glass slide can be imposed onto the NOMA microchip for another round of detection. Due to the disturbance of this glass changing step, it is reported that ~43% of the single cells in the open microwells would be lost in this process, even with adherent cells[37]. While for suspension cells, it would be much more challenging to keep single cells in the same microwells during the glass slide changing step when there is no adherent force between the cells and PDMS substrate.

To solve the significant cell loss problem, we innovatively designed the narrow-opening microwell to minimize cell loss during repetitive detection procedures. Due to the small opening and increased depth of PDMS microwells, the surface tension would increase significantly, which would help to confine the single cells trapped inside the microwells. We validated cell retention efficiency by changing glass slides on the top repetitively. We found that ~98% of adherent SCC25 tumor cells (**Figure 1E**) would be kept in the same microwells after the glass changing step, which is significantly higher than previous reports ($p<0.0005$, **Figure S3**). The retention results were verified by two different researchers, which showed no significant difference between each other (98% vs. 99%, $p=0.39$), demonstrating the robustness of the method. Notably, the excellent cell retention efficiency works not only for adherent cells but also for suspension cells, which was verified with U937 suspension cells (average diameter: ~10 μm). We found around 97% of the cells would be kept in the same microwells after the glass changing step (**Figure 1F**), and the result was also validated by two researchers independently (97% vs. 97%, $p=0.67$), further demonstrating the robust, excellent single cell retention efficiency of NOMA platform. A hypothetical comparison of the single-cell retention between the open microwell and the NOMA platform reveals that only 3% of single cells would be kept inside the

open microwells after six rounds of repetitive detection (**Figure 1G**). In comparison, 89% of single cells could still be retained in the NOMA microchip, demonstrating a significant improvement in single-cell retention efficiency.

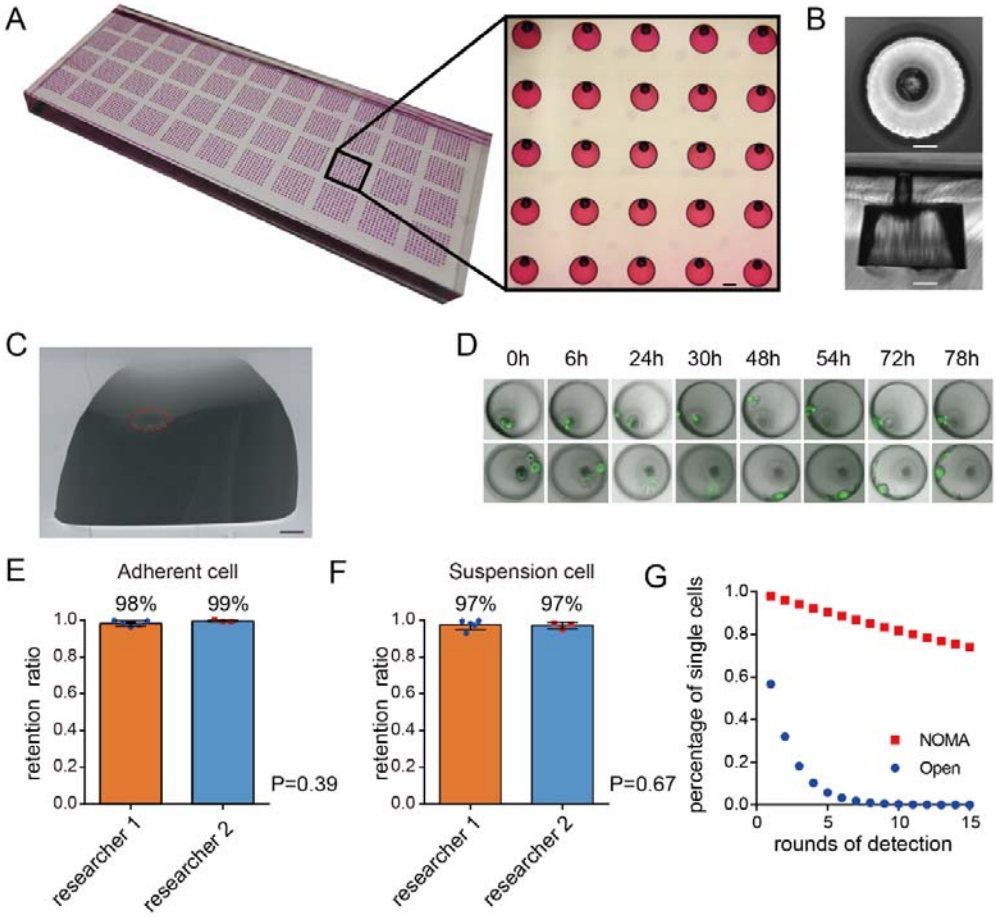

Figure 1. Design and characterization of the NOMA microchip. A. Photograph of a NOMA microchip and its enlarged view (filled with food dye to facilitate visualization). B. Optical micrograph showing the enlarged view of the individual microwell with a narrow opening, including the top and the cross-section views. The scale bar is 50 µm. C. SEM characterization of the cross-section view of the microwell with a narrow opening, in which the perforated microwell was circled. D. SCC 25 cancer cells (stained with membrane dye DiO) inside NOMA microchip showed excellent viability over days. E-F. Both adherent cell (E) and suspension cell (F) showed excellent single-cell retention efficiency during repetitive operation procedures, and the results were verified with two researchers independently. G. Hypothetical comparison between NOMA microchip and open microwells in cell

retention performance after repetitive detection procedures based on their respective cell retention efficiency.

Decoding the correlation of protein abundance between secreted EV and its donor cell from the same single cells

To demonstrate the NOMA microchip's use, we realized the sequential measurements of extracellular vesicles secretion and the corresponding protein of its parental cell from the same single cells. Extracellular vesicles are cell-derived nano-sized mediators regulating intercellular communications and delivering biological information molecules[38-40]. It is reported that the protein abundance of their surface proteins was dependent on their parental cells at the bulk level[41]. However, due to the presence of cell heterogeneity, whether this correlation still stands at the single-cell level is unknown due to the technical difficulty in measuring the extracellular vesicle secretion and the protein abundance of its parental cell from the same single cells [23]. We tried to use the NOMA microchip to generate information to answer the question.

We firstly verified their correlation at the bulk level using antibody-based EV capture/detection (**Figure 2A**). The antibodies targeting CD63, CD9, and CD81 were used, which are tetraspanins highly expressed in EV and have been widely used as proteomic markers for EV capture/detection[39, 42]. The bulk results with conditioned medium and its corresponding cell lysate showed an excellent correlation in proteomic markers between cell-derived EV and its donor cells with different cancer cell lines (SCC25, SCC6, MCF7) and two OSCC patient samples (Pearson correlation: CD63, 0.82; CD9, 0.72; CD81, 0.89; 0.94 when grouped), which is in agreement with previous reports[41]. We then applied the NOMA platform to distinguish their correlation at the same single-cell level, and the experimental procedures were illustrated in **Figure 2B**. Briefly, the EV secretion from single-cells was firstly captured with the antibody-coated glass slide sitting on the top of the microwells array for 18 hrs. Titration tests with EV standards were completed to characterize detection sensitivity (**Figure S5**&**Table S1**). Then the PDMS microwell

array was released and washed stringently to remove the residual EV in the microwells. A new glass slide coated with antibody targeting corresponding protein was applied onto the microwell array immediately after the cold SDS cell lysis buffer was used, during which over 98% of cells inside the microwells would be lysed within 15 minutes (**Figure S4**). The tetraspanin proteins within single cells would be released after lysis and be captured by the antibody immobilized on the top glass slide and further converted into fluorescence signals with ELISA like procedures for detection (**Figure 2C**).

The results showed that 48% of SCC25 single cells released $^{CD9+}$EV, and 62% of cells were positive in CD9 protein, which is in the same range (**Figure 2D**). However, Linear regression analysis revealed that CD9 protein abundance between secreted EV and its donor cells were poorly correlated with each other at the single-cell level (Pearson r=0.03) (**Figure 2E**). The low correlation was also found in the other two tetraspanins highly expressed in EV, CD63, and CD81 (Pearson r=-0.006, and 0.12 for CD63, and CD81, respectively) (**Figure 2F-G**). Further validation with primary cells from an OSCC patient sample also revealed that protein abundance between secreted EV and its donor cells were poorly correlated at the same single-cell level (Pearson r=-0.03, 0.05, and 0.02, for CD9, CD63, and CD81, respectively) (**Figure 2H-I**). We also observed similar results when secretions were captured and detected from single cells were cultured in microchips for 6 h and 12 h (Pearson r=-0.05, 0.002, **Figure S6**).

Although the mechanism to drive protein sorting into EV is complex and is still at the beginning of understanding, it has been demonstrated that the proteins would be produced, enriched, and sorted into EV from their parental cells during the EV formation [43], including the tetraspanins membrane proteins used in this study. Due to the enrichment and sorting of proteins into EV, the abundance of the corresponding proteins in the parental cells would be decreased, resulting in the low correlation in the protein between EV and its donor single cell. But they could have an excellent correlation at the population level due to cell secretion's stochasticity, which is evidenced by the bulk assay. These single-cell results indicate the intrinsic nature of

the low correlation of protein abundance between secreted EV and its donor cell at the same single-cell level and demonstrate the unique merit of the NOMA microchip to realize the same single-cell analysis sequentially.

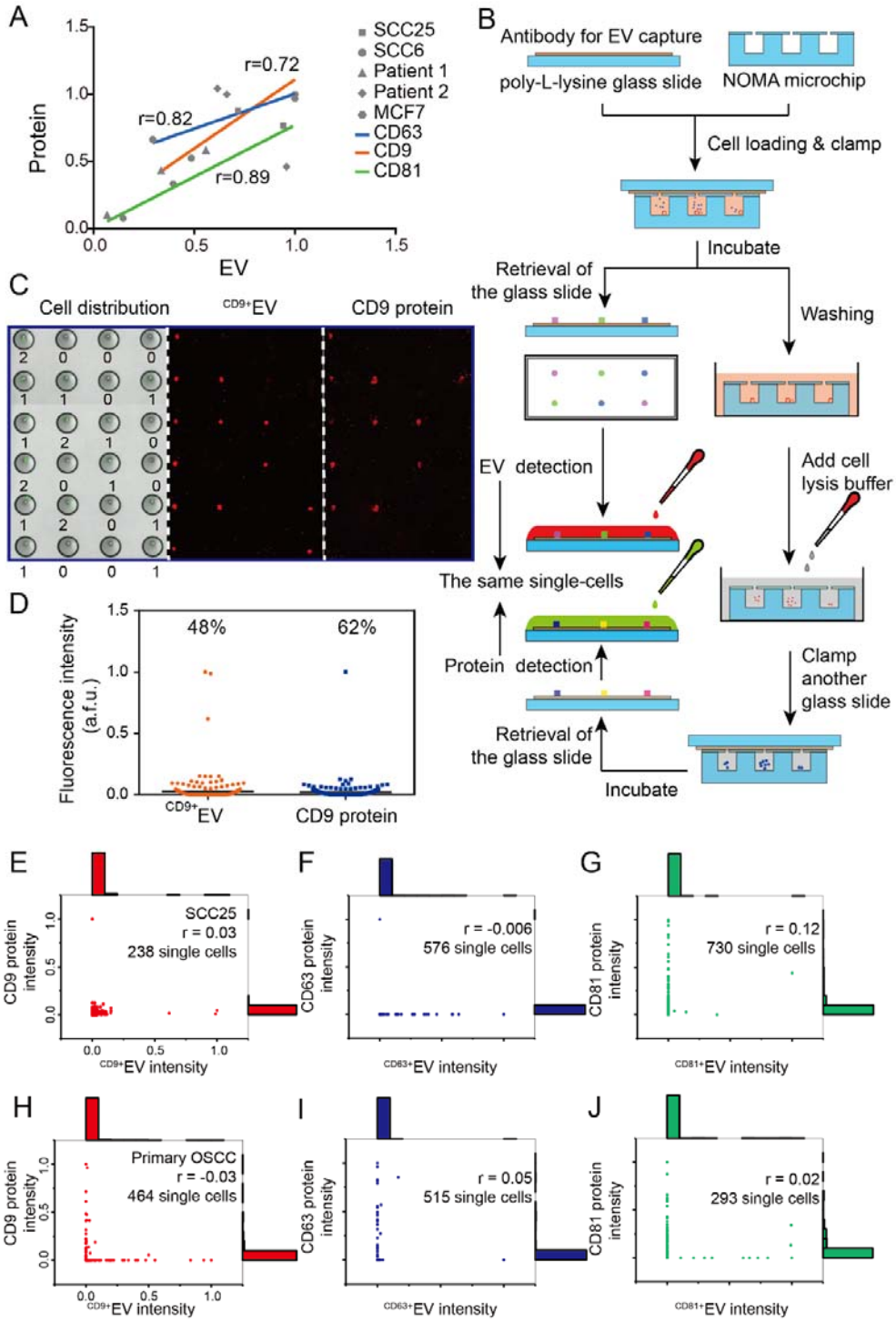

Figure 2. Analysis of the correlation of protein abundance between secreted EV and

their donor cell at the same single-cell level. A. Bulk analysis with cell culture medium showed an excellent correlation of protein abundance between secreted EV and their donor cell. B. Schematic illustration of the procedure to sequentially measure the protein abundance from secreted EV and their donor cells from the same single cells. C. Representative image showing the single cells and their corresponding sequential detection results. D. Scatter plots showing the $^{CD9+}$EV and CD9 protein detection from the same single cells. E-G. Mapping the correlation between the abundance of the CD9 (E), CD63 (F), CD81 (G) protein from the EV and its donor SCC25 cells. H-I. The correlation between the abundance of the CD9, CD63, CD81 protein from the EV, and its donor OSCC primary cells.

Measuring the secretion sequentially from the same single cells reveals the dominance of the digital secretion pattern

Profiling of cell secretion profiling, including cytokines, chemokines, etc., provides a direct way to evaluate the functional cell states for different types of cells, including immune cells, cancer cells[44-46]. Due to the dynamic characteristic of protein secretion, secretion detection from the same single cells at different time points is essential, which would provide unique information, besides the magnitude and intensity, to reveal the new heterogeneity in persistence underlying secretion. We then applied the NOMA platform for tracking the secretions from the same single-cells sequentially to show the time-resolved heterogeneity in secretion.

We firstly used macrophage cells in response to LPS stimulation as a model system to profile the protein secretion sequentially from the same single cells. Primary human monocytes from a healthy donor were isolated from PBMC with magnetic sorting and further differentiated into macrophage cells with GMCSF[18, 44]. Macrophage cells were challenged with 100 ng/mL LPS before the single-cell assay, which would activate the pathogen recognition by binding to the TLR4 receptor and stimulate the innate immune response against Gram-negative bacteria[17]. An antibody-coated glass slide was imposed on the PDMS microwells to profile protein secretion, fabricated by immobilizing antibodies associated with innate immune

responses, including IL-8, IL-6, onto poly-L-lysine coated glass slides under the static condition at room temperature. The antibody pairs used were titrated with corresponding recombinant proteins (**Figure S7**&**Table S1**). After measuring protein secretion from single macrophage cells for 4 hrs, we removed the antibody glass slide and then put another glass slide coated with the same panel of antibodies (or a different panel of antibodies) to measure protein secretion from the same single cells for another 4 hrs. We successfully obtained secretion data from 903 single cells at two-time points along with the LPS stimulation, which could be visualized with line graphs showing the oscillatory change of protein secretions from the same single cells (**Figure 3A**).

Interestingly, we found all single cells positive with protein secretion at either of two-time points could be classified into three secretion patterns based on their dynamic change: ON-OFF, OFF-ON, and ON-ON. For example, we observed that 39 single cells for IL-8 secretion during the first 4 hrs would be negative for IL-8 secretion in the second 4 hrs. Similarly, 40 cells positive for IL-8 secretion in the second 4 hrs were negative for IL-8 secretion in the first 4 hrs. Only six cells were positive in IL-8 secretion at both the first 4 hrs and the second 4 hrs. Similar secretion behaviors were also observed from IL-6 protein. These data suggest that individual cells would secrete proteins for relatively short periods, and protein secretions are dominated by digital secretion patterns (ON-OFF & OFF-ON, 93% for IL-8, 89% for IL-6). The results are consistent with a previous study that revealed the digital activation of transcription factor nuclear factor NF-kB in response to activation at the single-cell level[47]. Here we used the NOMA platform to demonstrate the stochastic oscillation pattern within single cells at the functional protein level, complementing the observation at the transcriptional level.

We also applied the NOMA microchip to profile the protein secretions from suspension cell U937 (**Figure 3B**) and OSCC cancer cells (**Figure S8**) to assess the broad applicability of the platform. We found the secretion patterns in these two types of cells could also be resolved into ON-OFF, OFF-ON, and ON-ON patterns, in which digital secretion patterns (ON-OFF& OFF-ON, U937 monocyte: 97% for IL-8, 86%

for IL-6; OSCC cancer cell: 82% for IL-8, 85% for IL-1b) dominated the secretions. Collectively, these results revealed the existence of three different secretion patterns among different types of cells, indicating the intrinsic dynamic feature of cell heterogeneity in protein secretion. These observations also revealed the dominant roles of digital secretion patterns in these cells, which are difficult to observe with single-cell analysis technologies based on a single snapshot measurement, demonstrating the unique merit of the sequential measurements from the same single cells with NOMA microchip.

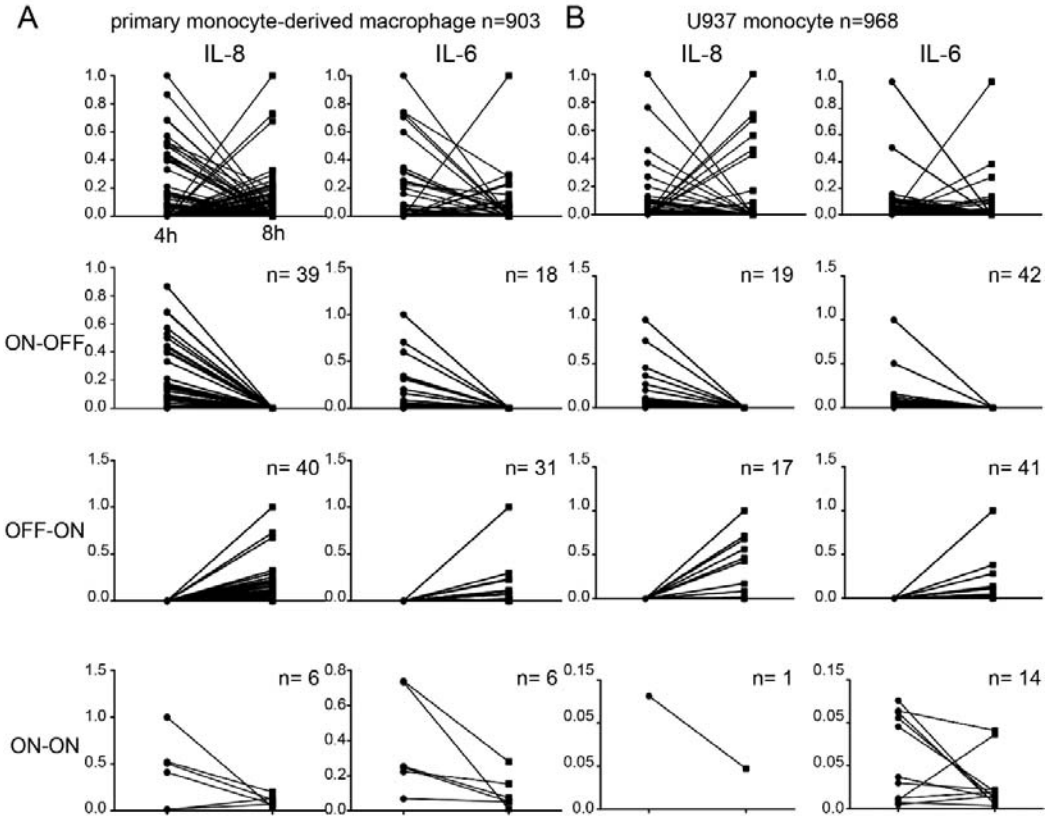

Figure 3. Line graphs showing the sequential readout of protein secretion information (IL-8, IL-6) from the same single cells (primary monocyte-derived macrophage(A) and U937 monocyte) revealed that protein secretion from single cells could be classified into three different patterns based on their change at two different time points: on-off, off-on, and all on.

Longitudinally tracking of the single-cell integrative secretions over days

The unique advantage with the NOMA platform is its high fidelity in single-cell retaining capability, making it possible to track the information from the same single over a long time, which is challenging to realize with existing technologies. To demonstrate, we used the platform for longitudinally tracking of the single-cell integrative secretion over days. Oral squamous cell carcinoma cell line (SCC25) was used as the model cell line, which would secrete cytokines or EVs continuously. After SCC25 cells were enclosed as single cells into microwells, the antibody-coated glass side was used to capture the secretions from these single-cells twice each day (4 hours incubation for each detection window), three days in total (**Figure S9**). To obtain integrative secretion information from the same single cells, we immobilized IL-8 antibody for cytokine capture and CD81 for EV capture simultaneously on the same glass slide. We successfully obtained IL-8 and $^{CD81+}$EV secretion data from 377 single-cells across six different time points, which could be summarized with line graphs (**Figure 4A-B**) or other analysis tools (**Figure S10**) to show the changes of secretion from each cell. Around 35% of cells released IL-8 secretion more than once during the detection time and most of the IL-8 secretion follows the digital pattern, which is consistent with our previous observation. Most of the single cells would only produce IL-8 protein during a 1-time window. A small population of cells (~ 2%) was quite active to secrete IL-8 protein more than three times, indicating the presence of "super secretors" within the cell population that could secrete proteins more persisting for an extended period. Similarly, we found 18% of cells produced $^{CD81+}$EV secretion more than once during the detection, and EV secretion was also dominated by the digital pattern. Only two cells (0.5%) were able to secrete $^{CD81+}$EV more than two times, further confirming the presence of persisting active secreting cells in the population. These results reveal the dynamic heterogeneity underlying cell secretion that a few cells could secrete cytokines or EVs multiple times, which could not be observed with snapshot measurements.

Previous studies have revealed polyfunctional immune cells within the cell

population are critical players in protective immunity and immunotherapy, demonstrating the significance of secretion heterogeneity. However, it is still difficult to evaluate the persistence of secretion at the same single-cell level. Besides, how the heterogeneity in the persistence of secretion would affect the cell function is yet to be explored. However, these studies are mainly hindered by the lack of research tools. The NOMA microchip that could realize the robust, high-efficiency single-cell retention demonstrated itself potentially be a useful tool to widely used for deep phenotyping dynamic heterogeneity and persistence of secretion. Besides, it is also possible to recover the single-cells of interest selectively from the NOMA microchip (**Figure S11**&**Movie S1**) and be further coupled with other single-cell manipulation or detection methods, such as genome editing or RNA-seq, etc., to obtain more information from the same single cells.

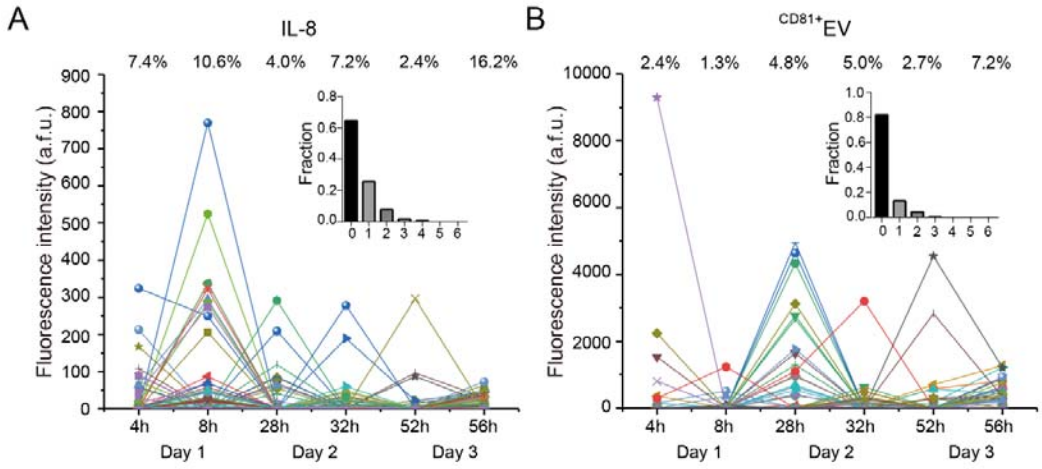

Figure 4. Longitudinally tracking of integrative secretions from single cells over days. Line graphs showing the change of individual secretions (A: IL-8; B: $^{CD81+}$EV) from 377 single cells over three days.

Conclusion

We developed a novel NOMA microchip to track the information from the same single cells, which realized the retention of over 97% of single cells with both adherent cells and suspension cells during repetitive detection procedures. The unique

merit of this single-cell microdevice is the robust, high-efficiency single-cell retention to keep single cells alive in the dedicated microwells after rounds of detection. This platform was further applied to measure the extracellular vesicle secretion and the protein abundance of its parental cell from the same single cells sequentially, which revealed that they were poorly correlated at the same single-cell level. With this platform, we also realized the sequential measurements of the same live single-cell to realize the tracking of single-cell secretion longitudinally, which revealed three different secretion patterns and the dominant roles of digital secretion pattern among different types of cells. The highly robust cell retention capability also makes it possible to recover the single-cells of interest selectively based on their secretion profiles and be coupled with other single-cell manipulation or detection methods for additional studies in the future. This platform represents a novel and powerful tool broadly applicable for different cell types, including immune cells (B cell, T cell), tumor cells (circulating tumor cells), for informative monitoring of the same single cells in basic and clinical research.

Supporting Information

This article contains supporting information online.

Acknowledgments

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Author contributions

Y Lu, MM Liu and YH Ji designed the research; MM Liu, YH Ji, FJ Zhu, X Bai, LM Li, XM Liu, Y Luo, TJ Liu, BC Lin and Y Lu performed research; Y Lu, MM Liu and YH Ji wrote the manuscript, with extensive inputs from all authors.

Conflict of interest

The authors declare no conflict of interest.

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Supporting information

NOMA: a high-throughput microchip for robust sequential measurements of secretions from the same single-cells

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Supporting methods

Cell culture

SCC25 (ATCC(American Type Culture Collection), USA) was cultured with MEM (HyClone) complete medium containing 10% (v/v) fetal bovine serum (Pan), 1% (v/v) penicillin-streptomycin solution (100 U/mL of penicillin G sodium, 100 U/mL of streptomycin) (HyClone) , 1% (v/v) MEM non-essential amino acid (Gibco). Human

monocytic U937 cells (ATCC, USA) were cultured in RPMI 1640 medium (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Invitrogen)) and 1% antibiotics (100 U/mL of penicillin G sodium, 100 U/mL of streptomycin), at 37□ and 5% CO_2 in the incubator. Human monocytes were isolated from PBMC using a magnetic-based monocyte isolation kit (Miltenyi Biotec) and differentiated into macrophages with GMCSF (50 ng/mL; R&D) for 3 d and then RPMI complete medium for 4 d. The OSCC primary cells were obtained from the primary tissues from the Affiliated Hospital of Dalian Medical University. The collection and use of human samples was approved by the Ethics Committee of Dalian Medical University. Cells were cultured at a laminar flow hood (37□ and 5% CO_2). The fetal bovine serum needs to be ultra-centrifuged at 100,000 x *g* at 4 ºC for 4 h before using to remove the interference of serum exosomes in single-cell EV secretion detection.

Device fabrication

The procedures to fabricate the NOMA microchip are as follows: 1) Fabrication of the hard molds for microwells by patterning SU8-3035 (Microchem) on silicon wafers with photolithography. 2) Spin coating PDMS (base: curing agent = 20:1) on the 30 μm-microwell mold by 1700 rpm to obtain perforated PDMS microwell film. Molding of PDMS microwell (200 μm diameter) with PDMS (5:1 ratio). 3) Align the PDMS microwell (200 μm diameter) slab with perforated PDMS microwell film and bake the assembly at 80 □ for 2 h to realize the irreversible bonding. After curing, the PDMS microchip was peeled off the mold, cleaned for further use.

Antibody immobilization on the poly-L-lysine coated glass slide

100 μL antibody solution (10 μg/mL) was added on the 1-mm-thick poly-L-lysine coated glass slide. Press the cover glass from left to right to distribute the antibody solution evenly on the glass and incubate it at 4 □ overnight. Disassemble the cover glass from the poly-L-lysine glass and block the glass slide with 1% BSA for 1 h at room temperature. After blocking, the glass slide was washed with DPBS, 50/50 DPBS/DI water, and DI water sequentially. Finally, dry the antibody glass slide with a slide centrifuge and store the antibody-coated slide at 4□ before use.

Single-cell detection procedures

After cell suspension was collected, they were stained with viability dye Calcium-AM

before adjusting the cell density to $1*10^5$ cells/mL. The PDMS NOMA microchip was treated with oxygen plasma for 2 min before use to render the surface hydrophilic. After plasma treatment, 1 mL cell suspension was added onto the NOMA microchip to let the cells settle down to the bottom of the microwell. A fresh cell culture medium was used for flushing excess cells from the surface of the chip. Then the antibody-coated glass slide and NOMA microchip were clamped together and incubated at 5% CO_2 incubator at 37□. After incubation, the glass slide captured with secreted protein/EV was removed from the clamp for detection. For time-resolved single-cell sequential secretion analysis, a new antibody-coated glass slide was imposed to another round of detection. For intracellular analysis, cold cell lysis buffer would be added onto the surface of the NOMA microchip before clamp it immediately with another antibody-coated glass slide to capture the intracellular information. The retrieved glass slide was incubated with a cocktail of detection antibodies for 1 hour and stained with streptavidin-APC (eBioscience, 1:100 dilution) afterward for another 30 min. Then it was washed thoroughly with DPBS, 50/50 DPBS/DI water, DI water sequentially. After drying with a slide centrifuge, it was scanned with a GenePix 4300A fluorescence scanner (Molecular Devices) for fluorescence readout.

Data analysis

Images containing cell number/position across NOMA microchips were captured by the Nikon Eclipse TiE microscope. The resulting secretion fluorescence signals were scanned by GenePix 4300A and analyzed with GenePix Pro software to add and align the microwells array template, followed by the extraction of fluorescence intensity results. The cell counts in the NOMA microchip can be matched with the extracted fluorescence intensity. The mean intensity plus 2×SD of zero cells was defined as the threshold to determine the positive secretion from single cells. Excel (Microsoft), Origin, or Graphpad Prism were used for data analysis.

Supporting figures

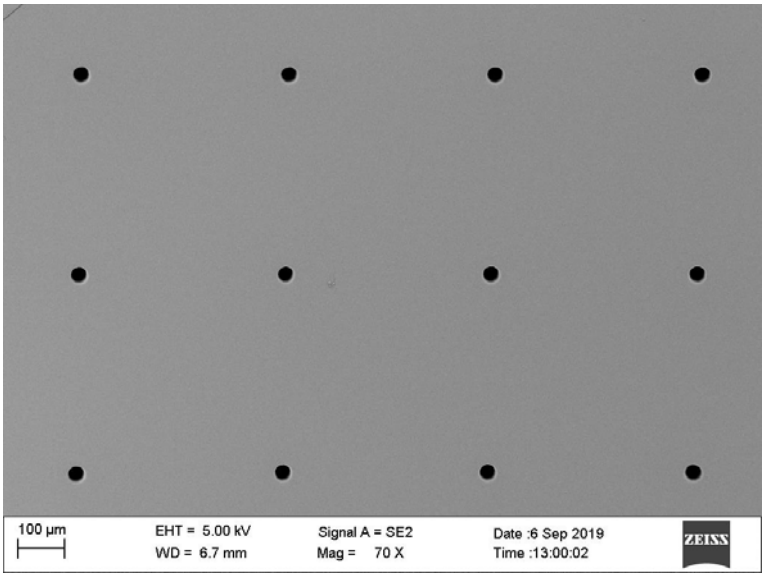

Figure S1. Characterization of perforated PDMS microwells (diameter: 30 µm) with SEM.

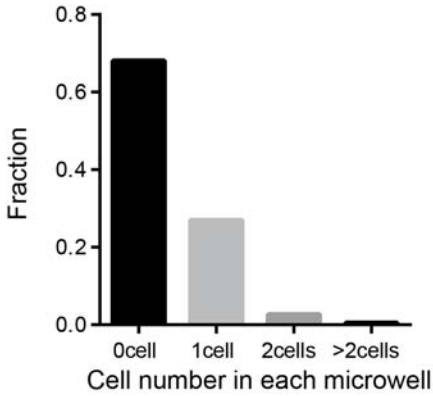

Figure S2. Distribution of the number of cells in 4000 microwells across the NOMA microchip

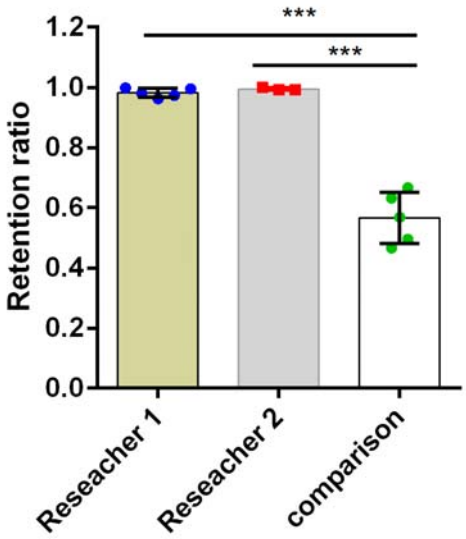

Figure S3. Comparison of cell retention efficiency between NOMA microchip and open-microwell based microchip during repetitive glass changing steps.

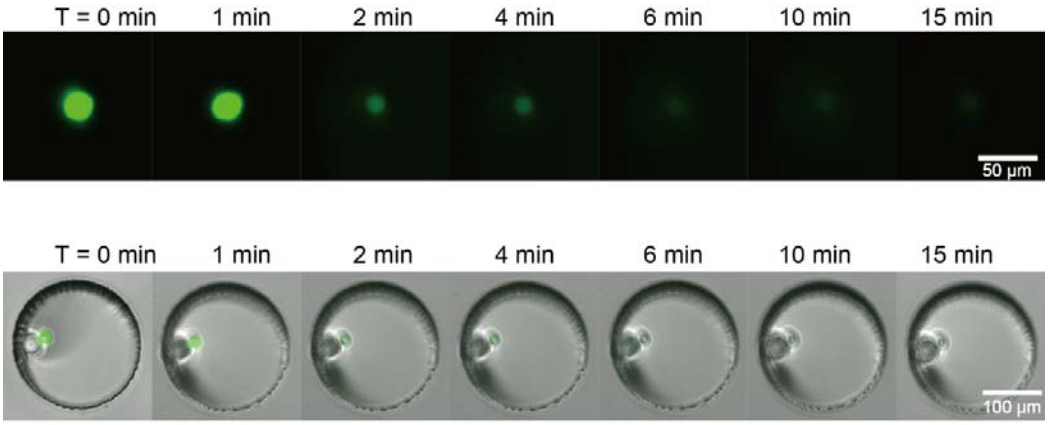

Figure S4. Time-lapse images showing the single-cell lysis process inside an individual microwell in the NOMA microchip, in which the single-cell was labeled with viability dye.

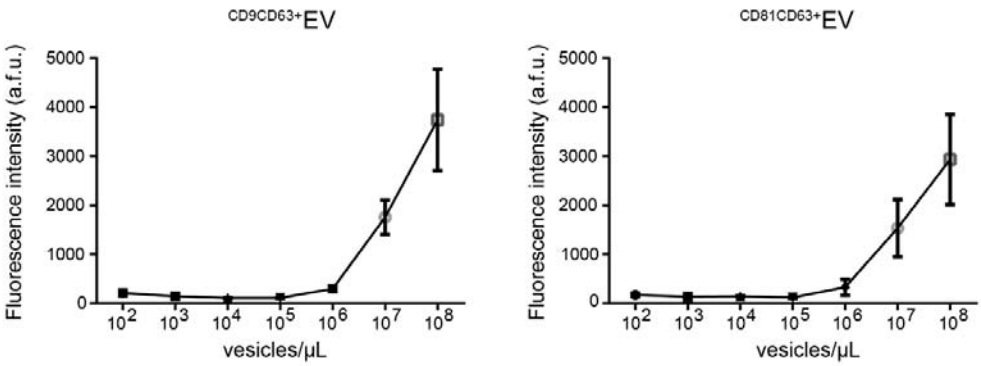

Figure S5. Sensitivity characterization of the antibody-based EV detection with EV standard (ExoStd™ Lyophilized Exosome Standard, Biovision, USA) reveals that the lowest detection concentration for EV is ~10^5 EVs/μL. Considering the volume of an individual microwell in the NOMA microchip is ~3 nL, the lowest detectable number of EV is ~ 300 vesicles/microwell.

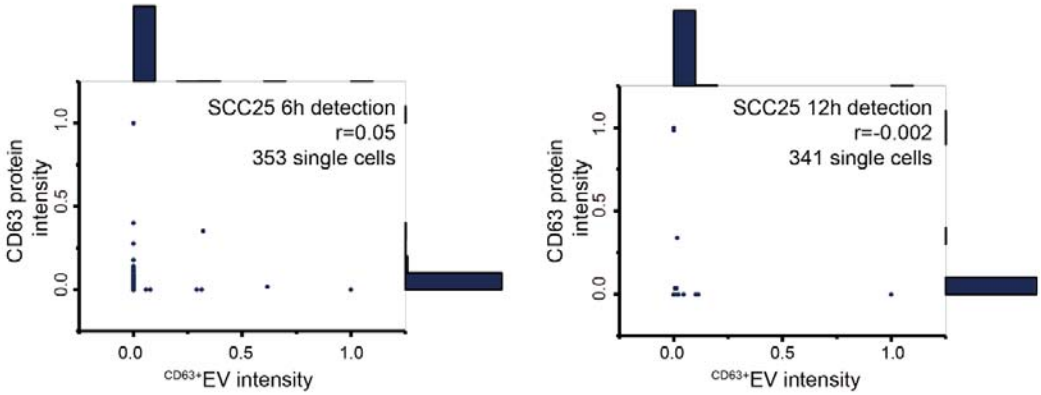

Figure S6. Scatter plots showing the $^{CD63+}$EV secretion detection and CD63 protein detection from the same single cells at different time points (6 h and 12 h).

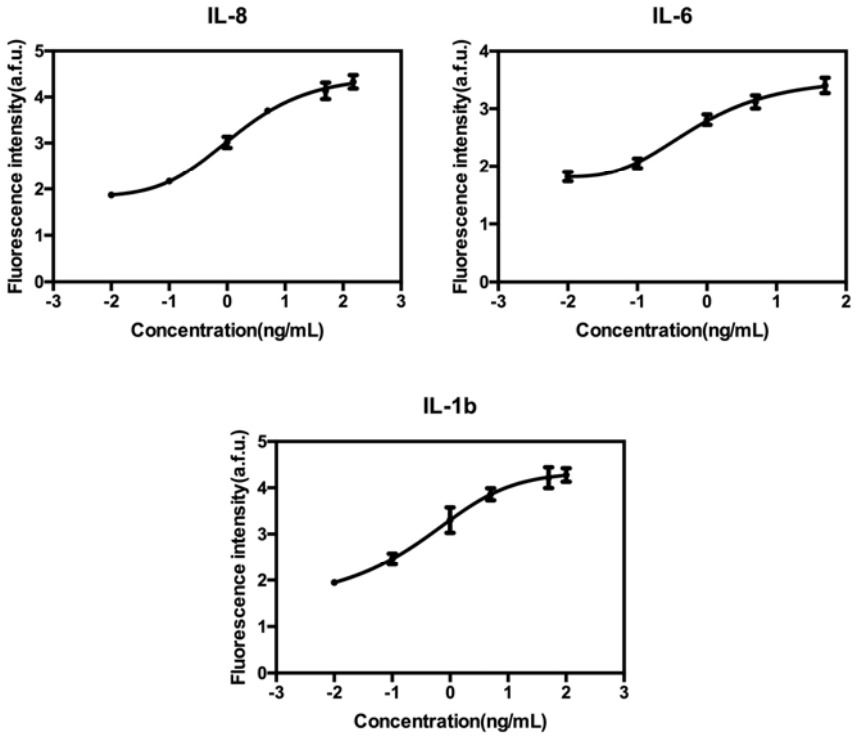

Figure S7. Calibration curves obtained with corresponding recombinant proteins (x, y axes: log-scaled, the intensities were averaged from 6 spots for each data).

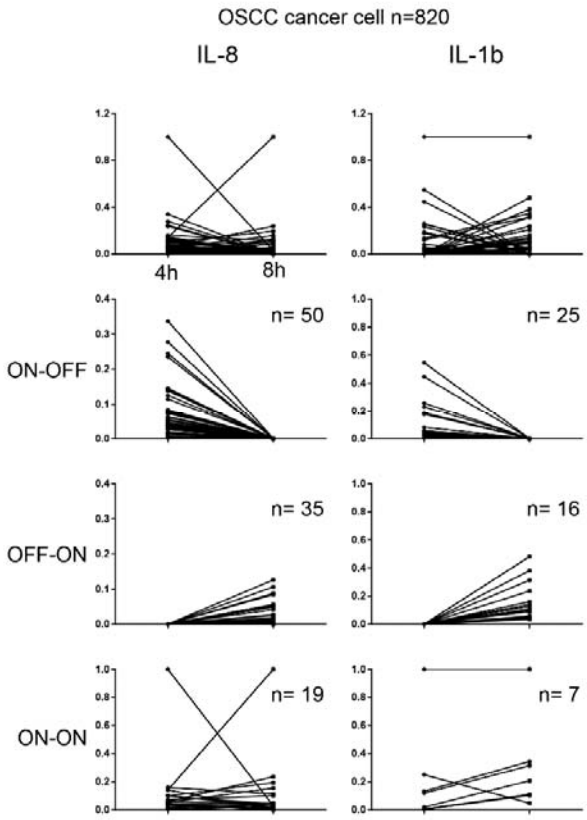

Figure S8. Sequential measurements of protein secretions(IL-8, IL-1b) from the same OSCC single cells (n=820) revealed that protein secretions from single cancer cells could also be classified into three secretion patterns based on their change: on-off, off-on, and all on, and the digital secretion patterns (on-off, off-on) dominated the secretion behaviors.

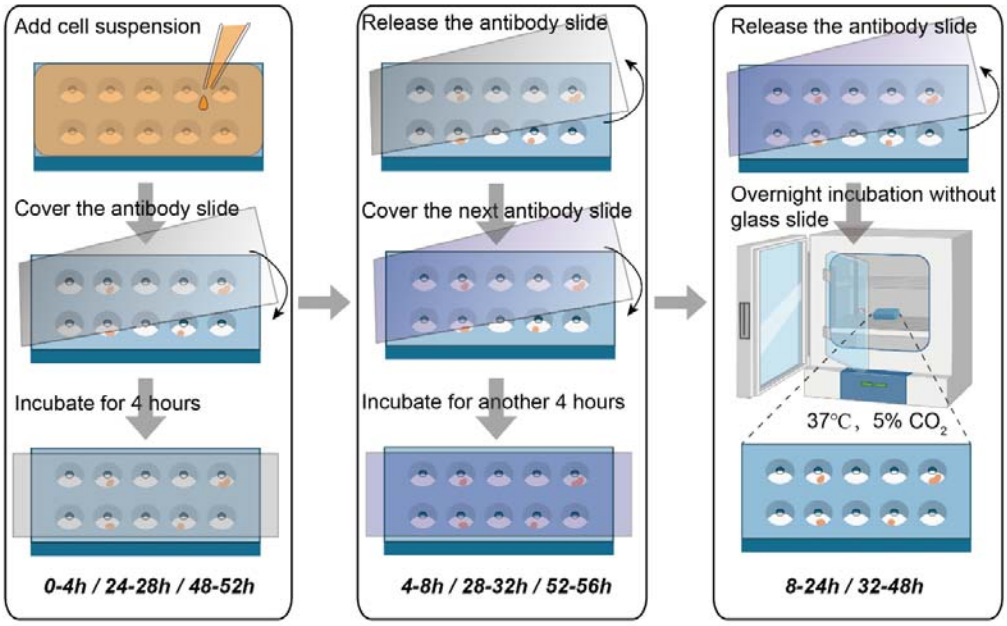

Figure S9. Illustration of the detection procedures to follow the secretions from the same single cells at different time points over three days.

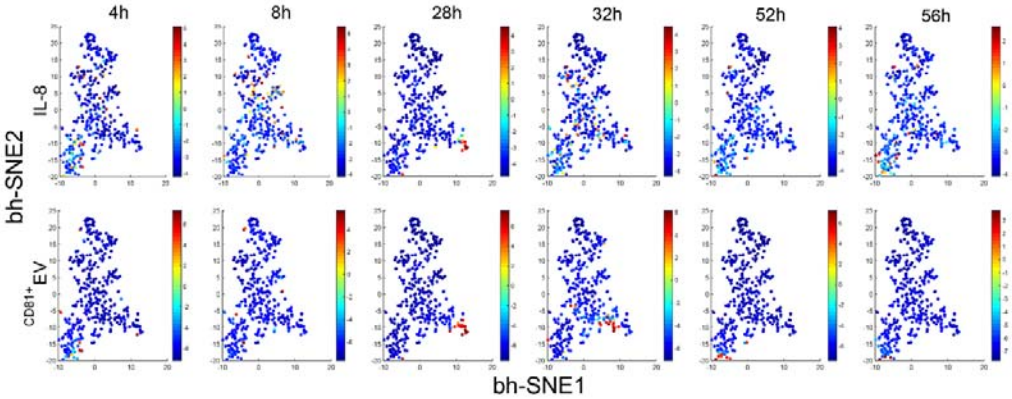

Figure S10. Visualized clustering analysis with viSNE to reveal the protein and EV secretions from the same single cells at different time points. All the single-cell data at six different time points with both protein secretion and EV secretion were combined into a unique dynamic single-cell data metric to be visualized with viSNE, which is a high-dimensional data analysis tool based on the t-Distributed Stochastic Neighbor Embedding algorithm. Interestingly, we found EV secretion and protein secretion are from into different cells, except for 28 h, during which they were nicely correlated with each other at this time point (Pearson r=0.89). This was not observed previously,

highlighting the value of a dynamic detection platform with the single-cell resolution to reveal how biological systems respond and evolve.

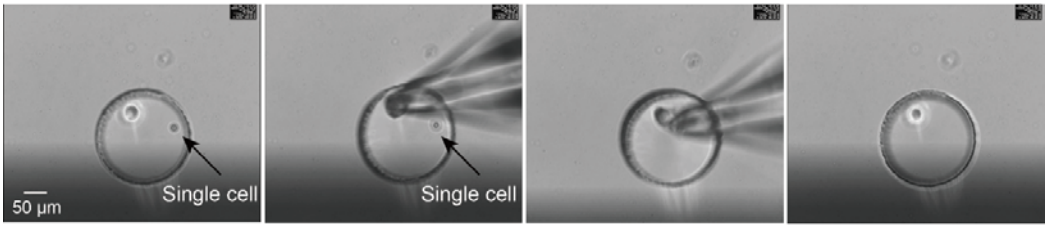

Figure S11. Video clips showing the single-cell retrieval processes from an individual microwell in the NOMA microchip.

Reagent	Company	Catalog number
Anti-human CD9	Millipore	CBL162
Anti-human CD63	Millipore	CBL553
Anti-human CD81	Novus biologicals	NB100-65805
Human IL-6	eBioscience	88-7106-88
Human IL-8	eBioscience	88-8086-88
Human IL-1b	eBioscience	88-7261-88
Biotin labeld anti-human CD63	Biolegend	353017-50
Biotin labeld anti-human CD9	Biolegend	312112
FITC labeld anti-human CD81	Biolegend	349502
Streptavidin-APC	eBioscience	17-4317-82
ExoStd™Lyophilized Exosome Standard	Biovision	M1043

Table S1. Summary of key reagents used in this study.

Movie S1. Single-cell retrieval from individual microwell in the NOMA microchip.