Droplet array-based platform for parallel optical analysis of dynamic extracellular vesicle secretion from single cells

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ABSTRACT: Extracellular vesicles (EVs) are essential intercellular communication tools, but the regulatory mechanisms governing heterogeneous EV secretion are still unclear due to the lack of methods for precise analysis. Monitoring the dynamics of secretion from individually isolated cells is crucial because, in bulk analysis, secretion activity can be perturbed by cell–cell interactions, and a cell population rarely performs secretion in a magnitude- or duration-synchronized manner. Although various microfluidic techniques have been adopted to evaluate the abundance of single-cell-derived EVs, none can track their secretion dynamics continually for extended periods. Here, we have developed a droplet array-based method that allowed us to optically quantify the EV secretion dynamics of >300 single cells every 2 hours for 36 hours, which covers the cell doubling time of many cell types. The experimental results clearly show the highly heterogeneous nature of single-cell EV secretion and suggest that cell division facilitates EV secretion, showing the usefulness of this platform for discovering EV regulation machinery.

Figure 1. Schematic illustration of a droplet array-based method for the optical quantification of single-cell extracellular vesicle secretion dynamics by time-lapse imaging.

Extracellular vesicles (EVs) are nanoscale lipid bilayer structures secreted from cells and enclose a variety of cargos, such as proteins and RNAs.1,2 Recent studies have revealed that EVs serve as essential tools for communication between cells by transferring diverse molecules and have implicated EVs in various diseases, such as cancer.3 To uncover the roles of EVs in intercellular communication and develop therapeutic approaches targeting EV-related diseases, understanding the mechanisms governing EV secretion is of critical importance. Specifically, understanding these mechanisms can lead to the development of methods to modulate EV secretion levels and thereby evaluate the contribution of EVs in cell–cell communication. Moreover, selective modulation of EV secretion from cancer cells has the potential to lead to strategies for suppressing cancer metastasis.4

To this end, critical regulators of EV secretion have been widely identified,4 yet little is known about the mechanisms that fine-tune EV secretion levels. For example, little is known about how dynamic biological processes (e.g., cell cycle, differentiation, and migration) affect EV secretion abundance. This is mainly because experiments have been performed on mixtures of EVs collected from cell populations, where the perturbation-coupled EV secretion fluctuation is buried in miscellaneous noise: first, cell–cell interactions can perturb EV secretion modes; second, adjacent cells can promptly take up secreted EVs; and last, EV secretion from each cell is rarely synchronized within the cell population, i.e., the magnitude and the timing are variable.

To overcome these issues, a single-cell culture platform is required to clarify the causal relationship between perturbation and changes in EV secretion levels. Additionally, single-cell
monitoring of EV secretion is essential for capturing heterogeneous secretion dynamics. Microfluidic platforms for single-cell EV analyses have been developed; however, each has its limitations. Several approaches enabled the detection of heterogeneity in EV populations from single cells, and others took time-lapse strategies for monitoring the secretion of EVs using antibody-based methods (5, 10) (also see the review (3)). However, none can continually track EV secretion dynamics from single cells for an extended period (longer than a general cell doubling time), which is critical for analyzing EV secretion machinery throughout the steps of the cell cycle.

Here, we integratively developed a droplet-based, parallel and scalable method to perform time-lapse fluorescence imaging of EV secretion dynamics from single cells for a long time with high temporal resolution (every 2 hours for 36 hours) under standard culture conditions. In this method, as shown in Figure 1, after encapsulating individual cells that secrete fluorescently labeled EVs inside 250 picoliter-sized droplets, we array thousands of them stationarily between microfabricated pillar arrays within a single device. This device is then soaked in oil to minimize solvent evaporation and placed in a stage-top incubator under an optical microscope. Inside the small volumes of the droplets, concentrations of the secreted EVs increase rapidly, allowing us to quantitatively monitor their dynamics by optical imaging and simple image analysis. Using the system developed, we demonstrate that CD9-positive EV secretion from >300 cells can be monitored every 2 hours for 36 hours, and the results intriguingly suggest that cell division facilitates EV secretion.

**MATERIALS AND METHODS**

**Plasmid.** The method used to prepare CD9-sfGFP in pSBbi-Hyg was as follows. A fragment encoding human CD9 (NM_001769) was PCR-amplified from pDB96 (13) by oKK236 and oKK237, digested with SfiI, and cloned into the corresponding site of pSBbi-Hyg (#60534, Addgene) to form an intermediate plasmid. Then, another fragment coding sfGFP was PCR-amplified from pHdSV40-sfFV-GCN4-sfGFP-VP64-GB1-NLS (#60904, Addgene) using oKK334 and oKK335, digested with KpnI-HF (R3142, New England Biolabs), and cloned into the corresponding site of the intermediate plasmid. The primer sequences were as follows (purchased from Fasmac): oKK236: 5′-attagctcttagccgccatcgcggcagagagaggagagcag-3′, oKK237: 5′-attagctgcttagccgccatcgcggcagagagaggagagcag-3′. The amino acid sequence of CD9-sfGFP is CD9 [MPVKGGTCKYKLLGTTGFGNFIFWLALGAVLAGILWRFD SQTKSIFEQTNNNSSFTYGVYILIGALMVMVFLGL GCAVQGCMQLCMGFGLFVIFAEIAEAWGYSKHDKVIKEVQEFYKDTYNKLTKEPDORVPAKNVMAXLCA CNCCGLAVGGEFISDPCPKDVLETLFVTKSCPDIAEVEFVDFNKF HIIAGVIGIAVVFMIIFMSMCILLAARXNEMV] – linker [LEFGGGGG] – sfGFP [SKGEEFLLGTVPVLPLEDGDGVNGHKFSVREGEGGAT NGKLTLEKCIITCKLRVLPVPWPLTTLTVYGVQFCFSRPD HMKHRDFIKSAMPEGVYQERTSIFKDDTGYTKRAYEVK FEGDLTVNLRCGDKFDGKEDGNILKHEYNFNHNHYVYI TADQKQNGIKANFKIRHVNEDGSVLADHYQNTPIG DGPVLLPNDHYLSTQSVLSDKPEKRHDMLVLEFVTA AGITHGMDELYK]
device used in the time-lapse imaging was fabricated by bonding the PDMS slab to a 35 mm glass-bottom dish after oxygen plasma treatment. The device was presoaked in FC-40 (3M) for more than 30 min and placed in a stage top incubator (STXG-WSKMX, TOAKI HIT). The incubator was set on an EVOS M7000, and images were automatically scanned every 2 hours.

**Image analysis.** The image analysis pipeline was developed using SciPy and scikit-image in Python 3. See the results section as the summary of analysis. The analysis scripts are available at https://github.com/solabtokyo/Hattori_et_al_2022.

**EV purification and quantification.** K562-CD9 cells were cultured in medium containing (10% FBS) for 3 days, and the medium was centrifuged at 2000 x g for 10 min at 4 °C to remove cells. The conditioned medium was ultracentrifuged at 210,000 x g (35,000 rpm) using a SW41Ti rotor (Beckman Coulter) for 70 min at 4 °C. The pellets were washed with 11 mL PBS (ultracentrifugation at 210,000 x g for 70 min at 4 °C) and then resuspended in PBS. Purified EVs were evaluated by nanoparticle tracking analysis with a NanoSight LM10 (Malvern Panalytical, equipped with a CCD camera and a 488 nm laser). Fluorescent particles were detected with a 500 nm longpass filter. Measurements were conducted 3 times (60 sec each) per sample.

**qRT–PCR.** RNA was extracted from cells using a FastGene™ RNA Premium Kit (FG-81050, NIPPON Genetics), and the concentration was measured by a NanoDrop One (Thermo Fisher Scientific). RNA was reverse transcribed with ReverTra Ace qPCR RT Master Mix with gDNA Remover (FSQ-301, TOYOBO). Primers were designed using Primer-BLAST and purchased from Eurofins Scientific. qRT–PCR was performed by a CFX Connect Real-Time System (BIO-RAD) using KOD SYBR qPCR Mix (KQD-201, TOYOBO).

Data were normalized to RPS18.

The primer sequences were as follows: human RPS18 forward – TAAAGGTTGGGCGGAAGAT, human RPS18 reverse – TGATCACACGTTTCCACCTCA, human CD9 forward – ACCATGCGGTCAAAGGAG, human CD9 reverse – AATCCCGGCAAGCCAGAAG, human CD63 forward – ACCACACTGCTTGATCCGT, human CD63 reverse – GGGACTCCGTTCCTCGCAT, sfGFP forward – GCGATGGCCCTGTCCTTTTA, sfGFP reverse – TAAGGGTGTGGGCCGAAGAT, human CD63 reverse – GGGACTCCGTTCCTCGCAT, sfGFP forward – GCGATGGCCCTGTCCTTTTA, sfGFP reverse – CCATGTGGTCACGCTTTTCG.

**RESULTS AND DISCUSSION**

**Development of platform for time-lapse monitoring and quantification of EVs inside droplets.** In this method, we formed and utilized a stationary array of more than three thousand 250 μL droplets within a microfluidic device under optical microscopy. The microfluidic device consists of an array of ~5000 micropillars, spaced 80 μm apart (Fig. 2A and B). Droplets were generated with a flow-focusing device and introduced to the pillar device in advance, thereby being held stationary between pillars in preparation for time-lapse imaging (Fig. 2C). We fully kept the micropillar array device soaked in less volatile oil (FC-40) to minimize water and oil evaporation for more than 36 hours of experiments. The soaked device was placed in a stage-top incubator to maintain standard cell culture conditions (37 °C, 5% CO2). We used a commercially available automated fluorescence microscope for the automatic acquisition of time-lapse fluorescent and phase-contrast images. In concrete, it took less than 5 minutes to image all the droplets in the device.
We aimed to analyze the single-cell EV secretions quantitatively and sensitively by encapsulating cells secreting fluorescent EVs inside the small droplets and measuring the increase in fluorescence intensity of the droplets. For this purpose, we prepared cells producing fluorescent EVs by transfecting the CD9-sfGFP gene into K562 cells. We first selected K562 cells stably expressing CD9-sfGFP by treatment with an antibiotic and then isolated a monoclonal cell line by limiting dilution (K562-CD9 cells). The overexpression of CD9 and sfGFP in the isolated cell line was confirmed by qRT-PCR, while the expression levels of CD63 as another EV subset marker (negative control), were comparable to those in the parental K562 cells (Fig. S1A). This established K562-CD9 cell line was encapsulated in droplets, and the secreted EVs were successfully detected (Fig. 2D). In parallel with EV quantification, we monitored cell viability by encapsulating SYTOX Orange (dead cell stain) inside the droplets and eliminated dead cells from the image analyses. Considering the potential leak of chemical compounds from the aqueous phase into the oil phase,\(^{20,21}\) we verified the availability of SYTOX Orange in droplets by restaining the cells extracted from droplets after quantifying SYTOX Orange signals in droplets. The cell viability in droplets was comparable to that in the extracted sample, confirming that SYTOX Orange stains dead cells accurately in droplets (Fig. S1B).

![Figure 3](image)

**Figure 3.** Monitoring of EV secretion dynamics. (A) Representative time-lapse fluorescence and phase-contrast (PC) images of two tracked droplets encapsulating K562-CD9 cells for 36 hours. (B) Temporal change of accumulated EV abundance in each droplet secreted from K562-CD9 cells, obtained by calculating the mean intensities of sfGFP signals outside the cell region inside droplets (N = 332). For calculating the abundance of secreted EVs, the mean intensities at each measurement point were subtracted by those at the initial measurement. In each droplet used in this graph, single cells were initially encapsulated and divided into two cells during the monitoring in general. (C) The total abundance of accumulated EVs measured at 24 hours. Single: droplets encapsulating single cells at 24 hours (N = 79). Divided: droplets encapsulating 2 cells at 24 hours (N = 253). (D) The total abundance of accumulated EVs measured at 24 hours, when cells were pretreated for 1 hour with or without 5 µM manumycin A. Mean and individual data are shown (N = 84). (E) The total abundance of accumulated EVs measured at 24 hours, when cells were co-encapsulated with or without 5 µM monensin. Mean and individual data are shown (N = 76). (F) The abundance of EVs accumulated in the last 2 hours at each measurement point (N = 20). Dots are shown when 2 cells were observed for the first time in the droplet after cell division. (D, E) All data were divided by the mean of the control sample. An unpaired two-tailed Welch’s t-test was used.
We computationally analyzed the time-lapse images using an in-house Python 3-based method (Fig. 2E). Each droplet and cell were segmented using phase-contrast and sfGFP images, respectively. Inside sfGFP-positive droplets (i.e., droplets containing the fluorescent cells), we obtained the abundance of secreted EVs by calculating the mean intensities of sfGFP signals outside the cell region and subtracting the background from them. The background was estimated by the mean intensities of blank droplets at each measurement. Droplets exhibiting high mean intensities of SYTOX Orange signals were omitted to exclude dead cells from the analysis. Droplets initially containing more than one cell were then also excluded from the analysis based on the cell area values. Droplets were tracked according to their positions in each image over time and used for subsequent analyses unless the droplets coalesced with their neighbors during measurements. We visually counted the number of cells in each droplet using phase-contrast images. Lastly, we also visually checked whether the cell region was correctly defined using green fluorescence images and, if not, removed the droplets from analyses.

**Observation of heterogeneous EV secretion and its dependence on cell division.** Leveraging this established EV quantification strategy, we successfully tracked the EV secretion dynamics of >300 single K562-CD9 cells for 36 hours, which is generally enough time for cells to proliferate (Fig. 3A). The results reveal that the EV secretion mode of every single cell—even if we use a single-clone cell line—is heterogeneous, and the cells do not always secrete EVs at a constant rate (Fig. 3B, C).

To verify and demonstrate that our platform detects signals of the secreted EVs and is useful for detecting the perturbation-coupled modulation of EV secretions, we up- and downregulated EV secretion by treating chemical compounds, respectively. First, we pretreated K562-CD9 cells with or without manumycin A, a canonical inhibitor of EV secretion, and incubated the cells in droplets for 24 hours without the compound. The quantification results indicated that manumycin A significantly suppressed fluorescent signals (Fig. 3D). Conversely, treatment with monensin, a widely used EV secretion activator, enhanced fluorescent signals (Fig. 3E). In both experiments, we only selected droplets encapsulating the single cell for analysis to exclude the involvement of cell division. These results confirmed that the accumulated sfGFP signals in droplets are derived from EVs, and our platform could detect the change in EV secretion levels induced by established regulators.

Furthermore, our analysis of the single-cell dynamics clearly showed that cells facilitate EV secretion when cells divide. Figure 3F shows representative results obtained by calculating the difference in the fluorescence intensities between every consecutive image taken every 2 hours. In this analysis, the representative 20 cells were randomly selected for visual clarity. When we co-plot dots reflecting the timing of cell division, which we defined when two cells appeared for the first time, intriguingly, we observed high secretion rates at these cell divisions in many cases. While we also observed the increased secretion several hours before the cell divisions (see two dots in Fig. 3F whose y values are approximately 0), we believe that the strong relationship between the EV secretion dynamics and the cell division is apparent.

Several previous studies have suggested that cell proliferation correlates with EV secretion, however, none has directly observed the relationship between cell division and EV secretion due to technical difficulties of measuring EV secretion specifically from dividing cells. With our single-cell EV quantification platform applicable for long-term continual monitoring, we successfully uncovered the potential contribution of cell division to EV secretion. Despite this observation, the precise mechanisms underlying this phenomenon remain to be elucidated. Since CD9 can be detected in both exosomes and ectosomes/microvesicles, the subtype of cell division-coupled CD9-positive EVs should be examined in a future study. Another candidate to consider is the midbody remnant, a recently proposed class of EVs secreted following cell division.

**Translating fluorescent intensity to absolute number of EVs.** While we have analyzed the EV secretion levels based on the fluorescence intensity, the absolute number of EVs is potentially valuable for comparing the EV secretion capacity among different cell types. Hence, we tested if we could perform absolute counting of the EVs secreted from each cell using the same device. To prepare a standard curve, we first purified EVs from K562-CD9 cell-conditioned medium and measured the particle number of the EV solution by nanoparticle tracking analysis with a NanoSight device. (Fig. S2). The concentration was $1.02 \times 10^{12}$ particles/mL, and the sfGFP-positive rate was 39.7%. Then we encapsulated the purified EVs at three concentrations ($8 \times 10^9$, $2 \times 10^{10}$, and $8 \times 10^{10}$ sfGFP particles/mL) in 250 pL droplets (Fig. 4A) and calculated signal intensities by a similar method to that in Figure 2. We used the least-squares method for standard curve fitting and confirmed that the mean fluorescent signal linearly correlates with the EV concentration on the order of $10^9$ particles/mL (Fig. 4B). Using these results, we quantified the absolute concentrations of the secreted sfGFP EVs from each K562-CD9 cell cultured for 24 hours where we omitted the droplets that contain 2 or more cells. Resultantly, the values varied between different single cells from approximately $5 \times 10^9$ to $7 \times 10^{10}$ particles/mL, equivalent to approximately $1.3 \times 10^9$ to $1.9 \times 10^9$ particles/cell (Fig. 4C).

![Figure 4](https://example.com/figure4.png)

Figure 4. Measurement of absolute EV numbers secreted from single cells. (A) Representative fluorescence images of droplets containing purified EVs at 3 different concentrations, arrayed in a micropillar array device. (B) A calibration curve calculated by the least-squares method (20 data points from each sample). $R^2$: coefficient of determination. (C) Quantified secreted EV abundance from K562-CD9 cells accumulated for 24 hours in droplets. Mean and individual data are shown ($N = 100$).
Discussions on future improvement of device performance. Our platform overcomes the challenge of culturing and phenotyping single mammalian cells in droplets for an extended period (> 1 day). We note that there have been efforts made to develop methods of single-clone monitoring in droplets for microorganisms, such as bacteria, yeast, and microalgae,18–31 as they proliferate fast. However, the dynamic phenotyping of mammalian cells in droplets has been performed only for a limited duration (up to 12 hours).22–34 In this work, we carefully optimized the conditions for culturing mammalian cells in droplets and accomplished the monitoring of their EV secretion dynamics for 36 hours. It is straightforward to further scale up our platform for tracking more than 1,000 cell-containing droplets (332 droplets in this paper) by enlarging the area of micropillar arrays in the device. Since the automatic imaging process completes within only 5 minutes for 332 droplets at each measurement, tracking more than 1,000 droplets is practical. On the other hand, it is also straightforward to prolong the total duration of observation beyond 36 hours by enlarging the size of droplets. This is because a limited amount of nutrients enclosed in the droplets primarily restricts the time of cell culture. However, the change in fluorescent intensity of droplets slows down as their sizes increase; hence, the trade-offs between the sensitivity in EV detection and the total duration of the measurement period has to be considered.

CONCLUSIONS

We developed a droplet array-based imaging platform for sensitively quantifying the dynamics of EV secretion from single cells for an extended period. The advantages of this method are as follows: (1) simple measurement solely by imaging, which allows us to perform frequent quantification with minimal operating cost and time, (2) minimal modification of culture conditions (e.g., no removal of EVs from FBS, no addition of antibodies, etc.), (3) a long measurement period (> 1 day), and (4) high specificity in detection of fluorescently labeled EVs (minimized contamination of other particles). Furthermore, we can extend our new approach to meet many needs, such as parallel tracking of the secretion of multiple proteins even at larger scales of thousands of cells. Using droplet sorting methods, those compartmentalized cells can also be selectively sorted to enrich a subset of cells based on the accumulated EV abundance. Such integration may extend this method to multimodal analysis, including optical and genomic multiplexed profiling.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website.
Supplementary Figures: qRT–PCR of the established cell line; cell viability in droplets; EV size profiles (PDF).

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENT

We thank all the members of the networked biophotonics and microfluidics group. This work was supported by JST, CREST grant number JPMJCR19H1, Japan, and by the Takeda Science Foundation, Uehara Memorial Foundation, and Japan Society for the Study of Obesity. Figure 1 was created with BioRender.com.

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