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Multiplexed Profiling of Single Extracellular Vesicles

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Abstract

Extracellular vesicles (EV) are a family of cell-originating, membrane-enveloped nanoparticles with diverse biological function, diagnostic potential, and therapeutic applications. While EV can be abundant in circulation, their small size (~4 order of magnitude smaller than cells) has necessitated bulk analyses, making many more nuanced biological explorations, cell of origin questions, or heterogeneity investigations impossible. Here we describe a single EV analysis (SEA) technique which is simple, sensitive, multiplexable, and practical. We profiled glioblastoma EV and discovered surprising variations in putative pan-EV as well as tumor cell markers on EV. These analyses shed light on the heterogeneous biomarker profiles of EV. The SEA technology has the potential to address fundamental questions in vesicle biology and clinical applications.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

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Additional figures and a supporting note as described in the text (PDF)

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Keywords

extracellular vesicle; exosomes; cancer; diagnostic; imaging

The shedding of small vesicles into circulation occurs in the majority of cancers.^{1,2} Extracellular vesicles (EV) are typically <1000 nm in size, occur at concentrations of up to 10^{11} vesicles/mL of peripheral blood in cancer patients, are fairly stable over time,³ and have been shown to contain proteins and nucleic acids reflective of those found in parent tumors.^{2,4} The vesicles differ in size, molecular composition, biogenesis, and function^{5,6} and include exosomes and microvesicles among other membrane vesicles.⁷⁻⁹ In certain cancers such as glioblastoma (GB), analysis of tumor-derived EV (tEV) afford an opportunity to longitudinally study tumor evolution and response to therapies in real time.^{10,11} EV have also been shown to have therapeutic potential.^{8,12-14}

Currently available and clinically viable diagnostics are all based on “bulk measurements” requiring 10^5 – 10^6 EV per biomarker to measure protein (*e.g.*, Western, ELISA) or 10^2 – 10^3 EV for the more sensitive methods (μ NMR, nPLEX).^{10,15} Since EV are also shed by normal host cells (hEV), current assays are thus invariably “contaminated” and unsuited for precise analyses of single vesicles.^{16,17} Yet each single EV analysis could be extremely valuable in studying EV biogenesis, tumor heterogeneity, rare tumor subtypes, phenotypic changes occurring during therapy, and hEV variations that occur concomitantly with tumoral changes. Because of the unmet need for single vesicle analysis, there has been recent interest in developing single vesicle analytical methods. Recent approaches have included optical trapping, Raman spectroscopy, and flow cytometry.^{18,19} However, multiplexed protein analysis in individual vesicles has been much more difficult.

In this paper we describe a single EV analysis (SEA) technique that is capable of robust, multiplexed protein biomarker measurement in individual vesicles. We first immobilize EV inside a microfluidic chamber and then perform on-chip immuno-staining and imaging. Given the nanometer-size scale of EV, the imaging cycling process has been adapted from multiplexed cyclic cell and tissue analysis.²⁰⁻²² We optimized a microfluidic system to stably capture EV and perform all staining steps on-chip, in-flow condition. We repeat imaging cycles for different target markers (three at a time) for multiple rounds. Multidimensional data analysis using methods such as tSNE (t-distributed stochastic neighbor embedding)^{23,24} can then be used to characterize groups of EV and ontogeny. In addition to serving as a biomarker discovery platform, SEA analysis of human EV shows

surprising findings on expression levels of cancer markers within and among individual vesicles.

RESULTS

SEA Technology

Figure 1a summarizes the SEA strategy. We biotinylated EV and captured them on the neutravidincoated glass surface of a microfluidic device (Figure 1b). This immobilization was necessary to keep EV spatially fixed during image acquisition. Using microfluidic chips allowed us to control experimental conditions (*e.g.*, flow rate, incubation time), which facilitated washing and staining steps, and minimized potential sample loss. The stationary EV were then stained using rounds of different fluorescent antibodies recognizing either ubiquitous EV markers (*i.e.*, tetraspanins; CD9, CD63, CD81) or putative GB tumor markers (*i.e.*, EGFR, EGFRvIII, IDH1, IDH1-R132, PDPN, PDGFRa, PDL1, PDL2) using a permeabilization buffer. Staining was performed using complementary fluorochromes (typically up to three colors). To analyze more than three markers simultaneously, we optimized a quenching protocol. After prior image acquisition, we introduced a H₂O₂ buffer (3% v/v) to the fluidic chip to quench fluorochromes for the next staining round (see Materials and Methods for details). For different fluorochromes, the fluorescence signal disappeared within 15 min of the quenching step (Figure 1c). The optimized sequences for a single imaging cycle were target labeling (30 min), imaging (1 min), and quenching (15 min). To analyze large numbers of these vesicles, we automated image processing and analysis (see Materials and Methods for details).

Assay Characterization

We first optimized the assay for single vesicle detection. We used a well characterized human GB model cell line (Gli36 wildtype; Gli36-WT). EV were harvested from conditioned cell culture media through differential centrifugation according to established protocols.^{11,25} This yielded pure vesicles in the size range of 50–200 nm as determined by nanoparticle tracking analysis and electron microscopy. We further filtered the samples through a 0.22 μ m pore membrane filter and immunostained using the SEA device. The average signal-to-noise ratio (SNR) of stained EV was ~3. Without the membrane filtration, we observed occasional bigger and brighter spots (SNR > 10) which could be from EV or protein aggregates. We also performed SEA using EV-depleted supernatants from ultracentrifugation; bright spots were barely observable, and, if any, its SNR was about 1.1 with a single camera pixel. We therefore used filtered samples and set the intensity cutoffs with SNR between 2 and 8 for EV analyses and the minimal pixel size of 3 (Supplementary Table 1).

We performed a series of control experiments to estimate signal variations. We first determined the overall imaging stability. Biotinylated fluorescent beads (diameter, 250 nm) were captured on the device surface and monitored over time (Supplementary Figure 1a); the observed temporal variation of the signal was <4%. We next assessed the effect of the cyclic imaging on the signal quality. We prepared aliquots of EV samples (technical replica) and subjected them to repeated washing/quenching steps before staining for CD63. The observed

mean fluorescent intensities were statistically identical ($p = 0.242$, one way ANOVA) among these samples (Supplementary Figure 1b). The result confirmed that EV maintained their structural and molecular integrity, which can be attributed to (i) the use of mild bleaching condition, (ii) chemical fixation of EV before staining, and (iii) the gentle flow rate during the process.

We next tested how the staining order affects molecular profiles. We used aliquots of EV samples and immunostained EV for CD63 in different staining orders: first, second (after EGFR labeling), third (after EGFR, CD81 labeling), and fourth (after EGFR, CD81, IDH1 labeling). The results showed a nearly identical CD63 profile (Supplementary Figure 1c), and no major effects of obscuring epitopes during rounds of staining were observed. We also determined the effects of biotinylation. Biotinylated EV were captured by the neutravidin-coated device, and native EV were captured based on CD63. EGFR expression level was statistically identical in both samples (Supplementary Figure 2). We also measured other sources for signal variation, including (i) physical noise, (ii) different imaging area, (iii) repeats of the same experiments, and (iv) repeated experiments with different batches of same cell line (Supplementary Table 2).

SEA of Cancer Cell-Derived EV

We further extended the SEA technique. We used three Gli36 cell lines previously described:^{10,26} Gli36-WT, Gli36-EGFRvIII with EGFRvIII overexpression, and Gli36-IDH1R132H with overexpression of human IDH1 R132H mutant protein. EV were harvested from conditioned cell culture media and membrane filtered. Then, collected EV were biotinylated for EV immobilization on the microfluidic chip surface. Figure 2a shows representative SEA images with Gli36-WT derived EV. We identified individual vesicles by staining EV with fluorescent streptavidin (Figure 2a, left). We varied EV concentrations and counted the number of captured EV (Supplementary Figure 3). The average capture rate per vesicle was 4.5%, which limits the chance for co-localization of multiple EV (*e.g.*, 0.02% for two EV). Typically 700–3000 EV were observed (20 $\times$) in the field of view of $4.3 \times 10^4 \mu\text{m}^2$. We also determined that the sample specimen did not affect measurement. Indeed, we were able to observe spiked EVs from two cell lines (Gli36-EGFRvIII and Gli36-IDH1R132H) in human serum samples (Supplementary Figure 4). We can detect 6.1% of EGFRvIII positive vesicles and 4.8% of IDH1R132H positive vesicles. Line scan (Figure 2b) shows high signal-to-noise and heterogeneity for the chosen markers on individual vesicles.

Comparison of EV from Isogenic Cell Lines

We next analyzed the raw intensity data for the expression of ubiquitous EV and tumor markers (Figure 3). For a given protein marker, we could identify two subpopulations, marker-negative and marker-positive, which can be separated by the intensity cutoff of 10^2 . It has been reported that EV prepared according to the protocol used here contain tetraspanins (CD9, CD63 and CD81).^{17,27,28} However, these measurements were made with bulk preparations, and data do not exist for single vesicles. As is summarized in Figure 3, distinct EV populations existed for each tetraspanin marker. Roughly half the vesicles had CD63 (54%) and a fraction expressed CD9 (4.8%) and CD81 (26%). With respect to tumor markers, we observed the following fractions of positive markers (Table 1): EGFR (71%),

EGFRvIII (6.5%), IDH1 (28%), PDGFRa (14%). For the remaining markers, the positive fraction (<4%) was below the threshold for statistical significance. For marker-positive EV subpopulations, the distribution profile had a similar full width at half-maximum (fwhm). In other words, only 20% of the EGFR positive EV fraction (71%) have either very high or very low levels of expression with the remainder falling in between. We further assessed the biomarker distributions in clones of Gli36 EV that were positive for EGFRvIII or IDH1-R132 mutation (Table 1 and Supplementary Figure 5). A somewhat surprising finding was that Gli36-EGFRvIII EV were far more positive for CD9 than EV from other cell lines. There were also differences in expression of tumor markers across the clones. As expected, a substantial fraction of Gli36-EGFRvIII EV were positive for EGFRvIII (67%), but only a much smaller fraction of Gli36-IDH1R132H EV were positive for mutant IDH1-R132 (9%). We also compared the profiling results between SEA and bulk ELISA measurements (Supplementary Figure 6). Mean fluorescent intensities, calculated from EV marker distributions, showed a good correlation ($R^2 = 0.89$) with bulk signals.

EV Heterogeneity

We next determined how many of the markers co-existed in vesicles (Figure 4a). We grouped EV according to different marker combinations that are mutually exclusive. Regardless of cell lines, >50% EV only express one or two of the 11 markers evaluated. Only 3.3% of WT vesicles exhibited five or more markers, while the portion was slightly higher for EV from Gli36-EGFRvIII and Gli36-IDH1R132 cells. Overall, for a given cell type, four EV marker subtypes accounted for >90% of the total population (Figure 4b). We further analyzed EV compositions according to marker combinations. We grouped EV by drawing a binary positive/negative gate on the individual marker expression histograms and consequently finding the combinations of positively expressed markers in the EV. Figure 4c summarizes the results. EV from Gli36-WT and Gli36-IDH1R132 showed similar compositions, but with nuanced differences: Gli36-WT EV had more groups associated with IDH1 expression and Gli36-IDH1R132 EV with IDH1R132. In contrast, Gli36-EGFRvIII EV had subgroups strongly clustered around EGFRvIII expression.

Clustering Analyses of Single EV

To visualize and compare populations of EV in an unbiased fashion, we mapped our raw intensity data set onto a two-dimensional plane using tSNE and computationally identified clusters of EV (Figure 5). This approach offers three advantages to the binary gating method discussed above. First, EV populations are identified in a data-driven manner using the measured signals, allowing marker expression levels (continuous values) to define populations, not merely their presence or absence. Second, this method identified 14 main populations, whereas the previous method produced 150 unique populations across the three cell lines. Third, tSNE allows for the visualization of high-dimensional data sets and the organization and scattering of data within its axes. We first created several tSNE mappings at various perplexity values ranging from 25 to 500 (Supplementary Figure 7). Next, the optimal number of clusters in each tSNE plot was determined by minimizing the proportion of ambiguous clustering (PAC) metric; the smallest cluster number that minimizes PAC was chosen to reduce redundancy. The Davies–Bouldin index (DBI) was used to evaluate the

clustering solution in each tSNE plot. A tSNE mapping at a perplexity of 100 and with 14 clusters had the lowest DBI and was selected for further analysis (Supplementary Figure 8).

Figure 5a shows 14 clusters from this unsupervised classification; each point represents a single EV. Clusters were ranked by their significance, which was defined as a ratio between the cluster size (*i.e.*, the number of points inside the cluster; Supplementary Table 5) and its DBI. The biomarker phenotype for each cluster (Figure 5b) qualitatively matched with major EV subgroups identified by the gating method (Figure 4c). The most clustered population (cluster 1) was positive for CD9 and EGFRvIII, which corresponded to the major EV subgroup in Gli36-EGFRvIII. Likewise, the second most significant population was CD63 and EGFR positive, which was mainly from Gli36-WT EV. Some markers are expressed in many populations (*e.g.*, CD63 is in eight populations), whereas others are present in much fewer (*e.g.*, PDGFR α , bright only in one population). Marker correlations were also apparent from this analysis; for example, CD63 and EGFR are expressed together in clusters 2, 11, and 14. tSNE maps also made it easier to identify and compare EV subgroups. In the tSNE plots of the individual cell lines (Figure 5c), in which data from other cell lines are shaded light gray, we could readily observe that Gli36-WT and Gli36-IDH1R132 EV are more similar to each other, as they share several large subpopulations (clusters 2, 4, 5, 6). Gli36-IDH1R132 has distinguishable subpopulations (*e.g.*, 8, 11, 14), and these groups have correlation with IDH1R132 expression. Gli36-EGFRvIII possesses several smaller, highly clustered, characteristic populations (clusters 1, 3, 7, 12) that are nearly absent in the other two cell lines.

DISCUSSION

GB is the most common and lethal primary malignant cancer of the central nervous system. Adult high-grade glioma tumor heterogeneity has four major subtypes based on core gene signatures: proneural (PN), mesenchymal (MES), classical (or proliferative), and neural.^{29–33} Although all subtypes have indistinguishably poor therapeutic response, individual subtypes appear to depend on distinct signaling and onco-metabolic pathways, and future therapeutic strategies will likely be based on tumor subtype. From a molecular standpoint, multiple signaling pathways are differentially activated or silenced under intricately converging and/or parallel interactions.³¹ Epidermal growth factor receptor (EGFR) amplification is the most common genetic abnormality, and EGFR overexpression occurs in up to 85% of cases.³⁰ Glioblastomas also often express EGFRvIII, a genomic/splicing deletion variant of EGFR that is constitutively active and highly oncogenic.^{34–36} In addition, malignant gliomas regularly overexpress both platelet-derived growth factor (PDGF-A) and PDGF receptor α (PDGFR α), both of which contribute to tumor progression *via* an autocrine or paracrine loop.³⁷

A major clinical challenge is the ability to measure drug responses at the molecular/cellular level. While imaging and the new RANO criteria^{38–40} are clinically useful, there continues to be a need for more sensitive and frequent response monitoring.⁴¹ While new imaging approaches can be quite sensitive, they are costly and often not amenable to the serial frequency required for fully informative measurements.^{42–44} As a result, an intense interest in tumor released materials (“liquid biopsy”) has emerged. The release of EV in particular

has been shown to be robust and has also furthered our understanding of cellular communication.⁴⁵ The relative abundance and composition of EV proteins serve as a fingerprint that indicates their cellular origin.^{4,46} Most EV contain cytoplasmic proteins (actin, tubulin, annexins) in addition to signal transduction proteins, heat shock proteins, tetraspanins, and major histocompatibility complex (MHC) class I molecules. Furthermore, recent findings indicate that EV also contain mRNA, non-coding RNAs (including miRNA), and DNA, which can be transferred to another cell and be functional in that new environment. This heterogeneous EV mixture presents a new challenge in evaluating both the range of information contained in a broad tumor genotype/phenotype profile and the body's physiologic response to the tumor.

In the laboratory setting, vesicles are often analyzed by electron microscopy, nanoparticle tracking analysis (NTA), PCR, flow cytometry, Western blotting, and ELISA. To be clinically useful, a number of newer technologies have been developed to isolate and profile EV in small clinical samples and within feasible time frames. Some of these isolation systems include microfluidic chips⁴⁷ and acoustic devices,⁴⁸ whereas the EV profiling systems include microNMR,¹⁰ electrochemical sensors,⁴⁹ integrated microfluidics,¹¹ and nPLEX.¹⁵ Irrespective of the technology, these molecular measurements are all based on EV populations. Even the most sensitive nPLEX technology still requires at least 3000 EV (but more commonly in the order of 10^4 EV) per measurement. These methods all report bulk properties from an ensemble of EVs, unable to discern any compositional heterogeneity. The SEA technology allows for obtaining a much richer information set, including the heterogeneity of biomarker expression, marker make-ups, and the presence of EV subpopulations. Importantly, single EV analyses will enable molecular identification of tumor-derived EV even in a vast biological background of host cell-derived EVs. It is also conceivable to detect the presence of certain cell types using EV as a surrogate. Such capabilities could be exploited to generate in-depth, multidimensional data sets, such as tSNE, or be used to render more straightforward readouts for clinical decision (*e.g.*, the fraction of tumor-derived EV, the most common tumor markers).

In the current SEA method, EV are first captured inside a microfluidic channel and target specifically stained with fluorescent antibodies. We then measure fluorescence intensity of individual EV only using low-magnification (20×) imaging. The method produces two data sets: (i) total vesicle counts and (ii) protein makeup based on fluorescent antibody staining. Because the vesicles are immobilized on the chip surface, achievable SNRs from each vesicle are generally much higher than when vesicles are free-floating in solution or under flow conditions.⁵⁰ Furthermore, the immobilization allows for restaining of captured vesicles to enable a high degree of multiplexed screening (>10 markers).

In the future, we plan to employ higher magnification imaging to measure both EV size and fluorescence signal. The SEA strategy can also be combined with positive or negative up-front enrichment methods. In this work, we focused on establishing an unequivocal baseline for detection by using cancer cell line-derived EV. Real blood samples, however, contain abundant host cell-derived EV that could confound tumor-EV analyses. One efficient way to improve the analytical accuracy is to perform immuno-selection: depleting EV that are

positive for host cell marker (*e.g.*, CD45, CD31, CD41, CD235a) or enriching EV that are positive for tumor markers such as IDH1.

Here we measured 11 different protein markers in a single vesicle, and this number could be further increased by additional rounds of staining. Increasing the number of independent measurements performed on each EV has the potential to reveal interdependencies among differentiation status, local environment, signal-transduction states, and phenotype that are not evident when the same measurements are made at the population level. Image acquisition is relatively fast (<1 s), and $>10^3$ EV are simultaneously analyzed in a single image acquisition.

We believe that the SEA platform will be a useful analytical tool for studying different types of extracellular vesicles (*e.g.*, exosomes, microvesicles, oncosomes, apoptotic bodies, and other membrane-bound vesicles)^{51,52} across different cell types (*e.g.*, normal, non-invasive cancer, and metastatic cancer cells) at the single-particle level. This bottom-up approach will likely uncover biological processes that are currently masked in bulk measurements. For example, the technology could be useful to enumerate varying EV types based on biogenesis, whether tumor-derived EV differ from host cell-derived EV, how EV change normal and tumor cell phenotypes to support tumor growth, how EV carry RNA based on SEA and RNA analysis, or how EV payloads are processed by the cellular vesicular machinery. Tumor cells may use a different type of vesicle biogenesis, and the protein content may give us clues about that process. Biogenesis could be very important, as several studies have hypothesized that thwarting tumor vesicle biogenesis could improve prognosis. Analyzing the protein content may reveal/indicate how EV promote tumor growth (*e.g.*, VEGF angiogenic proteins, immune suppressive proteins, proteases (*e.g.*, MT1-MMP) that digest the extracellular matrix, signaling ligands). Finally, there are different types of vesicles (exosomes, microvesicles, others), and it is currently unclear which proteins best distinguish them from one another at a single vesicle level. This could provide additional insight into different modes of biogenesis.

MATERIALS AND METHODS

Cell culture

Gli36-WT was acquired from ATCC. Gli36-EGFRvIII and Gli36-R132H were generated from Gli36-WT through lentivirus transduction (Leonora Balaj in the Breakefield lab). Each line was regularly tested for mycoplasma contamination. Gli36-WT, Gli36-EGFRvIII, and Gli36-R132H cells were grown in DMEM with 10% fetal bovine serum (FBS; Sigma) at 37 °C in a humidified atmosphere with 5% CO₂. Before EV collection, cells were grown in DMEM with 10% exosome-depleted FBS (Thermo Fisher Scientific) for 72 h.

Preparation of EV

Supernatant from cell culture media was centrifuged at $2000 \times g$ for 10 min to remove cell debris. Supernatant was then filtered through a 0.22 μ m pore size membrane to clear membranous debris. Supernatant was centrifuged again at $100,000 \times g$ for 70 min to isolate the putative exosome fraction. The pellet was washed in $1 \times$ PBS and then centrifuged at

100,000 $\times g$ for 70 min to pellet. Isolated exosomes were resuspended in 300 μ L of 1 $\times$ PBS and incubated with 333 μ M EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) for 30 min at room temperature. We used a 20-fold molar excess of sulfo-NHS-biotin to EV protein in approximately 0.5 mL volume. Approximately 4–6 biotins were expected to be incorporated per molecule. Excess biotin was then removed utilizing the Zebra Spin Desalting Column, 7K MWCO (Thermo Fisher Scientific) per the kit instructions. The prepared EV were filtered using 0.22 μ m centrifugal filter (Ultrafree, Millipore).

Antibody Preparation

EGFR-AF555, EGFRvIII, PD-L1-AF647, and PDGFR α -AF555 antibodies were purchased from Cell Signaling Technology, IDH1 and PD-L2-AF647 antibodies were from BioLegend, and IDH1-R132H antibody was from EMD Millipore. CD63 antibody was acquired from Ansell Corporation. CD9 antibody was acquired from Abcam. CD81 antibody was acquired from Santa Cruz Biotechnology. Vendor and clone information is summarized in Supplementary Table 3. IDH1, CD63, and CD81 antibodies were conjugated to Alexa Fluor 555 and EGFRvIII, IDH1-R132H, PDPN, and CD9 antibodies were conjugated to Alexa Fluor 647 utilizing the Alexa Fluor 555/647 Labeling Kits per kit instructions (Thermo Fisher Scientific). We used Alexa Fluor 488 for the streptavidin imaging channel and Cy5 channel for the quenching test.

Microfluidic Device for EV Capture

The device was fabricated using standard soft lithography technique. Cast molds were prepared by patterning epoxy-based SU8-2075 photoresist (Microchem) on silicon wafers *via* conventional photolithography. The fluidic device was then replicated by pouring polydimethylsiloxane (PDMS, Dow Corning) to the mold and curing the polymer on a hot plate (60 $^{\circ}$ C, 1 h). The cured PDMS structure and a glass substrate were oxygenplasma treated and irreversibly bonded. The fluidic channel height was about 100 μ m, and the dimension of the chamber was 5 $\times$ 10 mm². The fluidic device was flushed with 4% (v/v) solution of 3-mercaptopropyl trimethoxysilane (Sigma-Aldrich) in ethanol for 30 min (5 μ L/min), followed by 0.01 M GMBS (Sigma-Aldrich) in ethanol for 15 min (5 μ L/min). After each step, the device was rinsed with ethanol (5 min, 50 μ L/min). The fluidic chamber was then filled with 200 μ g/mL NeutrAvidin (Sigma-Aldrich) solution in 0.2% BSA (Sigma-Aldrich) for 1 h at 20 $^{\circ}$ C and rinsed with 0.2% BSA buffer (5 min, 50 μ L/min). We measured the capture efficiency by performing particle concentration analyses (Supplementary Figure 3). The SEA analysis allows measurement on EV particle concentrations in the range of 10⁷–10¹¹ EV/mL. Potential EVs clusters were distinguished by their spot size and intensity. To remove EV clusters, we filtered samples through 0.22 μ m pore membranes.

SEA Protocol

Experiments were performed on an inverted microscope (Nikon, Eclipse TE2000S) equipped with an sCMOS camera (Andor, Zyla). The following buffers were prepared: a washing buffer (0.2% BSA in PBS); an imaging buffer⁵³ containing 10 mM MES pH 6.5, 60 mM KCl, 0.32 mM EDTA, 3 mM MgCl₂, 10% glycerol, and 0.1 mg/mL acetylated BSA (Promega, R3961); and a quenching buffer²² prepared by mixing 2 volumes of 0.5 M

sodium bicarbonate, 7 volumes of DI water, and 1 volume of 30% (v/v) hydrogen peroxide. Biotinylated EV were captured on the neutravidin-coated surface of the microfluidic device. Then the fluidic chamber was filled with a fixation/permeabilization buffer (eBioscience) and incubated for 10 min at 20 °C. The chamber was then filled with an imaging buffer for 30 min. Next, fluorescently labeled antibodies were flown through the fluidic chamber (30 min, 2 μ L/min). Following the wash with the imaging buffer, fluorescent images were taken. After the imaging, the chamber was filled with the quenching buffer (15 min) and washed with the imaging buffer. At this point, EV were imaged again, and residual spots were excluded from further analyses. We then repeated the labeling, imaging, and quenching steps. The overall assay time was 70 min for EV capture and 60 min/cycle (see Supplementary Table 4 for details).

Image Processing

Image analyses were performed using ImageJ. We used the streptavidin imaging channel to create masks at EV locations. For each molecular target, the corresponding fluorescent micrograph was aligned using ImageJ plugins (Align slices in the stack). At each mask position, we obtained average pixel intensities. The signal was corrected by subtracting background signal surrounding the mask.

Dimensionality Reduction

We measured 3099 EV from Gli36-WT, 1324 EV from Gli36-EGFRvIII, and 840 EV from Gli36-IDH1R132. Single EV data were subsampled to 600 vesicles from each cell line and merged. The resulting merged, 11-dimensional, single vesicle data set was then mapped onto a two-dimensional space using t-distributed stochastic neighbor embedding (tSNE),²³ a nonlinear dimensionality method that emphasizes the preservation of local structure within a data set. To obtain the best embedding, 20 values (ranging from 25 to 500) of the “perplexity” parameter, a measure of the number of effective nearest neighbors, were tested. Due to the stochasticity of tSNE, visually disparate mappings may be produced with successive runs of the algorithm on equivalent data sets. As such, for each tested perplexity value, 10 tSNE mappings were created, and the solution with the minimum value of the Kullback–Leibler divergence reported by the algorithm was chosen. An optimal perplexity value and associated tSNE mapping were then chosen based on the degree of clustering in that visualization.

Cluster Analysis

To identify populations of EV in the tSNE visualizations, the link-based cluster ensemble approach was employed.⁵⁴ For each tSNE plot, connected-triple-based similarity matrices for 10–30 clusters were each computed using 50 K-means clustering runs. The optimal number of clusters for each tSNE plot was then assessed using the proportion of ambiguous clustering metric.⁵⁵ The similarity matrix for the optimal number of clusters in each tSNE mapping was used to produce a final clustering result for its respective mapping using a connected linkage hierarchical agglomerative clustering method. The clustering solutions for the tSNE plots at various perplexity values were then evaluated and compared to each other using the DBI, a ratio of within cluster scatter to between cluster scatter.⁵⁶ The perplexity

value and associated clustering solution with the lowest average DBI was chosen for further data analysis. All computational analyses were done using MATLAB R2016b.

Plate reader bulk measurement

Biotinylated EV were immobilized onto streptavidin-coated 96 well plates (Thermo Fisher Scientific) and blocked with $1 \times$ PBS containing 1% FBS and 1% BSA. Subsequently, antibodies (1 μ g/mL) were added, and samples were incubated overnight at 4 °C. Each well was washed 5 $\times$ with 100 μ L TBST buffer. Fluorescent signal was measured by a plate reader (Safire, Tecan).

Scanning Electron Microscopy

Immobilized EV were fixed inside a microfluidic chamber with Karnovsky's fixative. Fixed EV were washed with $1 \times$ PBS and dehydrated by injecting a series of increasing concentration of ethanol. Samples were then processed by a critical point dryer (Autosamdri 931, Tousimis) and coated with platinum and palladium (20/80) using a sputter coater (EMS300T-D, EMS). The samples were imaged with a scanning electron microscope (Ultra Plus FESEM, Carl Zeiss).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Single EV analysis (SEA). (a) Overview of the procedural steps. EV are biotinylated and captured on the device surface coated with neutravidin (Av). The stationary EV are then stained by fluorescent antibodies (up to three colors per step) and imaged by microscopy. Subsequently, fluorochromes are quenched, and the staining process is repeated for a different set of markers. The multidimensional data are then analyzed. (b) Photo of microfluidic chip for EV capture and imaging. Scale bar, 1 cm. (c) For the image cycling, we quenched fluorochromes (Alexa 488, Alexa 555, and Alexa 647) by injecting an oxidation buffer. The fluorescent signal disappeared within 15 min after the buffer injection.

Figure 2. Measurement of 11 markers using the SEA method. (a) EV from Gli36-WT cell line were biotinylated and captured on the device. Individual EV were detected through staining with fluorescent StAv (top left). For molecular profiling, EV were labeled with fluorescent antibodies against conventional EV markers (tetraspanins; CD9, CD63, CD81) as well as tumor markers (EGFR, EGFRvIII, IDH1, IDH1R132, PDPN, PDGFR α , PD-L1, PD-L2). Spots with circles indicate individual EV. To help visualize, EV were artificially color coded. (b) Line scans showing high signal-to-noise for the chosen markers in this example. Gray shading highlights EV positions. Scale bar, 5 μm . (Inset) Electron microscopy of EV immuno-gold stained for CD63. Scale bar, 100 nm.

Figure 3.

Marker expression profile. From SEA images of Gli36-WT EV, the population density functions were constructed. A total of 800 vesicles were analyzed. Note that not all tetraspanins are present in these vesicles. The mean expression levels of markers were corroborated by bulk measurements (ELISA). The density functions were normalized with the area under the curve equal to 100%.

Figure 4. Heterogenous marker expression per vesicle. (a) Number of markers detected on individual vesicles. Most EV expressed either single or two markers. (b) Cumulative distribution showed that >90% EV expressed four or less markers. (c) EV from Gli36-WT and Gli36-IDH1R132 showed similar compositions, whereas Gli36-EGFRvIII EV populations were distinct with CD63 being the most shared. Each marker group (*e.g.*, CD63•EGFR, EGFR) represents distinct EV subpopulation with no overlap.

Figure 5.

Dimensionality reduction and clustering analysis. (a) Two-dimensional tSNE mapping of the 11-dimensional data set with an optimized clustering solution of 14 unique clusters. (b) Heatmap of tSNE derived population fractions in each cell line and marker expression profiles. (c) Subset of tSNE mapping showing EV from a single cell line. Data from other cell lines are shaded light gray. Note the similarity between Gli36-WT and Gli36-IDH1R132 EV and the distinct clustering of Gli36-EGFRvIII EV.

Table 1Comparison of EV among Three Cell Clones^a

markers	parent cell line		
	WT	EGFRvIII	IDH1R132
CD9	0.048	0.72	0.083
CD63	0.54	0.39	0.45
CD81	0.26	0.083	0.5
EGFR	0.71	0.05	0.55
EGFRvIII	0.065	0.67	0.19
IDH1	0.28	–	0.2
IDH1R132	–	–	0.09
PDPN	–	–	0.06
PDGFR α	0.14	–	0.2
PD-L1	–	–	–
PD-L2	–	–	–

^aEV from Gli36-WT, Gli36-EGFRvIII, and Gli36-IDH1R132H cell lines were profiled. The fraction of EV that are positive for a given marker is listed (see Supplementary Figure 5 for density plots). Note the substantial differences in marker positivity.