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Ultrasensitive Quantification of Tumor mRNAs in Extracellular Vesicles with an Integrated Microfluidic Digital Analysis Chip

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Abstract

Extracellular vesicles (EVs) present a promising liquid biopsy for cancer diagnosis. However, it remains a daunting challenge to quantitatively measure molecular contents of EVs including tumor-associated mRNAs. Herein, we report a configurable microwell-patterned microfluidic digital analysis platform combined with a dual-probe hybridization assay for PCR-free, single-molecule detection of specific mRNAs in EVs. The microwell array in our device is configurable between the flow-through assay mode for enhanced hybridization capture and tagging of mRNAs and the digital detection mode based on femtoliter-scale enzymatic signal amplification for single-molecule counting of surface-bound targets. Furthermore, a dual-probe hybridization assay has been developed to enhance the sensitivity of the digital single-molecule detection of EV mRNAs. Combining the merits of the chip design and the dual-probe digital mRNA hybridization assay, the integrated microfluidic system has been demonstrated to afford quantitative detection of synthetic *GAPDH* mRNA with a LOD as low as 20 aM. Using this technology, we quantified the level of *GAPDH* and *EWS-FLII* mRNAs in EVs derived from two cell lines of peripheral primitive neuroectodermal tumor (PNET), CHLA-9 and CHLA-258. Our measurements detected 64.6 and 43.5 copies of *GAPDH* mRNA and 6.5 and 0.277 copies of *EWS-FLII* fusion transcripts per 10⁵ EVs derived from CHLA-9 and CHLA-258 cells, respectively. To our knowledge, this is the first demonstration of quantitative measurement of *EWS-FLII* mRNA copy numbers in Ewing Sarcoma (EWS)-derived EVs. These results highlight the ultralow frequency of tumor-specific mRNA markers in EVs and the necessity of developing highly sensitive methods for analysis of EV mRNAs. The microfluidic digital mRNA analysis platform presented here would provide a

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Supporting Information

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Notes

The authors declare no competing financial interest.

useful tool to facilitate quantitative analysis of tumor-associated EV mRNAs for liquid biopsy-based cancer diagnosis and monitoring.

Graphical Abstract

A microwell-patterned microfluidic digital mRNA analysis platform enables PCR-free, single-molecule detection of EWS-FLI1 fusion transcripts in EVs towards liquid biopsy-based non-invasive diagnosis of Ewing Sarcoma.

Introduction

Tissue biopsy of tumors is highly invasive, costly, and often impractical for repeated sampling during the course of therapy.¹ Since the past decade, liquid biopsy has emerged as a non-invasive alternative and promises to revolutionize disease diagnosis, surveillance, and treatment.^{2, 3} Extracellular vesicles (EVs) present a promising paradigm of liquid biopsy of tumors, as they are enriched of a selective subset of bioactive molecules from parental cells, including proteins, mtDNA, microRNAs, LncRNAs, and mRNAs representative of their cellular origin.^{4, 5} Circulating mRNAs are thought to be one of paradigm-changing markers for cancer diagnosis and therapeutic monitoring.⁶ Nevertheless, detection of mRNA in biological fluids is challenging, due to the rarity of specific mRNA targets and their susceptibility to enzymatic degradation.^{7, 8} EVs have been found to be a uniquely advantageous source of mRNAs in the circulation, as EV encapsulation protects mRNAs from degradation by exogenous RNases in biological matrices.^{9, 10} Recent studies have demonstrated the potential of the expression levels, expression patterns and mutations of EV mRNAs for diagnosis, subtype classification, assessment of treatment efficacy, and predicting drug resistance of cancer.^{11, 12}

Despite the promising clinical value, very limited progress has been made in quantitative measurements of mRNA contents of EVs, which can be in part attributed to the unique challenges in analysis of EV mRNAs. First, EVs were found to contain low levels of RNAs and mRNAs only account for a small fraction of total EV RNAs.^{13, 14} Even for miRNAs, a prominent type of EV RNAs, the most abundant species was quantified to be lower than 0.01 copies per vesicles on average.¹⁵ Moreover, in addition to full-length mRNAs, it is well documented that EVs carry fragmented mRNAs, which further increases the difficulty of quantitative detection.^{14, 16} Lastly, due to genetic heterogeneity of tumor, clinically significant mRNAs can be diverse and less frequent in EVs, such as EGFR mutation variants

in glioblastoma (GBM) among which oncogenic EGFRvIII could be of low prevalence.¹⁷ These properties of EV mRNAs make it highly challenging to accurately quantify the expression of specific gene transcripts in EVs.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR), the gold standard for mRNA analysis, have been widely adapted for exosome analysis. However, the assay workflow involves multi-step sample processing such as RNA extraction and reverse-transcription. Moreover, mRNA quantification by RT-qPCR is based on amplification of synthesized cDNAs, which leads to inaccurate quantification of mRNA copies and is prone to sequence-dependent bias.¹⁸ Numerous alternative methods, such as optical and electrochemical sensors, have been developed to confer simplified, less biased and/or highly sensitive detection of mRNAs.^{19–26} Distinct from these conventional analog assays, digital bioassays recently have attracted significant interests as they offered superior sensitivity via digitally counting single molecules of the analyte. In digital bioassays, target molecules are partitioned into a large number of discrete small compartments (fL to nL), most of which are loaded with none or one molecule. The reactions confined in such small volumes produce detectable concentration of the product, enabling digital counting of individual molecules to determine the concentration of the analyte. This concept of single-molecule detection has been successfully adapted to develop digital PCR (dPCR) for absolute quantification of genetic targets with greatly enhanced sensitivity and accuracy.^{27–30} Nonetheless, dPCR quantification of mRNA still share the intrinsic limitations associated with RT-PCR as discussed above. Digital ELISA for protein detection has also been accomplished using a Single Molecule Array (Simoa) technique,^{31–33} digital microfluidics,³⁴ and droplet arrays.^{35–38} Recently, the Simoa has been expanded to PCR-free digital detection of DNA and miRNAs based on the hybridization capture and detection assay.^{32, 39} In most of current digital methods, the analysis workflow is divided into two separated steps: 1) stochastic capture of single target molecules and tagging with detection probes using affinity microbeads, and 2) loading microbeads into an array of fL reactors for single-copy counting based on enzymatic signal amplification. The bead loading process compromises the multiplicity and throughput in comparison with the microarray format;⁴⁰ it also causes loss of beads and variable loading efficiency, which could lead to statistical variation at low concentration levels. Moreover, these methods mostly use oil phase for compartmentalization, which could pose challenges to large-scale integration and automation of multi-step assays as it is more difficult to precisely generate and process fL droplets than nL to pL droplets.^{28, 36, 41}

In contrast, we developed a mechanically configurable microwell-patterned microfluidic platform that combines the sample processing, i.e., target capture and tagging, and a PCR-free digital mRNA detection modality in one integrated workflow. A pneumatic control circuit was integrated to streamline the assay workflow and to precisely control the flow delivery and binding kinetics, which enhances the assay reproducibility.^{42, 43} The fL well-arrayed chip can be mechanically switched from the flow-through hybridization assay mode to the digital detection mode for absolute single-molecule counting of surface-bound targets. Compared to the bulk assays, the microfluidic flow-through hybridization assay enhances the binding kinetics and efficiency for specific mRNA capture and tagging.⁴⁴ Moreover, distinct from other digital assays using oil sealing, our chip uses a robust mechanical

mechanism to ensure reliable sealing of a large-area array of fL wells, enabling *in-situ* digital detection of surface-bound targets. This integrated workflow of target capture and detection addresses the aforementioned issues stemming from bead loading and oil sealing. Finally, to address the challenges in analysis of EV mRNAs, a dual-probe hybridization assay was evaluated to increase the chance to detect fragmented sequences, which further enhances the sensitivity of digital detection of EV mRNAs.

Using synthetic GAPDH mRNA, we demonstrated sensitive and quantitative detection of mRNA with a LOD of 20 aM. As a proof-of-concept for potential biomedical applications, the microfluidic digital assay chip was applied to directly detect *EWS-FLII* fusion transcripts in EVs derived from the peripheral primitive neuroectodermal tumor (PNET) cell lines. The Ewing Sarcoma Family of Tumors, including PNET, is the second most common pediatric osseous malignancy.^{45, 46} Ewing Sarcoma (EWS) is characterized by a genomic translocation resulting in the *EWS-Ets* oncogene.^{47, 48} Tissue biopsy is the gold standard to access this most important molecular determinants of the pathobiology of the EWS. Such invasive biopsy procedure raises unique challenges for childhood cancers compared to adult cancers, including major ethical issues like clinical equipoise.^{49, 50} Thus, developing liquid biopsy-based biomarkers and tests is particularly appealing in pediatric cancer. However, very few, if any, studies of *EWS-Ets* mRNAs in EWS-derived exosomes have been reported, due in part to the technical challenges in analysis of exosomes and mRNA.⁴⁸ Here we present for the first time quantitative measurement of the level of *EWS-FLII* mRNA in EWS-derived EVs. Our results showed an averaged level of 6.5 and 0.277 copies of the fusion transcripts per 10⁵ EVs for CHLA-9 and CHLA-258 cells, respectively. These findings highlight the ultralow frequency of tumor-specific mRNA markers in EVs and the necessity of developing highly sensitive methods for analysis of EV mRNAs. The microfluidic digital mRNA analysis platform presented here would provide a useful tool to facilitate quantitative analysis of tumor-associated EV mRNAs for liquid biopsy-based cancer diagnosis and monitoring.

Experimental Section

Reagents and Materials.

NH₂-PEG-biotin (MW 3400) was ordered from Laysan Bio Inc (Arab, AL, USA). (3-Aminopropyl) triethoxysilane (APTES), (3-Mercaptopropyl) trimethoxysilane (3-MPS), (3-Glycidyloxypropyl) trimethoxysilane (GOPS), glutaraldehyde (GL), formamide, and dextran sulfate were purchased from Sigma-Aldrich (St. Louis, MO). Human skeletal muscle total RNA, streptavidin conjugated β -Galactosidase (S β G), and Fluorescein di- β -D-galactopyranoside (FDG) were purchased from Thermo Scientific. All the DNA probes, PCR primers and RNA oligonucleotides were synthesized and purified by Integrated DNA Technologies, Inc. (IDT; Coralville, IA). 1 $\times$ phosphate-buffered saline (PBS) was obtained from Corning (Tewksbury, MA, USA). 20 $\times$ Saline-sodium citrate buffer (SSC (3 M NaCl, 0.3 M sodium citrate, pH~7.0)) was received from Sigma-Aldrich and then diluted into working concentration with deionized water. S β G and FDG were dissolved in PBS working solution (PBSW) at pH 7.4 which contain 0.5 mM DL-dithiothreitol solution, 2 mM MgCl₂, and 5% bovine serum albumin (BSA). All other reagents were used as received without

further purification. Deionized water (18.2 M Ω cm) used for all experiments was obtained from a Milli-Q system (Millipore, Bedford, MA).

Microfabrication.

Standard soft photolithography was used for the microfabrication of digital chip. The silicon mold for the pneumatic layer (50 μ m) was fabricated by SU-8 2025 photoresist (MicroChem). The detailed diameter of each procedure followed the manual of manufacture. For the fluidic layer, the mold was prepared by the two-step lithography. Briefly, the 20 μ m thick channel feature was first patterned with SU-8 2025 on silicon wafer. After pre-baking, UV exposure and post-baking, the microwells array with a thickness of 6 μ m was patterned on the top of first layer with SU-8 2010 photoresist. The prepared patterns were developed for 2 min and hard-baked at 200 $^{\circ}$ C for 2 h. All silicon molds were treated with trimethylchlorosilane by gas phase silanization under vacuum for 8 h, prior to making PDMS chip. To prepare the pneumatic layer, 30 g PDMS (Dow Corning, USA) mixture with a ratio of 7 (base material): 1 (curing agent) was poured on the mold. After cured in the oven at 70 $^{\circ}$ C for 2 h, PDMS was peeled off and cut into rectangular pieces, and access holes were punched for gas connection. For fluidic layer, 5 g PDMS with a ratio of 15:1 was spin coated over the mold at 800 rpms for 30 s and cured in oven. Both pneumatic layer and fluidic layer were treated with UV Ozone for 5 min, and then aligned manually under a stereomicroscope. The double layer chip was heated at 70 $^{\circ}$ C overnight to form permanent binding. The assembly was removed from the mold and holes were punched for fluid access.

Surface Modification of Chips.

Glass slides were pre-treated with piranha solution to activate the surface silanol groups, and then immersed into 2% APTES in 95% ethanol and reacted for 30 min. The amine groups modified glass was rinsed thoroughly with deionized water and baked at 80 $^{\circ}$ C for 2 h to cross-link the monolayer. The glass slide was then shaken in 2.5 % (v/v) glutaraldehyde in PBS for 2 h to introduce aldehyde functionality. Specific area of glass slide was immobilized with amine-conjugated single chain DNA probes via reductive amination reaction. Finally, PDMS chip was treated with UV Ozone for 3 min and bounded with probe modified glass.

Cell Culture and EV Isolation.

The Hs919.T cell line, derived from benign osteoid osteoma, was obtained from the American Type Culture Collection. CHLA-258, derived from a primitive neuroectodermal tumor (PNET) patient prior to treatment and CHLA-9, derived from a PNET patient who relapsed after chemotherapy, were obtained from the Children's Oncology Group (COG). All cell lines were cultured at 37 $^{\circ}$ C under a 5% humidified CO₂ atmosphere. CHLA-258 and CHLA-9 were maintained in Iscoves Modified Dulbecco's Medium (IMDM), supplemented with L-glutamine (3 mM), insulin, and transferrin (5 mg/ml each), selenium (5 ng/ml), and 20% heat-inactivated EV-free FBS (whole medium). Hs919.T was maintained in DMEM with high glucose with L-glutamine, supplemented with 20% heat-inactivated EV-free FBS (whole medium). Cell lines were cultured with 10% penicillin streptomycin to prevent bacterial growth/contamination.

The three cell lines were cultured in T175cm² flasks until cellular sub-confluency of ~70%. EVs were isolated by standard ultracentrifugation with a WX Ultra Series ultracentrifuge as previously published⁵¹. Briefly, conditioned media was collected and centrifuged at 2,500 rpm for 5 min to remove cellular debris. The supernatant was then centrifuged at 4 °C for 45 min at 10,000× g to remove microvesicles (10K pellet) and then at 100,000× g for 2 h to enrich exosomes (100K pellet). Both spins utilized the Fiberlight F40L rotor (Thermo Fisher). The 100K pellets were then washed once by resuspending in 10 mL of PBS and ultracentrifugation at 4 °C for 60 min at 110,000× g in a Fiberlight F65L rotor (Thermo Fisher). After aspiration of the supernatant, EV pellets were resuspended in 100 µL PBS, aliquoted and stored at –80 °C.

Extraction of Total RNA.

Total RNA was isolated from 100K EV pellets and cells using a combination of the RNAeasy kit (Qiagen) and Phase Lock Heavy Tubes (Quanta Bio). Briefly, ~ 6 × 10⁶ cells or 10–50 µg of EVs were lysed with 750 µL Qiazol after which 350 µL of chloroform was added and mixed by pipette. The slurry was transferred to a phase lock tube for phase separation. The resulting aqueous phase containing the RNA was processed according to the Qiagen protocol and purified RNA was eluted in either 50 µL (cells) or 30 µL (EVs).

Digital mRNA Assay.

The digital assay chip was incubated with 5% BSA containing 100 mM ethanolamine for 1 h to block non-specific adsorption sites prior to use. The treated chip was then flushed with 10 µL washing buffer (1× SSC, 0.1% Tween 20) at 50 °C for 10 min. The reaction solution was prepared by mixing the RNA sample and the detection probes in hybridization buffer (5× SSC, 0.2% Tween-20, 50% formamide, 10% dextran sulfate and 0.8 U RNase inhibitor). 60 µL of the mixture was denatured at 75 °C for 5 min, cooled down on ice immediately, and then flowed through the microfluidic chip for hybridization capture at 45 °C for 2 h. After hybridization, the chip was washed with 10 µL washing buffer at 45 °C for 10 min, then incubated with 15 µL SβG (20 ng/mL) for 30 min. Excess enzyme was washed away by 10 µL PBST (PBS with 1% BSA and 0.05% Tween-20), and FDG (500 µM) was loaded into the chip. The actuator was activated to press down and seal the microwells. Fluorescent images were taken after 10 min reaction. The captured fluorescent images were analyzed using ImageJ (<http://rsbweb.nih.gov>). For analog fluorescent images, the average intensity of fluorescent signal was read from software directly; for digital images, the number of positive microwells was measured following the standard protocol of cells counting. Briefly, the fluorescent images were converted into greyscale firstly, and then a threshold was set according to the histogram of signal intensity to distinguish positive signal from the background level in microwells. Using the threshold, the images were converted to binary black/white images to count the positive microwells. The size of each microwell is set to be 5-infinity to exclude small “noise” pixels.

RT-qPCR.

Total RNA was converted to cDNA using the Superscript IV VILO mastermix (Invitrogen) according to manufacturer’s instructions. ~1 µg of total RNA (cell lines) and 20–50 ng (EVs) of total RNA was used to generate cDNA. qPCR of the resultant cDNA was

performed on a CFX96 real-time thermocycler (Bio-Rad) using TaqMan gene expression master mix (Thermo Fisher) in 20 μ L reactions. We utilized a previously published *EWS-FLII* primer/probe set⁵²: forward primer, 5'-GTCAACCTCAATCTAGCACAGGG3'; reverse primer, 5'-CTGTCTGGAGAGCAGCTCCAG3'; and TaqMan probe, 5'-CTCCTACCAGCTATTCCTCTACACAGCCGACT3'. Commercial primers from IDT were used for RT-qPCR of *GAPDH* mRNA. Thermal cycling conditions were as follows: 50 °C for 3 minutes, 95 °C for 10 minutes, and 40 cycles of 95 °C for 15 s, 65 °C for 45 s. For PCR based quantification of *GAPDH* and *EWS-FLII* transcripts, PCR amplicons were isolated from DNA gel electrophoresis and their total concentration (in ng/ μ L) and 260/280 ratios were determined by Tecan NanoQuant plate reader. Using software freely available from URI Genomics and Sequencing Center (<http://cels.uri.edu/gsc/cndna.html>), we determined the copy number concentration of these amplicons. Serial dilutions of both *GAPDH* and *EWS-FLII* were prepared from this stock and used to generate standard curves. These curves were used to determine the concentration of unknown samples.

Results and Discussion

Chip Design and Characterization.

Distinctive from the droplet or bead-based digital assays,^{31, 36} our microfluidic platform was designed to integrate the sample processing, i.e., mRNA capture and tagging, with *in situ* PCR-free single-molecule detection in one integrated workflow. The chip was assembled with a pneumatic control layer, a fluidic channel layer, and the glass substrate patterned with the capture probe on the surface (Fig. 1a). The prototype device integrates three parallel units to demonstrate the scalability of the microfluidic design. The core component of each unit is a digital assay microchamber patterned with a rectangular array of 12000 microwells on the ceiling (Fig. 1b). The square wells are 10 μ m wide and ~6 μ m high, giving a volume of ~600 fL, with a center-to-center distance of 10 μ m. Capture probes are uniformly immobilized on the glass substrate surface inside the assay chambers using a patterning chip. A pneumatic chamber is located above each assay chamber to facilitate removal of air bubbles trapped in fL microwells using vacuum. The assay chambers were kept open while flowing the samples and detection probes through for hybridization capture and tagging of mRNAs on the glass surface (Fig. 1c, top). The flow injection was controlled by pneumatically actuating the on-chip microvalves with a stop-flow pumping sequence that we developed.⁴² Compared to continuous injection, this stop-flow pumping method has been shown to promote the efficiency and kinetics of surface binding and minimize the spatial bias of target capture across the assay chamber.⁵³ After the flow-through assay mode, the chip can be switched to the digital detection mode by using a mechanical actuator to press the assay chambers against the substrate (Fig. 1c, bottom). As mRNA molecules are randomly captured on the substrate surface, a fraction of surface-bound mRNA-enzyme complexes can be sealed into discrete fL wells in which single enzyme can produce sufficient concentration of dyes for fluorescence detection. Compared to the PDMS diaphragm valve structure operated by air or water pressure, the mechanical press is easy to operate and capable to deliver high force for reliable sealing of a large-area array of microwells without delaminating the multi-layer PDMS chip.

We first performed a photobleaching method to assess the sealing performance of the mechanical press. The microwell chamber was filled with a fluorescein solution, pressed by the mechanical press, and then exposed to an intense excitation light to photobleach the dye. Fluorescence images were recorded every 20 min to monitor the diffusion of fluorescein back into the bleached area. Fig. S1 in the Supporting Information (SI) shows no discernible leak of fluorescein into the dark area even after 1 h, indicating good sealing of microwells. To further assess the chip for digital detection, a robust streptavidin-biotin assay was implemented to capture and detect streptavidin- β -galactosidase (S β G), as illustrated in Fig. 2a. NH₂-PEG-biotin was uniformly immobilized on APTES and glutaraldehyde modified glass surface inside the assay chambers and S β G molecules were randomly captured on the biotin-patterned substrate surface via biotin-streptavidin interaction. When the microwell-patterned membrane was pressed tightly against the glass substrate, a fraction of surface-bound S β G molecules will be individually confined inside the sealed fL reactors for enzymatic fluorogenic detection, while others outside the microwells will be blocked. By real-time monitoring of the fluorogenic reactions in microwells, it was found that 10 min incubation provides the best signal-to-background ratio to identify the positive wells (Fig. S2). Titration of S β G was conducted using a series of 10 $\times$ diluted solutions in a concentration range of 0.3 aM to 1 fM (Fig. 2a). As expected, the fraction of positive microwells decreases along with the enzyme concentration. Histograms of the average fluorescence intensity of individual wells were plotted to quantify the positive wells. As demonstrated in Fig. 2b, the histogram obtained for detection of 1 fM S β G displays three major populations of the microwells, which can be attributed to the presence of 0, 1, and 2 S β G molecules, respectively. Based on the number of S β G injected into the chip (60 μ L, *i.e.*, ~36,000 molecules) and the ratio of surface areas covered by the microwells and the assay chamber (~0.34), the average occupancy was calculated to be ~1.0 molecule per well. The observed positive wells accounted for 61.6 % of the wells and the percentage of each population agrees closely with the theoretical values predicted by Poisson distribution (SI Table S1). The calibration curve for digital detection of S β G was plotted and compared to that of on-chip analog detection (Fig. 2c). For digital detection, the linear response was obtained in a range of 1 fM to 1 aM and the limit of detection (LOD) was calculated to be 0.3 aM, which is comparable with the Simoa assay.³¹ Compared to the LOD of analog detection (0.3 fM), our digital assay chip improves the sensitivity by approximately 1000 times.

Optimization of mRNA Hybridization Assay.

Our digital mRNA assay is based on the double-hybridization assay that has been widely used in DNA microarray and fluorescence *in-situ* hybridization.^{54–56} As illustrated in Fig. 3a, target mRNA is captured by the complementary DNA probes immobilized on the chip surface by specific hybridization and then tagged by two detection probes that recognize different sequences on the target. The reporter enzymes (S β G) bind onto the tripartite complex via strong streptavidin-biotin interaction and continuously convert the non-fluorescent FDG substrate into fluorescein to amplify the detection signal. Targeting *GAPDH* mRNA in a commercially available skeletal muscle total RNA standard, the hybridization assay parameters were optimized in the analog mode first, followed by the transition to the digital assay. The sequences of the capture and detection probes are detailed

in SI Table S2. Surface immobilization of capture probes was assessed using a FAM-labelled complementary oligo probe (SI Fig. S3). Three commonly used silane chemistries were examined for glass surface treatment, which showed the highest efficiency of oligo immobilization for APTES compared to 3-MPS and GOPS (SI Fig. S4). Thus, APTES chemistry was chosen for surface modification of the assay chamber. We evaluated various strategies to tag target mRNA with the detection probes by hybridization. As shown in Fig. 3b, mixing *GAPDH* mRNA with the detection probes prior to on-chip capture (pre-tagging) substantially enhances the detection sensitivity than tagging *GAPDH* mRNA that were first captured on the chip surface (post-tagging). This phenomenon can be due to the better hybridization efficiency in solution and extended reaction time for the pre-tagging approach compared to the post-tagging approach. An extra tagging step after the pre-tagging (combined tagging) does not further enhance the detection signal, confirming the high efficiency of the pre-tagging approach (Fig. 3b). The concentration of detection probe was optimized by testing a wide concentration range of 10 to 1000 nM, while keeping the concentration of *GAPDH* mRNA consistent. As shown in Fig. 3c, 100 nM detection probe affords the best signal-to-background ratio versus two other concentrations. Temperature is a critical factor for hybridization-based sensing of mRNAs, as increasing temperature will improve the specificity of hybridization but suppress binding efficiency. Indeed, when we increased the hybridization temperature from 35 °C to 50 °C, both non-specific background and specific binding were decreased and the optimal balance was obtained at 45 °C (Fig. 3d). Lastly, different buffers were compared for the hybridization reaction. Saline-sodium citrate (SSC) was found to yield a much better detection sensitivity than Tris-EDTA (TE) buffer (SI Fig. S5). However, the hybridization in SSC required 6 h incubation, which could lead to significant analytical variations due to the increased effects of non-specific adsorption, evaporation, and mRNA degradation over such long reaction time. Thus, we added 50% formamide and 10% dextran sulfate as exclusion reagents in 5× SSC buffer (SSCF) to enhance the interactions between surface probes and target molecules.⁵⁷ SSCF was seen to maintain the detection performance, while greatly reducing the assay time to 2 h.

We attempted to establish a dual-probe hybridization assay to enhance the detection sensitivity for EV mRNAs. When testing the analog detection of *GAPDH* mRNA (Fig. 3e), individual detection probes (100 nM DP1 and DP2) performed only slightly differently. As expected, an equimolar mixture of two probes (100 nM each) led to a nearly 2-fold increase in fluorescence signal, indicating good hybridization efficiency of the probes for quantitative mRNA detection. For the digital detection (Fig. 3f), the performance of individual detection probes was consistent with that in the analog assay. In contrast, the probe mixture yielded only a slightly higher percentage of positive wells than DP1. This is because the digital hybridization assay counts the number of discrete reactions for target quantification rather than the signal intensity in proportion to the amount of enzyme molecules. These results further verify the chip sealing performance and the dual-probe assay for digital quantification of single molecules.

Using the optimized assay conditions, we characterized our assay for absolute digital mRNA quantification using serial dilutions of a synthetic *GAPDH* mRNA standard (SI Table S3) of known concentration. The calibration curve of the digital assay was plotted in Fig. 3g, which

shows a limit of detection (LOD) of ~18 aM, calculated from the background level plus three standard deviations. For comparison, analog detection was also calibrated, yielding a much worse LOD of ~13 fM. The LOD obtained here is lower than a commercial digital assay system, Simoa,³⁴ and other existing chip-based digital assays (Table 1), which demonstrates the ability of our integrated chip to promote the sensitivity of digital bioassay. The dynamic range of digital detection is dictated by Poisson distribution in theory. As the average rate of positive wells is less than 10%, Poisson statistics predicts that the majority of microwells (>99%) contain at most one molecule (SI Table S1). Thus, the readout range between the limit of quantitation of our assay (1%) and 10% positive wells would afford a linear window for absolute quantification of mRNA. As shown in Fig 3h, our assay demonstrates excellent linearity (R^2 : 0.99) for the analysis of 0 to 1 fM of synthetic *GAPDH* mRNA, which corresponds to a range of 2% to 6% positive wells. Overall, these data suggest our microfluidic digital assay chip provide a sensitive and quantitative modality for analysis of EV mRNAs.

Ultrasensitive Detection of EV mRNAs.

As a proof-of-concept for potential biomedical applications, we adapted the microfluidic digital assay chip to directly detect *EWS-FLII* fusion transcripts in EVs derived from the peripheral primitive neuroectodermal tumor (PNET) cell lines. PNET are consistently associated with chromosomal translocation and functional fusion of the *EWSR1* gene to any of several structurally related transcription factor genes of the E26 transformation-specific (ETS) family.^{58, 59} The most frequent aberrations fuse portions of the *EWS* gene to either *FLII* (approximately 85%) or *ERG* (approximately 10%).^{60, 61} The detailed information of several common fusion types and their corresponding breakpoints was shown in SI Table S4. In this pilot study, we were focused on *EWS-FLII* fusion transcripts and three cell lines, CHLA-9 (*EWS-FLII* type 1), CHLA-258 (*EWS-FLII* type 3) and Hs919.T (benign osteoid osteoma as negative control) were employed.⁶² The probes designed for *EWS-FLII* transcripts were schematically illustrated in Fig. 4a with detailed sequences summarized in SI Table S2. The capture probe CP2 targets a sequence located on *FLII* exon 9 that are shared by the *EWS-FLII* fusion variants; the detection probes recognize the locations on the *EWS* exons.

We first optimized our digital assay for detection of *EWS-FLII* fusion transcripts using total RNA (0.1 $\mu\text{g}/\mu\text{L}$) extracted from the three cell lines. Individual detection probes and the probe mixtures were tested for the respective transcript targets, as displayed in Fig. 4b and c. The single-probe *EWS-FLII* assays resulted in comparable levels of positive counts, while the readouts of the dual-probe assays were slightly higher. This observation is consistent with the digital assay of *GAPDH* mRNA in Fig. 3e and f, verifying the effectiveness of the detection probes for the digital hybridization assay. To assess the specificity of the digital hybridization assay, we used the probes DP1 to DP4 to measure both *GAPDH* and *EWS-FLII* type 1 mRNAs in total RNA extracted from Hs919.T, CHLA-9 and CHLA-258 cells. As seen in Fig. 4d, high levels of *GAPDH* mRNA were detected in all three cell lines. *EWS-FLII* fusion transcripts were detected in two PNET cell lines as the probes DP3 and DP4 recognize the EWS exons on both type 1 and type 3 (Fig. 4a). In contrast, essentially no *EWS-FLII* mRNAs were detected for the control Hs919.T cells. Moreover, we attempted to

detect *EWS-FLII* fusions in both PNET cell lines using each set of two detection probes, respectively. As shown in Fig. 4e, our results showed a strong expression of *EWS-FLII* type 1 fusion and a very low level of readout for type 3 fusion in CHLA-9 cells. This is consistent with the design of DP5 and DP6 which recognize the EWS exons 8–10 that are depleted in the type 1 fusion mRNA (Fig. 4a). Both sets of the detection probes detected the type 3 fusion in CHLA-258 with the same levels of digital readout, indicating the reliable performance of our dual-probe hybridization assay. Collectively, these results demonstrated that our dual-probe hybridization assay affords highly specific detection of *EWS-Ets* fusion subtypes in EWS/PNET disease.

To assess the chip for absolute quantitative mRNA analysis, we measured *GAPDH* and *EWS-FLII* type 1 in serial dilutions of the total RNAs (1 $\mu\text{g}/\mu\text{L}$) from both PNET cell lines (Fig. 4f and g). The titration curves showed excellent linearity with $R^2 = 0.9988$ in all cases. Using the calibration curve established above, we calculated the concentration of *GAPDH* to be 62.8 pM and 127.0 pM and the concentration of *EWS-FLII* type 1 transcript to be 6.12 pM and 0.87 pM in 1 $\mu\text{g}/\mu\text{L}$ total RNA from CHLA-9 and CHLA-258 cell lines, respectively (Fig. 4h). The quantification results by the digital assay were further validated by the independent RT-qPCR analysis conducted at KUMC (see Experimental Section). The standard RT-qPCR analyses consistently reported significantly higher concentrations of the target mRNAs (Fig. 4h), which can be mainly attributed to the fact that RT-qPCR quantify cDNA copies synthesized by the RT reaction using hexamer primers. Indeed, previous studies have revealed that RT-qPCR analysis based on random priming of cDNA tends to overestimate mRNA concentration by up to 19 folds,⁶³ despite its advantages over other cDNA priming methods, such as higher sensitivity, better reproducibility, and less sequence bias.¹⁸ To compare the two methods, we normalized the expression level of *EWS-FLII* by *GAPDH*, as presented in Fig. 4i. The relative expression levels of *EWS-FLII* type 1 transcript measured by the digital hybridization assay agrees well with the RT-qPCR results for both cell lines. These findings should validate our PCR-free digital assay for accurate and reliable absolute quantification of mRNA in biological samples.

Lastly, we applied the microfluidic platform to quantify *EWS-FLII* transcripts in EVs derived from PNET CHLA-9 and CHLA-258 cells. While recent studies detected *EWS-FLII* mRNAs in EVs derived from Ewing Sarcoma cell lines^{64, 65}, quantitative analysis of the transcript variants in EVs has been challenging due to their low abundances. EVs were isolated from cell culture media using ultracentrifugation and characterized with SEM and NTA.^{66, 67} SEM imaging reported a representative cup-shaped morphologies of freeze-dried extracellular vesicles from the two PNET cell lines (Fig. 5a, b). The size and concentration of CHLA-9 and CHLA-258 derived EVs were measured by NTA (Fig. 5c) and the results were presented in SI Table S5. The purified EVs were lysed with lysis buffer and diluted with SSC buffer by ten times. The EV lysate was injected into our digital assay chip for mRNA detection without further sample pretreatment. The averaged abundance of *GAPDH* mRNA in CHLA-9 and CHLA-258 EVs were estimated to be 60.6 ± 3.3 and 43.5 ± 2.4 copies per 10^5 EVs, respectively; and the levels of *EWS-FLII* type 1 fusion transcript were 7.25 ± 0.97 and 0.277 ± 0.023 copies per 10^5 EVs, respectively (Fig. 5d). To validate these measurements, we normalized the levels of *EWS-FLII* mRNA against *GAPDH* and compared to the results obtained by the independent RT-qPCR analysis of total RNA

extracted from these EVs. As shown in Fig. 5e, these two methods reported the fairly consistent results for both EV types. The expression ratio between *EWS-FLII* and *GAPDH* mRNAs in EVs was found to be nearly the same as in their parent cells for both PNET cell lines (Fig. 5f). These data support the potential clinical utility of EVs as liquid biopsy as they carry mutant transcripts reflective of tumor cells of origin. It is worth noting that while both our chip-based digital assay and RT-qPCR are sufficiently sensitive to detect *EWS-FLII* mutations in PNET EVs, our method enables direct and absolute quantification with much less sample consumption and simplified workflow because of eliminating the steps of manual RNA extraction and cDNA synthesis.

Conclusion

A configurable microwell-patterned microfluidic digital bioassay chip was developed to enable absolute quantification of mutant mRNAs in tumor-derived EVs with high sensitivity and specificity. The device can be mechanically switched from the flow-through assay mode for enhanced hybridization capture and tagging of mRNAs to the digital detection mode based on femtoliter-scale enzymatic signal amplification for single-molecule counting of surface-bound targets. A dual-probe hybridization assay has been developed to enhance the sensitivity of the digital single-molecule detection of EV mRNAs. The integrated microfluidic system affords quantitative detection of synthetic *GAPDH* mRNA with a LOD as low as 18 aM. Using this technology, we quantified *EWS-FLII* fusion transcripts to be as low as 6.5 and 0.277 copies of per 10^5 EVs derived from CHLA-9 and CHLA-258 cells, respectively. These results suggest the microfluidic digital mRNA analysis platform as a potentially useful tool to facilitate quantitative analysis of tumor-associated EV mRNAs for liquid biopsy-based cancer diagnosis and monitoring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Configurable microwell-patterned microfluidic digital analysis platform.

(a) Design of the microfluidic chip that integrates a pneumatic control layer and a microwell-patterned assay chamber. Inset: microscopic photo of the microfabricated femtoliter wells. (b) Photograph of an assembled microfluidic digital analysis chip. The flow channels and the pneumatic lines were filled with blue dye and orange dye, respectively. (c) An electric actuator-based press was developed to mechanically switch the chip from the flow-through hybridization assay mode to the digital detection mode by sealing the microwells for single-molecule counting of surface-bound targets.

Figure 2. Characterization of the microfluidic digital analysis chip.

(a) Fluorescent images of detecting SβG diluted to a concentration range of 0.3 aM to 1 fM using a biotin-modified chip. Scale bars: 100 μ m. (b) Histogram of the average fluorescence intensity of individual wells for detection of 1 fM SβG. (c) Log-log plots for titration of SβG using the digital and analog detection mode, respectively. The dashed lines indicate the LOD calculated from the background level plus three standard deviation (S.D.).

Figure 3. Optimization of the microfluidic hybridization assay for digital detection of single mRNA molecules.

(a) Schematic illustration of the hybridization assay assisted by the enzymatic signal amplification for direct detection of single mRNA molecules without PCR amplification. (b) Evaluation of the performance of hybridization tagging of mRNA with the detection probes prior to on-chip capture, after capture or two approaches combined. (c, d) Optimization of the effects of detection probe concentration (c) and the assay temperature (d) on the signal-to-noise ratio of the hybridization assay. (e, f) Assessment of individual detection probes and their combination for detection of *GAPDH* mRNA in the analog (e) and digital detection mode (f). *GAPDH* mRNA in skeletal muscle total RNA was targeted for the experiments in (b-f). (g) Log-log calibration plots obtained by the digital and analog detection of synthetic *GAPDH* oligonucleotides, respectively. The dashed lines indicate the LOD, i.e., background plus three S.D. (h) Calibration curve for absolute quantification of synthetic *GAPDH* mRNA using the microfluidic digital assay. Error bars: one S.D. ($n = 3$).

Figure 4. Microfluidic digital detection of *EWS-FLI1* fusion transcripts in cells.

(a) Design of various capture and detection probes to discriminate *EWS-FLI1* type 1 and type 3 fusion transcripts. (b, c) Evaluation of the detection probes and their mix for digital detection of *EWS-FLI1* type 1 (b) and type 3 (c) transcripts, respectively. (d) Specific detection of *GAPDH* and *EWS-FLI1* type 1 mRNAs in total RNA extracted from a control cell line Hs919.T, and two PNET cell lines, CHLA-9 and CHLA-258. (e) Specific dual-probe detection of *EWS-FLI1* type 1 and type 3 fusion transcripts in CHLA-9 and CHLA-258 cells, respectively. (f, g) Quantitative detection of *EWS-FLI1* type 1 (f) and *GAPDH* (g) mRNAs in serially diluted total RNA extracted from CHLA-9 and CHLA-258 cells (1 $\mu\text{g}/\mu\text{L}$). (h) Comparison of microfluidic digital assay chip and RT-qPCR for quantifying the levels of *GAPDH* and *EWS-FLI1* type 1 transcripts in total RNA from CHLA-9 and CHLA-258 cells (1 $\mu\text{g}/\mu\text{L}$). (i) Comparison of the relative expression of *EWS-FLI1* type 1 mRNA normalized over *GAPDH* in CHLA-9 and CHLA-258 cells measured by microfluidic digital detection and RT-qPCR, respectively. Error bars: one S.D. ($n = 3$).

Figure 5. Digital quantification of mRNAs directly from EV lysates.

(a, b) SEM images of EVs isolated from the culture media of CHLA-9 (a) and CHLA-258 (b) cell lines. (c) NTA characterization of the sizes and abundance of CHLA-9 and CHLA-258 derived EVs. (d) Quantification of the levels of *GAPDH* and *EWS-FLI1* type 1 mRNA transcripts in CHLA-9 and CHLA-258 derived EVs measured by using the microfluidic digital assay chips. (e) Comparison of microfluidic digital assay chip and RT-qPCR for quantifying the relative levels of *EWS-FLI1* type 1 fusion transcript against *GAPDH* in CHLA-9 and CHLA-258 derived EVs. For RT-qPCR, cDNAs were synthesized using hexamer primers. (f) Donut charts comparing the levels of *EWS-FLI1* type 1 transcripts to *GAPDH* in CHLA-9 and CHLA-258 cells and their EVs, respectively. Error bars: one S.D. ($n = 3$).

Table 1.

Comparison of microfluidic digital assays for quantitative detection of DNA/RNA.

Technology	Characteristic of Assay	Target	Biological Samples	LOD	Reference
Self-Digitization Chip	One-step RT-PCR in microwells	mRNA	cell lysate	mRNA of single cell	30
NanoString	Surface capture and multiplexed fluorescent labelling by hybridization	mRNA	total RNA from cells	0.1–0.5 fM	26
Simoa	Bead-based capture and tagging in bulk assays, bead loading and fluorogenic detection in fL microwells	DNA	DNA from <i>S. aureus</i>	0.07 fM	31
		miRNA	Total RNA from cells & miRNA spiked in serum	0.58 fM	39
Microfluidic Digital Assay Chip	On chip surface capture and <i>in situ</i> fluorogenic detection in fL microwells	mRNA	Total RNA from cells and exosomes	0.018 fM	This study