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Nano-plasmonic exosome diagnostics

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Abstract

Exosomes have emerged as a promising biomarker. These vesicles abound in biofluids and harbor molecular constituents from their parent cells, thereby offering a minimally-invasive avenue for molecular analyses. Despite such clinical potential, routine exosomal analysis, particularly the protein assay, remains challenging, due to requirements for large sample volumes and extensive processing. We have been developing miniaturized systems to facilitate clinical exosome studies. These systems can be categorized into two components: microfluidics for sample preparation and analytical tools for protein analyses. In this report, we review a new assay platform, nano-plasmonic exosome (nPLEX), in which sensing is based on surface plasmon resonance to achieve label-free exosome detection. Looking forward, we also discuss some potential challenges and improvements in exosome studies.

Keywords

Exosome; Surface plasmon resonance; Extracellular vesicles; Cancer; Molecular diagnosis

The growing emphasis on targeted and personalized therapy concomitantly increases the need to analyze and monitor key cancer proteins and pathway activation[1–3]. Although tissue biopsies remain the gold standard, their invasiveness and limited sampling often present practical challenges with patient management[4].

Exosomes have emerged as a new class of cancer biomarker for clinical diagnostics[5, 6]. Exosomes are membrane-bound phospholipid vesicles (50–200 nm in diameter) that are

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actively secreted by cancer cells (Fig. 1). These vesicles carry cellular constituents of their originating cells, including transmembrane and intracellular proteins[7], mRNA[8], DNA[9], microRNA[10], lipids and metabolites, and can serve as cellular surrogates[11]. Combined with their large abundance and ubiquitous presence in bodily fluids (e.g., blood, ascites, urine)[5, 12, 13], exosomes offer significant advantages for cancer monitoring[14–16]. Namely, an exosomal assay can be robust and minimally invasive for repeated tests. As most tumor cells shed exosomes, the assay can also report relatively unbiased readouts of the whole tumor burden, less affected by the scarcity of the samples (e.g., circulating tumor cells to circulating DNAs) or intra-tumoral heterogeneity (e.g., fine-needle aspiration)[17]. Furthermore, the amount and molecular profile of cancer exosomes have been shown to correlate with tumor burden as well as treatment efficacy[17, 18]. A number of recent review articles have highlighted exosomes' role in diagnostics, cell-to-cell interactions and therapeutic opportunities[5, 6, 19–23]. Despite such clinical potential, routine exosome analysis is still a challenging task. Conventional methods (e.g., Western blotting, ELISA) require larger sample volumes (>500 μL per biomarker) and extensive processing (e.g., 3 hours with ultracentrifugation)[6, 24]. Such assays become impractical when multiple markers need to be profiled or the sample volume is inherently limited (e.g., cerebrospinal fluid)

Various exosome detection platforms have been introduced to overcome these challenges (Fig. 2, Table 1)[17, 18, 25–30]. Integration with microfluidics allows for exosome analyses in small volumes; adoption into novel sensing methods (e.g. surface plasmon resonance, magnetic resonance) generated exosomal assays with shorter assay time, higher sensitivity and higher throughput. Commercialized nucleic acid sensing technologies (e.g. RainDrop, NanoStrings) have been adapted for a variety of exosomal RNA components with high sensitivity. With the high sensitivity and throughput, these new technologies have shown great promise for both exosomal RNA and protein detection over conventional analytical methods.

We have been advancing miniaturized systems to facilitate exosome studies (Figure 3). These systems are comprised of two components: microfluidics to facilitate sample preparation and analytical tools for protein analyses. The microfluidic devices are designed to collect intact exosomes directly from biological samples, replacing ultracentrifugation or proprietary precipitation methods. The first developed device used a detachable membrane filter (0.1 μm pore) to size-selectively enrich exosomes from large sample volumes[31]; the next developed system was based on acoustic actuation, which enabled controllable size-cutoff and continuous, inflow filtration[32]. For protein analyses, we initially adopted the μNMR technology to magnetically profile exosomal proteins[17]. In μNMR, target proteins were labeled with magnetic nanoparticles, and changes in transverse relaxation of the samples were measured. The signal detection is robust against biological background, and the assay was demonstrated to benefit from such a well-established platform[33–36]. The μNMR assay, however, was difficult to scale up for high throughput detection. The task requires a large NMR-grade magnet to accommodate multiple NMR probes, and also entails labeling with magnetic nanoparticles. Recently, we developed a new assay system, termed nano-plasmonic exosome (nPLEX)[18] that could overcome these challenges. The nPLEX sensing is based on surface plasmon resonance (SPR) through periodic nanohole arrays,

wherein target-specific exosome binding on the array causes significant SPR signal changes. The system is scalable with a large number of sensing units (> 100) integrated into a single chip, and the assay is label-free (i.e., no need for secondary labeling with nanoparticles)[37, 38].

This special report will review this nascent nPLEX technology, assessing its sensor design, assay protocols, and clinical applications. We will specifically focus on nPLEX's capacity for fast, high-throughput exosome analyses, and also discuss directions to further improvements.

nPLEX Technology

Sensing principle

The nPLEX system comprises of periodic nanohole arrays made in an opaque gold (Au) film (Figure 4a). Light illumination to the nanohole arrays can excite strong electromagnetic fields, called surface plasmons (SPs)on the surface (Figure 4b), which lead to SP-mediated extraordinary optical transmission (EOT)[39, 40]. The transmission spectral peak positions are highly sensitive to the refractive index on the nanohole surface, and exosome binding to the nanohole surface (via affinity ligands) would red-shift the optical transmission peaks (Figure 4c). The amount of spectral shift correlates with the molecular mass density [41], which enables quantification of captured exosomes on the sensing surface. Because exosome binding itself induces a spectral shift, the nPLEX can detect exosomes in a label-free manner.

The nanohole-based plasmonic detection has unique advantages over conventional SPR systems (e.g., Kretschmann configuration[42]). First, a simple, collinear optical setup can be used for signal measurements[38, 43], and the system can be readily miniaturized[18, 44]. Second, the system is scalable for high throughput detection. The minimal array size for the EOT could be as small as 5-by-5 periodic nanoholes (foot print < 10 μm^2)[45], which allows for the integration of high density arrays (> 10^6 detection sites per cm^2)[46–48]. Such high density is difficult to achieve with the Kretschmann configuration. The large tilt angle of incidence could lead to optical aberration when a NA aperture is used to increase spatial resolution or defocusing when imaging arrays in a large area.

System design

The geometry of the nanoholes was optimized through 3-dimensional simulation to match the sensing range with the mean diameter of exosomes (~100 nm; Figure 5a). The nPLEX signal was measured by monitoring the transmission spectrum via a spectrometer setup. In this mode, individual arrays are sequentially scanned, and a spectral shift of resonance peak from exosome binding is detected. Although the spectrum-based measurements provide comprehensive information of the nanohole's optical characteristics, it could be time-consuming with large sensing arrays. For faster readout, we alternatively measured changes in transmission intensity at an excitation wavelength (Figure 5b). This intensity-based method could monitor multiple sensing arrays simultaneously, enabling high-throughput parallel measurements.

Figure 6a shows the first nPLEX prototype. The structure was patterned in a 200 nm-thick Au film on a glass substrate. We laid out a 12 × 3 array of sensing units with multi-channel microfluidics placed on top (Figure 6b). Each channel spanned over three sensing units for triplicate measurements. The sample volume per sensing unit was ~1 μL. For parallel measurements of nPLEX arrays, an intensity-based detection system integrated with miniaturized optics consisting of a laser diode and an image sensor was also developed (Figure 6c). This system can simultaneously monitor changes in the transmitted light intensities of 36 arrays for high-throughput parallel measurements.

Analytical nPLEX assay for molecular profiling

To impart molecular specificity, the nanohole surface was coated with different antibodies in each channel. Following antibody conjugation, exosomes were introduced and spectral shifts are measured before and after exosome binding. An IgG control channel was incorporated to measure the contribution from non-specific binding and its signal was subtracted from each target channel.

To determine the detection sensitivity, we functionalized the sensor surface with antibodies against CD63, a type III lysosomal membrane protein enriched in exosomes. Alternatively, other exosome-specific lysosomal membrane proteins (e.g. CD9, CD81) were also used[49]. Samples were prepared from CaOV3 (human ovarian carcinoma) cell lines, and their initial exosome concentrations were estimated by nanoparticle-tracking analysis (NTA). A pair of nPLEX sensors, functionalized with CD63 and control IgG antibodies respectively, were used to measure the relative spectral ($\Delta\lambda^{CD63}$) or intensity (Δp^{CD63}) changes against known exosome counts. The titration experiments established the limit of detection (LOD) of ~ 3000 exosomes (670 aM) with the label-free nPLEX assay (Figure 7a). The observed sensitivity based on the LOD was 10^4- and 10^2-fold higher than Western blotting and chemiluminescence ELISA, respectively.

To quantitatively detect exosome proteins, we functionalized the nPLEX sensors with antibodies against target markers and measured associated signals ($\Delta\lambda^{target}$ or Δp^{target}) from exosome capture. Next, we defined the expression level (ζ^{target}) of the target marker by scaling the marker-associated changes to those of CD63 (i.e., $\zeta^{target} = \Delta\lambda^{target}/\Delta\lambda^{CD63} \approx \Delta p^{target}/\Delta p^{CD63}$). Such normalization accounted for differences in exosome counts among samples and thereby reported the average expression level of a target marker per exosome[17, 18]. This method was applied to profile exosomes from different cell lines (CaOV3, OV90) for various extravesicular markers (Figure 7b). Expression levels were well-matched ($R^2 > 98\%$) between nPLEX and ELISA, verifying the accuracy of the developed nPLEX assay. In addition, the nPLEX assay could be adapted for downstream genetic analyses by releasing captured exosomes from the device using surface regeneration protocols[17, 18].

Clinical potential of exosomes

We first explored the correlation between exosomes and their parental cells. Multiplexed *in vitro* nPLEX screening showed good agreement of protein expression between exosomes and their parental cells across different ovarian cancer cell lines (Figure 8a). Such close

matching of molecular profiles between exosome and cells was previously identified in glioblastoma multiforme (GBM) cell lines using μNMR[17]. In addition, our nPLEX screening showed that EpCAM and CD24 were highly expressed in tested ovarian cancer cell lines.

Based on these results, the nPLEX system was applied to detect ovarian cancer exosomes in patient-derived ascites (Figure 8b). Thirty ascites samples were obtained: 20 patients were diagnosed with Stage 3 (n = 10) and 4 (n = 10) ovarian cancer and 10 control ascites patients were diagnosed with liver cirrhosis[18]. The study demonstrated that 1) unprocessed ascites contained large quantities (>10^9 per ml) of exosomes; 2) nPLEX was sensitive enough to detect exosomes directly isolated from ascites by simple syringe membrane filtration; and 3) the levels of EpCAM and CD24 per exosome were significantly higher in ovarian cancer patient samples than in control groups. For 30 samples tested, the detection accuracy was 97% using EpCAM and CD24 as diagnostic markers. The nPLEX screening was further used to evaluate the prognostic values of exosomes for treatment monitoring (Figure 8c). For ovarian cancer patients (n = 8) undergoing standard chemotherapy, the study demonstrated that the levels of exosomal EpCAM, CD24 or both decreased among responding patients, whereas levels of these markers increased in non-responding patients.

Expert commentary

Exosomes present new opportunities for cancer diagnoses and treatment monitoring. These vesicles abound in biological fluids and carry cell-specific cargos (lipids, proteins and genetic materials), which can be harnessed as a minimally invasive means to probe the molecular status of tumors. Significant technical developments are underway to channel exosome analysis into clinical settings: fluidic-based tools have been devised to facilitate sample preparation, and analytical platforms have been adapted to detect exosomes in clinical samples. Such efforts have started to unveiling tumor-associated exosomal fingerprints, particularly in RNA profiles (both coding and noncoding).

Exosomal protein analysis, on the other hand, still remains challenging. With the lack of universal amplification strategy (e.g., PCR), protein analysis generally requires large quantities of exosomes and often involves extensive sample processing. The nPLEX technology was developed to address these issues. The nPLEX's high sensitivity allows for quantitative measurements on small sample amounts; the detection is label-free to minimize assay time and potential sample loss/degradation; and the system is scalable to a large array for high-throughput assays.

Extended insight into exosomal proteins could help capture dynamic snapshots of tumors, that are hard to detect with genetic assays. Aberrant changes in cancer cells, in response to microenvironmental stress, are reflected in protein levels and its post-translational modification, which have significant effects on disease progression and therapeutic response. As such, the improved exosomal proteomic analyses, proffered by nPLEX, could pave the way for the potential use of exosomes as companion diagnostics and pharmacodynamic readouts.

We identify two immediate directions to further improve the nPLEX technology. First, the assay format needs to be developed to measure both extra- and intravesicular proteins. The initial nPLEX studies were limited to detecting transmembrane or lipid-bound proteins, since the assay was based on capturing whole exosomes on the device surface. Devising a new assay for intravesicular proteins is critical to probe the activation status of proteins as well as to measure cytosolic protein targets. Second, the clinical utility of nPLEX requires further validation under the auspices of larger clinical trials. The large datasets thus generated would aid in identifying key exosomal fingerprints for cancer. These efforts would establish nPLEX as a transformative platform facilitating cancer research and clinical practice.

Five-year view

The trajectories undertaken by the exosome field's development of first generation analytical tools parallel those of the more mature circulating tumor cell (CTC) research. Exosomes are abundant and stable in circulation. These advantages impart significant practical value on exosomes as noninvasive and unbiased surrogates for tissue-based biomarkers. As seen in cellular analyses, we envision that more advanced technologies for exosome enrichment and detection will be developed to ultimately enable high-throughput profiling of single exosomes. This will potentially lead to the identification of exosome subpopulations, highly specific to cancer, that could be prospectively explored in cancer clinical trials. Additional investigations will focus on whether testing of cancer exosomes could generate pharmacodynamic readouts. Coupled with the ready access of liquid biopsies, earlier 'go-no go' decisions could inform drug development. Improved understanding of the mechanisms driving exosomal signaling will accelerate the efforts to exploit exosomal targeting to deliver therapeutic payloads. This transition to theranostics could be a key step for the exosome field and usher in further attention from pharmaceutical stakeholders, among others. Such diverse opportunities create an exciting venue for exosome research – we anticipate an expanding pipeline of committed and accomplished junior and seasoned investigators across disciplines, along with increased funding opportunities for the next five years and beyond.

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Key issues

- Exosomes are membrane-bound vesicles that contain molecular constituents of their cell of origin, including proteins, nucleic acids, lipids and metabolites.
- Exosomes can serve as a minimally invasive biomarker for cancer diagnosis and treatment monitoring.
- Miniatured devices are being developed to expedite exosome isolation and its downstream analyses. These devices could shorten the hands-on assay time and minimize required samples volumes.
- The SPR-based nPLEX technology enables rapid, sensitive, label-free profiling of exosomal proteins.
- The nPLEX assay is quantitative, reporting the average expression level of target protein markers per exosomes.
- The detection platform is scalable for high throughput, automated detection.
- By changing the affinity ligands, the nPLEX platform could be used to detect exosomes from virtually any cell type, and hence could serve as a universal platform for exosome analyses.
- Intra-exosomal biology remains an area of active interest given its potential to generate novel pharmacodynamic readouts or therapeutic approaches

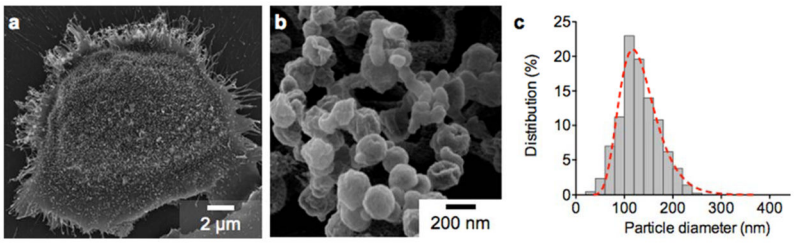

Fig. 1. Exosomes shed from ovarian cancer cell

(a) Electron microscopy image of a primary human ovarian cancer cell (CaOV3) confirms the avid release of membrane vesicles by the cell. **(b)** High magnification image shows that the vesicles on the cell surface assumed typical saucer-shaped characteristics of exosomes. **(c)** The size distribution of the exosomes, as characterized by the nanoparticle tracking analysis (NTA), ranges from 20 – 250 nm. Reproduced from Im H, Shao H, Park YI et al. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. Nat Biotechnol 2014;32:490–495 with permission from Nature Publishing Group, copyright 2014 [18].

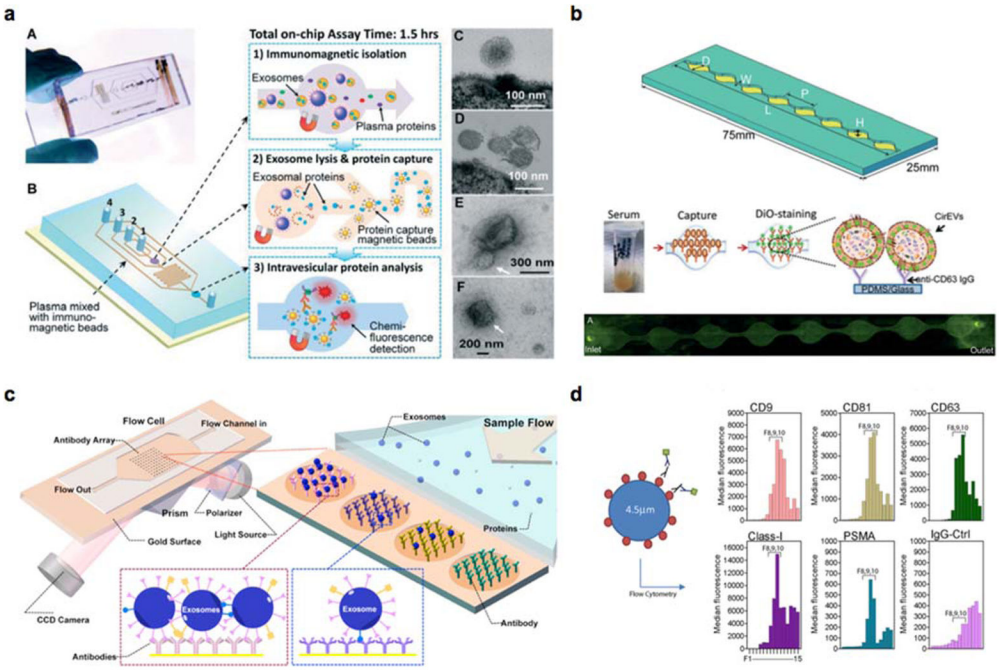

Fig. 2. New exosome sensing platforms

(a) An integrated microfluidic chip for exosome isolation, chemical lysis and exosomal protein analysis. Reproduced with permission from He M, Crow J, Roth M, Zeng Y, Godwin AK. Integrated immunoisolation and protein analysis of circulating exosomes using microfluidic technology. Lab Chip 2014;14:3773–3780, published by The Royal Society of Chemistry [26]. **(b)** A microfluidic device (ExoChip) for on-chip exosome capture and analysis. Reproduced from Kanwar SS, Dunlay CJ, Simeone DM, Nagrath S. Microfluidic device (ExoChip) for on-chip isolation, quantification and characterization of circulating exosomes. Lab Chip 2014;14:1891–1900 with permission of The Royal Society of Chemistry [27]. **(c)** A surface plasmon resonance imaging (SPRi) system for label-free exosome detection. Reprinted with permission from Zhu L, Wang K, Cui J et al. Label-Free Quantitative Detection of Tumor-Derived Exosomes through Surface Plasmon Resonance Imaging. Anal Chem 2014;86:8857–8864. Copyright 2014 American Chemical Society [29]. **(d)** Aptamer-based platform (SOMAscan™) for proteomic analysis of cancer exosomes. Reproduced with permission from Webber J, Stone TC, Katilius E et al. Proteomics Analysis of Cancer Exosomes Using a Novel Modified Aptamer-based Array (SOMAscanTM) Platform. Mol Cell Proteomics 2014;13:1050–1064 [28].

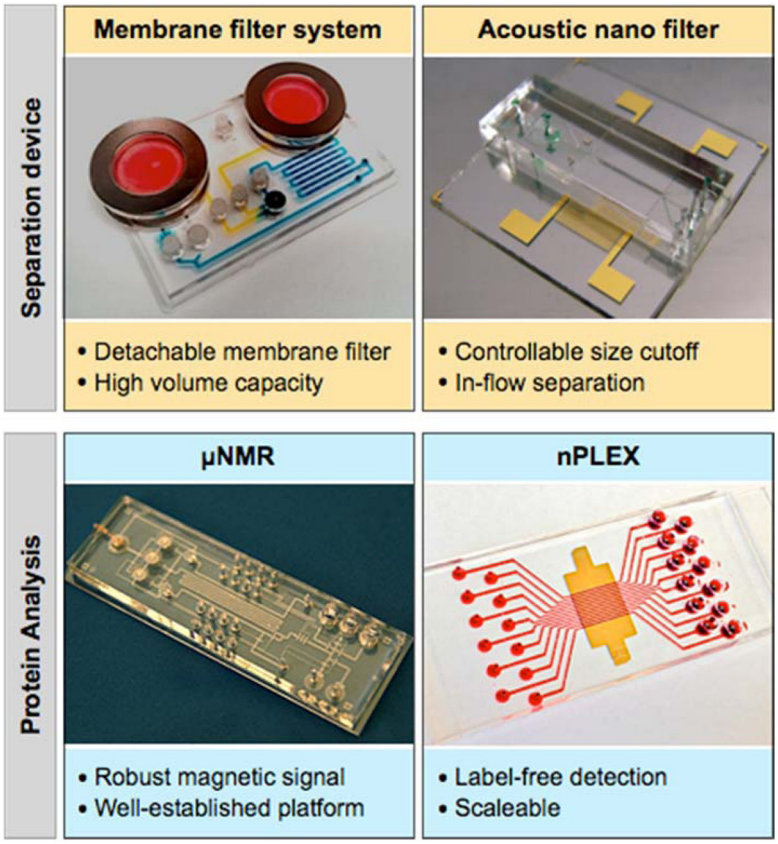

Figure 3. Miniaturized devices developed for exosome separation (top) and its protein profiling (bottom)

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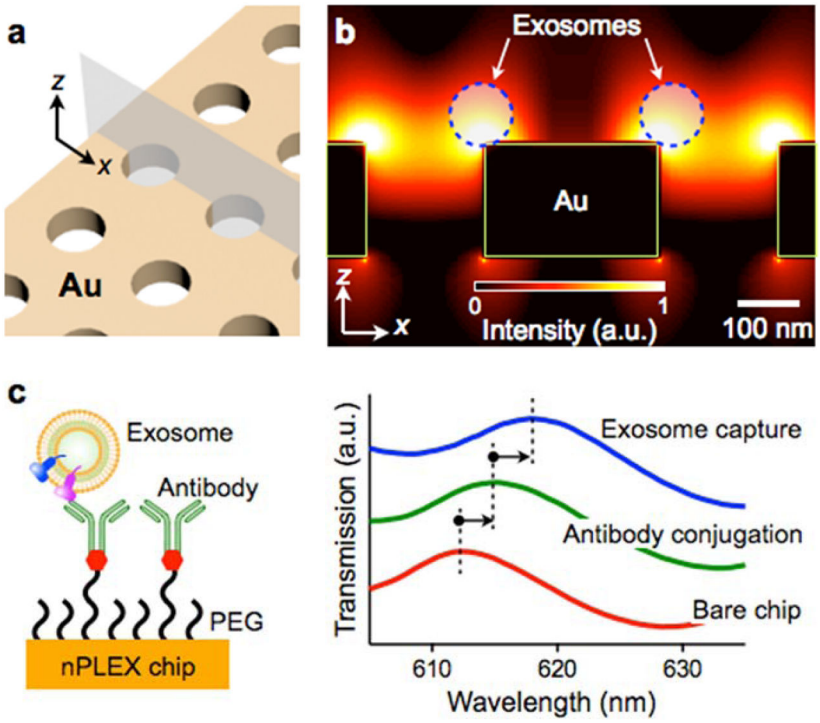

Fig. 4. nPLEX sensing principle

(a) A sensing site comprises a periodic nanohole array patterned in a gold film. **(b)** Finite-difference time-domain simulation shows the enhanced electromagnetic fields tightly confined near a periodic nanohole surface. The field distribution overlaps with the size of exosomes captured onto the sensing surface. a.u., arbitrary unit. **(c)** Antibodies were immobilized on the nPLEX chip, and exosomes were captured based on their expression of extravesicular markers (left). Antibody conjugation and exosome binding were monitored measuring spectral shifts via the nPLEX sensor (right). Reproduced from Im H, Shao H, Park YI et al. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. Nat Biotechnol 2014;32:490–495 with permission from Nature Publishing Group, copyright 2014 [18].

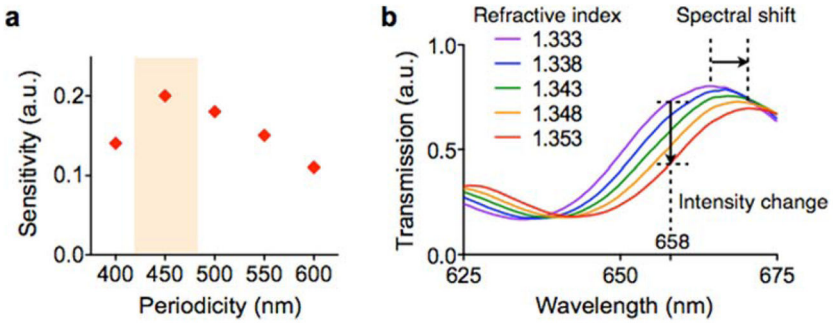

Fig. 5. Device design optimization

(a) The sensitivity of the nPLEX sensor was defined as Δλ/w, where Δλ and w are the shift and the width of SPR spectrum, respectively. The nanohole array with 450-nm hole-pitch showed the highest sensitivity for the detection of 100 nm exosomes. a.u., arbitrary unit. **(b)** Increase in the refractive index on the nPLEX surface induces a spectral shift (Δλ) of resonance peak to a longer wavelength. The increase of refractive index also causes intensity changes (Δ*p*) at a given wavelength (e.g., at 658 nm). Therefore, exosome binding can be detected by either tracking Δλ by spectrometry or Δ*p* by imaging. Reproduced from Im H, Shao H, Park YI et al. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. Nat Biotechnol 2014;32:490–495 with permission from Nature Publishing Group, copyright 2014 [18].

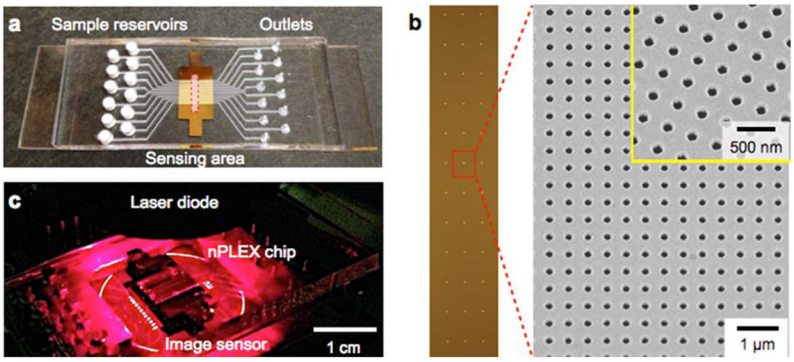

Fig. 6. First nPLEX prototype

(a) A photograph of the nanohole device integrated with microfluidics. A 12-channel fluidic cell was placed on top of a glass slide containing nanohole arrays. **(b)** A total of 36 measurement sites were arranged into a 12 × 3 array. Each measurement site had periodic nanoholes (right). The structure was patterned in a gold film (200 nm thick) deposited on a glass substrate. **(c)** A photograph of the miniaturized nPLEX imaging system. The nPLEX chip was located directly on an image sensor, which measured transmitted light intensities of the 36 sites simultaneously. Reproduced from Im H, Shao H, Park YI et al. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. Nat Biotechnol 2014;32:490–495 with permission from Nature Publishing Group, copyright 2014 [18].

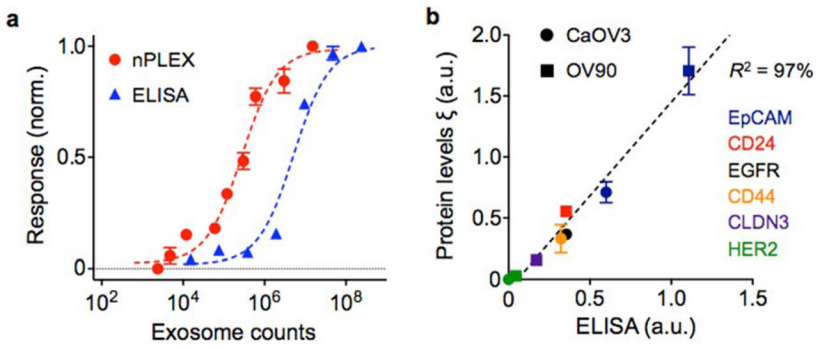

Fig. 7. Exosome quantification and protein profiling with nPLEX

(a) Exosomes isolated from human ovarian cancer cell line (CaOV3) were introduced onto a nPLEX sensor functionalized with CD63 antibody for exosomal capture. The nPLEX platform showed considerably higher sensitivity than ELISA. **(b)** Comparison between nPLEX and ELISA measurements. Exosomes isolated from human ovarian cancer cell lines were used. The expression level (ξ) was determined by normalizing the marker signal with that of CD63, which accounted for variation in exosomal counts across samples. All measurements were in triplicate and the data is displayed as mean ± s.d. Reproduced from Im H, Shao H, Park YI et al. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. Nat Biotechnol 2014;32:490–495 with permission from Nature Publishing Group, copyright 2014 [18].

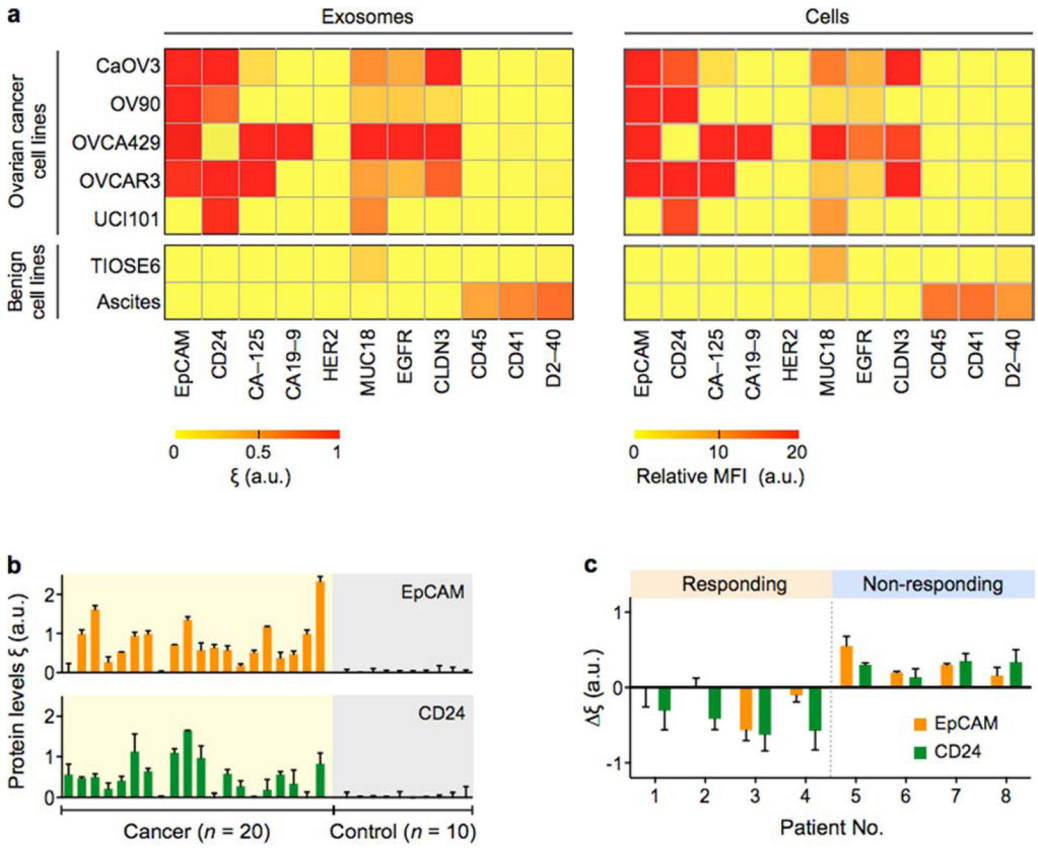

Fig. 8. Molecular profiling of ovarian cancer exosomes

(a) *In-vitro* study. Ovarian cancer associated markers (EpCAM, CD24, CA-125, CA19-9, HER2, MUC18, EGFR, Claudin3), immune host cell markers (CD41, CD45) and a mesothelial marker (D2-40) were profiled on both parental ovarian cells (**right**, using flow cytometry) and their derived exosomes (**left**, using nPLEX sensor). Exosomal protein profiles showed an excellent match with those of originating cells. A two-marker combination comprising EpCAM and CD24 could effectively distinguish cancer exosomes from benign exosomes. MFI, mean fluorescence intensity. **(b)** Ascites exosomes from ovarian cancer and non-cancer patients were evaluated by the nPLEX sensor. Cancer exosomes were captured on EpCAM and CD24-specific sensor sites, and the exosomal expression levels of these markers were measured. Ovarian cancer patients (n = 20) were associated with elevated EpCAM and CD24 expression, while non-cancer patients (n = 10) showed negligible signals. **(c)** Longitudinal nPLEX assays. Ascites samples were collected sequentially from ovarian cancer patients undergoing chemotherapy (n = 8). Measuring temporal changes in exosomal expressions of EpCAM and CD24 could distinguish treatment response. Reproduced from Im H, Shao H, Park YI et al. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. Nat Biotechnol 2014;32:490–495 with permission from Nature Publishing Group, copyright 2014 [18].

Table 1

Comparison of exosomal sensing platforms.

Method	Sensing principle	Target	Sensitivity	Throughput	Reference
nPLEX	SPR	Protein	~10^3 exosomes	High (>100 arrays)	18
μNMR	Magnetic resonance	Protein	~10^4 exosomes	Low (single channel)	17
ExoChip	Fluorescence	CD63+/Rab5+ EVs	0.5 pM	Low (three channels)	27
SOMAscan	Fluorescence	Protein	100 fM	Very high (1,129)	28
Microfluidic chip	Fluorescence	Protein	0.3 pg/mL	Low (single channel)	26
SPRi	SPR	Protein	5×10^7 exosomes/cm^2		29
BEAMing & digital PCR	Fluorescence	RNA	0.01% (mutant detection)	High	25
miRNA microarray/NanoString	Fluorescence	miRNA		Very high (> 1,000)	30

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