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Analyses of Intravesicular Exosomal Proteins Using a Nano-Plasmonic System

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Abstract

Extracellular vesicles (EVs), including exosomes, are nanoscale membrane particles shed from cells and contain cellular proteins whose makeup could inform cancer diagnosis and treatment. Most analyses have focused on surface proteins while analysis of intravesicular proteins has been more challenging. Herein, we report an EV screening assay for both intravesicular and transmembrane proteins using a nanoplasmonic sensor. Termed iNPS (intravesicular nanoplasmonic system), this platform used nanohole-based surface plasmon resonance (SPR) for molecular detection. Specifically, we i) established a unified assay protocol to detect intravesicular as well as transmembrane proteins; and ii) engineered plasmonic substrates to enhance detection sensitivity. The resulting iNPS enabled sensitive (0.5 μ L sample per marker) and high-throughput (a 10×10 array) detection for EV proteins. When applied to monitor EVs from drug-treated cancer cells, the iNPS assay revealed drug-dependent unique EV protein signatures. We envision that iNPS could be a powerful tool for comprehensive molecular screening of EVs.

Graphical Abstract

We report on an EV screening assay for both intravesicular and transmembrane proteins using a nanoplasmonic sensor. Termed iNPS (intravesicular nanoplasmonic system), this platform used nanohole-based surface plasmon resonance (SPR) for molecular detection.

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Supporting Information Available

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Surface plasmon resonance; Extracellular vesicles; Exosomes; Nanohole arrays; Biomarkers; Cancer

Analysis of extracellular vesicles (EVs) is emerging as a new biomarker tool to detect and manage clinical cancers. EVs are membrane-bound phospholipid vesicles actively secreted by cancer cells.^{1–7} EVs have been reported to contain molecular constituents of originating cells, including transmembrane and intracellular proteins,⁸ mRNA,⁹ DNA,¹⁰ and microRNA,¹¹ thus serving as potential cellular surrogates.^{12,13} Given their abundance and structural stability, EVs can reflect global tumor burden and heterogeneity, overcoming sampling biases.¹⁴ Moreover, the amount and molecular profiles of cancer-derived EVs correlate with tumor burden and treatment efficacy.

We previously developed a nanoplasmonic system (NPS) for quantitative EV protein detection.^{3,15} The system uses two-dimensional periodic nanohole arrays as a base sensing element; light illumination to the periodic nanoholes excites surface plasmons that mediate intense optical transmission.^{16,17} Such extraordinary light transmission is exquisitely sensitive to the nanohole surface condition, which we exploited to detect EVs. The first prototype system achieved $>10^2$ fold higher sensitivity than ELISA while using small sample volumes ($0.5\ \mu\text{L}$) per protein marker.³ Using cancer patient-derived samples, we further showed that cancer-specific EVs could be identified based on unique transmembrane protein signatures.^{3,15} This system, however, was based on capturing whole EVs, detecting only transmembrane proteins present on EV surfaces. Conversely, many proteins affected by drug treatment are cytosolic/intravesicular proteins.¹⁸ Specifically, most drugs directly target intracellular proteins, or affect them through signaling cascades following membrane protein binding. To use EVs as surrogate readouts for drug responses, it is thus necessary to advance a quantitative assay for intravesicular proteins.^{19–23}

Here, we report an important expansion of the NPS analytical capabilities. Termed iNPS (intravesicular nanoplasmonic system), this next generation system is now able to detect both transmembrane and intravesicular proteins on a single device with high sensitivity and throughput. Specifically, molecular targets in EV lysates are first captured on the plasmonic chip, and subsequently labeled with gold nanoparticles (AuNPs) for signal amplification. The resulting iNPS assay offers a single, unified assay format regardless of target protein location in EVs to promote throughput and automation. We applied iNPS to screen protein markers in cancer-derived EVs. Our results showed that EVs carry both membrane and

cytosolic proteins whose expression levels correlate with parental cells. Moreover, we observed that drug treatment modulated intravesicular protein levels, which could have implications for treatment monitoring through EV analysis.

RESULTS AND DISCUSSION

We first re-engineered the iNPS chip aiming for higher throughput and sensitivity. The basic plasmonic unit periodic nanohole arrays in a 100 nm-thick Au film. Light transmission through the periodic nanoholes is mediated by surface plasmon resonance excited on Au-dielectric interfaces (Au-water and Au-substrate, Fig. 1a). The resonance peak wavelengths of transmission spectrum through the nanoholes are thus related to the refractive indices of sensing medium (*i. e.* aqueous solution) and chip substrate. In the previous plasmonic system,³ we patterned nanoholes on an Au-deposited glass substrate. This structure, however, was found suboptimal with aqueous samples. Because glass has a refractive index ($n_s = 1.45$) close to that of water (1.33), the resonance peak from the Au-glass interface could overlap with an Au-water peak (Fig. 1b). The overlap broadens the optical spectrum of transmitted light, lowering the overall detection sensitivity.²⁴ We reasoned that substrates with higher n_s than glass could be used to increase the inter-peak distance. Finite-difference time-domain (FDTD) simulation showed a progressive peak separation with increasing n_s (Fig. S1). Indeed, when we implemented Au nanoholes on a SiN substrate ($n_s = 2.1$), the Au-water peak was well-isolated (Fig. 1c, *right*), facilitating much more robust spectral analyses.

To enable higher-throughput analysis, we devised a microarray-type detection system (Fig. 1d). The plasmonic chip had 100 sensing sites (a 10×10 array, Fig. S2), which were mounted on an automated scanning stage for spectral measurements. Because iNPS operated in a co-linear transmission mode, the optical setup was simplified; we built the system around a conventional upright microscope by adding a mini-spectrometer. To scale up the chip fabrication, we used interference lithography (Fig. S3);^{3, 25} the entire 4-inch Si/SiN wafer (SiN thickness, 200 nm) was patterned with nanoholes, and then diced into individual chips. The nanohole dimensions were 200 nm in diameter and 500 nm in periodicity in a 100 nm-thick Au film (Fig. 1e). The coefficients of variation of peak wavelengths within chips were measured to be $< 1\%$ (Fig. S4). A titration experiment with varying concentrations of EVs showed a detection limit of 10^4 EVs (Fig. S5).

The previous NPS iteration captured whole EVs by targeting transmembrane proteins. The size of EVs matched with NPS sensing range,³ which enabled high detection sensitivity. The method, however, is incompatible with intravesicular proteins. To detect both intravesicular and transmembrane proteins, we explored a new assay format, specifically adopting a “forward phase array” concept (Fig. 2a). We first lysed EVs to expose all proteins, and immuno-captured target proteins on specific detection sites. Protein capture alone after lysis generated negligible spectral shifts. To improve detection sensitivity, we labeled targets with AuNPs in a similar size of EVs (Fig. 2b). AuNP-labeling amplified electromagnetic fields through plasmonic coupling between particles and device surface.^{26, 27} FDTD simulation showed significant field enhancement with AuNP binding (~ 70 -fold in field intensity, Fig. 2c), when compared to binding of protein or a whole EV (diameter, 100 nm). Indeed, when

concentration-matched EV and AuNP samples were measured by iNPS, the spectral shifts were about 9-fold larger with AuNP binding (Fig. 2d).

Figure 2e shows the validation of the developed iNPS assay. The whole EV capture (Fig. 2e, *left*) detected proteins only on the EV surface (*i. e.*, EpCAM and CD63), missing the intravesicular protein (*i. e.*, AKT1). Applying EV lysates (10^8 EVs) generated small spectral shifts (< 0.2 nm) due to low molecular weights (< 25 kDa) of captured proteins (Fig. 2e, **middle**). Further labeling targets with AuNPs (100 nm), however, enabled the detection of both transmembrane (EpCAM and CD63) and intravesicular (AKT1) proteins (Fig. 2, *right*).

We next assessed the analytical performance of iNPS. First, EVs were collected from conditioned cell culture media, and lysed (see Supporting Information). EV lysates were then introduced to the plasmonic chip for target capture, wherein measurement sites were pre-functionalized with antibodies. We then coupled molecular-specific AuNPs to captured targets. Aliquots of samples were also processed by ELISA for comparison. Readouts from both assays showed a good match (Fig. 3a and Fig. S6; $R^2 = 0.8248$), confirming the analytical capacity of the new iNPS assay. The iNPS assay, however, required smaller amounts of samples (0.5 μ L per marker) compared to ELISA (100 μ L per marker). We further compared the expression of protein markers both in cells and EVs. Seven markers, representing intravesicular (AKT1, HSP90, HSP70, TSG101) and transmembrane (CD63, EpCAM, EGFR) proteins, were measured in ovarian cancer cell lines (OVCAR3, OV420, CaOV3) and a benign cell line (TIOSE). We used the iNPS assay for EV lysates, and flow cytometry for cells. Protein expression patterns in EVs correlated well with those observed in parental whole cells (Fig. 3b and Fig. S7).

We next applied iNPS to monitor EV protein profiles upon drug treatment. As a model drug, we used 17AAG, an HSP90 inhibitor, which is known to modulate intracellular proteins by inhibiting the protein folding activity of HSP90. Specifically, HSP90 inhibition dissolves HSP90, HSP70, and the HSF1 complex. Free HSF1 triggers up-regulation of HSP90 and HSP70, whereas AKT1 (HSP90 substrate) is down-regulated due to insufficient protein folding.^{28–31} We treated OV90 cells with either 17AAG (10 μ M, Fig. S8) or vehicles (DMSO). Following 48-hour treatment, both EVs and cells were harvested. EV profiles by iNPS affirmed the known mechanism (Fig. 4a). Both HSP90 and HSP70 expression increased, and AKT1 levels decreased in EVs, whereas the expression of non-HSP90 substrates (*i. e.*, EpCAM and CD63) were unaffected. In addition, we observed unexpected TSG101 decrease. These iNPS results were further confirmed by western blotting (Fig. 4b).

We extended the treatment experiments by using an EGFR inhibitor (gefitinib). We treated OV429 cells with or without gefitinib (20 μ M, Fig. S9) and collected EVs from media after 48 hours of treatment. The iNPS assay revealed unique changes in EV protein profiles. In concentration-matched EV samples, we unexpectedly observed significant increases in EGFR, EpCAM, HSP70, HSP90 and CD63 and decreases in TSG101 with drug treatment (Fig. 4c), which aligned with EV western blotting (Fig. 4d, *right* and Fig. S10). The cellular level of these proteins, however, were unchanged with gefitinib treatment (Fig. 4d, *left*), paralleling the working mechanism of the drug: gefitinib regulates EGFR phosphorylation,

not EGFR itself. Detecting phosphorylated EGFR (p-EGFR) with iNPS, however, was challenging; we found no good antibody pair for both p-EGFR capture and detection.

CONCLUSIONS

In summary, we developed the iNPS assay platform for comprehensive EV protein analyses. Benefiting from plasmonic engineering and nanoparticle-mediated signal amplification, the system achieves high detection sensitivity. It is also scalable for high-throughput and expanded EV screening; i) the new assay strategy can detect both transmembrane and intravesicular proteins in a single device format; and ii) the assay offers high throughput using a microarray-type iNPS chip while consuming small volumes of samples ($<0.5 \mu\text{L}$ per marker). Applying iNPS, we profiled a group of transmembrane and intravesicular proteins in EVs derived from ovarian cancer cells. We observed a good match in expression profiles between unperturbed cells and their EVs. When cells were perturbed with molecular drugs, however, EVs displayed drug-specific protein signature different from cells. Similar observations were reported for EVs from other cell lines and with different drugs.^{19–22} These suggest the need for further study on drug-mediated EV protein patterns to maximize EVs' value for treatment monitoring.

Certain aspects of the iNPS assay could be further improved. First, from time to time we observed spurious signals presumably caused by the binding of AuNP aggregates. We expect to minimize this artifact through AuNP engineering, particularly its surface chemistry.³² Second, the accuracy of the iNPS assay depends on finding a pair of antibodies for target capture and detection. Using antibody pairs could improve detection specificity. However, for some targets (*e. g.*, phosphor-EGFR), a good pair may not be readily available, which could limit the range of detectable targets. Therefore, antibody screening or selection of alternative affinity ligands (*e. g.*, aptamers) should be exploited. Similarly, the iNPS could be also applied to detect other types of EV biomarkers (*e. g.*, mRNA, miRNA) by oligonucleotides as affinity ligands. Long-term efforts to develop multiplexed assays for EV proteins and RNA biomarkers could render the iNPS a powerful tool to reveal and catalog such patterns and help attain significant clinical inroads.

EXPERIMENTAL SECTION

Cell culture

OV90, OVCAR3, OVCA420, and TIOSE6 cells were grown in RPMI-1640 medium (Cellgro). CaOV3 were cultured in Dulbecco's modified essential medium (Cellgro). All media were supplemented with exosome depleted 10% fetal bovine serum (FBS) and penicillin–streptomycin (Cellgro). All cell lines were tested and were free of mycoplasma contamination (MycoAlert mycoplasma detection kit, Lonza, LT07-418).

EV isolation

We used a standard method based on ultracentrifugation to harvest EVs from cell culture media. Cells at passages 1–15 were cultured in vesicle-depleted medium (with 5% depleted FBS) for 48 h. Conditioned medium from 10^7 cells was collected and centrifuged at $300 \times g$ for 5 min. Supernatant was filtered through a $0.2 \mu\text{m}$ membrane filter (Millipore) and

concentrated by $100,000 \times g$ for 1 h. After the supernatant was removed, the EV pellet was washed with PBS and centrifuged at $100,000 \times g$ for 1 h. The EV pellet was resuspended in PBS.

Antibody pair selection

We tested >4 antibody pairs for each EV marker and selected a pair that produced the highest iNPS signal. Isotype control antibodies were used for measuring background signals from non-specific binding, cross-reactions, and/or unknown variables. The net difference between antibody pair and isotype control antibodies was the signal for EV markers.

Biotinylation of labeling antibodies

Sulfo-NHS-biotin (10 mM, Pierce) solution in PBS was incubated with antibodies for 2 h at room temperature. Unreacted sulfo-NHS-biotin was removed using Zeba spin desalting column, 7K MWCO (Thermo Scientific). Antibodies were kept at 4 °C until use.

Preparation of antibody conjugated Au nanoparticles (AuNPs)

100-nm neutravidin coated AuNPs (Nanopartz) were mixed with biotinylated antibodies at room temperature for 1 h. Unbound antibodies are removed after centrifuge at $3000 \times g$ for 2 mins. Antibody bound AuNPs were washed with PBS twice and resuspended in PBS (1% BSA). Prepared particles were kept at 4 °C until use.

FDTD simulation

Three-dimensional, finite-difference time-domain (FDTD) simulations were performed by using a commercial software (FDTD solutions, Lumerical Solution Inc.). A unit cell consisted of a single hole with a 200-nm diameter made in a 100-nm thick Au film. Periodic boundary conditions in x - and y -directions were used for an infinite array of periodic nanoholes. A minimum grid size of 2 nm in a nonuniform mesh was used. The refractive indices of glass and silicon nitride substrates were set to 1.45 and 2.10, respectively. The complex dielectric constants for Au were obtained from the Palik's model.³³

iNPS chip preparation

Periodic nanoholes (220 nm in diameter and 500 periodicity) were partially patterned in a 200-nm thick nitride film on a double-polished 4-inch silicon wafer by interference lithography and reactive ion etching (RIE). A 60 nm nitride layer was remained in the nanohole patterns to protect the silicon during potassium hydroxide (KOH) etching. The other side of wafer was patterned through optical lithography and RIE etching to define 10×10 sensing arrays. Anisotropic etching of silicon in a KOH solution formed a free-standing nitride film on each sensing array. The remaining 60 nm nitride layer was then removed by RIE. An electron-beam evaporator was used to deposit a 100 nm gold film with a 2 nm titanium adhesion layer. The deposition rates were set to 2 (Au) and 0.5 (Ti) Å/sec. The size of each sensing arrays was $100 \mu\text{m} \times 100 \mu\text{m}$ and the arrays were separated by 2 mm. The entire chip size was 25×25 mm.

iNPS measurements

Antibody immobilization on the chip was performed based on the previous protocol.³ Briefly, the chip was first biotinylated by incubating with a 1:3 (v/v) mixture of two heterobifunctional polyethylene glycols (PEGs) (Biotin-PEG-SH, 1 kDa, Nanocs; Methyl-PEG-SH, 0.2 kDa, Fisher Scientific; 10 mM in PBS) overnight at room temperature. Neutravidin (50 µg/mL in PBS with 0.2% BSA, Thermo Scientific) was then used as a linker between the modified surface and biotinylated antibodies. Antibodies (10 µg/ml in PBS with 0.2% BSA) were dropped to individual sensing units (0.5 µL each) by using a nanoarray spotter (DigiLab, Inc) and kept for 45 min at room temperature with humidity. The chip with antibodies was measured by a spectrometer (USB4000-UV-VIS-ES, Ocean Optics, Inc.) for baseline spectra before incubating with EV lysate.

EVs (2×10^{11} /mL in PBS, 45 µL used) were lysed in RIPA buffer with phosphatase inhibitor (Thermo Scientific) for 15 min at 4 °C and diluted in 1.5% BSA (1:3 v/v). Half of the chip with 50 sensing units (12 different antibodies with 4 replicates) were covered with 200 µL of EV lysate in BSA for 1 hr, then washed with PBS. Antibody-conjugated AuNPs (2.3×10^9 /mL in 2% BSA) was spotted on each antibody-immobilized sensing unit (0.5 µL each) by using a microarray spotter and kept for 40 min at room temperature with humidity. The chip was then gently washed in PBS, and each sensing unit was measured in the same way performed for the baseline spectrum. The spectral shift from antibody-conjugated to AuNP captured sensing unit was calculated by a custom-built software program (MATLAB R2015a, MathWorks Inc.).

Enzyme-linked immunosorbent assay

CD63 and IgG1 antibodies (Ancell) were diluted to 5 µg/mL in PBS and added to the Maxisorp 96-well plate (Nunc), respectively, for overnight incubation at 4 °C. After being washed with PBS, a blocking solution with 2% BSA in PBS was added to the plate and incubated for 1 h at room temperature. Subsequently, $\sim 10^8$ EVs in 100 µL of PBS were added to each well for 1 h incubation at room temperature. After the blocking solution was removed, antibodies (1 µg/mL) against various markers were added to each well and incubated at room temperature for 1 h. Unbound antibodies were triple washed with PBS. Streptavidin–horseradish peroxidase (HRP) molecules were added to the each well for 1 h at room temperature. After being washed out with PBS, chemiluminescence signals were measured.

Flow cytometry

We prepared 1.5×10^6 cells per each antibody for flow cytometry experiments. Cells were fixed with a fix/perm solution (BD Science) for 30 min at 4 °C and then washed with PBS. Subsequently, cells were blocked with 1% BSA in PBS and then incubated with primary antibodies (4 µg/mL). After primary antibody incubation, cells were washed and incubated with fluorophore-conjugated secondary antibody (2 µg/mL; Abcam). After washing, fluorescence signals from the labeled cells were measured using BD LSRII flow cytometer (BD Biosciences). Measured mean fluorescent intensities were normalized using the following formula [(signal – IgG isotype control)/secondary]. Blocking and incubation with

antibodies (primary and secondary) were performed for 30 min each at room temperature. Every washing step comprised three 5 min washes at 300 g with PBS.

Western blotting

Cells (1×10^5 cells) and EVs (2×10^{11} /ml) were lysed in RIPA buffer containing protease inhibitors (Thermo Scientific). The protein concentrations were quantified using a bicinchoninic acid assay (BCA assay, Thermo Scientific). Protein lysates were resolved with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto polyvinylidene fluoride membranes (PVDF, Invitrogen) and immunoblotted with antibodies against HSP90 (k3720a, Biolegend), HSP70(4876, Cell signaling), AKT1 (Y89, Abcam), TSG101 (GTX118736, Genetex), EGFR (E114, Abcam) and EpCAM (MOC-31, Abcam). All antibodies were used at a 1,000-fold dilution (Cell Signaling). Following incubation with a HRP-conjugated secondary antibody (Cell Signaling), a chemiluminescence substrate was used for immunodetection (Thermo Scientific).

Drug treatment

Drug concentrations were determined based on AKT and pEGFR levels in OV90 and OV429 cell lines (Fig. S3 and S4). 1.5×10^5 cells were seeded in 6 well plate 24 h before compounds treatment. Various concentration of HSP90 inhibitor, 17-AAG (Selleck Chem) or EGFR inhibitor, Gefitinib (Selleck Chem) were added to OV90 or OV429 cells for 24 h or 48 h. Cells were washed with PBS and kept at -80°C until use. 100 μL RIPA buffer with haltTM phosphatase inhibitor (Thermo) was added to each well for cell lysis and scrapped for cell lysate harvest and centrifuged at $14,000 \times g$, 4°C for 5 mins. Supernatant was transferred to a new tube and protein concentration was measured with BCA kit (Thermo). Cell lysate was mixed with 4 $\times$ NuPAGE LDS sample buffer (Life Technologies) and boiled at 90°C for 5 mins. The protein was dissolved in were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto polyvinylidene fluoride membranes (PVDF, Invitrogen) and immunoblotted with antibodies against GAPDH (2118, Cell Signaling), HSP90 (k3720a, Biolegend), HSP70 (4876, Cell signaling), AKT1 (Y89, Abcam), EGFR (ab24293, abcam), and p-EGFR (ab5652, abcam). The concentration and incubation time of compounds were determined based on western blot data (17-AAG: 10 μM , 48 h; Gefitinib: 20 μM , 48 h).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, Becker A, Hoshino A, Mark MT, Molina H, Xiang J, Zhang T, Theilen TM, Garcia-Santos G, Williams C, Ararso Y, Huang Y, Rodrigues G, Shen TL, Labori KJ, Lothe IM, Kure EH, Hernandez J, Doussot A, Ebbesen SH, Grandgenett PM, Hollingsworth MA, Jain M, Mallya K, Batra SK, Jarnagin WR, Schwartz RE, Matei I, Peinado H, Stanger BZ, Bromberg J, Lyden D. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol.* 2015; 17:816–826. [PubMed: 25985394]
- Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, Molina H, Kohsaka S, Di Giannatale A, Ceder S, Singh S, Williams C, Soplop N, Uryu K, Pharmed L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T, Zhang H, Hernandez J, Weiss JM, Dumont-Cole VD, Kramer K, Wexler LH, Narendran A, Schwartz GK, Healey JH, Sandstrom P, Labori KJ, Kure EH, Grandgenett PM, Hollingsworth MA, de Sousa M, Kaur S, Jain M, Mallya K, Batra SK, Jarnagin WR, Brady MS, Fodstad O, Muller V, Pantel K, Minn AJ, Bissell MJ, Garcia BA, Kang Y, Rajasekhar VK, Ghajar CM, Matei I, Peinado H, Bromberg J, Lyden D. Tumour exosome integrins determine organotropic metastasis. *Nature.* 2015; 527:329–335. [PubMed: 26524530]
- Im H, Shao H, Park YI, Peterson VM, Castro CM, Weissleder R, Lee H. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. *Nat Biotechnol.* 2014; 32:490–495. [PubMed: 24752081]
- Jeong S, Park J, Pathania D, Castro CM, Weissleder R, Lee H. Integrated Magneto-Electrochemical Sensor for Exosome Analysis. *ACS Nano.* 2016; 10:1802–1809. [PubMed: 26808216]
- Liang K, Liu F, Fan J, Sun D, Liu C, Lyon CJ, Bernard DW, Li Y, Yokoi K, Katz MH. Nanoplasmonic quantification of tumour-derived extracellular vesicles in plasma microsamples for diagnosis and treatment monitoring. *Nat Biomed Eng.* 2017; 1:0021. [PubMed: 28791195]
- Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, LeBleu VS, Mittendorf EA, Weitz J, Rahbari N, Reissfelder C, Pilarsky C, Fraga MF, Piwnica-Worms D, Kalluri R. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature.* 2015; 523:177–182. [PubMed: 26106858]
- Shao H, Chung J, Balaj L, Charest A, Bigner DD, Carter BS, Hochberg FH, Breakefield XO, Weissleder R, Lee H. Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy. *Nat Med.* 2012; 18:1835–1840. [PubMed: 23142818]
- Graner MW, Alzate O, Dechkovskaia AM, Keene JD, Sampson JH, Mitchell DA, Bigner DD. Proteomic and immunologic analyses of brain tumor exosomes. *FASEB J.* 2009; 23:1541–1557. [PubMed: 19109410]
- Skog J, Wurdinger T, Van Rijn S, Meijer DH, Gainche L, Curry WT, Carter BS, Krichevsky AM, Breakefield XO. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol.* 2008; 10:1470–1476. [PubMed: 19011622]
- Balaj L, Lessard R, Dai L, Cho YJ, Pomeroy SL, Breakefield XO, Skog J. Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nat Commun.* 2011; 2:180. [PubMed: 21285958]
- Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvald JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol.* 2007; 9:654–659. [PubMed: 17486113]
- Kalra H, Simpson RJ, Ji H, Aikawa E, Altevogt P, Askenase P, Bond VC, Borrás FE, Breakefield X, Budnik V, Buzas E, Camussi G, Clayton A, Cocucci E, Falcon-Perez JM, Gabrielson S, Gho YS, Gupta D, Harsha HC, Hendrix A, Hill AF, Inal JM, Jenster G, Kramer-Albers EM, Lim SK, Llorente A, Lotvall J, Marcilla A, Mincheva-Nilsson L, Nazarenko I, Nieuwland R, Nolte-'t Hoen EN, Pandey A, Patel T, Piper MG, Pluchino S, Prasad TS, Rajendran L, Raposo G, Record M, Reid GE, Sanchez-Madrid F, Schiffelers RM, Siljander P, Stensballe A, Stoorvogel W, Taylor D, Thery C, Valadi H, van Balkom BW, Vazquez J, Vidal M, Wauben MH, Yanez-Mo M, Zoeller M, Mathivanan S. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. *PLoS Biol.* 2012; 10:e1001450. [PubMed: 23271954]

13. Santiago-Dieppa DR, Steinberg J, Gonda D, Cheung VJ, Carter BS, Chen CC. Extracellular vesicles as a platform for 'liquid biopsy' in glioblastoma patients. *Expert Rev Mol Diagn.* 2014; 14:819–825. [PubMed: 25136839]
14. Basik M, Aguilar-Mahecha A, Rousseau C, Diaz Z, Tejpar S, Spatz A, Greenwood CM, Batist G. Biopsies: next-generation biospecimens for tailoring therapy. *Nat Rev Clin Oncol.* 2013; 10:437–450. [PubMed: 23799370]
15. Yang KS, Im H, Hong S, Pergolini I, Del Castillo AF, Wang R, Clardy S, Huang CH, Pille C, Ferrone S, Yang R, Castro CM, Lee H, Del Castillo CF, Weissleder R. Multiparametric plasma EV profiling facilitates diagnosis of pancreatic malignancy. *Sci Transl Med.* 2017; 9:eal3226. [PubMed: 28539469]
16. Brolo AG. Plasmonics for future biosensors. *Nat Photon.* 2012; 6:709–713.
17. Dahlin AB. Sensing applications based on plasmonic nanopores: The hole story. *Analyst.* 2015; 140:4748–4759. [PubMed: 25675146]
18. Eder J, Sedrani R, Wiesmann C. The discovery of first-in-class drugs: origins and evolution. *Nat Rev Drug Discov.* 2014; 13:577–587. [PubMed: 25033734]
19. Lunavat TR, Cheng L, Einarsdottir BO, Olofsson Bagge R, Veppil Muralidharan S, Sharples RA, Lässer C, Gho YS, Hill AF, Nilsson JA, Lötvall J. BRAF^{V600} inhibition alters the microRNA cargo in the vesicular secretome of malignant melanoma cells. *Proc Natl Acad Sci USA.* 2017; 114:E5930–E5939. [PubMed: 28684402]
20. Montermmini L, Meehan B, Gamier D, Lee WJ, Lee TH, Guha A, Al-Nedawi K, Rak J. Inhibition of oncogenic epidermal growth factor receptor kinase triggers release of exosome-like extracellular vesicles and impacts their phosphoprotein and DNA content. *J Biol Chem.* 2015; 290:24534–24546. [PubMed: 26272609]
21. Lv LH, Wan YL, Lin Y, Zhang W, Yang M, Li GL, Lin HM, Shang CZ, Chen YJ, Min J. Anticancer drugs cause release of exosomes with heat shock proteins from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses in vitro. *J Biol Chem.* 2012; 287:15874–15885. [PubMed: 22396543]
22. van Dommelen SM, van der Meel R, van Solinge WW, Coimbra M, Vader P, Schifflers RM. Cetuximab treatment alters the content of extracellular vesicles released from tumor cells. *Nanomedicine.* 2016; 11:881–890. [PubMed: 27021928]
23. Shao HL, Chung J, Lee K, Balaj L, Min C, Carter BS, Hochberg FH, Breakefield XO, Lee H, Weissleder R. Chip-based analysis of exosomal mRNA mediating drug resistance in glioblastoma. *Nat Commun.* 2015; 6:6999. [PubMed: 25959588]
24. Cetin AE, Etezadi D, Galarreta BC, Busson MP, Eksioğlu Y, Altug H. Plasmonic Nanohole Arrays on a Robust Hybrid Substrate for Highly Sensitive Label-Free Biosensing. *ACS Photonics.* 2015; 2:1167–1174.
25. Yanik AA, Cetin AE, Huang M, Artar A, Mousavi SH, Khanikaev A, Connor JH, Shvets G, Altug H. Seeing protein monolayers with naked eye through plasmonic Fano resonances. *Proc Natl Acad Sci U S A.* 2011; 108:11784–11789. [PubMed: 21715661]
26. Hall WP, Ngatia SN, Van Duyne RP. LSPR Biosensor Signal Enhancement Using Nanoparticle-Antibody Conjugates. *J Phys Chem C.* 2011; 115:1410–1414.
27. Hong X, Hall EA. Contribution of gold nanoparticles to the signal amplification in surface plasmon resonance. *Analyst.* 2012; 137:4712–4719. [PubMed: 22950078]
28. Basso AD, Solit DB, Chiosis G, Giri B, Tschlis P, Rosen N. Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. *J Biol Chem.* 2002; 277:39858–39866. [PubMed: 12176997]
29. Nimmanapalli R, O'Bryan E, Kuhn D, Yamaguchi H, Wang HG, Bhalla KN. Regulation of 17-AAG-induced apoptosis: role of Bcl-2, Bcl-XL, and Bax downstream of 17-AAG-mediated down-regulation of Akt, Raf-1, and Src kinases. *Blood.* 2003; 102:269–275. [PubMed: 12623837]
30. Wang Y, McAlpine SR. N-terminal and C-terminal modulation of Hsp90 produce dissimilar phenotypes. *Chem Commun.* 2015; 51:1410–1413.
31. Workman P. Auditing the pharmacological accounts for Hsp90 molecular chaperone inhibitors: Unfolding the relationship between pharmacokinetics and pharmacodynamics. *Mol Cancer Therapeutics.* 2003; 2:131–138.

32. Park YI, Kim E, Huang CH, Park KS, Castro CM, Lee H, Weissleder R. Facile Coating Strategy to Functionalize Inorganic Nanoparticles for Biosensing. *Bioconjug Chem.* 2017; 28:33–37. [PubMed: 27792877]
33. Palik ED. Handbook of optical constants of solids. 1998

Figure 1. Detection of EV proteins with iNPS sensor

(a) The basic unit of the sensor is periodic nanoholes made in a thin gold film. Light transmission through the nanoholes is mediated by surface plasmon resonance excited on Au-dielectric interfaces shown by the Finite-difference time-domain (FDTD) simulation. (b, c) Transmission spectra of fabricated nanoholes with a glass substrate (b) and a silicon nitride substrate (c). (d) Photograph of iNPS sensor chip with 100 sensing sites. (e) A scanning electron micrograph of periodic nanoholes in the iNPS chip. The hole diameter is 200 nm and the periodicity is 500 nm. The Au film thickness is 100 nm.

Figure 2. New iNPS assay

(a) EVs are lysed to release molecular cargos. Each target is captured on the iNPS chip *via* affinity ligands, and further labeled with Au nanoparticles (AuNPs). Note that a single assay format is used both for transmembrane and intravesicular proteins. (b) Scanning electron micrograph showing AuNPs after iNPS assay steps. (c) FDTD electromagnetic simulation. AuNP on the iNPS surface concentrates electrical fields. Compared to a protein binding (left) or a whole EV binding (middle), the field intensity enhanced up to 70-fold with AuNP (right). (d) Measured signal enhancement. Compared to EV binding, the spectral shift was about 9-fold higher when the same concentration of AuNPs (100 nm) bound to the iNPS chip. (e) Validation of iNPS assay. AuNPs enables both membrane protein (EpCAM, CD63) and intravesicular protein (AKT1) detection with enhanced spectral shifts. The error bars represent the standard deviation of signals.

Figure 3. Assay validation

(a) iNPS and ELISA were used to detect intravesicular (HSP90, HSP70, TSG101) and transmembrane proteins (CD63, EpCAM, EGFR). The results from both methods showed a good match ($R^2 = 0.8248$). For iNPS assays, we used 10^8 EVs per marker and measured spectral shifts. Note that ELISA required 200-fold larger amount of samples than iNPS. (b) Protein profiling of ovarian cells and their secreting EVs. Transmembrane (CD63, EpCAM, EGFR) and intravesicular (AKT1, HSP90, HSP70, TSG101) protein levels were measured. Cellular protein expression of three ovarian cancer cell lines (OV420, CaOV3, and OVCAR3) and one normal cell lines (TIOSE6) were measured by flow cytometry. EVs secreted from those cells were analyzed by iNPS. Overall EV protein expression patterns were correlated with cellular expression patterns.

Figure 4. Drug response monitoring by EV protein analysis

(a) OV90 cells were treated with HSP90 inhibitor (17-AAG, 10 μ M) for 48 h in conditioned media. EVs secreted from OV90 were collected and the lysates were analyzed by iNPS (10^8 EVs per marker). Protein level fold changes after drug treatment were calculated from iNPS spectral shifts which were proportional to the amount of target proteins captured on the sensing surface. We observed the increase of HPS90 and HSP70, and the decrease of AKT1, which reflects the known working mechanism of the inhibitor. (b) 17-AAG mediated protein expression change of OV90 cell and EVs were monitored *via* western blotting. EV protein profiles matched with iNPS results. (c) OV429 cells were treated with 20 μ M EGFR inhibitor (gefitinib) for 48 h in conditioned media, and EVs were analyzed by iNPS. Significant increases in EGFR, EpCAM and CD63 were observed with EVs from drug-treated cells. The error bars represent the standard deviation of signals. (d) Gefitinib mediated protein expression change of OV429 cell and EVs were monitored *via* western blotting. Note the expression differences between cells and EVs upon treatment.