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WHITE PAPER

Extracellular vesicle quantification in complex matrices

White paper 5 | Version 2 | Kris Ver Donck, Filip Delport, Kim Stevens
Based on original publication: Yildizhan et al. (2021) Journal of Extracellular Vesicles. 10 (4)

Abstract

Fiber-optic surface plasmon resonance (FO-SPR) is a powerful tool that harnesses the performance of surface plasmon resonance (SPR) in an easy-to-use dip-in fiber-optic configuration. This white paper describes a sensitive FO-SPR assay for the specific detection and quantification of extracellular vesicles, a particularly challenging task due to the heterogeneity of EVs and the complexity of the matrices in which they are typically found, such as culture medium or blood plasma.

The bioassay buffers and EV-specific antibody pairs were optimized in developing the bioassay described here, which was capable of detecting EVs at 10^3- and 10^4-times lower concentrations than those typically found in human blood plasma from healthy donors and cancer patients, respectively (1). Furthermore, endogenous EVs in cell culture medium could be detected without prior purification or enrichment. Finally, MCF7 breast cancer cell EVs spiked in blood plasma were selectively captured on the FO-SPR surface using anti-EpCAM antibody.

We discuss how this FO-SPR sandwich bioassay can provide a fast, specific, and accurate alternative to other available methods of EV detection directly in crude samples, thereby avoiding bias from laborious manual purification steps. FO-SPR can simplify and improve EV quantification and analysis in this fast-developing field.

Extracellular vesicles (EVs) are gaining attention due to their potential as a minimally invasive method for the diagnosis, prognosis, and therapy of a wide range of diseases, including diabetes, cardiovascular disease, and cancer. They are nanometer-sized particles that are secreted by cells and carry lipids, proteins, nucleic acids, and metabolites in the intercellular matrices and into the blood. This allows the identification of disease-specific proteomic and genomic biomarkers from a simple blood sample.

Characterizing EVs, however, poses a range of challenges. They are highly heterogeneous in their size, origin, and molecular composition and the biofluids in which they are found are complex and, therefore, difficult to analyze reliably. There are multiple techniques available to analyze EVs, including ELISA, western blot, and flow cytometry, but many of them suffer from problems such as inaccuracy and bias from time-consuming sample purification such as ultracentrifugation, reliance on expensive or complex instruments, or an inability to process the complex matrices in which EVs are found.

SPR is a technique that has demonstrated great potential for EV analysis, with reported limits of detection of 10^7 – 10^8 particles/mL in purified samples in buffer. However, the most commonly used instruments are expensive and bulky and rely on microfluidics which makes them prone to clogging when using complex matrices. Furthermore, the microfluidics on these platforms pose a risk of cross-contamination and impede signal amplification techniques using gold nanoparticles (AuNPs) that can improve sensitivity.

FO-SPR offers a valuable alternative to conventional SPR and the other EV analysis methods mentioned above. The White FOx is a convenient benchtop FO-SPR instrument that combines the performance of microfluidic SPR devices with much reduced cost and complexity. Here, the gold layer used for SPR is applied to fiber-optic probes which can simply be dipped into the sample of interest. This approach eliminates the contamination and clogging issues often encountered when analyzing a series of crude samples with microfluidic instruments.

A FO-SPR method optimized to detect recombinant EVs (rEVs) (1) was tested to demonstrate its effectiveness in analyzing EVs in crude matrices without sample purification (2). First, the assay's effectiveness in detecting rEVs spiked in cell culture medium was determined. The next step was to test the assay's specificity in detecting MCF7 breast cancer EVs spiked in 100-fold diluted blood plasma. Anti-EpCAM was used to distinguish breast cancer cell EVs expressing this biomarker from all other EVs originating from healthy cells in the plasma samples used.

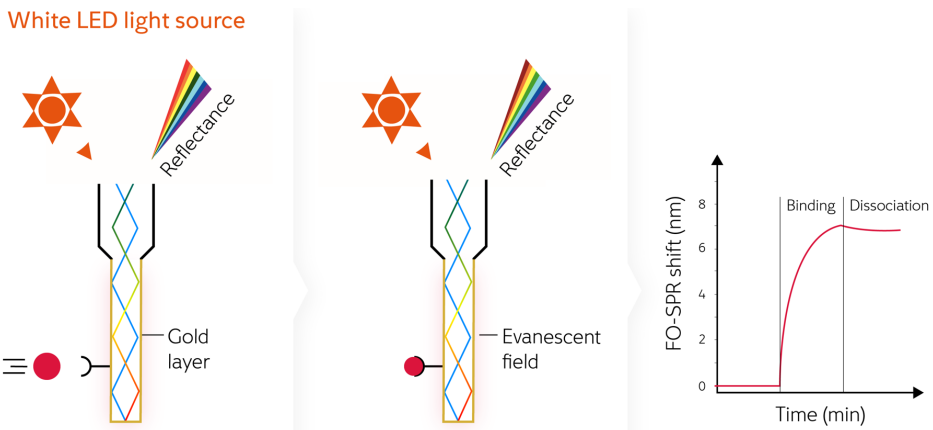
Materials and methods

What is FO-SPR?

FOx BIOSYSTEMS turned an optical fiber into a mass-sensitive sensor using the well-established SPR principle for biomolecular interaction analysis.

The SPR effect is achieved by coupling a white light source to the fiber-optic sensor probe. In this consumable probe, the light interacts with a gold layer and senses the refractive index up to 200 nm away from the outer surface. At the end of the probe, light is reflected back through the bifurcated fiber to a spectrometer. The resonance condition is monitored by tracking the wavelength at which the least light is reflected.

The FO-SPR sensor probe surface is coated with bioreceptor molecules which can bind to the target molecules of interest. This binding changes the refractive index, resulting in a wavelength shift that produces a sensor signal as shown in Figure 1. The sensor probe is simply dipped into the liquid sample to measure biomolecular interactions directly. Figure 2 shows a schematic assay buildup for a FO-SPR assay, including capture molecule immobilization with label-free EV detection, as well as a sandwich assay for enhanced sensitivity.



1. Analyte binds target.

2. Mass difference changes refractive index, altering the spectrum of reflected light.

3. Spectrometer readout is converted to real-time binding and dissociation curves.

Figure 1: The FO-SPR principle

Tools and reagents

To perform the procedures in this application you will need:

- White FOx instrument with FOx-SPR acquisition software and the FOx data processing tool
- Carboxyl probes for label-free quantification (FOx BIOSYSTEMS product nr: 30.0003)
- Microsoft Excel or other data calculation software for data handling and viewing
- Micro pipettes from 10 to 1000 µL with disposable tips
- On-desk refrigeration for protein solutions
- A microtube vortex mixer and centrifuge

Buffer/Reagent	Concentration
Dulbecco's modified Eagle's medium (DMEM) with 0.01 M Hepes, 1 mM sodium pyruvate, 10% heat-inactivated ED-FBS	1X
Media collected from MCF7 and HEK293 cell cultures for EV extraction	N/A
Capture antibodies: mouse anti-CD9 (# EX201-100), mouse anti-CD63 (# EX204-100) [Cell Guidance Systems], mouse anti-EpCAM (# 324202) [Biolegend], goat anti mouse IgG (anti-IgG) (# 31430) [Life Technologies]	Dissolve to 1 mg/mL stock
MES: 2-(N-morpholino) ethanesulfonic acid, pH 5.0, 5.5, 6.0, 6.5	50 mM
Sodium acetate, pH 5.2, 5.6	10 mM
Biotinylated detection antibodies: ^{B}anti-CD63 (# 353018), ^{B}anti-CD9 (# 312110) and ^{B}anti-CD81 (# 349514) [Biolegend]	Dissolve to 1 mg/mL stock
EDC: 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide	0.4 M in EDC/NHS
NHS: N-hydroxysuccinimide	0.1 M in EDC/NHS
PBS: Phosphate buffer saline, pH 7.4	10 mM
40 nm diameter gold nanoparticles coated with goat anti-biotin antibody [BBI Solutions]	OD 10
Bovine serum albumin (BSA)	

Table 1: Reagents and buffers. All solutions were prepared with deionized water purified by a Milli-Q Plus system.

Note: Special attention is required for EDC/NHS which is a highly reactive and short-lived reagent (EDC user guide, Sigma Aldrich) and should be prepared just prior to use. Separately dissolve NHS and EDC no more than 5 minutes before use, mix in equal quantities, and pipet into the designated wells immediately before use.

Alternatively, you can also prepare pre-dissolved aliquots of EDC and of NHS, respectively, and store them at -20°C until use as explained in the white paper number 1 about immobilization on carboxyl probes. Thaw one aliquot of each at room temperature for about 20 minutes before use and mix in the designated wells just before placing the reagent in the instrument.

EV isolation

MCF7 EVs and rEVs were separated from MCF7 and HEK293 cell culture media, respectively, via sequential centrifugation and filtration, as described by Geeurickx et al (1). The EVs were quantified and characterized using nanoparticle tracking analysis (NTA) to create particle size distribution plots and stored at -80°C until use.

Label-free and sandwich FO-SPR bioassay optimization to detect rEVs in buffer

To determine the optimal probe immobilization conditions for EV detection, FO-SPR probes were functionalized with three different EV-specific antibodies (anti-CD9, anti-CD63, and anti-EpCAM) diluted in three different buffers (50 mM MES pH 6, 10mM sodium acetate pH 5.2, and 10 mM sodium acetate pH 5.6). 10 mM sodium acetate at pH 5.6 was used for subsequent experiments as it gave the optimal FO-SPR shift for the three antibodies (2).

For a detailed method to optimize antibody immobilization to the FO-SPR probes, please consult white paper 1: "Carboxyl probe immobilization for label-free protein quantification".

The optimal protocol for label-free EV detection was determined by testing a range of detection buffer pH (50 mM MES pH 5, 5.5, 6, 6.5, and PBS pH 7.4). 50 mM MES pH 6 was used as a detection buffer for subsequent experiments as it provided the lowest variability in FO-SPR shift.

Real-time label-free monitoring of rEV binding over 20 minutes was tested using a range of rEV concentrations (0 – 2 x 10^9 particles/mL) to be used as a reference for subsequent investigations. Please note the lower concentrations will not produce a visible label-free signal and are progressed into a labelled assay for detection.

To further amplify the signal for this assay, the probes from the EV samples were incubated with 10 µg/mL of three different biotinylated detection antibodies (^{B}anti-CD63, ^{B}anti-CD9 and ^{B}anti-CD81) for 30 minutes and subsequently immersed in 1 OD goat anti-biotin conjugated gold nanoparticles suspended in PBS with 0.5% BSA for one hour without shaking.

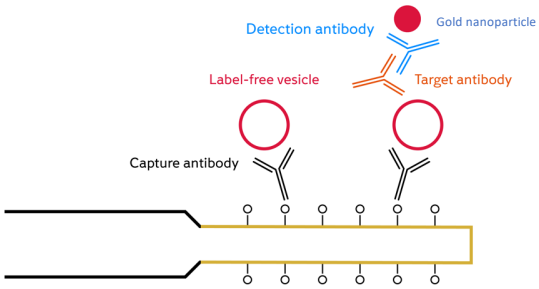


Figure 2: Schematic assay build-up of label-free and sandwich assays for EV detection and quantification.

Testing the specificity of rEV binding to the probe

In order to test the binding specificity of rEVs to the probe surface and to exclude non-specific surface charge effects, EV binding to probes functionalized with rEV-specific anti-CD9 was measured and compared to probes functionalized with anti-IgG serving as a negative control. To this end, both the SPR shift (Figure 3) and native fluorescence (not shown) were tested.

Detecting rEVs / EVs in cell culture medium and plasma

The method described above was used to detect:

1. rEVs at 2 × 10^9 particles/mL
2. Endogenous HEK293 EVs at 6.8 × 10^8 particles/mL
3. MCF7 EVs spiked in 100-fold diluted plasma samples at 2 × 10^9 particles/mL

rEVs [1.] and HEK293 EVs [2.] were both detected directly in DMEM cell medium supplemented with 10% ED-FBS using anti-CD63 as capture antibody and ^{B}anti-CD9 as detection antibody. MCF7 EVs [3.] were analyzed using anti-EpCAM as capture antibody and ^{B}anti-CD63 as detection antibody.

Data analysis

The experimental data were recorded using the White FOx, and further processed in MS Excel. The background signal (i.e., the FO-SPR signal obtained from blank measurements, using the same matrix without EVs added) was subtracted from all FO-SPR shifts prior to building calibration curves. Calibration curves were fitted across the measured concentration range by applying non-linear curve fitting using the GraphPad Prism specific binding equation: $Y = \frac{A*X}{B+X}$

The measured limits of detections (LODs) were determined as the blank signal plus 3 times the standard deviation of the blank signal. The signal to noise ratios (SNR) were calculated by dividing the specific signal by the background obtained for samples without EVs (0 particles/mL).

Results

rEV binding specificity

To determine whether the detection buffer pH could induce attraction to the sensor surface, thereby increasing both specific and non-specific EV binding, rEV binding to FO-SPR probes functionalized with rEV-specific anti-CD9 or anti-IgG (negative control) was compared via FO-SPR and fluorescence microscopy (image data not shown). The bioassay sensorgram indicated that rEV binding to anti-CD9 gave a signal six times greater than the negative control, anti-IgG (Figure 3), indicating specific rEV binding to the FO-SPR probe functionalized with rEV specific antibody.

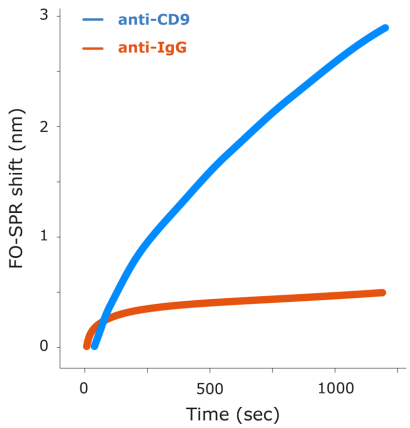


Figure 3: Comparison of rEV (3×10^9 particles/mL) label-free binding specificity to probes functionalized with specific (anti-CD9) and non-specific (anti-IgG) antibodies by FO-SPR shift. Adapted from Yildizhan et al. (2021).

Quantifying rEVs in buffer using a FO-SPR sandwich bioassay

The two best antibody pairs were determined to be anti-CD9/^{B}anti-CD81 and anti-CD63/^{B}anti-CD9 and these were used to build the calibration curves for a series of rEV concentrations (3.125 x 10^7 – 2 x 10^9 particles/mL) in buffer and a negative control of 0 particles/mL (Figure 4). The calibration curves showed that the FO-SPR biosensor successfully detected rEVs across the entire tested range for both antibody pairs, giving LODs of 3.125 × $10^7$and 3.459 × 10^7 particles/mL for anti-CD9/^{B}anti-CD81 and anti-CD63/^{B}anti-CD9 antibody combinations, respectively. These are 10^3 and 10^4 times lower than the expected physiological concentrations of EVs in healthy human plasma and cancer patients, respectively.

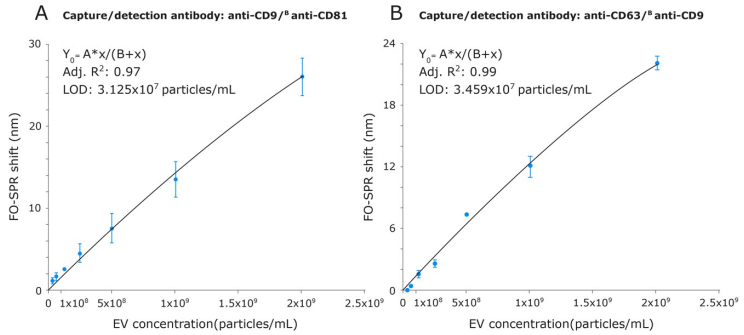


Figure 4: FO-SPR shifts for a range of rEV concentrations down to 3.1x10^7 particles/mL using (a) anti-CD9 / ^{B}anti-CD81 and (b) anti-CD63 / ^{B}anti-CD9 capture/detection antibody pairs. Error bars represent standard deviation (n=3). Adapted from Yildizhan et al. (2021).

Detecting EVs in complex matrices using a FO-SPR sandwich assay

The effectiveness of the developed assay for detecting EVs in complex media was first investigated by using the anti-CD63/^{B}anti-CD9 combination to detect rEVs (2 x 10^9 particles/mL) spiked in DMEM cell culture medium with 10% ED-FBS. This gave a signal to noise ratio (SNR) of 28.65, showing that rEV detection was specific, even in this complex matrix (Figure 5a).

To further test the assay, endogenous EVs were detected directly in HEK293 cell culture supernatant (DMEM medium supplemented with 10% ED-FBS) at a concentration of 6.8 x 10^8 particles/mL as determined by NTA. This investigation showed a SNR of 34.50 (Figure 5b).

Finally, to assess the assay's ability to detect disease related EVs among a population of EVs from healthy cells, a biomarker not commonly found in EV populations from healthy cells was chosen as a model system. EVs from the breast-cancer MCF7 cell line, which express the tumor-specific EpCAM biomarker, were spiked into blood plasma and investigated using the anti-EpCAM/^{B}anti-CD63 capture/detection antibody pair, together with AuNPs in a sandwich bioassay configuration. The SNR (31.39) and measured FO-SPR shifts demonstrated the successful detection of 2 x 10^9 particles/mL (Figure 5c). Although these results cannot be compared with the two other models tested in this section because of the differing bioassay settings, antibodies, and protein expression levels for these EVs, they still demonstrate FO-SPR's excellent potential for detecting cancer-specific biomarkers for the quantification and molecular profiling of cancer-derived EVs, to be confirmed and elaborated on in further research.

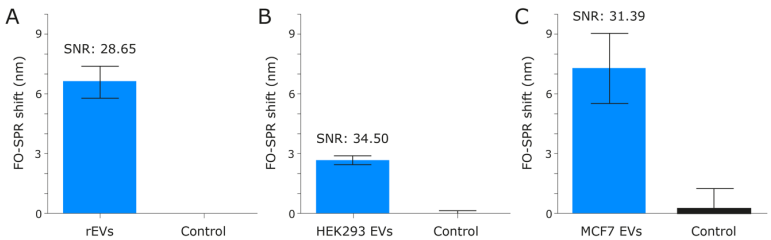


Figure 5: FO-SPR shifts obtained when detecting (a) rEVs spiked in DMEM cell culture medium with 10% ED-FBS, (b) endogenous HEK293 EVs at 6.8 x 10^8 particles/mL in the same medium, (c) MCF7 EVs spiked at 2 x 10^9 particles/mL in 100-fold diluted blood plasma. Capture and detection antibodies: (a) + (b): anti-CD63 / ^{B}anti-CD9, (c) anti-EpCAM / ^{B}anti-CD63. Adapted from Yildizhan et al. (2021).

Conclusions

The reliable detection and quantification EVs is becoming increasingly desirable due to their potential in disease diagnosis. However, their heterogeneity and the complexity of the matrices in which they are found, together with a lack of reference EV for assay development, and the bias introduced from time-consuming and difficult-to-scale sample purification steps, cause difficulties for many bioanalytical methods.

FO-SPR provides a unique opportunity to detect EVs directly in complex matrices, with minimal processing, thanks to its dip-in configuration. In this white paper, we describe a FO-SPR assay capable of detecting EVs with LODs 10^3 times lower than the expected physiological concentration of EVs in healthy human plasma and 10^4 times lower than EV concentration in plasma of cancer patients (1). FO-SPR, therefore, has a great potential for sensitive EV analysis, particularly for analyzing EV subpopulations with specific biomarkers, which are typically at a much lower concentration in patient samples.

The high sensitivity was achieved using gold nanoparticles functionalized with anti-biotin antibodies, in combination with two capture / detection antibody pairs (anti-CD9/^{B}anti-CD81 and anti-CD63/^{B}anti-CD9). These pairs were used to generate calibration curves to determine the LOD values of 3.125×10^7 and 3.459×10^7 particles/mL, respectively.

The optimized assay was robust enough to be able to detect three different EVs (rEVs, HEK293 endogenous EVs and MCF7 breast cancer cell EVs) in two different complex matrices (cell culture medium and 100-fold diluted blood plasma). Specifically, using an anti-EpCAM antibody allowed the distinction of MCF7 EVs from EVs without this biomarker from healthy cells.

FO-SPR shows remarkable sensitivity for EV detection and quantification. Its dip-in probe configuration allows it to measure directly in complex matrices like cell culture medium and blood plasma, furthermore, it can even distinguish cancer-specific EVs from those originating from healthy cells using a cancer-specific biomarker. Combined with its real-time monitoring, fast time to result, parallel measurements and ease of operation, FO-SPR shows huge potential for EV research where other methods have proven sub-optimal.

The authors gratefully acknowledge the MeBioS research group at KU Leuven and their partners at Ghent University and KU Leuven, for their elaborate research on FO-SPR applications in crude samples published in reference (2) which provided the basis of the method and results shared in this white paper.

References

1. E. Geeurickx et al. (2019). The generation and use of recombinant extracellular vesicles as biological reference material. Nature Communication 10, 1–12.
2. Y. Yildizhan et al. (2021) FO-SPR biosensor calibrated with recombinant extracellular vesicles enables specific and sensitive detection directly in complex matrices. Journal of Extracellular Vesicles. 10 (4), e12059; DOI: 10.1002/jev2.12059

Related white papers

White paper 1: Carboxyl probe immobilization for label-free protein quantification, describes how to optimize immobilization to the carboxyl FO-SPR probes.

White paper 2: Sensitive protein quantification in crude serum samples: comparison of FO-SPR and ELISA, describes a sensitive sandwich FO-SPR protocol for quantifying proteins in complex matrices.

White paper 3: Sensitive protein quantification in blood, dried blood spots, serum and plasma, describes a ten-minute FO-SPR protocol for quantifying proteins in complex matrices, including whole blood.

White paper 4: Label-free protein quantification in bioproduction samples, describes how to set up a reproducible label free FO-SPR assay and calibration curve.

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