Multiplexed Phenotyping of Small Extracellular Vesicles Using Protein Microarray (EV Array)

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Abstract

The Extracellular Vesicle (EV) Array is based on the technology of protein microarray and provides the opportunity to detect and phenotype small EVs from unpurified starting material in a high-throughput manner (Jørgensen et al., J Extracell vesicles 2:1–9, 2013). The technology was established to perform multiplexed phenotyping of EVs in an open platform. This protocol outlines the microarray printing procedure followed by the steps of capture and detection of small extracellular vesicles from plasma/serum or cell culture supernatants. The principles of data treatment and analysis are thoroughly described as well.

Key words Protein microarray, Extracellular vesicles, Exosomes, Capture antibodies, Surface antigens, Multiplexed phenotyping

Abbreviations

dAF488	Dextran coupled with Alexa Fluor 488
sEV	small Extracellular vesicle
IgG	Immunoglobin gamma
ON	Overnight
PBS	Phosphate-buffered saline
RT	Room temperature

1 Introduction

Extracellular vesicles (EVs) are released from healthy as well as diseased cells to facilitate cellular communication. They have a wide variety of names including exosomes, microvesicles, and microparticles. Several methods exist to characterize the protein composition of EVs either related to a surface marker phenotype or to the proteins present in the EV cargo as reviewed by Revenfeld et al. [1]. While the EV phenotype is particularly important in the determination of cellular and subcellular origin, it can in

combination with a protein cargo analysis also provide clues about the functionality of the EVs.

The EV Array is based on the technology of protein microarray and allows the detection and phenotyping of sEVs from unpurified starting material in a high-throughput manner [2]. The technology was developed to perform multiplexed phenotyping of sEVs in an open platform. Protein microarrays are well accepted as powerful tools in the search for antigens or antibodies in various sample types [3, 4] and constitutes basis for a high-throughput method. This provides the opportunity to track interactions and activities of proteins in a large scale. A great advantage of protein microarray is that a large number of proteins can be tracked in parallel. Using the EV Array, it is at present possible to phenotype for at least 60 markers simultaneously [5]. In addition, the technique offers a rapid and highly sensitive method consuming only small quantities of samples and reagents. An outline of the laboratory procedures, requirements, and time frames for the EV Array is illustrated in Fig. 1a.
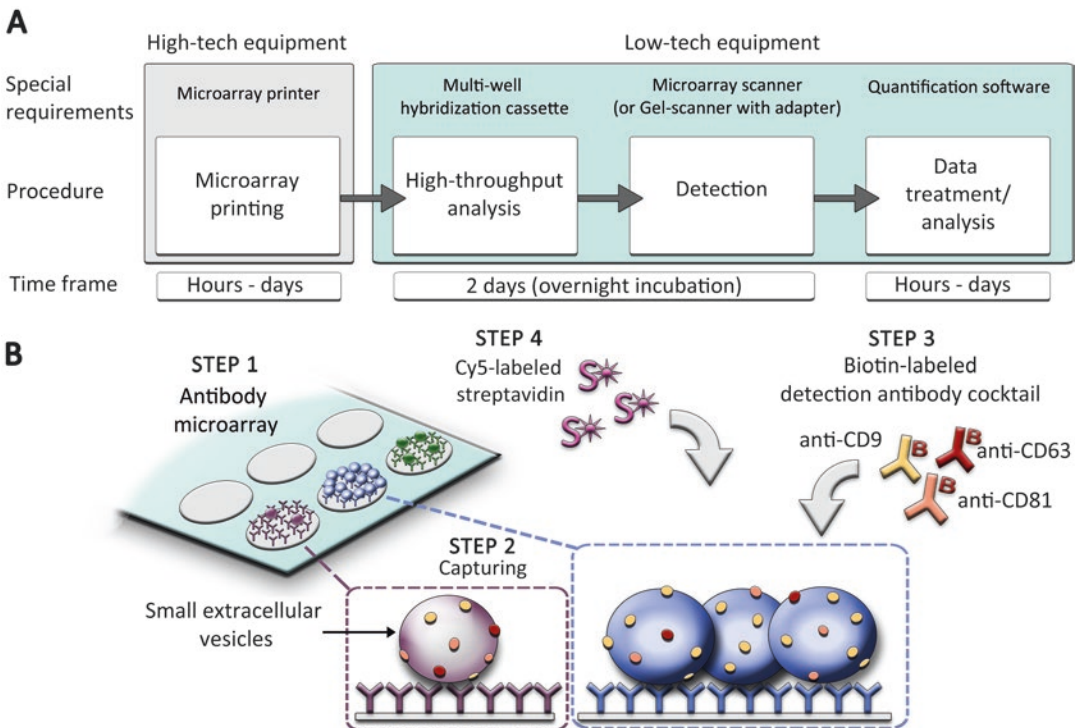

Fig. 1 (**a**) Schematic view of the laboratory procedures, requirements, and time frames for the EV Array. (**b**) Graphical sketch of the EV Array procedure. *STEP 1*: Epoxy-silane-coated microarray slides are the basis for the EV Array. *STEP 2*: EV containing samples (plasma/serum or cell culture supernatants) are applied, and the sEVs are captured onto the slides depending on their specific surface antigens. *STEP 3*: A cocktail of biotin-labeled detection antibodies is applied. Here illustrated with a cocktail of antibodies against the exosomal markers CD9, CD63, and CD81. *STEP 4*: Fluorescently labeled streptavidin is added prior to detection of the captured sEVs. *The time needed for printing and data analysis depends on the number of analytes and samples to be analyzed

Micro-sized spots of capturing antibodies against known EV surface antigens are printed in a customized setup using microarray printing technology (Fig. 1b STEP 1). The EV Array is printed on standard epoxysilane-coated microarray slides in patterns fitting into each well of a multi-well cassette. The use of a 96-well multi-well cassette allows the EV Array to be performed as a high-throughput procedure using a minimum of sample. A maximum of 100 μL of vesicle-containing sample can be applied to each well (Fig. 1b STEP 2). The captured vesicles are detected using a cocktail of biotinylated antibodies against the tetraspanins CD9, CD63, and CD81 (Fig. 1b STEP 3). These markers are regarded as exosomal markers in general [6–8], which is the type of EV that the EV Array is designed to analyse, though other sEVs of the same size are captured as well. This particular antibody combination is applied to ensure that all captured exosomes are detected. If wanted, the focus can easily be changed from exosomes to another type of sEV by replacing the detection antibodies, though subsequent optimization may be needed. Afterward, fluorescently labeled streptavidin is used to quantify the amount of exosomes captured on each spot (Fig. 1b STEP 4). The readout is a fluorescence signal for each individual microarray spot. The method is semiquantitative and determines the phenotype of sEVs for up to 60 protein markers simultaneously.

The EV Array is optimized as a high-throughput analysis where 20 samples can be analyzed simultaneously on each microarray slide. The technique features an overnight (ON) incubation to capture the sEVs and consequently the method can be completed in 2 days. As outlined, the development of the EV Array technology leaves the possibilities open to simply change the detection antibodies in order to detect other populations or subpopulations of sEVs as, e.g., tissue factor-bearing vesicles (Fig. 1b STEP 3). Hereby an open platform is generated, which is easy to transform and optimize further in a research and clinical perspective.

As clarified in Fig. 1a, specific laboratory requirements are necessary to perform an EV Array. The microarray epoxy-coated slides, which are custom-printed with the antibodies against the targeted EV marker proteins, are the basis of the Array. For laboratories without access to microarray printing technologies, several commercial companies offer custom-made microarray slides. To use the EV Array in a high-throughput manner, a simple multi-well cassette to allow multiple Arrays on each slide is required, and to detect the fluorescent signals, either a microarray scanner or laboratory gel scanner is needed.

Using a method such as the EV Array to phenotype sEVs has shown a great potential in diagnostics as highlighted by Jakobsen et al. [9] and Sandfeld-Paulsen et al. [10] where the EV Array was used to phenotype plasma sEVs from NSCLC patients and other lung-diseased controls. By establishing a multi-marker model, it was possible to separate the cancer patients with up to 75.3% accuracy.

2 Materials

Prepare all solutions using purified deionized water (sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (RT) (unless otherwise indicated). When handling the microarray slides, powder-free gloves should be used and touching should be limited to the edges of the slide.

2.1 Printing of Microarray

1. SpotBot Extreme Protein Microarray Spotter (Arrayit Corporation, CA, USA).

2. 946MP4 Pin (Arrayit Corporation, CA, USA).

3. Nexterion® Slide E, epoxysilane-coated glass slides with barcode (SCHOTT Nexterion, Germany).

4. Phosphate-buffered saline (PBS): 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, and adjust to pH 7.4 with HCl. Sterile filter with 0.22 μm filter.

5. Glycerol, minimum 99 % (*see* **Note 1**).

6. Dextran coupled with Alexa Fluor 488 (dAF488), 10.000 MW, anionic, fixable (Life Technologies Corp, USA) (*see* **Note 2**).

7. Capture antibodies of interest: Diluted to 50–300 μg/mL in PBS containing a final glycerol content of 5 % and 50 μg/mL dAF488 (store at –40 °C).

8. Positive control: 50–100 μg/mL biotinylated IgG in PBS containing a final glycerol content of 5 % and 50 μg/mL dAF488 (store at −40 °C).

9. Negative control: PBS containing 5 % glycerol and 50 μg/mL dAF488 (Store at –40 °C).

10. Microarray 384-well microplate (Arrayit Corporation, CA, USA).

11. InnoScan 710 AL microarray scanner (Innopsys, France).

12. Mapix (microarray analysis software, Innopsys, France).

2.2 EV Array Analysis

1. Peristaltic pump.

2. High-Throughput Wash Station (Arrayit Corporation, CA, USA).

3. Magnetic stirrer.

4. Blocking buffer: 50 mM ethanolamine, 100 mM Tris, 0.1 % (v/v) SDS, pH 9.0. Add 3.05 mL ethanolamine solution, 12.1 g Tris, and 5 mL of 20 % SDS solution to approximately 900 mL of water. Mix and adjust pH with NaOH. Add water up to a total of 1 L solution. Sterile filter with 0.22 μm and store at 4 °C.

5. Wash buffer: PBS (*see* Subheading 2.1) containing 0.05% Tween®20 (500 μL per 1 L PBS). Sterile filter with 0.22 μm and store at 4 °C.

6. Multi-well microarray hybridization cassette 4×24 or 1×24 (Arrayit Corporation, CA, USA).

7. Adhesive tape for ELISA plates or similar.

8. Orbital shaker.

9. Rectangular plate, four chambers, transparent, non-treated (Nunc, Thermo Fisher Scientific, MA, USA).

10. Detection antibodies (anti-human CD9/biotin, CD63/biotin, and CD81/biotin, Ancell Corporation, MN, USA; Catalog # 156-030, 215-030, and 302-030, respectively). Antibodies are diluted freshly at 1:1500 with wash buffer (3 mL per slide).

11. Streptavidin Cy®5 conjugate (Life Technologies Corp, USA): The streptavidin Cy®5 conjugate is diluted freshly at 1:1500 in 3 mL wash buffer per slide.

12. Microarray High-Speed Centrifuge (Arrayit Corporation, CA, USA).

13. InnoScan 710 AL microarray scanner (Innopsys, France).

14. Mapix (microarray analysis software, Innopsys, France).

3 Methods

3.1 Printing of Microarray

1. Place the prepared printing solutions in a microarray microplate; 10 μL antibody or control solutions are sufficient.

2. Give the plate a short spin (1 min) to remove air bubbles (*see* **Note 3**).

3. Place the microplate, epoxy-coated slides and one contact-printing pin in the SpotBot Extreme Microarray Printer and manufacture the EV Arrays using the 21-well Multiple Microarray Format function (*see* Fig. 2a and **Note 4**).

4. Keep the temperature at 15–18 °C and the humidity at 55–65% during the printing procedure by using the appertaining cooling bath and humidity controller, respectively (*see* **Note 5**).

5. Use an uncoated glass slide for disposal of 20 preprints after which each printing solution is printed as triplicate in each sub-array (*see* Fig. 2b and **Note 6**).

6. Before applying the next antibody solution, perform a washing procedure on the pin, as recommended by the manufacturer.

7. Quality control of the printing is manually performed using the InnoScan microarray scanner and the Mapix software with the settings of 532 nm at 10 V, PTM at 100%, and a 20 μm resolution (*see* Fig. 2c and **Note 7**).
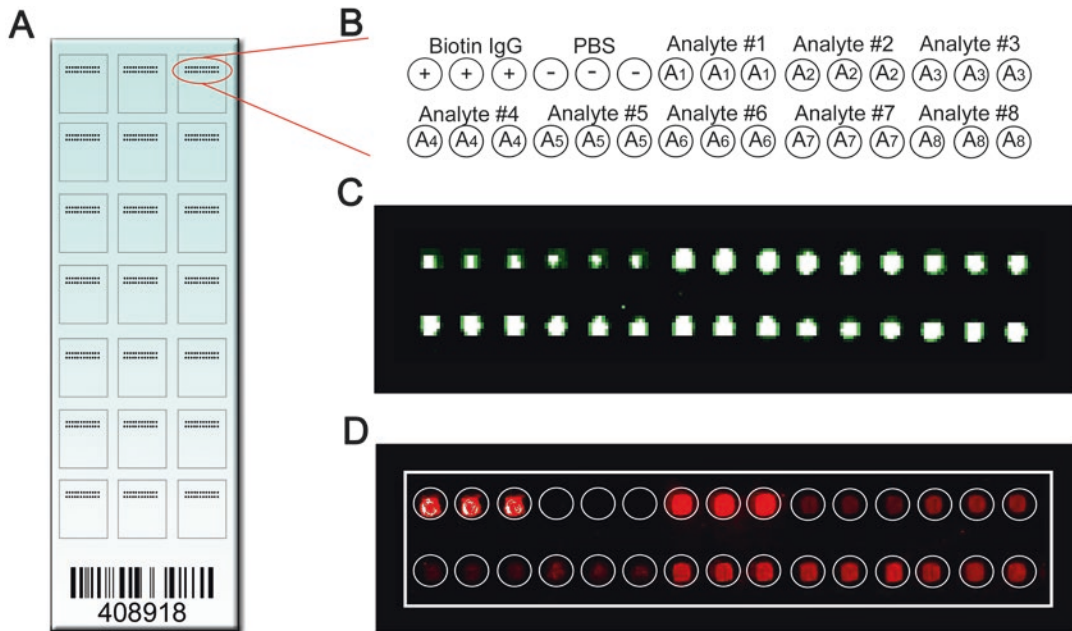
Fig. 2 (**a**) Bar-coded microarray glass slide (7.5 × 2.5 cm) printed in a 21-well setup as sketched out. (**b**) Outline of the printed analytes and controls in triplicates. Here sketched out with eight different analytes. (**c**) Quality control of the printed controls and analytes. Prior to analysis, the quality is validated by scanning at a resolution of 20 μm at 532 nm, a setting of 10 V, and a PMT at 100 %. (**d**) Scanning after capture and detection of sEVs. The scanning is performed at 635 nm, a setting of 10 V, and a PMT of 60 %. During data analysis a created *.GAL file is used to locate the printed spots as illustrated with the *white frame* and *spot circles*

8. Use the SpotBot software to generate a *.GAL file for the data analysis (*see* **Note 8**).

9. The EV Array slides are stored in closed containers at RT ON or longer until performance of the analysis (*see* **Note 1**).

3.2 EV Array Analysis

1. Place the EV Array slides into the gasket in the High-Throughput Wash Station placed on a magnetic stirrer set to 120 rpm.

2. Add 450 mL blocking buffer using the peristaltic pump at a speed of 5 mL/min (*see* **Note 9**).

3. After addition of blocking buffer, the slides are left to block for additional 30 min.

4. Exchange the blocking buffer with 450 mL of wash buffer and set the magnetic stirrer to 200 rpm, which is the setting for the remainder of the analysis.

5. Let the slides wash for 15 min.

6. Place the slides in the hybridization cassette (up to four slides per cassette) (*see* **Note 10**).

7. Load the samples of interest in the individual wells. Samples should be diluted in wash buffer (*see* **Notes 11** and **12**).

8. One well per slide should contain a blank/negative control (wash buffer) (*see* **Note 13**).

9. Seal the hybridization cassette with self-adhesive tape or similar to avoid evaporation.

10. Place the hybridization cassette on an orbital shaker set to 450 rpm for 2 h at RT and afterward incubate the cassette at 4 °C ON (no agitation is needed).

11. Empty the casette and carefully remove the slides from the hybridization cassette and place them in the High-Throughput Wash Station filled with wash buffer for 15 min.

12. Place the slides in the rectangular four chambers plate (one slide in each chamber) together with 3 mL of the detection antibody cocktail per chamber.

13. Place the chamber on an orbital shaker and incubate for 2 h at RT.

14. Wash the slides in the High-Throughput Wash Station for 15 min.

15. Place the slides in another rectangular four chambers plate with 3 mL of the diluted streptavidin Cy®5 conjugate per slide.

16. Incubate on an orbital shaker for 30 min under cover at RT (*see* **Note 14**).

17. Wash the slides in wash buffer for 15 min under cover, exchange the wash buffer with water and wash for additionally 15 min.

18. Dry the slides using the Microarray High-Speed Centrifuge for 20–30 s.

19. Load the slides into the microarray scanner (*see* **Note 15**).

20. Scan the slides at a resolution of 5 μm at 635 nm using 10 V and a PMT at 60 %.

21. Open the *.tif file together with the respective *.GAL file in the Mapix software.

22. Locate all the printed spots either manually or automatically (*see* **Note 16**) using a fixed spot size (e.g., Ø135 μm).

23. Calculate the total spot intensity for each spot and use the save function to generate a *.txt file (*see* Fig. 3).

24. Import the *.txt file contents into Microsoft Excel or another mathematical program, and use the values of total intensity to calculate the relative intensity (RI) for each analyte and each sample (block) (e.g., *see* **Note 17** of calculation).

$$RI_{\text{Analyte}_y;\text{Block}_x} = \text{Log2}\left(\frac{\dfrac{\sum \text{Analyte}_y \text{Block}_x}{N} - \dfrac{\sum \text{Analyte}_y \text{Block}_{\text{Blank}}}{N}}{\dfrac{\sum \text{neg control, Block}_x}{N}} \right)$$

where *N* indicates the number of spot replicates (triplicates).

Block	Column	Row	ID	Name	F635 Total Intensity
1	1	1	Control	pos	13811532
1	2	1	Control	pos	14673881
1	3	1	Control	pos	15579693
1	4	1	Control	neg	49133
1	5	1	Control	neg	45979
1	6	1	Control	neg	52196
1	7	1	Analyte	#1	377353
1	8	1	Analyte	#1	421072
1	9	1	Analyte	#1	426949
1	10	1	Analyte	#2	91207
1	11	1	Analyte	#2	92161
1	12	1	Analyte	#2	98970
1	13	1	Analyte	#3	106520
1	14	1	Analyte	#3	109357
1	15	1	Analyte	#3	116220
⋮	⋮	⋮	⋮	⋮	⋮
21	7	1	Analyte	#1	5685
21	8	1	Analyte	#1	5426
21	9	1	Analyte	#1	5549
⋮	⋮	⋮	⋮	⋮	⋮

Fig. 3 Example of the content of a *.txt file generated after analysis of the EV Array. *Column 1* designates the block number/sample well in the 21-well format. *Columns 2* and *3* indicate the spot placement inside the block. *Columns 4* and *5* give the ID and name of the printed analyte (as indicated in the *.GAL file). The *last column* gives the total intensity of the spot scanned at 635 nm

4   Notes

1. The addition of glycerol ensures that the printed antibodies will stay in solution on the EV Array slides for up to one month at RT. Dried out spots will alter the shape of the processed spots and the signal cannot be accounted as reliable.

2. Addition of dAF488 provides the possibility to perform a quality control of the printed spots using the InnoScan microarray scanner. The dextran molecules do not adhere to the epoxy-coated slides; hence, it will not disturb the following analysis.

3. The centrifuge used is an Eppendorf 5810 centrifuge with rotor A-4-62 and a microplate adapter.

4. The 21-well Multiple Microarray Format function is a software setup that allows for subarray prints, which fit into the 4×24 (or 1×24) multi-well hybridization cassette used in the following EV Array analysis. The use of the 21-well setup instead of the 24-well setup is chosen to avoid printing on the barcode, which may cause the very delicate pins to block. Slides without barcode can be used and will allow printing in a 24-well setup.

5. If the humidity exceeds 75%, there is a major risk that the printing compartment will steam up, which will cause the spots

to blur together. The limits of the steam formation may vary depending on the humidity and temperature parameters in the surroundings.

6. The chosen pin type will deliver approx. 1 nL solution in each spot. The diameter of the spots is approx. 135 μm though it may vary depending on the constituents of the used antibody.

7. Pins used for microarray contact printing are very delicate due to the tiny sample channels, which in the given pin type will take up approx. 250 nL printing solution. Therefore, even though the printing compartment is a closed box and even if great care is taken when the printing is prepared, small dust particles will find their way into the compartment when placed in a non-clean room lab. This will occationally disturb the spotting procedure and therefore needs to be outlined. Scanning at 532 nm makes it easy to point out if spots are missing and, consequently, the print will be discarded in the following analysis.

8. The GenePix Array List (GAL) file describes the size and position of blocks (one per sample well) and the layout of analytes (spots) printed in them, together with the names and identifiers of the printed spots. The *.GAL files can also be generated manually using, e.g., Microsoft Excel.

9. Addition of blocking buffer is a critical step; if the blocking solution is added too fast, it will result in deformed spots (rocket shaped), which may interfere with each other and make the analysis unusable. In the following steps of the analysis, it is no longer necessary to add buffer in this gentle manner.

10. Make sure the hybridization cassette is closed thoroughly or leak may occur. Avoid the slides to dry out on the printed side at any time during the analysis. This will cause a high background signal/fluorescence to appear.

11. Plasma/serum samples: In most cases 10 μL of sample in 90 μL wash buffer is the best compromise of signal versus background. In some cases (low expressed analytes), it is relevant to add more sample, e.g., 20 μL sample and 80 μL wash buffer, but one should note that increasing the amount of sample most likely will increase the background signal, which is described in [5]. The use of different blood collection tubes and the effect of various preanalytical treatments is outlined in [11]. Furthermore, other kinds of bodyfluids have been tested as well and is described in [12].

12. Cell culture supernatant: If the supernatant of a given cell line is to be analyzed for the first time, it is recommended to concentrate it 20–100 times using a 100 K filter (e.g., Amicon Ultra-15 centrifugal filter unit with Ultracel-100 membrane, Merck Millipore). When loaded onto the EV Array, it should be diluted at least 1:1 in wash buffer depending on its concentration. A negative control/culture media control should be included.

When the given cell culture supernatant is characterized 100 μL of non-concentrated, undiluted sample is sufficient. Usually only a small subset of markers is positive when analyzing cell culture supernatant.

13. To streamline the data analysis, it is recommended to use the same well as the negative control (wash buffer) each time. As a standard well number 21 has been used.

14. To avoid the Cy5® fluorescence signal to decay, the slides should be protected from light during the incubation and washing steps.

15. A normal gel scanner (e.g., Typhoon, GE Healthcare) or similar fluorescence scanner can be used instead of the specialized microarray scanner.

16. Using automatically detection of blocks and spots a manually validations is necessary.

17. For this example (*see* Fig. 3), the negative control (wash buffer) is placed in well 21.

$$RI_{Analyte_{\#1};Block_1} = Log2\left(\frac{\dfrac{\sum Analyte_{\#1}Block_1}{N} - \dfrac{\sum Analyte_{\#1}Block_{21}}{N}}{\dfrac{\sum neg\, control,\, Block_1}{N}}\right)$$

$$RI_{Analyte_{\#1};Block_1} = Log2\left(\frac{\dfrac{(377.353 + 421.072 + 426.949)}{3} - \dfrac{(5.685 + 5.426 + 5.549)}{3}}{\dfrac{(49.133 + 45.979 + 52.196)}{3}}\right)$$

$$RI_{Analyte_{\#1};Block_1} = Log2\left(\frac{408.458 - 5.553}{49.103}\right) = 3.04$$

Acknowledgments

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