Identifying extracellular vesicle subpopulations by Free Flow Electrophoresis

Miriam Zentgraf[1], Zeldali Lourens[1], Jan Van Deun[1], Giuseppe Di Fonzo[1], Markart Meckel[3], Gerhard Weber[3], Andreas Baur[1]
[1]Friedrich-Alexander University Erlangen-Nürnberg and University Hospital Erlangen, Department of Dermatology, Erlangen, Germany
[2] Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany
[3]FFE Service GmbH, Feldkirchen, Germany, www.ffeservice.com

1. Introduction

Extracellular vesicle (EV) driven cellular exchange plays an important role in maintaining normal bodily homeostasis. Charge is known to be one of the important factors influencing EV uptake by the receiving cell and EV charge arises from their expression of different surface molecules like proteins, lipids and glycans. Here we describe a standard workflow to identify differently charged EV populations by Free flow electrophoresis (FFE), which is typically used for the separation of various and complex protein mixtures. First, FFE Quality Control (QC) was done by performing two electrophoretic pre-tests to confirm the quality of discrimination of ionic analytes, followed by an additional performance test in order to test the quality and reproducibility of electrophoretic separation. After passing all QC tests, we used FFE to separate purified EVs derived from SK-MEL-37 cells, a melanoma cell line.

2. Methods

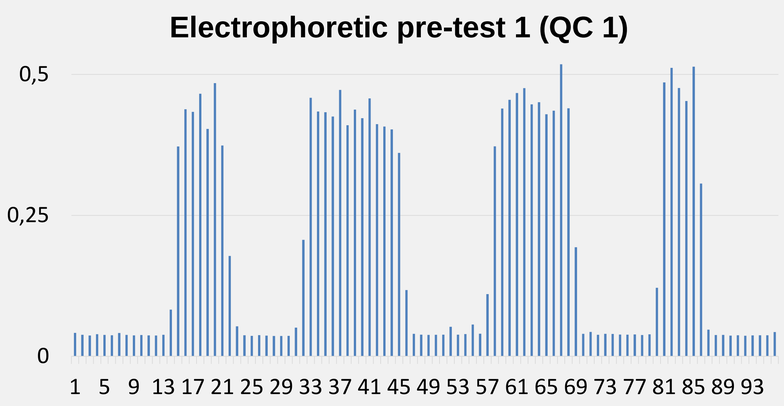
SK-MEL-37 melanoma cells were cultured in RPMI 1640 media supplemented with L-Glutamin, Penicillin/Streptomycin and 10% fetal calf serum. Cell culture supernatant was harvested after 24 hours and concentrated via ultrafiltration. EV isolation was then done by OptiPrep™ Density Gradient Ultracentrifugation (DG), in order to get highly pure vesicles.
For separating the purified EVs via FFE, a separation protocol for continuous FF-ZE-pH processing was performed using several separation media, which differ in conductivity and pH to form a pH gradient. Particle concentration and size measurements of SK-MEL-37 EVs, before and after FFE separation, was done by Nanoparticle tracking analysis (NTA). Additionally, relevant FFE EV fractions were pooled and analyzed regarding their tetraspanin expression (CD63, CD81, CD9) and differences in zeta potential.

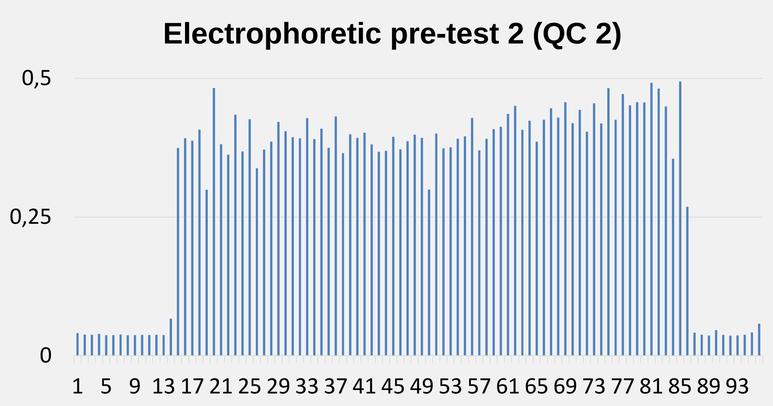
3. Results

1. FFE Quality Control

Purpose of test

Electrophoretic pre-tests (QC 1, QC 2) are part of the FFE quality control (QC) procedures to ensure proper FFE instrument set up and media composition. The stripe output of QC 1 and QC 2has always to be uniform and with clear borders.



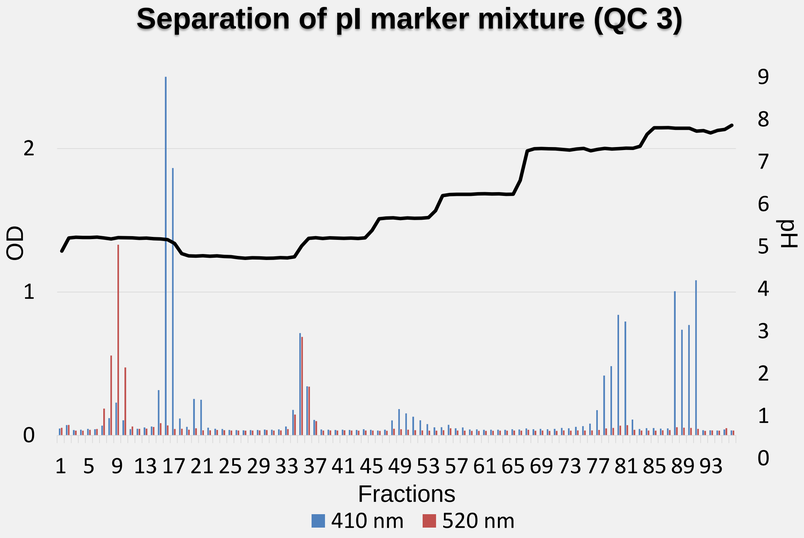


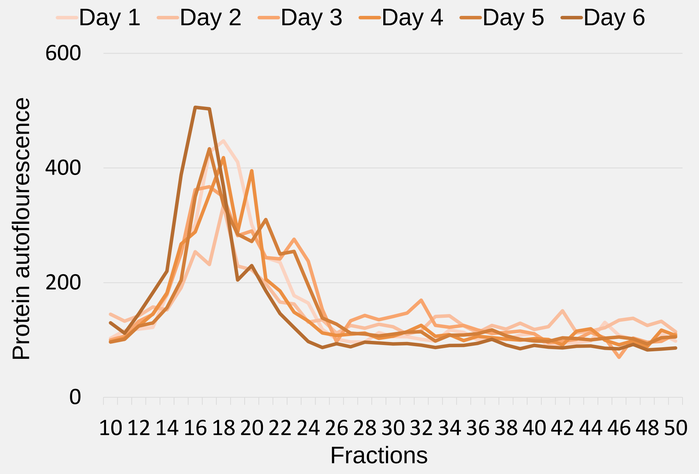
Result

Electrophoretic pre test 1 – QC 1:
Right tubing setup was confirmed by measuring the stripe-profile in the micro titer plate at absorbance 520 nm.
Electrophoretic pre test 2 – QC 2:
No blocked tubes in continuous and uniform stripe area, also verifying right tube and pump adjustments.

2. FFE performance

Checking the FFE performance (QC 3, QCad) includes the testing with separation media, stabilization media, electrolytes, IEF pI-marker mixture and purified control EVs. This is done to evaluate the quality of the pH gradient created for an experiment and to compare the sample reproducibility between different FFE tests.



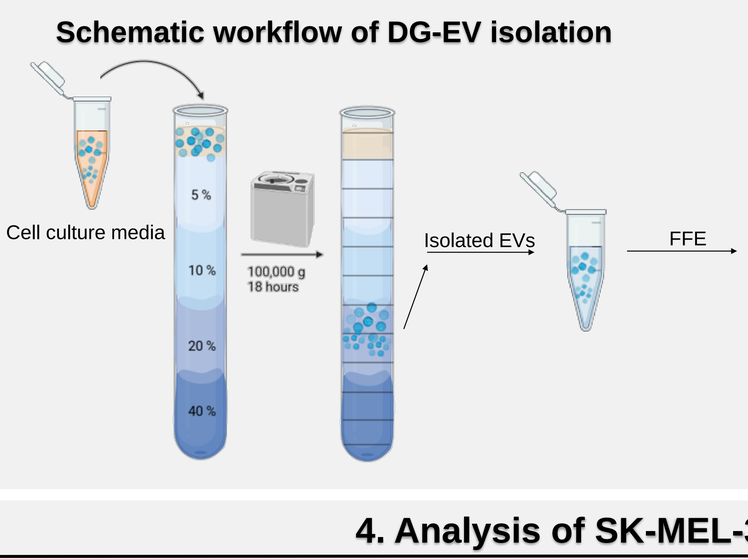


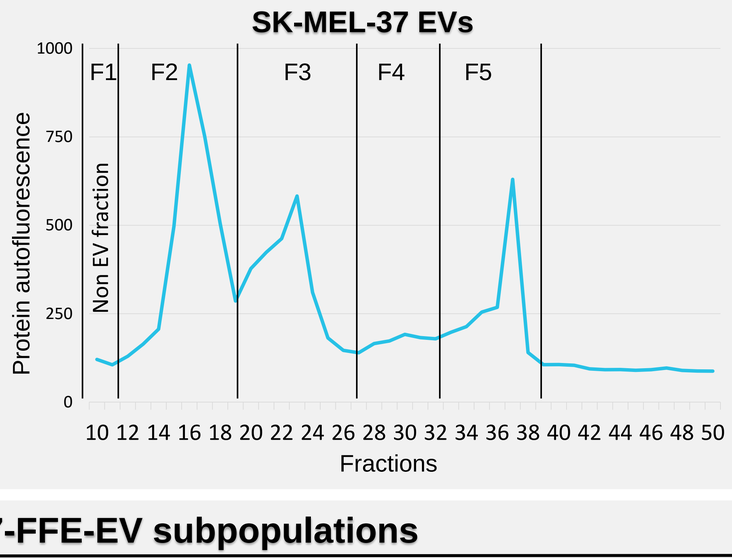
Result

pI marker – QC 3: A known mixture of analytes was separated and deflected towards their isoelectric point (pI) where they stop migration and focus. This is due to the pH gradient created by the IEF buffers, which flows through the electric field.
EV control – QCad: Daily separated purified control EVs showed similar FFE protein pherogram profiles confirming sample reproducibility.

3. Melanoma cell line DG-EVs can be separated into distinct subpopulations by FFE

To get highly pure EVs, non-ionic iodixanol-based OptiPrep™ Density Gradient Ultracentrifugation was the EV isolation method of choice.
After FFE separation of SK-MEL-37 EVs, fractions of interest were then pooled for further analysis regarding their peak profile shown in the protein autofluorescence channel.



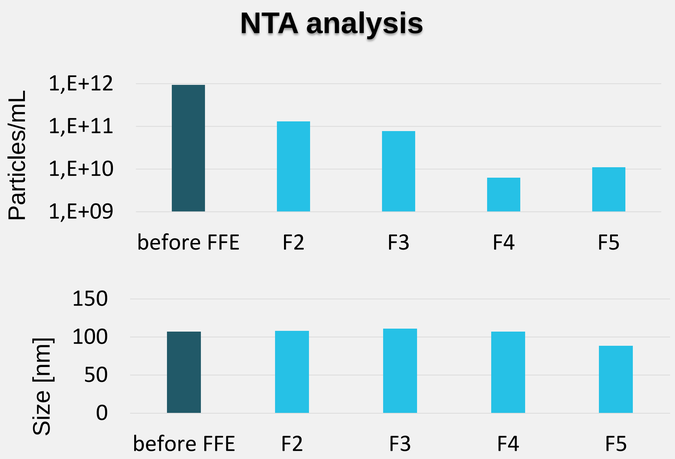


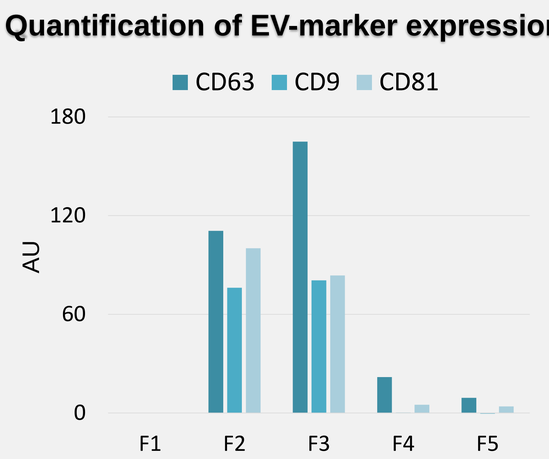
Result

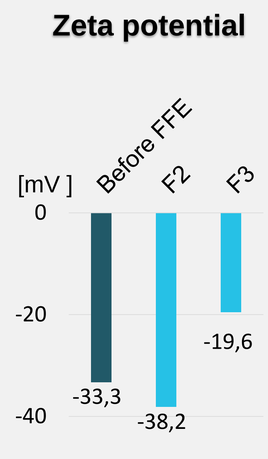
SK-MEL-37 EVs FFE protein pherogram profile showed several distinct peaks and pooled into four subpopulations (F2 -F5) regarding their differences in surface charge.
F1 was used in later experiments as a non EV control, e.g. for the quantification of EV expression markers.

4. Analysis of SK-MEL-37-FFE-EV subpopulations

In order to determine the particle concentration and size of SK-MEL-37 EVs before and after FFE separation, NTA analysis was performed. To investigate differences in surface charge and composition of FFE-EV-fractions, zeta potential and tetraspanin expression was quantified.







Result

NTA analysis of FFE-EV fractions showed a reduction in EV-concentration after FFE, with highest particle concentrations in F2 and F3, whereas EV size hardly changed. In Addition, the highest tetraspanin expression was found in F2 and F3. Zeta potential measurement revealed most negatively charged EVs in F2.

4. Conclusion and Outlook

Here we show that FFE can be used to separate OptiPrep™ DG isolated SK-MEL-37 EVs into several distinct subpopulations by their difference in surface charge. These results highlight the importance of charge based EV characterization and its use as a potenital biomarker health and disease states.

5. Acknowledgements

Conflict of interest: G. Weber, is CEO, M. Meckel is employee of FFE Service GmbH, the manufacturer of the FFE instrument