Free Flow Electrophoresis can display the degree of depletion of proteins from EVs from supernatant of cell culture of Mesenchymal stem cells via various standard techniques, like Ultracentrifugation (UC), precipitation with PEG + UC and tangential ultrafiltration (TFF)

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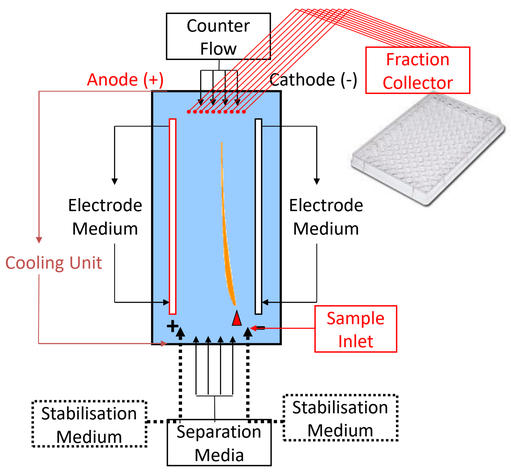
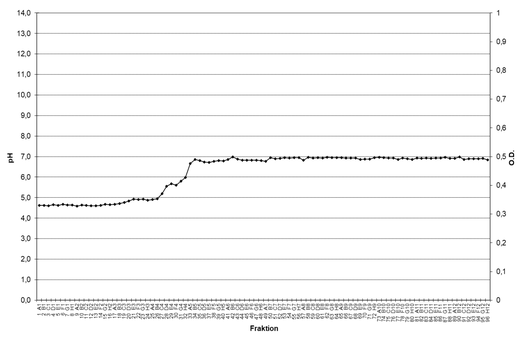
Overview: Free-flow electrophoresis (FFE) is a **matrix-free** electrophoretic separation technique, that separates analytes, ranging in molecular weight from metal ions to whole cells, according to **minimal differences in charge** and isoelectric point (pI). It can be used to obtain micro- to milligram amounts of purified samples for research purposes with excellent resolution. Matrix-free operation in combination with short separation time and cooled environment makes FFE's an ideal tool for preserving the fragile three-dimensional structure of particulate material. The technique is compatible with a variety of electrophoretic modes like IEF, iZE etc. FFE is extremely versatile and can be tailored to the customer's separation needs.
A wide range of protocols already exists for the separation of diverse samples like peptides, protein isoforms, multiprotein complexes, ribosomes, liposomes, nanoparticles, cells, and DNA origami. Here we show its use for the purification of extracellular vesicles from supernatants of cell culture, purified by standard techniques of enrichment of EVs, as Ultracentrifugation (UC), PEG-precipitation +UC and Tangential Flow filtration (TFF).

Principle of FFZE-pH, continuous Free Flow Zone Electrophoresis





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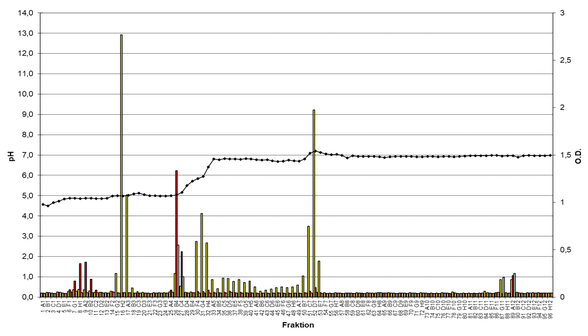
Preformed pH-steps of media



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pH-profile during process

Free Flow Electrophoresis Instrument

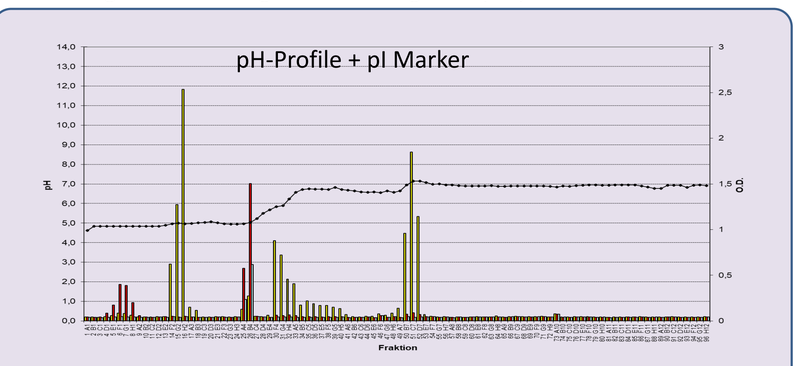
Introduction:

Currently, it remains a challenge to prepare extracellular vesicles (EVs) to high purity, especially those from body liquids, such as plasma. Neither fractionation by density nor by size alone is sufficient to separate EVs from all contaminants e.g. high and low density lipoprotein (HDL/LDL) and other material. For now, a time consuming combination of two methods (density and size separation) is required to enrich EVs to high purity at the expense of time and low recovery.
Free Flow Electrophoresis is a well-established (semi-) preparative method to separate analytes with inherent difference of charge density and/or difference of pI-value.

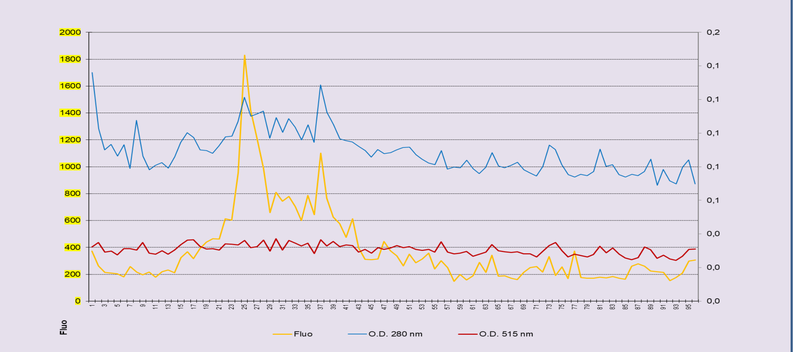
A **Free Flow Zone Electrophoresis (FF-ZE-pH) protocol** has been developed for the purification and isolation of EVs from supernatant of cell culture, using a set of buffer media of different pH-values ranging from pH 4.6 to pH 7. The sample capacity is scalable from 30 µl of sample volume per single experiment (FF-IZE-pH) up to 5 ml/h (Continuous process of FF-ZE-pH).
The 96 fractions from the FFE-instrument were collected and the 96 cavities were analyzed. First a performance test was done with the FFE-instrument before the final experiments with the samples of supernatants from cell cultures (please see the pherogram "pH-profile + pI Marker"). The actual samples were injected with a flow rate of 5 ml/h and the quality of the FFE-separation was analysed by measurement of fluorescence (280/350 nm), uV-280 nm and absorbance at 510 nm. The FFE-fractions for the final analysis with AMNIS were collected inside MTPs, made from Polypropylene.

According imaging flow cytometry analyses with EV specific antibodies on an AMNIS ISXII platform, EVs subtypes were detected with different charge densities across the area of electrophoretic migration. Only some subtypes of EVs were free of proteins, others were still associated with proteins. Upon combining FFE with subsequent ultrafiltration (300 KD-UF-membranes) even high amounts of contaminating protein can be removed from obtained samples (Please see. "Fast workflow for high purification of small EVs, based on the combination of FFE and Ultrafiltration"

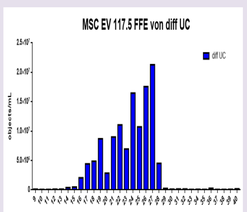
Separation of small EVs from supernatant of cell culture of Mesenchymal stem cells (MSCs-EVs), prepurified via differential centrifugation including ultracentrifugation (UC)



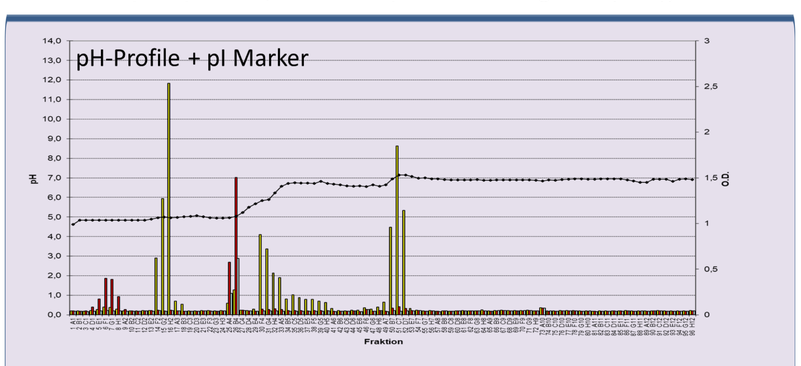
FFE-Pherogram (Autofluorescence, absorbance at 280 and 510 nm)



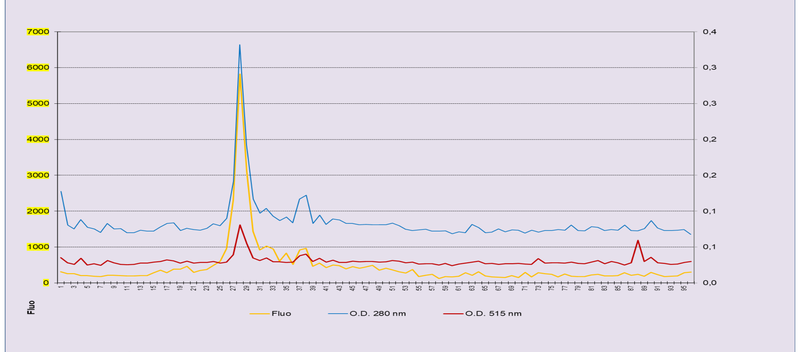
AMNIS-data 8464-11



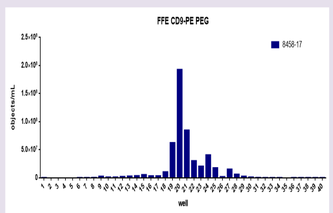
Separation of small EVs from supernatant of cell culture of Mesenchymal stem cells (MSCs-EVs), prepurified via precipitation with PEG precipitation, followed by ultracentrifugation (PEG))



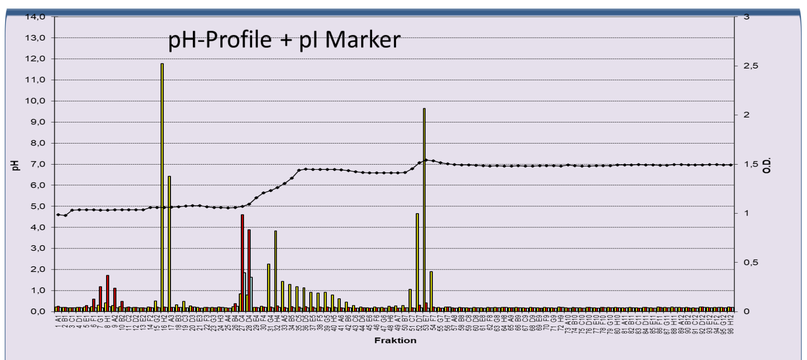
FFE-Pherogram (Autofluorescence, absorbance at 280 and 510 nm)



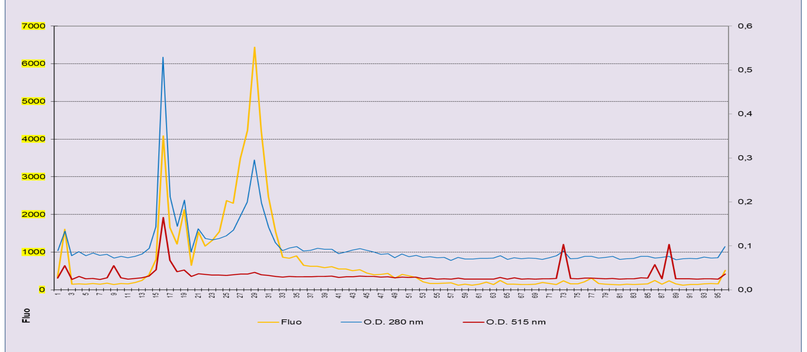
AMNIS-data



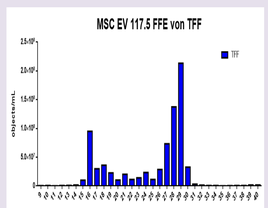
Separation of small EVs from supernatant of cell culture of Mesenchymal stem cells (MSCs-EVs), prepurified via tangential flow filtration (TFF)



FFE-Pherogram (Autofluorescence, absorbance at 280 and 510 nm)



AMNIS-data



Conclusion: FFE demonstrates that EV samples obtained with different preparation methods vary regarding the complexity of EVs and contaminating proteins. Furthermore the results demonstrates that none of the methods removes non-EV associated proteins appropriately. Notably, if required, the amount of contaminating protein can significantly be reduced obtained FFE-EV samples are additionally cleaned by ultrafiltration.

Key Features: FFE

- Matrix free separation, ideal for separation of particulate material and for conserving protein activity/protein complexes
- Excellent reproducibility from run to run and day to day
- Fast and sensitive detection of separation quality via protein fluorescence
- Compatible with many other downstream techniques, e.g. SDS-, IEF- and 2D-GE, HPLC, MS,
- Use of protein autofluorescence at 280 nm for the analysis directly in 96 well MTPs:
  Fast and very sensitive detection down to 0.5 ng/ml

Key Features: Separation of EVs

- Various protocols for processing the vesicles as anions
- Fast separation up to 5 ml pure cell culture/h
- Fast workflow: FFE + UF with 300 KD membrane
- Isolation of sub types of vesicles
- Minimal dilution of vesicles during the FFE-process
- Rapid quality control method for large scale purification batches
- Scalable as a first purification step for purification of EVs from protein free cell culture supernatant
- Scalable as a second purification step for drug application
- Compatible with any "downstream-analytics"
- Optional: precise determination of zeta-potential of EVs

Conflict of interest:

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