FRIEDRICH-ALEXANDER UNIVERSITÄT ERLANGEN-NÜRNBERG
FACULTY OF MEDICINE

Universitätsklinikum Erlangen

P-05 Free flow electrophoresis setup and experimental workflow for separating human plasma-derived proteins and extracellular vesicles

Miriam Zentgraf[1], Simon Staubach[2], Jan van Deun[1], Markart Meckel[3], Johann Bauer[4], Gerhard Weber[3], Bernd Giebel[2], 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, w.ffeservice.com

[4]SiHaTho GmbH, Biedenkopf, Germany

FFE Service GmbH

1. Introduction

Free flow electrophoresis (FFE) is applied for the separation of complex mixtures of proteins or/and small particles based on their different surface charges. It is performed while specimens are flowing in a fluid lacking stabilizers like a gel or matrix. Therefore, it needs careful preparation of the device and the fluids as well as thorough examination of the interplay of device and fluids.

Here we describe set up and workflow which enables an FFE to separate different proteins and extracellular vesicles (EVs) together present in a human fluid. The ultimate goal of this FFE preparation is to apply it successfully for characterization and separation of EV subpopulations. To work for this project it is important to get as large quantities of fractionated EVs as are required to carry out Western Blot (WB) analyzes. We chose the FFE regime of free flow zone electrophoresis through a pH gradient (FF-ZE-pH) and tested two separation protocols (SP 1 and SP 2) in regard to their advantages and disadvantages.

2. Methods

Prior to any kind of FFE experiment pH and conductivity of all separation media are carefully adjusted. Then the laminar flow of applied media and samples is tested to confirm the quality of discrimination of ionic analytes. Subsequently, formation of a pH gradient is tested before running a pooled human plasma sample in order to see the quality of electrophoretic separation.

After passing all tests, the FFE instrument can be used for separating complex protein mixtures from many different kinds of human samples which contain EVs in addition to soluble proteins.

If SP 1 is performed, three to four separation media with larger pH differences are used for separation and FFE-fractions, containing the analytes, are collected in 30 wells of a 96-microtiterplate (MTP-96).

If SP 2 is performed, six to seven separation media with smaller pH differences are used for separation and FFE-fractions, containing the analytes, are collected in 70 wells of an MTP-96.

3. Results

Set up and tests

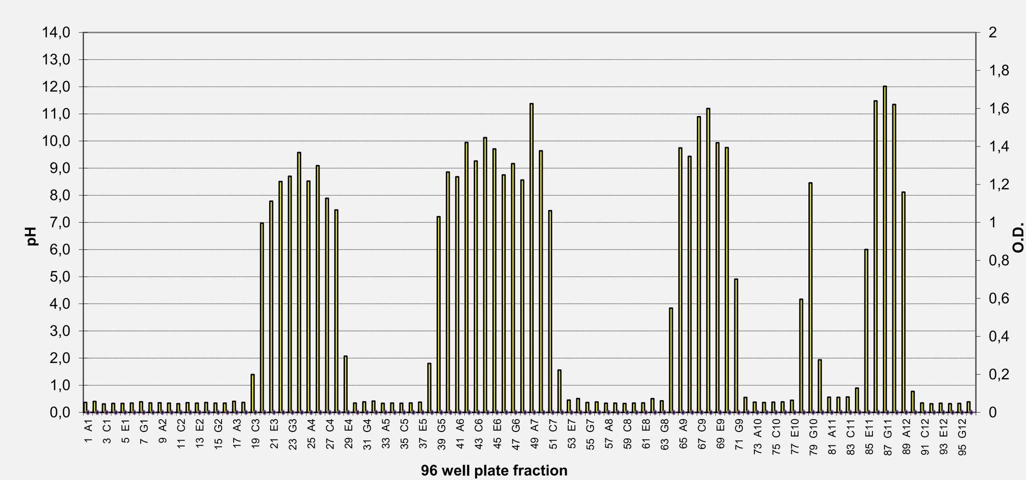
Separation protocol 1 (SP 1)

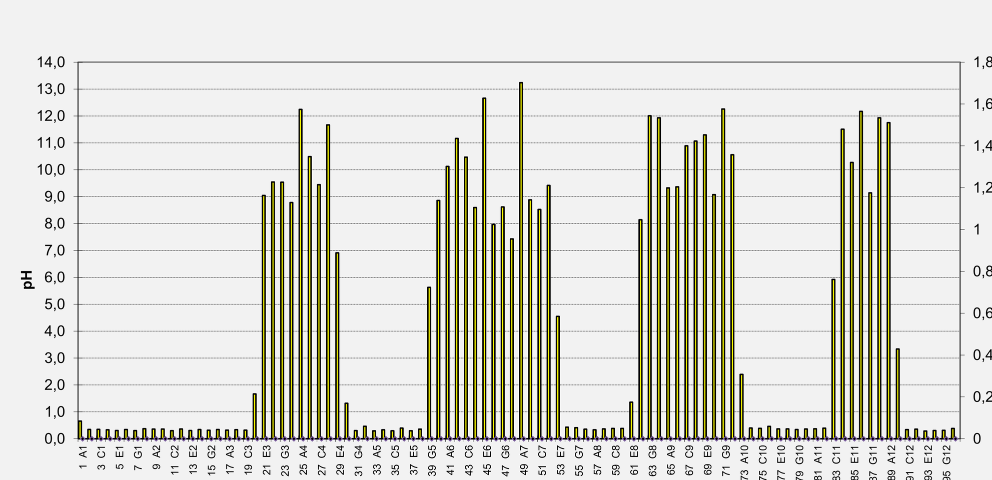
Separation protocol 2 (SP 2)

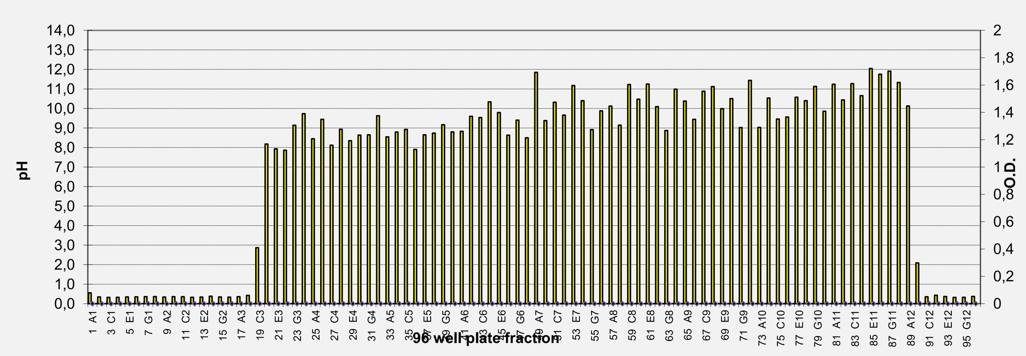
Result

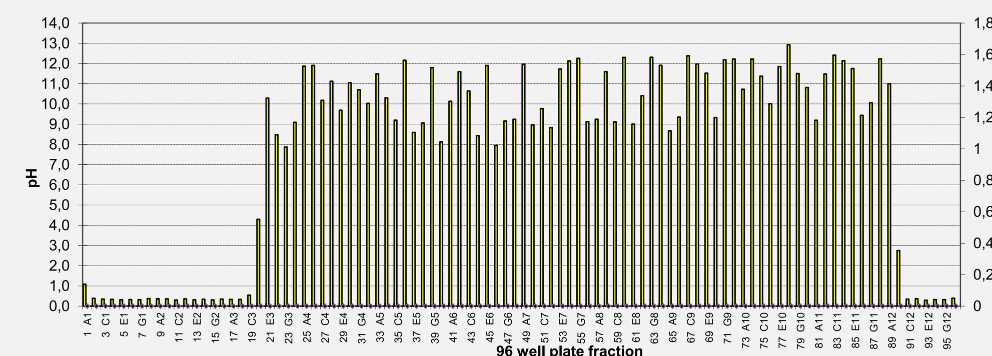
1. Electrophoretic pre-tests

Prior to each FFE experiment several quality control (QC) procedures have to be carried out to ensure proper FFE instrument set up and media composition. QC 1a and 1 b as well as QC 2 are performed before current is switched on. QC 1: Dyes dissolved in destilled water are injected in the chamber via up to 5 inlets and the optical density of each collected fraction is determined in order to see if the stripe output is uniform and with clear borders.







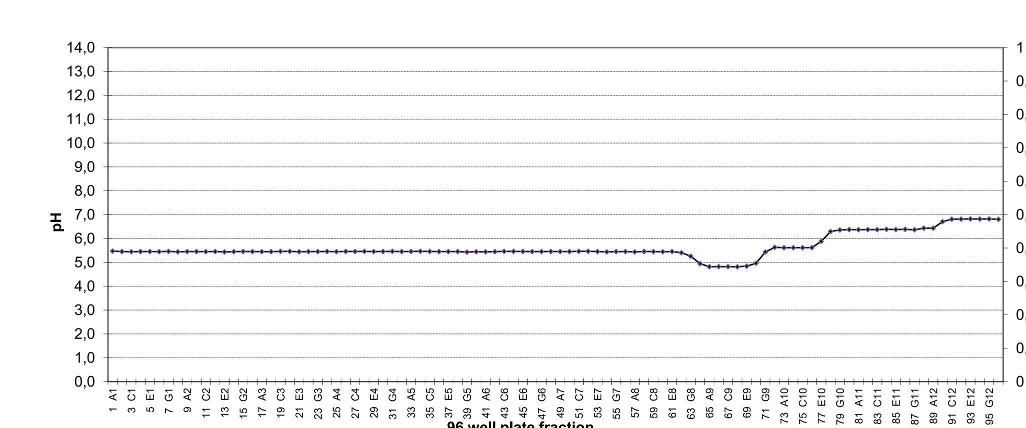


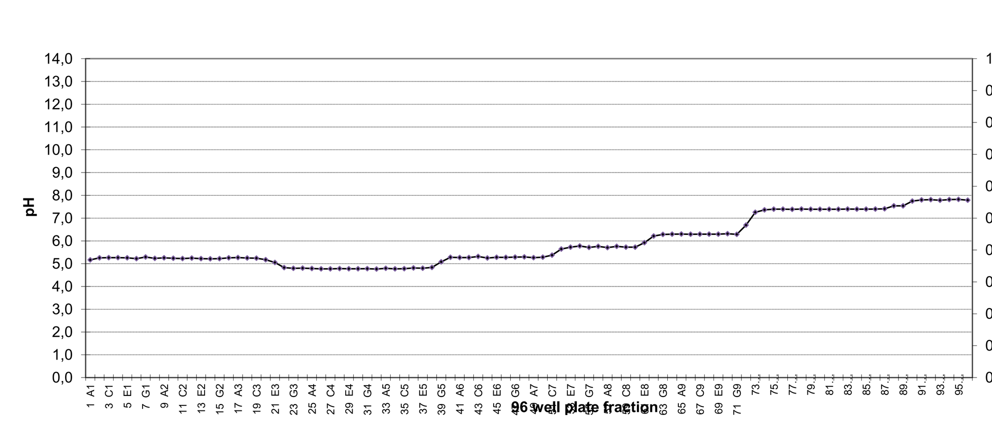
QC 1 – Electrophoretic pre test 1:
Upper panel: Each of the stripes (SP1 five stripes, SP2 four stripes) filled the dedicated wells in the MTP-96 confirming the right tubing setup and laminar flow.

Lower panel: Stripe area is uniform and continuous. This indicates that no tubes are clogged and verifies right tube and pump adjustments.

2. pH values of separation media

QC 2 Check of media: Conductivity, pH are checked before the injecting buffers into the FFE chamber. The media of different pH are arranged according the different pH- values and will be injected into the separation cell. Without voltage applied all 96 fraction leaving the separation cell are collected within a MTP and the pH-values will be measured by a robotic system. The resulting pherogram of pH-alues will be compared with the pherogram of the performance test QC 4 (below), checking the relative positions of the pH-steps within the pherograms

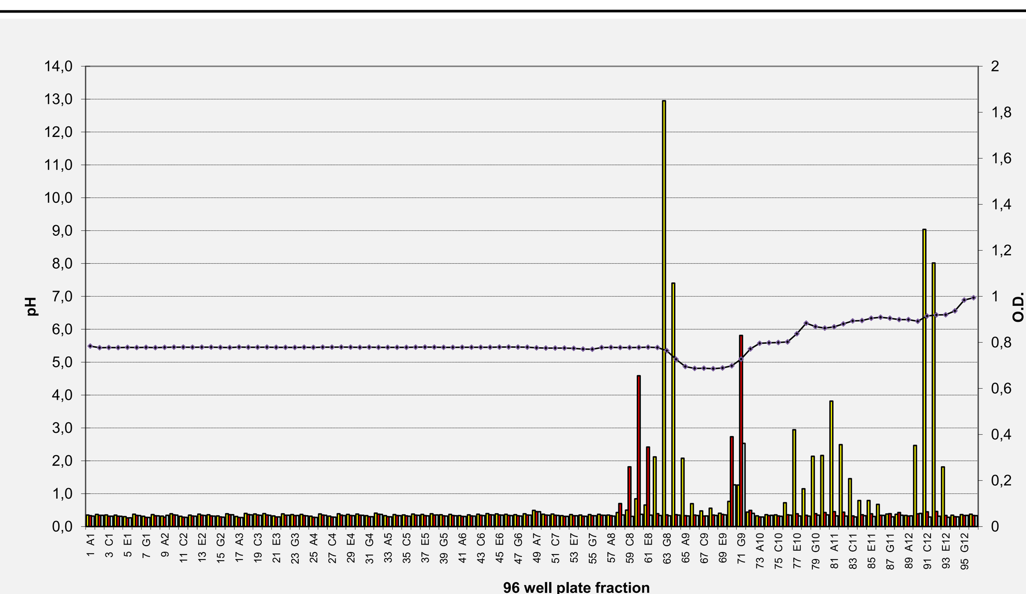


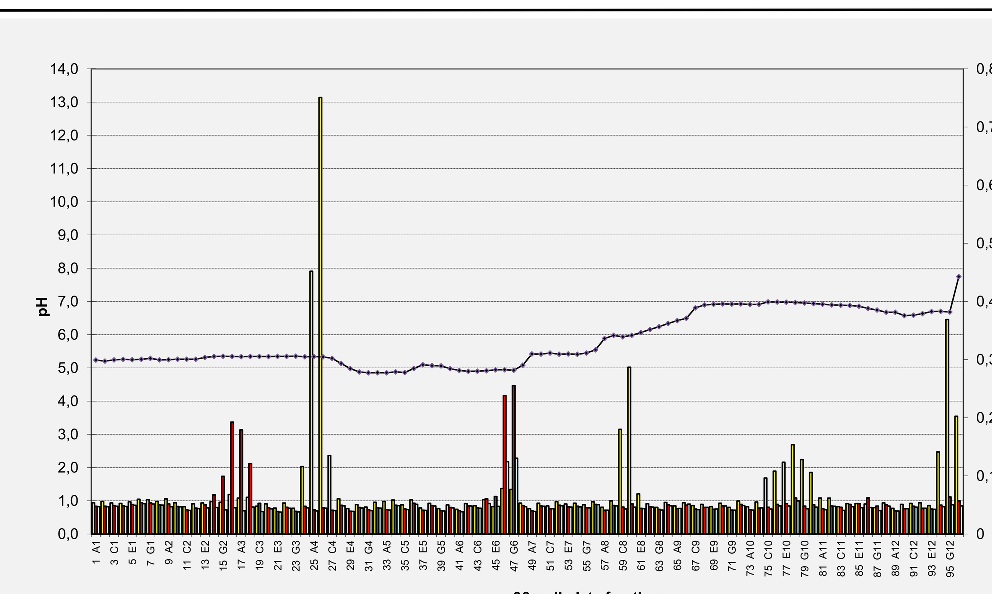


QC 2 – Electrophoretic pre test 2:
The pH curves represented here show the different sets of separation media used for SP 1 (three) and SP 2 (six). pH values ranging from 4.8 up to 8 are found in the media opposite to their site of injection.

3. FFE performance test

QC 3 (FFE-performance test): QC3 is the first check with high voltage applied. A mixture of IEF-pI-marker will be used as sample. The resulting pherograms will describe the performance of the FFE-instrument. The comparison of the pherogram of QC 3 and the pherogram of QC 2 and the check of the positions of the pH-steps will indicate , that the chemicals of the separation media and the pH-values of separation media will perform well under the electrophoretic conditions.

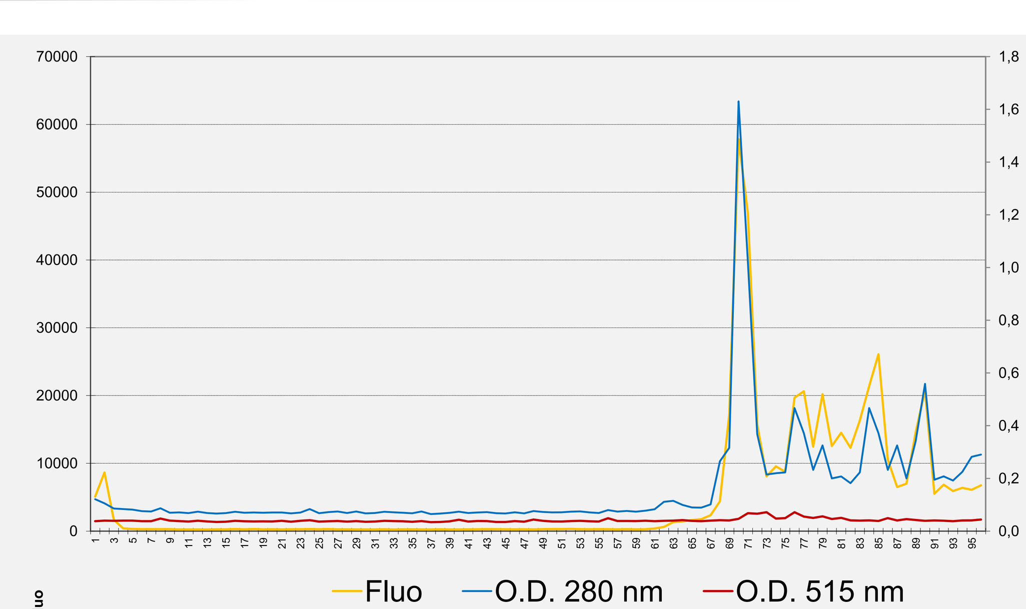


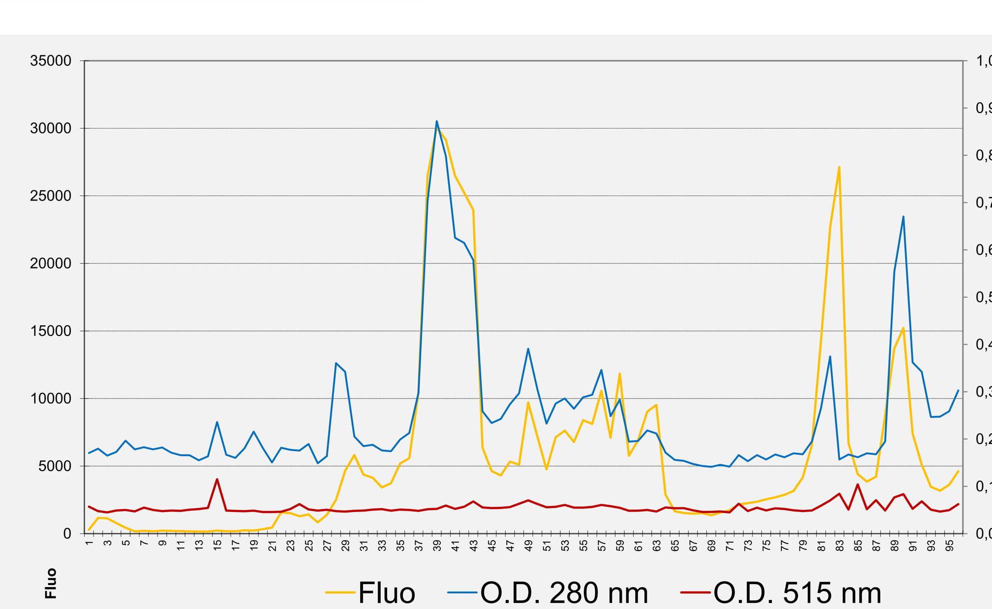


QC 3: In both types of ZE-separation media, used for SP 1 and SP 2 respectively, the pI markers injected in the separation chamber deflected under current towards their isoelectric point (pI) where they stop migration and focus, respectively. According to the different setups of media, pI-markers are spread over 30 fractions, if SP 1 is applied and over the whole area of separation, if SP 2 is applied.

4. Pooled human plasma profiles after FFE separation

Q 4: A second check comprises an electrophoretic separation of a pool of human plasma to test the compatibility of the FFE-separation process as well of separation media with the requirements for the native separation of biopolymers as well of bioparticles. like exosomes. In addition, the protein pherogram of this pooled plasma has to agree with earlier recorded pherograms of pooled human plasma to ensure a stable FFE separation system.





FFE-pherograms of pooled human plasma were photometrically determined with three different wavelengths to detect protein (fluorescence 280/360 nm), precipitates (O.D. 515 nm) as well as turbidity (O.D. 280 nm). After comparing the measurements with earlier experiments, it was stated that protein profiles were reproducible.

4. Conclusion for next step

After proper setup and performance tests both protocols SP 1 and SP 2 are applicable to separate human plasma proteins. While in the SP 1 procedure a greater part of applied plasma is collected in a well, with the help of SP 2 human plasma proteins are separated into several more subfractions

5. Acknowledgements

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

FAU FRIEDRICH-ALEXANDER UNIVERSITÄT ERLANGEN-NÜRNBERG
FACULTY OF MEDICINE

Universitätsklinikum Erlangen

P-05 Application of FFE for the fractionation of melanoma cell produced exosomes

(Scale up of FFE-process)

Miriam Zentgraf[1], Simon Staubach[2], Jan van Deun[1], Markart Meckel[3], Johann Bauer[4], Gerhard Weber[3], Bernd Giebel[1], Andreas Baur[1]

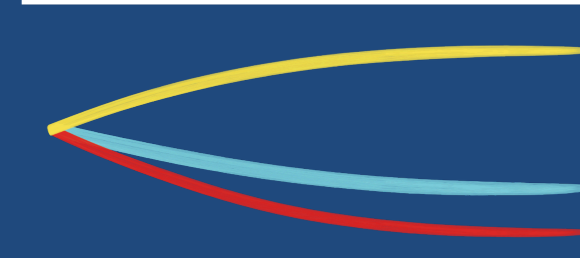
[1]Friedrich-Alexander University Erlangen-Nürnberg and University Hospital Erlangen, Department of Dermatology, Erlangen, Germany

[2] Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

[3]FFE Service GmbH, Feldkirchen, Germany, www.ffeservice.com

[4]SiHaTho GmbH, Biedenkopf, Germany

FFE Service GmbH



1. Introduction

In the study described here it is of interest to see whether FFE and its separation protocols can be applied to separate different proteins and extracellular vesicles (EVs) present together in plasma of melanoma patients. In this context enrichment of EVs is of special interest. As shown in earlier studies (see presentations P01-P04) a successful sub-fractionation of exosomes included in human plasma could be proved with the help of AMNIS technology. However, for profound identification and investigation of melanoma specific EVs circulating in the plasma of melanoma patients further analytical methods need to be applied. Amongst them are methods targeting defined proteins of EVs with antibodies. One of such methods is Western blotting (WB). WB is applied to detect proteins of interest within a great number of different proteins. This means that applying WB can unveil whether EV specific proteins surrounded by a great amount of soluble plasma proteins are sub-fractionated when plasma of melanoma patients is electrophoresed.

The problem of searching EV bound proteins by WB, however, is that much larger quantities of separated EVs in each fraction are needed as compared to AMNIS.

2. Methods

To collect quantities of EV bound proteins in the various FFE fractions which are large enough for WB, we extended the time period of separation in a way that allows increase of FFE throughput without attenuation of separation accuracy. If separation time is prolongated, it is important to make sure that the whole procedure remains stable over a time of at least 1 hour. This means that pherograms recorded at the begin and the end of a separation experiment must be identical.

Applying FFE protocol SP 1, when three to four separation media with larger pH steps were used, the fastest plasma proteins migrate within 1.5 minutes of electrophoresis-separation time over less than 50 % of the separation area and focus.

Applying FFE protocol SP 2, when six to seven separation media with smaller pH steps were used the fastest plasma proteins migrate within 5 minutes of FFE separation time over the whole separation area before they focus.

In order to increase the throughput the time period of separation was extended for SP1 and SP2.

2. Results

Separation protocol 1 | **Separation protocol 2**

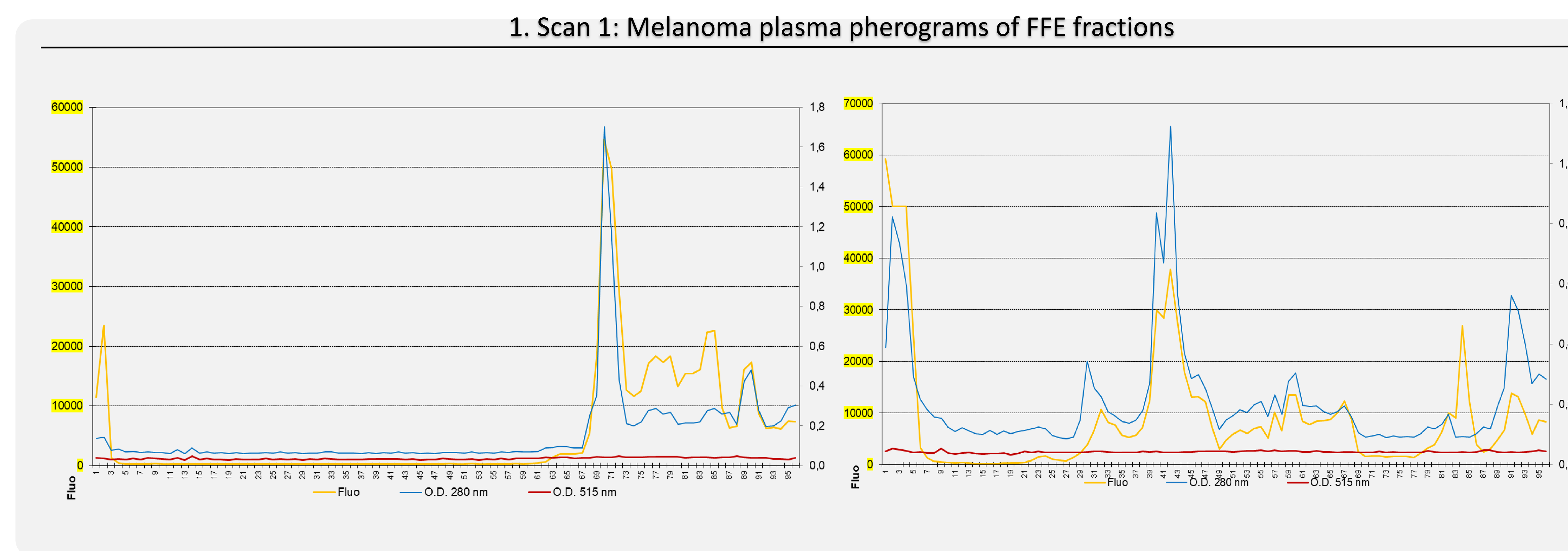
FFE-Throughput

Following the test expements for the FFE-instrument, plasma of melanoma patients was electrophoresed according to FF-ZE-pH protocol 1 (left column) and protocol 2 (right column), respectively.

In a first approach the concentrations of plasma proteins collected in each MTP-96 well were determined. For this purpose light absorption at 280 nm, and autofluorescence at 595 nm were measured for each well. In addition, light scattering was determined at 510 nm. Pherograms shown in the first and the third panels indicate that the electrophoretic migration behavior of proteins of the plasma of melanoma patients is similar to that of the proteins from the plasma of healthy donors shown in the fourth panel of page 1. Still some differences can be detected.

The electrophoretic analysis of plasma proteins is considered as an upstream step of the analysis of fractionated exosomes from plasma of melanoma patients. The actual step of exosome investigation is taking aliquots from each well, in which components of electrophoresed plasma had been collected. Then the aliquots, which contain free proteins and exosome bound proteins, are either labeled by anti CD9 for imaging flow cytometry (IFCM) performed semiquantitatively at the AMNIS Imaging Stream X II platform or are subjected to SDS gel electrophoresis for subsequent Western blotting.

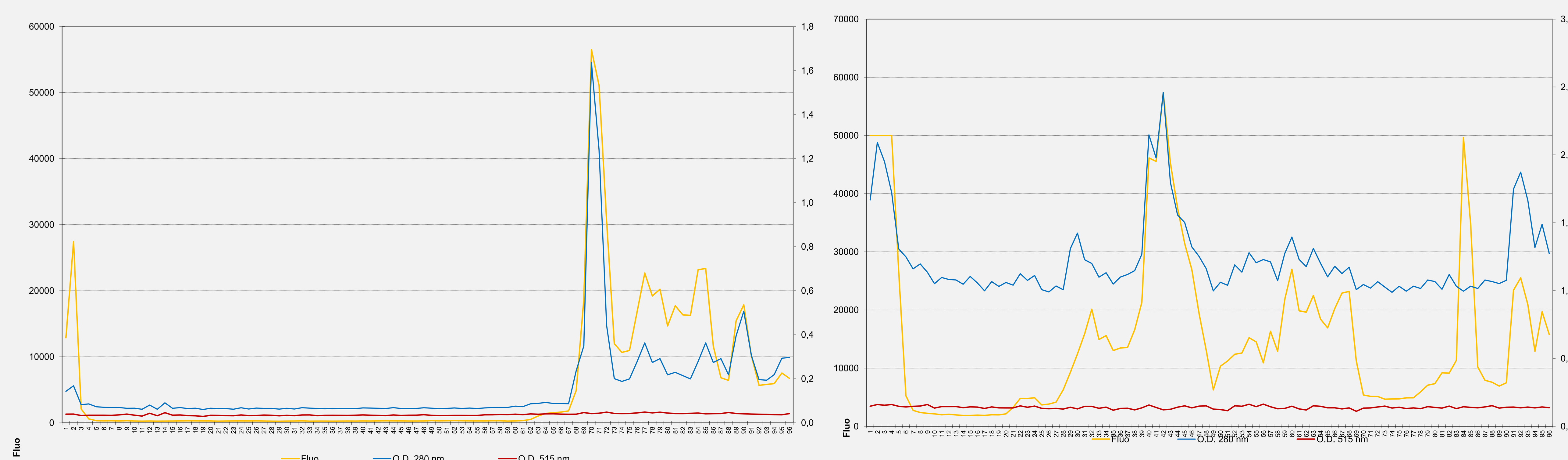
1. Scan 1: Melanoma plasma pherograms of FFE fractions



2. Workflow in between Scan 1 and Scan 2

Times:	Workflow: Collection of FFE-fractions:	Times:
1.5 minutes	1. Protein pherogram: Collection in UV transparent MTPs	5 minutes
2 x 1.5 minutes	1. Collection in 2 or more PP-MTPs	2 x 5 minutes
16 minutes	1. Collection in Deep well plate with 2 ml cavities	50 minutes
1.5 minutes	1. Repeated protein pherogram: collection in UV transparent MTPs	5 minutes
….	1. Repeated collection in Deep Well plate	…..
….	1. Repeated protein pherogram: Collection in UV transparent MTP	……
	1. Repetition of cycle…..	

3. Scan 2: Repeated melanoma plasma pherograms of FFE fractions



Result

Plasma separation according to FF-ZE-pH protocol SP 1 is faster (left column on pages 1 and 2) because the separated components of a sample are collected in 30 fractions only. Therefore, EVs detectable by AMNIS in wells where proteins are seen can be collected within 1.5 minutes, if one antibody (eg. anti CD9) is tested. For further tests, MTP-96s are changed after each 1.5 minutes collection time (see 2., middle column). Considerably larger quantities of plasma must be separated, if EV bound proteins will be analyzed by WB. If plasma is fractionated according to protocol 1 with the aim to use the fractions for WB, deep well plates with 2 ml cavities are used to collect separated plasma components within 16 minutes (see 3., middle column). To prove FFE stability a 1.5 minutes pherogram is recorded at the end of an experiment (see 4., middle column).

Plasma separation according to FF-ZE-pH protocol 2 takes more time (right columns on pages 1 and 2) because the separated components of a sample are distributed over 70 fractions. In this case separation of plasma sufficient for one AMNIS analysis needs 5 minutes (see 1., middle column, right side), and separation for one WB needs at leas 50 minutes (see 3., middle column, right side).

4. Conclusion and Outlook

Even if the time period of separation is extended for more than an hour, protein pherograms recorded at the begin and the end of the experiment are equal. This is an indicator for the quality of subfractionation of EVs.

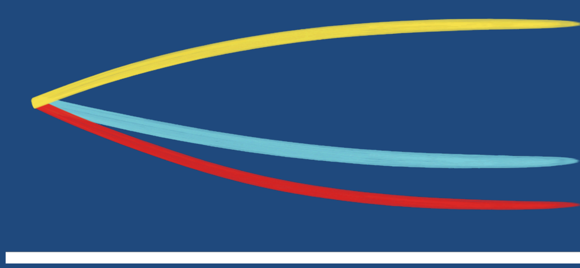
5. Acknowledgements

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

FRIEDRICH-ALEXANDER UNIVERSITÄT ERLANGEN-NÜRNBERG
FACULTY OF MEDICINE

Universitätsklinikum Erlangen

P-05 Analyzing extracellular vesicles from melanoma plasma using a combination of free flow electrophoresis, imaging flow cytometry and Western blot



Miriam Zentgraf[1], Simon Staubach[2], Jan van Deun[1], Markart Meckel[3], Johann Bauer[4], Gerhard Weber[3], Bernd Giebel[1], 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
[4]SiHaTho GmbH, Biedenkopf, Germany

1. Introduction

A preliminary photometrical evaluation of FFE separation fractions displays differences in charge density of plasma proteins. A subsequent imaging flow cytometry (AMNIS) analysis of particular components in each well combines the charge densities of plasma proteins with knowledge about the differences in charge density of EVs.

In the plasma of many patients suffering melanoma cancer EV levels are considerably upregulated. These EVs can contain a diverse array of factors to affect target cells and disease development. Detection and analysis of EVs with the help of FFE provide potential prognostic biomarkers of great importance.

Here we compare the two standard protocols SP 1 and SP 2 for continuous FF-ZE-pH processing looking whether they are applicable for investigation of EVs from plasma of a melanoma patient by further examination, especially by WB.

2. Methods

Plasma samples from melanoma patients comprising soluble proteins and EVs were separated by FF-ZE-pH according to the two different protocols SP 1 and SP2. Both, soluble proteins and EVs together were separated according to their different charge surface densities and the various fractions were collected in different cavities of 96-deep-well plates.

The total collection time of separated plasma in a 96 deep well plate was about 20 min if SP 1 was applied and about 1 h in case of SP 2 use.

Antibodies against EV specific antigens were then used to examine fractions of interest via imaging flow cytometry analysis and Western blot method.

3. Results

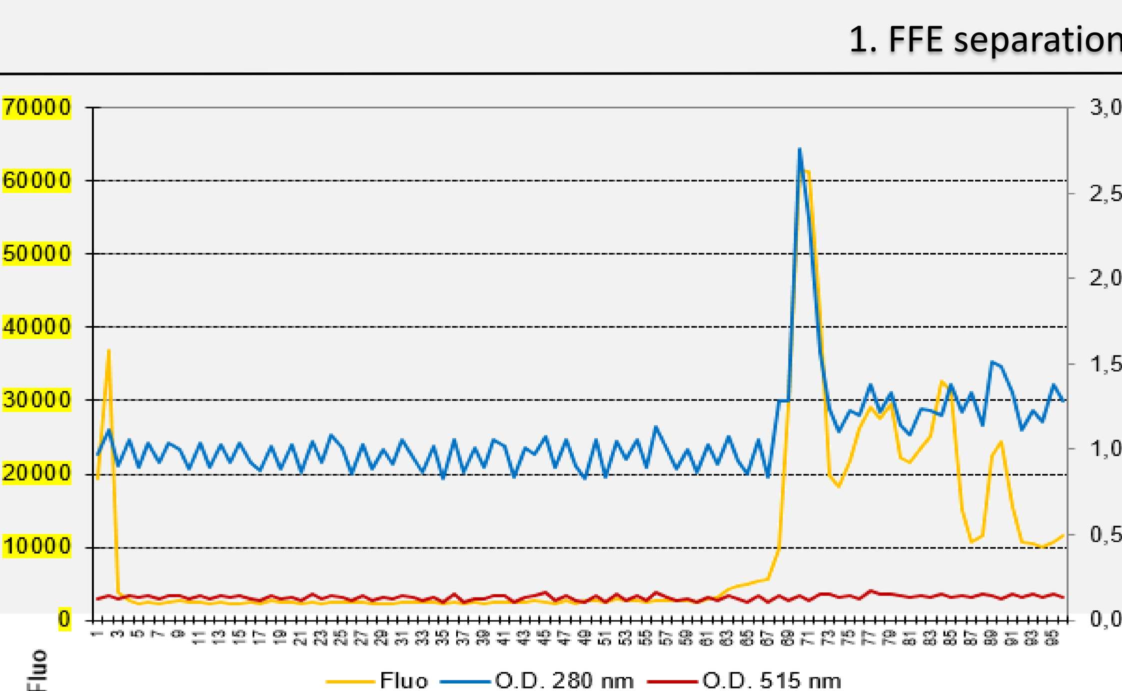
Western Blot analysis

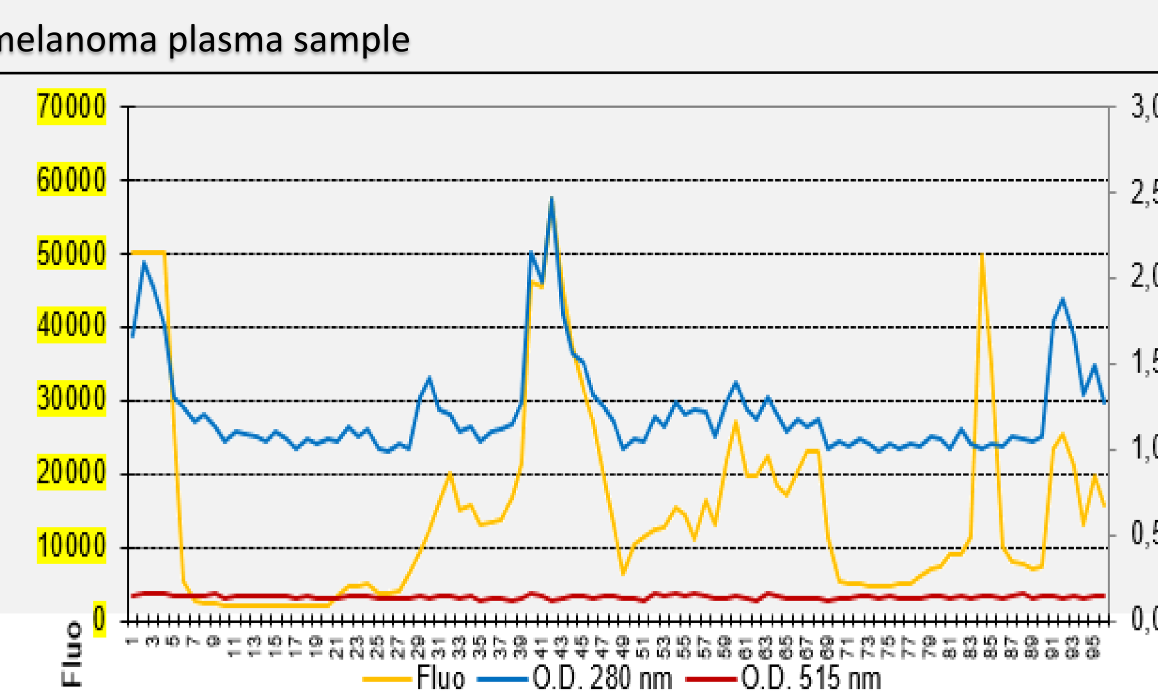
Samples of plasma of melanoma patients were fractionated using the two different FFE protocols SP1 and SP 2. Then soluble proteins collected in the various fractions were determined photometriccally with three different wavelengths to detect protein (fluorescence 280/360 nm), precipitates (O.D. 515 nm) and turbidity (O.D. 280 nm).

Separation protocol 1 (SP 1)

Separation protocol 2 (SP 2)

1. FFE separation of a melanoma plasma sample





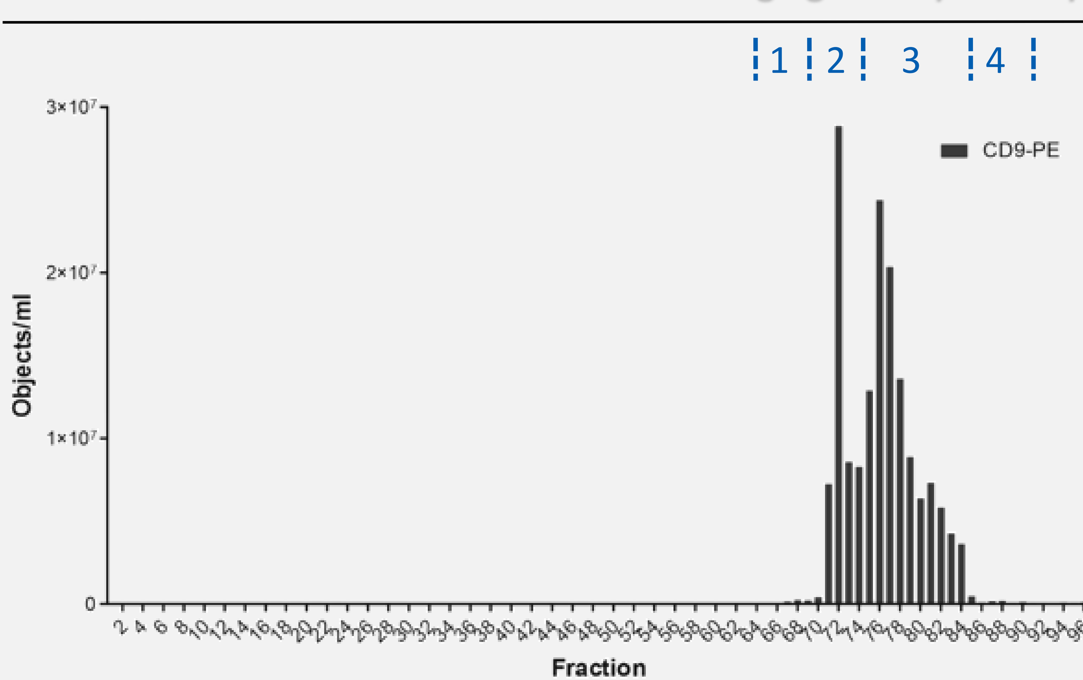
Result

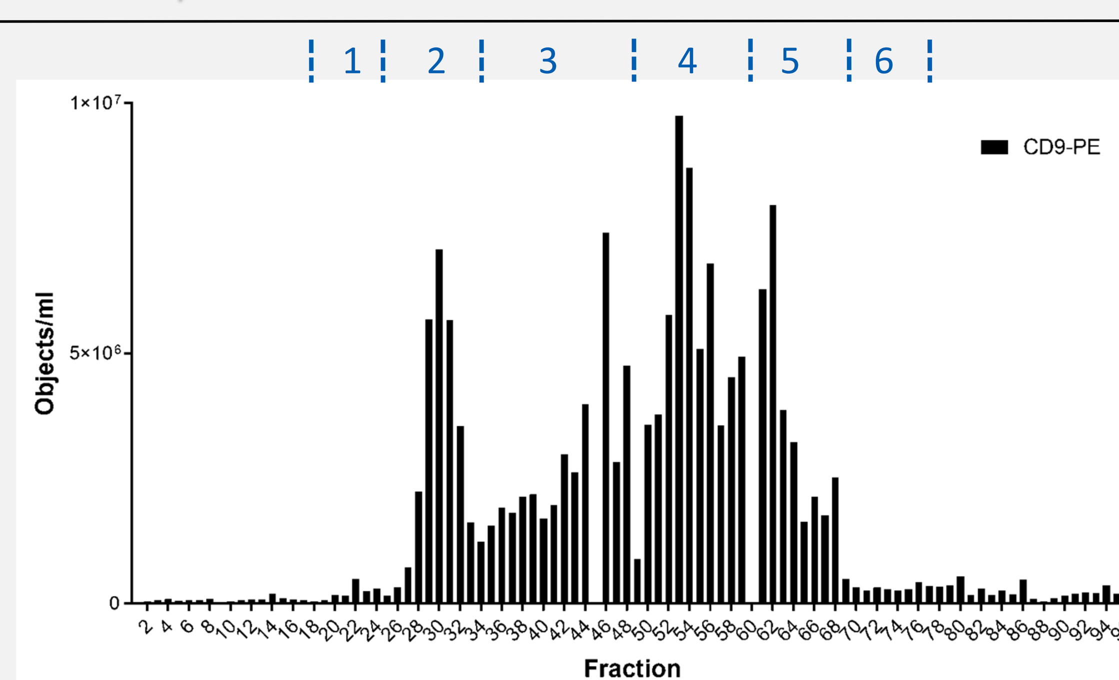
After applying SP 1 distinct melanoma plasma protein peaks were detected in wells ranging ranging from fraction 67 to 92, whereas after application of SP 2 proteins were detected spreading across the whole separation area (fraction 27 to 90). This latter separation resulted in more clearly isolated protein peaks.

To identify EVs in FFE fractions, aliquots were taken from each well and AMNIS was chosen first to analyse the aliquots in regard to CD9, a common EV marker.

A subsequent attempt to apply WB for analysis of each well content failed, because the various fractions did not contain enough EVs to detect their proteins by this method.

2. Imaging flow cytometry analysis of CD9-PE positive FFE fractions





When the FFE fractions were analyzed by the AMNIS technology the results indicated that CD9-PE signals concentrated in fractions 71-84 with maxima at fractions 72 and 76, if analysis was performed after application of SP 1 and that CD9-PE signals are spread across a larger area (faction 26-70) with several maxima at fractions 30, 46, 53 and 63, if analysis was performed after application of SP 2. These results suggest that application of SP 2 enables to separate possible EV subpopulations.

Therefore, FFE fractions were collected in deep well plates and FFE fractions, for which a preceding analyses of their soluble proteins and CD9 bearing particles indicated greater quantities of EVs, were pooled into the four (F1-F4) or six groups (F1 – F6) depending on the protocol used for separation. Afterwards Amicon® Ultra-15 centrifugal filters (10K) were used for concentrating the proteins via centrifugation at 4000 g constricting the fluids of each fraction to a final volume of 200 µl. These were applied to SDS gel electrophoresis and wet electro-blotting (WB) was performed for qualitative protein transfers over a broad molecular weight range.

Then anti-CD63 and anti-CD9 antibodies were used for EV-marker detection .

3. Pooled and concentrated FFE fractions

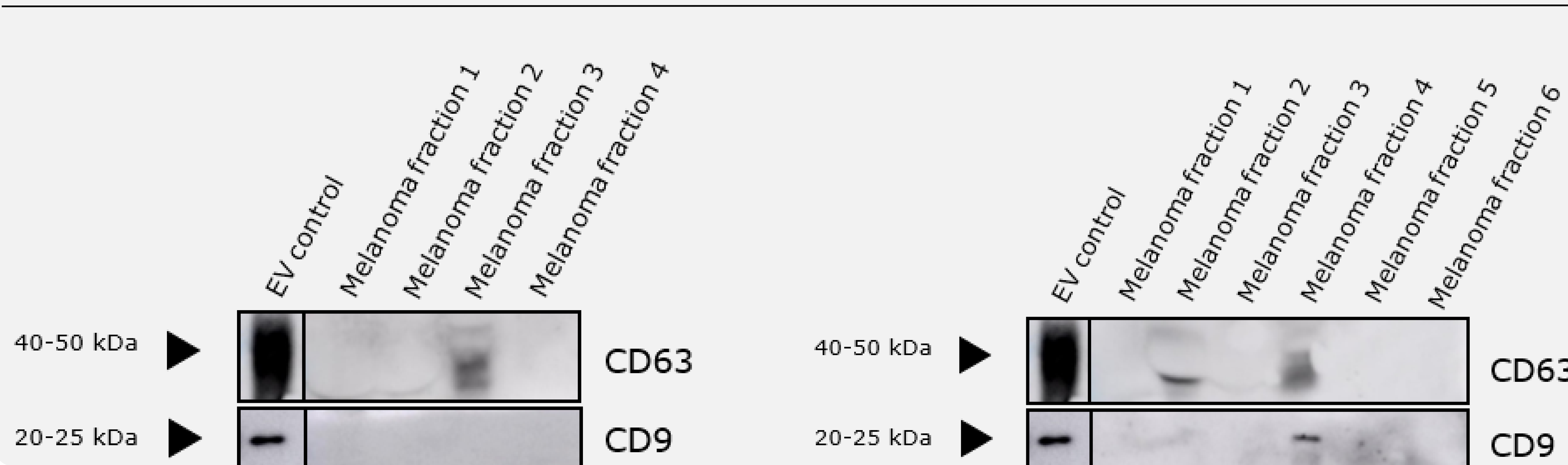
	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

SP 1: Fractionated melanoma plasma was pooled into four groups (F1-F4).

SP 2: Fractionated melanoma plasma was pooled into six groups (F1-F6).

4. Western blot analysis of FFE fractions



Western blot analysis revealed CD63 and CD9 antigens on control EVs (EV control) as well as in separated pools of FFE fractions. Only pool 3 was positive for CD63, if melanoma plasma fractionated according to FFE SP 1 was analyzed.

Pool 2 showed an unspecific signal, whereas in pool 4 CD63 and CD9 antigens were detected, if melanoma plasma fractionated according to FFE SP 2 was analysed.

4. Conclusion and Outlook

The proof of the EV markers CD63 and CD9 by AMNIS and Western blotting confirmed that both FFE protocols are applicable to fractionate EVs from melanoma plasma. Now it is of interest to separate EVs from soluble proteins. Ultracentrifugation and ultrafiltration as well as modified protocols of Western Blotting are considered suitable to further characterize various subpopulations according to their different surface charges.

5. Acknowledgements

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