Assessment of different sample preparation routes for mass spectrometric monitoring and imaging of lipids in bone cells via ToF-SIMS

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In ToF-SIMS analysis, the experimental outcome from cell experiments is to a great extent influenced by the sample preparation routine. In order to better judge this critical influence in the case of lipid analysis, a detailed comparison of different sample preparation routines is performed—aiming at an optimized preparation routine for systematic lipid imaging of cell cultures. For this purpose, human mesenchymal stem cells were analyzed: (a) as chemically fixed, (b) freeze-dried, and (c) frozen-hydrated. For chemical fixation, different fixatives, i.e., glutaraldehyde, paraformaldehyde, and a mixture of both, were tested with different postfixative handling procedures like storage in phosphate buffered saline, water or critical point drying. Furthermore, secondary lipid fixation via osmium tetroxide was taken into account and the effect of an ascending alcohol series with and without this secondary lipid fixation was evaluated. Concerning freeze-drying, three different post-processing possibilities were examined. One can be considered as a pure cryofixation technique while the other two routes were based on chemical fixation. Cryofixation methods known from literature, i.e., freeze-fracturing and simple frozen-hydrated preparation, were also evaluated to complete the comparison of sample preparation techniques. Subsequent data evaluation of SIMS spectra in both, positive and negative, ion mode was performed via principal component analysis by use of peak sets representative for lipids. For freeze-fracturing, these experiments revealed poor reproducibility making this preparation route unsuitable for systematic investigations and statistic data evaluation. Freeze-drying after cryofixation showed improved reproducibility and well preserved lipid contents while the other freeze-drying procedures showed drawbacks in one of these criteria. In comparison, chemical fixation techniques via glutar- and/or paraformaldehyde proved most suitable in terms of reproducibility and preserved lipid contents, while alcohol and osmium treatment led to the extraction of lipids and are therefore not recommended. © 2015 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution 3.0 Unported License. [<http://dx.doi.org/10.1116/1.4915263>]

I. INTRODUCTION

Reliable and reproducible mass spectrometry imaging of biological samples requires careful sample and surface preparation, which reduces analytical artifacts to a minimum. While this has been well solved for inorganics, the SIMS imaging of biological cells is still in an infant stage—not at least because of the critical need for proper sample preparation. Therefore, the current paper focuses on the development of an optimal protocol for the sample and surface preparation of cells with a specific analytical target, i.e., the systematic SIMS characterization of lipids in human mesenchymal stem cells (hMSCs).

Lipids belong to a group of structurally complex molecules since, in contrast to DNA or proteins, they are not

composed of similar repeating units.¹ This complexity accounts for the involvement of lipids not only in obvious functions such as energy storage and the formation of lipid membranes, but also in a variety of homeostatic processes and disease states.^{1–3} On cellular level, lipids act as cell signaling molecules and are involved in the regulation of various cellular functions such as proliferation, apoptosis, inflammation, immunity, and even DNA modulation.⁴ Lipids and their metabolism affect pathological processes like atherosclerosis,⁵ osteoporosis,⁶ cancer,⁷ and diabetes⁸ and are of potential value as novel biomarkers for such diseases on the one hand and for cellular processes like chondrogenesis,⁹ on the other hand. It is thus of strong interest to explore lipidomic pathways in order to understand molecular relationships and especially disease mechanisms. In this context, cell culture (model) experiments play a crucial role. And in general human cell culture systems are regarded as suitable

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tool to replace animal studies, which often produce data hardly transferable to the human system.¹⁰ However, complete cellular lipid profiles, “lipidomes”, cannot be analyzed with a single experimental approach and are analytically demanding.^{1,11,12}

At present, only a limited number of studies focus on systematic SIMS characterization and imaging of lipids in complex biological samples, compare (Ref. 13). Especially for cell cultures, this approach has yet not been explored in depth.⁹ As ToF-SIMS analysis requires vacuum conditions, cell samples cannot be analyzed directly in the living or native state.^{14,15} Sample preparation is thus a crucial factor to maintain sample integrity, and it has to be well adapted to the analytes of interest.^{14,16} A suitable preparation routine must preserve the native state concerning morphology,¹⁷ chemical composition and distribution of the analytes,^{14,17} remove excess salts from culture media^{17,18} and be reproducible.¹⁹ Systematic cell studies require that the preparation routine is applicable to large numbers of samples with high reproducibility in order to obtain reliable statistics. Ideally, the samples should be storable after preparation in order to reproduce experimental results in a later stage of the study if necessary.

A detailed survey and summary of existing routes for the sample preparation of cells will be given in Sec. I A, as this will be important to better understand our experimental strategy. In Sec. III, we present detailed results on a comparative study applying these sample preparation techniques, and finally evaluate the different preparation protocols.

A. Sample preparation techniques for cells

Chemical fixation and cryofixation are two well-known and quite different groups of sample preparation techniques for cell cultures. Chemical fixation is achieved by use of an aldehyde, e.g., glutaraldehyde (Glu) or “formalin”/paraformaldehyde (PFA), that cross-links the amine groups of (membrane) proteins via imine bonding^{20,21} or methylene bridges,²² respectively. It enables the analysis of samples in a dry state.

Cryofixation preserves the native state of cells by immobilizing all components instantly²³ while it prevents osmotic effects,²⁴ delocalization, and loss of small ions.^{25,26} During cryofixation, the sample is plunge-frozen into liquid propane or ethane, as their high heat capacity enables fast cooling. It leaves the sample in a frozen-hydrated (FH) state with preserved sample integrity as amorphous ice is formed instead of ice crystals that would disrupt the sample morphology.²⁷ Necessarily the sample has to be analyzed on a cooled sample holder or it has to be freeze-dried.

In literature, freeze-fracturing (FF) of frozen-hydrated cells is considered as the “gold-standard”¹⁶ because it preserves even ions and molecules with high diffusivity and volatility and exposes the cell interior directly.¹⁴ However, it is not routinely used due to the technically challenging procedure,¹⁷ high failure rates²⁸ and also because it lacks

reproducibility.²⁹ Instead systematic investigations, especially those with a focus on lipids, still rely on other methods like glutaraldehyde fixation^{30,31} or gelatin embedding.⁹

In 2009, Malm *et al.* compared cryofixation to chemical fixation via glutaraldehyde and freeze-drying (FD) to alcohol drying.²⁶ In one preparation, osmium tetroxide fixation was performed prior to alcohol drying. Both fixations were stated to retain lipid distributions and large-scale cell morphology. An extraction of lipids by alcohol application and some retention of membrane lipids due to osmium tetroxide fixation was observed. However, osmium tetroxide fixation was always followed by alcohol drying and was shown to produce undesirable interference peaks in negative spectra. Brison *et al.* compared different preparation routines with focus on depth profiling and obtained the best results with plunge-freezing followed by freeze-drying.³² In 2011, Fletcher *et al.* compared formalin fixed and freeze-dried cells to nonfixed frozen-hydrated and freeze-fractured ones.¹⁷ While they recommend frozen-hydrated analysis because of higher signal levels and localization of smaller species, they also state that both methods retain characteristic lipid distributions around the nucleus. Tucker *et al.* compared different preparations and the use of gold coating and chemical fixatives/stabilizers such as paraformaldehyde, formaldehyde, and glycerol for cultured neurons.³³ Gold coating proved beneficial to enhance signal intensities for higher m/z ions and PFA and glycerol were stated to be suitable for analysis. Robinson and Castner compared different preparations and had also a focus on depth profiles.¹⁸ In comparison to freeze-drying, it was suggested that formaldehyde fixation does not remove any molecular species to an extent detectable via ToF-SIMS. Analysis of frozen-hydrated samples showed increased yields for larger mass fragments.

Generally, only a few references that deal with sample preparation of cell cultures or similar samples for SIMS include a closer look on lipids.^{17,18,26,31,33} Usually, only some lipid fragments are considered^{17,26,33} or the investigation was carried out only in the positive ion mode,^{26,33} while important lipid fragments such as fatty acids can only be seen in the negative ion mode.¹⁸ Published comparisons are not comprehensive, e.g., Robinson and Castner¹⁸ only analyzed formalin fixed cells; fixation via glutaraldehyde and secondary fixation via osmium tetroxide was not considered, and parameters of the freeze-drying procedure remain unclear. In particular, fixation by osmium tetroxide should be tested, as it is a known procedure to stain and fix tissues for electron microscopy based on the fixation of unsaturated lipids^{34,35} and it might prevent lipid extraction during sample preparation. The important point of reproducibility is usually not taken into account although an improved reproducibility of cell preparation and SIMS analyses is essential for the reliable evaluation of data by multivariate analysis (MVA) methods, such as principal component analysis (PCA), and the unequivocal identification of biomolecules. MVA has become an indispensable tool for analysis of complex ToF-SIMS data because it helps to identify chemical differences

between samples and to decide whether certain compounds are present in a sample.³⁶ PCA is also chosen to evaluate the different sample preparation routes shown in Fig. 1 according to their lipid profiles. The interested reader is referred to Refs. 36–40 for detailed information on the working principle and the mathematical background of the PCA method.

Aim of the present study is to provide a comprehensive comparison of sample preparation routines for the analysis of biological cells via ToF-SIMS with focus on the analysis of lipids.

II. EXPERIMENT

A. Isolation of human mesenchymal stem cells

The experiments were approved by the local ethical committee of the Justus-Liebig-University of Giessen. hMSCs were extracted from the head of the femur received from the Department of Trauma Surgery, University Hospital of Giessen-Marburg (UKGM; Giessen, Germany), which was sawn into disks. The compacta was detached with bone scissors and the spongiosa was cut into small pieces. At humidified atmosphere (5% CO₂, 37 °C), three to four of these pieces were put into a petri dish and covered with a medium consisting of Minimum Essential Medium α (Gibco, USA) supplemented with 20% fetal bovine serum (Biochrom, Germany) and 1% Penicillin/Streptomycin (Gibco, USA). Cells were cultured with medium change every 2 days till a density of 80% was reached. Then the bone pieces were removed and cells were detached with 5 ml TripleE (Gibco, USA). All cells used were provided from one donor to avoid donor–donor differences.

B. Cell culture

Twenty thousand cells of the third passage were seeded onto approx. $1 \times 1 \text{ cm}^2$ ($7 \times 4 \text{ mm}^2$ for freeze-fracturing) silicon wafers⁴¹ for 7 days while the culture medium was regularly changed every two to three days. Prior to cell seeding, the silicon wafers were cleaned with demineralized water, acetone, and ethanol.

C. Ammonium acetate rinsing solution and rinsing

To form a 150 mM solution, ammonium acetate (AAc) was dissolved in Milli-Q water (TOC: 2 ppb, resistivity: 16.6 M Ω cm) and brought to pH 7.4 with diluted ammonium hydroxide.²⁸ The rinsing solution was used to remove excess salts from cell culture. For rinsing, the silicon wafer covered with cells was dipped into the solution followed by gently moving it forwards and backwards in the solution for about 30 s.

D. Chemical fixation

PFAPBS: For chemical fixation with paraformaldehyde, the wafers seeded with cells were washed two to three times with phosphate buffered saline (PBS) at 37 °C before they were fixed in 4% PFA-solution (Merck, Germany) for 15 min at room temperature. The PFA-solution was provided in 0.1 M sodium phosphate buffer (NPP) (Roth, Germany) at pH 7.2. After fixation, the cells were washed two to three times with 0.1 M NPP, once with PBS and then stored into PBS at 4–8 °C. Prior to SIMS analysis, the cells were rinsed with 150 mM ammonium acetate solution and subsequently air dried.

FIG. 1. Overview of the investigated sample preparation routes aiming at lipid analysis of cells via ToF-SIMS. A short name for the respective sample route is given below the figure. Abbreviations: Glu, glutaraldehyde; PFA, paraformaldehyde; AAc, ammonium acetate; CPD, critical point drying; FD, freeze-drying; FF, freeze-fracturing; and FH frozen-hydrated.

GluPFAPBS: The preparation was carried out likewise with a 3% fixative consisting of 2% PFA and 1% glutaraldehyde (Plano, Germany) in 0.1 M NPP.

GluPBS: Fixation with glutaraldehyde was carried out analogously with a 2.5% glutaraldehyde solution in 0.1 M NPP as fixative (20 min fixation).

GluH₂O: Instead of washing with PBS after fixation, cells were washed two times with Milli-Q water and then put into Milli-Q water and stored at 4–8 °C.

Prior to analysis, the wafers were simply air dried.

1. Secondary osmium tetroxide fixation

GluOsPBS/GluOsAlc(CPD): Secondary fixation of lipids via osmium tetroxide was performed by transferring the glutaraldehyde fixed cells for 15 min into 1% osmium tetroxide solution. The solution was prepared from pure osmium tetroxide (Roth, Germany) that was diluted in double distilled water to give a 2% solution, which was mixed in a 1:1 ratio with 0.1 M NPP shortly before use. After fixation, the cells were washed six times with NPP while vessels were changed after the third time. A few hours later, another washing step followed and before any alcohol application the cells were washed again.

2. Ascending alcohol series

GluAlc(CPD)/GluOsAlc(CPD): For dehydration via ascending alcohol series, the chemically fixed cells were transferred into 30%, 50%, 70%, 80%, and 96% ethanol solutions (prepared from $\geq 99.8\%$, p.a., Roth, Germany) two times 7 min each. In a final step, they were transferred four times for 10 min into pure ethanol and consequently stored in pure ethanol. Glu(Os)Alc samples were then air-dried and analyzed dehydrated. Glu(Os)AlcCPD samples were dried via critical point drying (CPD) as follows.

3. CPD

GluAlcCPD/GluOsAlcCPD: For critical point drying, a CPD 030 Critical point dryer (Baltec, Principality of Liechtenstein) was used. First, the cell samples were cooled to 10 °C in ethanol. Then, liquid CO₂ as medium was introduced and the system was given 7 min for equilibration before the medium was ejected. This procedure was repeated 5–9 times until no ethanol odor could be detected at the outlet. After that, temperature was increased with 3.2 K/min to 41 °C to produce supercritical CO₂ which could slowly be led out and dried the samples. The procedure was performed two times; once with osmium stained samples and once with nonosmium stained samples to avoid cocontamination. The so dried samples were stored at 4–8 °C and analyzed dehydrated.

E. Cryofixation

1. Rapid freezing (plunge-freezing)

FD/FF/FH: the medium was removed and the fresh cells were rinsed with ammonium acetate solution as described above. The sample was then submerged into liquid propane

which was produced by leaking propane into a liquid nitrogen cooled Polytetrafluoroethylene beaker like described in Ref. 18 with a setup similar to that shown in Ref. 42. Samples were then transferred into liquid nitrogen where they were stored in HistoMailerTM boxes (Biocision, USA).

2. Freeze-fracturing of cells

FF: The cells were rinsed, plunge-frozen, and attached to a self-constructed open spring-loaded copper fracture device similar to that one presented by Lanekoff *et al.*⁴³ The device was closed, introduced into the prechamber, evacuated, and then inserted into the main chamber where the spring snapped open after manually applying force by use of an attachment on the sample transfer rod. Care was taken that the sample remained in the frozen-hydrated state throughout the whole procedure. Analysis was thus performed in the frozen-hydrated state.

3. Frozen-hydrated preparation of cells

FH: The preparation routine was similar to that described by Piowar *et al.*⁴⁴ and according to Robinson and Castner.¹⁸ First, the medium was removed. Then, cells were rinsed with ammonium acetate and plunge-frozen as described above. Afterwards the cells were transferred frozen-hydrated into the SIMS prechamber precooled at –160 °C at a pressure of 10^{–7} mbar. After sample introduction and prechamber evacuation to about 10^{–7} mbar, the sample holder temperature was about –150 °C. The holder was then heated to –80 °C with a rate of 5 K/min. It was held at this temperature for at least 30 min. (up to 2 h) to sublimate excess water from the cell surface. After heating, it was recooled to –150 °C and introduced into the main chamber for analysis in the frozen-hydrated state.

F. Freeze-drying

GluFD/GluN₂FD/FD: Freeze-drying was performed the day after chemical fixation for GluFD and GluN₂FD preparations and immediately after cryofixation via plunge-freezing for FD preparation. Drying was performed in a commercial Gamma 1–20 freeze-dryer (Christ, Osterode, Germany). First, the cell samples were transferred into the chamber that was precooled to –30 °C and cooled for about 2 h at normal pressure. Then, the pressure was lowered to 0.011 mbar for 6 h. After that, the temperature was increased to –15 °C for 2 h and to 0 °C for another 2 h. Finally, the pressure was reduced to 0.009 mbar while the temperature was increased to 30 °C for at least 10 h. The so dried samples were stored at 4–8 °C and analyzed in the dehydrated state.

G. Optical images

For optical imaging, the 2D mode of a PLox 3D optical profiler (Sensofar, Terrassa, Spain) equipped with a blue light-emitting diode at 460 nm wavelength was used.

H. ToF-SIMS analysis

Analysis was performed on a TOF.SIMS 5 instrument (ION-TOF, Münster, Germany) using a pulsed 25 keV Bi₃⁺

TABLE I. List of the lipid peaks and fragments observed in the negative mode, based on lipid peaks known from literature (see References). The theoretical and measured mass-to-charge ratio, calculated mass deviation, formula, species, assignment, trivial name, and references are given.

<i>m/z</i>	<i>m/z</i> theoretical	<i>m/z</i> measured	Deviation/ (ppm) ^a	Formula	Species	Assignment	Trivial name	Literature
227	227.2017	227.2077	27	C ₁₄ H ₂₇ O ₂ [−]	[M−H] [−]	FA(14:0)	Myristic acid	47
253	253.2173	253.2308	53	C ₁₆ H ₂₉ O ₂ [−]	[M−H] [−]	FA(16:1)	Palmitoleic acid	47 and 48
255	255.2330	255.2406	30	C ₁₆ H ₃₁ O ₂ [−]	[M−H] [−]	FA(16:0)	Palmitic acid	47 and 48
279	279.2330	279.2405	27	C ₁₈ H ₃₁ O ₂ [−]	[M−H] [−]	FA(18:2)	Linoleic acid	47
281	281.2486	281.2506	7	C ₁₈ H ₃₃ O ₂ [−]	[M−H] [−]	FA(18:1)	Oleic acid	47 and 48
283	283.2643	283.2684	15	C ₁₈ H ₃₅ O ₂ [−]	[M−H] [−]	FA(18:0)	Stearic acid	47 and 48

^aThe ToF-instrument's resolution leads only to two significant decimals but calculating a mass deviation with only two decimals results in odd values being mostly zero which is why four decimals were taken into account for calculations.

primary ion beam in high current bunched mode with a primary ion dose of 10^{12} ions/cm² in order to not exceed the static limit. An area from $100 \times 100 \mu\text{m}^2$ to $300 \times 300 \mu\text{m}^2$ was analyzed with 128×128 pixels. The target current was adjusted to avoid detector saturation. Three samples of each

preparation routine were analyzed on five different spots in both, positive and negative, ion mode. Data evaluation was performed using Surface Lab 6.4 (ION-TOF GmbH, Münster, Germany). Internal mass calibration was performed by use of the signals CH[−], CH₂[−], OH[−], C₂H[−], C₄H[−], PO₂[−],

TABLE II. List of the lipid peaks and fragments observed in the positive mode, based on lipid peaks known from literature. The theoretical and measured mass-to-charge ratio, calculated mass deviation, formula, assignment/species, and references are given.

<i>m/z</i>	<i>m/z</i> theoretical	<i>m/z</i> measured ^a	Deviation (ppm)	Formula	Assignment/species	Literature
15	15.0229	15.0233	25	CH ₃ ⁺	PC ^b -fragment	30
27	27.0229	27.0227	8	C ₂ H ₃ ⁺	PC-fragment	30
29	29.0386	29.0386	1	C ₂ H ₅ ⁺	PC-fragment	30
30	30.0338	30.0341	9	CH ₄ N ⁺	PC-fragment but also amino acids	30, 53, and 54
41	41.0386	41.0376	24	C ₃ H ₅ ⁺	PC-fragment	30
43	43.0542	43.0537	12	C ₃ H ₇ ⁺	PC-fragment but also amino acids	30 and 54
44	44.0495	44.0488	15	C ₂ H ₆ N ⁺	PC-fragment but also amino acids	30, 53, and 54
53	53.0386	53.0359	50	C ₄ H ₅ ⁺	PC-fragment	30
55	55.0542	55.0528	26	C ₄ H ₇ ⁺	PC-fragment	30
56	56.0495	56.0486	16	C ₃ H ₆ N ⁺	PC-fragment but also amino acids	30 and 53
57	57.0699	57.069	15	C ₄ H ₉ ⁺	PC-fragment	30
58	58.0651/	58.0635/	28	C ₃ H ₈ N ⁺	PC-fragment but also amino acids	30 and 53
59	59.0685	59.0730	77	¹³ CC ₂ H ₈ N ⁺	PC-fragment	30
	59.0730		1	C ₃ H ₉ N ⁺		
60	60.0808	60.0805	5	C ₃ H ₁₀ N ⁺	PC-fragment but also amino acids	30, 53, and 54
67	67.0542	67.0535	11	C ₅ H ₇ ⁺	PC-fragment	30
68	68.0495	68.0488	10	C ₄ H ₆ N ⁺	PC-fragment but also amino acids	30 and 53
69	69.0699	69.0708	13	C ₅ H ₉ ⁺	PC-fragment	30
70	70.0651	70.0680	41	C ₄ H ₈ N ⁺	PC-fragment but also amino acids	30, 53, and 54
72	72.0808	72.0829	29	C ₄ H ₁₀ N ⁺	PC-fragment but also amino acids	30, 53, and 54
74	74.0964	74.1004	54	C ₄ H ₁₂ N ⁺	PC-fragment	30
81	81.0699	81.0716	21	C ₆ H ₉ ⁺	PC-fragment	30
82	82.0651	82.0672	25	C ₅ H ₈ N ⁺	PC-fragment but also amino acids	30 and 54
83	83.0855	83.0875	24	C ₆ H ₁₁ ⁺	PC-fragment	30
84	84.0808	84.0826	22	C ₅ H ₁₀ N ⁺	PC-fragment but also amino acids	30 and 54–56
85	85.1012	85.1036	28	C ₆ H ₁₃ ⁺	PC-fragment	30
86	86.0964	86.0998	39	C ₅ H ₁₂ N ⁺	PC-fragment but also amino acids	30 and 54–56
88	88.1121	88.1093	32	C ₅ H ₁₄ N ⁺	PC-fragment	30
91	91.0542	91.0477	72	C ₇ H ₇ ⁺	PC-fragment but also amino acids	30 and 53
93	93.0699	93.0659	43	C ₇ H ₉ ⁺	PC-fragment	30
95	95.0855	95.0826	31	C ₇ H ₁₁ ⁺	PC-fragment	30
97	97.1012	97.1004	8	C ₇ H ₁₃ ⁺	PC-fragment	30
98	98.0600	98.0600	0	C ₅ H ₈ NO ⁺	PC-fragment	30
100	100.0757	100.0778	21	C ₅ H ₁₀ NO ⁺	PC-fragment	30
102	102.0913	102.0870	43	C ₅ H ₁₂ NO ⁺	PC-fragment	30

TABLE II. (Continued)

m/z	m/z theoretical	m/z measured ^a	Deviation (ppm)	Formula	Assignment/species	Literature
104	104.1070	104.1051	18	$C_5H_{14}NO^+$	PC-fragment	30
150	150.0678	150.0611	45	$C_5H_{13}NPO_2^+$	PC-fragment	30
166	166.0628	166.0566	37	$C_5H_{13}NPO_3^+$	PC-fragment	30
168	168.0784	168.0695	53	$C_5H_{15}NPO_3^+$	PC-fragment	30
182	182.0577	182.0553	13	$C_5H_{13}NPO_4^+$	PC-fragment	30
184	184.0733	184.0802	37	$C_5H_{15}NPO_4^+$	PC-fragment	30
190	190.0628	190.0565	33	$C_7H_{13}NPO_3^+$	PC-fragment	30
194	194.0941	194.0806	69	$C_7H_{17}NPO_3^+$	PC-fragment	30
196	196.0733	196.0786	27	$C_6H_{15}NPO_4^+$	PC-fragment	30
198	198.0890	198.0931	21	$C_6H_{17}NPO_4^+$	PC-fragment	30
206	206.0553	206.0492	29	$C_5H_{14}NPO_4Na^+$	PC-fragment	30
210	210.0890	210.0937	23	$C_7H_{17}NPO_4^+$	PC-fragment	30
212	212.1046	212.1018	13	$C_7H_{19}NPO_4^+$	PC-fragment	30
224	224.1046	224.1019	12	$C_8H_{19}NPO_4^+$	PC-fragment	56
226	226.0839	226.0783	25	$C_7H_{17}NPO_5^+$	PC-fragment	30
238	238.0839	238.0879	17	$C_8H_{17}NPO_5^+$	PC-fragment	30
240	240.0995	240.0921	31	$C_8H_{19}NPO_5^+$	PC-fragment	30
246	246.0866	246.0843	9	$C_8H_{18}NPNaO_4^+$	PC-fragment	30
252	252.0632	252.0451	72	$C_8H_{15}NPO_6^+$	PC-fragment	30
254	254.0788	254.0710	31	$C_8H_{17}NPO_6^+$	PC-fragment	30
256	256.0945	256.0730	84	$C_8H_{19}NPO_6^+$	PC-fragment	30
282	282.0737	282.0589	53	$C_9H_{17}NPO_7^+$	PC-fragment	30
369	369.3516	369.3142	101	$C_{27}H_{45}^+$	Cholesterol $[M+H-H_2O]^+$	49 and 50
385	385.3465	385.3025	114	$C_{27}H_{45}O^+$	Cholesterol $[M-H]^+$	50

^aThe ToF-instrument's resolution leads only to two significant decimals but calculating a mass deviation with only two decimals results in odd values being mostly zero, which is why four decimals were taken into account for calculations.

^bPC is an abbreviation for phosphatidylcholine.

C_6H^- , $C_{18}H_{35}O_2^-$ for negative ion mode and CH_3^+ , $C_2H_5^+$, $C_3H_7^+$, $C_4H_9^+$, $C_5H_{15}NPO_4^+$ for positive ion mode. Average mass resolution ($m/\Delta m$) was about 5000 at peak C_4H^- (m/z 49.01) and about 5000 at peak $C_4H_9^+$ (m/z 57.07), respectively. Values of m/z are given dimensionless as recommended by IUPAC (Ref. 45) even though m generally represents the unified atomic mass unit in u.

I. PCA

PCA was performed by use of NESAC/BIO toolbox (Spectragui) version 2.7.⁴⁶

1. PCA of lipid related peak sets

PCA is a valuable tool to discriminate (groups of) samples according to their variance in surface chemistry. However, to properly evaluate the spectral variance between samples associated to the molecules of interest—here being lipids—it is

indispensable to select an adapted peak set of mass peaks originating from intrinsic sample molecules.^{30,36} Otherwise, PCA might emphasize the variance according to contaminant or preparation induced peaks (embedding media, fixatives, etc.). To select such a peak set, some spectra from samples representing all preparation routes were screened for known lipid derived peaks. For the negative ion mode, the mass signals given in Refs. 47 and 48 were used, whereas for the positive ion mode, the mass signals given in Refs. 30, 47, and 49–52 were applied. From the initial screening and comparison, we selected the lipid related peak sets for negative and positive ion mode presented in Tables I and II, respectively. The peak set limited to fatty acids in negative ion mode was chosen due to the fact that we analyzed cell membranes which are mainly composed of phosphatidylcholines. These lead mostly to fatty acid signals in the negative ion mode meaning that other lipid signals usually present in fatty tissue have quite low signal intensities, if they are present at all. As

FIG. 2. Optical images of two chemically fixed samples (GluPFAPBS and GluH₂O), FD, FH preparation, and FF. The first three images were taken dehydrated prior to measurement while FH as well as FF images were taken dehydrated after measurement as required by these sample preparations.

already stated by Anderton *et al.*,³⁰ lipid-related peaks with m/z 201–300 are usually more specific but have lower discriminant power in the spectra due to their lower intensities. Therefore, we chose to take into account all possible lipid peaks in positive ion mode, also those with lower m/z , while keeping in mind that some peaks might be more specific than others when it comes to data interpretation. In Table II, we tried to highlight this fact by exemplarily pointing out which peaks could also originate from amino acids while there might still be other parent molecules, especially for fragment ion peaks in the very low mass range.

The resulting spectra data form a matrix where the rows represent the different samples and where the columns correspond to the individual mass peaks. Prior to PCA, these data were scaled by square root mean centering but no normalization procedure was applied to prevent misinterpretation of the raw data. In comparison to other cell signals, lipids have low count rates and are extremely heterogeneously expressed throughout the different preparations. In general, the presented spectra from various preparations differ to a large extent. Typical normalization procedures like normalization to the total intensity or the sum of selected peaks would thus, in some cases, accentuate noise and, in any case, minimize real effects introduced externally by different preparation routines. Furthermore, normalization was not necessary because external effects like instrumental conditions were in this case negligible.

However, the data experienced quasi-intrinsic normalization because the primary ion dose was 10^{12} ions/cm² for all spectra and sample areas were adjusted to $150\ \mu\text{m}^2$ in order to have a comparable total ion dose for all spectra.

III. RESULTS AND DISCUSSION

A. Results from optical images

All sample preparations led to visibly intact cells, as can be seen from exemplary confocal microscope images in Fig. 2. Images of GluPFAPBS, GluH₂O, and FD preparations are shown dehydrated prior to measurement. As this is obviously impossible for FH and FF preparations, which require frozen-hydrated measurement, these images were taken after measurement and subsequent defrosting at ambient conditions. Freeze-fractured cells look slightly different from other preparations owing to the fact that the cell interior is exposed.

To evaluate the impact of the different sample preparation routes outlined in Fig. 1 on the presence of lipids, a comparison was performed by PCA with peak sets in both, negative and positive ion modes, consisting of lipid derived mass peaks (compare Table I and Table II). To get a general impression of the different preparation routes' mass spectra, the interested reader is referred to Figs. 1 and 2 of the supplementary material.⁵⁷

B. Comparison of chemical fixations including ascending alcohol series and secondary lipid fixation via osmium tetroxide

First, the influence of an ascending alcohol series (GluAlc/CPD, GluOsAlc/CPD) and secondary lipid fixation

via osmium tetroxide staining (GluOsAlc/CPD, GluOsPBS) was evaluated in comparison to simply chemically fixed samples. Alcohol dehydration was used to evaluate secondary lipid fixation via osmium tetroxide as properly fixed lipids are expected to not be extracted by application of alcohol.²⁶

PCA in negative ion mode was performed on the lipid related peak set shown in Table I. Results are presented in Fig. 3. The scores plot on PC1 and PC2 (a) shows a clear separation of all preparations with exception of an overlap of samples fixed by glutaraldehyde and paraformaldehyde. This shows that both chemical fixatives lead to similar results. PC1 captures the major variance with 94% and the scores on

FIG. 3. Scores and loadings plots on PC1 and PC2 resulting from PCA in negative ion mode with the peak list containing literature known lipid peaks on chemical fixations including ascending alcohol series and secondary lipid fixation via osmium tetroxide staining. Scores plot on PC1 and PC2 is shown in (a). The ellipses marked by a solid line represent the 95%-confidence interval (Ref. 58). (b) and (c) The loadings plot on PC1 and PC2, respectively. PC1 separates alcohol treated samples from remaining samples and loadings plot on PC1 (b) shows reduced lipid content in all samples treated with alcohol.

FIG. 4. Peak area data of m/z 255.24 [FA(16:0)] for chemical fixations including ascending alcohol series and secondary lipid fixation via osmium tetroxide staining.

PC1 separate the samples having experienced an ascending alcohol series from all other preparations.

As can be seen in the loadings plot on PC1 (b), there is a decrease of all lipid peaks in those samples treated with alcohol showing that even after osmium tetroxide fixation lipids can be extracted by subsequent use of alcohol. Even if osmium fixation is not followed by alcohol treatment (GluOsPBS preparation), no advantage over simple chemical fixation becomes obvious. GluOsPBS has only slightly lower negative scores on PC1. This confirms observations from Belazi *et al.*³⁴ who observed the extraction of phospholipids during the osmium staining procedure. As phospholipids are the major constituent components of cell membranes and should thus mainly contribute to the observed lipid/fatty acid peaks, this is

the most reasonable explanation for the loss of fatty acid peaks.

Regarding PC2, one has to keep in mind that only PC1 is truly reflective of the original data because PC2, as a subsequent PC, is calculated from the scaled covariance matrix after PC1 has been subtracted from the data set.³⁶ With this information, it is comprehensible that the m/z 255 signal [FA(16:0)] in GluOsPBS is higher than in alcohol treated samples but not really higher than in Glu/PFAPBS samples as can be seen from the peak area data in Fig. 4. Another serious disadvantage of using osmium tetroxide are osmium induced mass peaks in the negative ion mode, compare Fig. 5, which can be seen in a m/z -range higher than 300 and have already been reported in literature.^{26,34} These peaks are likely to interfere with cell signals in this mass range and make osmium tetroxide unsuitable for detection of higher molecular fragments. However, in the positive mode, there are no such interfering osmium induced peaks, at least not to such an extent.

For the positive ion mode, PCA results of the literature derived lipid peak set (given in Table II) is shown in Fig. 6. From the scores plot on PC1 and PC2 (a) it is clearly visible that PC1 which captures 75% of the total variance is again responsible for discrimination according to alcohol treatment. The loadings plot on PC1 (b) gives information about the chemical background of this discrimination. Apparently, samples without alcohol treatment express higher concentrations of highly specific fragments of phosphatidylcholines like m/z 166, 184, 206, 224, whereas alcohol treated samples express preferentially fragments in the low mass region below m/z 100. Those peaks are usually seen in lipid analysis but they are rather unspecific and can thus result from other species such as peptides, amino acids, and others. As an example, m/z 30 and 70 have preferably origin from glycine and proline, respectively.^{59,60}

FIG. 5. Comparison of spectra from samples with [upper row, (a) and (b), GluOsPBS] and without [bottom row, (c) and (d), GluPBS] osmium tetroxide in the negative ion mode and in mass ranges of $0 < m/z < 800$ (left) and $300 < m/z < 750$ (right), respectively. Osmium induced peaks that are likely to cause spectral interferences with cell signals are observed in a mass range of $m/z > 300$.

FIG. 6. Scores and loadings plots on PC1 and PC2 resulting from PCA in positive ion mode with the peak list containing literature known lipid peaks on chemical fixations including ascending alcohol series and secondary lipid fixation via osmium tetroxide staining. Scores plot on PC1 and PC2 is shown in (a). The ellipses marked by a solid line represent the 95%-confidence interval. (b) and (c) The loadings plot on PC1 and PC2, respectively. PC1 separates alcohol treated samples from remaining samples and loadings plot on PC1 (b) shows elevated content of specific phosphatidylcholine peaks, i.e., m/z 166, 184, 206, and 224, in all samples without alcohol treatment.

After all, an ascending alcohol series seems to always extract lipids, even after osmium treatment. Both sample treatments should thus be avoided for lipid analysis.

C. Simple chemical fixation in comparison to different cryofixation methods; freeze-drying, freeze-fracturing, and frozen-hydrated preparation

As alcohol treatment and osmium tetroxide fixation did not yield benefits for lipid analysis, these preparations were discarded. In the next step, “simple” chemical fixation was compared to different freeze-drying procedures (GluFD, GluN₂FD, FD), FF and “simple” FH preparation as depicted

in Fig. 1. The resulting PCA for the negative ion mode is shown in Fig. 7. The scores plot on PC1 and PC2 (a) reveals that “GluN₂FD”-samples spread over all four quadrants, which means that this preparation route is less reproducible than the other preparations.

This might be a result of the nitrogen freezing and successive water condensation on the cell surface and has thus to be perfected to be usable. Furthermore, it is visible that PC1, which captures the highest variance with 99%, separates chemical fixations (Glu/PFA/GluPFA/PBS) and the freeze-drying procedure which was performed without chemical fixation (FD) from the remaining procedures (GluFD, GluN₂FD, FH, and FF). The loadings plot on PC1 (b) shows

FIG. 7. Scores and loadings plots on PC1 and PC2 resulting from PCA in negative ion mode with the peak list containing literature known lipid peaks on simple chemical fixations in comparison to different cryofixation methods; freeze-drying, freeze-fracturing, and frozen-hydrated preparation. Scores plot on PC1 and PC2 is shown in (a). The ellipses marked by a solid line represent the 95%-confidence interval. (b) and (c) The loadings plot on PC1 and PC2, respectively. PC1 separates “Glu/PFA/GluPFA/PBS and FD” from the remaining samples, and the loadings plot on PC1 (b) shows that this is due to an elevated lipid content in the former samples.

that the elevated lipid content in the former preparation routes causes this separation.

For the positive ion mode, the results of the PCA performed with the lipid related peak set shown in Table II are shown in Fig. 8. A clear grouping of samples according to “chemical fixation,” “freeze-drying,” “frozen-hydrated preparation,” and “freeze-fracturing” is visible in the scores plot on PC1 and PC2 (a). PC1 captures a variance of 94% and mainly discriminates the chemical fixations from all other preparations with exception of parts from the freeze-drying. The loadings plot on PC1 (b) shows that chemical fixations have a higher occurrence of all peaks captured by the lipid related peak set in positive ion mode. As FF and FH samples

FIG. 8. Scores and loadings plots on PC1 and PC2 resulting from PCA in positive ion mode with the peak list containing literature known lipid peaks on simple chemical fixations in comparison to different cryofixation methods; freeze-drying, freeze-fracturing, and frozen-hydrated preparation. The scores plot on PC1 and PC2 is shown in (a). The ellipses marked by a solid line represent the 95%-confidence interval. (b) and (c) The loadings plot on PC1 and PC2, respectively. PC1 separates Glu/PFA/GluPFA/PBS and FD from the remaining samples and loadings plot on PC1 (b) shows an elevated lipid content in the former samples.

FIG. 9. Peak area data of m/z 184.08 (phosphocholine head group) for simple chemical fixations in comparison to different cryofixation methods; freeze-drying, freeze-fracturing, and frozen-hydrated preparation.

have the highest positive scores on PC1, this means that lipid peaks are merely present in these samples. Exemplary, peak area data for m/z 184, a known fragment of the phosphocholine head group, are plotted in Fig. 9, showing intensities that can be interpreted as “random noise” for FH and especially for FF samples. The same phenomenon accounts for negative spectra as can be seen for m/z 279.24 in Fig. 10.

This could also explain why the FF sample preparation appears to have high reproducibility in terms of the dimension of the 95%-confidence interval in scores plots (a) in Figs. 7 and 8 as a result of un-normalized PCAs in both, negative and positive, ion modes. It leads to the conclusion that indeed the lipid signals are not generated reproducibly by freeze-fracturing and that lipid signals are not observed in these spectra in a regular manner. To test this hypothesis, normalization to the internal cell signal CH^- was tested prior to PCA in negative ion mode. This procedure is supposed to represent the un-normalized data quite well if cell and lipid signals are generated in a reproducible manner, i.e., with relatively constant proportions. This procedure is known from literature⁶¹ and useful in cases where the variance of the

FIG. 10. Peak area data of m/z 279.24 [FA(18:2)] for simple chemical fixations in comparison to different cryofixation methods; freeze-drying, freeze-fracturing and frozen-hydrated preparation.

remaining peaks relative to the one chosen for normalization is presumed to adequately describe the chemical information. As expected, this normalization procedure reflected the results of the un-normalized PCA presented in Sec. III B comparing chemical fixations [Glu/PFA/GluOs-PBS, Glu(Os)Alc(CPD)] quite well, see Fig. 3 in supplementary material.⁵⁷

Contrary to that, in agreement with our hypothesis, it gave totally different results for the un-normalized PCA presented in Sec. III C which compared chemical fixation to different cryofixation methods [Glu/PFA/GluPFA-PBS, Glu(N₂)FD, FD, FH, and FF].

The 95%-confidence interval of FF widened very much. This emphasizes that emergence, intensity, and ratio of the mass peaks contained in the lipid peak list are not reproducible. Thus, normalizing to a cell signal like CH⁺ very clearly shows this irreproducibility, leading to large spreading in the resulting scores plot.

After all, it is comprehensible that FF sample preparation shows less lipids because the cells are fractured which leads to an exposure of the cell interior, while other preparation techniques examine the cell membrane. Irreproducibility of FF was already stated earlier in literature²⁹ and might be due to different fracture planes in the cells. However, it makes FF unsuitable for systematic investigations.

D. Comparison of chemical fixations

As a next step, different chemical fixations were assessed. Therefore, chemical fixation via glutaraldehyde, via paraformaldehyde and via a mixture of both, always with subsequent PBS treatment (GluPBS, PFAPBS, and GluPFAPBS), and a glutaraldehyde fixed sample with subsequent Milli-Q water treatment (GluH₂O) were compared via PCA. Results for the negative ion mode are presented in Fig. 11. The scores plot on PC1 and PC2 (a) shows a large overlapping part of all preparations which means that there is high chemical resemblance. However, combination with the loadings plot on PC1 (b) reveals that there is a slight tendency to higher lipid contents when the fixative paraformaldehyde is involved, i.e., GluPFAPBS-fixation gives slightly better results than simple GluPBS fixation and PFAPBS fixation shows the highest lipid content. This can be seen by decreasing negative scores on PC1 in scores plot (a).

Interestingly, GluH₂O fixation has a slightly increased lipid content (slightly higher negative values on PC1) compared to GluPBS fixation meaning that subsequent water treatment could be interesting. However, this could also be due to the matrix effect because subsequent water treatment is thought to better remove salt excess from the cell culture and therefore provides a slightly different matrix, which could enhance lipid intensities.

The loadings plot on PC2 (c) in Fig. 11 shows that for unsaturated fatty acids like *m/z* 281 [FA(18:1)], the intensities are slightly higher in GluH₂O samples than for saturated fatty acids like *m/z* 283 [FA(18:0)] (compare also Fig. 4 in supplementary material).⁵⁷ However, PC2 only captures a

FIG. 11. Scores and loadings plots on PC1 and PC2 resulting from PCA in negative ion mode with the peak list containing literature known lipid peaks on chemically fixed preparations. Scores plot on PC1 and PC2 is shown in (a). The ellipses marked by a solid line represent the 95%-confidence interval. (b) and (c) The loadings plot on PC1 and PC2, respectively. According to the combination of scores plot (a) and loadings plot on PC1 (b), the lipid content of PFAPBS and parts of GluPFAPBS and GluH₂O is elevated in comparison with simple GluPBS-fixation.

variance of two per cent meaning that this is only a hint that needs to be addressed in detail. Though for both, saturated and unsaturated fatty acids, subsequent water treatment in GluH₂O seems to extract less lipids than subsequent PBS treatment in GluPBS.

For the positive ion mode, Fig. 12 shows the result of the PCA that compares different chemical fixations with the lipid related peak set. The scores plot on PC1 and PC2 (a) shows a large overlapping part of all preparations which means that in large parts the preparations are comparable according to their lipid profile. However, GluPFAPBS samples are more likely to have lower negative scores on PC1, which accounts for higher occurrence of most lipid peaks

FIG. 12. Scores and loadings plots on PC1 and PC2 resulting from PCA in positive ion mode with the peak list containing literature known lipid peaks on chemically fixed preparations. Scores plot on PC1 and PC2 is shown in (a). The ellipses marked by a solid line represent the 95%-confidence interval. (b) and (c) The loadings plot on PC1 and PC2, respectively. Large overlapping parts in scores plot (a) result from comparable lipid profiles. However, GluPFAPBS samples show lower negative scores on PC1, which accounts for nearly all lipid peaks of the peak set as can be seen from loadings plot on PC1 (b).

from the positive peak set, which can be seen in comparison with the loadings plot on PC1 (b).

IV. SUMMARY AND CONCLUSIONS

Sample preparation is crucial for a successful analysis of lipids. Several factors have to be taken into account to obtain meaningful results for a certain purpose, and the best choice of the sample preparation routine depends on the analytes of interest, the number of samples, the requested reproducibility and others. With a focus on lipid detection for systematic investigations in negative and positive ion mode, several sample preparation routes for cell cultures of hMSCs are

evaluated in this study. In the negative ion mode the relevant mass peaks were primarily fatty acids/lipid fatty acid tail groups, whereas the relevant mass peaks in the positive ion mode were mainly caused by phosphatidylcholine fragmentation. Thus, in the chosen mass range, mass signals in the negative mode are more likely to be specific than in the positive mode.

An extensive comparison of the different preparation techniques was performed with the help of PCA and revealed clear advantages of chemical fixation techniques via glutaraldehyde and/or paraformaldehyde over other preparation routes like freeze-drying, freeze-fracturing, and frozen-hydrated preparation in terms of elevated lipid contents and reproducibility.

As expected, alcohol treatment is not advisable for lipid analysis because it extracts all fatty acids and reduces the intensity of specific phosphatidylcholine fragments in the negative and positive ion mode, respectively. Even secondary lipid fixation via osmium tetroxide staining could not prevent cells from this lipid extraction by subsequent treatment with alcohol. It also introduced known problems reported earlier in literature^{26,34} like spectral interferences in the negative ion mode and has thus no benefits for lipid analysis via ToF-SIMS. Concerning freeze-drying, GluN₂FD preparation turned out to be less reproducible, especially in the negative ion mode, while GluFD preparation indicated extraction of lipids in both ion modes. Thus only freeze-drying after plunge freezing without chemical fixation (FD) proved to be beneficial in terms of lipid content and reproducibility. The results were more promising in the negative than in the positive ion mode and still inferior to chemical fixation. Frozen-hydrated preparation showed an acceptable reproducibility. Nevertheless, lipid detection was inferior to chemical fixation and freeze-drying procedures. With freeze-fracturing, only remains of lipid peaks could be observed in both ion modes, which might be due to the exposure of the cell interior instead of the cell membrane. In general, frozen-hydrated measurement provides a different matrix due to the high water content, which could account for different peaks and peak intensities and therefore affect lipid signal detection. However, at a second glance, freeze-fracturing itself revealed to have a lack of reproducibility making it incompatible with the aim of systematic investigation. This does not mean that freeze-fracturing is in general of no use for cell analysis with ToF-SIMS. On the contrary, methods involving frozen-hydrated measurement, here FF and FH, are a valuable tool to perform analysis of mobile compounds or to examine the localization of specific molecules in proof-of-concept experiments. However, if the aim of analysis is a systematic investigation, which needs a larger number of comparable samples to be measured, and especially when statistic tools are involved, FF should not be the method of choice. Instead, chemical fixation has proven to be useful for that purpose. For lipid analysis, paraformaldehyde fixation or a mixture of paraformaldehyde and glutaraldehyde is recommended with subsequent “storage” in phosphate buffered saline or even Milli-Q water as it has proven beneficial in

this study. The analysis of lipids originating from cell membranes appears to be a good indicator for the condition of cells in general. We hope that this extensive analysis will be of use to decide for the best preparation route for the systematic analysis of cell cultures.

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