Storage of cell samples for ToF-SIMS experiments—How to maintain sample integrity

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In order to obtain comparable and reproducible results from time-of-flight secondary ion mass spectrometry (ToF-SIMS) analysis of biological cells, the influence of sample preparation and storage has to be carefully considered. It has been previously shown that the impact of the chosen preparation routine is crucial. In continuation of this work, the impact of storage needs to be addressed, as besides the fact that degradation will unavoidably take place, the effects of different storage procedures in combination with specific sample preparations remain largely unknown. Therefore, this work examines different wet (buffer, water, and alcohol) and dry (air-dried, freeze-dried, and critical-point-dried) storage procedures on human mesenchymal stem cell cultures. All cell samples were analyzed by ToF-SIMS immediately after preparation and after a storage period of 4 weeks. The obtained spectra were compared by principal component analysis with lipid- and amino acid-related signals known from the literature. In all dry storage procedures, notable degradation effects were observed, especially for lipid-, but also for amino acid-signal intensities. This leads to the conclusion that dried samples are to some extent easier to handle, yet the procedure is not the optimal storage solution. Degradation proceeds faster, which is possibly caused by oxidation reactions and cleaving enzymes that might still be active. Just as well, wet stored samples in alcohol struggle with decreased signal intensities from lipids and amino acids after storage. Compared to that, the wet stored samples in a buffered or pure aqueous environment revealed no degradation effects after 4 weeks. However, this storage bears a higher risk of fungi/bacterial contamination, as sterile conditions are typically not maintained. Thus, regular solution change is recommended for optimized storage conditions. Not directly exposing the samples to air, wet storage seems to minimize oxidation effects, and hence, buffer or water storage with regular renewal of the solution is recommended for short storage periods. © 2016 American Vacuum Society. [<http://dx.doi.org/10.1116/1.4940704>]

I. INTRODUCTION

In recent years, time-of-flight secondary ion mass spectrometry (ToF-SIMS) has become a technique of increasing interest for a wide range of biological samples.¹ In comparison to other mass spectrometry imaging techniques like matrix-assisted laser desorption/ionization (MALDI) or desorption electrospray ionization (DESI), a much better lateral resolution of about 200–500 nm can be achieved. This makes ToF-SIMS a promising technique for the analysis of small biological features in tissue sections and cell samples.¹

Systematic ToF-SIMS studies on biological samples are highly dependent on reproducible sample preparation techniques. Previous studies on cell analysis have compared various preparation methods focusing on different aspects in order to maintain sample integrity and to reduce analytical artifacts.^{2–12} Such studies are necessary to ensure that differences in measured spectra are due to true sample differences (e.g., “healthy” versus “diseased” cells) and not sample preparation-induced artifacts. It was inter alia revealed that frozen-hydrated analysis of freeze-fractured cells is the gold-standard for adequate detection of diffusible elements in cells.⁶ Still, this method struggles with problems in terms of complexity, reproducibility, and applicability to a large

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number of samples.^{3,11,13} For these purposes, chemical fixation and freeze-drying have proven to be beneficial.^{11,14,15} In addition, to obtain reliable results from biological samples, it is important to minimize their natural intrinsic variance by ensuring comparable conditions at all times prior and during measurement. For example, in the case of cells that constantly proliferate, samples should be harvested simultaneously to attain the same cultivation time and thus confluence, cell counts, differentiation time, passage, phenotype, age, etc. Moreover, to obtain statistically relevant results, it is recommended to analyze at least three samples of each sample group.¹⁶ These requirements will produce a large number of cell samples that cannot be analyzed at once. Therefore, sample storage is unavoidable. Moreover, it is well-known that sample storage is required during clinical studies because patient samples are usually not provided at the same time.¹⁷ Finally, sample storage is crucial when experiments have to be repeated or verified or when samples are shipped between different laboratories.

Various storage procedures can be applied, depending on the sample preparation routine used. Prior to analysis, cryofixed samples can be stored easily in liquid nitrogen or frozen at -80°C .^{6,18} These are common ways to ensure that biological samples are preserved. However, after initial measurement, cryostorage can be complicated if the samples are exposed to a humid atmosphere. Once the samples are defrosted, sample integrity will be compromised. Furthermore, humid atmosphere easily leads to a layer of condensed water on the cells. This condensed water cannot be removed readily or reproducibly prior to further frozen-hydrated analysis. Water condensation/defrosting can be avoided by handling the samples in dry, inert atmosphere (Ar/N_2) at all times. This, however, is difficult to achieve with standard instrumentation. One approach to deal with this issue was the development of the glovebox-equipped J105 3D Chemical Imager by Fletcher *et al.* in collaboration with Ionoptika, Ltd.¹⁹ Storage and analysis of multiple replicates of different sample groups for systematic studies has, however, not been reported for this system.

In addition to cryostorage, other storage conditions can be roughly categorized as “wet” and “dry” storage. Dry storage conditions are achieved after air-drying chemically fixed samples or with more complex procedures like critical point drying (CPD) or freeze-drying. During critical point drying, the cell morphology is maintained after replacing water by an organic solvent such as acetone or ethanol. To prevent loss of lipids during solvent replacement and to fix the membranes, osmium tetroxide is typically used as secondary chemical fixation agent.^{15,20} CPD has been used as drying method for cells prior to ToF-SIMS analysis in several cases,^{15,21,22} e.g., for the detection of strontium in cells by Kokesch *et al.*²³ Freeze-drying is typically used to obtain dry samples without the need of chemical fixation. Once the samples are dried, handling is very easy, and the risk of cell dissolution is eliminated. However, direct exposure of cells to oxygen bears the risk of oxidation and enzymes, such as lipases, might still be active.^{24,25}

Under wet storage conditions, after chemical fixation, cell samples are stored in a solution, e.g., water and/or buffers. After a secondary lipid fixation with osmium tetroxide, cell samples can also be stored in organic solvents like ethanol. Wet storage is convenient in terms of sample handling but may cause problems with bacteria and fungi because fully sterile conditions are difficult to maintain. For example, bacterial contamination during storage has been reported before for platelet samples.²⁶ Additionally, cell dissolution is possible when cells are placed in a liquid.

While many publications do not provide further information on storage, dry storage of cells was, for example, applied by Malm *et al.* in Fluoroware containers prior to ToF-SIMS analysis.¹⁵ Dry storage in a desiccator has been reported by Gazi *et al.* prior to FTIR microscopy and, for neurons, by Tucker *et al.* prior to ToF-SIMS measurements.^{27,28} Wet storage in PBS was described for flow cytometry by Aust *et al.*⁸ From literature studies in other fields, we know that several factors and processes can damage cell samples. Such factors include high temperature, introduced oxidation, deamidation, glycation or nitrosylation processes, high salt concentrations, and others.²⁴ With gas chromatography, Castro *et al.* studied changes in the cell membrane of *Lactobacillus bulgaricus* during storage after freeze-drying and reported on the decrease of the cell membrane fatty acid content.²⁵

So far, the effects of different storage procedures in combination with specific sample preparations remain largely unknown. In our recent publication, we focused on sample preparation induced differences on human mesenchymal stem cell samples (hMSCs).¹¹ We have learned that, compared to other preparation routines, analysis of chemically fixed and cryofixed freeze-dried cells leads to reproducible spectra with good and reproducible signal intensities of the cell membrane lipids. In the following study, we will investigate storage introduced trends on these differently prepared cell samples. To the best of our knowledge, this has not been addressed in previous literature. Specifically, the question of how long and under which conditions fixed cell samples can be stored until the sample integrity is compromised remains unanswered. Therefore, this study examines different storage procedures for human mesenchymal stem cell samples. Dry and wet storage procedures are compared after application of several sample preparation techniques. The leading question is if changes in the membrane composition can be observed after a storage period of 4 weeks. More detailed, major effects introduced by exposure to air when dry storage is performed are analyzed and compared to wet storage. The aim is to find a procedure that reduces degradation artifacts to a minimum.

In a first step, different drying procedures are compared, while in a second step, the focus is laid on wet storage in different solutions after initial fixation via glutaraldehyde. Specifically, for dry storage, air-dried chemically fixed cells (using solely glutaraldehyde), and critical point dried (CPD) glutaraldehyde- and osmium tetroxide-fixed cells are compared to freeze-dried samples. The latter were fixed by

cryofixation, and no chemical fixation step was involved. For wet storage procedures, glutaraldehyde-fixed cells stored in buffer or water were compared to glutaraldehyde- and osmium tetroxide-fixed cells stored in ethanol. Even after secondary fixation, every alcohol treatment bears the risk of lipid loss. Away from this obvious effect on lipids, the influence of alcohol treatment and CPD storage on the integrity of the membrane proteins is worth being addressed. To access the degradation process of lipids and proteins during storage, lipid and amino acid ToF-SIMS signals are evaluated and compared via principal component analysis (PCA). Our results suggest that wet storage is beneficial for the preservation of the cell membrane integrity, while dry storage leads to undesirable degradation effects. We are convinced that this study provides valuable information to researchers in the (SIMS) cell biology community who plan to analyze multiple replicates of fixed cells that must be stored prior to analysis or during ongoing studies.

II. EXPERIMENT

The cell surface of fixed hMSC samples was analyzed via ToF-SIMS immediately after preparation and after a 4 week storage period at 4 °C. The integrity of the cell membrane was taken as an indicator of the overall preservation of the cells' integrity. Positive ion mode spectra were evaluated and compared via PCA with a peak set consisting of amino acid- and lipid-derived peaks, as proteins and lipids, especially phospholipids, are the main constituents of the cell membrane.²⁹ In the negative ion mode, fatty acid tail groups of the lipids can be easily detected, while amino acids are hard to detect. However, it was a particular interest of this study to also examine amino acid peaks to see possible degradation effects on these species. This was especially valuable for preparations that reduced the overall lipid content through alcohol treatment. Hence, to be able to examine both analytes simultaneously, the positive ion mode was chosen.

All experimental details prior to the storage procedure are described in detail in our recent publication, which deals with different fixation and sample preparation methods for cells.¹¹ In continuation of this work, the present study focuses on sample storage. In Secs. II A–II F, the sample preparation methods will therefore only be described briefly. A graphical summary of all applied sample preparation techniques and subsequent storage procedures is given in Fig. 1.

A. Isolation of human mesenchymal stem cells

The local ethical committee of the Justus-Liebig-University of Giessen approved the experiments. The bone marrow-derived human mesenchymal stem cells used for this experiment were extracted from the head of a femur of one single donor provided from the Department of Trauma Surgery, University Hospital of Giessen-Marburg (UKGM; Giessen, Germany).

FIG. 1. Overview of all investigated sample preparation routines and subsequent storage procedures. Acronyms for the respective sample routes are given in the figure. AAC, ammonium acetate; Alc, alcohol/ethanol; CPD, critical point drying; FD, freeze-drying; Glu, glutaraldehyde; PBS, phosphate buffered saline; and Os, osmium tetroxide.

B. Cell culture

Cell culture was carried out for 7 days with 20 000 cells of the third passage seeded onto ca. $1 \times 1 \text{ cm}^2$ silicon wafers that were cleaned with demineralized water, acetone, and ethanol prior to cell seeding. The culture medium consisted of minimum essential medium α (α MEM, Gibco, USA), supplemented with 20% fetal bovine serum (Biocrom, Germany) and 1% penicillin/streptomycin (Gibco, USA) and was changed regularly every 2–3 days. This led to a homogeneous monolayer of cells on the silicon wafers for analysis.

C. Ammonium acetate rinsing solution

For rinsing the cells, a 150 mM ammonium acetate solution in Milli-Q water (TOC: 2 ppb, resistivity: 16.6 M Ω cm) at pH 7.4 was used to remove excess salts from the cell culture.

D. Chemical fixation and secondary treatments

GluPBS/GluH₂O/GluOsAlc(CPD): For chemical fixation, the wafers seeded with the cells were washed and then fixed in a 2.5% glutaraldehyde buffer solution, as described in detail by Schaepe *et al.*¹¹ The first analysis was performed as soon as possible after preparation. Until first analysis, all samples (GluPBS/GluH₂O/GluOsAlc) were stored wet in

their respective solution (PBS, H₂O, and ethanol). After initial analysis, the samples were stored (at 4 °C) either dry at atmospheric pressure or in PBS, water, or ethanol.

1. Secondary osmium tetroxide fixation

GluOsAlc(CPD): Osmium tetroxide fixation for secondary lipid fixation was performed using a 1% osmium tetroxide solution (prepared from pure osmium tetroxide; Roth, Germany).

2. Ascending alcohol series

GluOsAlc(CPD): Glutaraldehyde and OsO₄ fixed cells were transferred sequentially to 30%, 50%, 70%, 80%, and 96% ethanol solutions (prepared from ≥99.8%, pa., Roth, Germany) for 7 min, each. Finally, they were placed four times for 10 min into pure ethanol. GluOsAlc samples were stored in pure ethanol while GluOsAlcCPD samples were dried via critical point drying.

3. Critical point drying

GluOsAlcCPD: A CPD 030 Critical point dryer (Baltec, Principality of Liechtenstein) was used. First, the cell samples were cooled down to 10 °C in ethanol. As medium, liquid CO₂ was introduced, and the system was given 7 min for equilibration before the medium was ejected. This procedure was repeated five to nine times until no ethanol odor could be detected at the outlet. Afterwards, the temperature was increased to 41 °C, using a heating rate of 3.2 K/min. The as-produced supercritical CO₂ was slowly released, and the as-dried samples were stored at 4 °C under atmospheric pressure.

E. Cryofixation

1. Rapid freezing (plunge-freezing)

After stopping the cell cultivation, the medium was removed, and the cells were rinsed in an ammonium acetate solution, as described above. Afterwards, the samples were plunged into liquid propane and transferred into liquid nitrogen for storage until the freeze-drying procedure.

F. Freeze-drying

Freeze-drying (FD) was performed as soon as possible after cryofixation via plunge-freezing. Therefore, the samples were transferred into the precooled (−30 °C) chamber of a commercial Gamma 1–20 freeze-dryer (Christ, Osterode, Germany) and cooled for 2 h at atmospheric pressure. The samples were cooled at a pressure of 0.011 mbar for 6 h. Finally, the temperature was gradually increased to 30 °C at a pressure of 0.009 mbar. The samples were kept under these conditions for at least 10 h.

G. ToF-SIMS analysis

ToF-SIMS data were acquired using a TOF.SIMS 5 instrument (ION-TOF, Münster, Germany) equipped with a pulsed 25 keV Bi primary ion source. Data were collected in

spectrometry mode (hc-bu) with Bi₃⁺-clusters in the positive ion mode. Areas between 100 × 100 μm² and 250 × 250 μm² were analyzed with 128 × 128 pixels. The target current was adjusted to avoid detector saturation. The total dose density was 1 × 10¹² ions/cm² to stay below the static limit. To evaluate the different storage procedures depicted in Fig. 1, five spots on each of three samples from each sample group were measured immediately after preparation and after a storage period of 4 weeks at 4 °C in their special environment.

Data evaluation was performed with the software SURFACE LAB 6.5 (ION-TOF GmbH, Münster, Germany). For internal mass calibration in the positive ion mode, signals CH₃⁺, C₂H₅⁺, C₃H₇⁺, C₄H₉⁺, and C₅H₁₅NPO₄⁺ were used. Average mass resolution ($m/\Delta m$) was 4000 at peak C₄H₉⁺ (m/z 57.07). Values of m/z are given dimensionless as recommended by IUPAC, even though m generally represents the unified atomic mass unit in u.³⁰

H. PCA and experimental strategy

PCA is a multivariate data analysis method, well explained in the literature. The interested reader is referred to Refs. 16, and 31–35 for detailed information on the principles of the PCA method. Briefly, PCA is a multivariate method that considers the overall variance within a data set. It is used to find similarities or differences between samples and sample groups. Here, it was used to facilitate interpretation, identify grouping of the samples, and, therefore, be able to better judge reproducibility of the performed procedures.

As cell lipid membranes mainly consist of lipids, especially phospholipids and cholesterol, and have embedded proteins, all literature known lipid- and amino acid-related signals from Refs. 14, and 36–42, detected within m/z 0–400 of our samples, were chosen to form the peak list presented in Table I.²⁹ No normalization procedure was applied to prevent misinterpretation of the raw data, but the data were square root mean-centered. The different sample preparation techniques applied lead to very different spectra because of different matrices caused by alcohol-, osmium-, PBS-, or water-treatment or because of the storage itself. These spectral influences are real effects that should be considered during data analysis and are the main interest of this study. They are explicitly not due to measurement influences such as variations in the primary ion current or other instabilities of the instrument. Typical normalization procedures like normalization to the total ion intensity or sum of selected peaks were tested on the data before interpreting the data without normalization. These normalization procedures were not consistent with any obvious trends seen in the raw data. Thus, normalization would mask real effects and moreover accentuates noise in some cases. However, the spectra experienced some quasi-intrinsic normalization by a constant ion dose of 1 × 10¹² ions/cm² per spectrum, and additionally, all sample areas were adjusted to 150 × 150 μm² for comparable total ion doses in all spectra. This procedure was necessary because we had to vary our analysis area to avoid detector saturation as described in Sec. II G. A constant ion dose on

TABLE I. Detailed information on the peak list used for PCA in the positive ion mode. Lipid- and amino acid-related peaks and fragments known from the literature were used. The theoretical and measured mass-to-charge ratio, formula, assignment/species, and references are given.

<i>m/z</i>	<i>m/z</i> theoretical	<i>m/z</i> measured	Formula	Assignment/species	Reference
15	15.0229	15.02	CH ₃ ⁺	PC ^a -fragment	14
27	27.0229	27.02	C ₂ H ₃ ⁺	PC-fragment but also from proteins	14 and 42
29	29.0386	29.04	C ₂ H ₅ ⁺	PC-fragment	14
30	30.0338	30.03	CH ₄ N ⁺	PC-fragment but also amino acid “Gly”	14, 38, 39, and 41
41	41.0386	41.04	C ₃ H ₅ ⁺	PC-fragment but also from proteins	14 and 42
43a	43.0291	43.03	CH ₃ N ₂ ⁺	Amino acid fragment “Arg”	39 and 41
43b	43.0542	43.05	C ₃ H ₇ ⁺	PC-fragment but also from proteins	14 and 42
44	44.0495	44.05	C ₂ H ₆ N ⁺	PC-fragment but also amino acids “Ala/Cys”	14, 38, and 41
53	53.0386	53.04	C ₄ H ₅ ⁺	PC-fragment	14
55a	55.0178	55.02	C ₃ H ₃ O ⁺	Amino acid fragment “Tyr”	41
55b	55.0542	55.05	C ₄ H ₇ ⁺	PC-fragment but also from proteins	14 and 42
56	56.0495	56.05	C ₃ H ₆ N ⁺	PC-fragment but also several amino acids	14, 38, and 42
57	57.0699	57.07	C ₄ H ₉ ⁺	PC-fragment but also from proteins—may partly overlap with:	14 and 42
	57.0573		C ₃ H ₇ N ⁺	Amino acid fragment “Lys”	41
58	58.0651	58.06	C ₃ H ₈ N ⁺	PC-fragment	14
59a	59.0478	59.05	CH ₅ N ₃ ⁺	Amino acid fragment Arg	41
59b	59.0685	59.07	¹³ CC ₂ H ₈ N ⁺	PC-fragment	14
	59.0730		C ₃ H ₉ N ⁺		
60a	60.0444	60.04	C ₂ H ₆ NO ⁺	Amino acid fragment “Ser”	39 and 41
60b	60.0808	60.08	C ₃ H ₁₀ N ⁺	PC-fragment	14
61	61.0106	61.01	C ₂ H ₅ S ⁺	Amino acid fragment “Met”	39 and 41
67	67.0542	67.05	C ₅ H ₇ ⁺	PC-fragment	14
68	68.0495	68.05	C ₄ H ₆ N ⁺	PC-fragment but also amino acid “Pro”	14, 38, 39, and 41
69a	69.0335	69.03	C ₄ H ₅ O ⁺	Amino acid fragment “Thr”	39
69b	69.0699	69.07	C ₅ H ₉ ⁺	PC-fragment but also from proteins	14 and 42
70a	70.0287	70.03	C ₃ H ₄ NO ⁺	Amino acid fragment “Asn”	39
70b	70.0651	70.07	C ₄ H ₈ N ⁺	PC-fragment but also amino acids “Arg/Leu/Pro”	14 and 41
71	71.0128	71.01	C ₃ H ₃ O ₂ ⁺	Amino acid fragment Ser	39 and 41
72a	72.0444	72.05	C ₃ H ₆ NO ⁺	Amino acid fragment Gly	41
72b	72.0808	72.08	C ₄ H ₁₀ N ⁺	PC-fragment but also amino acid “Val”	14, 38, 39, and 41
74a	74.0600	74.06	C ₃ H ₈ NO ⁺	Amino acid fragment Thr	39 and 41
74b	74.0964	74.10	C ₄ H ₁₂ N ⁺	PC-fragment	14
81	81.0699	81.07	C ₆ H ₉ ⁺	PC-fragment	14
82	82.0651	82.07	C ₅ H ₈ N ⁺	PC-fragment	14
83a	83.0491	83.05	C ₅ H ₇ O ⁺	Amino acid fragment Val	39 and 41
83b	83.0855	83.09	C ₆ H ₁₁ ⁺	PC-fragment	14
84a	84.0444	84.05	C ₄ H ₆ NO ⁺	Amino Acid fragment “Glu/Gln”	39 and 41
84b	84.0808	84.08	C ₅ H ₁₀ N ⁺	PC-fragment but also amino acid Lys	14 and 41
85	85.1012	85.10	C ₆ H ₁₃ ⁺	PC-fragment	14
86	86.0964	86.10	C ₅ H ₁₂ N ⁺	PC-fragment but also amino acids “Leu/Ile”	14, 38, 39, and 41
88	88.1121	88.11	C ₅ H ₁₄ N ⁺	PC-fragment	14
91	91.0542	91.05	C ₇ H ₇ ⁺	PC-fragment but also from proteins	14 and 42
93	93.0699	93.07	C ₇ H ₉ ⁺	PC-fragment	14
95	95.0855	95.08	C ₇ H ₁₁ ⁺	PC-fragment	14
97	97.1012	97.10	C ₇ H ₁₃ ⁺	PC-fragment	14
98	98.0600	98.06	C ₅ H ₈ NO ⁺	PC-fragment	14
100	100.0757	100.08	C ₅ H ₁₀ NO ⁺	PC-fragment—may partly overlap with:	14
	100.0869		C ₄ H ₁₀ N ₃ ⁺	Amino acid fragment Arg	41
102	102.0913	102.09	C ₅ H ₁₂ NO ⁺	PC-fragment	14
104a	104.0528	104.05	C ₄ H ₁₀ SN ⁺	Amino acid fragment Met	38 and 41
104b	104.1070	104.11	C ₅ H ₁₄ NO ⁺	PC-fragment	14
110	110.0713	110.07	C ₅ H ₈ N ₃ ⁺	Amino acid fragment “Arg/His”	38, 39, and 41
115	115.0502	115.05	C ₄ H ₇ N ₂ O ₂ ⁺	Amino acid fragment Gly	41
120	120.0808	120.08	C ₈ H ₁₀ N ⁺	Amino acid fragment “Phe”	39
130	130.0651	130.06	C ₉ H ₈ N ⁺	Amino acid fragment “Trp”	38 and 39
136	136.0757	136.07	C ₈ H ₁₀ NO ⁺	Amino acid fragment Tyr	38 and 39
150	150.0678	150.06	C ₅ H ₁₃ NPO ₂ ⁺	PC-fragment	14

TABLE I. (Continued.)

<i>m/z</i>	<i>m/z</i> theoretical	<i>m/z</i> measured	Formula	Assignment/species	Reference
166	166.0628	166.05	C ₅ H ₁₃ NPO ₃ ⁺	PC-fragment	14
168	168.0784	168.07	C ₅ H ₁₅ NPO ₃ ⁺	PC-fragment	14
182	182.0577	182.05	C ₅ H ₁₃ NPO ₄ ⁺	PC-fragment	14
184	184.0733	184.08	C ₅ H ₁₅ NPO ₄ ⁺	PC-fragment	14
198	198.0890	198.09	C ₆ H ₁₇ NPO ₄ ⁺	PC-fragment	14
206	206.0553	206.05	C ₅ H ₁₄ NPO ₄ Na ⁺	PC-fragment	14
224	224.1046	224.09	C ₈ H ₁₉ NPO ₄ ⁺	PC-fragment	43
226	226.0839	226.08	C ₇ H ₁₇ NPO ₅ ⁺	PC-fragment	14
246	246.0866	246.08	C ₈ H ₁₈ NPO ₄ Na ⁺	PC-fragment	14
369	369.3516	369.30	C ₂₇ H ₄₅ ⁺	Cholesterol [M+H–H ₂ O] ⁺	36 and 37
385	385.3465	385.29	C ₂₇ H ₄₅ O ⁺	Cholesterol [M–H] ⁺	37

^aPC: phosphatidylcholine.

different analysis areas results however in different total doses. For comparability, each spectrum was leveled to a total dose of 2.25×10^8 ions. This is the resulting total dose on the mostly used analysis area of $150 \times 150 \mu\text{m}^2$ with an ion dose of 1×10^{12} ions/cm². PCA was carried out using the NESAC/BIO toolbox (SPECTRAGUI) version 2.7.⁴⁴

The preparation routines described above were chosen to evaluate which solution-based procedure is best suited for the storage of hMSCs and if dry storage may be an even better option, regarding ease of handling and sample preservation. The different drying procedures were selected to see if it makes a difference how the sample is dried before dry storage. The freeze-drying procedure without chemical fixation was conducted as comparison, to find out if chemical fixation itself has any effects on storage. At this juncture, the overall aim was to identify a proper way to store fixed samples by introducing minimal altering.

III. RESULTS AND DISCUSSION

The optimal cell storage conditions were identified by comparing cell surface spectra of the hMSC samples immediately after preparation and after a storage period of 4 weeks. Moreover, the effects of wet and dry storage protocols on sample integrity were explored (as depicted in Fig. 1). For completeness, representative mass spectra for all storage procedures in the mass ranges from *m/z* 0–200 to *m/z* 200–400 after the storage period of 4 weeks are given in supplementary Figs. 1 and 2, respectively.⁴⁵ It can be stated that sample preparation and storage have an influence on the resulting surface spectra of hMSCs. The spectra were evaluated with the help of PCA by the use of the peak list presented in Table I, as described in Sec. IIH, while the focus was first laid on dry storage and then on wet storage procedures. The results will be presented in Secs. IIIA–IIIC

A. Comparison of different drying methods and dry storage

First, three different drying methods for a dry storage procedure and their overall effects on hMSC cell membrane

integrity are compared: simple air-drying (GluPBS-0w/-dry4w2x), complex freeze-drying (FD-0w/-4w2x), and critical-point-drying (GluOsAlcCPD-0w/-4w2x). In this nomenclature, “0w” stands for the initial analysis at time point “0” weeks, while “4w2x” indicates a second measurement after 4 weeks of storage. The results of the PCA in the positive ion mode with the lipid- and amino acid-related peak set of Table I are presented in Fig. 2. It is clear from Fig. 2(a) that principal component 1 (PC1) mainly informs on sample preparation-induced spectral differences. The GluOsAlcCPD-0w/-4w2x samples dominate PC1. The loadings plot on PC1 [cf. Fig. 2(b)] reveals that the negative scores are mainly related to lipid peak losses. This means that osmium tetroxide “fixation” does not show the expected fixative effect and lipids are still extracted during this preparation. We have discussed this phenomenon and the general differences between the sample preparation techniques and their influences on the initial cell surface spectra in more detail in our recent publication.¹¹ In this evaluation, we will thus mainly focus on trends seen in the PCA that can be attributed to storage. Nevertheless, to understand these results, it is important to be aware of the effects of these dissimilar sample preparation techniques. This is especially important for all alcohol and osmium treated samples where the lipid content is already initially very low and the main focus should for these samples be laid on the intensity changes of amino acid peaks. We performed PCA once without the osmium- and alcohol-treated samples to verify that including these samples does not have any effect on our final results and conclusions drawn from this PCA (see supplementary Fig. 3). The PCA leads to a scores plot that mirrors the original one that contains contributions from osmium- and alcohol-treated samples, but still shows the same characteristic peaks of the samples not treated with osmium/alcohol. In the following, we will just focus on the PCA, including these osmium- and alcohol-treated samples, as this does definitely not influence the results drawn on the nonalcohol-treated samples.

With regard to the scores plot of PC1 versus PC2 [cf. Fig. 2(a)], all procedures tend to have lower scores on PC1

FIG. 2. PCA results of samples from different dry storage procedures are shown by scores and loadings plots on PC1 and PC2. A peak list for the positive ion mode, containing lipid- and amino acid-derived peaks, was used. The scores plot of PC1 vs PC2 is shown in (a). The ellipses marked by a solid line represent the 95% confidence interval (Ref. 39). (b) and (c) show the loadings plot on PC1 and PC2, respectively. Samples from all procedures reveal lower scores on PC1 after a storage period of 4 weeks. Connected to the loadings on PC1, this finding can be attributed to reduced lipid signal intensities of the higher mass region.

after the storage period of 4 weeks. The loadings plot in Fig. 2(b) shows that lower/negative scores on PC1 are mainly related to the low mass region. As can be seen from the peak list (cf. Table I), this region comprises mainly amino acid-related peaks (m/z 30, 43.03/43a, 59.05/59a, and 70.07/70b). Regarding lipids, this low mass region can probably be attributed to lipid fragmentation, and thus degradation. In contrast, higher/positive scores cover the higher mass region and are related to more specific lipid signals (m/z 104.11/104b, 166, 184, 206, 224, 246, 369, and 385). This shows that the cell membrane lipids degrade when cell samples are stored dry.

FIG. 3. Peak area plot of (a) m/z 184.08 ($C_5H_{15}NPO_4^+$ —phosphocholine head group) and (b) m/z 104.05/104a ($C_4H_{10}SN^+$ —fragment of the amino acid methionine) for samples from different dry storage procedures. Drying procedure from left to right: air-dried, freeze-dried, and critical-point-dried. After a storage period of 4 weeks, the signal intensities decrease in all sample groups, indicating lipid- and amino acid-degradation in samples of all dry storage procedures.

In order to compare the various drying methods, we had a closer look at the signal intensities for the PC head group fragment at m/z 184.08 [Fig. 3(a)]. Overall, the plot suggests that lipid degradation is minimal when critical-point-drying (GluOsAlcCPD-0w/-4w2x) is employed. In addition, the change of the CPD samples on PC1 after 4 weeks is not as extreme as in the air- and freeze-dried samples. Still, the CPD-method does not efficiently preserve lipids. The samples prepared in this manner already have low overall lipid content directly after fixation and drying (GluOsAlcCPD-0w), as can be seen from the initial intensity of the PC head group fragment at m/z 184.08 in Fig. 3(a). Moreover, our results when monitoring the signal at m/z 184.08 show that lipid degradation takes place in all dry storage procedures. Lipid degradation is also evidenced when monitoring the peak area plots for another phosphocholine fragment at m/z 93.07 ($C_7H_9^+$) and cholesterol at m/z 369.30 ($C_{27}H_{45}^+$ —Cholesterol [$M+H-H_2O$] $^+$) [cf. supplementary Figs. 4(a) and 4(b)]. This shows that lipid degradation is not limited to phospholipids but includes other cell membrane components. For instance, the peak area plot of the amino acid

FIG. 4. PCA results of samples from different wet storage procedures are shown by scores and loadings plots on PC1 and PC3. A peak list for the positive ion mode, containing lipid- and amino acid-derived peaks, was used. The scores plot of PC1 vs PC3 is shown in (a). The ellipses marked by a solid line represent the 95% confidence interval (Ref. 39). (b) and (c) show the loadings plot on PC1 and PC3, respectively. After a storage period of 4 weeks, the alcohol-stored samples show lower scores on PC1, while the water stored samples remain largely unchanged and those from PBS storage express even slightly higher scores on PC1. When related to the loadings on PC1 (b), higher scores on PC1 are mainly due to increased lipid signal intensities of the higher mass region.

fragment of methionine ($C_4H_{10}SN^+$) at m/z 104.05 (104a in the loadings plots) in Fig. 3(b) indicates that protein/amino acid degradation also takes place. Just as well, the signal intensity for the methionine fragment decreases significantly in all dry storage procedures.

In terms of reproducibility, based on the 95% confidence intervals outlined in the scores plot in Fig. 2(a), it is evident that all preparations lead to distinct and reproducible signal intensities both immediately after preparation and after

storage. Certainly, dry preparations are more likely to produce samples with less variance. Samples stored under wet conditions may always be altered and influenced by the solvent and surrounding solutes. That is also why the GluPBS-0w samples, which were measured directly after the initial drying of the samples, show larger variations. However, taking the observed individual localization of all sample (i.e., preparation) groups in the scores plot, we believe the results are reproducible enough for systematic studies.

In conclusion, dry storage does not preserve the cell membranes efficiently. Cell degradation is evidenced in the signal intensity losses of specific higher mass lipid- and amino acid-fragments. Therefore, when preservation of the cell membrane integrity is crucial, dry storage should be avoided for cell culture sample storage. In this case, another method with less degradation impact on the samples is needed.

B. Comparison of wet storage procedures after chemical fixation with glutaraldehyde

Three different wet storage methods and their effects on the cell membrane integrity were explored after chemical fixation with glutaraldehyde: (1) storage in PBS buffer (GluPBS-0w/-4w2x) compared to (2) storage in Milli-Q water (GluH₂O-0w/-4w2x), and (3) storage in pure ethanol after secondary lipid fixation with osmium tetroxide (GluOsAlc-0w/-4w2x).

The results of the PCA from wet storage with the lipid- and amino acid-related peak set from Table I are shown in Fig. 4. The higher/positive scores on PC1 are mainly due to increased intensities of the higher mass region fragments. This can be derived from the loadings plot on PC1 [Fig. 4(b)]. As mentioned in Sec. III A, these peaks are mostly specific lipid fragments in the higher mass region when compared to the used peak list (cf. Table I, m/z 104.11/104b, 166, 184, 224, 369, and 385).

The overall PCA of the various wet storage procedures reveals significant sample preparation-induced differences [see Fig. 4(a)]. Interestingly, the PCA separates all samples treated with osmium tetroxide and alcohol (GluOsAlc-0w/-4w2x) from all other wet preparation and storage procedures and clusters them on PC1. The negative loadings on PC1 shown in Fig. 4(b) suggest that this separation is due to lipid extraction. Therefore, osmium and alcohol treated samples mainly show amino acid-related signals (m/z 30, 43a, 59a, 70a/b, 120, 130, and 136). For detailed information on these sample preparation induced differences, the interested reader is however referred to our recent publication.¹¹ To be certain that inclusion of the osmium- and alcohol-treated samples does not influence our results, we performed PCA once without these samples. The result of this PCA is illustrated in supplementary Fig. 5. The corresponding scores plot of PC1 vs PC2 [cf. supplementary Fig. 5(a)] basically looks like a cutout of the one including the alcohol treated samples (cf. Fig. 4). The loadings do not contain the characteristic peaks for the alcohol treated samples. Hence, their appearance is

FIG. 5. Peak area plot of (a) m/z 130.06 ($C_9H_8N^+$ —tryptophan fragment) and (b) m/z 184.08 ($C_5H_{15}NPO_4^+$ —phosphocholine head group) for samples from different wet storage procedures. Storage conditions from left to right: in PBS, in Milli-Q water, and in ethanol/alcohol. After a storage period of 4 weeks, signal intensities of the presented lipid- and amino acid-signal increase for PBS- and water-storage but decrease for alcohol-storage.

slightly different. Still, the plots do not reveal any new or different information for the samples stored in aqueous solution. This is why the following evaluation will be based on the PCA that includes the osmium- and alcohol-treated samples.

With the focus on storage trends, the scores plot of PC1 vs PC3 in Fig. 4(a) shows that cell samples stored in ethanol/alcohol have lower scores on PC1 after storage compared to the initial measurement. The changes (even if small) prove a degradation with regards to the lipids and especially the amino acids after the storage period. The peak area plots of the tryptophan fragment at m/z 130.06 ($C_9H_8N^+$) and the signal of the phosphocholine head group at m/z 184.08 ($C_5H_{15}NPO_4^+$) [cf. Figs. 5(a) and 5(b)] also demonstrate such degradation since both signal intensities decrease after storage in ethanol.

The samples stored in PBS-buffer or water are not statistically different from each other on PC1, suggesting that these samples (and resulting spectra) are comparable after storage (GluPBS-4w2x and GluH₂O-4w2x). Interestingly, however, some storage trends are still evident for these two aqueous storage procedures. Cell samples stored in buffer or water

tend to show an opposed behavior to the alcohol-stored samples, i.e., they express higher lipid signal intensities after 4 weeks. For water-stored samples, this becomes visible in the scores plot of PC1 vs PC3 in Fig. 4(a). After storage, water-stored samples (GluH₂O-4w2x) are clearly separated on PC3. When PCA is performed without the alcohol-treated samples, this effect is already pointed out on PC2 instead of PC3, as can be seen in supplementary Fig. 5(a). PBS-stored samples show slightly higher scores on PC1 after 4 weeks [cf. Fig. 4(a) - GluPBS-4w2x]. The corresponding loadings plots in Figs. 4(b) and 4(c) illustrate that high (positive) scores on these two PCs (PC1 and PC3) are connected to highly specific cholesterol (m/z 369, 385) and phospholipid signals (m/z 166, 184, and 224). These increasing lipid signal intensities after storage are also illustrated by the peak area plot of the phosphocholine head group at m/z 184.08 in Fig. 5(b). The intensity of this specific lipid signal increases for PBS- and water-storage but decreases for alcohol-storage. In supplementary Fig. 7, this is also shown for the phospholipid fragment at m/z 93.07 ($C_7H_9^+$) and cholesterol as another lipid species at m/z 385.29 ($C_{27}H_{45}O^+$ —Cholesterol [$M-H$] $^+$).

Figure 5(a) illustrates that the effect of increasing signal intensities after storage also emerges for proteins/amino acids. This is emphasized by the peak area distributions of m/z 130.06 ($C_9H_8N^+$), the specific fragment of the amino acid tryptophan, which has a negative loading on PC1. The basic difference for amino acid-related peaks seems to be the following. Even after 4 weeks of storage and increasing intensities in the buffer- and water-stored samples, the intensities remain still higher in the alcohol-stored samples. This might be due to the simple fact that this preparation routine (GluOsAlc-0w/-4w2x) preserves proteins better than lipids. Thus, if the main constituent of the cell membrane, the lipids, are extracted, the ionization probability for proteins becomes comparably higher. This, in turn, leads to higher amino acid total signal intensities within the changed matrix.

In summary, buffer (PBS) and Milli-Q water storage seem to preserve the cell membrane adequately, and signal intensities were even higher after the storage period of 4 weeks. These increasing intensities are probably due to a changing matrix that enhances the ionization of protonated fragments and will be discussed in detail in Sec. III C.

C. Possible causes for observed changes and how to cope with them for analysis and data evaluation

In Secs. III A and III B, it was shown that lipid and amino acid signals decrease during dry storage and wet storage in alcohol, while they increase in cell samples stored in buffer or water.

In our opinion, for dry storage, matrix effects can be neglected as possible reason for the observed degradation effects. One has to keep in mind that exactly the same samples were measured again after 4 weeks. Any matrix change during that period should be a result of salt deposition from the surrounding air or diffusion of some diffusible molecules

to the surface. Salt accumulations are known to alter and attenuate the ionization efficiency of biomolecules.^{15,46} In biological tissue samples, diffusion to the surface has, for instance, been observed for cholesterol.⁴⁷ However, there was neither an increase of typical salt cation signal intensities such as Na^+ or K^+ nor were the cholesterol signal intensities increased after 4 weeks [cf. supplementary Fig. 4(b)]. Still, there was an overall decrease in the signal intensities, while the patterns of the spectra themselves looked quite comparable. We also exclude instrumental variations as the reason for this behavior, because within the same storage period, the signal intensities increased for some wet storage procedures as explained above. Therefore, the observed intensity decreases can indeed be attributed to real degradation effects.

Conversely, we doubt that the increasing signal intensities in aqueous wet storage procedures observed in Sec. III B are real effects. Clearly, the fact that lipid and amino acid signals increase after a storage period of 4 weeks does not point to an actual increase of these analytes in the sample. Instead, an explanation for this effect can probably be found in matrix effects. A buffered, or even better, a very pure aqueous environment might, to some extent, wash away cell culture medium residues like inorganic salts that prevent ionization of the regarded analytes. This would lead to higher ionization probabilities and thus yields of the specific lipid peaks in the higher mass region (in particular of the protonated fragments), rather than actually increasing their concentration. This hypothesis is strengthened by the peak area plots shown in Fig. 6 that depict (a) m/z 224.09— $\text{C}_8\text{H}_{19}\text{NPO}_4^+$ and (b) m/z 246.08— $\text{C}_8\text{H}_{18}\text{NPO}_4\text{Na}^+$, which are two differently ionized fragments of the same molecule. The first one is the protonated fragment, while the second one is the sodium adduct. Contrary to the sodium adduct that shows decreased intensities in all wet storage procedures after 4 weeks, the protonated fragment shows increased intensities after aqueous PBS- and water-storage, which is, according to our hypothesis, the result of a changing matrix and, therefore, ionization probability.

In conclusion, all tested storage and preparation techniques were demonstrated to have some influence on the samples and resulting cell surface spectra. Among these influences, some were more beneficial than others. In particular, the observed degradation effects for dry storage are undesirable and dry storage should consequently be avoided. Wet storage bears other risks that have been pointed out in Sec. I and might lead to higher variances between samples. Still, the advantages of wet storage in buffer or water outweigh these disadvantages, which seem to play only a minor role. In this study, aqueous wet storage has proven to be advantageous and more suitable for cell culture samples.

In this PCA study, no normalization procedure was applied to explicitly point out sample preparation- and storage-induced differences on the cell surface spectra. In order to obtain comparable spectral data from cell samples also after storage and to minimize the evaluated undesired effects of this study, we do however highly recommend the

FIG. 6. Peak area plot of (a) m/z 224.09 ($\text{C}_8\text{H}_{19}\text{NPO}_4^+$ —PC-fragment) and (b) m/z 246.08 ($\text{C}_8\text{H}_{18}\text{NPO}_4\text{Na}^+$ —PC-fragment) for samples from different wet storage procedures. Storage conditions from left to right: in PBS, in Milli-Q water, and in ethanol/alcohol. After a storage period of 4 weeks, the signal intensities of the protonated PC-fragment (a) increase for storage in PBS and water but decrease for storage in alcohol, while the signal intensities of the sodium adduct (b) decrease for all storage procedures.

application of a normalization procedure prior to data interpretation for systematic studies. This implies that the optimal sample preparation procedure for the regarded system has already been defined beforehand. Normalization to the total ion intensity or the sum of selected peaks, depending on the peak list that is used, should be able to computationally eliminate the investigated storage effects. This should be effective under the assumption that the total lipid- and amino acid-content stays comparable in the wet-stored cell samples and that it is solely the matrix and thus ionization efficiency that changes.

IV. SUMMARY AND CONCLUSIONS

ToF-SIMS analysis of hMSCs' cell membranes was carried out after initial preparation and after a storage period of 4 weeks. Various storage procedures under wet and dry conditions were compared. The integrity of the cell membrane was taken as an indicator for the overall preservation of the cells. All cell surface spectra were compared via PCA in combination with a lipid- and amino acid-related peak list, consisting of literature known peaks, to facilitate a qualitative interpretation of molecular changes during storage and

between the different storage procedures. First, different drying techniques regarding the used fixation method were compared. Air-dried chemically fixed cells, critical point dried cells after secondary osmium tetroxide fixation, and also cryofixed and freeze-dried cells showed a decrease of specific lipid- and amino acid-signals in the higher mass range after 4 weeks of storage under atmospheric pressure at 4 °C. This leads to the assumption that degradation of the cell membrane takes place regardless of the drying technique. Probably, this degradation takes place due to oxidation and enzymatic cleavage effects.

In an attempt to avoid this degradation, chemically fixed cells were stored wet in order to prevent direct exposure to air/oxygen. Solely glutaraldehyde fixed cells were stored in buffer or water, whereas secondary-fixed cells were stored in ethanol. After 4 weeks, the cells stored in ethanol also showed a decrease in their lipid- and amino acid-profile. Interestingly, the specific lipid- and amino acid-signals increased in the glutaraldehyde-fixed cells that were stored in buffer or water. Here, the ionization probability seems to increase due to a change in the surrounding matrix, leading to an enhancement of protonated signals from the cell membrane.

However, the results from this study suggest that all tested storage techniques had an impact on the cell surface spectra. Thus, spectra acquired before and after prolonged storage should not be directly compared and, in order to get comparable results, a normalization procedure should be applied. Normalization to the sum of selected peaks should lead to comparable results in wet stored samples. Note that this normalization will only be valid under the assumption that the total lipid and amino acid content stays comparable and only the matrix and ionization efficiency change.

Furthermore, because ToF-SIMS is a vacuum technique, we cannot exclude that the vacuum itself has an influence on the samples and resulting spectra. Therefore, it would be valuable to compare the results presented here with a nonvacuum technique such as DESI or AP-MALDI. It would be also interesting to see if storage in a humidity-free desiccator could, to some extent, reduce degradation effects of dry storage and if chemically fixed cells can somehow be transferred into a −80 °C freezer without disadvantageous impacts on the cell morphology.

In summary, we deduce that cell samples should best be stored chemically fixed and wet in buffer or, even better, a highly pure aqueous environment at reduced temperature (4 °C). However, that type of storage bears the risk of cell dissolution and bacterial contamination. Therefore, the solution should be changed regularly in order to obtain optimal results.

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