Chapter

Intact Cell Mass Spectrometry for Embryonic Stem Cell Biotyping

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Abstract

Stem cells represent a unique cell type that is capable of self-renewal and differentiation into somatic cell types. Since the derivation of human embryonic stem cells and induced pluripotent stem cells, enormous potential has been recognized for disease modeling, drug development and regenerative medicine. Both embryonic stem cells and induced pluripotent stem cells possess the ability to differentiate into all three germ layers, hence they are naturally prone to respond to various differentiation stimuli. These inherent cellular fluctuations, which can result in risky phenotypic instability, must be addressed prior to introduction of these cells to human medicine, since they represent one of the major biosafety obstacles in the development of bio-industrial or clinical-grade stem cell cultures. Therefore, there is an ongoing need for novel robust, feasible and sensitive methods for determination and confirmation of the otherwise identical cells status, as well as for the detection of hidden divergences from their optimal state. A method of choice can be the intact cell mass spectrometry. Here we show how it can be applied in routine quality control of embryonic stem cell cultures.

Keywords: intact cell mass spectrometry, whole cell mass spectrometry, embryonic stem cells, cell culture, culture adaptation, differentiation, quality control

1. Introduction

Embryonic stem cells (ESCs) emerged as an amazing cell biology phenomenon several decades ago. Their derivation represents a milestone in understanding of fundamental molecular and cellular processes during early embryonic development, as well as differentiation mechanisms in somatic cells. Capacity of self-renewal and unlimited differentiation make the human ESCs (hESCs) a promising tool for regenerative medicine, tissue engineering, bio-industry, pharmacological modeling and pollutant testing. However, *in vitro* cultured ESCs suffer from inherent instability, and may develop unwanted properties over time, such as propensity to cancer development or failure of the functional phenotype. Accordingly, the stability of hESCs in long term cultures represents an essential prerequisite for a safe use in medical or industrial fields. Robust, sensitive and feasible tools are therefore required for efficient quality control of ESCs culture.

The beginnings in Mass Spectrometry (MS) development in biology correspond with the discovery of soft ionization techniques, such as Electrospray Ionization (ESI) and Matrix-Assisted Laser Desorption-Ionization (MALDI) by John Fenn (1988) and Koichi Tanaka (1988), respectively, who have been awarded with the Nobel Prize in chemistry in 2002 for these findings. MALDI MS coupled with Time-Of-Flight (TOF) analysis has been then successfully introduced to many fields beyond analytical chemistry, including proteomics, metabolomics, clinical microbiology and structural biology. MALDI TOF MS allows precise identification, structural analysis and quantification of various, predominantly soluble, analytes. MALDI TOF MS can generate molecular or spectral patterns that can be assigned to specific cell types or states, when intact cells are used as the analyte. Moreover it provides sufficient discrimination capacity due to a high variability in molecular species, constituting the whole spectrum a biomarker even without preceding peak identification.

2. Embryonic stem cells and the need of quality control

Shortly after fertilization, early human embryo is composed of blastomeres - equal undifferentiated cells, which have an unlimited potential to develop into any type of embryonic as well as extraembryonic tissue. When the embryo reaches the 8-cell stage, its blastomeres start forming intercellular junctions, maximizing the contact with each other, in a process called compaction. Already in the 16-cell formation, called morula, cells with no contact with the outer environment - the inner cell mass (ICM) - can be recognized. Prior to implantation, morula develops a small cavity, becoming a blastocyst, in which ICM further proliferates and forms embryoblast. Cells of embryoblast, still being pluripotent, differentiate rapidly and build bilaminar germ disc with the distinct layers - hypoblast and epiblast. Epiblast cells represent the essential developmental source for principal embryonic germ layers - ectoderm, mesoderm and endoderm [1].

Mammalian ESCs are derived from undifferentiated ICM of early preimplantation blastocyst. While the embryoblast represents an ephemeral stage in embryonic development, the ESCs, when transferred into long term *in vitro* culture share some characteristics with an immortalized cell line. Specific conditions of the *in vitro* culture prevent differentiation of the ESCs into the embryonic structures, and allow ESCs to keep the molecular machinery necessary for the maintenance of pluripotency and unlimited cell divisions [2].

Development of differentiated structures from embryonic precursor cells is a tightly regulated process. Pluripotent cells, which have not differentiated properly in their developmental time window, or persist ectopically, can form peculiar tumors – teratomas. Teratomas contain regions with haphazard accumulation of mature tissues, such as hair, cartilage, bone, or teeth. Despite the inherent abnormality of teratomas, they were a unique model, which contributed significantly to understanding of embryonic pluripotency and differentiation principles. Indeed, the first cell lines that harbored traits of stem cells were derived from mouse testicular teratocarcinomas [3]. These cells share many features with primordial germ cells, thus they, at the time, served as the first principle model of cell plasticity and embryonic differentiation. Genuine embryonic stem cells were derived from 3.5 dpc mouse blastocyst in 1981 [4], and the long-term culture conditions preventing differentiation were adopted from teratocarcinomas culture protocols [5]. Later, soluble factors critical for maintaining the pluripotency of mouse ESCs (mESCs), e.g. mLIF – mouse leukemia inhibitory factor, and BMP4 – bone morphogenetic factor 4, were identified and allowed mESCs propagation [6, 7].

The embryonic development of primates including humans, however, differs from embryonic development of rodents. That is why the derivation of human embryonic stem cells has not been achieved until seventeen years later by James Thompson [8]. Currently, hundreds of hESC lines and their derivatives are available through curated stem cell repositories and biobanks [9]. A breakthrough in stem cell research came in 2006 when genetic regulators essential for induction of pluripotency were discovered by Shinya Yamanaka [10]. These transcription factors can reprogram fully differentiated somatic cells, so they obtain undifferentiated stem cell-like phenotype. Such human induced pluripotent stem cells (hiPSCs) provide a genuine stem cell model with no ethical burden associated with hESCs [11]. hiPSCs essentially complement the portfolio of cellular models for description of crucial molecular events during embryogenesis, tissue differentiation and cancer development. Nowadays, ESCs derived from embryoblasts and hiPSCs derived by reprogramming of somatic cells represent an important biological model and hold the promise to various clinical applications, including regenerative medicine, tailor-made cell therapy and drug testing [12, 13].

The state of pluripotency is developmentally unstable and requires specific molecular machinery to maintain the stem cell phenotype [14, 15]. Developmental trajectories during normal embryogenesis are determined soon, so the complex differentiated structures evolve even in a very early embryo. Sophisticated micro-environment of *in vitro* cell cultures, which is designed and optimized to stimulate unlimited propagation of stem cells and preservation of their full differentiation capability [16], is different from the conditions within the developing embryo. Protocols which represent the golden standard involve additional "feeder" cell layer, which supports cultured stem cells (**Figure 1A**).

Mouse embryonic fibroblasts (MEFs), human preputial fibroblasts, oviduct lining or fetal smooth muscle cells are commonly used as a feeder layer. Feeder cells provide necessary intercellular contacts and produce extracellular matrix (ECM) along with soluble factors, in order to recreate, to certain extent, the microenvironment of a blastocyst [17]. However, co-culture of hESCs with feeder cells bears the risks of carry-over contamination and induction of the immune reaction in a patient being exposed to antigens derived from animal feeder layer upon grafting.

Culture protocols with animal additives are therefore not suitable for the direct use in humans. hESCs culture on defined surfaces coated with ECM mixtures or pure protein layers in complex medium supplemented with essential cytokines

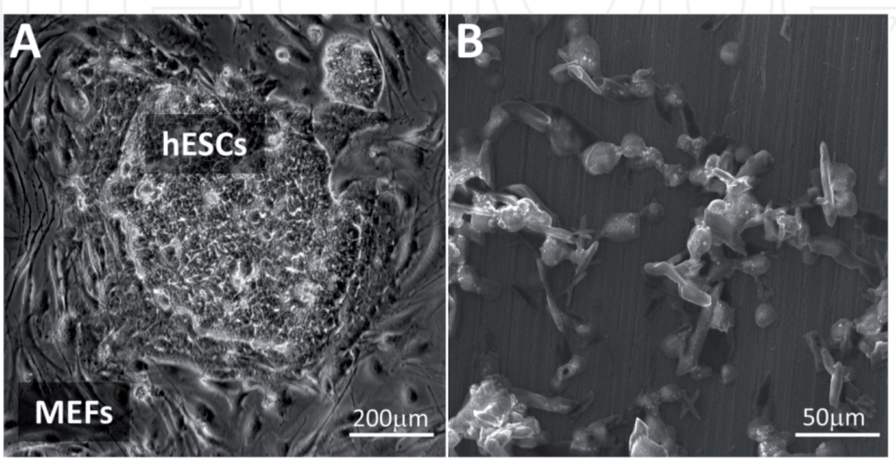


Figure 1.
(A) Single colony of hESCs co-cultured with feeder fibroblast layer (MEFs) as visualized by light microscopy. (B) Scanning electron micrograph of intact hESCs mixed with matrix containing acidified sinapinic acid and spotted on the target plate of the MS instrument.

(e.g. bFGF, activin) may be a solution, if combined with rigorous quality control of other culture parameters (**Table 1**) [18, 19].

2.1 Phenotype shifts and culture adaptation in hESCs

In prolonged culture, pristine hESCs adapt to a two-dimensional, biochemically and structurally less complex, in comparison to a blastocyst, microenvironment [20]. However, the introduction into the *in vitro* culture inevitably induces selection: hESCs clones, which fit the artificial culture conditions the best, are being preferentially selected. Particularly those are clones which divide regularly with optimal doubling time, show reduced sensitivity to programmed cell death, are resistant to passaging method and cell stress arising in culture. Despite stringent culture protocols, such clones can acquire non-random chromosomal aberrations, alterations of epigenetic landscape and changes in gene expression [21–24]. Often, genes which participate in tumor formation or dissemination are involved [25], similarly to those in undifferentiated stem cell lines derived from teratocarcinomas [26].

Darwinian selection in long-term hESC cultures, accordingly, generates cells which remarkably differ from pristine hESCs. Such culture-adapted cells can obtain hazardous phenotype [27], which is similar to a malignant cancer cell line, while keeping normal levels of transcription factors (e.g. Oct 3/4 and Nanog) and other stemness-related molecular markers (e.g. SSEA-4 and Alkaline Phosphatase) [28]. Paradoxically, such significant changes in hESCs, which have acquired the adapted phenotype, can stay unnoticed if cell morphology or expression of stemness factors are not affected. Thus culture-adapted cells can escape routine quality control, which is usually based on visual evaluation or monitoring of a several selected molecular markers. Incompatible cellular alterations are quickly eliminated *in vivo*, typically by the complete rejection of the embryo. *In vitro*, however, aberrant clones may become dominant in culture and disable the safe use of the particular hESCs for clinical or biotechnological applications.

At the moment, there is no routinely applicable method which can reveal hidden shifts in hESCs phenotype or to confirm general stability of a cell culture. The golden standard for authentication of *in vitro* cell lines, e.g. ones derived from individual patients' tumors, is based on analysis of short tandem repeats (STRs). Repetitive sequences dispersed throughout the genome can provide a unique genetic profile and effectively reveal potentially misidentified cell lines [29]. However, STRs analysis is uninformative if used for specific cell culture modifications, such as co-culture of two cell types, analysis of differentiation stages within a single cell line, use of more cell subtypes derived from an individual. Similarly, techniques used for analysis of batch to batch variability, purity of cells, genome or proteome changes, as well as methods focused on a limited panel of biomarkers are not

- microbial and viral contamination
- cell line cross-contamination
- carry-over of animal products in culture
- karyotype instability
- activation of oncogenes and risk of tumorigenicity in patient
- acquisition of immunogenicity and risk of graft rejection in patient
- failure of differentiation into functional phenotype

Table 1.
Risk assessment in clinical grade hESCs culture.

suitable for quality control in routine applications, because they are not informative and often are laborious and costly [30–32].

Successful implementation of pluripotent stem cells into clinical trials requires stringent assurance of the cell product quality and safety, including development of formal methodology for every step of the cells derivation and culture processes. Presence of an unwanted phenotype or deviation from the optimal state of cells in culture may inevitably result in failure of the product and a harm to a patient. Routinely used methods easily reveal microbial and viral contamination, cell mis-identifications and chromosomal aberrations which occur in the culture. However, subtle phenotypic shifts or unapparent alterations cannot be detected by microscopy, narrow focused screening for molecular markers or other conventional methods.

Recently, we have been working on the introduction of techniques used in bioanalytical chemistry, chemometrics and in complex data analysis into stem cell field. We have demonstrated that method of choice can be the Intact (Whole) Cell MALDI TOF Mass Spectrometry coupled with sophisticated statistics. Intact Cell MALDI TOF MS is sufficiently robust, sensitive, and cost-effective approach for monitoring of long-term cultures stability and differentiation trajectories of ESCs, therefore it has a potential to complement the portfolio of quality control tools in clinical or bio-industrial applications [33].

3. Intact cell MALDI TOF MS

MALDI TOF MS employs the laser energy to desorb and ionize molecules of an analyte from the crystallized mixture with the matrix, and subsequently separates the resulting ions according to mass-to-charge (*m/z*) ratio. The organic matrix enhances energy transfer to analyte, preserves the structure of the ionized molecules, e.g. peptides, proteins or other biomolecules, and allows their precise structural analysis and identification. In cell biology, MALDI TOF MS is one of the preferred methods for proteomic analysis in a broad range of samples, such as purified or fractioned extracts of cells or tissues. The MS-based proteomics uses protein fragmentation for identification and further generation of a list of unique peptide or protein signatures in wide range of *m/z* values [34]. However, the methodological complexity and the character of the data output may limit the use of traditional proteomics in routine quality control of stem cell cultures, even if coupled with transcriptomics or (meta)genomics.

Even when the intact (whole) cells are used as an analyte, MALDI TOF MS can generate rich spectra without the need of previous cell lysis, fractionation or protein extraction. Mass spectra contain signals for small proteins and peptides, and a variety of other low-mass molecules, including metabolites. Analysis of specific spectral (peak) signatures has been successfully introduced to clinical microbiology, where MALDI TOF MS enables the rapid discrimination, or "biotyping", of bacterial species without the necessity of complex sample processing [35, 36]. Generally the same approach - utilization of relevant spectral patterns as inputs for further processing and analysis [33] - can be used for discrimination of cancer cells [37, 38] or abnormal stem cells in long-term cultures, even in high-throughput setup [39, 40]. Intact Cell MALDI TOF MS was used to identify spectral signatures of glial cells and their classification to astrocyte, microglia and oligodendrocyte type [41]. Principal component analysis then revealed informative peaks for deeper spatial analysis using mass spectrometry imaging in whole brain sections. Similarly, mass spectra have demonstrated to contain sufficient information to reveal the immunophenotype and activation state of immune cells, [42–45] or to classify distinct mammalian cell lines [46, 47]. Moreover, MS can reveal changes associated with molecular phenotype, which occur within cell lines and sublines of common genetic

origin. Such approach has been used recently by Povey et al., who demonstrated discrimination of neuroblastoma cell lines sensitive to chemotherapy [48], or by Cadoni et al. who classified ovarian cancer cells sensitive or resistant to cisplatin, based on phospholipid patterns generated by MS [37].

3.1 Intact cell MALDI TOF MS of hESCs

The first step of the preanalytical sample processing is the enzymatic or manual harvesting of hESCs under visual microscopic control. Next, cell clusters are enzymatically disaggregated and washed in isotonic buffers (e.g. phosphate buffered saline, PBS) to remove residual culture medium and additives. PBS has been reported not to interfere with MALDI TOF MS significantly [49]. However, we observed that it may induce random quenching of ionization and decreased intensity of peaks. Therefore, we have added an additional wash with MS fully compatible buffers, such as ammonium acetate [41] or ammonium bicarbonate (ABC) [33], to our protocol, to remove traces of PBS in order to improve mass spectra quality. After cell number assessment, cell are resuspended in 150 mM ABC to desired concentration. Dry cell pellets can be cryostored (at −80°C or lower) with no significant impairment of mass spectra quality.

The MS protocol for hESCs biotyping (fingerprinting) follows the established proteomic or microbiological workflow. Dependent on cell type, instrumentation type and matrix composition, we use typically 1000-25,000 cells per measurement in routine analysis. Cell number can be, though, reduced to several hundred in an optimized experimental design. Cells can be directly placed onto a steel target plate or on transparent indium-tin oxide (ITO) coated glass slides. The ITO coated glass slides enable correlative microscopic analysis in parallel to the MS. In addition, they can be used as a substrate for culture of adherent cells [50].

Sinapinic acid (SA) or α-cyano-4-hydroxycinnamic acid (CHCA) acidified with trifluoroacetic acid are used as a matrix predominantly. SA and CHCA generate uniform-sized crystals, in which cells can be embedded regularly (**Figure 1B**). Although, other matrices, such as 2,5-dihydroxybenzoic acid (DHB) or 2-mercaptobenzothiazole (MBT) can also provide informative output, they form long, needle-like crystals distributed over the target spot unevenly, and therefore are more suitable for solubilized samples.

Routinely, we analyze samples in linear positive mode in *m/z* range of 2-20 kDa, using the usual range of laser energy. Some of the dominant peaks, which have already been partially identified [41, 47, 51], are regularly observed also in hESCs. They correspond to modified histones, thymosin and presumably to ribosomal or other small structural proteins, and can provide an immediate verification of mass spectrum quality.

Processing of the mass spectrum prior to statistical analysis includes reduction of raw data matrix, smoothing of the spectrum, alignment of peaks, baseline subtraction and finally detection of peaks. Average spectrum is then calculated from technical replicates and used to generate a final dataset of *m/z* values with assigned intensities in mV or relative arbitrary units [33, 52, 53].

4. Data analysis

4.1 Mass spectrum as a biomarker

Mass spectrum recorded in a wide range of *m/z* values contains hundreds of charged molecular entities, which together form a spectral profile, or “fingerprint”

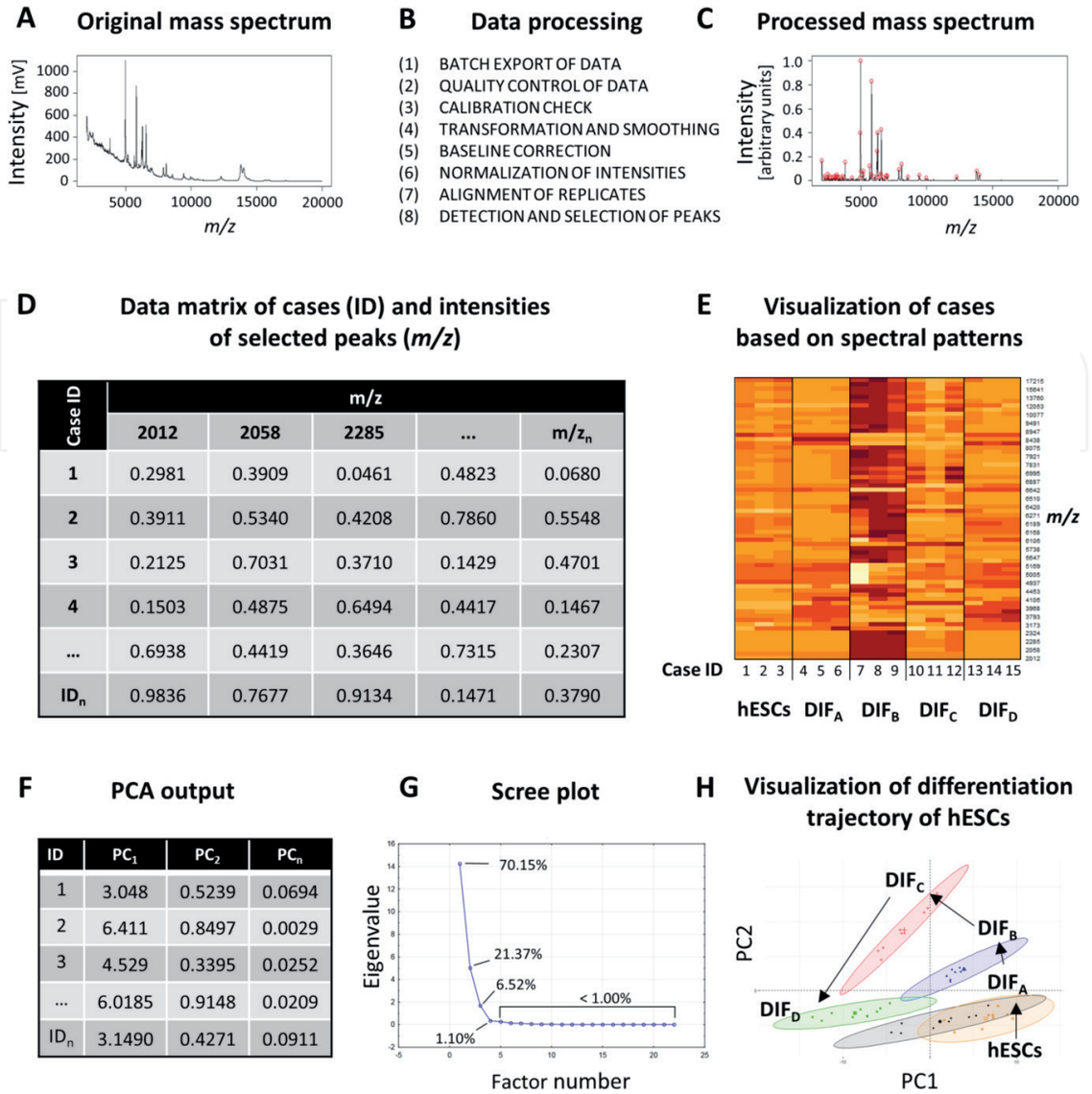
that can be uniquely assigned to a specific cell type, phenotype or state. However, MALDI TOF mass spectra generated from ionized molecules desorbed from the intact cells are complex and depend strongly on the experimental conditions and preanalytical errors, such as matrix choice, hardware setup and even operator skills. Despite the technical variability, individual mass spectra assembled to a correctly processed dataset may serve as input data for sophisticated mathematical analysis. After the reduction of the unwanted inconsistency, informative patterns in mass spectra can be identified. Finally, processed spectral dataset can be organized in two-dimensional array of cases and intensities of selected peaks. Before statistical analysis is applied to the spectral dataset, preliminary examination of data quality is required. Such rigorous control of data quality includes verification of reproducibility, meticulous calibration and elimination of apparent technical errors or outliers.

Mass spectra of complex biological samples usually contain numerous peaks with rather low intensities and low signal-to-noise ratio. Therefore, the peak detection and recognition is dependent on precise calibration. Where appropriate, we do recommend using the clusters of isotopically pure elements, such as nanoparticles of gold (gold clusters) or black and red phosphorus as calibration standards [54, 55], next to commercially available peptide standards. Mono-isotopic calibrants provide well defined peaks corresponding accurately to predicted mass, allowing proper peaks alignment. Besides, they do not suffer with occasionally problematic indication of isotopic envelope of high mass peptides or proteins.

For evaluation of mass spectra similarity of technical replicates or experimental cohorts, mathematical approaches used in proteomics or metabolomics can be applied. Correlation analysis (e.g. Pearson's correlation, Spearman's correlation, Kendall rank correlation or cosine correlation) can provide a quantitative output that can globally evaluate the similarity of mass spectra [56].

Another relevant factor, which can interfere with the outputs of statistical analysis, is the presence of outlier values in the dataset (case) or within the mass spectrum (peak intensity). Despite the precise laboratory work, outlier values are inevitable and are probably associated with stochastic MALDI effects, as have been already described in bacterial Intact Cell MS [57]. One of the classical procedures, which allows to reveal outliers within the data, is provided by factor analysis and includes careful following of the rank of the data matrix by computing eigenvalues. The number of non-zero eigenvalues (rank of the matrix), visualized in a scree plot, immediately gives the crucial information related to the sample, such as the number of recognizable data groups in mass spectra (e.g. cell types or experimental conditions). In case of two data groups, the rank should equal to two. The presence of outliers is thus indicated by an increased value of the rank [58, 59].

The multivariate evaluation and validation can identify relevant groups or classes within the spectral data. The principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) are commonly used. PCA reduces dimensionality of the spectral data, and defines new vectors - principal components, which maximize the variance. Besides, it enables visual observation of the recognized groups of the samples. PCA is an unsupervised method with minimal bias, and its performance is optimal when the intra-group variability is significantly lower than the inter-group variability. It is a well-established tool for processing of complex spectral data, e.g. in proteomics or microbiology [60, 61]. PLS uses different mathematical model than PCA for the distinction of groups. It represents a supervised discriminant analysis, which involves the group information in the algorithm. PLS can provide an excellent discrimination, however, it can suffer from inherent tendency to over-fit the data and identify the clusters even in a uniform spectral dataset. The validation on independent data is therefore recommended [62]. The workflow of data processing in routine analysis of hESCs is summarized in **Figure 2.**



Case ID	m/z				
	2012	2058	2285	...	m/z_n
1	0.2981	0.3909	0.0461	0.4823	0.0680
2	0.3911	0.5340	0.4208	0.7860	0.5548
3	0.2125	0.7031	0.3710	0.1429	0.4701
4	0.1503	0.4875	0.6494	0.4417	0.1467
...	0.6938	0.4419	0.3646	0.7315	0.2307
ID_n	0.9836	0.7677	0.9134	0.1471	0.3790

ID	PC_1	PC_2	PC_n
1	3.048	0.5239	0.0694
2	6.411	0.8497	0.0029
3	4.529	0.3395	0.0252
...	6.0185	0.9148	0.0209
ID_n	3.1490	0.4271	0.0911

Figure 2.
(A) Example of raw mass spectrum generated from intact cells by MALDI TOF MS, (B) data processing workflow, (C) processed mass spectrum, (D) spectral dataset consisting of individual cases (ID_1-ID_n) and assigned values of peak intensities at defined m/z*, (E) heat map graphically visualizing the dataset containing spectral data of hESCs and four differentiation stages (DIF_{A-D}), (F) example of the output matrix of PCA with recalculated coordinates, (G) scree plot visualizing the number of significant factors contributing to the variability in the dataset, (H) PCA-based visualization of the differentiation trajectory of hESCs progressing towards endodermal phenotype through the four differentiation stages (DIF_{A-D}). Adapted with permission from [63].*

4.2 Classification by machine learning

Artificial Neural Networks (ANNs) represent a non-linear mathematical model, which resembles a brain neural architecture, and possess "learning" and "generalization" abilities. For this reason, ANNs belong to a group of artificial intelligence methods with wide spectrum of complex applications, ranging from purely scientific to industrial or clinical. ANNs utilize diverse types of input data, which are processed in the context of previous training history on a defined sample database to produce a relevant output [64]. The unique chemical fingerprints generated by intact cell mass spectrometry allow the ANN to classify the samples even without preceeding identification of relevant peaks. Successful application of ANNs or any other machine-learning algorithms requires building-up a database of spectral patterns specific for individual cell types, phenotypes or states. This has been successfully achieved in clinical microbiology, however, in eukaryote biology, the

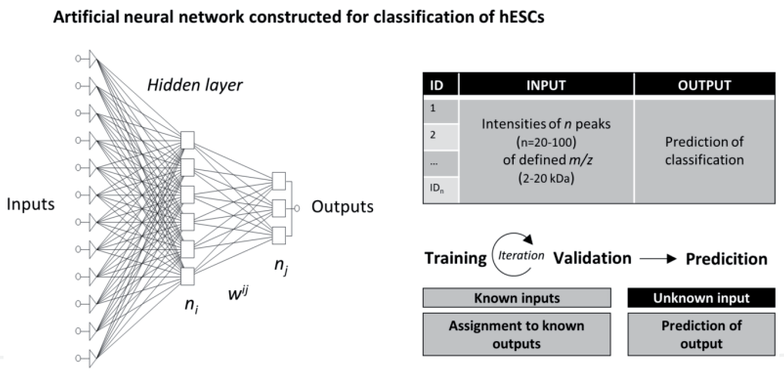


Figure 3.
Architecture of the representative artificial neural network used for prediction of hESC phenotype (output) using peak intensities arranged in a defined spectral matrix (input). Adapted with permission from [63].

complexity of cellular composition and cell plasticity in general represents a major issue. Nevertheless, for the "in-house" databases of well-defined cell models and conditions of their handling and analysis, the Intact Cell MALDI TOF MS coupled with ANNs is a powerful and robust approach that can be easily adapted to any specific application (**Figure 3**).

5. Applications of intact cell mass spectrometry in quality control of embryonic stem cell cultures

Monitoring of clinical-grade stem cells during manipulation, banking or quality control by appropriate tools is the essential prerequisite for their application. We hypothesized that different cell and tissue types or their different states may vary in levels of numerous small molecules, metabolites or peptides and proteins. An unambiguous and unbiased chemical fingerprint obtained by MS can thus reflect such divergences with high sensitivity. In addition, spectral patterns can serve as a highly informative input for subsequent statistical analysis and classification.

To test this hypothesis we used a mouse model of primary hyperoxaluria I - a congenital disorder that affect enzymatic machinery of glyoxylate metabolism. Primary hyperoxaluria I causes oxalate deposits to localize in liver and kidneys, and ultimately lead to hepatorenal failure and extrarenal manifestation of the disease. Alterations of chemical composition within the tissue microenvironment of hyperoxaluric animals can be translated into specific patterns in mass spectra. A dataset, composed of peaks and their corresponding intensities obtained from diseased and healthy animals, was used as an input for cluster and classification analysis and machine learning (ANN) prediction. Spectral patterns clearly distinguished samples from healthy and hyperoxaluric animals and, in parallel, the ANN correctly predicted the category based solely on mass spectrum fingerprint [65].

Generally the same approach can be used for rapid discrimination of cells occurring in stem cell cultures. Mass spectra from pure populations of mESCs, hESCs, and mouse embryonic fibroblasts (MEFs) contain enough information to distinguish the cell types by cluster analysis. Interestingly, these spectral profiles are not lost even in case of mixed populations of two cell types, such as in cross-contaminated cell cultures. Therefore, they can serve as a basis for quantitative estimation of the individual cell types in the mixture. To model such scenario, a broad panel of binary suspension mixtures containing hESCs and MEFs or hESCs and mESCs in defined ratios was prepared. Mass spectra were recorded, processed and the spectral patterns assigned to known quantities of cells in suspension. Resulting dataset then represented calibration data matrix, suitable for quantitative

prediction by ANNs. Indeed, well-trained and validated network predicted the number of contaminating cell type with high accuracy in otherwise homogenous and morphologically uniform mixtures. The precision of prediction was dependent on a number of peaks and corresponding peak intensities included in the data matrix. The apparent, dominant peaks with high intensity, did not contribute significantly to the classification. Their exclusion had a negligible effect on the ANN performance, suggesting that there is rather a cumulative effect of minor, but informative peaks, essential for the analysis output [53].

In the long term cultures of hESCs, abnormal clones with the origin in pristine hESCs are inevitably being generated, selected and, finally, expanded. The CCTL 14 hESC line, which has been propagated over hundreds of passages *in vitro* suffers

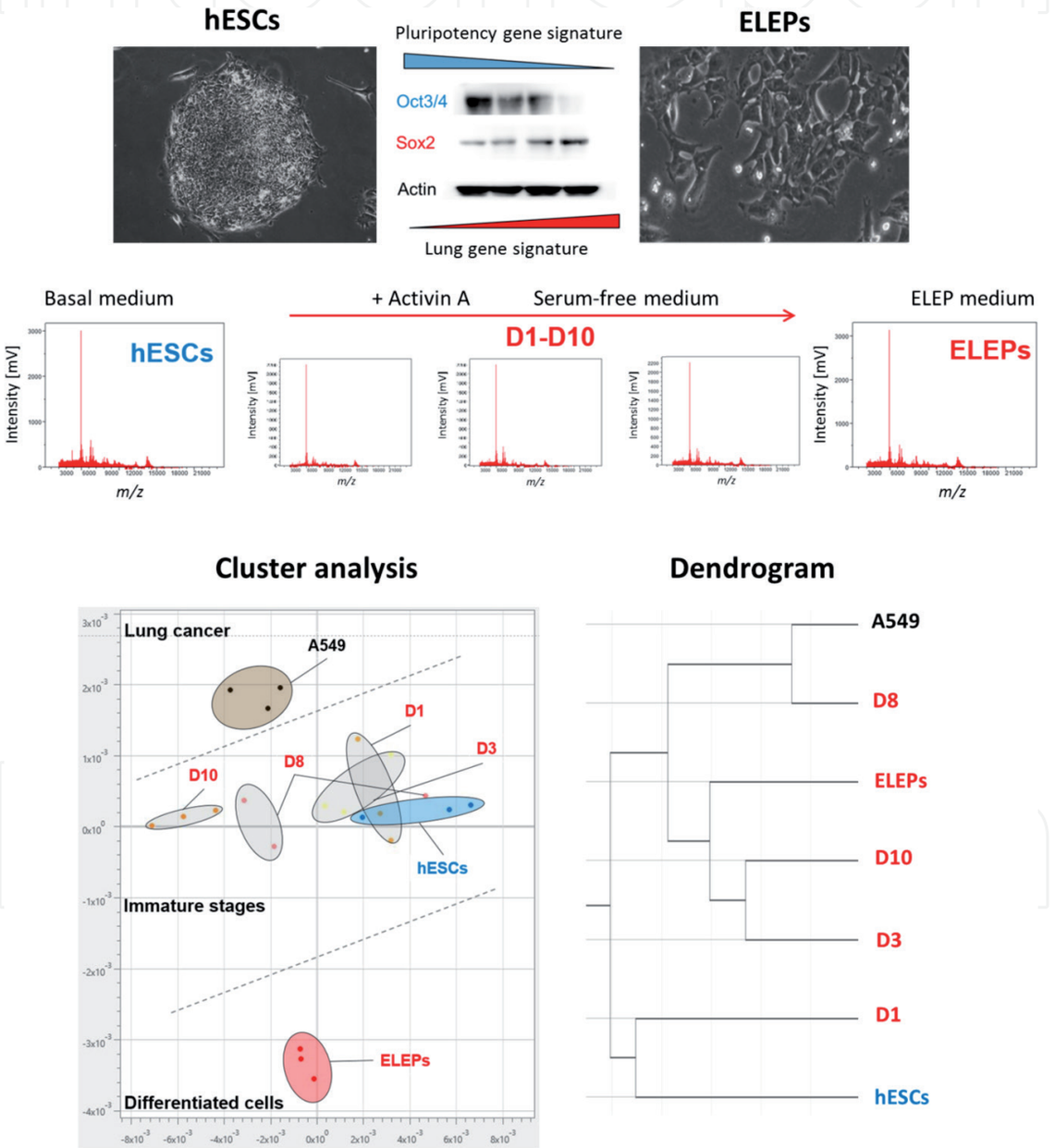


Figure 4.
Monitoring of differentiation of hESCs into early lung progenitors (ELEPs) by intact cell MALDI TOF MS. hESCs induced to ELEP differentiation lose gradually the gene signatures associated with pluripotency and acquire the phenotype of lung progenitors. Differentiation trajectory of cells goes through several immature stages and reaches the final, differentiated stage approximately at day 21. Samples for intact cell MALDI TOF MS were collected prior to induction of differentiation (hESCs) at days 1, 3, 8 and 10 (D1, D3, D8, D10) and finally at the final stage (ELEPs). The lung cancer cell line A549 was used as lung-associated, but abnormal sample class. Cluster analysis, such as PCA or a dendrogram, based only on spectral patterns shows clear separation of classes and indicate that ELEPs represent indeed a distinct cellular entity [66].

from chromosomal abnormalities and shows an increased expression of CD30 and CD44 surface molecules, previously shown to be associated with acquisition of the adapted phenotype and karyotype instability [67]. In addition, adapted, high passage hESCs proliferated with reduced doubling time, and showed lower sensitivity to apoptotic stimuli and decreased capability of differentiation, than pristine hESCs. All the low (P29), mid (P72) and high passage (P269) cells maintained high and constant expression of stemness markers, e.g. Sox-2, and also share the identical morphology [33].

We recorded mass spectra from intact P29, P72 and P269 hESCs cultured under identical conditions, processed and assembled into the dataset containing 255 *m/z* values with assigned peak intensities. The mass spectra showed a high level of visual similarity, however the Pearson's correlation analysis revealed disparities in spectral patterns between P29, P72 and P269 cells. Principal component analysis then correctly discriminated P29-pristine and P269-adapted cell to individual distant cluster, while the P72-transition population cluster has been located and scattered between them. Therefore, such a robust dataset of spectral fingerprints recorded from intact hESCs contains sufficient information to distinguish normal and aberrant cells with otherwise identical morphology and expression of stemness markers. Next, we used the same approach to analyze CCTL 14 hESC line stimulated to differentiation. Generally, cell differentiation means acquisition of novel phenotypic properties, and morphological hallmarks, as well as various molecular mechanisms, involved in cell functioning. Retinoic acid (RA) is a potent inducer of embryonic patterning and neurogenesis *in vivo*, so hESCs induced to the differentiation by RA acquire the neuronal phenotype within several weeks. Mass spectra generated from cells treated with RA for only 24 hrs, contained spectral regions which allowed detection of such early differentiation events even prior to development of clear morphological hallmarks [33].

To test a routine, pre-clinical application, we used Intact Cell MALDI TOF MS to monitor the differentiation of hESCs to early lung progenitors (ELEPs). ELEPs, being the direct precursors of lung cells (pneumocytes), represent an important resource for tissue engineering, pollutant testing and pharmacological analyses. ELEPs gradually differentiate from hESCs under specific culture conditions through several stages (D1-D10). Differentiating immature cells lack an unambiguous marker, which would distinguish them from ELEPs. In addition, during differentiation process aberrant cells with unwanted properties may appear in culture. Intact

+ Feasible, affordable, and robust technique
+ Intuitive, straight-forward approach
+ Unbiased and marker-free classification
+ Discrimination of unapparent, yet critical alterations in stem or progenitor cells, which are not detectable by other techniques
+ Stringent culture protocols and defined SOPs in clinical grade hESCs laboratories allow precise pre-analytical phase
– Inherent instrumental and technical variability of MALDI TOF MS
– Necessity to build own specialized database of spectral patterns
– Calibration sensitivity
– Nontrivial processing and analysis of complex data

Table 2.
Advantages (+) and limits (–) of Intact Cell MALDI TOF MS in quality control of hESCs culture in clinical grade laboratory.

Cell MALDI TOF MS was able to distinguish individual immature stages from hESCs and from ELEPs, as well as from lung cancer cell line (**Figure 4**).

In summary, Intact Cell MALDI TOF MS coupled with advanced statistics provides an efficient tool for revealing aberrant cells in culture or following differentiation trajectories of pluripotent stem cells and progenitors. The advantages and limits of Intact Cell MALDI TOF MS in quality control of clinical grade stem cell cultures are summarized in **Table 2**.

6. Conclusions

Intact Cell MALDI TOF MS reliably discriminates functionally different, but otherwise identical types or subtypes of stem cells of common genetic origin. Moreover, it reveals aberrant or differentiating clones of clinically relevant stem cells or committed tissue progenitors. Coupling the outputs of Intact Cell MALDI TOF MS with sophisticated statistics, such as cluster analysis or machine learning, may provide a feasible and easy-to-use routine tool for quality control of pluripotent stem cells and progenitors long-term cultures.

Acknowledgements

LM is Brno PhD Talent Scholarship holder, funded by the Brno City Municipality, and junior researcher supported by Faculty of Medicine, Masaryk University (ROZV/28/LF/2020) and by Masaryk Memorial Cancer Institute (00209805). Dr. Anna Mac Gillavry Danylevska, Ph. D. is acknowledged for critical reading of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Funding

This work was supported by Ministry of Health of the Czech Republic (grant no. NV18-08-00299) and by Masaryk University (MUNI/A/1421/2019, MUNI/A/1382/2019).

List of abbreviations

ABC	ammonium bicarbonate
ANN	artificial neural network
CHCA	α-cyano-4-hydroxycinnamic acid
dpc	days post conception
DHB	2,5-dihydroxybenzoic acid
ESI	electrospray ionization
ELEPs	early lung progenitors
ECM	extracellular matrix
hESCs	human embryonic stem cells
hiPSCs	human induced pluripotent stem cells

ICM	inner cell mass
MALDI TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MBT	2-mercaptobenzothiazole
MEFs	mouse embryonic fibroblasts
mESCs	mouse embryonic stem cells
PCA	principal component analysis
PLS-DA	partial least squares discriminant analysis
SA	sinapinic acid
STR	short tandem repeats.

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