RESEARCH ARTICLE

Analysis of ion currents in mass spectrometric profiles using glioblastoma tissue [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: The development of direct ambient ionization methods makes way for fast mass-spectrometry profiling of biological samples, which has great potential in medicine. Those methods, unlike traditional mass spectrometric analysis with chromatographic separation, are not able to take into account inter-ion interaction, ion suppression, and matrix effect due to the absence of chromatographic separation of the mixture components. So dynamics of ion current during direct ambient ionization mass-spectra is governed by the component micro-extraction and electrospray ionization influenced by the geometry of the sample, its position, and internal heterogeneity. Despite the progress in mass-spectrometry of biological samples, not much is known about the influence of sample type and structure on its molecular profile peculiarities.

Methods: In this work, we propose to use analysis of the correlation between individual ion currents for a better understanding of ion current variability sources and grouping ions of high biological importance. Several fragments of glioblastoma tissue from a single patient are used for these purposes.

Results: Ion currents have different dynamics considering different ions in different fragments. The correlation of two selected ion currents could be positive or negative for single fragment measurement. Correlations have persistent or alternating signs in different fragments for two selected ions. The spread of correlations of each pair of ion currents is calculated for evaluation of the signs' stability.

Conclusions: We were able to group ions according to the primary reason for their variabilities such as micro-extraction, mass-spectrometry measurement, or specimens' properties. Such grouping

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would allow the development of more reliable and reproducible methods of mass-spectrometry data analysis and improve the accuracy of results of its application in medicine.

Keywords

mass spectra, ion current, microextraction processes, ambient ionization

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Introduction

New techniques for rapid tissue profiling in mass spectrometry have made it possible to develop methods for express analysis of biological substances^{1–7}. Many approaches to integrate mass spectrometry into the clinical routine have been published^{8–12}. Interpretation and analysis of mass spectra of complex mixtures of biological molecules are still a rather difficult task due to the enormous variety of molecule types contained in biological tissues and their concentrations^{13,14}. The LC-MS/MS analysis, which is considered a gold standard between methods for the accurate identification of complex mixture components, is time-consuming and cannot be used as a method for rapid analysis¹⁵. Therefore, for rapid analysis, one has to use direct mass spectrometry without preliminary chromatographic separation of mixtures. Neurosurgery is one of the areas that requires rapid analysis of biological tissues dissected during procedure¹⁶ to determine the boundaries of the tumor and control the volume of the excised tissue. It was shown that this problem could be solved using the mass spectrometric approach¹⁷.

Primary brain tumors account for 1.4% of all cancers and 2.4% of all cancer-related mortality in the United States. Approximately 20,500 newly diagnosed cases and 12,500 deaths are associated with primary malignant brain tumors each year¹⁸. According to recent reports, glial tumors are considered the most common primary brain tumors. They represent 31% of all brain and central nervous system (CNS) tumors diagnosed in the United States, and 81% of all malignant brain and CNS tumors^{19,20}. Glial tumors can be divided into different subtypes based on morphological and genetic data following the WHO classification²¹.

It should be noted that all glial tumors can be very heterogeneous at the morphological level not only in between patients but even in within-patient due to the relatively random mixture of more benign and more aggressive parts²². This variability can become a serious problem for the rapid, accurate, and objective diagnosis of both the tumor border and its level of malignancy. The task of detecting tumor cells is most important in the border area since the undissected tumor cell may contribute to recurrence in the future²³.

During the transfer of the results of biological experiments into clinical practice a number of questions needs to be addressed²³, including a study of the stability of the mass spectrometric characteristics of the sample and the reproducibility of the results under variable external (mode, range, temperature, resolution, time) or internal (sample characteristics, biological variability) conditions. For this analysis, the stability of the whole spectrum structure and the analysis of the dynamics of individual ion currents are of interest.

The study of the dynamics of individual ion currents is widely used in the analysis of mass spectrometric images^{24–27}. In this work, using the analysis of the dynamics of ion currents, we investigated the influence of the internal heterogeneity of the sample, as well as the processes of ion suppression and extraction on the interpretation of mass spectrometric profiles of glioblastoma samples.

Methods

Signal acquisition

The study used a Thermo LTQ Orbitrap XL high-resolution mass spectrometer, which allows measurements in both high- and low-resolution spectra. For mass spectrometric profiling of the samples, extraction using an integrated cartridge (ICE)²⁸ was used, followed by electrospray ionization. Mass spectra were measured according to a previously developed protocol²⁹. In this work, we used only low-resolution spectra in the range of 100–2000 m/z in both polarities.

Sample collection

A tissue sample dissected at the border of the tumor was provided by the N.N. Burdenko neurosurgery center and analyzed following the protocol approved by the ethical committee of the N.N. Burdenko neurosurgery center (order 40 from 12.04.2016, revised with order 131 from 17.07.2018). Signed informed consent was obtained from the patients before surgery, in which it was specifically noted that all removed tissues could be used for further research. The study was conducted in accordance with the 2013 edition of the Declaration of Helsinki. All procedures were carried out according to the relevant guidelines and regulations approved by the N.N. Burdenko neurosurgery center. Written informed consent for publication of the patients' details was obtained from the patients.

The tissue sample was split into two parts. The first was characterized by hematoxylin and eosin staining and further immunohistochemical analysis to determine the diagnosis and grade of malignancy. As a result of the analyses carried out, the diagnosis “glioblastoma, IDH-1 wild type, without MGMT mutation” was obtained. The second part was frozen and kept at -80°C until mass spectrometry measurements were taken. Glioblastoma tissue was divided into six fragments, and a mass spectrometric profile was measured for each of them. Measurements were carried out continuously by alternating modes of polarity and range and repeated twice for 10 minutes. Thus, the interval between repetitions of the measurement in each condition was approximately five minutes.

Data analysis

Mass spectra were processed in a similar way to previously described^{23–30}. So, mass spectra were converted to vectors with 1 m/z binning width and were normalized to total ion current. Previously we calculated similarity spectra matrices (SSM)^{29,30}, where each matrix element was calculated as a cosine measure between two mass spectra (vectors), and these mass spectra (scans) were taken from a whole measurement. Thus, SSM demonstrated the similarity between scans of single measurement and/or different measurements. In this paper, we calculate similarity measure matrices, where similarity measure is Pearson's R correlation between ion currents of different m/z , and ion currents are continuously sampled from scan to scan and from measurement to measurement.

Also, we calculated the spread of correlations for different tissue samples and each m/z . The matrices of correlation spread were created in the following way: the correlation matrix of

ion currents was calculated for each fragment of tissues; each value of matrices (R) was rounded to -1, 0, or +1 by the binning with bin boundaries -0.2 and 0.2 respectively; the difference between maximum and minimum R in the set of six fragments formed the values of matrices of correlation spread.

All calculations and visualizations were made using code written by the authors using MATLAB (available in the *Underlying data*³¹).

Results

Correlation matrices of ion currents are shown in **Figure 1** and **Figure 2** for negative and positive ion modes, respectively. The top and the right panels of the figure show the average spectrum for all measurements, the left panel represents the similarity matrices. So, the right and the top panels are the aggregated mass spectrum of the spectra of all 6 measurements normalized to the total ion current. The m/z range on the axis of the spectra corresponds to the range on the axes of the similarity matrix,

i.e. the values of the axes of the similarity matrix and the spectra coincide.

Several regions of the high ion correlation represented by compact red squares could be seen in the negative ion current correlation matrix (**Figure 1**). Within these regions, ion currents have a correlation close to unity, which indicates the similarity in dynamics regardless of the relative intensity of the ion current in samples. Correlation values close to the negative unity indicate a negative linear dependence of the intensities of a pair of ions in all samples.

Interestingly, there are two groups of ions in the approximate ranges of 700–900 m/z and 1500–1700 m/z . Ions in these groups are correlated both within and among themselves. The blue stripes at the boundaries of these red squares indicate a negative correlation of these groups with ions in the ranges of 100–700 m/z and 900–1500 m/z .

Figure 1. Similarity matrix of ion currents in negative mode with aggregated mass spectra.

Figure 2. Similarity matrix of ion currents in positive mode with aggregated mass spectra.

The correlation matrix for positive ions (Figure 2) contains significantly fewer areas of high correlation on the main diagonal of the similarity matrix. This means that ions close to each other in mass in the positive ion mode behave more independently than negative ions. Nevertheless, several correlated squares on the main diagonal with a correlation between 0.2 and 0.4 can be recognized in Figure 2. The highest correlated ions group in all samples are located around 1500–1600 m/z. Contrary to the small number of high positive correlation values, the high negative correlation values are more common in the matrix of similarity of ion currents in positive polarity spectra.

Correlation matrices in Figure 1 and Figure 2 were built using all 6 fragments of the biological sample. In this case, the orientation of the fragment in the cartridge during measurement, as well as the internal heterogeneity of the sample, can affect the value of the correlation. Figure 3 and Figure 4 demonstrate the dynamics of the currents of several pairs of negative and positive ions, respectively. Figure 3 and Figure 4 represent the mutual

distribution of ion currents. Figure 3 and Figures 4 A,C,E,G show a time course for pairs of ion currents grouped by fragments, where dashed lines separate repetitions of measurements for each fragment, whereas different glioblastoma fragments are separated by vertical lines. Ion currents are shifted to zero mean and normalized to the standard deviation (z-normalization). Figure 3 and Figures 4 B,D,F,H are scatter-plots for the corresponding pairs of ion currents. The coordinate of each point is determined by the value of the ion intensities normalized to the total ion current in a single mass spectrum.

Both pairs of ions with a high positive correlation (“correlating”, Figures 3A, 3C, Figures 4A, 4C) and with a noticeable negative correlation (“anti-correlating”, Figures 3E, 3G, Figures 4E, and 4G) were selected. For clarity, the measurements for individual fragments are separated by a solid line, and repetitions within one fragment are separated by a dotted line. Figure 3A and Figure 4A demonstrate the dynamics of pairs of ion currents whose correlations are positive in all fragments.

Figure 3. Representations of ion currents in negative mode. Data correlations are above the graphs.

Figure 4. Representations of ion currents in positive mode. Data correlations are above the graphs.

Figure 3B and Figure 4B prove this statement since the cloud of points is strongly elongated along the $y=k \cdot x$ line. Ion pairs that are shown in Figure 3C and Figure 4C also have a positive total correlation. However, the correlation of ion currents in the fifth and sixth fragments in Figure 3C and the second and fifth fragments in Figure 4C is negative.

Unlike the previous two pairs, the ion currents of the pair shown in Figure 3E and Figure 4E have a negative total correlation,

which can be seen from the descending shape of the point clouds in Figure 3F and Figure 4F. In this case, the correlation of currents in the first and fourth fragments in Figure 3E and the third fragment in Figure 4E is positive.

The spread matrices shown in Figures 5B and 5D were constructed for visual assessment of the presence of ions with alternating correlations in various fragments in addition to the correlation matrices (Figures 5A and 5C the same as

Figure 5. Matrices of correlation values spread for ion currents in different fragments' mass spectra.

Figure 1 and Figure 2). Most of the anticorrelating regions for negative ions (blue stripes in Figure 5A) are alternating signs (corresponding red stripes in Figure 5B). Some of the low-mass ions have spread close to 1, despite the relatively high overall correlation. This means that these ions showed a correlation close to zero for some of the fragments. The similarity matrices of ion currents for individual fragments, which were used to calculate the spread of correlations, are presented in Figures S3 and S4.

Discussion

The ions in the matrices (Figure 1 and Figure 2) are ordered by the m/z ratio (along the axis of matrices). The understanding of conformity of ion currents with the obtained mass spectrometric profiles is convenient in Figure 1 and Figure 2. However, ion currents can be reordered, for example, using hierarchical clustering methods so that the most similar ions are found in the closest rows (and columns) (Figures S1 and S2). This method of analysis allows for exploring different groups of ions. For example, groups of isotopes and adducts can be combined into one feature, hence, the dimension of the mass spectrum feature space can be reduced. Such reduction is of crucial importance for the application of machine learning

techniques since the performance of many algorithms is degraded with the growth of the number of features and the presence of highly correlated features. It also should be taken into account that both adducts and isotopes represent the same pool of molecules, so redistribution of the concentration into a number of features obscured the overall dynamics of the ion current and makes interpretation of the spectra more difficult. The most common distance between the closest ions is equal to one for positive ions and one or two for negative ions (Figures S1C and S2C). The presence of chlorine adducts in negative mode causes this effect, as chlorine adducts give a significant isotopic peak at a distance of $2m/z$ from the monoisotopic peak. Such peaks with similar ion currents dynamics and 1 or 2 m/z difference form about 10% of all peaks in both polarities.

Some of the pairs of ion currents have persistent signs of correlation, i.e. the correlation has the same sign for all measured fragments (Figure 3A, Figures 4A,E). Adducts and isotopic peaks belong to such pairs. At the same time, ions that are neither isotopes nor adducts, but have a high positive persistent correlation of ion currents (Figure 3A, Figure 4A), can be a result of coordinated metabolic processes, for example,

intermediates of one metabolic pathway. The identification and detailed analysis of such groups of ions in different fragments of the sample can provide additional information for the characterization of the sample itself. Thus, groups of ions with a high positive correlation of ion currents, whose sign does not change from fragment to fragment, are functionally coupled: either as adducts/isotopes or as biochemically related molecules.

Strictly negative persistent values of the correlation of ion currents (Figure 4E) indicate the ion suppression, the matrix effect. Such groups of ions could be associated with the measuring characteristics of the mass spectrometer.

Ion pairs with alternating signs of correlation values are associated with sample heterogeneity and extraction specifics of corresponding substances. So, two substances in one fragment were extracted in the same way from similar locations, whereas the regions of high concentration of these substances could be located differently in the other fragment, for example, on the surface and deeper inside the fragment. In this case, in the second fragment, the first substance might have been exhausted by the time the second substance could just start to be actively extracted. Examples of such pairs can be seen in Figures 3E, 3G in the second fragment, when the ion intensities change take place at the repetition border. It should be noticed that due to the peculiarities of the measurement protocol, the time between two repeated measurements of one sample is equal to five minutes, which is enough for a significant change in the ion concentration during the extraction process. The study of the internal heterogeneity of the sample would be very interesting since the sample was located at the border of the tumor and thus contained both tumor tissues and reactively modified brain tissues of the transitional region.

Ion currents of a pair of molecules can be either correlated or anticorrelated within one fragment, whereas the trend can be stable or alternating in different fragments. These factors allow the statement of the reason for the dynamics of ion currents: ion suppression, the sample position, or the functional relationship of molecules.

The alternating-sign correlation is rare in the positive mode compared to the negative ion mode, if we compare the correlation of the corresponding ion currents in different fragments (Figure 5D). Thus, the effects associated with the specifics of extraction, heterogeneity of the sample, and the location of the sample in the ion source are quite rare for the positive mode. At the same time, the alternating correlation of ion currents between different fragments dominates in the negative mode, which indicates the manifestation of such effects. Consequently, the biopsy tissues contain large quantities of molecules that are detected in the positive mode, and these molecules are rather evenly distributed within the sample. The effects associated with the geometry of the sample and its internal heterogeneity are visible in the negative ion mode. This confirms earlier observations that positive ions better describe inter-patient variability, while negative ions reflect intra-sample variability²⁹.

Thus, all pairs of ions between that have a significant correlation value can be divided into three groups:

1. The persistent positive or negative relationship caused by the processes of measuring the mass spectrum (isotopes and adducts or ion suppression respectively);
2. Positive biochemical relationship caused by the relationship of molecules concentrations in one tissue type, for example, due to a common precursor;
3. Sign-alternating relationship caused by the position of different tissue types with different molecules concentrations in the sample and their orientation to the eluent flow.

We will refer to the three types of relationships described above as measure-, biochemical-, extraction-related coupling. All types of these couplings are present in mass spectra, but they can be distinguished. For example, if the difference m/z is equal to 1 or the mass of the adduct and the overall correlation is positive and has a constant sign, then this is a positive measure-related coupling; positive persistent correlation with an unobvious difference in mass — is a biochemical-related coupling candidate; negative persistent correlation with an unobvious difference in mass — negative measure-related coupling; alternating correlation in different fragments — extraction-related coupling.

Thus, we have three types of coupling, two signs for each coupling type. Four of these six cases can be identified with high accuracy by analysis of ion currents using mass spectrometric profiling, and the other two require additional identification of molecules and their mapping to metabolic networks. For example, the difference between a positive measure-related coupling due to the presence of a chlorine adduct and a biochemical-related coupling due to the presence of two lipid species, which fatty acid residues differ by one double bond, can be reliably revealed only by the identification of molecules through the LC-MS/MS analysis.

The study of biochemical-related coupling is of great interest. The ratio of saturated and unsaturated lipids could be changed due to metabolic reprogramming during the development of the tumor¹⁷. So, the differences in the population of fatty acids between healthy and tumor tissues become noticeable. This can lead to significant ion currents correlations due to common precursors — an altered set of fatty acids. For example, if stearate is present in excess, lipids containing C18 will be at sufficiently high concentrations and, hence, the correlation between the peaks will reflect an excess of C18 within a particular tissue type, despite the actual class of lipids (phosphoserines, cardiolipins, ethanolamines, etc.). If we assume that the microextraction processes for similar molecules have similar dynamics, then their ion currents will have a high correlation. Thus, biochemical specifics appear as a positive relationship of ion currents. Moreover, these ion currents can be affected by the extraction and geometric variability, which leads to a low absolute value of the overall correlation, although the correlation of ion currents in different fragments stays positive.

The mass spectrometric profiles of glial tumors in the negative mode consist mainly of ions, whose dynamics reflect the specifics of microextraction and geometry of a particular sample. In the positive mode, mass spectra consist mainly of evenly distributed ions that are not significantly affected by the geometry of the sample, and processes of microextraction are constant in various fragments of tumor tissue. It is worth noting that ions in the positive and negative modes correspond to different molecules and even classes of molecules.

Methods created in the laboratory should be proven to be stable for the transition from laboratory studies to routine clinical mass spectrometric analysis. Routine clinical practice requires a reduction in the analysis time, therefore, recently, ambient ionization MS methods have been widely developed. Ambient ionization, unlike traditional LC-MS/MS analysis, allow measurements in the shortest possible time (minutes for the analysis vs hours for chromatography). At the same time, markers of a particular tissue found using LC-MS/MS can disappear from the ambient MS data due to the ion suppression, which can be found through analysis of [Figures 5A and 5B](#) or [Figures 5C and 5D](#). The indicator of ion suppression is the case, where both values of ion currents correlation and correlation spread matrices are closed to zero (twice blue pixels). Thus, an understanding of the processes of ion suppression makes it possible to predict the behavior of markers found by LC-MS/MS analysis under conditions of ambient ionization setup. Aggregation of ions that behavior correlated due to measurement procedure (isotopes, adducts, etc.) is another possibility to simplify the analysis of mass spectrometric profiles. Such groups of ions are well distinguished using hierarchical clustering ([Figures S1 and S2](#)): [Figures S1C and S2C](#) demonstrate high peaks at 1m/z and 2m/z, which indicates the isotopic structure and the presence of chlorine adducts in the negative ion mode or saturated bonds in lipid tails.

Conclusion

The currents of ions forming the mass spectrometric profiles obtained from glial tumors are grouped according to their similarity with each other due to several factors that include the specifics of measuring, extraction, and biochemical processes.

The dynamics of ions in the mass spectrometric profiles of glioblastoma samples obtained in the positive mode proves that the bulk of ions corresponds to molecules uniformly distributed

in the sample volume in a relatively stable concentration. At the same time, the profiles obtained in the negative mode contain ions with high heterogeneity of the spatial distribution, which is confirmed by a large number of pairs with an alternating sign of correlation. This makes the negative range the preferred choice when looking for markers of glial tumors. On the other hand, this fact allows us to conclude that marker lipids are differently distributed within the sample.

Data availability

Underlying data

Zenodo: Data and code for analysis of ion currents in mass spectrometric profiles using glioblastoma tissue, <https://doi.org/10.5281/zenodo.4309077>³¹.

This project contains the following underlying data:

- Dataset of mass spectrometric profiles of glioblastoma tissue fragments.
- Software file for figures replication.

Extended data

Zenodo: Figure S1. Sorted similarity matrix of ion currents for negative mode, <https://doi.org/10.5281/zenodo.4308954>³².

Zenodo: Figure S2. Sorted similarity matrix of ion currents for positive mode, <https://doi.org/10.5281/zenodo.4308956>³³.

Zenodo: Figure S3. Similarity matrix of ion currents in separated fragments of tissues for negative mode, <https://doi.org/10.5281/zenodo.4308958>³⁴.

Zenodo: Figure S4. Similarity matrix of ion currents in separated fragments of tissues for positive mode, <https://doi.org/10.5281/zenodo.4308962>³⁵.

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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Joris Meurs

Exhaled Biomarkers and Exposure Group, Department of Analytical Chemistry & Chemometrics,, Institute for Molecules and Materials, Radboud University, Nijmegen, The Netherlands

The authors present to use of ion currents to assess MS profiles of glioblastoma tissue. I have some suggestions for further improvement of the article:

- Please add more detail to the figure caption to make the figures easier to understand.
- I was wondering why the authors used the correlation of the average spectrum to assess and not the variability in ion intensity between the repeats.
- Brief details should be added on the mass spectrometry analysis (extraction solvent, mass resolution).
- It was not clear what measure in the 'spread matrices'.
- The authors could elaborate more on the use of the method for clinical practice.
- How do the results reflect on the immunostaining?
- How were the ions chosen for correlation (Figure 4)? Were these randomly chosen, are these fragments/clusters, or biologically-related?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Analytical chemistry, mass spectrometry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 30 July 2021

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Vladimir Frankevich

National Medical Research Center for Obstetrics, Gynecology and Perinatology, Ministry of Healthcare of the Russian Federation, Moscow, Russian Federation

The study is aimed to determine the causes of the ion current dynamic. This question is very actual for ambient ionization mass spectrometry since there are numerous attempts to make predictions as classification or regression tasks by mass spectrometric profiles obtained with different ambient ion sources. This paper demonstrates an approach to differ the internal heterogeneity of the sample, extraction processes, and measuring process by the mutual dynamics of the ion currents. The research is based on the correlation calculation between ion currents. It was clearly demonstrated that some of the ions are “correlating” with each other, while others are “anti-correlating” or not sufficiently interconnected. The results are justified enough. The data is full enough to reproduce the results. The core of the method is simple but appears to be effective in the task of grouping ions by the nature of their dynamics. The paper has a high level of novelty and could be applied in the analysis of ambient mass spectrometry data.

However, some minor revisions would improve the readability and quality of the paper:

1. The paper would benefit from English correction.
2. The resolution of the spectra should be noted.

3. The sentence describing the spread of correlations should be rewritten in a better manner.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: mass spectrometry, clinical mass spectrometry, metabolomics, instrumentation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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