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Imaging and differentiation of mouse embryo tissues by ToF-SIMS

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Abstract

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) equipped with a gold ion gun was used to image mouse embryo sections and differentiate tissue types (brain, spinal cord, skull, rib, heart and liver). Embryos were paraffin-embedded and then deparaffinized. The robustness and repeatability of the method was determined by analyzing ten tissue slices from three different embryos over a period of several weeks. Using principal component analysis (PCA) to reduce the spectral data generated by ToF-SIMS, histopathologically identified tissue types of the mouse embryos can be differentiated based on the characteristic differences in their mass spectra. These results demonstrate the ability of ToF-SIMS to determine subtle chemical differences even in fixed histological specimens.
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Keywords: ToF-SIMS; Mouse embryo; Paraffin-embedded; Image PCA

1. Introduction

Formalin-fixed paraffin-embedded (FFPE) tumor samples are routinely used for disease diagnosis and are one of the most important and most abundant sources of clinical samples available in medical centers and medical schools. New technologies for analyzing these samples that could be used to improve tissue-based diagnosis, predict response to specific modes of treatment, and aid in prognosis decisions have the potential to greatly improve decisions about therapeutic strategies.

Beyond the conventional histopathological methods, little has been done to develop new methods to analyze FFPE tissues. Jaremko et al. has applied MALDI-ToF MS to the genotyping of low quality DNA obtained from FFPE tissues [1]. However, no studies have been done to investigate the distribution of small molecules in FFPE tissues. We are using bioimaging time-of-flight secondary ion mass spectrometry (ToF-SIMS) to image tissues by their secondary ions in deparaffinized mouse embryo sections. We then differentiate these tissues based on differences in small molecules remaining after paraffin-embedding and fragments of the tissue proteins. These experiments serve as a preliminary study for further investigation of human FFPE samples from tumor and normal tissues.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a surface-sensitive technique that allows the detection and localization of the chemical composition of sample surfaces. The instrument uses a finely focused (∼300 nm), pulsed primary ion beam to desorb and ionize molecular species from a sample surface. The resulting secondary ions are accelerated into a mass spectrometer, where they are analyzed for mass by measuring their time-of-flight from the sample surface to the detector. Displaying the mass spectra that were collected from the sample surface generates chemical images. The resulting ion images contain a mass spectrum in each pixel of the 256 × 256 pixels in an image. These mass spectra are used to create secondary ion images that reflect the composition and distribution of sample surface constituents.

Using ToF-SIMS technology, several groups have been successful in identifying intracellular distributions of specific biological ions such as sodium, potassium, calcium and membrane lipid fragments [2–11]. Quong et al. showed that in human breast cells exposed to the carcinogen PhIP, the target compound was found in detectable amounts within the outer leaflet membrane of the cells [12]. Similarly, analysis of yeast cells that had

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been exposed to the drug clofazimine demonstrated the presence of the drug within the cells [8].

Very little has been done to apply ToF-SIMS imaging to cancer or tissue-specific problems. Mouse brain tissue slices have been analyzed using gallium, indium, or gold cluster as a source of primary ions [13–15]. Nygren and Borner demonstrated localization of phosphocholine, galactosylceramide, and cholesterol in rat brain slices using a bismuth cluster ion source [16–18]. Imaging cancer cells grown in culture has also been shown [19–21]. These studies illustrate the ability of ToF-SIMS to sensitively analyze and localize small molecules and large molecule fragments in cells and tissues.

A strength of the ToF-SIMS technique arises from the data generated; each ion image provides a highly detailed mass spectral map of the sample being analyzed. However, the spectra of biological samples are extremely complex and difficult to interpret. This complexity is derived from the contribution of secondary ions that are generated from fragmentation of larger molecules within the sample and matrix effects that influence the secondary ion yield depending on the chemical environment of the surface being sampled [22]. Further, because most of a cell's mass is comprised of proteins, which are composed of only 20 fairly homogenously distributed amino acids, there is a lack of unique peaks among different biological samples. In fact, mass spectra obtained from different types of biological materials qualitatively appear very similar. Therefore, data reduction and pattern recognition statistical analysis techniques must be used to differentiate similar biological materials.

Principal component analysis (PCA) is commonly used to identify similarities and differences in ToF-SIMS spectra and classify spectra into groups [19,22–26]. PCA is a standard, unsupervised multivariate statistical technique which reduces a large data matrix to a few manageable variables called principal components (PCs). Principal components represent linear combinations of the original data and capture the greatest variation in the data set. By plotting the resulting 'scores' and 'loading' plots, the relationship between samples and variables can be visualized and easily interpreted. Wagner and Castner have used PCA and singular value decomposition to successfully cluster ToF-SIMS mass spectra generated from samples of single proteins and from samples of alkanethiol self-assembled monolayers, adsorbed onto gold substrates [22,27–29]. Statistical analysis of ToF-SIMS spectra has also been employed to distinguish three species of freeze-dried yeasts based on membrane phospholipids [11] and to discriminate four yeast strains based on composite spectra from samples of yeast cultures [30]. Vegetative Bacillis cells were discriminated from spores based on ToF-SIMS analysis of phospholipid fragments [25]. We have shown that ToF-SIMS imaging and PCA can differentiate whole cells and homogenates of three carcinoma-derived human breast cancer cell lines (MCF-7, T47D and MDA-MB-231) [21].

This study extends the analytical capabilities of ToF-SIMS and PCA by imaging and differentiating histopathologically identified tissues from 16-day-old FFPE mouse embryos. In this study we demonstrate differentiation of six tissue types and show the reproducibility and robustness of the analysis. These experiments provide the foundation for work with human FFPE tissues and suggest new uses for ToF-SIMS for molecular pathology.

2. Experimental

2.1. Animals

Female C57BL/6BAC mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and bred with male C57BL/6BAC mice to generate the embryos used in this experiment. The animals were maintained on a 12-h dark/light cycle in a temperature and humidity controlled room. The care of the animals was in accord with the Lawrence Livermore National Laboratory (LLNL) Institutional Animal Care and Use Committee (IACUC) committee guidelines. Animals are anesthetized with isoflurane and killed through cervical dislocation. Embryos were harvested using standard techniques [31].

2.2. Mouse embryo tissue slice preparation

Three 16-day-old mouse embryos from three different dams designated with the numbers 1 to 3 were fixed in 4% paraformaldehye for 36 h and embedded in paraffin blocks using standard techniques [32]. Four-micron thick saggital slice sections were cut from each embryo using a Leica RM2165 microtome and were designated as sections 1 through 9. A fourth slice was cut from the third embryo and was designated as section 10. The sections were placed on 1.2 cm × 1.2 cm silicon (Si) wafer substrates and incubated at 40 °C for overnight. The samples were then deparaffinized and dehydrated using xylene and 100% ethanol. The samples were allowed to air dry and were stored in vacuum at 1E−4 Torr for 24 h before ToF-SIMS analysis.

A fourth mouse embryo was prepared as described above for optical imaging. A 4 μm section was cut and stained with hematoxylin and eosin (H&E) using standard immunohistochemical techniques [32]. The sample was placed on a glass slide and evaluated by the pathologist, Dr. Lu. The identified tissues in this sample were used as a reference for the ToF-SIMS analysis.

2.3. ToF-SIMS analysis

ToF-SIMS measurements were conducted on a PHI-TRIFT III instrument (Physical Electronics USA, Chanhassen, MN) equipped with a gold liquid metal ion gun (Au LMIG). The ion gun was operated at 22 kV and in an unbunched mode. Positive ion SIMS analyses were done utilizing Au^+ ions at room temperature, with a pulsed, low-energy electron gun providing charge neutralization. For the tissue differentiation experiment, six tissue types were selected from section number 10: skull, rib, brain, spinal cord, heart and liver. Tissues were identified based on the pathologist's designation of similar tissue regions in the H&E stained section. ToF-SIMS measurements were conducted over a 300 μm × 300 μm area for 5 min; one average mass spectrum was reconstructed for that specific region of the tissue section. Ten measurements were recorded for each tissue type. The mass spectra were calibrated using common hydro-