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Quantitative imaging of deuterated metabolic tracers in biological tissues with nanoscale secondary ion mass spectrometry

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ABSTRACT

In the field of secondary ion mass spectrometry at nanometer scale (NanoSIMS), configuration of parallel detectors to routinely measure isotope ratios in sub-100 nm domains brings classical stable isotope tracer studies from the whole tissue level down to the suborganelle level. Over the past decade, the marriage of stable isotope tracers with NanoSIMS has been applied to a range of fundamental biological questions that were largely inaccessible by other means. Although multiplexed measurement of different stable isotope tracers is feasible, in practice there remains a gap in the current analytical capacity to efficiently measure stable isotopes commonly utilized in tracer studies. One such example is the measurement of deuterated tracers. The most obvious approach to measuring deuterium/hydrogen isotope ratios is at mass 2/1. However, the radius of the magnetic sector limits concomitant measurement of other masses critical to multiplexed exploration of biological samples. Here we determine the experimental parameters to measure deuterated tracers in biological samples using the C_2H^- polyatomic ion species (C_2D^-/C_2H^-) while operating the NanoSIMS at a reduced Mass Resolving Power of 14,000. Through control of the sputtering parameters, we demonstrate that there is an analytical window during which the C_2D^-/C_2H^- isotope ratio can be measured with sufficient precision for biological studies where the degree of D-labeling is typically well above natural abundance. We provide validation of this method by comparing the C_2D measurement of D-water labeling in the murine small intestine relative to measurements of native D/H conducted in the same analytical fields. Additional proof-of-concept demonstrations include measurement of D-water, D-glucose, and D-thymidine in biological specimens. Therefore, this study provides a practical template for deuterium-based tracer studies in biological systems.

1. Introduction

Since Schoenheimer pioneered stable isotope methodology to elucidate the dynamic nature of intermediate metabolism [1,2], stable isotope tracers have been utilized to study a wide array of complex metabolic processes in model organisms and humans [3,4]. The standard analytical approach is to track label incorporation by isotopic enrichment in tissue or other biological samples with isotope ratio mass spectrometry (IRMS). As is common with analytical methods utilized in biological research, IRMS-based studies require pooling cells to obtain sufficient material for analysis, even with instrumental modifications designed to analyze small sample sizes [5]. The assumption of normality inherent to analyses of pooled cells may obfuscate biologically significant heterogeneity, providing rationale for analytical methods capable of providing quantitative measurements at the level of the single cell.

NanoSIMS is a form of secondary ion imaging mass spectrometry that allows measurement of isotope ratios at the level of a single cell [6,7], and therefore is a significant advance over classical tracer studies conducted with bulk sample analyses. An ion probe is scanned over a sample surface, resulting in the sputtering of atoms and small molecular fragments, representative of the surface layers. The ionized fraction is guided by ion optics to a double-focusing mass spectrometer where instead of being scanned, the magnetic

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field is kept constant, allowing for simultaneous measurement of several secondary ions from the same nanovolume. Seven detectors are positioned on trajectories of varying radii, facilitating measurement of different isotopic variants, and in turn isotope ratios, with routine lateral resolution below 100 nm. When merged with stable isotope tracer methodology, the NanoSIMS instrument reveals dynamic functional information in individual cells and organelles in their natural microenvironment, an approach that has illuminated fundamental metabolic pathways in microbes [8–11], plants [12,13], and mammalian model organisms [14–16].

To date, the majority of studies utilizing NanoSIMS imaging of stable isotopes have been conducted with molecules tagged with ^{15}N or ^{13}C, despite extensive precedent for utilization of deuterated compounds in classical tracer studies. A limitation when the instrument is configured to directly capture hydrogen ions is that the upper mass limit of detection, as constrained by the radius of the magnetic sector, prevents simultaneous acquisition of heavier mass images of interest. Even when the first detector is positioned at a lower radius beyond the manufacturer's specifications to enable analysis up to mass 27 [17], additional ions of interest cannot be measured, including $^{31}P^-$ and $^{32}S^-$ that provide crucial histological information or halogenated tracers [14–16]. The polyatomic ratio of C_2D^-/C_2H^- has previously been utilized as a proxy for D/H for analysis of non-biological samples [18]. However, the mass resolving power required to correctly isolate C_2D^- from its closest isobars was achieved by utilization of a narrower, custom slit. Achievement of such high mass resolution comes at the significant cost of reduced ion transmission, an issue of concern for large-scale biological studies. Here we demonstrate that operation of the NanoSIMS at reduced mass resolving power combined with control of the sputter time allows for the quantification of the C_2D^-/C_2H^- ratio with sufficient sensitivity and precision for routine biological applications.

2. Experimental section

2.1. Materials

Stable isotope tracers, consisting of ^2H-thymidine, ^{15}N-thymidine, ^2H-water, and ^2H-glucose, were obtained from Cambridge Isotope Laboratories, Inc. Paraformaldehyde and osmium tetroxide utilized for tissue fixation and Epon resin used for tissue/cell embedding were obtained from Electron Microscopy Sciences, Inc.

2.2. In vivo *tracer studies*

Male mice on C57Bl/6 genetic background were injected with tracers by intraperitoneal injection. For the thymidine labeling studies, mice received twice daily 500 μg injections for a total of 3 days. ^2H-glucose (50 mg) was administered by intraperitoneal injection twice daily for 24 h prior to sacrifice. ^2H-water was administered as a single load of 250 μl, followed by daily injections of 60 μl for a total of 3 days. For the human D-water study, the volunteer ingested 400 ml of sterile ^2H-water over 15 min, followed by 60 ml daily for 2 additional days, after which peripheral blood was sampled.

2.3. Sample preparation

All samples were fixed with 4% paraformaldehyde. Post-fixation with osmium tetroxide was utilized for adipose tissues. Due to its dark staining effect, osmium was also used for white blood cell analyses to assist with location of the cell pellet during embedding and cutting. All samples were embedded in EPON resin, sectioned (0.5 μm), and mounted on silicon chips. Samples were coated with a 15 nm layer of gold, prior to NanoSIMS analysis to prevent charging, which often occurs with resin-embedded biological samples.

2.4. NanoSIMS

Analyses were performed on the NanoSIMS 50L (Cameca) at the Brigham and Women's Center for NanoImaging, where multiplexed labeling experiments now represent the majority of the analytical load, including measurement of deuterium as described here, in conjunction with ^{13}C, ^{15}N, and/or halogens like ^{81}Br. The standard analysis utilizes a 16 keV cesium ion beam for the analysis of negatively charged secondary ions. Through deflection plate peak jumping [18], the 7 detectors can be configured to record 9 masses quasi-simultaneously. This enables the routine acquisition of $^{12}C_2^-$, $^{12}C^{13}C^-$, $^{12}C_2H^-$, $^{12}C_2D^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$, $^{31}P^-$, $^{32}S^-$, and a higher mass such as $^{81}Br^-$ [19,20]. Specifics regarding the detector configurations are summarized in Supplemental Table 1. Measurement of C_2D^- and CN^- or C_2H^- and $^{12}C^{13}C^-$ on the same detectors is reliable because the detector responses (measured as the pulse height distribution) to these specific poly-atomic species exhibit minimal difference. When tuning for peak switching, the detectors are adjusted such that the secondary ion beam arrives at the anode within the deflection voltage range where the secondary ion counts are stable. This is particularly important for switching between C_2D^- and CN^-, where 15 V must be applied to the electrostatic plates positioned in front of the detectors in order to span both species. Therefore, by alternating the voltage applied to the plates with successive acquisitions, two different series of images are generated (Supplemental Fig. 1). The detectors are aligned such that series 1 acquisition includes: $^{12}C_2^-$, $^{12}C_2H^-$, $^{12}C_2D^-$, $^{12}C^{15}N^-$, $^{31}P^-$, $^{32}S^-$, leaving the 7th detector to capture a higher mass (*e.g.* ^{81}Br). The series 2 acquisition includes: $^{12}C_2^-$, $^{12}C^{13}C^-$, $^{12}C^{14}N^-$,$^{12}C^{15}N^-$, $^{31}P^-$, and $^{32}S^-$ with the 7th detector capturing the same species as in series 1 (*e.g.* ^{81}Br). For each series, the acquisition time is set independently to optimize the counting statistics for the given masses of interest. For species of high yield such as $^{13}C^{12}C^-$ and $^{12}C^{15}N^-$, for example, we acquire images at a rate of 500 μs/pixel (256 × 256 pixels), in contrast to 2000 μs/pixel for the series containing C_2D^-.

2.5. OpenMIMS

Images were visualized and analyzed, using a custom plugin to ImageJ, called OpenMIMS. The OpenMIMS 3.0 version incorporates a new "interleave" function, which processes image files acquired in the peak-switching, multiple-series mode. In particular, images of the same ionic species, but collected in different series can be merged into a single stack in the order in which they were acquired (Supplemental Fig. 1). It is particularly useful to compress image data from different series in this fashion (obtained by peak jumping) when imaging low-yield ions (^{31}P, ^{81}Br), thereby enhancing image quality.

3. Results and discussion

3.1. Widespread use of deuterated tracers provides rational for their measurement with NanoSIMS

Stable isotope tracers are utilized in a variety of applications; however, they represent a unique tool for *in vivo* studies, including in humans, because they are innocuous. A PubMed search for "stable isotope," limited to human studies, underscores this point, demonstrating a time-dependent increase in human stable isotope tracer studies (Fig. 1). Of studies on this curated list, deuterium-based tracers were the most commonly utilized (43.4%), followed closely by ^{13}C studies (43.0%), and ^{15}N studies (8.8%) to a much