Quantitative Imaging of Cells with Multi-Isotope Imaging Mass Spectrometry (MIMS) – Nanoautography with Stable Isotope Tracers

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Abstract

We describe some technical aspects of the application of Multi-Isotope Imaging Mass Spectrometry (MIMS) to biological research, particularly the use of isotopic tags to localize and measure their incorporation into intracellular compartments. We touch on sample preparation, on image formation, on drift correction and on extraction of quantitative data from isotope ratio imaging. We insist on the wide variety of sample types that can be used, ranging from whole cells prepared directly on Si supports, to thin sections of cells and tissues on Si supports, to ultrathin TEM sections on carbon coated grid. We attempt to dispel the myth of difficulties in sample preparation, which we view as a needless deterrent to the application of MIMS to the general biological community. We present protocols for the extraction of isotope ratio data from mass images. We illustrate the benefits of using sequential image plane acquisition followed by the application of an autocorrelation algorithm (nanotrack) to remove the effects of specimen drift. We insist on the advantages to display the isotope ratios as Hue Saturation Intensity images.

Keywords: MIMS, Isotope Ratios, Hue-Saturation-Intensity, Cells, 15N, Turnover

1. Introduction

Magnetic sector secondary ion mass spectrometry (SIMS) has made many important contributions in a number of research and commercial areas including semiconductor, geological, astrophysical and materials sciences [1-7]. A new generation of SIMS offers exceptional capabilities to biomedical research [8]. These capabilities include the simultaneous detection of several atomic masses (parallel imaging), together with a high mass separation (M/ΔM >8000) at high transmission (70-80%), and a high lateral resolution (~ 35 nm). They allow one to proceed beyond the pioneering work [9-12].

For the first time, the possibility exists to both see (at high resolution) and measure (with high precision) molecules or elements labeled with stable isotopes in subcellular compartments of individual cells.

We use this new instrument (the factory prototype of the Cameca NanoSIMS) to develop multi-isotope imaging mass spectrometry (MIMS), a technique combining SIMS analysis, tracer methods and quantitative imaging techniques for the quantitative analysis of cubic
nanometer volumes at definite locations in cells and tissues. MIMS provides a kind of nanoautography that may use any element of the periodic table as a tag, that is quantitative, fast, and permits multiple tagging. Tagging cells with stable isotopes, particularly tagging the DNA, may provide a means for very long-term and risk-free identification of cells.

While the spatial resolution of MIMS is high, the depth resolution is a few atomic layers. The ability to image and analyze the first atomic layer at the surface of cells may open a world of new studies, from antigen presentation to cell-to-cell communication.

MIMS exceptional capabilities should make it a major player in the array of physical methods for biomedical research.

2. Samples and Sample Preparation

The number of different types of biological materials that can be examined by MIMS is essentially limitless. The only restriction on MIMS samples is that they must be capable of withstanding an ultrahigh vacuum. We have examined a wide variety of sample types, from human hair cross-sections [13], to ultrathin (90 nm) TEM sections mounted on C-coated Cu grids [14], to whole cells such as adipocyte, endothelial and fibroblast cells grown directly on Si supports [15-17].

Unfortunately, we still observe a great deal of often uninformed debate over appropriate methods for sample preparation, particularly involving chemical fixation. Such discussions are to the detriment of the rapid application of MIMS to biological research. In fact, there is an immense body of high quality studies that use chemical fixation to localize proteins for light and electron microscopy. It has been and is being used by the likes of George Palade (1974 Nobel Prize for Medicine), Keith Porter, Morris Karnovsky and Gunter Blobel (1999 Nobel Prize for Medicine). Also, as an example, in the August 2005 issue of the Journal of Cell Biology, all of the research articles requiring the cellular localization of one or more proteins employed chemical fixation [18-27].

Cryogenic methods, however, may be used for studies involving diffusible ions or fast-diffusing small molecules. We have discussed cryogenic methods and wish to underscore that to prevent remodeling of water crystals in the cells, we use a custom built freeze drier that maintains samples at -80°C [28].

In general, tissue and cell samples are chemically fixed, embedded in Epon and sectioned with an ultramicrotome. The sections (200-500 nm) are placed on Si supports, and then inserted into the MIMS 16-window custom made sample holder (figure 1). We have found that keeping the sections in a heated vacuum oven until loading into the instrument minimizes charging during analysis.

We have begun to analyze ultrathin sections (70-90 nm) mounted on 100 mesh honeycomb Cu grids. These sections are first observed with TEM and then analyzed with MIMS. Thus we are able to acquire two sets of image data on the identical region of the sample providing both high resolution histological

![Figure 1: Top face of custom made 16 window sample holder. Microtome sections on Si chips are located in each of the 16 windows. Arrow: points to a thin section on a silicon chip.](image)
(TEM) and chemical information (MIMS).

For identification of regions of interest (ROI), we map the samples extensively before introduction into the instrument. They are photographed using differential interference contrast reflection microscopy with a Nikon Eclipse E800 microscope (figure 2a and 2b). We record the (x,y) coordinates of several reference points across the 5 cm diameter sample holder using a micrometric stage and controller (LUDL Electronic Products, Hawthorne NY) driven by ISee software (ISee Imaging Systems, Raleigh NC). These reference points are converted to MIMS stage coordinates through a custom processing software.

3. MIMS Analysis

We use a primary beam Cs\(^+\) ions with a net impact energy of 16 keV. The current can be measured by a Faraday cup in the primary column (Fcp), or directly at the sample location by moving a Faraday cup into the sample position (Fco). The relationship between the size of the objective diaphragm, the value of Fcp, and the corresponding value for Fco, measured over two years are given in table 1.

<table>
<thead>
<tr>
<th>Objective Diaphragm</th>
<th>D1-1</th>
<th>D1-2</th>
<th>D1-3</th>
<th>D1-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (μm)</td>
<td>500</td>
<td>300</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>Mean Fco Beam Current (pA) per nA of Fcp Beam Current</td>
<td>0.387</td>
<td>0.146</td>
<td>0.043</td>
<td>0.023</td>
</tr>
<tr>
<td>Standard Deviation</td>
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<td>0.014</td>
<td>0.006</td>
<td>0.005</td>
</tr>
<tr>
<td>Number of Measurements</td>
<td>6</td>
<td>7</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 1: Relationship between the measured Fcp and Fco beam currents. The Fco current is expressed in pA per nA of Fcp current.

After analysis of a sample begins, we wait for a period of time for the secondary ion current to become constant. This happens when the Cs\(^+\) implantation reaches a steady state, where the amount of Cs\(^+\) implanted equals the amount of Cs\(^+\) that is sputtered [29]. We have performed systematic studies of secondary ion emission as a function of Cs\(^+\) implantation in an Epon-embedded bullfrog.
Figure 3: Secondary ion count rates for $^{12}\text{C}$ (blue), $^{13}\text{C}$ (red), $^{12}\text{C}^{14}\text{N}$ (yellow) and $^{12}\text{C}^{15}\text{N}$ (green) as a function of time acquired from a bullfrog cochlear sample. The primary beam at the sample (Fco) was 0.62 pA. (a): Scanning field is 2 μm x 2 μm. (b): Scanning field is 4 μm x 4 μm.

saccular sample (see figure 3). We acquired the data using 2 μm x 2 μm (figure 3a) and 4 μm x 4 μm (figure 3b) scanning fields. The primary beam current (Fco) was 0.62 pA. We observed a steady state secondary ion current after bombarding with a dose of Cs’ primary ions equal to $5 \times 10^{16}$ atoms/cm$^2$. This dose was obviously obtained faster over a small area (figure 3a) than a larger one (figure 3b).

In ion image studies, we are fortunate that the $^{12}\text{C}^{14}\text{N}$ image have enough contrast to allow us to recognize the essential anatomical features (figure 4 shows an example of a section of mouse intestine). The brightness of an image area depends, overall, upon the local concentration of the considered element, the implanted cesium concentration and the sputtering rate. A given area may appear brighter either because the sputtering rate is higher when the ionization yields are saturated or because the sputtering rate is lower if the ionization yield is still sensitive to the cesium implanted concentrations (lower sputtering yields lead to higher implanted cesium concentrations which lead to higher ionization yields). As shown in figure 5, zymogen granules, which are densely packed digestive enzyme proteins are strongly emitting $^{12}\text{C}^{14}\text{N}$.

Note that the Zn signal is coming from a few individual granules (parallel images in figure 5c and 5d). This may indicate heterogeneous distribution of carboxypeptidase in the granules, similar to our findings for amylase and chymotrypsine measured in individual zymogen granules using ultramicrofluorescence chemistry [30]. The high contrast and spherical shape of the zymogen granules are also useful in that they provide us with a means of studying the primary beam astigmatism.

The parallel imaging of an Epon-embedded section of bullfrog saccula is shown in figure
6. The $^{12}\text{C}^{14}\text{N}$ image of the stereocilia shows striking contrast, whereas in the $^{12}\text{C}$ image they are barely discernible from the background. Carbon atoms have an electron affinity of about 1.2eV which makes the ionization probability fairly sensitive to local concentrations of implanted cesium.

![Figure 6: Parallel MIMS images of stereocilia from a bullfrog saccular sample for (a): $^{12}\text{C}^{14}\text{N}^{-}$. (b): $^{12}\text{C}$ secondary ions. The field is 8 μm x 8 μm. (with D. Corey and D-S. Zhang, Harvard Medical School).](image)

From the low contrast variations in $^{12}\text{C}$ images and taking into account that carbon is the main constituent and rather uniformly distributed, one may infer that it is very likely that sputtering yields are evenly distributed. The electron affinity of $^{12}\text{C}^{14}\text{N}$ clusters is about 3.6 eV so that their ionization probability reaches its highest level for much lower cesium concentrations and is therefore less sensitive to small variations in the sputtering yields. As a consequence, $^{12}\text{C}^{14}\text{N}$ images have a good chance, in a first approximation, to give a fair representation of the local concentration of nitrogen.

![Figure 5: MIMS images from a section of human pancreas showing zymogen granules (ZG) and acinar cells (AC). (a): $^{12}\text{C}^{14}\text{N}^{-}$ image from a 52 μm x 52 μm field. Acquisition time was 1 minute. (b): $^{12}\text{C}^{14}\text{N}^{-}$ image from a 12 μm x 12 μm field. Acquisition time was 16 minutes. (c) and (d): Parallel acquisition of $^{12}\text{C}^{14}\text{N}^{-}$ and $^{16}\text{O}^{64}\text{Zn}$ images. The field is 6 μm x 6 μm. The acquisition time was 1 minute.](image)

In steady-state conditions, the sputtered material leaving the sample must have the same composition than the original sample. Yet, the processes associated with sputtering can be seen as producing a kind of "local stirring" in which most atoms do not move far from their original position (range of atomic cascades) but in which their relative positions may be greatly different in the superficial layers before they are expelled from the sample. Nonetheless, the intensity of the CN$^{-}$ ion should be proportional to the product of the respective concentrations of carbon and nitrogen in the superficial layers. Because carbon concentration is much higher than that of nitrogen, CN$^{-}$ intensities will mainly depend upon local nitrogen concentrations. It should be noted that the $^{12}\text{C}$ intensities may be slightly lowered by the presence of nitrogen because the channel of CN production may remove carbon atoms from the other emission channels. This might be the reason for the small contrast observed in $^{12}\text{C}$ images.
We have recently found that CN ions are formed by the recombination of C and N [31]. We also noted a strong effect of the local atomic environment of the $^{13}$C label when studying isotope ratios obtained from a series of $^{13}$C- and $^{15}$N-labeled polyglycine samples where the atomic position of the label was known. When the $^{13}$C label is double-bonded to the O atom in the polyglycine molecule, the $^{13}$C/$^{12}$C, $^{13}$C$^{14}$N/$^{12}$C$^{14}$N and $^{13}$C$^{15}$N/$^{12}$C$^{15}$N isotope ratios decrease by 40-50%.

Atomic mixing occurs in dimensional volumes at the atomic scale and thus does not affect usual biological studies. For example, the boundary between the cytoplasm and the nucleus remained relatively sharp after imaging the same field for several hours. Rat embryo fibroblasts were pulsed with both bromodeoxyuridine (BrdU), a specific marker of DNA, and $^{15}$N-labeled uridine, a specific constituent of RNA. There was no discernible difference between the first (not shown) and the last pair of $^{12}$C$^{15}$N-$^{81}$Br images. In the last pair, despite the long bombardment, the nuclear boundary, marked with the $^{81}$Br from the BrdU incorporated in the DNA remained sharply separated from the cytoplasm (figure 7b). Also the core of a nucleolus that contains essentially RNA was strongly labeled with $^{15}$N-Uridine (figure 7a).

The results presented in figure 7 not only illustrate the negligible effect of atomic mixing, but also demonstrate that although the method is destructive by essence, practically it is not. We have recently estimated that the sputter rate for one adipocyte was 0.3 nm/minute at a beam current ($F_{co}$) of 2 pA (see also text for figure 12). In general, we find that samples are very stable under the beam: successive images and measurements at the same location are equivalent. Practically, one can study the same field many times and obtain information at a variety of resolutions and/or for a variety of atoms.

For certain experiments on whole cells, one may remove material from the sample surface quickly in order to study a variety of depths within the cell. We refer to this as “shaving” the cells. This is accomplished by operating under conditions giving high primary ion beam currents (for example by removing the objective diaphragm). As an example, in a study of integrin clustering, anti-integrin antibodies labeled with $^{127}$I were bound to endothelial cells and the dorsal cell surface analyzed by MIMS. The $^{12}$C$^{14}$N- and $^{31}$P- mass images of the whole endothelial cell are shown in figure 8 (top row). Two color-coded overlay images with $^{12}$C$^{14}$N shown in red and $^{12}$C in green, and $^{12}$C$^{14}$N shown in red and $^{31}$P as green are also presented (top row). A striking difference in gross composition is revealed. The area over the nucleus and the meaty part of the cytoplasm are intensely red, indicating a high nitrogen content. The wide area stretched at the periphery is lamellipodia, that we found relatively rich in phosphorus and poor in nitrogen.
The lack of P and C in the nucleus area of the cell prompted a “shaving” of the cell to expose a deeper area for analysis. The resultant $^{12}\text{C}^{14}\text{N}$, $^{12}\text{C}$, $^{31}\text{P}$ and $^{127}\text{I}$ mass images are shown in the bottom row of figure 8. This area was then re-analyzed at higher spatial resolution. The results are shown in figure 9. We can now observe a high P signal at the
nuclear membrane (likely heterochromatin) and also inside the nucleus, in particular two bright structures which are likely nucleoli. Also note the absence of iodine signal in the nuclear area. Over the course of this study, we found micro-sized domains approximately 1 μm in size very rich in hydrogen (see arrow in figure 10a and line scan in figure 10b). This suggests the possibility of macromolecular lipidic domains over or within the nucleus.

4. Quantitative Imaging

4.1 Generalities

In our laboratory, MIMS allows us to make a major effort to study the dynamics of the major biochemical constituents at the intracellular level. We continue with MIMS the fundamental studies of the Schoenheimer school [32] that used stable isotopes and mass spectrometry with whole tissues. We locate and measure the excess over its natural abundance of an isotope tagging precursor molecule. The excess of the isotope tag is evaluated by reference to the value of the natural abundance isotope ratio for the species of interest. Initially, we used a stationary beam for measuring the isotope ratios. Soon we found that calculating the isotope ratios out of selected areas of parallel images provided many advantages: an excellent preservation of the sample, a high precision of the measurements, and an easy comparison among different locations on the same image.

For example, simultaneous images of the distributions of $^{12}\text{C}$, $^{13}\text{C}$, $^{12}\text{C}^{14}\text{N}$ and $^{12}\text{C}^{15}\text{N}$ are obtained by scanning the Cs$^+$ primary ion beam stepwise across the stationary sample. The beam current and diameter, dwell time per pixel, and step size are adjusted according to the size of the area being analyzed. A step raster of 256 by 256 or 512 x 512 pixels is normally used. In general, we adjust the beam diameter to be approximately twice the size of a pixel.

In the scanning mode, the number of counts for each detected mass emitted from each pixel is stored as a 16-bit integer. Gray scale secondary-ion images for each of the selected masses are obtained after analysis by reconstructing the data onto a pixel array.

To obtain a statistically meaningful number of counts for mass images that have a low count rate (such as $^{12}\text{C}^{15}\text{N}$), very long counting times per pixel are required. For images acquired at the highest spatial resolution, small amounts of specimen or stage drift can lead to blurring of the image. In order to correct for drift, we now split the total acquisition time of a single image into short duration repeated acquisitions of a series images. This stack of images are then brought into registration by applying our autocorrelation function among
successive images with a custom “nanotracking” software. The stack of images corrected for registration are added and provide us with a “sum” image. An example of the power of this method is shown in figure 11 from studies on protein turnover in bullfrog saccula. The effectiveness of the nanotracking procedure to bring the stereocilia into registry is clearly apparent.

From data obtained using the nanotracking software we have determined the average drift rate of our instrument in both the X- and Y-directions from numerous acquisitions ranging in duration from 50 to 1452 minutes over a period of a few months. The results are outlined in table 2.

Acquiring a series of planes in a sequential manner opens the door for full 3D volume rendering. We have begun using this capability in the study on the cellular transport of free fatty acids (FFA) by measuring the distribution of $^{13}$C in cultured adipocytes incubated with $^{13}$C oleic acid [15]. The image shown in figure 12 is a 3D reconstruction of 1100 $^{13}$C/$^{12}$C ratio image planes acquired over a period of 200 hours (8 days, 8 hours). It is clear that there are a number of distinct $^{13}$C-enriched droplets (represented by the gold color in the figure) below the sample surface.

4.2 Identification of Regions of Interest (ROI) using Hue Saturation Intensity (HSI) Images

In order to locate ROI that have accumulated the precursor label $^{15}$N (or any other label), we derive the ratio image $^{12}$C$^{15}$N/$^{12}$C$^{14}$N and the ratio image $^{13}$C/$^{12}$C from a pixel-by-pixel division of the parallel images. In the absence of any added isotope, ratio images should appear flat across the entire analysis field with a value

<table>
<thead>
<tr>
<th></th>
<th>X- Direction</th>
<th>Y- Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Drift Rate (nm/minute)</td>
<td>2.02</td>
<td>1.66</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>4.52</td>
<td>3.05</td>
</tr>
</tbody>
</table>

Table 2: Mean drift rate in the X- and Y-direction determined from nanotracking data collected from 77 experiments.
Figures 13a-c show an example taken from images of a Si grid. Figures 13a and 13b are $^{28}\text{Si}$ and $^{30}\text{Si}$ MIMS images, respectively, in an area with a highly emitting defect in the Si. The resulting $^{30}\text{Si}/^{28}\text{Si}$ ratio image is presented in figure 13c and shows a flat field, illustrating how the isotope ratio over the field is equivalent to the terrestrial ratio irrespective of the morphological and topographical regions from which the secondary ions were emitted. Another example of this is from a microdissected whole cochlear hair cell. A strong three-dimensional depth of field in the $^{13}\text{C}^{14}\text{N}$ (figure 13d) and $^{12}\text{C}^{15}\text{N}$ (figure 13e) images might make it more susceptible to instrumental fractionation. The ratio image shown in figure 13f, however, shows a completely flat field in the region of the cell with mean $^{15}\text{N}/^{14}\text{N}$ equivalent to the terrestrial ratio. The region outside the cell marked by ROI's #7 and #8 are over the Si chip and therefore carry a much smaller number of CN⁻ counts explaining the contrast observed in the ratio image. Yet, the ratio is equivalent to the terrestrial value.

Images acquired with MIMS have a dynamic range of 16 bits, and the resulting ratio images generate far more information than can be easily displayed using simple gray level methods. In order to show high dynamic range ratio images and to de-emphasize values resulting from data with few counts, we have developed a method based on a hue saturation intensity transformation (HSI) [33, 34] of the ratio image and derived from a calcium ratio imaging procedure [35]. The hue codes for the ratio value and the intensity at a given hue code for the number of ions detected. A schematic diagram showing the relationship between the isotope ratio value and its reliability, the hue and the intensity is shown in figure 14a. The HSI ratio display allows us to take full advantage of our increased perception of color and of the quantitative information contained in each mass image. It enables identification of ROI with significant excess of the $^{15}\text{N}$ label (or any other label) by a means independent of visual recognition of expected histological structures. We have created additional plug-ins for the free ImageJ image processing software [36] that allows to read MIMS image files and to create HSI images [37]. These plug-ins are located in the NRIMS website [38]. An example of an application of the $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N}$ HSI transform image is presented in figures 14b and 14c.
Figures 14b and 14c are of a section of shipworm gill exposed to seawater equilibrated with $^{15}$N. Examination of the $^{12}$C$^{15}$N/$^{12}$C$^{14}$N- HSI transform image clearly show areas of significantly different incorporation of $^{15}$N.

A second example is from a study of protein turnover in mouse cochlea fed with a $^{15}$N-leucine diet (figure 15). The image shows a cell from the Reisner membrane with four red blood cells (RBC). The HSI $^{12}$C$^{15}$N/$^{12}$C$^{14}$N ratio image again displays areas of different $^{15}$N incorporation. It is likely that this particular cell had matured while the mouse was under the $^{15}$N-leucine diet. Observations such as this will certainly open the door for the study of individual cells in complex populations.

### 4.3 Isotope Ratios from ROI's

It is important to stress that the great advantage of isotope ratio analysis is that many potential pitfalls are canceled out. Guided by the HSI images, ROI can be selected, and the isotope ratios can be calculated from the mass images in two ways: the ratios of the means [[$^{15}$N mean] / [$^{14}$N mean], or the mean of the ratios. The error due to counting can be evaluated from Poisson statistics. The image in figure 16a is from a control sample of a mouse kidney. The mean of all 14 ROI's in figure 16a is 0.367% with a standard deviation of 0.006. The coefficients of variation of $^{12}$C$^{14}$N and $^{12}$C$^{15}$N for the largest (ROI #11) and smallest (ROI#13) ROI assuming Poisson statistics are given in table 3. The counting precision of the data is essentially a function of the counting error on $^{12}$C$^{15}$N because the number of counts is much smaller than for $^{12}$C$^{14}$N.

<table>
<thead>
<tr>
<th>ROI #</th>
<th>No. of Counts</th>
<th>Standard Deviation</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{12}$C$^{14}$N</td>
<td>11</td>
<td>5.31 x 10$^6$</td>
<td>2304</td>
</tr>
<tr>
<td>$^{12}$C$^{15}$N</td>
<td>11</td>
<td>1.91 x 10$^4$</td>
<td>138</td>
</tr>
<tr>
<td>$^{13}$C$^{14}$N</td>
<td>13</td>
<td>5.98 x 10$^3$</td>
<td>773</td>
</tr>
<tr>
<td>$^{13}$C$^{15}$N</td>
<td>13</td>
<td>2.18 x 10$^3$</td>
<td>47</td>
</tr>
</tbody>
</table>

Table 3: The coefficients of variation of $^{12}$C$^{14}$N and $^{12}$C$^{15}$N for ROI#11 and #13 assuming Poisson statistics.
From the nitrogen isotope ratios determined within the ROI’s, we can calculate the percent nitrogen renewal as: 100*(\text{Tissue $^{15}N/^{14}N$ ratio} - \text{Control diet ratio})/ (\text{Experimental diet ratio} - \text{Control diet ratio}). Nitrogen renewal is expressed as the corresponding percentage of full incorporation of labeled dietary nitrogen. Zero percent represents a ratio equivalent to the terrestrial ratio, while 100 percent represents a ratio identical to that in the experimental diet.

A $^{12}$C$^{14}$N$^{-}$ image from a section of mouse intestine upon which eleven ROI’s have been drawn is shown in figure 16b. The mouse had been fed a diet supplemented with $^{15}$N-leucine. The isotope ratio levels in ROI’s #2 through #11 all show elevated $^{15}$N/$^{14}$N ratios and the renewal of $^{15}$N was determined to be 100%. ROI #1, however, located in the embedding resin and only about 300 nm in size, gave a mean value for the ratio of 0.365%, the terrestrial value that one would expect. Furthermore, this tiny region is located only ~270 nm removed from the highly labeled sample, indicating that no redistribution of the $^{15}$N label has occurred at this scale during sample preparation or analysis.

We can estimate the volume sputtered and detectability limit. Take a field analyzed of 6$\mu$m x 6$\mu$m, a primary Cs beam current intensity $\equiv 0.4$pA, a dwell time per pixel of 20ms, a number of pixels of 256 x 256, a sputtering efficiency of 5 target atoms/Cs$^+$, an atomic layer thickness of 2.55 x 10$^{-4}$ $\mu$m, an atomic density of 6 x 10$^{10}$ atoms/$\mu$m$^3$, then the total sputtered volume is 0.27 $\mu$m$^3$, and the sputtering rate 0.4nm/min or 1.5 atomic layer/min. For a probability of detection of 95%, 3 ions have to be emitted; assuming a useful ionization yield of 1 x 10$^{-2}$ ion/atom, then the minimum detectable is 1.85 x 10$^{-8}$ or $\equiv 20$ppb.

Poisson statistics can be used to confirm a uniform distribution of a label within a region of interest. In a Poisson distribution the variance is equal to the mean [39]. In figure 17, eight ROI have been drawn on the $^{12}$C$^{15}$N$^{-}$ image of the sterocilia from a bullfrog.
saccula sample. ROI #1-3 are from regions overlapping the stereocilia and the embedding resin, while the other ROI's are completely within the stereocilia or otolithic membrane (ROI #7). From each region of interest we have extracted the mean and variance of the counts for both $^{12}$C$^{15}$N- and $^{13}$C-. The results, expressed as $(\text{Mean-Variance})/\text{Mean}$ are given in table 4. In ROI #1-3, a large deviation from Poisson statistics is observed for the $^{12}$C$^{15}$N- counts where the region of interest is deliberately drawn to overlap the embedding resin. Note that a similar deviation is not, however, observed for the $^{13}$C- counts. Since no $^{13}$C was deliberately added to this sample, its distribution is uniform across the whole field.

### Table 4: Results of the ROI analysis from the regions shown in figure 17.

<table>
<thead>
<tr>
<th>ROI</th>
<th>$^{12}$C$^{15}$N-Mean-Variance/Mean (%)</th>
<th>$^{13}$C-Mean-Variance/Mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33.9</td>
<td>-0.1</td>
</tr>
<tr>
<td>2</td>
<td>38.7</td>
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<tr>
<td>3</td>
<td>50.9</td>
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</tr>
<tr>
<td>4</td>
<td>7.8</td>
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<tr>
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</tr>
<tr>
<td>6</td>
<td>2.7</td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
<td>0.8</td>
<td>2.6</td>
</tr>
<tr>
<td>8</td>
<td>-0.5</td>
<td>-0.2</td>
</tr>
</tbody>
</table>

5. Conclusions

With MIMS we can obtain quantitative images of stable (or radioactive) isotope tags at high lateral resolution, well within intracellular domains, at an extremely high depth resolution of the order of a few, if not a single, atomic layer and with a precision that is easily within a few percent, which is more than satisfactory for most biological experiments. The technique can be used with animal tissue or with isolated cells from a tissue culture preparation using conventional methods. Sample preparation is not nearly as problematic as many believe. We have outlined some improvements that we have made in our laboratory to both the efficiency of the analysis as well as the quality of the results (for example, the use of nanotrack to minimize stage or specimen drift problems where long acquisition times are required). We have demonstrated the power of HSI transform images for defining ROI in subcellular compartments of volumes smaller than 1 $\mu$m$^3$.

With these advances, we have been able to apply MIMS to a variety of biological problems that could not be solved by other means. Among them, we are currently studying protein turnover in the chick tectorial membrane. The values of the $^{12}$C$^{15}$N/$^{12}$C$^{14}$N isotope ratios reveal significant new protein incorporation by the acellular tectorial membrane. The images of the $^{12}$C$^{15}$N/$^{12}$C$^{14}$N isotope ratios show that the mechanism of incorporation is a transfer from the apices of supporting cells to the basal layer of the tectorial membrane [40].

In studies on protein turnover in bullfrog saccula, we observed, contrary to results obtained from gene overexpression methods, a slow renewal of the stereocilia proteins. Such studies have the advantage of being performed in undisturbed adult animals in metabolic equilibrium [41].

We have also demonstrated that intracellular symbiotic bacteria of a shipworm fix nitrogen within the cells of a specialized symbiont-bearing organ after exposure of the

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Figure 17: $^{12}$C$^{15}$N image showing stereocilia from a bullfrog saccula sample. The field is 7 $\mu$m x 7 $\mu$m. (with D. Corey and D-S. Zhang, Harvard Medical School).
shipworms to $^{15}$N-equilibrated seawater [14].

Through experiments on free fatty acid transport in adipocytes we have demonstrated that FFA have been pumped into adipocytes against their electro-chemical potential by an energy dependent process [15].

Multi-isotope imaging mass spectrometry has moved well beyond a very interesting technique in its infancy to a mature and powerful method for biomedical research. It opens the field of “nanoautography” using the large pool of stable isotope tracers.

Acknowledgments

This work was supported in part by the NIH under Grant 5P41EB001974-05 and Grant 5RO1DC004179-03. The authors would like to express their thanks to the dedicated and thorough work of Jason Lebeau in flying amongst the data reduction software, and to Adam Cohen for winning the fight against the instrument.

References

[38] Additional ImageJ plug-ins for opening raw MIMS images and creating HSI images available at http://www.nrims.harvard.edu