Gold-ISH: A nano-size gold particle-based phylogenetic identification compatible with NanoSIMS

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Abstract

The linkage of microbial phylogenetic and metabolic analyses by combining ion imaging analysis with nano-scale secondary ion mass spectrometry (NanoSIMS) has become a powerful means of exploring the metabolic functions of environmental microorganisms. Phylogenetic identification using NanoSIMS typically involves probing by horseradish peroxidase-mediated deposition of halogenated fluorescent tyramides, which permits highly sensitive detection of specific microbial cells. However, the methods require permeabilization of target microbial cells and inactivation of endogenous peroxidase activity, and the use of halogens as the target atom is limited because of heavy background signals due to the presence of halogenated minerals in soil and sediment samples. Here, we present “Gold-ISH,” a non-halogen phylogenetic probing method in which oligonucleotide probes are directly labeled with Undecagold, an ultra-small gold nanoparticle. Undecagold-labeled probes were generated using a thiol-maleimide chemical coupling reaction and they were purified by polyacrylamide gel electrophoresis. The method was optimized with a mixture of axenic ¹³C-labeled *Escherichia coli* and *Methanococcus maripaludis* cells and applied to investigate sulfate-reducing bacteria in an anaerobic sludge sample. Clear gold-derived target signals were detected in microbial cells using NanoSIMS ion imaging. It was concluded that Gold-ISH can be a useful approach for metabolic studies of naturally occurring microbial ecosystems using NanoSIMS.

Keywords

Gold-ISH, NanoSIMS ion microprobe, Undecagold

Introduction

As most microbes remain non-cultivable, the ability to link microbial phylogeny and metabolic activity at the single-cell level would be a significant advantage for understanding the metabolic functions of environmental microorganisms. Isotopic labeling of microorganisms and subsequent phylogenetic identification using fluorescence *in situ* hybridization (FISH) of rRNA-targeted probes (i.e. microautoradiography (MAR)-FISH and secondary ion mass spectrometry (SIMS)-ISH) can directly link metabolic activity and phylogeny with single-cell resolution (see review, [38, 39]). The MAR-FISH technique, introduced in 1999, utilizes radioisotopic substrates to label metabolically active cells and has been applied to studies of many habitats in order to enhance understanding of microbial hydrogen, carbon, sulfur, and phosphate uptake [28, 31]. The use of a SIMS-based analytical approach was introduced approximately 10 years ago in environmental microbiology for matching the chemotaxonomic and phylogenetic signatures of microbes that metabolize methane under anoxic conditions [30]. Following the recent development of a NanoSIMS ion microprobe with a high spatial resolution of ~50 nm, isotope probing studies at a single-cell resolution have become more popular for exploring microbial carbon, nitrogen, and sulfur metabolism [7, 12, 23, 25, 26]. Methods for simultaneous isotopic measurement and phylogenetic identification of single microbial cells were reported in 2008 by three laboratories [4, 22, 26]. These methods used halogen elements because of their high ionization yields and relatively low biomass abundance [11]. SIMSISH, developed by Li et al. [22], uses oligonucleotide probes containing 5'-iodo-2'-deoxycytidine instead of 2'-deoxycytidine. However, each probe used in the SIMSISH approach can only label a limited number of halogen atoms and thus may not detect microorganisms with low ribosomal (r)RNA content. This is a widely recognized problem with FISH-based detection of environmental microbes [3]. More sensitive approaches, such as enhanced elemental labeling (EL)-FISH and halogen *in situ* hybridization (HISH)-SIMS, developed by Behrens et al. [4] and Musat et al. [26], respectively, use

horseradish peroxidase (HRP)-mediated tyramide signal amplification to deposit a large number of fluorophores containing fluorine (F) or bromine (Br) (e.g. 544Br, BODIPY TMR-X, and Oregon Green 488-X) atoms into cells. Successful application of these approaches has been reported [4, 15, 26] but is limited to when permeabilization of cells allows for penetration of HRP-labeled probes and inactivation of endogenous peroxidases is successfully achieved. However, it often still faces problems associated with cell permeabilization and endogenous peroxidase inactivation, especially when targeting archaeal cells [20]. In addition, the presence of halogenated minerals in soils or marine sediments can cause high background during halogen detection with SIMS.

Gold, which has a comparable ionization yield to halogens [40, 41], represents an alternative element for use in SIMS. As gold has a relatively low natural abundance in biomass [11], it produces a lower material-induced background signal. In addition, gold signals can be enhanced by inorganic deposition of the metal ions, a process known as gold enhancement or autometallography [6]. Gold nanoparticle applications were first developed for use in the field of histology and often involved colloidal gold particles (~15 nm) conjugated to IgG for immunological localization of target proteins [5]. In environmental microbiology research, Nanogold and Undecagold have been used for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) with hapten-labeled probes and gold-conjugated antibodies [10, 13, 19, 35]. Recently, a Nanogold-coupled antibody was used to detect methyl-CoM reductase using NanoSIMS [23], and it demonstrated that Nanogold was a useful material for probing individual cells. Nevertheless, the currently published methods for Nanogold or Undecagold labeling of microbial cells involve immunological reactions, which introduce additional permeabilization-related problems for detection. Also, the preparation of appropriate antibodies for immunological labeling of microbial cells is sometimes problematic.

Alternatively, gold particles can be directly labeled to oligonucleotides. Mirkin et al. demonstrated the modification of alkene thiol-functionalized oligonucleotides with 13-nm colloidal

gold particles for sequence-specific reversible aggregation, which was visualized by a change in the color of the solution [24]. Using much smaller particles, Alivisatos et al. described the conjugation of Nanogold particles (1.4-nm diameter) with oligonucleotides via a maleimido-thiol coupling reaction that was used as a candidate nanofabrication material [1]. Several years later, Dubertret et al. applied this conjugation technique to create a new type of molecular beacon by using surface-modified Nanogold as the fluorochrome quencher [9]. These pioneering studies were advanced further by de la Torre et al., who demonstrated the better hybridization properties of Nanogold-oligonucleotides compared with unmodified nucleotides [8].

In this study, a method termed “Gold-ISH” was developed for the phylogenetic identification of environmental microorganisms using NanoSIMS. In Gold-ISH, oligonucleotides were directly labeled with ultra-small gold particles via activated linker arms [1, 8, 9, 16]. The labeled oligonucleotides were then used for ISH followed by detection using NanoSIMS ion imaging analysis. Undecagold was selected for gold labeling of microorganisms instead of Nanogold for several reasons: 1) Undecagold appears to be more stable than Nanogold under the conditions used in ISH [14], and 2) the diameter of Undecagold particles is smaller than that of Nanogold particles (0.8 nm vs. 1.4 nm, respectively), and therefore Undecagold more readily penetrates the microbial cell wall during ISH. Gold-ISH combined with direct NanoSIMS imaging can complement previously published halogen-based ISH techniques, especially for halogen-rich environments, thus providing deeper insights into microbial metabolism in various environmental settings.

Materials and Methods

Sample preparation. *Escherichia coli* JM109 and *Methanococcus maripaludis* JCM13030 were cultivated in pure culture. *Escherichia coli* JM109 was incubated with ¹³C-labeled glucose (Chlorella Industry) and ¹⁵N-labeled ammonia (NLM-467, SI Science) as the sole carbon and

nitrogen sources, respectively. The cultures were fixed with paraformaldehyde (2% final concentration), and stored in ethanol/PBS (1:1) at -20°C . An environmental sample, represented by anaerobic granular sludge, was taken from a laboratory-scale upflow anaerobic sludge-blanked reactor used to treat artificial wastewater. The sludge sample was washed with phosphate buffer and dispensed into 50 mL of medium containing lactate and sulfate (30 mM and 20 mM, respectively) in 122-mL vials. The sludge concentration was adjusted to 26 g volatile suspended solids L^{-1} . Following pre-incubation, the sludge sample was washed with phosphate buffer and inoculated into fresh medium containing ^{13}C -labeled lactate ($^{13}\text{CH}_3\ ^{13}\text{CH}(\text{OH})\ ^{13}\text{COONa}$, CLM-1579; SI Science) and sulfate. Samples were then taken from the vial, fixed with paraformaldehyde (2% final concentration), and stored in ethanol/PBS at -20°C . The sludge samples were dispersed by ultrasonication (1 min twice on ice with an output power of 1, UH-50, SMT, Tokyo, Japan) prior to ISH.

Undecagold-labeled probes. Two specific probes targeting the 16S rRNA gene were used in this study: EUB338 (5'-GCT GCC TCC CGT AGG AGT-3') and SRB385 (5'-CGG CGT CGC TGC GTC AGG-3') [2]. Probes were synthesized and labeled with a thiol group at the 5' end and Cy3 at the 3' end. The probes were then mixed with mono-maleimide-functionalized Undecagold (Nanoprobes Inc.) for labeling at the 5' end via a thiol-maleimide or thiol-gold coupling reaction (Fig. S1). Labeled oligonucleotides were purified by polyacrylamide gel electrophoresis (PAGE). Synthesis and custom labeling were carried out by Tsukuba Oligo Service (Tsukuba, Japan).

***In situ* hybridization of Undecagold-labeled probes.** Appropriate amounts of each sample were mixed with 200 μL of 0.01% SDS solution. The samples were filtered using carbon-coated polycarbonate filters and then air-dried. The sludge samples were then treated with lysozyme (0.1 mg L^{-1} in 100 mM Tris-HCl, pH 8.0, and 50 mM EDTA, pH 8.0) for 20 min at room temperature.

After washing in TNX buffer (100 mM Tris-HCl, 150 mM NaCl, 0.05% Triton X-100) followed by ultra-pure water, the filters were covered with hybridization buffer (20 mM Tris-HCl, 0.9 M NaCl, 1% [w/v] blocking reagent, 0.01% SDS, and 0–40% formamide) containing 0.5 μ M probe. The formamide concentrations used for EUB338 and SRB385 were 10% and 20%, respectively. The pH of the hybridization buffer was adjusted to 7.2. Following incubation for 4 h at 40 °C, the filters were incubated in pre-warmed washing buffer (20 mM Tris-HCl, 0.9 M NaCl, 0.01% SDS, and 0–40% formamide) for 15 min at 42 °C [21]. The filters were briefly washed with ultra-pure water to remove the washing buffer and then air-dried. A piece of the filter was cut, mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes, Inc., Eugene, OR, USA) and examined using epifluorescence microscopy by observing the signals derived from the 3' Cy3 label. The remainder of the filter was used for NanoSIMS ion imaging analysis.

NanoSIMS ion imaging analyses of experimental samples. Experimental samples were analyzed by raster ion imaging in an AMETEK CAMECA NanoSIMS 50L ion microprobe at the JAMSTEC Kochi Institute for Core Sample Research, Japan. A focused primary Cs⁺ beam of ~0.8 pA for carbon isotopic analysis (hereafter referred to as setting 1) and 2–3 pA for Au abundance analysis (setting 2) was rastered on samples over 20 × 20 to 28 × 28 μ m² areas. Negative secondary ions of ¹²C (EM#2), ¹³C (EM#3), ¹²C¹⁴N (EM#4), and ³²S (EM#5) for setting 1 were measured using four electron multipliers (EMs) in multidetection mode at a mass resolving power of ~9,000 that is sufficient to separate all relevant isobaric interferences (i.e., ¹³C on ¹²C¹H). After the measurement of carbon isotopes in the sample, the magnetic field was changed for ¹²C¹⁴N and ¹⁹⁷Au abundance analysis (setting 2). Simultaneous measurement of carbon isotopes, ¹²C¹⁴N, ³²S, and ¹⁹⁷Au was impossible because of mechanical difficulties with the electron multiplier trolleys; therefore, ¹²C¹⁴N and Au abundance measurements were carried out followed by a separate carbon isotopic measurement. For Au abundance analysis (setting 2), negative secondary ions of ¹²C¹⁴N (EM#1)

and ^{197}Au (EM#7) were measured using two EMs in multidetection mode at a mass resolving power of $\sim 4,500$ in order to collect more secondary ions due to the low abundance of Au in the samples. Detailed settings, including target isotopes, trolley positions and B-field, are summarized in Table 1. Each run was initiated after stabilization of the secondary ion beam intensity following pre-sputtering for approximately 5–10 min with a strong primary ion beam current. Each imaging run repeatedly scanned (30–40 times) the same area, with individual images consisting of 256×256 pixels, depending on the measured area, and having a dwell time of 2,000–3,000 μs for setting 1 and 5,000–10,000 μs for setting 2. The total acquisition time ranged from 1–2 h for setting 1, and 2–7.5 h for setting 2. It should be noted that $^{12}\text{C}^{14}\text{N}$ cps and the image of the sample were monitored before and after Au abundance measurement, whether or not the sample was lost after measurement, with relatively strong primary ion beam irradiation. Since the environmental samples might show a more complicated mass spectrum of approximately mass 197, care was taken to ensure that there were no isobars for each measured isotope.

Standard *E. coli* samples were prepared in order to have different carbon isotopic ratios of 1 (natural), 2.0, and 10.9% enriched in ^{13}C relative to ^{12}C in order to evaluate instrumental mass fractionation for carbon isotopes, as well as for finding target mass peaks (^{12}C , ^{13}C , $^{12}\text{C}^{14}\text{N}$, and ^{32}S).

Recorded images and data were processed using either CAMECA WinImage software (AMETEK) or IDL-based NASA JSC imaging software for NanoSIMS [18]. Different scans of each image were aligned in order to correct image drift during acquisition. Final images were generated by adding the secondary ion counts of each recorded secondary ion from each pixel over all scans.

Results

Generation of Undecagold-labeled oligonucleotide probes. Modification of oligonucleotides with smaller nanoparticles is particularly advantageous for ISH-based approaches, in which

penetration of modified oligonucleotides into the cell is the critical step for successful staining. In this study, an attempt was made to use an even smaller nanoparticle, Undecagold (0.8 nm). Mono-maleimide-functionalized Undecagold was conjugated with thiol-linked oligonucleotides. PAGE analysis of the conjugation product showed multiple bands (Fig. S2, lane A), all of which were larger than the source oligonucleotides. Thiol residues are known to covalently bind to gold colloids through thiol adsorption chemistry [24]; therefore, it was presumed that the bands appearing on the gel corresponded to the Undecagold conjugates, with multiple oligonucleotides being formed through direct binding of thiol-oligonucleotides to the Undecagold particles. To maximize the number of gold particles for FISH staining, the band that was expected to be a single oligonucleotide labeled with Undecagold was excised (Fig. S2, lane B), the product purified, and it was used for ISH. Oligonucleotide probes were also labeled with Cy3 at the 3' end, allowing verification of specific hybridization signals by epifluorescence microscopy prior to NanoSIMS analysis. It should be noted that although we tried conjugation of mono-*Sulfo*-NHS-Undecagold with amino-linked oligonucleotides, it was not successful.

Identification of specific microorganisms using ISH with Undecagold-labeled probes and NanoSIMS ion imaging. There have been no reports of the use of probes directly labeled with Undecagold for ISH. The stability of Undecagold has been tested by other researchers under the following conditions: (a) high NaCl concentrations (at least 1 M) at near neutral pH, (b) 4-h incubation at up to 100 °C, either in PBS or 20 mM phosphate (pH 7.4) with 0.5 M NaCl, and (c) heating at 100 °C in the presence of 1.3% SDS for 5 min [14]. However, there is no available information on the stability of Undecagold-labeled probes in any concentration of formamide solution.

Based upon available information, control of hybridization stringency was first attempted by controlling the concentration of NaCl in the hybridization buffer; however, sufficient specificity

could not be obtained in a FISH trial using *E. coli* and *M. maripaludis* cells (data not shown). Thus, it was decided to use conventional hybridization conditions involving formamide. As discussed below, using this approach it was possible to detect gold signals by NanoSIMS analysis and it was found that formamide could be used for ISH of Undecagold-labeled oligonucleotide probes.

As a proof-of-concept experiment, pure cultures of ^{13}C -enriched *E. coli* and non-enriched *M. maripaludis* cells were mixed and then hybridized with the 5'-Undecagold and 3'-Cy3-labeled EUB338 probes. Specific fluorescent signals were first confirmed using fluorescence microscopy (Fig. 1A). A high background was observed under UV excitation (DAPI images) due to, most likely, a carbon-coated membrane. Subsequently, Undecagold-derived gold signals were detected in *E. coli* cells by ion imaging using NanoSIMS, and this result was confirmed by comparison to ^{13}C -enriched signals (Fig. 2A). As the absolute count number in NanoSIMS varies depending on the analytical conditions, the gold signals were compared by dividing the number of gold signal counts by the number of CN^- -ion counts. The average $^{197}\text{Au}^-/^{12}\text{C}^{14}\text{N}^-$ ratio in *E. coli* cells was $25.9 \pm 5.5 \times 10^{-5}$ ($n = 24$, 1σ). This ratio was significantly different from the average $^{197}\text{Au}^-/^{12}\text{C}^{14}\text{N}^-$ ratio in *M. maripaludis* cells ($2.4 \pm 0.4 \times 10^{-5}$, $n = 4$, 1σ ; Welch t test, $p < 0.01$), and thus, the signal-to-noise ratio for this experiment was 10.8. This was greater than that of standard FISH with the halogen-containing EUB338 probe in which three thymine bases were substituted with 5FI-dU [4]. This is because a single Undecagold-labeled oligonucleotide probe carries eleven gold atoms. However, it should be noted that the signal-to-noise ratio of 10.8 was equivalent to the 9.4 signal-to-noise ratio of fluorescent signals.

As an advanced application, Gold-ISH analysis of a microbial community was conducted in a granular sludge sample. In anaerobic wastewater treatment processes, sulfate reducers are the primary bacterial group that degrades lactate or other fatty acids in granular sludge. Granules from laboratory-scale sludge samples were incubated with ^{13}C -labeled lactate and sulfate to label lactate-utilizers under sulfate-reducing conditions. Following hybridization with the

Desulfovibrionales-targeting SRB385 probe, specific fluorescent signals were obtained from sphere-shaped cells (Fig. 1B). Using NanoSIMS analysis, Undecagold-derived gold signals were detected in a location identical to that of the ^{13}C -enriched region (Fig. 2B), indicating that *Desulfovibrionales* was the main lactate-utilizer in this environment. The average $^{197}\text{Au}/^{12}\text{C}^{14}\text{N}^-$ ratio in the detected cells was in the range $9.9\text{--}13.3 \times 10^{-5}$, which was still significantly higher than the noise signal observed in the *M. maripaludis* analysis (Welch *t* test, $p < 0.05$). The average background signal from other microbial components in the granule samples was $1.5 \pm 0.4 \times 10^{-5}$, which was sufficiently low to permit specific detection of the target (Welch *t* test, $p < 0.05$). The carbon isotopic ratio for the *Desulfovibrionales* cells was 0.17–0.23 (^{13}C relative to ^{12}C), which corresponded to an incorporation rate of 5.8–7.5 fg-C per cell per 4-h incubation period (34.8–45 fg-C per day) based on an assumption of approximately 40 fg-total C per cell [29]. The values indicated that the doubling time of the *Desulfovibrionales* cells under the experimental conditions employed was approximately 1 day. The doubling times of pure cultures of *Desulfovibrio* are reportedly 5–16 h when lactate and sulfate are provided [27]. Considering the differences in the surrounding environmental conditions between a pure culture and granular sludge, and differences in species diversity and doubling time, it can be said that the doubling time of *Desulfovibrionales* determined in our experiment was reasonably consistent with that determined in the pure culture study. This is useful information for validating the potential load of carbon compounds in wastewater, which is important for maintaining optimal operational conditions. In summary, these results indicated that Undecagold was stable under the parameters used in this study and can thus be used for ISH studies with NanoSIMS ion imaging in order to examine the metabolism of environmental microorganisms.

Discussion

This study demonstrated that an ultra-small nanoparticle, Undecagold, could be used to label

oligonucleotide probes for specific hybridization targeting rRNA within whole fixed cells. Although the signal-to-noise ratio of this technique was low compared with other halogen-based approaches using HRP-labeled probes (i.e. EL-FISH and HISH-SIMS) and two-step analysis is needed for NanoSIMS, the Gold-ISH technique described can complement previously published halogen-based ISH techniques [4, 22, 26] for NanoSIMS analysis, especially for the examination of marine sediments in which halogenated minerals interfere with specific detection. In addition, Gold-ISH has several advantages over previously published methods in terms of probe size. Undecagold particles have a small size of only 0.8 nm [14], which is almost equivalent to the size of commonly used fluorescent dye molecules and requires milder or no permeabilization. In contrast, the HRP molecules used in EL-FISH or HISH-SIMS are approximately 5–6 nm in diameter. As a result, HRP-labeled probes usually require permeabilization procedures prior to hybridization [21, 33, 34, 36]. In addition, the inactivation of endogenous enzymatic activity is a critical step for successful HRP-based ISH [17, 32, 33]. Therefore, many researchers still try to develop appropriate permeabilization and inactivation methods for samples of interest. Another advantage of Gold-ISH is that it introduces less carbon and nitrogen from foreign sources into the microbial cells than EL-FISH and HISH-SIMS, which deposit thousands of tyramide-dye conjugates into the target cells.

Our Gold-ISH approach showed sufficient signals for the detection of lactate-utilizing *Desulfovibrionales* in an anaerobic granular sludge sample. As described above, the signal-to-noise-ratio of Gold-ISH was higher than that of standard FISH with halogenated oligonucleotide probes, but it was much less than those of CARD-FISH-based approaches. Thus, it is expected that, unlike EL-FISH and HISH-SIMS, the current Gold-ISH could have difficulties to analyze microorganisms with low rRNA contents in oligotrophic environmental samples. Therefore, the method should be further improved to have higher sensitivity. An attractive feature of gold nanoparticles is autometallographic signal amplification (i.e. gold enhancement), which does not

rely on an enzymatic reaction. After autometallographic signal amplification, gold signals will be visible even by light microscopy [37] and the number of gold atoms should be sufficient for detection by the real-time imaging mode in NanoSIMS. Our preliminary trial using biotinylated probes and Nanogold-streptavidin [10, 19, 35] dramatically increased the gold abundance (more than 500-fold) within the cells but this method has a permeabilization problem and introduces a foreign source of additional carbon and nitrogen atoms associated with Nanogold-streptavidin, which can alter the isotopic composition of microbial cells. Autometallographic signal amplification with Undecagold-labeled probes can overcome these issues but it is still in the development stage because less signal amplification and higher background (low signal-to-noise ratio) have been noted. Gérard et al. [13] reported that silver enhancement of ultra-small gold (≤ 0.8 nm) for detection under SEM and TEM, indicating autometallographic signal amplification of Undecagold, is possible. One important reason for this low signal-to-noise ratio might be due to the non-specific binding of oligonucleotides to a supporting membrane. Non-specific binding and/or oligonucleotide probes remaining on the membrane are well-known issues in FISH staining. The use of other supporting materials (e.g. silicon wafers) [22] or performing the hybridization in a suspension could be considered for reducing background noise during gold enhancement and these attempts are ongoing as future development items. Although further methodological improvement is still necessary, staining microorganisms via Gold-ISH can help our understanding of microbial metabolism by facilitating linkage with microbial phylogenetic analyses of complex microbial communities.

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Table 1. Trolley settings for two-step analysis with a NanoSIMS ion microprobe.

		Setting 1	Setting 2
	B-field (G)	1228.5	2725.3
	Radius (mm)	Measured isotopes	Measured isotopes
Trolley 1	240.0		$^{12}\text{C}^{14}\text{N}$
Trolley 2	362.9	^{12}C	
Trolley 3	377.6	^{13}C	
Trolley 4	534.4	$^{12}\text{C}^{14}\text{N}$	
Trolley 5	592.8	^{32}S	
Trolley 6	N/A		
Trolley 7	663.6		^{197}Au

Figure legends

Fig. 1. Epifluorescence micrographic images collected after Gold-ISH. Each double panel depicts DAPI (left) and probe staining (right). A: a mixture of *E. coli* and *M. maripaludis* cells stained with Eub338. B: a sample of anaerobic granular sludge stained with SRB385. The scale bar represents 10 μm .

Fig. 2. (A) Gold-ISH/NanoSIMS ion images of a mixture of axenic ^{13}C -labeled *E. coli* and *M. maripaludis* cells hybridized with EUB338, and (B) an anaerobic sludge sample hybridized with SRB385. (a) $^{12}\text{C}^{14}\text{N}$ ion image; (b) ^{197}Au ion image after gold-ISH; (c) $^{13}\text{C}/^{12}\text{C}$ isotopic ratio image. The colored bars indicate the total ion counts for (a) and (b) and the isotopic ratio of ^{13}C relative to ^{12}C (the terrestrial ratio of $^{13}\text{C}/^{12}\text{C}$ is 0.0112372) for (c). The scale bars represent 5 μm (A) and 3 μm (B). The $^{12}\text{C}^{14}\text{N}/^{197}\text{Au}$ ratio is not shown since the region without cells had a large noise ratio because both the $^{12}\text{C}^{14}\text{N}$ and ^{197}Au counts were low.

Figure 1

Figure 2