Winter 2015

Application of Lipid Styryl Dye for Staining Intracytoplasmic Membranes in Gram-Negative Bacteria

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Honors Research Project

Submitted to

The Honors College

Abstract:

Intracytoplasmic membranes are structures that form within cells which help facilitate a variety of different metabolic processes. This feature of intracellular membranes makes them particularly valuable for studying compartmentalization and cell dynamics in bacteria. In the past, transmission electron microscopy has been the primary method for imaging bacteria with intracytoplasmic membranes. Because transmission electron microscopy takes images of a cell in fixed slices, it's impossible to follow a cell's growth and development over time. Fluorescence microscopy is a particularly effective method of measurement that can combat these issues when evaluating live bacterial cells. Here, standard biochemical laboratory procedures were used for the growth and development of two methanotrophs, *Methylomicrobium alcaliphilum* 20Z and *Methylosinus trichosporium* OB3b, and a photosynthetic purple non-sulfur bacterium, *Rhodobacter sphaeroides* 2.4.1. Fluorescence microscopy was used to monitor and evaluate these cells, and subsequently quantify the data obtained. The data obtained from this research project helped develop a rapid assessment method for intracellular membrane identification. It also helped clarify both how and why these intracellular membranes form.

Acknowledgements:

I would like to thank the Department of Chemistry and the University of Akron for allowing me to pursue my degree in chemistry. I would also like to thank my readers, Dr. Aliaksei Boika, Dr. Leah Shriver, and Dr. Adam Smith for their comments and suggestions in the writing process of this report. One of our graduate students, Kyle Whiddon, has also been extremely helpful in assisting me with my research and how to use the various laboratory equipment.

I would especially like to thank Dr. Michael Konopka for allowing me to join his group and take part in interesting work, alongside great people. I would also like to thank him for his guidance in the field of chemistry and the opportunities that lie ahead of me. My time as a chemistry major has been much easier because of his help.

A special thanks to my parents, siblings, and close friends is also deserved for their continuous support and help through my time as an undergraduate student. Without their help, attaining my degree would have been a much more difficult process.

1. Introduction:

Intracellular membranes (ICM) are structures that facilitate a number of different metabolic pathways. These functions can range from nitrogen fixation[1] and methanotrophy[2] to photosynthesis.[3] They have even been implicated in cell growth and division.[4] This is made possible through the inclusion of membrane bound enzymes in bacteria. These ICM are primarily derived from the inner membranes of bacteria and can exist as invaginations, stand-alone vesicles, or lamella in the cytoplasm.[1-3] Different organisms have distinct ICM features; *Methylomicrobium alcaliphilum* 20Z (*M. alcaliphilum* 20Z) for example, have stacked, disc-like ICM structures whereas *Rhodobacter sphaeroides* has vesicular ICM structures.[2,3]

In the past, transmission electron microscopy (TEM) has been the primary analytical technique for identifying ICM in bacteria. Through the utilization of TEM imaging in conjunction with cell-lysis techniques, valuable information about the structural and functional aspects of ICMs has been obtained.[5] TEM offers excellent, detailed images of bacterial cells (Figure 1), but this method is incapable of following cell dynamics in real-time.[5,6] Fluorescence microscopy, and the use of lipid styryl dyes, is a novel method that is being used to evaluate bacteria in real-time.[7] While TEM imaging requires cell fixation, fluorescent methods have the capability of studying molecular interactions in living and growing bacterium.

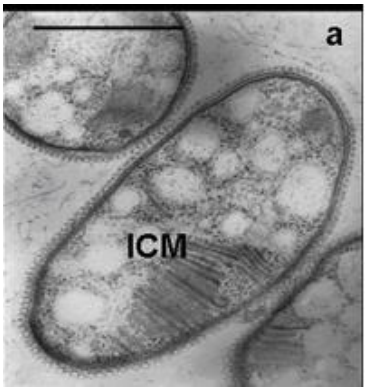
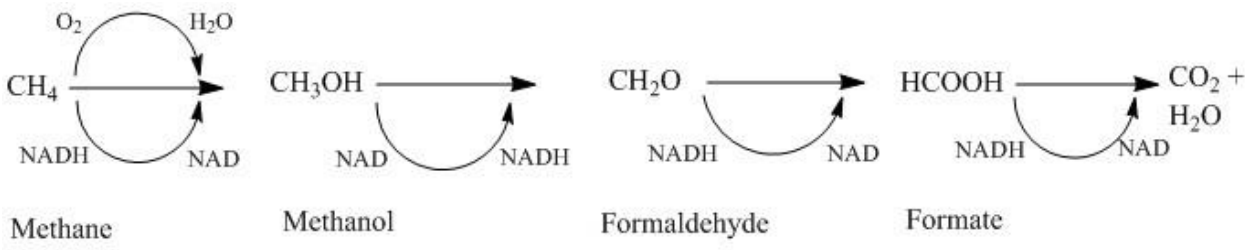


Figure 1: TEM image of an ICM containing *Methylomicrobium alcaliphilum 20Z.* Taken from reference 8.

Methylotrophs are a unique type of Gram-negative bacteria which utilize single-carbon compounds such as methane, methanol, methylated amines, or even halogenated amines as their sole source of carbon and energy.[9,10] Methanotrophic bacteria are found in a number of different aquatic and earthbound ecosystems, as well as extreme environments with wide pH ranges and intense thermal conditions such as hot springs.[10,15] Because of their resilient nature, these bacteria have generated tremendous amounts of interest due to the growing concerns of global warming and climate change. Methane is emitted into the environment through a number of natural sources as well as man-made sources such as fossil fuel production, agriculture, and landfills.[11] Since methanotrophs naturally consume methane, a greenhouse gas that is 20 times more potent than CO_2, these species could play a valuable role in methane mitigation.[12] They even have the potential for various new applications such as waste water treatment and the bioremediation of chlorinated pollutants.[13,16] However, a better understanding of these cells is still required before significant advancements can be made.

The growth of methanotrophs not only depend on carbon sources, but also a number of different micronutrients such as copper. All aerobic methanotrophs use the enzyme, methane monooxygenase (MMO) to covert methane to methanol through the process of oxidation

(Scheme 1).[4,9] However, there are two types of MMO – soluble methane monooxygenase (sMMO) which is found in the cytoplasm of the cell, and particulate methane monoxygenase (pMMO) which is membrane bound. Both forms of MMO can be found in some methanotrophs such as *Methylosinus trichosporium* OB3b whereas others such as *M. alcaliphilum* 20Z only encode for pMMO.[10] Bacteria that encode for MMO provide the opportunity to elucidate of the relationship between the presence of copper, methane oxidation, and ICM formation.[4,5,16]



Scheme 1: Methane oxidation cycle used by methanotrophic bacteria.

Photosynthetic bacteria such as the purple non-sulfur bacterium, *Rhodobacter \sphaeroides* 2.4.1 are a type of bacteria which depend on light energy for ICM development and respiration.[3,14] These species use bacteriochlorophyll protein complexes, integrated in their ICM, as a way to collect solar energy and initiate the electron-transfer process of photosynthesis.[3,17] However, the intensity of the light is extremely important to the growth and development of these bacteria. Low levels of light requires highly efficient photosynthetic membranes whereas high levels of light can damage the cells.[3]

Purple bacteria fall under two categories: purple non-sulfur bacteria and purple sulfur bacteria. Purple non-sulfur bacteria utilize an organic electron-donor such as succinate, malate, or elemental hydrogen whereas purple sulfur bacteria take advantage of elemental sulfur or sulfide as their electron-donor for the process of respiration.[14,18] Photosynthesis is a redox

reaction process which harvests light energy to generate complex organic molecules from simpler ones such as CO_2 in plants, or sulfur in purple sulfur bacteria.[14] Purple bacteria contain three types of molecular complexes which are involved in photosynthesis: antennae or light-harvesting complexes, reaction centers, and cytochrome complexes.[18] Each of these is very important in the bacterium's ability to utilize light energy. These cellular components are housed in the ICMs of these species. Because of this, there is a direct correlation between the presence of light and the subsequent formation of ICMs (Figure 2). Without these ICMs, these bacteria are incapable of harvesting and utilizing light energy for respiration.

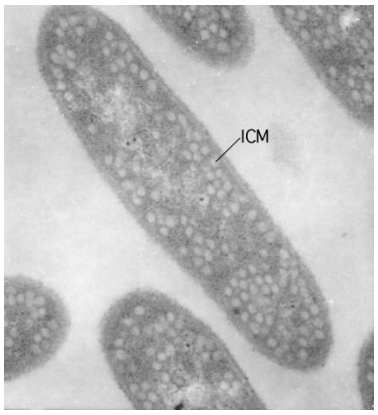


Figure 2: TEM image of *R. Sphaeroides* bacterium with labeled ICM structures. Take from reference 19.

Here, I focused on the growth and imaging of two methanotrophic species, *M. alcaliphilum* 20Z and *M. trichosporium* OB3b, as well as a purple non-sulfur bacterium, *R. Sphaeroides* 2.4.1, using a Nikon A1+ confocal fluorescence microscope. A rapid method of ICM assessment was used to label the bacteria and identify regions of interest. This method was utilized on a couple of different bacteria types that form ICMs. Following the successful imaging of the bacteria, the Nikon NIS elements software package was used to analyze and subsequently quantify the data in Microsoft Excel (Figure 3). This approach was taken to develop a widely applicable, rapid analysis method to ultimately gain a better understanding of cell dynamics.

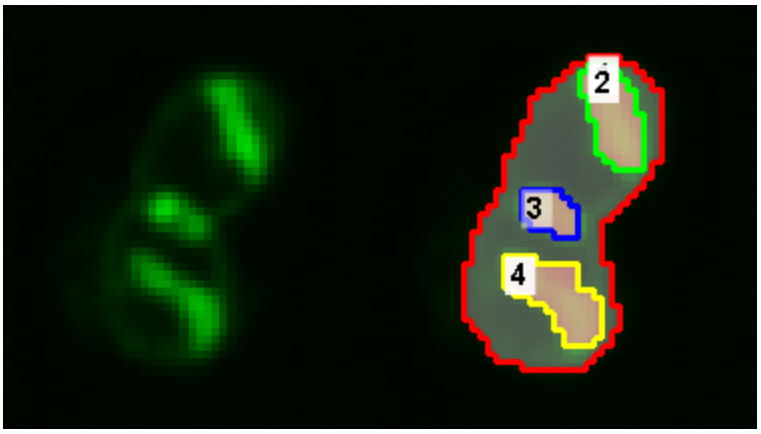


Figure 3: Fluorescent imaging of a *M. alcaliphilum* 20Z cel prior to, and after evaluating it with the NIS elements software. A threshold measurement is first taken to label the entire cell area and labeled as a region of interest. A second threshold measurement is then taken to label the ICM structures as regions of interest. Excel is subsequently used to quantify the % ICM coverage.

2. Experimental Methods:

2.0 General chemical information

All chemicals were used as received and of molecular biology grade – Sigma Aldrich.

2.1 Methanotrophic growth conditions (nitrate mineral salt media)

Both *M. alcaliphilum* 20Z and *M. trichosporium* OB3b were initially grown aerobically at 30^{o}C in nitrate mineral salt media, 3% salinity (NMS-3); 1.0 KNO_3, 1.0 $MgSO_4$ x $7H_2O$, 0.02 $CaCl_2$, 30.0 NaCl (g/L of nanopure water). The resultant media was autoclaved and 20 mL of phosphate buffer, pH 6.8 (2.72 g KH_2PO_4 and 5.36 g Na_2HPO_4 per 500 mL of nanopure water), 50 mL of carbonate buffer, pH 9.5 (350 mL of 1 M $NaHCO_3$ and 150 mL of 1 M Na_2CO_3), and 2 mL of trace element solution per 1 L of media were added. Methanol (0.5%) was the carbon source used for growth.

2.2 Methanotrophic growth conditions (modified nitrate mineral salt media – P-media)

Both *M. alcaliphilum* 20Z and *M. trichosporium* OB3B were grown aerobically at 30^{o}C in a modified nitrate mineral salt media, 0.0%, 0.75%, and 3.0% salinity (P-0 media, P-0.75, media and P-3.0 media, respectively); 1.0 KNO_3, 0.2 $MgSO_4$ x $7H_2O$, 0.02 $CaCl_2$, 0.0 NaCl (P-0), 7.5 NaCl (P-0.75), or 30 (P-3.0) (g/L of nanopure water). The resultant media was autoclaved and 20 mL of phosphate buffer, pH 6.8 (5.44 g KH_2PO_4 and 5.68 g Na_2HPO_4 per 1 L of nanopure water), 40 mL of carbonate buffer, pH8.6-9.0 (75.6 g $NaHCO_3$ and 10.5 g Na_2CO3 per 1 L of nanopure water), and 1 mL of trace element solution per 1 L of media was added. A carbon source, either methane (100 mL) or methanol (0.2%) was used for growth.

2.3 Trace element solutions (NMS-3, and P-medium)

Trace element solutions were prepared for both the NMS-3 and P-mediums with slight variations. For NMS-3: To ~300 mL of nanopure water, 0.50 g Na_2EDTA, 1.0 g $FeSO_4$ x $7H_2O$, 0.40 g $ZnSO_4$ x $7H_2O$, 0.015 g $MnCl_2$ x $4H_2O$, 0.10 g $CaCl_2$ x $6H_2O$, 0.30 g $CuSO_4$ x $5H_2O$, 0.010 g $NiCl_2$ x $6H_2O$, 0.025 g Na_2MoO_4 x $2H_2O$, and 0.015 g H_3BO_3 was added and dissolved, then diluted to 500 mL before autoclaving and set aside in a 4^{o}C refrigerator. For P-medium: To ~300 mL of nanopure water, 2.50 g Na_2EDTA, 1.0 g $FeSO_4$ x $7H_2O$, 0.15 g $ZnSO_4$ x $7H_2O$, 0.015 g $MnCl_2$ x $4H_2O$, 0.10 g $CaCl_2$ x $6H_2O$, 0.60 g $CuSO_4$ x $5H_2O$, 0.15 g Na_2O_4W x $2H_2O$, 0.025 g $NiCl_2$ x $6H_2O$, 0.025 g Na_2MoO_4 x $2H_2O$, and 0.015 g H_3BO_3 was added and dissolved, then diluted to 500 mL before autoclaving and set aside in a 4^{o}C refrigerator.

2.4 R. Sphaeroides 2.4.1 *growth conditions*

R. Sphaeroides 2.4.1 were grown up under two different conditions and two separate mediums – Luria broth (LB), and minimal media. LB growth: To 1 L of nanopure water, 10 g of

select peptone 140, 5 g of select yeast extract, and 5 g of NaCl was added. The solution was autoclaved and cells were cultured in the dark and grown aerobically, without light at 37^{o}C. Minimal media: To 1 L of nanopure water, 1.2 g of NH_4Cl, 0.2 g of $MgSO_4$ x $7H_2O$, 0.07 g of $CaCl_2$ x $2H_2O$, and 10 mL of trace element solution was added. The resultant media was autoclaved and to the media, 1.5 mL of vitamin solution, 15 mL of 1 M phosphate buffer (pH – 6.7), and 10 mL of 1 M succinate was added. Following the addition of the remaining solutions, the media was purged with N_2 for 1 hour to make anaerobic and the resultant culture was grown in light, anaerobically at room temperature.

2.5 Cell quantification of a liquid culture

Optical density (OD_{600}) measurements were used to evaluate the extent of cell growth within a culture. Using an Implen P330 Nanophotometer, absorbance spectra were taken using an OD_{600} measurement. Measurements of the same cultures were carried out over a period of time. From the values obtained, doubling-times were calculated for log-phase growth of *M. alcaliphilum* 20Z, grown in NMS-3 medium, 0.5% methanol, and copper (equations 1 and 2).

1. $G = t/n$
2. $G = t/3.3\log(b_f/b_o)$

From the equation above, t is the elapsed time from the initial measurement and n is the number of generations which can be derived to $3.3\log(b_f/b_o)$ where b_o and b_f are the initial and final OD_{600} measurements respectively, and G is the calculated doubling time.

2.6 General culture preparation and plating

Cultures of *M. alcaliphilum* 20Z, *M. trichosporium* OB3b, and *R. sphaeroides* 2.4.1 were prepared in their respective medium under desirable growth conditions. From freezer stocks, cultures were first streaked out onto agar plates and then sub-cultured into test tubes and flasks with the use of inoculating loops. Samples were collected from the culture when they had reached an optimal imaging range (0.2-0.8 OD_{600}) and were then labeled with FM 1-43. The set of cells (500 μL) was added to an epi tube along with the dye (10 μL) and agitated. The sample was allowed to sit for 10-15 minutes to completely label the contents. From the sample, 10 μL of stained cells were placed onto a plate and covered with a cover slide soaked in polylysine (0.1%). The FM 1-43 dye was typically used because the image quality was often brighter and clearer (a vibrant, lime-green fluorescence) than that of FM 4-64 dye, another common fluorescent dye (pale purple fluorescence). Caution was taken with the stained cells to prevent exposure to excessive amounts of light. This could lead to photo-bleaching.

2.7 Microscope imaging and quantification of ICM development

A Nikon A1+ confocal microscope was used for fluorescent imaging. The fluorescent compound, FM 1-43, was used throughout experimentation for labeling. The dye has a characteristic excitation wavelength and emission wavelength of 479nm and 598nm respectively. Cells were cultured and grown up in the desired conditions. The cells were evaluated using the fluorescein isothiocyanate (FITC) setting. Images were optimized to reduce signal-to-noise ratio by adjusting the light (*hv*), cell size, and pinhole.

Using the Nikon elements software program, data analysis was performed on the images taken. A minimum fluorescence threshold was first obtained, covering the entire area of the cell and marked as a region of interest; this area was identified as the cell in whole. A second

threshold measurement was obtained for the ICMs present inside of the cell's outer membrane and marked as a region(s) of interest. After exporting the raw data into excel, the ICM area(s) was compared against the area of the entire cell and multiplied by 100 to obtain a % area of ICM coverage. Bins of 5% windows were created and compared against the frequency (the total number of cells containing % ICM coverage that fell within these regions).

2.8 TEM cell fixation

The specimen was placed in a 1.5% glutaraldehyde/media (P-.75 and P-0 for methanotrophs; LB and minimal media for purple non-sulfur bacteria) solution to preserve the cells for about a half hour. The sample was washed three times with the buffered media at 10 minute intervals to remove the glutaraldehyde. The cells were placed into a vial along with 2% osmium tetraoxide (OsO_4) and buffered media for 1 hour to stain the electrons in the cells. The OsO_4 and buffered media were removed with 3 washes of distilled water at 10 minute intervals. Caution was taken when handling OsO_4. Staining was done with uranyl acetate for approximately 30 minutes in the dark. The specimen was dehydrated with acetone using an acetone dessicator. This was done overnight and stored over molecular sieves to remove the water and replace it with acetone. A few drops of 100% acetone were added to see if there was swirling. The old acetone was replaced with new 100% acetone 3 times. The sample was infiltrated with a 9:1 acetone-epoxy plastic mixture. The vial was left in the hood overnight to evaporate off the acetone and the process was repeated a second time. The cells were placed into a mold and covered with plastic. The mold was then placed into a $60^{o}C$ oven for 48 hours to harden the plastic.

3. Results and Discussion:

3.1 NMS-3, plus-copper M. alcaliphilum 20Z *growth rate*

The growth rate of *M. alcaliphilum* 20Z was determined through the analysis of six different cultures. Measurements were obtained over the course of about two days until the cultures reached stationary phase; log phase was reached in approximately 24 hours, where the doubling rate (the time required for the cell culture to double) was determined. The results obtained were comparable to literature values (see table 1 for the doubling time of cultures 1-6).[15]

M. alcaliphilum 20Z sample	Doubling rate (hours)
Culture 1	7.94
Culture 2	7.84
Culture 3	7.92
Culture 4	7.78
Culture 5	9.29
Culture 6	9.02
Average	8.30

Table 1: Displays the 6 cultures and their growth rates for 20Z in NMS-3 media.

Once log phase was reached, OD_{600} measurements were taken every two to three hours over the course of the day. Upon evaluating two data points from the readings taken, a doubling rate of ~7.5-9.0 hours was consistently observed (data points for culture 1).

Time surpassed (hours)	OD_{600} measurement
0	0.035
20	0.191
22	0.228
24	0.275
26	0.329
45	0.927
48.5	0.862

Table 2: Displays the time surpassed and the corresponding OD_{600} measurement of culture 1.

3.2 NMS-3, plus-copper M. alcaliphilum 20Z *ICM analysis*

Batch cultures of *M. alcaliphilum* 20Z grown in NMS-3 medium, 0.5% methanol, and plus-copper conditions were first analyzed at mid log phase (~0.2-0.5 OD_{600}). These cells grown in plus-copper conditions showed clear evidence of ICM formation through the presence of fluorescent regions located within the cell's inner membrane (Figure 4). While it is unclear as to what the exact mechanistic role copper plays in the formation of these physiological structures, it has been consistently observed that there is a direct correlation between the presence of copper and the formation of ICMs in methanotrophic bacteria. This too, was observed for *M. alcaliphilum* 20Z in plus-copper growth conditions. As expected, those cells grown in minus-copper conditions lacked the formation of ICMs.

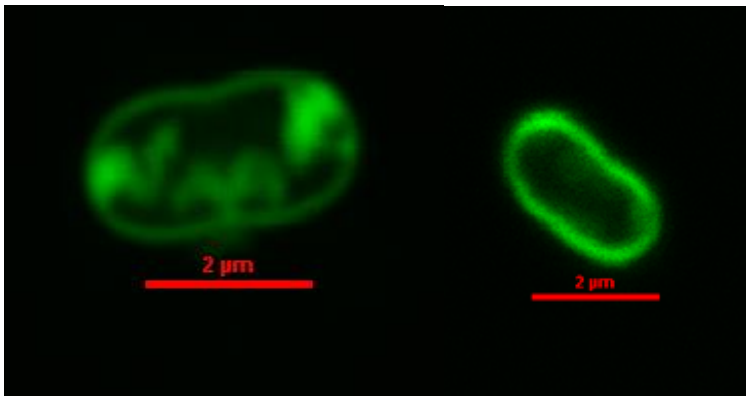


Figure 4: Fluorescent imaging of two *M. alcaliphilum* 20Z cells. The image on the left was grown in the presence of copper with clear signs of ICM formation. The image on the right was grown in the absence of copper and does not contain ICMs.

The percent of ICM coverage in NMS-3 grown *M. alcaliphilum* 20Z varied, with the majority of the cells having the most ICM area coverage between 30-45% (Figure 5). However, in all of the NMS-3 samples analyzed, there were always a small percentage of cells that did not contain ICM structures. On some occasions, cultures would contain more cells without ICMs than others but on average, each culture would contain around 15% without ICMs. It was unclear as to what the exact reasoning was behind this. One theory is that the high salt content of the NMS-3 (where the 3 stands for 3% NaCl) stressed the cells during growth and hindered the formation of ICM structures. This same theory could also explain why NMS-3 grown *M. alcaliphilum* 20Z had a much slower doubling time than the P-media; the P-media is essentially the same as NMS-3 with the exception of a few additional microelements and an altered amount of phosphate buffer added. The other theory behind this phenomenon is that the NMS-3 grown *M. alcaliphilum* 20Z were imaged outside their optimal OD_{600} range. When cells reach stationary phase, a phase of limited growth and development, space is limited and resources are low. The formation of ICMs at this stage could be counterproductive for the survival of the bacteria. In

this phase, the culture was also not efficiently stained due to the large amounts of cells present. This made it difficult to decipher between the presence or absence of ICMs.

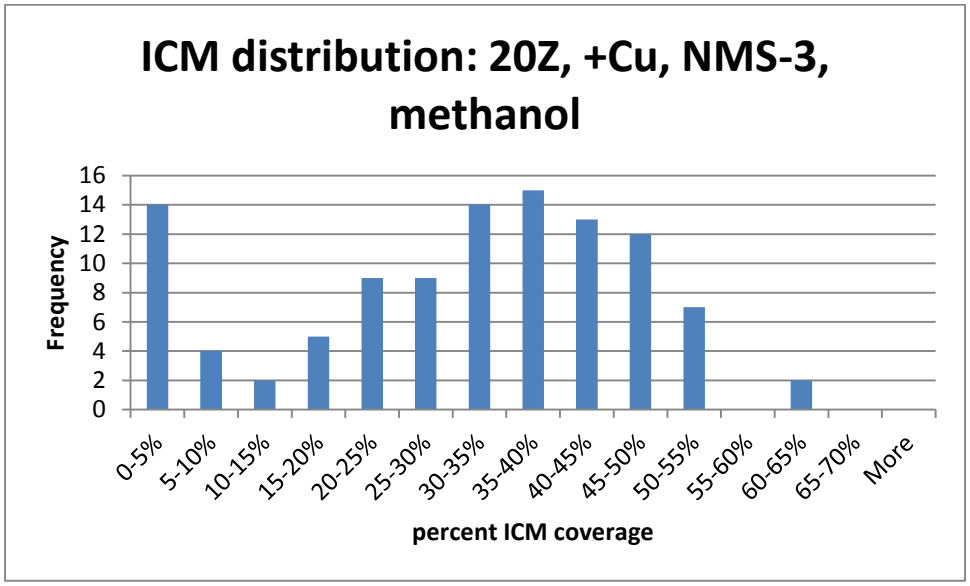


Figure 5: The percent ICM distribution and their corresponding frequencies (times observed during imaging) for NMS-3 grown, plus-copper *M. alcaliphilum* 20Z at 30°C.

3.3 P-.75 media, plus-copper M. alcaliphilum 20Z growth and ICM analysis

Batch cultures of plus-copper *M. alcaliphilum* 20Z was grown in P-.75 media with both methane and methanol carbon sources and imaged at mid-log phase (0.3-0.8 OD_{600}; P-medium 20Z grew much quicker and to higher OD_{600} measurements than in NMS-3 medium). While doubling rates weren't determined for 20Z grown in P-.75 medium, cultures did grow at accelerated rates and to higher OD_{600} levels than their NMS-3 medium counterparts. A 1/50 dilution subculture was necessary to reach log phase in one day for imaging with NMS-3 medium whereas a 1/250 dilution subculture was necessary to reach log phase in one day with P-.75 medium. Furthermore, cell death in NMS-3 grown 20Z was observed when the culture had reached an OD_{600} level somewhere around 1.2-1.3. In P-.75 medium, stationary phase often

exceeded the UV instrument's measurement window. As a result, this yielded a wider optimum imaging range (0.3-0.8 OD_{600}) – imaging done at cells past this range would typically result in dull fluorescence because of the vast amount of cells present in the sample.

Different carbon sources (methane or methanol) were used with the 20Z to see if there were any variations in ICM formation. The alteration in carbon source was evaluated for the purpose of long-term cell dynamic studies. A flow through system was purchased that was compatible with the Nikon fluorescence microscope. This system would be used to monitor the growth of bacteria from lag phase, up through log phase, and in to stationary phase. Methane would prove as a much more viable source than methanol, because of its gaseous phase at room temperature.

However, while the growth rate did appear quicker for those grown in methane (100 mL) versus those grown in methanol (0.2%), the % ICM coverage within the cells were not different (Figures 6 and 7). This was expected because the formation of ICM, as identified through TEM imaging, was found to be copper dependent. Because both methane and methanol are part of the methane oxidation cycle, it was determined that either source would be efficient in growing and developing the methanotrophic bacteria. This was particularly valuable because it suggested that the same ICM results would be obtained using either methane or methanol as the carbon source; some methanotrophic bacteria favor growth in one source over the other, which could prove problematic if methane was used in the flow through system.

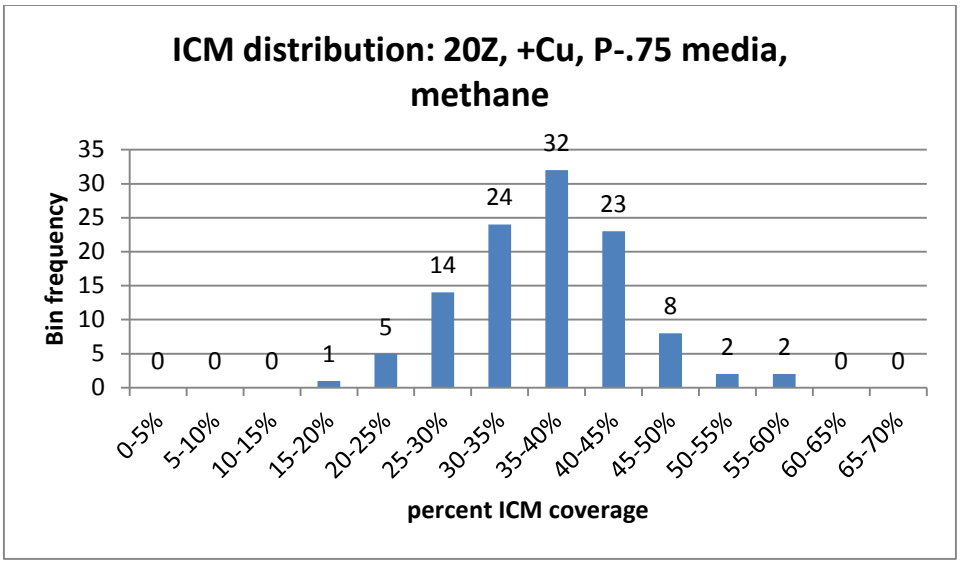


Figure 6: The percent ICM distribution for plus-copper 20Z grown in P-.75 media with methane as the carbon source.

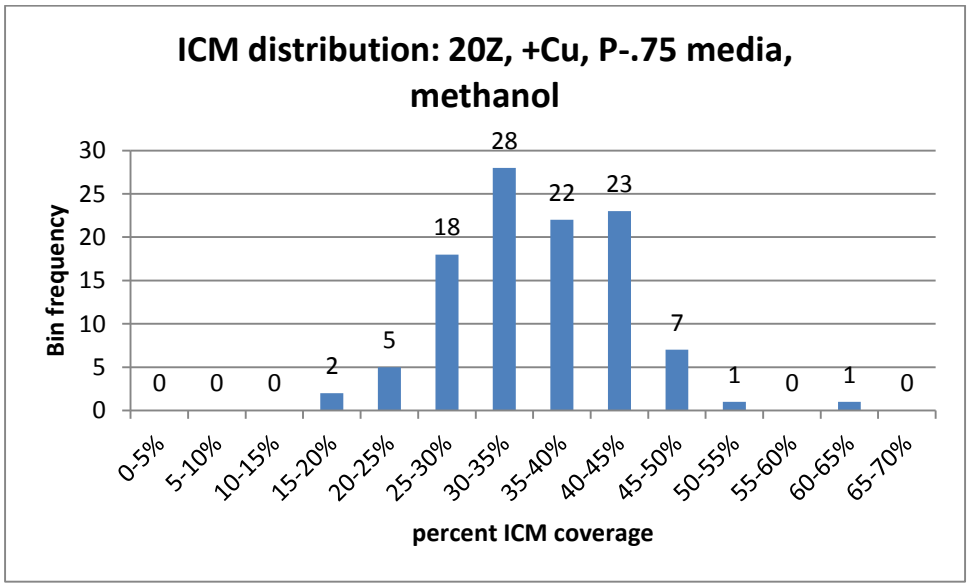


Figure 7: The percent ICM distribution of plus-copper *M. alcaliphilum* 20Z grown in P-.75 media with methanol as the carbon source.

3.4 P-.75 media, plus-copper M. alcaliphilum 20Z growth in dye and ICM analysis

Batch cultures of plus-copper *M. alcaliphilum* 20Z were grown with dye in P-.75 media and methane as the carbon source before being imaged at mid log phase (0.3-0.8 OD_{600}). This approach was taken to validate that the typical method of staining the cells immediately prior to imaging was just as efficient as growing them within dye. Further, this method was used to ensure that all of the ICMs were being labeled with the method of cell staining prior to imaging and analysis. It was observed that the cells grown in the presence of dye yielded identical percent ICM distributions as those which were stained in log phase, prior to microscopic evaluation (Figure 8). Both methods used, whether it was staining the cells directly before imaging or growing the cells up in dye, were efficient routes for ICM labeling. This reinforced that the development of a rapid method of assessing ICM formation in single bacterial cells was achieved.

However, it was noted that the presence of dye was particularly unfavorable to the growth of 20Z. Several different concentrations of dye were attempted – 500 μL (the concentration of dye used to stain the bacteria immediately prior to imaging), 125 μL, 75 μL, 30 μL (the concentration used for successful imaging), and 10 μL. In some instances, not enough dye was present (10 μL) and the cells did not fluoresce enough to obtain useable images; in other cases, too much dye was present and the cells did not grow and divide (500 μL and 125 μL). Even when the desirable amount of dye was determined (30μL), the overall cell size was slightly smaller than normal and growth rates were typically slower.

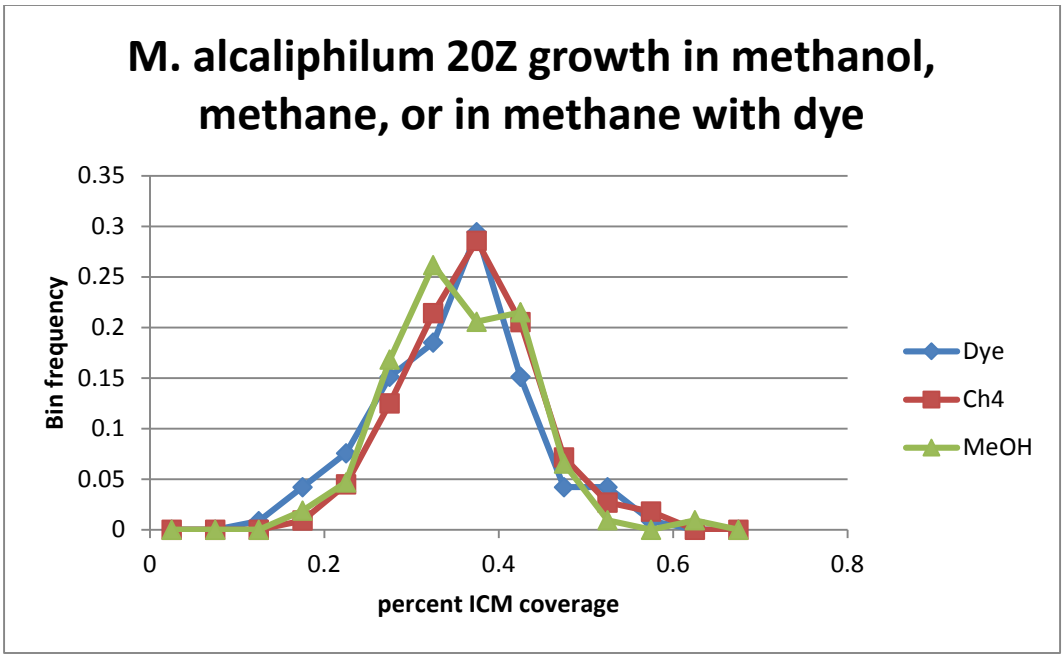


Figure 8: The percent ICM distribution for *M. alcaliphilum* 20Z grown in P-.75 medium with dye (and methane), and without dye (with methane and with methanol) in the presence of copper at 30°C.

As seen in the figure above, the ICM distribution for each of the three growth methods is nearly identical. For the methane grown, methanol grown, and growth in dye sets, a total of 112, 107, and 120 images were obtained and analyzed respectively. The cells typically contained between 30-45% ICM coverage, with the majority of the cells falling into the 35-40% bin. While there is a slight variance observed in the methanol grown *M. alcaliphilum* 20Z, statistical analysis using ANOVA methods in Microsoft Excel showed that this variation was not statistically different compared to that of the methane grown 20Z or the 20Z grown in dye.

3.5 R. sphaeroides 2.4.1 *growth and analysis in LB medium*

Batch cultures of *R. sphaeroides* 2.4.1 were grown in LB medium and dark, aerobic conditions and imaged at mid-log phase (0.2-0.5 OD_{600}). These growth conditions were used to develop *R. sphaeroides* 2.4.1 without ICMs. These purple, non-sulfur bacteria need the presence

of light as a source of energy for the electron-transfer process of the photosynthesis. With the presence of light, ICMs in the form of vesicles would be observed within the cells inner membrane. By growing these cells in a dark environment, it voided the cells capability of forming ICM (Figure 9).

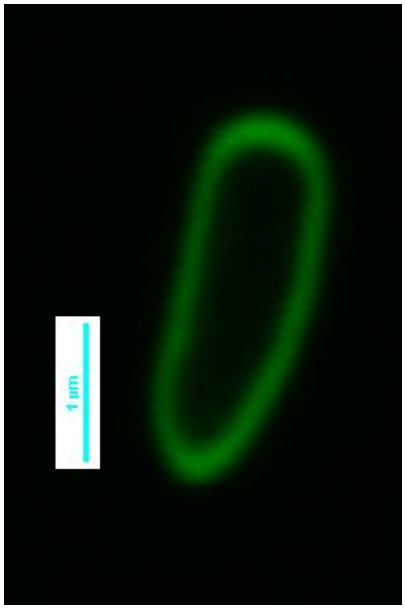


Figure 9: Fluorescent image of an *R. sphaeroides* 2.4.1 cell without ICMs grown in LB medium and dark, aerobic conditions.

After analysis of the *R. sphaeroides* 2.4.1 data, nearly all of the cells did not have ICMs (Figure 10). The majority of cells that did contain some ICMs were from the first culture analyzed. This particular culture was prepared in the dark, and wrapped in aluminum foil. A crimp flask was used and the aluminum foil was applied after the cells were added as an effort to not disturb the bacteria. While efforts were taken to avoid the presence of light, it's possible that these cells could have been subjected to even minimal amounts of energy. Because these bacteria scavenge for light sources, even the smallest amount of exposure could initiate the growth of ICM to harvest energy. All other *R. sphaeroides* 2.4.1 cultures were prepared in the dark and

placed into the 37°C incubator with a tin foil covered window; this prevented all light from entering while the door was shut. As a result, almost every cell analyzed from these cultures did not show any ICM formation.

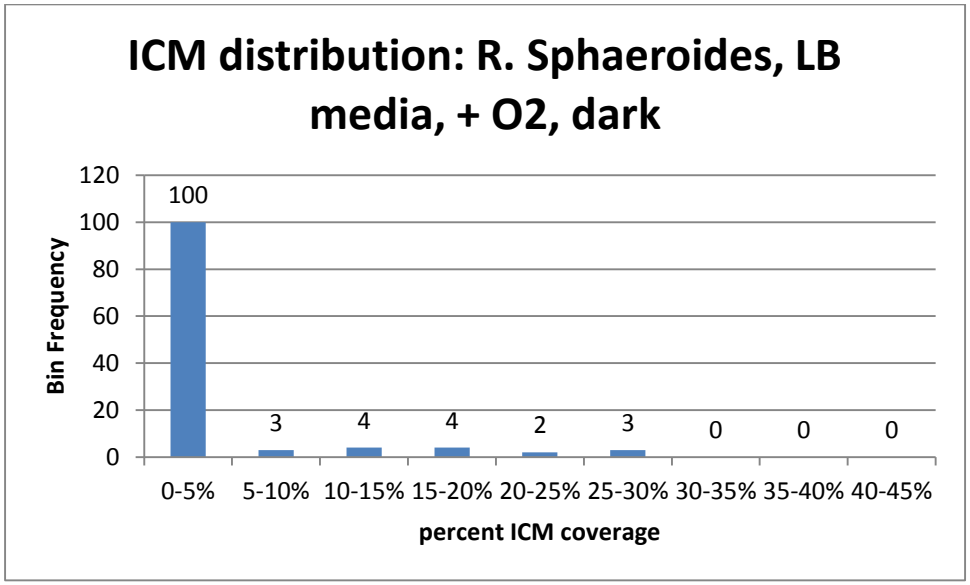


Figure 10: The percent ICM distribution for *R. sphaeroides* 2.4.1 grown in LB media and dark, aerobic conditions at 37°C.

One issue with imaging the *R. sphaeroides* 2.4.1 and subsequently analyzing the data is the fact that these particular cells tend to grow near and stick to one another. It was typically easy to distinguish between the membranes but in some cases, it was not. Another issue with some of these cultures was that the cells would stick to both the polylysine coated slide as well as the polylysine cover slip. As a result, cells would be present at two different imaging ranges and sometimes it appeared as though they would overlap, owing to brighter areas of fluorescence. This was problematic because these could have potentially been mistaken for ICM when they were actually two separate cells. To minimize this potential source of error, adjustments were

made by using a cover slip that was not coated in polylysine or imaging was simply performed on the cells without these overlapping regions.

3.6 R. sphaeroides 2.4.1 *growth and analysis in minimal media*

Batch cultures of *R. sphaeroides* 2.4.1 were grown in minimal media and the presence of light, anaerobic conditions at room temperature before imaging at log phase. These cultures showed clear signs of ICM formation through the observed fluorescent regions located within the cell's inner membrane (Figure 11). The presence of these ICMs was necessary for the bacteria to harvest light energy and initiate the photosynthetic process. Consistent, fluorescence light intensity was used to control this condition and initiate the formation of these membranes.

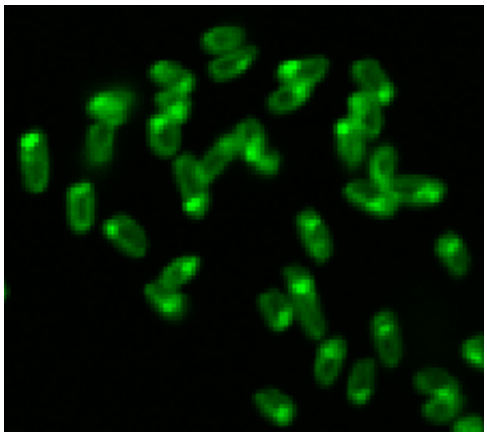


Figure 11: Fluorescent image of a grouping of *R. sphaeroides* 2.4.1 bacterial cells imaged after 5 days of growth in + ICM conditions. Nearly all cells observed in this particular culture contained ICMs.

Optimal imaging ranges were achieved between two to three days using a 1/250 dilution subculture. Because these species were prepared in 125 mL crimp flasks, it took a much longer period of time to reach log phase before any image analysis could be completed. Regardless, each of the four cultures analyzed showed identical results – nearly all cells contained ICMs. However, it should be noted that these cells' ICM do not fully occupy the cytoplasm as shown in

the TEM image earlier (Figure 2). This could be due to alterations in the light intensity used or the growth stage when each culture was imaged.

All cultures of *R. sphaeroides* 2.4.1 were quantified both quantitatively and qualitatively to suggest that these species were successfully labeled and contained ICMs. Most samples that were evaluated contained large amounts of cells, making the quantitative analysis a little more difficult. Nonetheless, 10 images containing numerous cells from three of the cultures were quantified (Figure 12). These results showed clear signs of ICM formation.

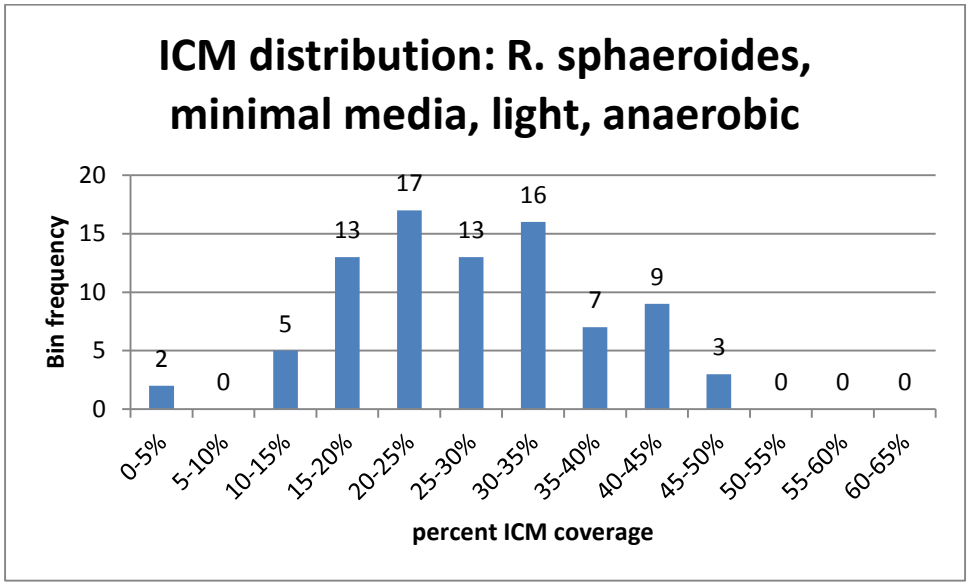


Figure 12: The percent ICM distribution for *R. sphaeroides* grown in minimal media and light, anaerobic conditions at room temperature.

3.7 OB3b growth and ICM analysis in P-0 media

Initial attempts were made at growing batch cultures of *M. trichosporium* OB3b and analyzing them at mid-log phase (0.3-0.8 OD_{600}) with limited success. These cultures were prepared in P-0 media and methane was the carbon source of choice. The cells were first imaged straight from an agar plate by re-suspending them in a minimal amount of media. All of these

cells (8 images taken) showed signs of ICM formation (Figure 13). However, after several attempts at growing these in liquid P-0 media, limited amounts of cells showed any signs of ICM – nearly 40 images had been taken from a number of different cultures. It was unclear as to the reasoning behind why these cells did not form ICMs. Continuous efforts have been taken at growing the OB3b cells in conditions that would suggest ICM formation as well as those that would not suggest the formation of ICMs.

Figure 13: Fluorescent image of an OB3b cell taken straight from an agar plate with clear signs of ICM formation. Methane was used as the carbon source.

4. Conclusion:

Through the utilization of fluorescence microscopy and lipid styryl dyes, the ability to develop a widespread, rapid method of ICM assessment is certainly achievable. It was reinforced that the presence of copper has a strong tie to the metabolic process of oxidation and subsequent formation of ICMs in methanotrophic bacteria, despite a known mechanism. Continual efforts at identifying and evaluating ICM formation in methanotrophic bacteria can help in the understanding of this systematic process.

However, the use of fluorescence microscopy is farther reaching that just methanotrophic bacteria. This method was also able to rapidly identify the absence, or presence, of ICMs in a photosynthetic, purple non-sulfur bacterium, *R. sphaeroides* 2.4.1. Additional work is being performed on another type of bacterium, *Gluconobacter oxydans*, a glycerol oxidation species, to further reinforce this rapid method of ICM assessment.

The rapid method of fluorescent labeling used to assess ICM formation was both widely applicable and extremely efficient for all bacteria. Both methanotrophic species evaluated, *M. alcaliphilum* 20Z and *M. trichosporium* OB3b, as well as *R. Sphaeroides* were successfully imaged, analyzed, and quantified accordingly with the Nikon NIS elements software package. Ongoing projects with this technique include further work on minus-copper methanotrophic growth to determine the threshold for ICM formation in these species, additional growth and assessment of *Gluconobacter oxydans*, TEM images of each cell species in plus-ICM and minus-ICM conditions, the determination of the subcellular architecture of pMMO, and ultimately, a better understanding of cell dynamics as a whole.

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Appendix 1: Safety Considerations

The Konopka group is involved in biophysical research, conducted in a biosafety level one laboratory. Common safety precautions taken when performing laboratory work include the use of proper personal protective equipment (PPE) such as gloves, a lab coat (when essential), and safety glasses (when essential). Non-pathogenic, non-infectious bacteria were typically handled on a daily basis in either a biosafety fume hood, or using common biochemical laboratory techniques on an open bench top (i.e. Bunsen burner, ethanol wash) to prevent contamination. When finished with disposable equipment and gloves, they were discarded in their appropriate waste designations. Glassware containing any cells were diluted with a 50/50 mixture of bleach in water and sat for no less than a half an hour before being poured down the drain; this ensured that all bacteria was killed.

Other safety procedures followed in the Konopka lab were much less frequently used; these included the use of the UV setting in the biosafety hood, use of the methane tank, and acid washes for the removal of microelements from glassware. The latter two were encountered more often than the former. Use of the methane tank required training from the safety hygiene office and familiarity with standard operating procedures (SOP). Work with methane was also performed in a well-ventilated chemical hood to avoid any unnecessary release of the flammable gas. Caution was also taken when using the explosion-proof vacuum pump, as to avoid the risk of generating any spark or static while using the methane tank.

Acid washes were also used on a few occasions. Because nitric acid (3 M) was required to remove microelements such as copper from glassware, laboratory coats, a rubber apron, safety goggles, nitrile gloves and rubber gloves were required. These washes were carried out in a well-

ventilated chemical hood and in the confinement of a plastic bin. The acidic wash water generated was subsequently neutralized with sodium bicarbonate and tested with pH paper to verify neutrality before removing it as waste.

Several additional precautions were taken during the fixation portion of TEM imaging. Because some of the materials were extremely hazardous and or toxic to the environment such as OsO_4, severe caution was taken when handling and disposing this chemical; a special contained housed the used material before safety services properly handled the wate. Additional PPE was also required when handling OsO_4, such as a chemical fume hood, nitrile gloves, and splash resistant goggles. Acetone was also used in the fixation process which is an extremely flammable solvent, capable of igniting a flash fire. Preventative measures were taken to avoid the generation of any static or heightened temperatures.