Currently, cellular metal content is studied by removing the cells from their culture media (either by centrifugation or filtration), washing with fresh media solution, and then acid-digesting them for analysis by ICP-MS. This methodology gives the total metal or particle content in a given number of cells rather than on a per-cell basis. As such, the metal concentration of an individual cell relies on the assumption that all cells accumulate the same amount of ionic or nanoparticulate metal. This assumption is not always true, as demonstrated by techniques such as transmission electron microscope (TEM), scanning electron microscope (SEM), and fluorescent tracking. These microscopy techniques allow visualization of NP uptake into cells but are time consuming and prone to artifacts. TEM and SEM are qualitative, and labelling may give false positives where the label-NP complexes are not persistent.

**Introduction**

The uptake of metals into individual cells is of interest to both environmental and human health studies, whether the metal is dissolved or exists as nanoparticles (NPs).
These limitations (i.e., inability to quantify ionic or NP on a per-cell basis) can be overcome through the use of Single Cell ICP-MS (SC-ICP-MS), a new technique based on Single Particle ICP-MS (SP-ICP-MS)\(^8,9\), allowing larger numbers of cells to be examined in a more accurate and quantitative manner. Similar to SP-ICP-MS, SC-ICP-MS is based on the ability to completely ionize a single cell in the plasma and measure the resulting ion content.

One critical requirement for both SP-ICP-MS and SC-ICP-MS is the ability of the instrument to acquire data at high speed in order to fully capture the signal from the ionized cell or nanoparticles. Dwell times lower than 75 µs are essential for the accurate detection of such events\(^10\). With this in mind, SC-ICP-MS allows for the rapid determination of metal or NP content within individual cells (at levels as low as attograms (ag) per cell) and the number of cells containing metal. With knowledge of the total number of cells in a given sample (as determined by hemocytometry or flow cytometry), the percentage of cells containing metal can be ascertained, resulting in increased information available compared to other techniques. With such capabilities, SC-ICP-MS is a powerful new technique to quantify the cellular uptake and depuration rates of NPs (or dissolved metals), allowing exposure studies to be conducted on a smaller number of cells than possible with other techniques, considerably reducing cell expense.

This work will focus on the use of SC-ICP-MS to monitor the uptake of ionic and nanoparticulate Au into individual fresh water algae cells (Cyptomonas ovata).

**Experimental**

**Samples and Sample Preparation**

Cell cultures were prepared at concentrations of 200,000 cells/mL and exposed to either ionic Au or Au NP (60 nm NPs, NIST 8013) at various concentrations, as shown in Table 1. Each exposure study was run in triplicate at 20 °C for up to 74 hours with a light:dark cycle of 12 hours light and 12 hours dark.

During the exposure, 1 mL aliquots were removed periodically for analysis. Prior to analysis, the cells were separated from the exposure media and washed with media three times. Each wash cycle consisted of centrifuging the cells for 15 minutes at 300 g-force and re-suspended in 1 mL of fresh culture media (containing no NP or ionic Au). A schematic of the sample preparation cycle is shown in Figure 1. After the three washes, the cell recovery was 43.8 ± 8.6%.

**Instrumental Conditions**

All analyses were carried out on a PerkinElmer NexION® ICP-MS using the Syngistix™ Single Cell Application Software Module for data collection and processing\(^11\).

The instrumental conditions used are shown in Table 2, and the Single Cell Software Module is shown in Figure 2. Since cells are typically larger than the aerosol droplets which are passed to the plasma, a conventional spray chamber limits their transport to the plasma. To overcome this limitation, the new, proprietary Asperon™ single cell spray chamber was used. The Asperon spray chamber was developed specifically to increase transport efficiency of cells to the plasma. This is accomplished by modifying the spray chamber design and incorporating new flow patterns within the spray chamber. Specifically, a dual make-up gas inlet is positioned to create a tangential flow to the spray chamber walls to prevent cells from colliding with and sticking to the walls. In addition, an inner tube has microchannels where some of the makeup gas is diverted. The purpose of these channels is to prevent liquid deposition in the flow path. Finally, a laminar flow is incorporated for maximum transport of the cells.

**Standards**

Calibrations were performed with both ionic/dissolved and NP standards. The ionic calibrations were performed with 1, 2, and 3 ppb Au, while the NP calibrations used 10, 30, and 60 nm Au NPs (NIST 8011, 8012, and 8013, respectively), prepared at 50,000 part/mL. All standards were prepared in the algae media to matrix-match the cell suspensions. Transport efficiency was determined using the 60 nm Au NPs.

![Figure 1. Schematic of sample preparation for SC-ICP-MS analysis.](image-url)

**Table 1. Exposure Studies.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell Concentration (cells/mL)</th>
<th>Au Ionic Concentration (ppb)</th>
<th>Au NP Concentration (part/mL)</th>
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<tbody>
<tr>
<td>Cell Control</td>
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</tr>
<tr>
<td>NP Control</td>
<td>0</td>
<td>0</td>
<td>200,000</td>
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<tr>
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<tr>
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<td>Ionic Treatment-3</td>
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</table>
Results and Discussion

Before analyzing the cells for Au NP uptake, the effect of Au on the cells themselves must be determined. This was accomplished by exposing cells to different concentrations of ionic/dissolved gold and different concentrations of Au NPs. The cell concentrations were then monitored over 74 hours using a hemocytometer. As shown in Figure 3, there was no significant difference in cell concentration between the exposed and control cells for both the ionic (Figure 3A) and NP (Figure 3B) Au exposures. As a result, Au does not impact cell concentrations.

Cell Viability

The cell line used in this study is *Cryptomonas ovata*, which has a size range of 20-30 µm. The aspiration of the cells through the nebulizer can subject them to high pressures, which are dependent on the nebulizer, sample flow rate, and nebulizer gas flow. To ensure that the cells were not damaged during nebulization, a variety of sample uptake and nebulizer gas flow rates were evaluated by counting the cells before and after the nebulization process using light microscopy. Figure 4 shows that 100% of the cells were intact at a sample flow rate of 100 µL/min with a nebulizer gas flow of up to 0.5 L/min. Therefore, under the preferred running conditions, all of the cells will enter the spray chamber fully intact.
Removing NPs from the Cell Media

It is important to confirm that the signal measured from the washed cells is due to the metal within the cells themselves and not from residual metal left over from the original exposure. Thus, it is important to know that no NPs persist in the cell media after the final wash cycle. To check this, the cells were washed three times with fresh media, as described in Figure 1. To monitor the NP content of the media, the supernatant of each cellular wash cycle was analyzed by SC-ICP-MS. As shown in Figure 5, the NP content decreases over the three wash cycles to reach zero particles detected after the third wash. The table in Figure 5D shows the number of particles counted in each wash cycle. These results show that after three washes, any signals must originate from the cells themselves.

Because the cell-exposure studies are carried out over 74 hours, the studies shown in Figure 5 were repeated at various times over the exposure time. The results always mimicked those in Figure 5, with the size distribution peak of the Au NPs remaining constant: no broadening or shifting to larger or smaller sizes was observed, nor was any ionic Au detected. Therefore, NPs are not dissolving or aggregating in the exposure period. The significance of these results is that if the size or size distribution of the NPs from the cells differ from those in the supernatant, then it can be concluded that these changes result from transformations occurring within the cell.
SC-ICP-MS Controls

As shown in Table 1, three control experiments were performed in SC-ICP-MS on solutions containing only algae media, only non-exposed algae cells, and only Au nanoparticles (60 nm, 50,000 part/mL), with typical responses shown in Figure 6. The responses from the cell and media controls (Figures 6 A and B) show that no Au NPs are detected, while the 60 nm Au NIST NPs in media show a Gaussian distribution around 1800 ag/particle (Figure 6 C). Assuming spherical shapes and knowing the molecular mass of Au, 1800 ag correspond to the mass of a 56.26 nm Au spherical NP, which is in agreement with the 56 nm certified TEM value for the Au NIST standard. The particle concentration from this distribution is 49,500 part/mL, which is 99% of the NPs added (50,000 part/mL). These results demonstrate that any Au measured from the exposure studies results from Au NPs being taken up by the cells and does not originate from non-exposed cells. In addition, the results confirm that the algae media does not affect the size distribution of the Au NPs.

NP Uptake Into Cells

One of the main advantages of SC-ICP-MS is the ability to determine not just the number of cells that contain NPs, but also the percentage of those cells which contain single or multiple NPs. In Figures 7 A-D, there is a main peak around 1700 ag which is due to a single NP within the cells (labeled 1NP/1C = 1 particle/1 cell). Since a single 60 nm Au NP corresponds to ≈ 1800 ag (as discussed earlier), then a mass of ≈ 1700 ag Au measured from a cell corresponds to a single 60 nm Au NP in the cell. This slight reduction in mass is believed to result from the NP being within the cell, which will be explored by microscopy in a future study. As the exposure time increases from two hours (A) to 74 hours (D), the presence of multiple NPs per cell can be seen as peaks appearing at 3400 ag (2 NPs) and 5200 ag (3 NP), marked as 2NP/1C and 3NP/1C in A-C, respectively.

With the ability to determine the number of NPs per cell, the percentage of cells containing various numbers of NPs can be tracked over time and as a function of the NP concentration in the algae media. Figure 8 shows the number of NPs per cell both as a function of time (2 to 74 hours) and as a function of the initial NP concentration in the media solution. As the exposure time increases, the number of cells with 1 NP increases, as would be expected. Also, a higher percentage of cells contain a single NP as the concentration of NPs in the media increases from 200,000 to 600,000 part/mL. A similar trend is seen in the number of cells containing 2 NPs and 3 NPs in the 600,000 part/mL exposure. However, with the 200,000 part/mL, the number of cells that have taken up two or more nanoparticles is too small to draw any conclusions with regard to the effect of exposure time on uptake rate.

Figure 6. Typical responses for the cell control (A), algae media control (B), and the 60 nm Au NIST control in algae media at a concentration of 50,000 part/mL (C).

Figure 7. The signal collected from cells showing the increase of cells containing Au metal over 74 hours along with an increase of cells containing more than one particle. Exposure times of 2 hours (A), 28 hours (B), 53 hours (C), and 74 hours (D). 1NP/1C = 1 NP per cell; 2NP/1C = 2 NPs per cell; 3NP/1C = 3 NPs per cell.
Uptake of Ionic Au Into Cells
To determine the cellular uptake of ionic Au, algae cells were exposed to dissolved Au concentrations of 1, 2, and 3 µg/L for up to 74 hours, with sample aliquots being drawn at 2, 28, and 74 hours. As shown in Figure 9A, there is an apparent decrease in the average amount of Au per cell (expressed as ag/cell) over time, which is independent of initial Au concentration. However, as demonstrated in Figure 9B, the % of cells containing Au increases over time and with initial Au exposure concentration. These data suggest that there is a cellular mechanism that limits the amount of Au taken into the cell, which is dependent on the Au concentration in the cellular media.

Conclusion
This work has shown the ability of SC-ICP-MS to measure the concentration of both ionic and nanoparticulate metals in algal cells. The percentage of cells containing one NP increases as a function of both time and exposure concentration, while the number of cells containing 2 and 3 NPs/cell only increases in the highest initial exposure concentration. However, when exposed to ionic Au, the amount of Au/cell does not increase with time, although the number of cells containing Au did increase with time and exposure concentration.

These results demonstrate that SC-ICP-MS can, for the first time, quantify the number of cells containing metals or NPs and relate exposure concentration to dose. In order to obtain statistically meaningful results, a large cell population must be studied. In the past, this was very time consuming, but with the advent of SC-ICP-MS, a large number of cells can be measured in a short time period. Accuracy is also increased due to the fast data acquisition rates of the NexION ICP-MS, only available with dwell times of less than 100 µs. With its single cell detection capabilities, the NexION ICP-MS offers a unique opportunity to study the uptake of metals into cells and can also be used to determine the intrinsic metal content of the cells themselves in their natural environment.

The examples shown in this study have considerable implications for the development of regulations governing nanoparticle concentrations in the environment. For instance, the European Union is considering number concentration as the appropriate metric on which to regulate nanoparticles in REACH. Currently, there are no other techniques capable of quantifying these metrics.
References


Consumables Used

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