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Single-Cell Elemental Analysis Using Inductively Coupled Plasma Mass Spectrometry

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Abstract

Single-cell elemental analysis using inductively coupled plasma-mass spectrometry (ICP-MS) is a rapidly developing field within metallomics, offering the ability to quantify elemental contents in individual cells. Pioneering works have developed diverse sample introduction systems coupled with ICP-MS, enabling high-throughput, precise, and accurate elemental analysis at the single-cell level. These advancements have facilitated applications across medicine and biology, providing insights into elemental metabolism and toxicity. Two prominent approaches have emerged: fast time-resolved analysis of cell suspensions, applicable to a broad range of cell types (bacterial, fungal, plant, and mammalian cells), and laser ablation (LA) for generating aerosols from individual cells. LA is particularly well suited for adherent cultured cells and allows for selective analysis based on cell morphology and size. These complementary techniques provide powerful tools for elucidating the complex interplay between elements and biological systems.

Keywords: fast time-resolved analysis, ICP-MS, laser ablation, microdroplet generator, single cell

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Introduction

Living organisms rely on a suite of elements for essential biological functions. While organic molecules are primarily composed of hydrogen, carbon, nitrogen, and oxygen, trace elements, collectively known as minerals, play critical roles in a wide array of processes. Major minerals, including calcium (Ca), phosphorus (P), sulfur (S), sodium (Na), magnesium (Mg), and potassium (K), contribute to diverse functions. Ca, Na, Mg, and K primarily function as electrolytes, regulating fluid balance and facilitating signal transduction. P is covalently incorporated into nucleic acids and lipids, while S is a key component of protein thiol groups, influencing protein structure and function. Trace minerals, including the transition metals iron (Fe), copper (Cu), zinc (Zn), chromium (Cr), cobalt (Co), and molybdenum (Mo), the chalcogen selenium (Se), and the

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halogen iodine (I), are essential for maintaining the structure and catalytic activity of numerous enzymes. Because of their diverse involvement in biological processes, imbalance in mineral intake or metabolic disorders can have significantly adverse effects at the organismal level.

The critical roles of minerals in biological systems are evident at the cellular level as well as the organismal level. Intracellular P content, for instance, doubles prior to DNA replication [1]. Similarly, changes in intracellular elemental content are observed during cell differentiation [2], and excessive Ca transport to mitochondria can trigger cell death [3]. These examples underscore the crucial involvement of minerals in essential cellular phenomena such as transcription, translation, metabolism, homeostasis, and programmed cell death. Consequently, elemental analysis at the single-cell level has emerged as a powerful technique in metallomics, offering insights into these critical processes.

High-resolution elemental imaging analysis using particle induced X-ray emission (PIXE) and scanning fluorescnece X-ray microscopy (SFXM) provides insights into intracellular distribution and localization of elements [4, 5]. In contrast, inductively coupled plasma (ICP)-based techniques offer high-throughput and quantitative elemental analysis, serving as complementary methods to high-resolution imaging for elucidating intracellular metabolism and the functions of elements. ICP is a powerful ionization source due to its ability to efficiently atomize and ionize a broad range of elements. It offers excellent sensitivity and a wide dynamic range for atomic emission spectrometry (ICP-AES) or mass spectrometry (ICP-MS), both of which are widely used for elemental analysis of biological samples. Recent advances in ICP-MS technology include high matrix tolerance interfaces and collision/reaction cells. These developments have significantly enhanced the detection and quantification of trace minerals in complex biological matrices. These advancements have also paved the way for single-cell elemental analysis using fast time-resolved data acquisition. This technique enables the measurement of elemental content in individual particulate matter, such as nanoparticles (single-particle ICP-MS, spICP-MS) and, importantly, single cells (single-cell ICP-MS; scICP-MS). Introducing particles or cells individually into the ICP, where their constituent elements are ionized and recording the resulting ion signals at 1 ms or shorter intervals make it possible to resolve elemental contents in individual cells. This fast time-resolved approach is crucial because the signals generated from a single cell are typically shorter than 1 ms.

Laser ablation (LA) for sample introduction is an alternative approach to fast time-resolved analysis. LA generates aerosols from solid samples, which are introduced into the ICP-MS, providing two-dimensional elemental distribution data and insights into tissue-level elemental metabolism. While the generated aerosols disperse spatially during transport to the ICP over several seconds, unlike fast time-resolved analysis, which maintains particle/cell integrity until ionization within the ICP, conventional signal integration times (10–100 ms) can be used to resolve signals from individual cells. LA offers several advantages, including eliminating the need for cell suspension preparation for adherent cells and enabling selective analysis based on cell size and morphology. However, the relatively long washout time inherent to LA can limit throughput. Recent development of two-volume cells for rapid washout has significantly mitigated the limitation in LA-based single-cell analysis [6]. Furthermore, the use of galvanometric optics in the solid-mixing method enhanced the accuracy of quantification, ranging from trace to major elements [7].

Both fast time-resolved analysis and LA-based analysis offer powerful capabilities for single-cell elemental analysis, potentially significantly advancing single-cell research in metallomics. More precisely, scICP-MS is a promising technology for elucidating elemental metabolism in toxicology, advancing drug discovery in pharmaceutical sciences, and improving prognosis and diagnosis in medicine. In this review, we provide a comprehensive overview of these complementary approaches and discuss their principles, advantages, limitations, and recent applications.

Single-cell analysis by fast time-resolved ICP-MS

In ICP-MS analysis, the dwell time, or integration time, for detecting specific ions can be set independently for each element or isotope. Typically, ICP-MS allows for dwell times of 10 ms or longer. While extending the dwell time increases the total number of detected ions, leading to improved precision, it does not always enhance the signal-to-noise ratio significantly. This is because the background signal also accumulates during the extended

integration period, potentially offsetting the benefits of increased ion counts. The development of ICP-MS systems capable of setting dwell times shorter than 10 ms has revolutionized single-particle and single-cell analysis. By enabling fast time-resolved data acquisition, these systems can accurately capture transient signals generated from individual nanoparticles or cells [8]. When samples are introduced into the ICP, their constituent elements are atomized and ionized, subsequently entering the mass spectrometer. Due to the relatively small number of ions generated from a single particle or cell, fast time-resolved data acquisition is crucial to accurately capture the transient signals and extract meaningful elemental information. The acceleration of ions by several hundred volts at the extraction lenses results in the formation of a tightly confined spatial cluster, known as an "ion cloud" or "ion plume", which rapidly traverses the detector within a fraction of ms. Consequently, the use of conventional dwell times (>10 ms) can result in the simultaneous detection of multiple particles/cells, ultimately leading to content overestimation. While reducing sample density can mitigate this issue, it compromises sample throughput. To overcome such a challenge, setting the dwell time to a value shorter than the detection period of a single ion cloud/plume, typically ranging from 0.2 to 0.6 ms [9], enables high-throughput signal acquisition. Nevertheless, even in the fast time-resolved mode, simultaneous detection may still occur, especially at higher particle or cell densities. Therefore, it is essential to ensure proper sample dispersion and maintain an appropriate density in the suspension to minimize the likelihood of such events.

Cell suspension preparation

It is vital to prepare a well-dispersed cell suspension while maintaining the structural integrity of the cells in fast time-resolved analysis. Adherent cells require careful dispersion and filtration to isolate individual cells while floating cells can be more readily prepared as single-cell suspensions. In this regard, Á-Fernández García et al. have demonstrated the potential of using single cells isolated from solid tissues by enzymatic digestion for scICP-MS analysis [10]. Our preliminary investigations have shown that human leukemia K562 cells maintain structural integrity in isotonic solutions like phosphate-buffered saline (PBS) and saline (0.9% sodium chloride) but swell in hypotonic solutions like Tris (tris(hydroxymethyl)aminomethane) buffer or rupture in ultrapure water (data not shown). Chemical fixation has been used to preserve cell structure in ultrapure water [11, 12], but our findings suggest that this approach can lead to the leakage of intracellular elements [13]. Bacterial, fungal, and plant cells, with their rigid cell walls, may exhibit greater resistance to osmotic stress than mammalian cells. Moreover, elemental contamination from the cell suspension solution can significantly impact baseline signals in fast time-resolved analysis. Consequently, the use of low-purity reagents should be avoided during sample preparation. High salt concentrations can give rise to several analytical challenges, including nebulizer clogging, interface contamination, mass spectrometric interference, and non-mass spectrometric interference (matrix effects). Preparing isotonic solutions with simple chemical compositions using high-purity reagents is recommended to minimize these issues.

Quantification protocol

The fast time-resolved analysis enables the acquisition of transient signals, approximately 0.5 ms in duration, from single cells. We can evaluate the elemental content within individual cells by analyzing these transient signals. One potential method involves using reference particle materials containing a known mass of the target element [14]. When these particles are analyzed, the detected transient signals are affected by ion loss within the mass spectrometer during the transmission process. Assuming that the transmission efficiency of the target element is similar for both reference particle materials and cell samples, the elemental content in a cell (m_c) can be calculated using the sensitivity factor (α; signal intensity per elemental mass) determined from the reference particle materials. This method provides a straightforward approach to quantitative analysis using particulate substances that resemble cells in size and morphology. However, the availability of reference particle materials containing the target element may be limited, particularly for less common elements.

An alternative approach for calculating elemental content relies on the sensitivity factor derived from the signal intensity of ion solutions [14]. While this method may appear similar to the reference particle method, there are

significant differences in the behavior of particles and ionic solutions introduced through the nebulizer. Elements contained within particles are not subject to the same losses as those introduced in solution form and are detected as transient signals. In contrast, ions in the aerosol are subject to losses during transport from the nebulizer to the ICP and are detected as continuous signals rather than transient signals. Therefore, when utilizing a sensitivity factor obtained from ionic solutions, the following steps are necessary: (i) determine the transport efficiency (f) of the ions in the aerosol from the nebulizer to the ICP, (ii) measure the ion solution containing the analyte element to calculate the sensitivity factor (α) using transport efficiency (f), and (iii) calculate the elemental content by dividing the transient signal from the cells by the sensitivity factor (α). The detailed calculation is outlined in Equation (1), where I_c, I_s, and I_b represent the signal intensities from the cells, the ionic solution of the analyte element, and the blank solution, respectively, t_d denotes the dwell time, C_s indicates the concentration of the ionic solution, and v is the sample introduction rate.

$$m_c = \frac{I_c}{I_s - I_b} \times t_d \times f \times C_s \times v \quad (1)$$

We have determined elemental contents in yeast (*Saccharomyces cerevisiae*), green alga (*Chlamydomonas reinhardtii*), and rat red blood cells, ultimately validating the quantification procedure for single-cell analysis [15]. **Fig. 1a** presents the signal profiles of P. For all cell types, dozens of transient signals were detected within a single second of the time-resolved analysis (TRA) profile (**Fig. 1a**). These signals, with a duration of approximately 0.5 ms, strongly suggest that they originated from individual cells. We quantified the intracellular P content using the sensitivity factor derived from ionic P solutions. These values were comparable to those obtained from the bulk concentration analysis following acid digestion (**Fig. 1b**). These findings highlight the potential of fast time-resolved analysis to provide unprecedented insights into cellular heterogeneity and the role of essential elements in cellular processes.

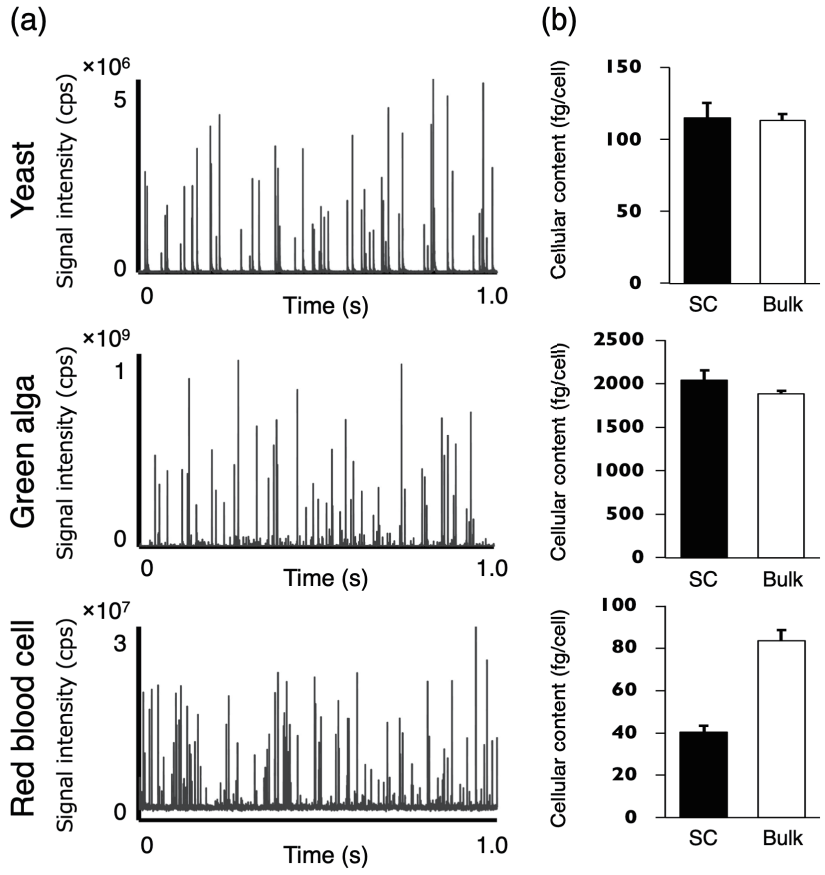

Fig. 1. Comparison of intracellular phosphorus (P) content determined by single-cell and bulk analysis (Data from reference [15]). (a) Time-resolved analysis data for ^{31}PO with a signal integration time of 0.1 ms. (b) Comparison of P content determined by fast time-resolved single-cell (SC) ICP-MS and bulk solution nebulization ICP-MS following acid digestion (bulk analysis). The higher P content observed in the bulk analysis of red blood cell suggests potential contamination from lysed red blood cells and/or serum components.

Applications in bacterial cells

Given the diversity of bacteria, including environmental and pathogenic bacteria, gut microbiota, and bacteria used in food processing, they represent promising targets for scICP-MS analysis. Gomez-Gomez et al. investigated the uptake of tellurium (Te) nanoparticles, which are potential environmental contaminants, by *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) [16]. Hellmann et al. also focused on environmental contaminants such as cadmium (Cd), cerium (Ce), and uranium (U) and evaluated the potential of *Streptomyces coelicolor* for bioremediation [17]. Moreover, Xu et al. utilized scICP-MS to investigate the antimicrobial activity of Cu ions against *Legionella pneumophila* [18]. Similarly, Liang et al. employed scICP-MS to detect pathogenic bacterial strains such as *E. coli*, *S. aureus*, *Listeria monocytogenes*, *Shigella dysenteriae*, and *Vibrio parahaemolyticus* using metal-labeled polyclonal antibodies specific to each strain [19].

E. coli is a well-established model organism for recombinant protein production. Accordingly, we explored the feasibility of applying single-cell elemental analysis to evaluate the expression of recombinant proteins in *E. coli* [20]. Given the limitations of directly detecting organic molecules using ICP-MS, we labeled proteins with metal ions using a sequence of six histidine residues (His-tag) fused to the protein terminus. The His-tag sequence has a high affinity for divalent transition metal ions such as nickel (Ni) and Co. We utilized a plasmid vector encoding a His-tagged red fluorescent protein, mCherry. To validate the results of ICP-MS, mCherry expression levels were also assessed by flow cytometry. As shown in **Fig. 2a**, the fluorescence intensity from flow cytometry revealed a time-dependent increase following protein expression induction, indicating increased mCherry expression within the cells. Like the results from flow cytometry, scICP-MS analysis showed an increase in the signal intensity of Co from individual cells following induction (**Fig. 2b**). Collectively, these findings highlight the potential of scICP-MS to provide quantitative insights into recombinant protein expression at the single-cell level in *E. coli*, thereby aiding the optimization of protein production.

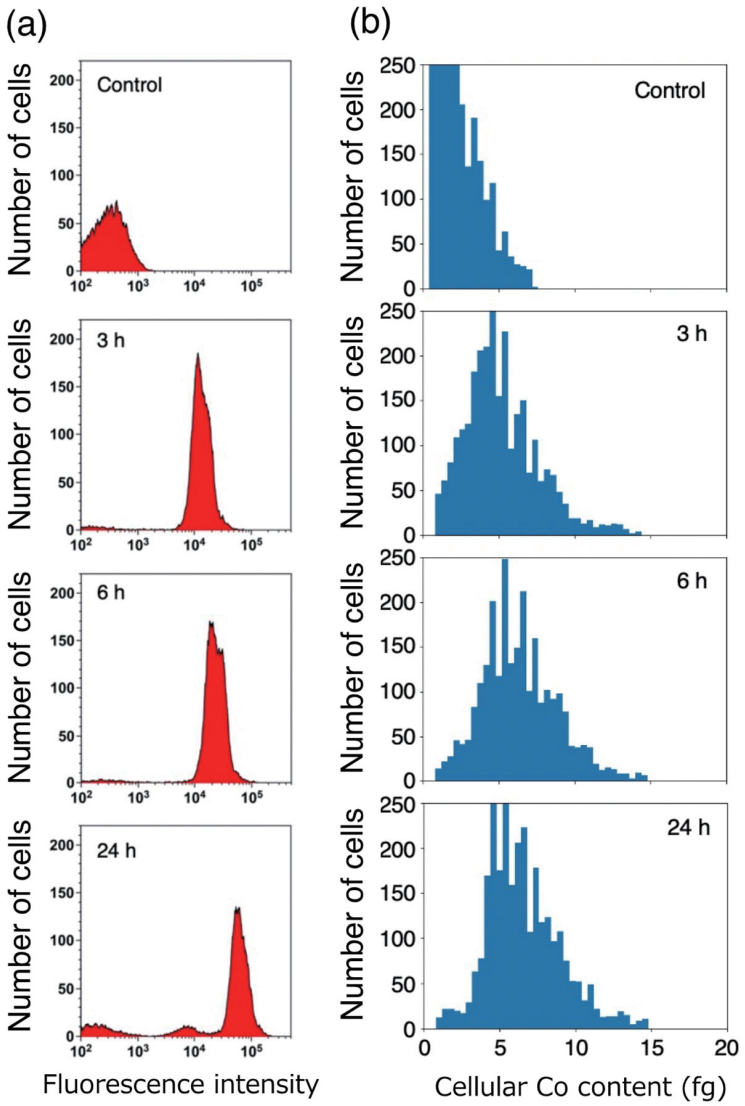

Fig. 2. **Correlation of mCherry protein expression measured by flow cytometry and scICP-MS (Data from reference [20]).** *E. coli* cells were induced to express mCherry for 0 (control), 3, 6, and 24 h. mCherry expression was quantified by (a) flow cytometry based on fluorescence intensity and (b) scICP-MS by measuring cobalt (Co) signal intensity.

Applications in mammalian cells

Mammalian cultured cells are the most commonly used cell models in biological research, serving as a preclinical or alternative model to *in vivo* experiments for assessing biological responses,

metabolism, and toxicity. From an animal welfare perspective, cultured cell experiments are expected to expand their role in a broader range of research fields, reducing the reliance on animal models.

Recent research has focused on biomedical applications, including the uptake of nanoparticles potentially used as nanomedicine or drug delivery system (DDS) carriers [21–23]. Studies have also evaluated cellular absorption of metal-containing pharmaceuticals such as platinum-based anticancer agents [24–26], gadolinium-containing anticancer drugs [24], and arsenic-based pharmaceuticals for leukemia [27]. These studies demonstrate the expanding role of scICP-MS analysis with cultured cells in medical and pharmaceutical research.

Several research groups have developed microfluidic-based sample introduction systems for efficient and controlled delivery of cultured cells to the ICP-MS instrument, ultimately validating elemental quantification employing the protocol described in the previous section [28–30]. Furthermore, developing hyphenated techniques integrating fluorescence detection prior to ICP-MS has enabled simultaneous spectroscopic and mass spectrometric analyses at the single-cell level. These techniques provide complementary information on cellular elemental composition and function [31, 32]. These hyphenated techniques have facilitated detailed studies of intracellular processes at the single-cell level. For instance, the impact of Ag ion and AgNP exposure has been assessed by quantifying GFP-labeled metallothionein and Ag concentrations within individual cells [33]. This approach has also been extended in mammalian cells to combine fluorescence-activated cell sorting (FACS) with scICP-MS. Gutierrez-Romero et al., for instance, used this combined approach to isolate apoptotic, necrotic, and viable human ovarian cancer cells (A2780 and OVCAR-3) by FACS and subsequently analyze cisplatin prodrug uptake in each cell population [26].

Despite significant advances in single-cell analysis technologies and their applications, a key analytical challenge persists, particularly for mammalian cultured cells. These cells are inherently fragile owing to their large size compared with microorganisms and the lack of a rigid cell wall. This fragility can significantly impact analytical performance. For example, we previously demonstrated that the human myelogenous leukemia K562 cell structure is damaged during nebulization, leading to lower transport efficiency than similarly sized silica particles (**Fig. 3**) [13]. To address these challenges, we employed a microdroplet generator, a technique previously employed in various cell types, including yeast [34], bovine red blood cells [35], and mouse lung cells [36]. Efficient desolvation

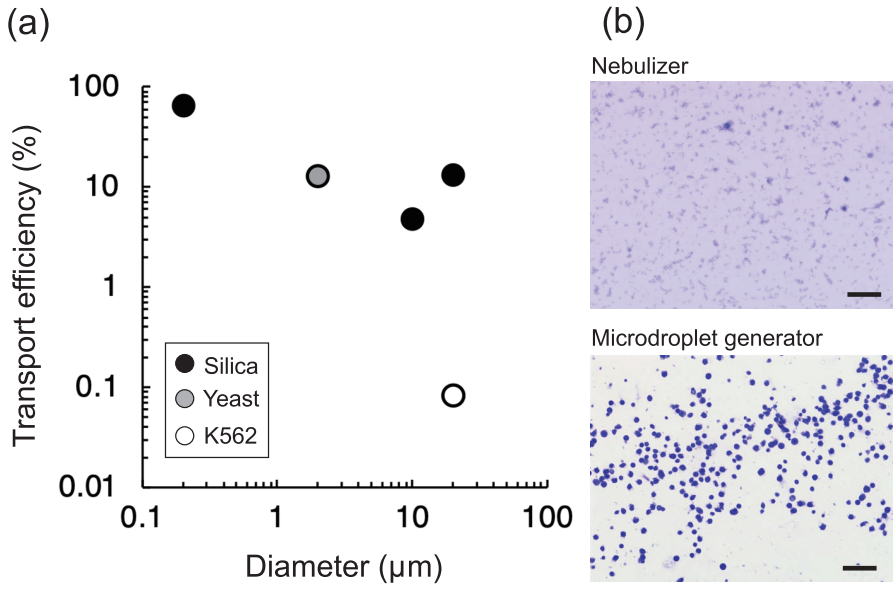

Fig. 3. **Effect of nebulization on mammalian cell transport and integrity (Data from reference [13]).** (a) Comparison of transport efficiency from the nebulizer to the ICP for differently sized silica particles, yeast, and K526 cells. The significantly low transport efficiency observed for K562 cells is attributed to cell disruption caused by the pneumatic nebulizer. (b) Preservation of cell structure using a microdroplet generator for sample introduction, contrasting with the disruptive effects of nebulization. Scale bars represent 150 μm.

of the generated droplets is crucial for achieving high ionization efficiency, and this is typically accomplished using nitrogen or helium gas [37–39], sometimes in conjunction with heating devices [36] or membrane desolvators [35]. Although we did not utilize these additional desolvation methods, the microdroplet generator alone proved sufficient to maintain cell integrity (**Fig. 3b**), allowing for efficient signal acquisition from mammalian cultured cells [13].

Single-cell LA-ICP-MS analysis

Laser ablation (LA) has become a versatile analytical technique for directly sampling solid samples, providing valuable spatial elemental distribution data across diverse scientific disciplines. Its applications span various fields, including geochemistry, archeology, materials science, environmental science, food science, forensic science, and life science. In biological studies, LA is frequently employed in the two-dimensional elemental mapping of plant and animal tissues, offering insights into the distribution and concentration of elements within specific anatomical regions [40]. In single-cell LA analysis, individual cells are ablated to produce aerosols. Because of the relatively long washout times associated with LA, which are influenced by the sample chamber volume and transport tube length, a fast time-resolved acquisition mode is not required to resolve signals from individual cells. Consequently, the analytical throughput of LA-based single-cell analysis is generally lower than that of techniques employing fast time-resolved measurements. However, LA offers a significant advantage: the ability to selectively target and measure cells based on specific size and morphological characteristics, providing unique insights into heterogeneous cell populations.

Cell sample preparation

Cell immobilization is crucial for achieving stable ablation and high analytical throughput for effective single-cell LA analysis. Adherent cultured cells can be analyzed directly after removing the culture medium and washing with a rinse solution (e.g., saline). Generating a cell suspension and employing microfluidic devices for cell alignment can further enhance throughput [41]. Alternatively, piezo-acoustic microarrayers offer another effective approach for preparing well-aligned single-cell samples on a substrate, providing precise control over cell positioning [42].

Quantification protocol

Quantification in LA-ICP-MS typically relies on calibration using solid standard materials. However, matrix effects, arising from differences in composition between the standard and the biological sample, can significantly influence aerosol particle size and elemental composition, leading to inaccuracies [43]. The limited availability of certified reference materials specifically designed for biological matrices exacerbates this issue further [44]. In-house standards, such as homogenized tissues [40] or gelatin [45] spiked with known element concentrations, provide a partial solution in single-cell LA-ICP-MS. Mervič et al. reported that correcting for ablated volume is effective for quantitative LA analysis even when using non-matrix matched standards [46]. Galvanometric optics enable the generation of calibration curves across appropriate concentration ranges, from trace to major components, by mixing standards with low and high elemental concentrations [7]. They also facilitate the homogeneous mixing of solid samples with different matrices, allowing for the application of the standard addition calibration method using commercially available glass standards [47]. However, applying standard addition method to single-cell samples remains challenging, as single-cell samples are entirely ablated in a single analysis. Consequently, accurate quantification of elemental content in single-cell LA-ICP-MS remains a considerable analytical hurdle.

Cellular applications

While early applications of LA for single-cell elemental analysis were limited, its use has been steadily increasing. Studies investigating the cellular uptake of gold nanoparticles [48] and cisplatin [49] in mammalian cultured cells have demonstrated the technique's potential. Theiner et al. employed two-dimensional mapping on a blood smear, evaluating the elemental content of individual blood cells [50]. Their findings revealed distinct elemental

signatures, with red blood cells exhibiting high Fe content and white blood cells characterized by high P content, demonstrating the capability of LA to differentiate cell types based on their elemental composition.

Using LA-based scICP-MS analysis, we recently investigated the changes in elemental content during cell differentiation [51]. Rat adrenal pheochromocytoma PC12 cells, upon treatment with nerve growth factor (NGF), differentiate into neuron-like cells. However, the expression of differentiation marker proteins remains low after five days of NGF treatment. By distinguishing mature and less mature neuron-like cells by size, we performed separate LA-ICP-MS analyses. Our results revealed a clear correlation between cell size and elemental content: large differentiated cells exhibited significantly higher levels of essential elements than undifferentiated PC12 cells, whereas small differentiated cells showed elemental content comparable to undifferentiated cells (**Fig. 4**).

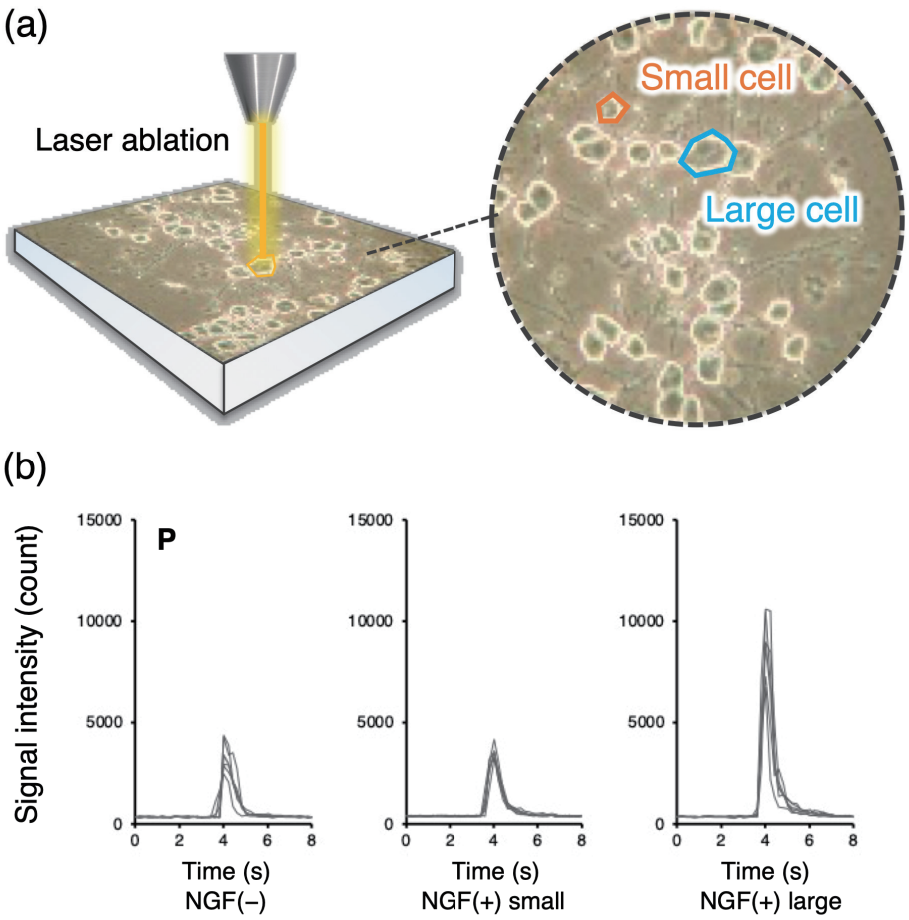

Fig. 4. **Single-cell-based selection and analysis of PC12 cells by LA-ICP-MS (Data from reference [51]).**
(a) Schematic diagram illustrating the selective measurement of PC12 cells by size as a differentiation marker. (b) Phosphorus (P) signal intensity measured by LA-ICP-MS. Phosphorus content in small differentiated cells was comparable to that observed in undifferentiated cells.

Conclusion

We have presented an overview of single-cell elemental analysis using ICP-MS. While fast time-resolved analysis offers broad applicability across various cell types and has facilitated numerous applications, the impact of nebulization on the structural integrity of fragile mammalian cells has not been adequately addressed. Therefore, the development and implementation of standardized sample introduction systems, such as those employing

microdroplet generators, are crucial for the reliable analysis of these cell types. Although LA applications in single-cell analysis are currently limited, ongoing instrumental advancements are promising and offer the potential for robust quantitative single-cell elemental analysis.

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