Serial Review

Copper Biology in Health and Disease
Guest Editor: Hirokazu Hara

Recent advances in copper analyses by inorganic mass spectrometry

Yasumitsu Ogra,* Yu-ki Tanaka, and Noriyuki Suzuki

Laboratory of Toxicology and Environmental Health, Graduate School of Pharmaceutical Sciences, Chiba University, 1-8-1 Inohara, Chuo-ku, Chiba 260-8675, Japan

(Received 24 December, 2021; Accepted 24 January, 2022; Released online in J-STAGE as advance publication 31 March, 2022)

Copper (Cu) participates in the biological redox reaction in the body, and its deficiency is fatal to the body. At the same time, Cu is extremely toxic when it exists in excess. Thus, the body has to tightly and spatiotemporally regulate the concentration of Cu within a physiological range by several groups of Cu-regulating proteins. However, entire mechanisms underlying the maintenance of Cu homeostasis in body and cells have not fully understood. It is necessary to analyze Cu itself in a body and in a cell to reveal the Cu homeostasis. In this review, recent advances in the analytical techniques to understand the Cu metabolism such as speciation, imaging and single-cell analysis of Cu were highlighted.

***Key Words*: copper, speciation, imaging, laser ablation, ICP-MS**

Copper (Cu) is an essential metal for living organisms that is required as a cofactor of redox-regulating enzymes, such as superoxide dismutase (Sod1), lysyl oxidase, tyrosinase, dopamine β-hydroxylase, and ceruloplasmin.[1,2] Cu is also a harmful metal in body and cells. Cu in the body is present in the form of either mono- (cuprous, Cu^{+}) or divalent (cupric, Cu^{2+}) state. Cuprous ions are readily oxidized to cupric ions and Cu cannot exist in the form of cuprous ions without being coordinated by appropriate ligands. In other words, Cu in the monovalent form readily reduces chemicals as in the case of the production of ROS. Thus, Cu is tightly and spatiotemporally controlled by several Cu-regulating factors. However, entire mechanisms underlying Cu homeostasis in body and cells have not fully understood, and many researchers are tackling to reveal the mechanisms at molecular level. Molecular-biological techniques to analyze genes and proteins provided many new insights in the research field. In addition, the techniques which detect Cu directly are expected to pave a road toward further insights.

In this review, recent advances in techniques for understanding the Cu metabolisms, in particular, applications using inductively coupled plasma mass spectrometry (ICP-MS), were highlighted. In our opinion, there are three advanced applications of ICP-MS. The first is ICP-MS hyphenated with high performance liquid chromatography (LC-ICP-MS), namely speciation. Speciation is not newly developed technique, however, the recent improvements of ICP-MS in terms of sensitivity, specificity, and robustness pave a road to new application of speciation analysis. Second, laser ablation (LA) is used for the sample introduction of ICP-MS. This technique makes elemental imaging possible. The third is single cell-ICP-MS (SC-ICP-MS). Recently, ICP-MS with a fast time-resolved mode has been developed, and has been applied to the analysis of particle materials such as nanoparticles (NPs) and living cells. We summarize some applications of these three advanced ICP-MS to reveal the Cu metabolism (Fig. 1).

Speciation

According to the recommendation of International Union of Pure and Applied Chemistry interdivisional working party, "speciation" is described as the distribution of an element amongst defined chemical species in a system.[3] The term of "speciation analysis" denotes the analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample.[3] For the Cu speciation, ICP-MS has some advantages over other detectors in terms of the sensitivity and the discrimination of Cu isotopes, i.e., ^{63}Cu and ^{65}Cu. Indeed, Cu speciation in biological samples by LC-ICP-MS was reported.[4–7] However, early LC-ICP-MS has an inevitable disadvantage. It requires a substantial volume of sample at μl level when a conventional size of HPLC column is used. Under the condition when a conventional LC-ICP-MS is adopted, the injection volume and flow rate are 20–200 μl and 0.6–1.0 ml/min, respectively, in our previous experiments. Since the flow rate of a conventional HPLC is comparable to the flow rate of sample introduction into an ICP-MS, the eluate can be directly introduced into an ICP-MS without splitting or addition of sheath flow. This is simultaneously a strong point and a weak point. Namely, although the direct introduction does not reduce the sensitivity of ICP-MS, such a large volume of sample requirement limits an applicable sample to an early conventional LC-ICP-MS. Indeed, massively acquirable samples such as blood plasma, tissue extract, and urine have been analyzed.

Recent ICP-MS has been improved in terms of sensitivity, it can be hyphenated with a narrow bore HPLC column without severe decrease in sensitivity. In addition to suppressing the diffusion of the sample during separation in narrow bore HPLC (compared to conventional HPLC), a small volume of eluent is efficiently introduced into the ICP by a micronebulizer and a high efficient spray chamber. The introduction system contributes to avoid the decrease in the sensitivity of ICP-MS detection. Narrow bore LC-ICP-MS was developed to analyze a minute amount of tissue extract, and the relationship between the amount

*To whom correspondence should be addressed.
E-mail: ogra@chiba-u.jp

doi: 10.3164/jcbn.21-170

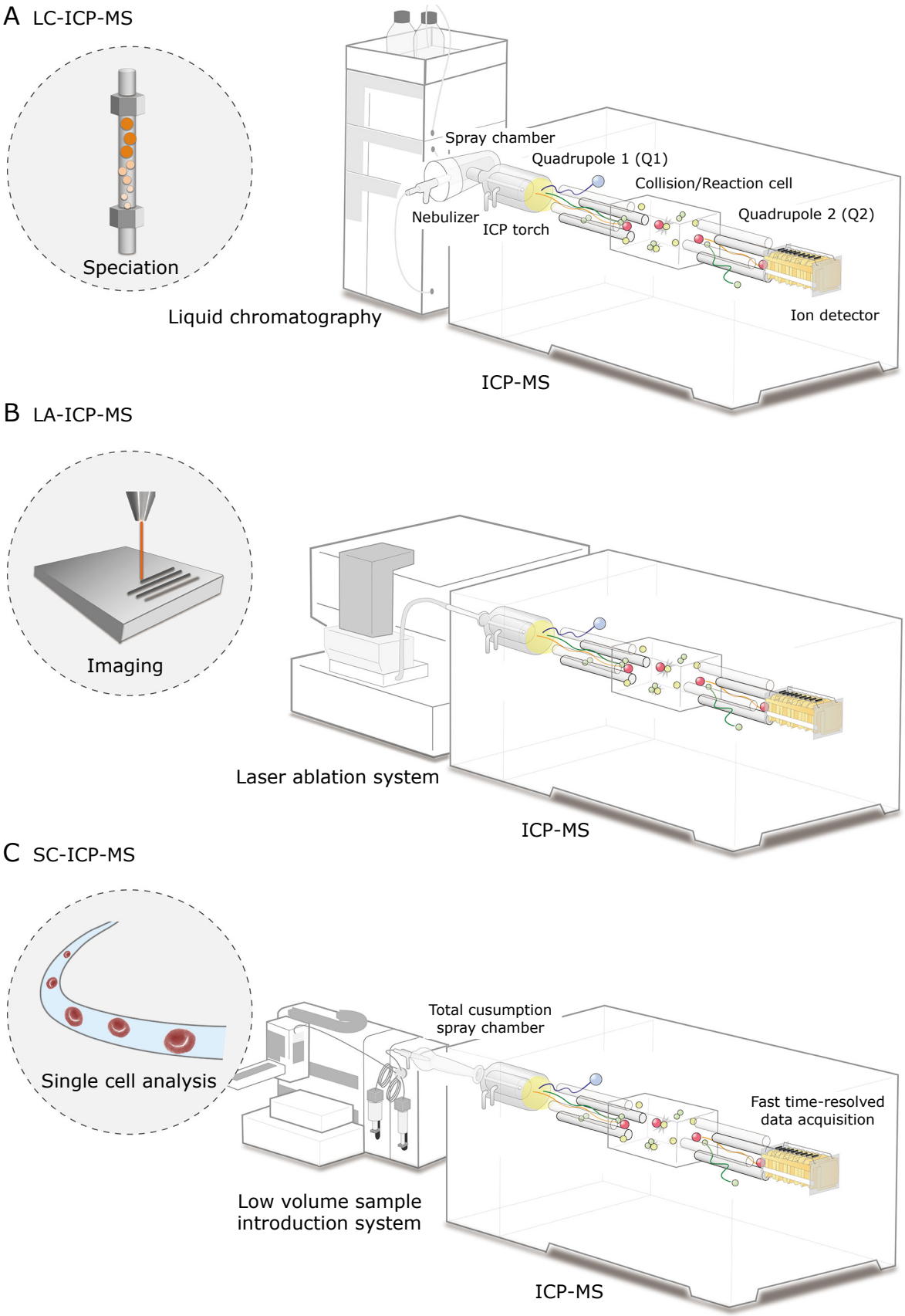

Fig. 1. Advanced techniques using an inductively coupled plasma mass spectrometer for copper analysis in biological samples. Speciation by LC-ICP-MS (A), Imaging by LA-ICP-MS (B), and Single cell analysis by SC-ICP-MS (C).

of Cu in the metallothionein-bound form (Cu-MT) and MT mRNA expression was evaluated to reveal Cu metabolism in the mutant animal model.[8,9] The hemizygote bearing a mutation in Atp7a located on the X chromosome, i.e., the male blotchy mouse shows typical symptoms of Cu deficiency. Due to the Cu deficiency, the mouse shows severe growth retardation and dies before weaning. Thus, the organs from this neonatal mouse are too small to be analyzed by a conventional LC-ICP-MS. The narrow bore LC-ICP-MS was operated under the following conditions; the injection volume and the flow rate were 1.0–5.0 µl and 40 µl/min, respectively. The results for the Cu distribution in a cytosolic fraction obtained by the narrow bore LC-ICP-MS showed that the male blotchy mouse presented the systemic Cu deficiency except the intestine and the kidneys.[10] The kidney of blotchy mouse accumulated Cu in the form bound to MT comparing to the control mouse (Fig. 2A). Contrary, the liver of blotchy mouse showed Cu deficiency (Fig. 2B). In a normal neonate, Cu is accumulated in the liver in a form of bound to MT.[9] Cu bound to MT was specifically lowered in a blotchy neonate although Cu bound to superoxide dismutase 1 (SOD1) was not altered. These results indicate that MT acts as a cellular pool of Cu in organs because Cu is preferably delivered to a Cu enzyme rather than MT. As indicated above, Cu speciation is a useful technique to reveal the Cu metabolism.

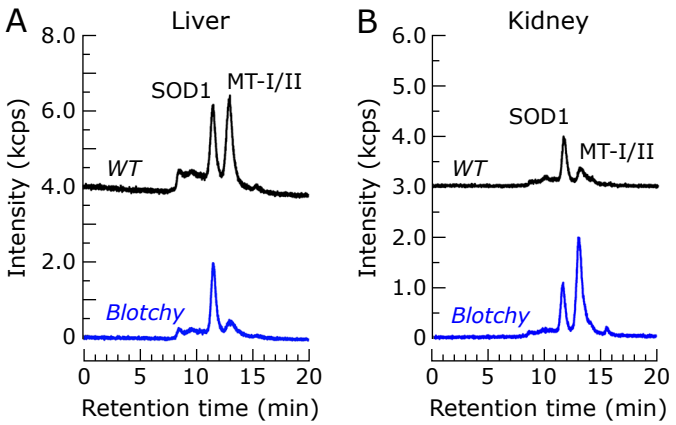

Fig. 2. Elution profile of Cu in the supernatant of wild type and blotchy mutant (Atp7a deficient) mice. The tissue supernatant was prepared from the liver (A) and the kidney (B) of wild type (upper lines) and blotchy (lower lines) mice.

Imaging

Bioimaging of metals, i.e., mapping the distribution of metals in tissue specimen and a cell is an effective technique to reveal biological significance of metals. The techniques to perform metal bioimaging are primarily divided into two categories. One is the techniques using a chemical probe which specifically react with an individual metal. We reported that the Cu (I) distribution in the cells bearing knockdown of Atox1 or Commd1 were determined by a Cu (I)-specific fluorescent probe, CS1.[11,12] Since Professor Okuda reviews the Cu imaging probes in this Serial Review, please read his article for further information.

The other is the technique using specific analytical instruments. For instance, laser ablation coupled with an ICP-MS (LA-ICP-MS), scanning X-ray fluorescence microscopy (SXFM) and secondary ion mass spectrometry (SIMS) belong to the category. Recently, there are several excellent reviews for LA-ICP-MS,[13–15] SXFM, and SIMS.[16–18] Some researches focused on Cu imaging. The Cu distribution was analyzed by LA-ICP-MS.[19–23] The Cu, Zn, and Fe distributions in the fibroblasts established from Atox1-deficient mice were visualized by SXFM.[24] These techniques require the special instrumentations, e.g., a synchrotron source for SXFM and a laser ablation system with an interface to ICP-MS for LA-ICP-MS, although they are more specific to each metal than chemical probes. However, LA-ICP-MS can be more easily set up than other instrumental techniques, thus, it is expected that LA-ICP-MS becomes a more easily accessible technique for metal imaging.

Single Cell Analysis

Recent ICP-MS can be operated in a fast time-resolved mode, and this mode is applicable to single cell- (SC-)ICP-MS. In this analytical mode, cell suspension is directly introduced into ICP through a specially customized nebulizer and spray chamber. The cells are decomposed, atomized, and finally ionized in ICP, then plume of the ions derived from a single cell passes detector within 1 ms, which is quite shorter period than signal integration time used in a conventional ICP-MS measurement (10–100 ms). The contents of the endogenous element of dry yeast were successfully measured by SC-ICP-MS.[25,26] SC-ICP-MS gives us elemental contents in a cell as a histogram (Fig. 3). For mammalian cell characterization, the cellular Cu content in human RBCs was determined.[27] Culture cells such as A549, HeLa, and 16HBE have been investigated by SC-ICP-MS analysis, and several elements such as P, S, Fe, Zn, Cu, and Mn

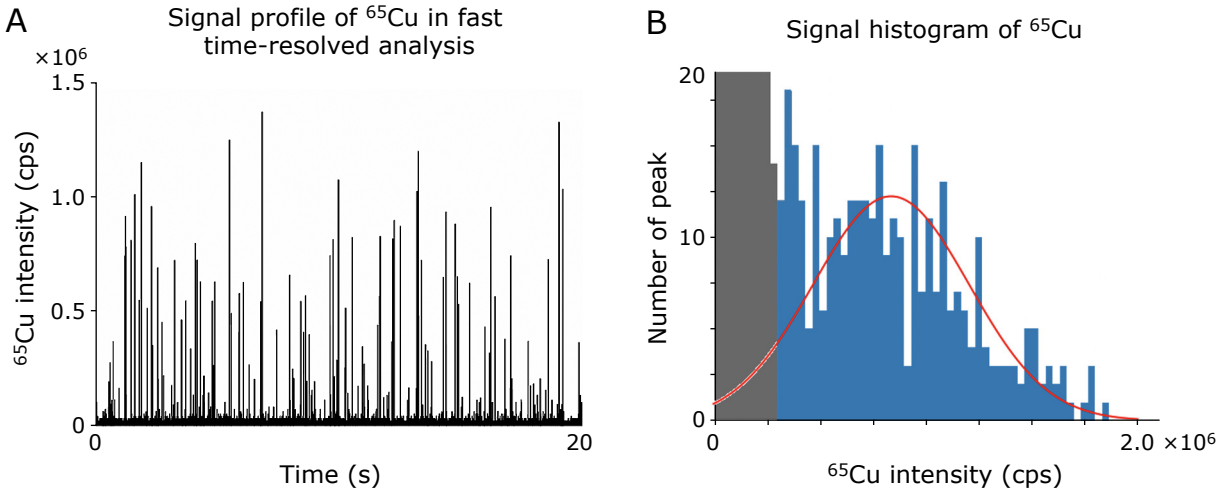

Fig. 3. Transient signals and histogram of metal content obtained by SC-ICP-MS.

doi: 10.3164/jcbn.21-170

have been quantitatively measured in the cells.[28] SC-ICP-MS was also used to evaluate the effects of Cu-based algaecide on the toxic algae, *Microcystis aeruginosa*.[29] The toxic effects of arsenic (As) were evaluated using *Chlamydomonas reinhardtii* and A549 cells.[12,30] SC-ICP-MS has also been used to detect inorganic NPs or quantum dots in biological tissue and cells.[31,32] Namely, elemental data (i.e., signal intensity and number of signals) are useful to evaluate the metabolism and the transportation mechanism of nano-sized materials. Therefore, the development of a metal analysis technique for the analysis of single cells would be useful to study both the physiological and nutritional importance and toxicological effects of metal NPs. This technique is called single particle (SP-)ICP-MS. However, it is not readily feasible to detect NPs in biological samples consisting of complicate matrices. SP-ICP-MS for biological samples is mainly reported for plant samples. For instance, isotopically enriched Cu, silver, and zinc oxide NPs in *Arabidopsis thaliana* were detected by SP-ICP-MS.[33] Although SC-ICP-MS should be more sophisticated, it could be a potential emerging technique for metabolism of metals.

Conclusion

The instrumental techniques for Cu biology are continuously developing. The miniaturized speciation could be evolving to single-cell-speciation, namely, the technique that allows analyzing entire Cu-containing species in a single cell. The development of LA-ICP-MS in terms of spatial resolution and sensitivity is one of the fruitful topics for both analytical chemists and biologists. Further advanced techniques may provide novel insights into the research field of Cu biology.

Acknowledgments

This study was financially supported by JSPS KAKENHI grant (Nos. 19H01081, 19K16346, 19H05772, and 21H04920).

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Kim BE, Nevitt T, Thiele DJ. Mechanisms for copper acquisition, distribution and regulation. *Nat Chem Biol* 2008; **4**: 176–185.
2 Balamurugan K, Schaffner W. Copper homeostasis in eukaryotes: teetering on a tightrope. *Biochim Biophys Acta* 2006; **1763**: 737–746.
3 Templeton D, Ariese F, Cornelis R, *et al*. Guidelines for terms related to chemical speciation and fractionation of elements. Definitions, structural aspects, and methodological approaches. *Pure Allp Chem* 2000; **72**: 1453–1470.
4 Inagaki K, Mikuriya N, Morita S, *et al*. Speciation of protein-binding zinc and copper in human blood serum by chelating resin pre-treatment and inductively coupled plasma mass spectrometry. *Analyst* 2000; **125**: 197–203.
5 Mestek O, Komínková J, Koplík R, Zima T, Miskusová M, Stern P. Speciation of Cu, Se, Zn and Fe in blood serum of hemodialysed patients. *Sb Lek* 2002; **103**: 23–27.
6 Van Campenhout K, Infante HG, Adams F, Blust R. Induction and binding of Cd, Cu, and Zn to metallothionein in carp (*Cyprinus carpio*) using HPLC-ICP-TOFMS. *Toxicol Sci* 2004; **80**: 276–287.
7 Wuilloud RG, Kannamkumarath SS, Caruso JA. Speciation of nickel, copper, zinc, and manganese in different edible nuts: a comparative study of molecular size distribution by SEC-UV-ICP-MS. *Anal Bioanal Chem* 2004; **379**: 495–503.
8 Miyayama T, Ogra Y, Osima Y, Suzuki KT. Narrow-bore HPLC-ICP-MS for speciation of copper in mutant mouse neonates bearing a defect in Cu metabolism. *Anal Bioanal Chem* 2008; **390**: 1799–1803.
9 Tanaka YK, Ogra Y. Evaluation of copper metabolism in neonatal rats by speciation analysis using liquid chromatography hyphenated to ICP mass spectrometry. *Metallomics* 2019; **11**: 1679–1686.
10 Miyayama T, Ogra Y, Osima Y, Suzuki KT. Narrow bore HPLC-ICP-MS for speciation of copper in mutant mouse neonates bearing a defect in Cu metabolism. *Anal Bioanal Chem* 2008; **390**: 1799–1803.
11 Miyayama T, Hiraoka D, Kawaji F, Nakamura E, Suzuki N, Ogra Y. Roles of COMM-domain-containing 1 in stability and recruitment of the copper-transporting ATPase in a mouse hepatoma cell line. *Biochem J* 2010; **429**: 53–61.
12 Miyayama T, Suzuki KT, Ogra Y. Copper accumulation and compartmentalization in mouse fibroblast lacking metallothionein and copper chaperone, Atox1. *Toxicol Appl Pharmacol* 2009; **237**: 205–213.
13 Becker JS, Jakubowski N. The synergy of elemental and biomolecular mass spectrometry: new analytical strategies in life sciences. *Chem Soc Rev* 2009; **38**: 1969–1983.
14 Becker JS, Matusch A, Palm C, Salber D, Morton KA, Becker JS. Bioimaging of metals in brain tissue by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) and metallomics. *Metallomics* 2010; **2**: 104–111.
15 Becker JS, Zoriy M, Matusch A, *et al*. Bioimaging of metals by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). *Mass Spectrom Rev* 2010; **29**: 156–175.
16 Fahrni CJ. Biological applications of X-ray fluorescence microscopy: exploring the subcellular topography and speciation of transition metals. *Curr Opin Chem Biol* 2007; **11**: 121–127.
17 McRae R, Bagchi P, Sumalekshmy S, Fahrni CJ. *In situ* imaging of metals in cells and tissues. *Chem Rev* 2009; **109**: 4780–4827.
18 Boxer SG, Kraft ML, Weber PK. Advances in imaging secondary ion mass spectrometry for biological samples. *Annu Rev Biophys* 2009; **38**: 53–74.
19 Hachmöller O, Zibert A, Zischka H, *et al*. Spatial investigation of the elemental distribution in Wilson's disease liver after d-penicillamine treatment by LA-ICP-MS. *J Trace Elem Med Biol* 2017; **44**: 26–31.
20 Müller JC, Lichtmannegger J, Zischka H, Sperling M, Karst U. High spatial resolution LA-ICP-MS demonstrates massive liver copper depletion in Wilson disease rats upon Methanobactin treatment. *J Trace Elem Med Biol* 2018; **49**: 119–127.
21 Kysenius K, Hilton JB, Paul B, Hare DJ, Crouch PJ. Anatomical redistribution of endogenous copper in embryonic mice overexpressing SOD1. *Metallomics* 2019; **11**: 141–150.
22 Feng L, Wang J, Li H, Luo X, Li J. A novel absolute quantitative imaging strategy of iron, copper and zinc in brain tissues by Isotope Dilution Laser Ablation ICP-MS. *Anal Chim Acta* 2017; **984**: 66–75.
23 Wang LM, Becker JS, Wu Q, *et al*. Bioimaging of copper alterations in the aging mouse brain by autoradiography, laser ablation inductively coupled plasma mass spectrometry and immunohistochemistry. *Metallomics* 2010; **2**: 348–353.
24 McRae R, Lai B, Fahrni CJ. Copper redistribution in Atox1-deficient mouse fibroblast cells. *J Biol Inorg Chem* 2010; **15**: 99–105.
25 Groombridge AS, Miyashita S, Fujii S, *et al*. High sensitive elemental analysis of single yeast cells (*Saccharomyces cerevisiae*) by time-resolved inductively-coupled plasma mass spectrometry using a high efficiency cell introduction system. *Anal Sci* 2013; **29**: 597–603.
26 Liu Z, Xue A, Chen H, Li S. Quantitative determination of trace metals in single yeast cells by time-resolved ICP-MS using dissolved standards for calibration. *Appl Microbiol Biotechnol* 2019; **103**: 1475–1483.
27 Cao Y, Feng J, Tang L, Yu C, Mo G, Deng B. A highly efficient introduction system for single cell- ICP-MS and its application to detection of copper in single human red blood cells. *Talanta* 2020; **206**: 120174.
28 Wang H, Wang B, Wang M, *et al*. Time-resolved ICP-MS analysis of mineral element contents and distribution patterns in single cells. *Analyst* 2015; **140**: 523–531.
29 Shen X, Zhang H, He X, *et al*. Evaluating the treatment effectiveness of copper-based algaecides on toxic algae Microcystis aeruginosa using single cell-inductively coupled plasma-mass spectrometry. *Anal Bioanal Chem* 2019; **411**: 5531–5543.

30 Meyer S, López-Serrano A, Mitze H, Jakubowski N, Schwerdtle T. Single-cell analysis by ICP-MS/MS as a fast tool for cellular bioavailability studies of arsenite. *Metallomics* 2018; **10**: 73–76.
31 Zheng LN, Wang M, Wang B, *et al*. Determination of quantum dots in single cells by inductively coupled plasma mass spectrometry. *Talanta* 2013; **116**: 782–787.
32 Wang H, Wang M, Wang B, *et al*. Interrogating the variation of element masses and distribution patterns in single cells using ICP-MS with a high efficiency cell introduction system. *Anal Bioanal Chem* 2017; **409**: 1415–1423.
33 Nath J, Dror I, Landa P, Vanek T, Kaplan-Ashiri I, Berkowitz B. Synthesis and characterization of isotopically-labeled silver, copper and zinc oxide nanoparticles for tracing studies in plants. *Environ Pollut* 2018; **242 (Pt B)**: 1827–1837.

doi: 10.3164/jcbn.21-170