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In situ Elemental Analysis of Frog Eggs and Oocytes by LA-ICP-MS

[1]Hannigan, R., [2]Machaca, K., and [2]Sun, L; [1] Arkansas State University Dept. of Chemistry, State University AR 72467; hannigan@astate.edu; [2] University of Arkansas Medical School Dept. of Physiology, Little Rock AR 72205; kamchaca@uams.edu

ABSTRACT

Laser ablation (Nd:YAG 266 nm) coupled with a dynamic reaction cell quadrupole based ICP-MS (LA-DRC-ICP-MS) was used to quantify changes in elemental distribution during fertilization of *Xenopus laevis* eggs. Changes in the distribution of Ca^{2+} and Na^{+} are known to occur rapidly after activation to prevent polyspermy. However previous studies have relied on whole egg digestion, membrane ionophoresis, or confocal microscopy. While these techniques provide information about broad changes in, for example, Ca^{2+} distribution within the membrane at the time of fertilization these techniques do not allow for in situ analysis of elemental distributions at prior to, during, and soon after activation. LA-ICP-MS allows for rapid in situ elemental analysis and quantification of concentrations within the egg and stimulated oocytes. We focused our experiments on the distribution of major ions (e.g., Na^{+} and Ca^{2+}) and minor ions (Cu^{2+} and Fe (total)) prior to and after stimulation in both the animal and vegetable poles. We used a "matrix matched" standard (Lake Superior Fish Tissue) and the carbonate standard (MACS-1) for calibration and quantification. Data was collected from three 50 um spots each pole of each egg/oocyte/standard at 25% energy, 20Hz, and a scan rate of 100 ms. The DRC-ICP-MS conditions were optimized to obtain the best signal-to-noise and signal-to-background ratios using the tissue standard. Our data indicate that this method provides unique insights into the ionic changes occurring withi
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INTRODUCTION

Laser ablation ICP-MS is routinely used for the determination of major, minor, and trace element concentrations in solid media. More and more this technique is applied for studies of spatial variability in the chemistry of solid biological tissues such as fish ear stones (otoliths; Campana, 2005), corals (e.g., Swart and Grottoli, 2003), and (e.g., Uryu et al., 2003). More recently the capabilities of LA-ICP-MS to spatially resolve chemical variability in softer biological tissues has been explored (e.g., Harden and Hannigan, 2004; Harden, 2004; Legrand et al., 2004).

The development of LA-ICP-MS for the study of elemental variations in soft biological tissues has been limited by the need for the biological community to be able to quantify the concentrations of the analytes of interest. In many cases the relative abundances of metals provide limited to no information regarding fundamental biological processes.

The purpose of this study was to develop an LA-ICP-MS method that would provide quantitative information regarding the concentration of Ca, Cu, and Zn to allow the elucidation of cell cycle progression during oocyte maturation.

SIGNIFICANCE

Cell cycle progression is essential for the development of multicellular organisms and when disrupted leads to serious consequences such as cancer. Transition metals are essential for cellular survival as they are structural elements of many important proteins, yet their concentration needs to be carefully controlled because they are toxic at high doses (Vallee and Falchuk, 1993). Finney and O'Halloran (2003) argue that regulation of Cu^{2+} and Zn^{2+} is dynamic and that these metals play no other non-structural roles. Cellular concentrations of these metals are low and are difficult to measure using traditional solution based atomic spectroscopic methods. Much more is known about Ca^{2+} signaling due to the higher cellular abundance of this metal and the development of fluorescent dyes and detection systems (Tsien, 1981; Poenie et al., 1985; Grynkiewicz et al., 1985).

Cellular free transition metal concentrations are undetectable experimentally arguing that these metals are bound to their target proteins or chelated and transported by carrier proteins. Given that Cu and Zn are known to play a dynamic role in regulation it is critical that the cellular distribution of these metals be quantified.

METHODS

Taking advantage of the large size of *Xenopus* oocytes (Figure 1) we developed a highly sensitive and reproducible method that allows us to map the transition metal content at the subcellular level over time. We analyzed the animal and vegetal poles of unfertilized eggs and stimulated oocytes using a DRC II ICP-MS (PerkinElmer) and a 266 nm Nd:YAG laser ablation system (Cetac Technologies) (Figure 2). Instrument parameters are shown in Table 1.

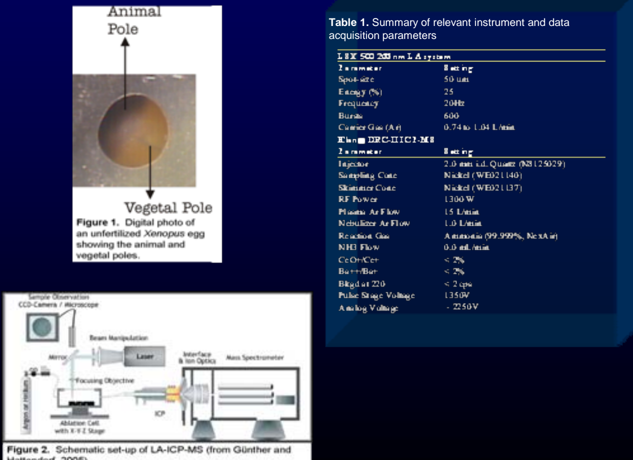


Figure 1. Digital photo of an unfertilized *Xenopus* egg showing the animal and vegetal poles.

Table 1. Summary of relevant instrument and data acquisition parameters

LSX 500 266 nm LA system	
Parameter	Setting
Spot-size	50 um
Energy (%)	25
Frequency	20Hz
Bursts	600
Carrier Gas (Ar)	0.74 to 1.04 L/min
Elan DRC-II ICP-MS	
Parameter	Setting
Injector	2.0 mm i.d. Quartz (N8125029)
Sampling Cone	Nickel (WE021140)
Skimmer Cone	Nickel (WE021137)
RF Power	1300 W
Plasma Ar Flow	15 L/min
Nebulizer Ar Flow	1.0 L/min
Reaction Gas	Ammonia (99.999%, NexAir)
NH3 Flow	0.0 mL/min
CeO+/Ce+	< 2%
Ba++/Ba+	< 2%
Bkgd at 220	< 2 cps
Pulse Stage Voltage	1350V
Analog Voltage	- 2250V

Figure 2. Schematic set-up of LA-ICP-MS (from Günther and Hattendorf, 2005).

RESULTS

In order to quantify metal concentrations in the oocytes we used a matrix matched standard, NIST 1946 (Lake Superior Fish Tissue). This tissue is freeze-dried and kept at -80°C until used. The solid frozen tissue was ablated directly. To ensure accuracy the tissue was also calibrated using solution-based ICP-MS. The measured solution-based NIST 1946 metal values were within < 5% of the reported values. For use in ablation the NIST 1946 tissue standard was run as an unknown and calibrated against NIST 612 (glass) and MACS-1 (USGS carbonate). LA-ICP-MS measurements of the metals of interest (Ca, Cu, Zn, Pb, and Mn) were found to be within 8% of the reported NIST 1946 values.

Initial tuning of the ion lens was undertaken monitoring C-12 in the Ar carrier gas, followed by fine-tuning during ablation of NIST 612. Laser operating conditions were optimized for a maximum signal at Ce-140 and for best analytical precision. Data were acquired with analyses performed in triplicate. No pre-ablation was performed since this can result in the loss of volatile elements such as lead (Williams and Jarvis, 1993).

Oocytes were fixed in ethanol immediately before laser ablation, because living oocytes lose cellular integrity following laser ablation. In contrast a fixed oocyte can withstand multiple laser ablations without any general deleterious effects (Figure 3).

LA-ICP-MS Calibrations

Figures 4 shows the calibrations of NIST 1946 for the analytes of interest. The calibrations represent the counts in each of three 50um ablations (35-s integration). The blank intensities are based on measurements of the gas blank prior to ablation (15-s integration). We selected Zn-66 as the internal standard. Using this isotope of Zn both short-term precision, indicated by relative errors and long-term reproducibility were improved. Internal standardization correct not only for variable signal intensity caused by inconsistent quantities of material being ablated but also for the general loss of signal sensitivity through time, and is therefore essential for reliable quantitative determination. as its concentration in eggs and oocytes is well constrained and is ubiquitous throughout the sample as well as at similar concentrations to that found in the NIST 1946 fish tissue standard.

Detection limits were calculated from LA-ICP-MS measurements of the gas blank and NIST 612/1946 (Table 2). LA-ICP-MS intensities were converted to concentrations based on linear interpolation using GeoPro (Figure 5).

Table 2. Detection limits of LA-ICP-MS using gas blanks and NIST 612/1946.

Element	Instrumental[a]
Ca	0.027
Mn	0.0014
Cu	0.006
Fe	0.068
Zn	0.008
Pb	0.0015

[a] Calculated from 3 replicate analyses of gas blanks using NIST 612 as a response standard

Element	Analytical[b]
Ca	0.018
Mn	0.0018
Cu	0.008
Fe	0.053
Zn	0.01
Pb	0.025

[b] Calculated from 10 replicate analyses of NIST 1946 using NIST 612 as a response standard

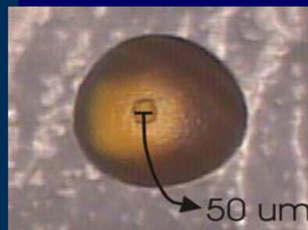


Figure 3. 50 um spot on vegetal hemisphere of a stimulated fixed egg.

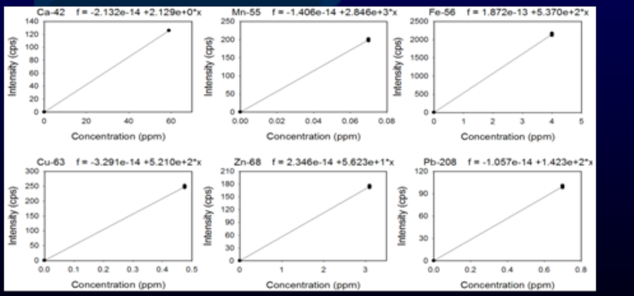
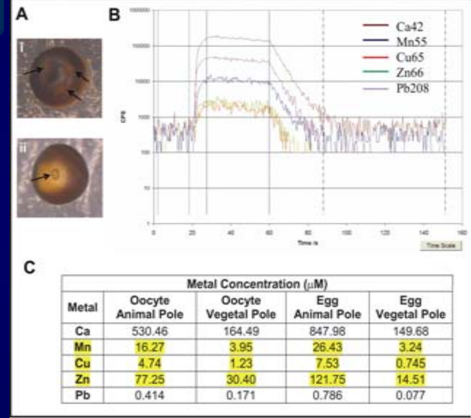


Figure 4. Calibration of NIST 1946 fish tissue. The blank is measured as a gas blank. Each sample calibration point is an average of signal of three individual 50um spots.

DISCUSSION AND CONCLUSION

We successfully used LA-ICP-MS to study the metal content of *Xenopus* oocytes (Figure 5). The results of this study show differences in the spatial distribution of metals between the animal and vegetal hemispheres as well as between eggs and oocytes. Focusing our attention on Zn, Cu, and Mn, it is compelling that the concentrations of these ions is higher in the animal versus the vegetal hemisphere (Figure 5 C, highlighted). The animal pole in the egg is the site of sperm entry and where the arrested metaphase II resides, and thus where the maternal and paternal pronuclei migrate and unite. The vegetal hemisphere is the storage site for yolk granule, the energy source of for the growing embryo. Although transition metal concentrations are higher (2-3 fold) in the animal vs. vegetal pole of the immature oocyte, this difference is greatly accentuated in the egg (on the order of 10 fold). It is tempting to speculate that this concentration of transition metals in the animal pole may reflect the localization of the cellular machinery that is important for fertilization and early embryogenesis, which may be enriched in transition metals. However, a more detailed and complete analysis is required to obtain a comprehensive coverage of the cortical region of the oocyte and egg.

Future studies will involve the mapping of transition metal concentrations in internal regions of *Xenopus* oocytes and eggs. Although it is clear from the data presented in Figure 5 that the LA-ICP-MS technique is applicable to *Xenopus* oocytes we are constantly improving this methodology. Specific method development efforts are currently focusing on optimizing laser energy, spot size, wavelength (266 nm vs. 213 nm) and standardization (internal and external).



Metal	Metal Concentration (μM) Oocyte Animal Pole	Oocyte Vegetal Pole	Egg Animal Pole	Egg Vegetal Pole
Ca	530.46	164.49	847.98	149.68
Mn	16.27	3.95	26.43	3.24
Cu	4.74	1.23	7.53	0.745
Zn	77.25	30.40	121.75	14.51
Pb	0.414	0.171	0.786	0.077

Figure 5. LA-ICP-MS analysis of *Xenopus* oocytes and eggs. **A.** Images of oocyte animal pole (i) and vegetal pole (ii) after laser ablation and ICP-MS analysis. Oocytes and eggs were ablated using a 50 um spot which is visible on the surface of the samples (arrows). This allows multiple ablations on the same pole without affecting total cellular integrity. **B.** Example of raw data (intensity vs. time). Blank signal is isolated between the left-most two dashed lines. Sample intensity is isolated between the middle solid lines, and the matrix signal is isolated between the right-most dashed lines. Each sample was isolated identically with blank intensities from 2 to 18 seconds, sample from 25 to 60 seconds and matrix from 85 to 150 seconds. **C.** Summary of metal concentrations of the animal and vegetal hemispheres of both oocytes and eggs. Oocytes are the immature cells arrested in G2 and eggs are the cells that have completed maturation and are arrested in Metaphase II. Zn-66 was used as an internal standard for these experiments. These results represent the average of 5 eggs and 5 oocytes.

REFERENCES

1. Campana, S.E. 2005. Otolith elemental composition as a natural marker of fish stocks. P. 227-245. In: Cadrin, S.X., K.D. Friedland and J.R. Waldman [eds]. Stock Identification Methods. Academic Press, N.Y.
2. Finney, L.A. and O'Halloran, T.V. 2003. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science 300, 931-936.
3. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. 1985. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440-3450.
4. Günther, D. and Hattendorf, B. 2005. Solid sample analysis using laser ablation inductively coupled plasma mass spectrometry. Trends Anal. Chem., In Press.
5. Harden, W. 2004. Development of a Standard Method for Laser Ablation Inductively Coupled Plasma Mass Spectrometric Analysis of Human Hair. Arkansas State Univeristy MS Biology, Unpubl. Thesis.
6. Harden, W. and Hannigan, R. 2004. Analysis of Trace Metal Content in Human Hair Using Laser Ablation Inductively Coupled Plasma Mass Spectroscopy (LA-ICP-MS). FBI Laboratory Symposium on Forensic Toxicology and Joint Meeting of the Society of Forensic Toxicologists (SOFT) & The International Association of Forensic Toxicologists (TIAFT). Platform presentation. Aug 28 – Sept 3. Washington DC. [abstract].
7. Legrand, M., Lam, R., Jensen-Fontaine, M., Salin, E.D., and Chan, H.M. 2004. Direct detection of mercury in single human hair strands by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). J. Anal. At. Spectrom. 19, 1287 – 1288.
8. Swart, P.K. and Grottoli, Æ A. 2003. Proxy indicators of climate in coral skeletons: a perspective. Coral Reefs, 22, 313–315.
9. Poenie, M., Alderton, J.M., Tsien, R.Y., and Steinhardt, R.A. 1985. Changes of free calcium levels with stages of the cell division cycle. Nature 315, 147-149.
10. Tsien,R.Y. 1981. A non-disruptive technique for loading calcium buffers and indicators into cells. Nature 290, 527-528.
11. Uryu, T., Yoshinaga, J., Yanagisawa, Y., Endo, M., and Takahashi, J. 2003. Analysis of lead in tooth enamel by laser ablation-inductively coupled plasma-mass spectrometry. Anal. Sci. 19, 1413-1416.
12. Vallee, B.L. and Falchuk, K.H. 1993. The biochemical basis of zinc physiology. Physiol Rev. 73, 79-118.
13. Williams, J. G. and Jarvis, K. E. 1993. Preliminary assessment of laser ablation inductively coupled plasma mass spectrometry for quantitative multi-element determination in silicates. J. Anal. Atom. Spectrom. 8, 25-34. 1993.

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