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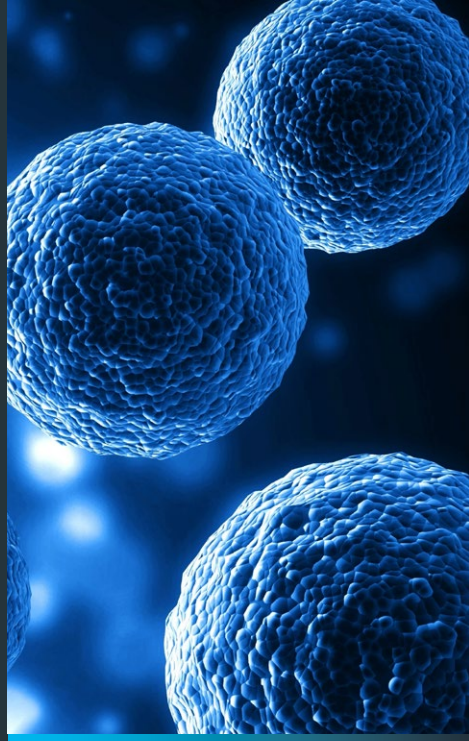

Quantification of copper content in Peripheral Blood Mononuclear Cell (PBMC) one Cell at a time using Single Cell ICP-MS

CASE STUDY

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INTRODUCTION

Copper (Cu) is present in all organisms and is one of the essential elements necessary for survival, as it is important for development and maintenance of many organs. Copper is also involved in the formation of red blood cells, the absorption and utilization of iron, the metabolism of cholesterol and glucose, and the synthesis and release of important proteins and enzymes [1]. These enzymes, in turn, produce cellular energy and regulate nerve transmission, blood clotting, and oxygen transport. Copper stimulates the immune system to fight infections, repair injured tissues, and to promote healing. Copper also helps to neutralize free-radicals, which can cause severe damage to cells [2].

On the other hand, copper can catalyze oxidation-reduction (redox) reactions, which can inadvertently lead to the production of reactive oxygen species (ROS) [3], which can result in cellular damage. For this reason, copper content is strictly regulated in the organism by a very efficient and complicated homeostasis mechanism that controls the uptake, distribution and excretion of the element. Present data indicates that the current model of cellular Cu homeostasis is a “skeleton” that requires many new details [4]. A disorder or imbalance in the homeostasis mechanism can lead to abnormalities in the functioning of an organism and can be connected with some diseases (e.g. inflammation, asthma, ageing processes, cancer) [5].

The metal content of cells is traditionally estimated by the total digestion of a cell population followed by analysis with ICP-MS. This type of analysis gives an average value of metal content per cell over the entire cell population [6]. However, direct measurement of the copper concentrations in individual cells has not been possible due to instrumental limitations. Recent advances in single cell ICP-MS (SC-ICP-MS) allow for the metal concentration of individual cells to be measured directly on an individual cell basis.

In this study we use SC-ICP-MS to measure the Cu content in Peripheral Blood Mononuclear Cells (PBMCs). PBMCs are any peripheral blood cell having a round nucleus; it is a population of blood cells that is composed mostly of lymphocytes and monocytes. The average diameter of PBMC cells is 7 µm. PBMC cells are easy to isolate and are widely used in research in the field of new drug and vaccine development as well as in immunology, oncology and epidemiology [7].

EXPERIMENTAL SAMPLE PREPARATION

PBMC cells were isolated from whole blood directly after the blood was taken. Whole blood was centrifuged for 10 minutes at 1000 g , with the break off, in a Leucosep™ tube (GREINER BIO-ONE) with addition of Ficoll paque PLUS (GE Healthcare). This is a hydrophilic polysaccharide that separates layers of blood, and during gradient centrifugation, it separates the blood into a top layer of plasma, followed by a layer of PBMCs and a bottom fraction of polymorphonuclear cells (such as neutrophils and eosinophils) and erythrocytes.

Figure 1. **NexION 2000 ICP-MS equipped with PerkinElmer Single Cell Sample Introduction Kit and Single Cell Aut osa mpler.**

After centrifugation the PBMC layer was transferred into new 50 mL autosampler tubes and washed twice in PBS. Directly before measuring with SC-ICP-MS, the cells were centrifuged (300 g, 10 minutes) and the cell pellet was suspended in Ringer's solution. The cell concentration was measured using a hemocytometer and diluted accordingly to ensure a cell number of around 150,000 cells for SC-ICP-MS measurements.

INSTRUMENTATION

The measurements were performed on a NexION 2000 ICP-MS equipped with PerkinElmer Single Cell Sample Introduction Kit, the Single Cell Micro DX Autosampler (Figure 1), and dedicated Syngistix Single Cell v. 2.5 software package.

Table 1. **ICP-MS Conditions**

Parameter	Value
Measured Element	^{65}Cu
Measurement Mode	Standard
Nebulizer	MEINHARD® HEN
Spray Chamber	Asperon™
Injector	2.0 mm id Quartz
Sampler and Skimmer cones	Nickel
Autosampler	Single Cell Micro DX
RF Power	1600 W
NEB Gas Flow	0.45 L/min
Makeup Gas Flow	0.7 L/min
Dwell Time	50us
Sample Analysis Time	150 s
Sample uptake rate	0,01 mL/min
Sample Flush	0 s
Ready Delay	160 s
Wash Delay	140 s
Software	Syngistix Single Cell v 2.5

The Single Cell kit contains the Asperon™ Spray Chamber, which is specifically designed for increased transport of micron sized particles and cells into the plasma compared to traditional introduction systems. The spray chamber together with high efficiency microflow nebulizer ensures that the highest number of cells reach plasma intact.
ICP-MS operating conditions are summarized in Table 1.

The sample was introduced using the Single Cell Micro DX Autosampler. The autosampler is designed to efficiently agitate samples prior to analysis to ensure that cells are suspended and consistently deliver small-volume samples at micro flow rates using syringe-driven sample delivery. This configuration decreases sample uptake and wash times. All the parameters necessary for proper functioning of the Autosampler were set according to the appropriate SOP [8].

The use of the Single Cell Micro DX Autosampler was more efficient and less time consuming than the traditional peristaltic pump. With the Single Cell Autosampler, typical sample-to-sample analysis times are 7 minutes, in comparison to experiments using the peristaltic pump and sample probe where typical sample to sample analysis times are 18 minutes.

Additionally, at the low flows required for SC analysis (10-20 µL/min), the peristaltic pump caused pulsations, which resulted in uneven sample delivery into the nebulizer. The use of syringes on the Single Cell Micro DX Autosampler solves this problem.

STANDARDS AND CALIBRATION

The transport efficiency of the system was measured using Calibration beads standard (EQ™ Four Element Calibration Beads, Fluidigm) prepared in Ringer's Solution at a concentration of 165 000 parts/ mL and was found to be around 20 %. A standard curve was generated using 1, 2 and 3 µg/L copper standards from the stock solution and is shown in Figure 2. R^2 for calibration curve used in the experiment was 0.99959.

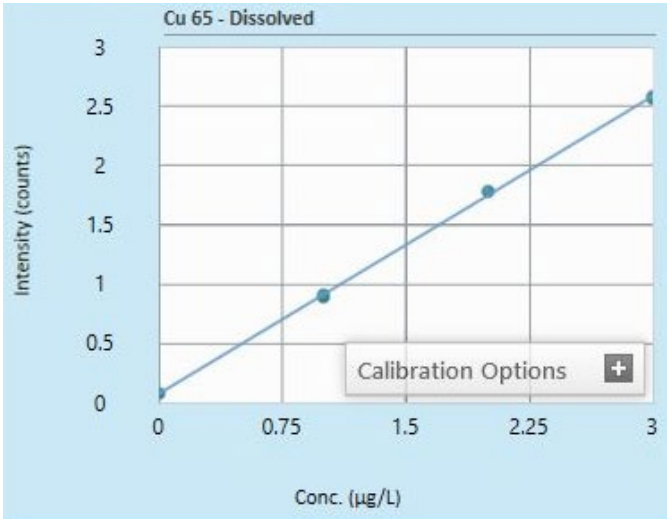


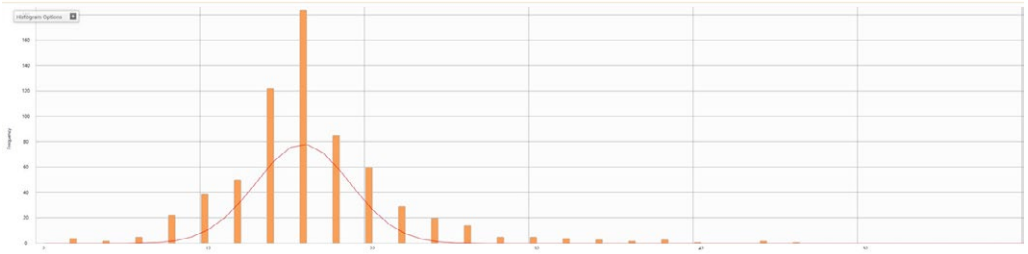
Figure 2. Calibration curve

RESULTS AND DISCUSSION

A sample was measured in duplicate, with the results appearing in Table 2. The results demonstrate excellent repeatability of the methodology.

A typical real time signal and real time peak area histogram can be seen in Figure 3. Each peak in the real time image corresponds to a single cell, while the real time histogram gives an idea about the distribution of the amount of copper in each cell (Fig 3A). The histogram is in peak area and is converted into ag in the results window (Fig 3B).

Figure 3. A. Analysis window with real time signal B. Histogram of copper content converted to ag/ cell



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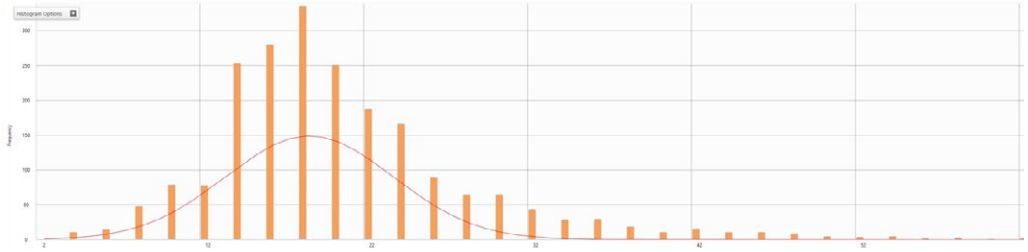

B

Figure 4 A,B. Histograms of copper concentration for each measured sample (Bin size=1 ag)

Table 2. Results Summary

Parameter	Unit	Measurement 1	Measurement 2	RSD
Most Frequent Mass	ag	20	20	0
Most Frequent Mass (from the histogram)	ag	21	20	3,45
Frequency of Most Frequent Mass		164	141	
Mean Mass	ag	21	21	0
Number of Peaks		619	605	
Mean Intensity	counts	10.44	10.3	0,95
Cell concentration	Cells/mL	198 600	121 000	

To the best of our knowledge this is the first attempt of successful measurement of copper concentration in PBMC cells on cellular level.

The accurate analysis of the copper concentration on the cellular level could be helpful in assessing which cell type is a reliable biomarker to accurately evaluate Cu exposure [1]. Relevant biological effects associated with mild to moderate copper deficiency and copper excess are still unknown, and identification of markers of those changes is difficult to identify. In the range of the copper homeostasis area the markers tested are not suitable to detect mild changes of copper metabolism [9]. Measurement one cell at a time using single cell ICP-MS can be the answer for this challenge.

Moreover, many cancer types exhibit increased intratumoral copper and/or altered systemic copper distribution, that could lead to the development in some new cancer therapy strategies concerning copper levels. Another approach is to use of copper to replace platinum in coordination complexes currently used as mainstream chemotherapies as copper potentially has reduced toxicity toward normal cells. In addition, cancerous cells can adapt their copper homeostatic mechanisms to acquire resistance to conventional platinum-based drugs and this mechanism can be used to re-sensitize cancer cells to these drugs [3].

CONCLUSION

This work demonstrates that Single Cell ICP-MS is able to quantify the amount of copper in individual PBMC cells. The method provides also the distribution of Cu per cell in PBMC cell population.

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