ESI: Quantification of metals in single cells by LA-ICP-MS: Comparison of single spot ablation and imaging

ESI-1 Cell culture

Swiss albino mouse fibroblast cells (3T3, DSMZ, Braunschweig, Germany) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS Superior) (FBS and DMEM from Biochrom AG, Berlin, Germany) and grown under standard conditions (37°C and 5 % CO2). For the experiments the cells were grown for 24 h on sterile coverslips (Menzel Gläser GmbH, Braunschweig, Germany, 20 x 20 mm) directly on 4-well Lab-Tek™ chamber slices (Thermo Fisher Scientific, Langenselbold, Germany). The cells were washed three times with phosphate buffered saline (without calcium and magnesium (PBS w/o Ca/Mg, Biochrom AG, Berlin, Germany) and immediately fixed with Histofix (Carl Roth GmbH, Karlsruhe, Germany). After fixation the cells were washed again with PBS w/o Ca/Mg (three times). The cells can be stored up to 2 months at 4 °C in buffer.

ESI-2 Validation of the spotter

Validation of the arrayer system with liquid ICP-MS was performed with the flexible membrane from Whatman. The reproducibility of the spotting process was checked on different days and the delivered volume of the pin was determined (0.61 nL ± 0.14 nL). For determination of the delivered volume a nitrocellulose membrane was printed with 10 grids (a grid consists of 4 x 4 spots each). These generated grids were cut out; the membrane pieces were dissolved in concentrated HNO3, diluted with water (purified to 18.2 MΩ using a Millipore Milli-Q water system) and measured against a standard calibration series with an ICP-SF-MS (Element XR, Thermo Fisher Scientific, Germany). Furthermore, the relative spot-to-spot standard derivation was determined to be less than 10 % by LA-ICP-MS.

ESI-3 Element XR operation and data acquisition

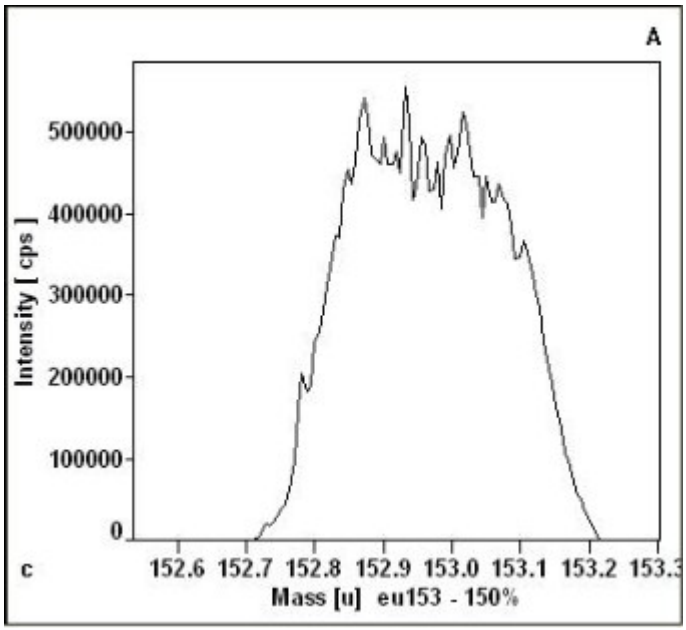
The ICP-MS operating conditions are listed in ESI Table 1 and the data acquisition parameters for singleplex and multiplex measurements in ESI Table 3, respectively.

ESI Table 1: Operating conditions of the ICP-MS (Element XR, Thermo) for LA- coupling.

ICP-MS operating conditions	
Mass resolution	400
RF plasma source:	1350 W
Plasma gas flow:	16 L min^{-1} Ar
Auxiliary gas flow:	1,1 L min^{-1} Ar
Sample gas flow:	0,6 L min^{-1} Ar
Scan type:	E Scan

To be able to measure a LA-ICP-MS signal in multiplex mode, one needs to sample the peak of a single laser shot with all elements. The used ICP-MS is not able to measure multiple elements simultaneously and is designed for the measurement of constant signals with a high precision. The manufacturer provides a preset measurement method for constant signals which takes 1.25 s for each measured isotope, see ESI Table 2. That would be longer than the transient signal of a single laser shot produced by the used LA platform (~1s). For the acquisition of transient signals, the instrument manufacturer provides an optimized method which only takes 0.1 s. To be able to measure multiple elements, this method was further optimized in our group based on Latkoczy et al.[1], to be able to measure one element within 0.01 s. In the following the relevant parameters will be introduced briefly along with the optimizations made:

The used Element XR has a reversed Nier-Johnson geometry of the mass analyzer and is usually operated in E-Scan mode, because masses can be selected very fast by changing the acceleration voltage and ESA voltage, while the magnetic field, which has a higher settling time, is left constant[2]. An E-Scan can resolve the mass peak of an isotope. ESI Figure 1 shows the resolved mass peak of 153Eu.



ESI Figure 1: Resolved mass peak of 153Eu in a mass window of 150% (screenshot from instrument tuning window)

Each isotope's mass peak has a specific width, which width corresponds to a *mass window* of 100%. For the measurement of constant signals, such a mass peak would be typically resolved completely by using a mass window bigger than the mass peak (e.g. 125%, see ESI Table 2). Thereby it can be ensured that always the same fraction of the mass peak is used as *integration window* (e.g. the 60% in the center), even if the mass calibration is shifted a little bit. For the low-resolution mode, it is a reasonable assumption that the mass calibration is stable enough to skip the time intense peak searching because the mass peaks are broad and have a flat top. Thereby for transient signals the *peak search window* doesn't exist (0% of the mass window) and the integration window equals the mass window. To decrease the likelihood of sampling outside of the flat top of the mass peak, the mass window was narrowed from 20% to 5%, if compared to the preset Element XR method.

The *sample time* (=integration time at a specific mass) of 50 ms for constant signals is lowered considerably for transient signals to 10 ms for the preset method and 2 ms for the optimized method.

Another parameter affecting the analysis time is the number of *samples per peak.* This exact number will be sampled for a mass window of 100%; but if the mass window is different from 100%, the samples taken per peak change proportionally. To still sample the peak at multiple positions on the flat top, the samples per peak are increased from 20 for constant signals to 50 for the preset method of transient signals to 100 for the optimized method.

Using these parameters, one can calculate the total time needed to measure one isotope, the so-called *segment duration*, the following equation applies:

$$segment\ duration = mass\ window \times samples\ per\ peak \times sample\ time$$

ESI Table 2: Comparison of Element XR methods.

		Preset by Thermo Scientific		Customized for multiplexed
		constant signal	transient signal	LA-Imaging
Sample Time	/ s	0.050	0.010	0.002
Mass Window	/ %	125	20	5
Samples per Peak		20	50	100
Integration Window	/ %	60	20	5
Peak Search Window	/ %	60	0	0
Segment Duration	/ s	1.250	0.100	0.010

To measure at a specific mass, a specific mass close to the desired mass is selected by the magnetic field, the so-called *magnet mass*. The E-Scan mode is limited to a maximum mass range of ±30% around the magnet mass.

For the first seven isotopes of the multiplex measurement a magnet mass of 149.920 Da is selected automatically by the instrument and for the biggest two isotopes a higher magnet mass of 174.940 is chosen, respectively. Usually, a minimum *settling time* of 1 ms is required between the measurement of two isotopes. Only if the magnet mass is changed, a higher settling time is needed. These settling times were optimized to low values of 100 ms for the jump from 193Ir to 150Nd and to 14 ms for the jump from 172Yb to 175Lu. The segment durations of the nine elements and their corresponding settling times add up to 211 ms to measure all isotopes, but their actual measurement takes an *acquisition time* of 220 ms. The overhead of 9 ms is needed for data treatment. This optimized acquisition time ensures that each peak of a single laser shot gets sampled multiple times.

In case of singleplex measurements, the magnet mass is constantly set to 174.940 Da for the measurement of 193Ir. Still, a minimal settling time of 1 ms is needed.

The sample time was increased ten times from 2 ms to 20 ms for two reasons. Firstly, the data treatment for one element takes 3 ms, what means that only during 10 ms out of 13 ms, the signal would be acquired (77% of the time). Secondly, since the data is anyways acquired quasi-continuously, such a high time resolution is not needed and data reduction by increasing the sampling time is advantageous. The acquisition time for singleplex measurements adds up to 103 ms, what is still enough to nicely resolve a measurement.

ESI Table 3: Data acquisition method of the ICP-MS (Element XR, Thermo).

ICP-MS Data Acquisition								
	Element	Settling Time ms	Magnet Mass Da	Mass Window	Samples Per Peak	Sample Time ms	Segment Duration ms	Acquisiton Time ms
Single-element	193Ir	1	174.940	5%	100	20	100	103
Multi-element	150Nd	100	149.920	5%	100	2	10	220
	153Eu	1	149.920	5%	100	2	10	
	159Tb	1	149.920	5%	100	2	10	
	162Dy	1	149.920	5%	100	2	10	
	165Ho	1	149.920	5%	100	2	10	
	166Er	1	149.920	5%	100	2	10	
	172Yb	1	149.920	5%	100	2	10	
	175Lu	14	174.940	5%	100	2	10	
	193Ir	1	174.940	5%	100	2	10	

Data acquisition and processing timing

Depending on the number of runs (data points) acquired by the ICP-MS, time is needed for processing and saving. If the ICP-MS software is still processing data, no new data can be acquired if a trigger signal is received from the LA platform. Thus, usually a wash-out time of 5 s after each line is added on the LA platform, to prevent the omission of a complete line scan. For imaging of calibration standards very lengthy line scans (660 s) were performed, which required an elongated time for data processing. Thus, an unusual lengthy wash-out time was used after each line scan (> 1 minute). Despite all imaging line scans were recorded using the elongated wash-out time for automation purposes, a washout time of 5 s was used for throughput calculations (multielement 1625 µm x 1000 µm image of cells: laser warm up (5 s) + data acquisition (49 s; including 4 s gas blank) + 5 s wash out time + 1 s relocation of LA-stage = 60 s per line scan; equaling ~25 min for the complete image).

ESI-4 Comparison of different measurement modes

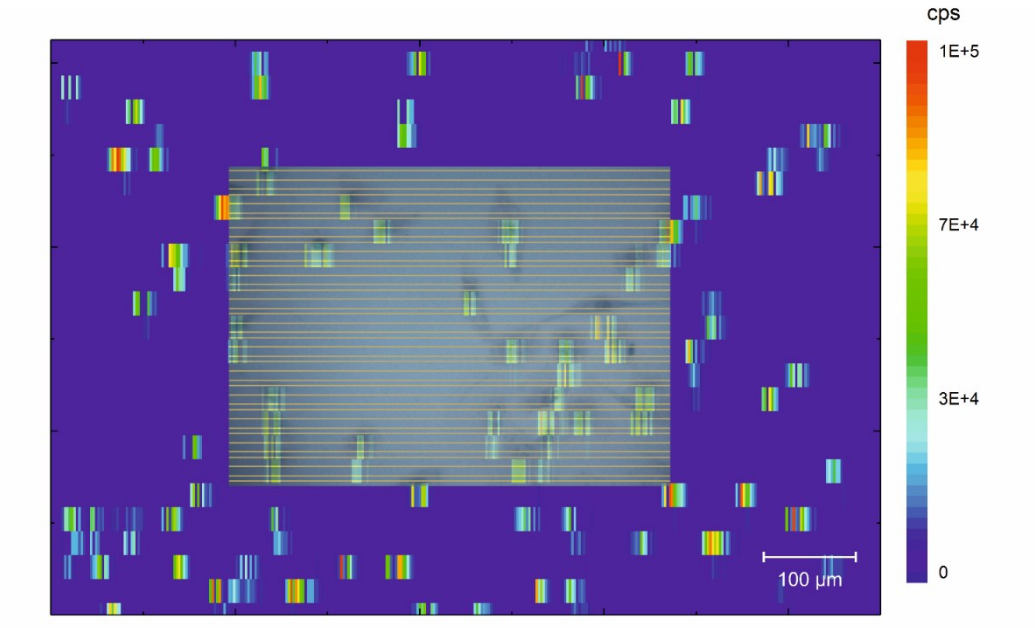
Quantitative comparison of metal content distributions.

In this supplementary section, we present data from repeating the same experiment as presented in the main paper with the modification that we only measure the single isotope ^{193}Ir, see single-element data acquisition parameters, ESI Table 3. Such an experiment can best be compared with data achieved by measuring a single isotope of a metal in a cell in suspension in the so called sc-ICP-MS mode.

Single-element imaging

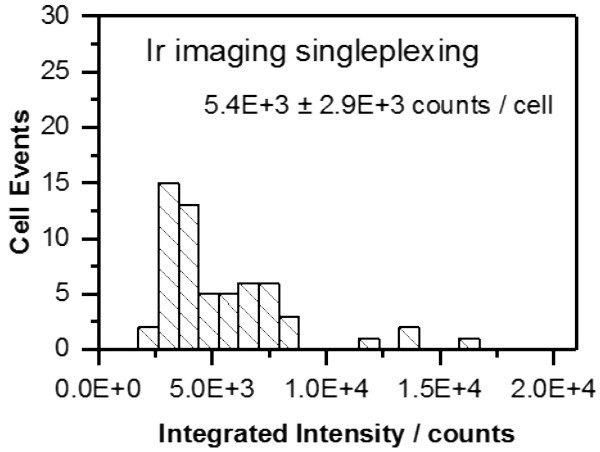
Single-element imaging of ^{193}Ir was performed using the same laser ablation parameters like in multi-elemental imaging, see main text Table 1, for optimized single-element ICP-MS data acquisition

parameters see ESI Table 3. Acquired raw data was transformed into images using the same procedure as for multi-elemental imaging. Overlays of ^{193}Ir distribution images and the corresponding bright field image were prepared in CorelDraw, see ESI Figure 2.



ESI Figure 2: Overlay of singleplex LA-ICP-MS image of 193Ir and the brightfield image. Yellow lines in the brightfield image indicate individual line scans with a laser spot diameter of 30 µm in a line to line distance of 25 µm.

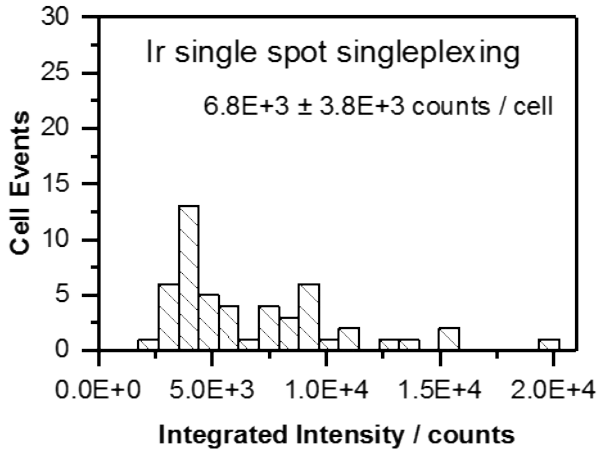
If compared to the overlay from multi-element ^{193}Ir imaging in main text Figure 1 a), the two images look very similar. Darker regions in the middle of cells, corresponding to the nuclei, gave higher signals as well. Between single-element and multi-element imaging of Ir-DNA-intercalator, no qualitative difference is visible. Signals of 59 cells were integrated on a single cell basis as in multi-element imaging, see procedure described in main text section 2.4. Cells were found to comprise of 52 ± 14 pixels, while the smallest cell had 20 and the biggest cell 96 pixels. Compared to multi-element imaging (27 ± 12 pixels) the cells have approximately twice the number of pixels. This is a consequence of different acquisition times per pixel: in single-element mode the acquisition time is only 103 ms per pixel, whereas the acquisition time in multi-element mode is approximately twice as big with 220 ms. Each integrated intensity value was corrected by a blank intensity calculated according to the number of pixels. The average blank signal of a pixel was calculated by averaging over an area without cells (243 pixels) and was found to have acceptable low values of 63 ± 46 counts. Compared to multi-element imaging (18 ± 25 counts) the background per pixel is about three times higher. Most likely, this is a consequence of a slightly unequal distribution of the background on the glass slide. However, the background was considered approximately stable within the imaged area. Moreover, the background is in the order of only one percent of the Ir intensity per cell, and accordingly the influence on the quantitative results is limited. Cells were found to have a background corrected integrated intensity of $5.4 \times 10^3 \pm 2.9 \times 10^3$ counts / cell, agreeing well with the numbers obtained from the multi-element measurements ($5.3 \times 10^3 \pm 2.8 \times 10^3$ counts / cell). ESI Figure 3 shows the resulting histograms of integrated Ir intensity.



ESI Figure 3: Histogram of ^{193}Ir intensity per cell for single-element imaging.

Single element single spot analysis

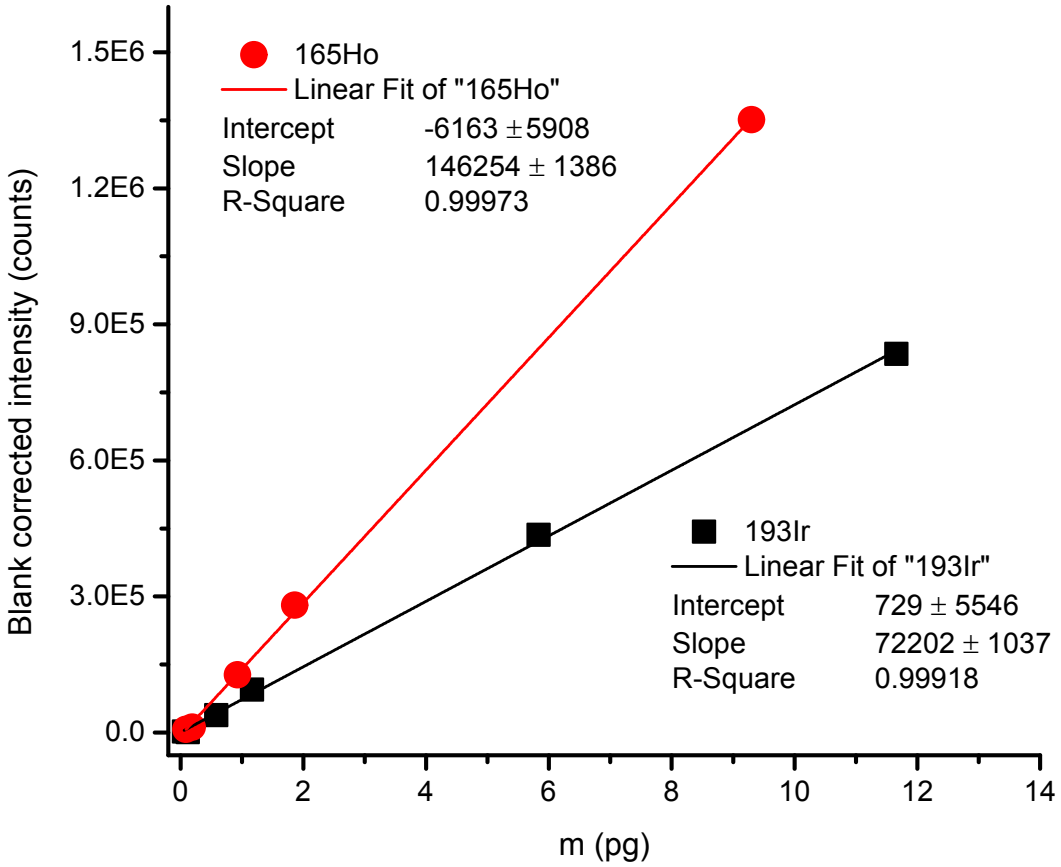
Single-element single spot analysis of single cells was performed using the same optimized LA-parameters as for multi-element single spot analysis, see main text section 2.4, and ICP-MS data acquisition parameters optimized for single-element analysis, see ESI Table 3. We measured the Ir intensity of 51 cells and ablated as well at 11 spots without a cell to obtain a blank Ir intensity. Acquired signals were integrated for each cell and each blank, see section 2.4. The integrated blank intensities were averaged to 1017 ± 46 counts for Ir, that means the background was found to be in the same order compared to multi-element single spot analysis (~25% higher). From each integrated intensity of a single cell we subtracted the mean blank intensity which gave an integrated mean intensity of 6.8 x 10^3 ± 3.8 x 10^3 counts. This results in average signal to noise ratio of ~148:1. The resulting histogram of Ir intensity per single cell is shown in ESI Figure 4. The distribution looks like two almost separated Gaussians, having their maxima at ~4 x 10^3 counts and ~8 x 10^3 counts.



ESI Figure 4: Histogram of Ir intensity per cell for single-element single spot analysis.

ESI-5 Digestion of cell samples for liquid ICP-MS

For the control experiment, cells were grown in a 12-well plate for 24 hours. To determine the number of cells per well after 24 h, three replicate wells were trypsinized for 6 minutes at 37 °C, aliquots were stained with Trypan blue and a cell number per well of 60E+3 ± 14E+3 was determined using a C-chip (Biochrom AG, Berlin, Germany). The other wells were fixed and staining using Ir-intercalator and mDOTA(Ho) was performed as described in section 2.2. Instead of an ethanol series at the end, the cells were digested using 800 µl Trypsin solution (Biochrom AG, Berlin, Germany) for 1 h at 37 °C. Until analysis the samples were stored at -20 °C in Eppendorf-tubes.



ESI Figure 5: Calculated trend line for 165Ho from 0.09 pg to 9.8 pg (the highest concentration of 18.6 pg was an outlier) and for 193Ir from 0.09 pg to 18.6 pg Ir

References

1. C. Latkoczy and D. Gunther, *Journal of Analytical Atomic Spectrometry*, 2002, **17**, 1264-1270.
2. T. Prohaska, J. Irrgeher, A. Zitek and N. Jakubowski, *Sector Field Mass Spectometry for Elemental and Isotopic Analysis*, Royal Society of Chemistry, 2014.