Three-dimensional reconstruction of the distribution of elemental tags in single cells using laser ablation ICP-mass spectrometry *via* registration approaches

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Abstract

This paper describes a workflow towards the reconstruction of the three-dimensional elemental distribution profile within human cervical carcinoma cells (HeLa), at a spatial resolution down to 1 µm, employing state-of-the-art laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) instrumentation. The suspended cells underwent a series of fixation/embedding protocols, and were stained with uranyl acetate and an Ir-based DNA-intercalator. *A priori*, laboratory-based absorption micro-computed tomography (µ-CT) was applied to acquire a reference frame of the morphology of the cells and their distribution before sectioning. After CT-analysis, a trimmed $300 \times 300 \times 300\ \mu m^3$ block was sectioned into a sequential series of 132 sections with a thickness of 2 μm and subjected to LA-ICP-MS imaging. A pixel acquisition rate of 250 pixels s^{-1} was achieved, in combination with a bidirectional scanning strategy. After acquisition, the two-dimensional elemental images were reconstructed using the timestamps in the laser log file. The synchronization of the data required an improved optimization algorithm, which forces the pixels of scans in different ablation directions to be spatially coherent in the direction orthogonal to the scan direction. The volume was reconstructed using multiple registration approaches. Registration using the section outline itself as a fiducial marker resulted into a volume, which was in good agreement with the morphology visualized in the µ-CT volume. The 3D µ-CT volume could be registered to the LA-ICP-MS volume, consisting of 2.9 x 10^7 voxels, and the nucleus dimensions in 3D space could be derived.

Keywords: *laser ablation LA, inductively coupled plasma-mass spectrometry ICP-MS, X-ray fluorescence spectrometry XRF, HeLa cells, 3D, multimodal registration*

Introduction

Laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) is an elemental probe characterized by limits of detection at the sub-$\mu g\ g^{-1}$ level, a lateral resolution down to sub-μm level and mass spectra with a straightforward interpretation that permit one to immediately draw qualitative and even semi-quantitative conclusions.[1,2] Visualizing the distribution of endogenous trace elements and bioconjugated metal tags (*e.g.*, secondary antibodies labeled with lanthanides) at the single cell level has become a key application of this field, complementing data from other modalities. Owing to the introduction of low-dispersion ablation cells and laminar flow aerosol transport systems, sample throughput was drastically improved and intracellular imaging made feasible at lateral resolutions in the order of 1 µm.[3,4] Recently, Pisonero *et al*.[5] demonstrated the presence of Cd-based quantum dots and Au nanoparticles in intracellular structures of human cervical carcinoma (HeLa) and mouse embryonic fibroblast (NIH-3T3) cells, respectively, *via* 2D single-cell LA-ICP-MS imaging. One of the limitations of the single-cell LA-ICP-MS imaging approaches applied thus far is the limited vertical resolution. It can be challenging to distinguish signals from different cell compartments, as typically the laser beam penetrates through the entire cell, and the signal originating from the cytosol is monitored at the same time the nucleus is traversed. For microbiologists, highly sensitive and quantitative imaging tools to track cell compartments and metal-labeled compounds such as antibodies or proteins, are very important to expand the understanding of the intra-cellular processes that occur under environmental stress, disease, and a variety of other conditions. The ability to pinpoint the position of metals in relation to specific 3D structures within the cell is important to gain a better insight as to why these metals are at a specific location.

Due to the matrix-dependent ablation rates in LA-ICP-MS, large 3D volumes can only be constructed from a series of 2D images originating from a collection of sequential sections of the sample, similar to approaches in serial 3D imaging mass spectrometry (IMS). In the reconstruction, elemental or molecular ion images of adjacent sections are stacked and aligned through registration to reflect the true 3D morphology of the sample. Furthermore, multimodal (co-)registration approaches exist, in which information from multiple techniques is combined. Registration is a process in which the transform is calculated to correlate spatial positions of different sets of image data, based on a degree of similarity. This approach has been utilized in neuroimaging *via* IMS for the automated alignment of the molecular ion images relative to i) anatomic brain atlases, *e.g.*, the Allen Brain Atlas, or ii) histochemical microscopy information.[6-10] The registration of multiple modalities allows one to generate a multi-channel image in which correlations between modalities can be clearly demonstrated.[11] Several imaging modalities can be combined with serial 3D IMS, such as infrared (IR) imaging, positron emission tomography (PET), computed tomography (CT), single-photon emission CT (SPECT) or (functional) magnetic resonance imaging (MRI).[12-14,11,15] However, these macroscale registration approaches, which often operate based on edge-detection algorithms, have mostly been demonstrated to work for elemental imaging of objects $> 1\ mm^3$ in volume. In LA-ICP-MS, sequential registration has only been applied in a few cases, due to practical limitations in terms of measurement time, and access to appropriate software for reconstructing these volumes.[16,1] In order to tackle the challenge of single-cell 3D imaging, a multimodal registration methodology, previously developed to study a small crustacean *Ceriodaphnia dubia via* alignment of a set of 2D element distribution images using a 3D micro-absorption CT dataset, has been adapted and refined in this work to allow for imaging at an intracellular scale.

Human cervical carcinoma cells were chosen as a study object in the context of this study for the following reasons: (i) the size of the cell is approximately $20\ \mu m$, which is a typical size of a cell within the human body, (ii) all 132 sections can be imaged within an acceptable total time span of approximately 50 h (<20 min / section) when aiming at the highest lateral resolution available on state-of-the-art instrumentation, (iii) HeLa cell lines are frequently used as human cancer cell lines to evaluate the efficacy of chemotherapeutic drugs, and (iv) HeLa cells can be cultured easily. The concept of 3D single-cell imaging is not new, Duenas *et al.* demonstrated MALDI-MSI imaging of phospholipid classes in a zebrafish embryo ($\sim 600\ \mu m\ \emptyset$) at a resolution of 5 µm, however, the scale of the HeLa cells is an order of magnitude smaller.[17] 3D ToF-SIMS has potential for 3D MSI through depth profiling, though this technique is highly limited in terms of sample throughput due to modest sputter speeds in combination with differences in sputter rate across the cell, and only a limited range of compounds can be detected.[18-20]

Typically, the concentration differences of endogenous elements in cells are too small to distinguish individual cell compartments, hence, biomarkers (*e.g.*, immuno-labelling using metal-coded affinity tags) are routinely applied in proteomics to distinguish cell features.[21] Simultaneously, these tags are used for phenotyping *via* mass-tag cellular barcoding (MCB) and counterstaining. Metal-based oxidizing species such as RuO_4 and OsO_4 can also be used for counterstaining.[22] Metallo-intercalators can be used for cellular DNA detection as they insert themselves using their planar aromatic ligand between nucleotide base units. Iridium intercalators have been proposed by Frick *et al.* as a potential macroscale internal standard, apart from Iodine. In this work, they are used to locate and describe the bounds of the nuclear envelope of HeLa cells.[23]

Non-destructive high-resolution 3D imaging techniques such as two-photon, confocal, super-resolution (PALM,STORM, and STED) optical fluorescence microscopy, nano-CT and transmission electron microscopy (TEM) are typically used to describe the internal 3D morphology of cells, as widefield microscopy techniques encounter difficulties in providing an accurate description of internal structures, even when their outer surface is transparent, due to the presence of photons outside the focal plane, and the relatively limited spatial resolution of 200 nm. However, TEM and nano-CT are not suitable for imaging a large trimmed block of embedding medium. Optical slicing using fluorescence microscopy is a powerful tool, but bleaching, interaction of the fluorophore probes with live systems, non-linear response, and limited multiplexing restrict this approach. In this work, micro-absorption CT was applied to the non-sectioned sample. Most cellular structures have a similar (electron) density to common embedding media, such as Spurr's resin, polysaccharose, and LR-White, as they are composed of carbon-based light-weight polymers. The native difference in the X-ray attenuation coefficient of the medium and cells is hence too small to achieve sufficient contrast during exposure of the specimen *via* lab-sources. This work describes an approach to enhance the contrast in µ-CT through metal staining, and demonstrates an approach to track the 3D distribution of Ir in the nuclei of single cells using LA-ICP-MS whilst linking this to the morphology of each cell as imaged by µ-CT.

Materials and Methods

HeLa cells (IRC-TC) were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and L-glutamine (200 mM) at 37 °C in a humidified atmosphere containing 5% CO_2. Cell cultures were routinely tested for *Mycoplasma* contamination. Confluent HeLa cells were trypsinized with 0.25% (w/v) Trypsin in 0.53 mM EDTA solution, washed and resuspended in DMEM. HeLa cells in suspension (80% confluent) were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature. A 2.5 µM Cell-ID™ Ir intercalator solution (Fluidigm™ Corporation, South San Fransico, CA, USA) was added (0.5 mL of working solution per $1x10^6$ cells/ tube) for 25 min. Cells were concentrated in low-melting agarose 2% and washed 3 times with sodium cacodylate buffer, followed by a bulk staining with 1% uranyl acetate ($UO_2(CH_3COO)_2 \cdot 2H_2O$) for 1 h at 4 °C while rotating. Cells were subsequently dehydrated through a graded ethanol series stepwise over 3 days at 4 °C, followed by embedding in LR-White (hard grade, London Resin). Polymerization was performed in a Leica EM AFS at 37 °C under UV light. For µ-CT analysis, the excess of LR-White was trimmed away with a glass knife on a Leica UC7 ultramicrotome to produce a cuboid shape with a cross-section of approximately $300 \times 300\ \mu m^2$, outlining the sample exterior, in order to minimize X-ray scatter, resulting in an enhanced signal-to-noise ratio for the reconstructed µ-CT image**.** After µ-CT analysis, the block containing the cells was sectioned into 132 adjacent sections of 2 µm thickness using a Leica UC7 ultramicrotome. Each section was placed in a water droplet on a Starfrost® microscope slide and numbered according to the order in which they were sectioned.

The LA-ICP-MS setup comprises an Analyte G2 Excimer-based 193 nm ArF* laser ablation system (Teledyne Photon Machines, Bozeman, MT, USA) coupled to an Agilent 7900 quadrupole-based ICP-mass spectrometer (Agilent Technologies, Tokyo, Japan). For the 3D imaging runs, only a single mass channel was monitored (^{193}Ir), using an extremely short total scan cycle time (4 ms) to achieve a high rate of pixel acquisition (250 pixels s^{-1}). With edge-to-edge ablation of the 1 µm spot, a voxel size of $1 \times 1 \times 2\ \mu m^3$ was achieved (though a round Gaussian crater profile is assumed due to diffraction effects). Owing to the limited section thickness and high laser energy density, the biological material

could be removed quantitatively using a single shot / area. The LA system was equipped with ablation chamber, based on the ablation cell described in a previous paper from Van Malderen *et al*.[24].The ablation chamber used consists of an enclosed volume, with an inner cup of a tube cell design. The beam is focused through a window on top of the tube cell. The sample moves laterally relative to the cup using piezo-electric motors. This ablation cell was connected to the ICP torch *via* the aerosol rapid introduction system (ARIS)[25], a low-dispersion mixing bulb developed at Ghent University and commercialized by Teledyne Photon Machines (Bozeman, MT, USA). The low-dispersion aerosol transport in the ablation cell and ARIS components greatly reduce the effects of pulse-to-pulse mixing at laser-pulse repetition rates of 250 Hz, as is demonstrated in the results of the experiment below . The LA and ICP-MS settings (Table 1) were optimized at the start of each experiment (after 1 h of warm-up) while ablating NIST SRM 612 (Trace elements in glass, National Institute of Standards and Technology, Gaithersburg, MD, USA) with a 5 µm laser beam spot to achieve high signal intensity for $^{24}Mg^{+}$, $^{89}Y^{+}$, $^{115}In^{+}$ and $^{238}U^{+}$, whilst maintaining low oxide levels ($^{238}U^{16}O^{+}$/ $^{238}U^{+}$ <1.5%), low elemental fractionation ($^{238}U^{+}/^{232}Th^{+} \approx 1$), and low gas background levels (evaluated *via* the signal intensities for $^{16}O_2{}^{+}$, $^{15}N^{16}O^{+}$).

Table 1 LA and ICP-MS settings

Energy density $[J\ cm^{-2}]$	3.5
Repetition rate $[Hz]$	250
Scan speed $[\mu m\ s^{-1}]$	250
Number of shots per position	1
Beam waist diameter $[\mu m]$	1
Cup-Sample distance $[\mu m]$	240
Image XY dimensions $[\mu m$ x $\mu m\ ]$	467 × 472
Mask shape	*Round*
He carrier gas flow rate $[L\ min^{-1}]$	300 (cup) + 300 (chamber)

X-ray absorption micro-computed tomography. The µ-CT scanner of the HERAKLES instrument (UGCT and XMI, Ghent University, Belgium) is equipped with an open-type microfocus X-ray tube with a high-resolution transmission target (X-RAY WorX, Garbsen, Germany).[26] Voltages can be set between 20 and 100 kV, the filament current can be set from 50 to 1000 µA with the restriction that the tube power should not exceed 80 W (max. target power is 3 W). For this work, a voxel size of ~1 μm^3 could be achieved by optimizing the sample-source distance. A Photonic Science VHR CCD sensor (Photonic Science™, Millham, UK) equipped with a GdOS:Tb scintillator was used to detect the transmitted X-rays. The CCD detector contains 4008×2672 pixels of $9 \times 9\ \mu m^2$ with a $20\ MHz$ read-out. A total of 1491 projections (0.24° steps) were recorded with a scanning time of 1 s per projection.

Data pre-processing and reconstruction of the 3D LA-ICP-MS dataset. Laser log files and ICP-MS files were synchronized and the areas were reconstructed using HDIP software (Teledyne Photon Machines, Bozeman, MT, USA).

The machine time offset represents the time between a laser firing event and the response for this event in the MS data readout. This time difference has two components:1) the time difference between the system time of the computer responsible for the laser firing and motion control, and the system time of the computer handling the MS data acquisition, and 2) the time that it takes for the ejected particles to reach the detector, which comprises the aerosol transfer time delay, *i.e.* the total time that it takes for the aerosol to arrive in the ICP, for the ions to travel from the plasma towards the detector, and for the detection event to take place (see **Fig. 1**A). A 5V 50Ω TTL signal is used to start an experimental run on the both computers, yet both computers register a different time at which the run was started, as the system time on the computers is different, and the moment that the time is registered depends on different pieces of the software, which run independently on both computers. If the time that it takes for the ejected particles to reach the detector changes during a run, the machine time offset changes during a run. Normally, the total ion beam (TIB) can be used to determine the machine time offset, by gradually shifting the laser computer system time and calculating the sum of the TIB during the time that the laser would be firing.[27] The machine time offset for the maximum sum of the TIB generally represents the optimal offset. In many experiments, signal responses generated when scanning the substrate and the samples are higher compared to the gas background at statistically significant levels. This type of experiment results in a TIB curve as displayed by curve A (black dash-dotted line) in **Fig. 2**. Curve B (green line) represents a situation in which only a low signal background is generated when scanning the substrate, in that case, a small but singular maximum sum of the TIB can be found at the correct machine time offset. However, if there is an extremely low signal-to-noise ratio when scanning the laser over the substrate, the starting time of firing the laser, stated in the log file, does not accompany an increase of detector response. Hence, there is no clear maximum in the TIB curve (curve C, blue dashed line), as the optimization curve displays a flat-top peak when the signal from the sample is distributed within the lasing time. This is often the case when single cells are imaged on their microscope slide substrate at high lateral resolution, as the targets are not present in all points of the ablation area. When all lines are scanned in the same direction, this produces a unidirectional translation of the image, which can be corrected for during image post-processing. However, for bidirectional scans, each odd scan shifts in the opposite direction to the even scans. This leads to severe signal skew effects, which are difficult to correct for once the image has been reconstructed (see **Fig. 1**B).

Therefore, an algorithm was created in Python 3.6 to iteratively improve the accuracy of the synchronization between the laser ON/OFF signal and the detector signal, based on another criterion: the signals of pixels in a column of an image are expected to be coherent, as features of similar intensity cover multiple pixels in the column. Nelder-Mead (downhill simplex) and COBYLA optimization were applied to minimize the sum of the signal differences in a column of pixels, *i.e.* orthogonally to the scan direction. *A priori*, the data underwent a background correction using the gas blank. After reconstruction of the areas, the dimensions of these areas were rescaled based on the laser log file information. The 132 images were written to a 3D data stack in their predefined order. Within this stack, the sections have a random orientation relative to each other. In the following step, the images in the stack were aligned through registration based on affine transformations.

Results and discussion

Machine time offset refinement

The Nelder-Mead and COBYLA optimization were applied in different runs on all 132 sets of data for each section. In an initial step, each ICP-MS file was roughly synchronized using a known delay time between the system time of both

computers of 600 ms. Every set of data consists of a vector of 3×10^5 datapoints. In a first instance, a rough optimization consisting of a maximum of 50 iterations was performed (maximum step 0.1 s, minimum step 0.01 s), after which a finer optimization was performed (maximum step 0.002 s, minimum step 0.0005 s, maximum iterations 20). This process was time-consuming (4 s per iteration), as for each iteration an image was reconstructed from the vector based on the laser log times. Four different seed values were used (-0.105, -0.2, -0.29, -0.43 and -0.49 s) to avoid convergence on local maxima. The value is expected to be negative here as it takes a few 100 ms for the aerosol to reach the plasma, this is known as the aerosol transfer delay time. The reconstruction takes into account the position of every scan, and produces a 2D image by pickling the datapoints within each lasing timeframe, and placing them on the correct position and size of the grid through interpolation. The calculation of the sum of absolute differences when traversing the columns is fast by comparison (<10 ms). **Fig. 3** exemplifies a reconstruction artefact which occurs when the machine time offset is >0 or <0. When the reconstruction is correct, the outlines of the nuclei become very defined (**Fig. 3**B). Due to the severity of these artefacts, careful optimization is required. Given appropriate seed values, both Nelder-Mead and COBYLA algorithms converge onto the same optimum value, though COBYLA generally needs significantly less iterations to reach the convergence criterion, as the algorithm operates using a known, fixed step size and can hence be optimized better, as demonstrated in **Fig. 4**. Local minima are present for offsets which are shifted by the time needed to ablate a single line (apart from random minima), therefore it is necessary to start the algorithm with different seed values. Failure to succeed might result in a loss of data and spatial shift of the image, as one scan line on the top or bottom of the image might not be taken into account during the reconstruction.

Bidirectional scanning in a raster pattern significantly enhances sample throughput, particularly at high pixel acquisition rates (>100 pixels s^{-1}), as it removes the time needed for the stage to travel back to one of the sides of the ablation area. For example, the total run time per image was reduced from approximately $2000\,s$ down to around $1100\,s$. Overall, this reduces the total experiment run time from >73 h down to ~46 h (accounting for the time for ICP-MS warm-up and retuning). Further improvements in the pixel acquisition rate are necessary (500 Hz) to make this type of experiments possible in <24 h. In this work, the pixel acquisition rate was limited by the maximum design frequency of the laser (300 Hz), in combination with the desire to avoid aliasing effects by operating at an integer dwell time.[28] The reconstructed volume encompasses data from 2.9×10^7 spatial positions, which is a large amount of sampling positions compared to recently published works.[29]

Comparison of registration methods for 3D image reconstruction

3D LA-ICP-MS data registration approaches reported in literature are based on the sequential registration of neighboring slices (sequential slice registration – SSR), and operate under the assumption that shared features are present in neighboring slices and that the relative positions of these features can be extrapolated along the Z-axis. A previous study showed that SSR registration of each Z-slice relative to adjacent Z-slices failed to mimic the correct vertical orientation of the slices, as slight shifts in the alignment caused drift over multiple slices. The information on the curvature of the sample object along its boundary orthogonal to the cutting plane is lost in the cutting process.[11] In the same study, correlative slice registration (CSR) of a Z-slice in the LA-ICP-MS data volume relative to the corresponding slice in the CT data volume was applied. This strategy requires a way to correlate the X-ray attenuation as observed by CT and the elemental distribution visualized using LA-ICP-MS. As a polychromatic source is deployed, and the elemental distribution is unknown *a priori*, insufficient information is present to characterize all photoionization and scattering effects. Normally, this would mean that there was

no path to correlate the X-ray attenuation, or electron density, to an elemental distribution. During the sample preparation, a uranyl acetate stain, containing the abundant isotope ^{238}U, was applied onto the HeLa cells. The large total absorption cross-section for an electron-dense nuclide as ^{238}U, along with its relatively high concentration, results in a situation in which U significantly increases the X-ray absorption in the cellular structures, providing sufficient contrast in the CT image to visualize these cells. Thus, its distribution reflects that of the cells within the block. In this case, the entity detected *via* the first modality (Ir), LA-ICP-MS, does not directly correlate with the electron density (or the distribution of uranyl acetate), however, a rough registration can be performed, as the position of the uranyl stain also reflects the positions of the nuclei. In order to verify this, one of the sections was imaged with ICP-MS data acquisition settings configured to detect both ^{193}Ir and ^{238}U. From the result in **Fig. 5**, it can be concluded that the center of mass position for both stains in a single cell is similar, but that the uranyl stain is also present at the boundaries of the nuclei. Sectioning the sample in the plane orthogonal to the block's vertical center axis has the advantage that the number of degrees of freedom (DOF) of the transformation can be reduced to 3: 2 translational and 1 rotational DOF. In this case, the scaling parameter that links both modalities together is known, as it can be deduced from the pixel step size in the coordinate systems used in each individual system. It can be noted that CT has a superior depth resolution in this experiment (a factor of two higher), but the lateral resolution is comparable. In feature-based registration (FBR), a set of fiducial markers (keypoints) is required within the image; this can be a distinguished feature in the image, which is present at a fixed location. As the sample block is trimmed to have straight edges, the location of the edges of the block is reproducible and near-identical in their position in the reconstructed volume. Haris corner detection is a rotation-invariant registration algorithm which was applied here to solve this problem (*via* its implementation in OpenCV).[30,31] Modern algorithms like Scale-Invariant Feature Transform would also be applicable here.[32] The data were registered with SSR, FBR, and CSR.

Table 2 summarizes the target and reference images for the various registration approaches. It is important to note that SSR and FBR can be applied within the LA-ICP-MS dataset, while CSR requires an additional modality.

Table 2 Summary of all registration approaches used in this work.

Registration approach	Abbr.	Target	Reference
Sequential slice	SSR	Intensity distribution	Intensity distribution in the previous slice
Feature-based	FBR	Keypoints on the intensity images	Keypoints on a template shape
Correlative slice	CSR	Intensity distribution	Intensity distribution of secondary modality, in the corresponding slice

CSR has shown two distinct advantages over SSR and FBR; (i) each slice is correlated to their matching slice in CSR (which provides a better correlation with the morphology), and (ii) CSR will correctly reflect the sample orientation, as can be seen in **Fig. 6**. With multimodal registration *via* CSR, a correct reconstruction can be obtained without the use of artificial fiducial markers included within the embedding medium. Manual mechanical sectioning of the samples requires a skilled operator, as handling and inventorisation of the sections is a delicate process. A small minority of the sections (<4) shows signs of severe warping/overlap/missing pieces as it is very challenging to avoid some damage for a large number of sections (>100). The damage to these sections will induce propagating errors in the SSR approach. When comparing the

CT slice with the FBR result in **Fig. 6**, it can be seen that this approach also produces a correct reconstruction, without the need for a second modality, which simplifies the workflow. In this case, the potential to sharpen the image through mid-level image fusion of the CT and LA-ICP-MS volumes is limited, as the spatial resolution of both techniques in this experiment is similar. The CT image allows to cross-validate that all cells detected by LA-ICP-MS are also detected in the CT image. **Fig. 7** shows a two-channel composite of single slices of the LA-ICP-MS volume after CSR, and the corresponding slice of the CT volume. Identical cell positions are identified by both modalities, across all slices (which can be observed in Online Resource 1 in the Electronic Supplementary Information).

3D cell characterization

The Ir intercalator stain binds to the nuclear DNA of the HeLa cells; hence, its distribution is thought to reflect the nucleus position and volume. In work from Hermann *et al.* (2017), the same intercalator stain was applied to the nuclei of Swiss albino mouse fibroblast cells (NIH-3T3) and human lung epithelial cells (A549); the spatial resolution of the resulting LA-ICP-MS images was limited to ~6-10 µm as a result of the use of legacy equipment, which made it difficult to distinguish the outlines of the nuclei.[33] Like the authors of this work pointed out, the use of the latest generation hardware dramatically increases resolution, contrast, and throughput, as demonstrated in this work. The stack of elemental images was exported to a stack of 16-bit TIFF files with known boundaries and imported in Fiji[34] (Fiji is just ImageJ, https://fiji.sc/), after which the 3D object counter, a plugin of the JACoP toolset[35], was run with the following settings: the threshold minimum object size was set to 200 voxels with no upper limit, threshold value set to 28/255 (after 8-bit conversion of the stack). Statistical data were extracted from the population of cells and are displayed in **Fout! Verwijzingsbron niet gevonden. Fig. 8** and **Fig. 9**. Gaussian fitting of the density histogram allows one to extract the average distance from the centre to the surface of the nucleus of $6.7 \pm 1.7\ \mu m$, and an average nuclear volume of $1.3 \pm 0.3 \times 10^3\ \mu m^3$ assuming the cells to be spherical. As expected, there is some variance in the results, due to the natural variation and manually setting the threshold for the shape detection algorithm. The algorithm does have a bias towards the larger nuclei; it is possible that a larger signal cluster was occasionally assigned to one nuclear volume, although it represented several neighboring nucleï closely spaced together. The nuclear volume is highly dependent on the cell cycle phase and can vary from $540 - 1780\ \mu m^3$.[36] The nuclear volume found in this work lies within this range. Other works have reported values in this range; for example, Monier *et al*. found an average nuclear volume of $697 \pm 299\ \mu m^3$.[37]

Conclusions

In this work, LA-ICP-MS 3D imaging at the intracellular level *via* image registration was demonstrated. Given appropriate data handling, the shape of 3D structures as small as the nuclei of HeLa cells can be reconstructed using metal-based tags, such as the Ir-based intercalator used here. The intercalator distribution was visualized in a $467 \times 472\ \mu m^2$ region in <1100 s using a 1 µm diameter spot at 250 spatially resolved pixels s^{-1} using a bidirectional scanning scheme. Although the acquisition of the total set of 132 images in a time span of 50 h makes LA-ICP-MS still comparatively slower than several other imaging tools, the methodology and instrumentation employed here permit a highly efficient measurement process, which yields an improved throughput over many other current works in the field. The machine time offset can be iteratively refined in a situation where the substrate does not provide a signal above the gas background. FBR serves as a viable alternative when multimodal co-registration methods based on µ-CT absorption data are not available (CSR). For larger samples, LA images and lab-based µ-CT volumes can be pooled to generate insights on the relationship between the morphology and elemental distribution, and provide cross-validation of the volume reconstruction, but on a scale smaller than $10\ \mu m$, the combination of both modalities on a intracellular scale remains challenging unless lab-based X-ray sources

with higher brilliance (*e.g.*, liquid metal jet sources) are employed. Further highly desired improvements in spatial resolution (down to 500 nm and below) and pixel acquisition rates would make LA-ICP-MS imaging competitive with microprobes of synchrotron beamlines and allow visualization of smaller cell structures (mitochondria, endoplasmic reticulum), at higher sensitivity. The signal-to-noise ratio observed for the mean ^{238}U response inside of the cell nuclei, originating from the uranyl acetate stain, is sufficient for further reductions in the beam spot profile without compromising image contrast. More generally, this work demonstrates that the distribution of metal-based tags can be reconstructed in 3D on the intracellular level, permitting access to additional parameters, *e.g.*, the nuclear volume. Similar 3D imaging strategies can be applied to intracellular tissue imaging, which would allow a better insight in the 3D distribution of biomarkers within tissue structures. This would be interesting in the case of imaging mass cytometry, where biomarkers related to intra- and inter-cellular signaling could be tracked. The volumetric information would provide an additional metric; this increase in the parameterization of single-cell measurements, combined with MCB phenotyping, could lead to improved insights, for example into the antibody distribution across single cell compartments. FBR could be easily applied to the reconstruction of 3D images of tissue structures, as it relies on the sharp outer boundaries of the tissue block; CSR on the other hand would struggle, as there are only slight differences in X-ray absorption within a tissue block.

Electronic supplementary information

Online Resource 1 This movieclip shows every frame of the dataset acquired by LA-ICP-MS and 3D renderings of the combined slices for both the LA-ICP-MS and CT datasets.

Online Resource 2 This image displays HeLa cells stained with DAPI (blue) and alpha-tubulin (green) and visualized using an LSM confocal microscope.

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Potential conflict of interest disclosure

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Figure captions

Fig. 1 This scheme visualizes the concept of a machine time offset and its effect on the reconstructed image. In diagram A a simplified representation of the order of events that happen during signal acquisition is drawn. The time segment a) is the initial offset due to different system times and software delays, whilst time segment b) represents the second part of the offset due to the detection delay of the ablation event. Diagram B displays a situation where a machine time offset error, indicated by label c), causes image artefacts in the reconstruction of the image. The reconstruction step is labeled d).

Fig. 2 Curves displaying the iterative optimization process that can be used to find the correct machine time offset in a conventional approach

Fig. 3 Reconstructed grey-scale elemental images of a single 2D slice with different machine time offsets. A) Reconstructed image when a machine time offset of 0.2 s is used. Figure inset shows a zoomed-in region of the image. B) Reconstructed image when a machine time offset of 0 s is used (*i.e.* laser ON/OFF and ICP-MS timetrace are synchronized)

Fig. 4 Graphical representation of the progress of both optimization algorithms to find the machine time offset

Fig. 5 Example of a two-channel 2D elemental image of a cell block section for ^{193}Ir (red channel) and ^{238}U (green channel), obtained *via* LA-ICP-MS. The yellow color is obtained by superposition of red and green. This analysis was performed in a separate run, and is not part of the 3D dataset

Fig. 6 YZ and XZ cross-sections of the reconstructed, registered CT and LA-ICP-MS 3D volumes. The colormap has a logarithmic scale. (A) LA-ICP-MS Ir distribution using the FBR algorithm. (B) LA-ICP-MS Ir distribution using the SSR registration. (C) LA-ICP-MS Ir distribution using the CSR registration. (D) CT absorption cross-sections of the embedded sample, thresholded to optimize the contrast between the cells and the background.

Fig. 7 Single slice of joined LA-ICP-MS / CT stack of a cell block section. The image contains two channels: ^{193}Ir distribution (red channel) and CT reconstructed image (green channel), obtained via LA-ICP-MS and µ-CT analysis, respectively. The yellow color is obtained by superposition of red and green.

Fig. 8 Density histogram of the distance between the center and the surface of the nucleus

Fig. 9 Density histogram of the volume of the nucleus

440