NanoSIMS analysis of an isotopically labelled organometallic ruthenium(II) drug to probe its distribution and state *in vitro*

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Electronic Supplementary Information (ESI†)

CONTENTS

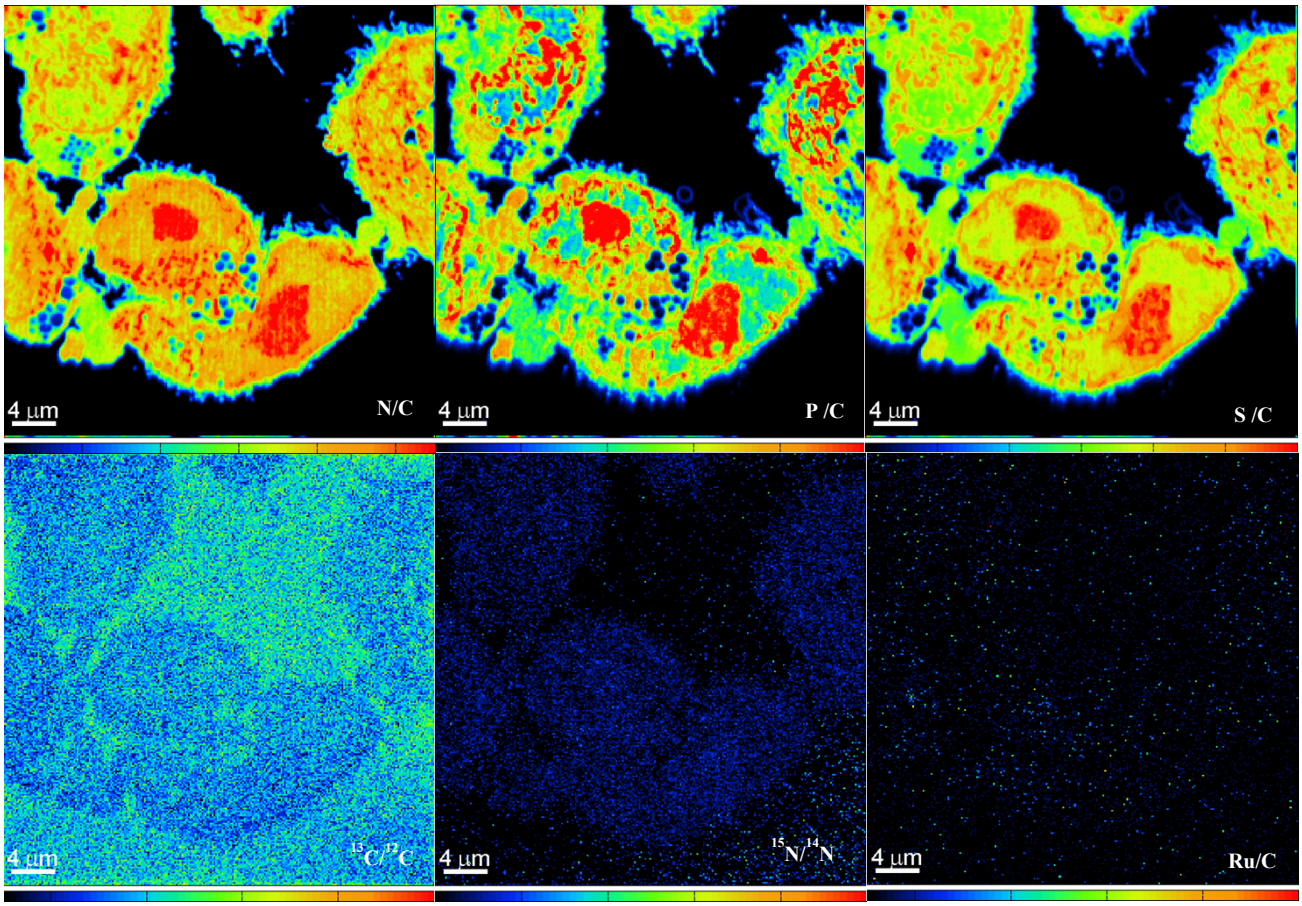

Fig S1. Secondary ion maps of $^{14}N^{12}C^-$/ $^{12}C_2^-$, $^{31}P^-$/ $^{12}C_2^-$, $^{32}S^-$/ $^{12}C_2^-$, $^{13}C^{12}C^-$/$^{12}C_2^-$, $^{15}N^{12}C^-$/ $^{14}N^{12}C^-$ and $^{102}Ru^-$/ $^{12}C_2^-$ (figure labels have been simplified) in untreated A2780CR cells. No enrichments of ^{15}N, ^{13}C, or ^{102}Ru are observed.

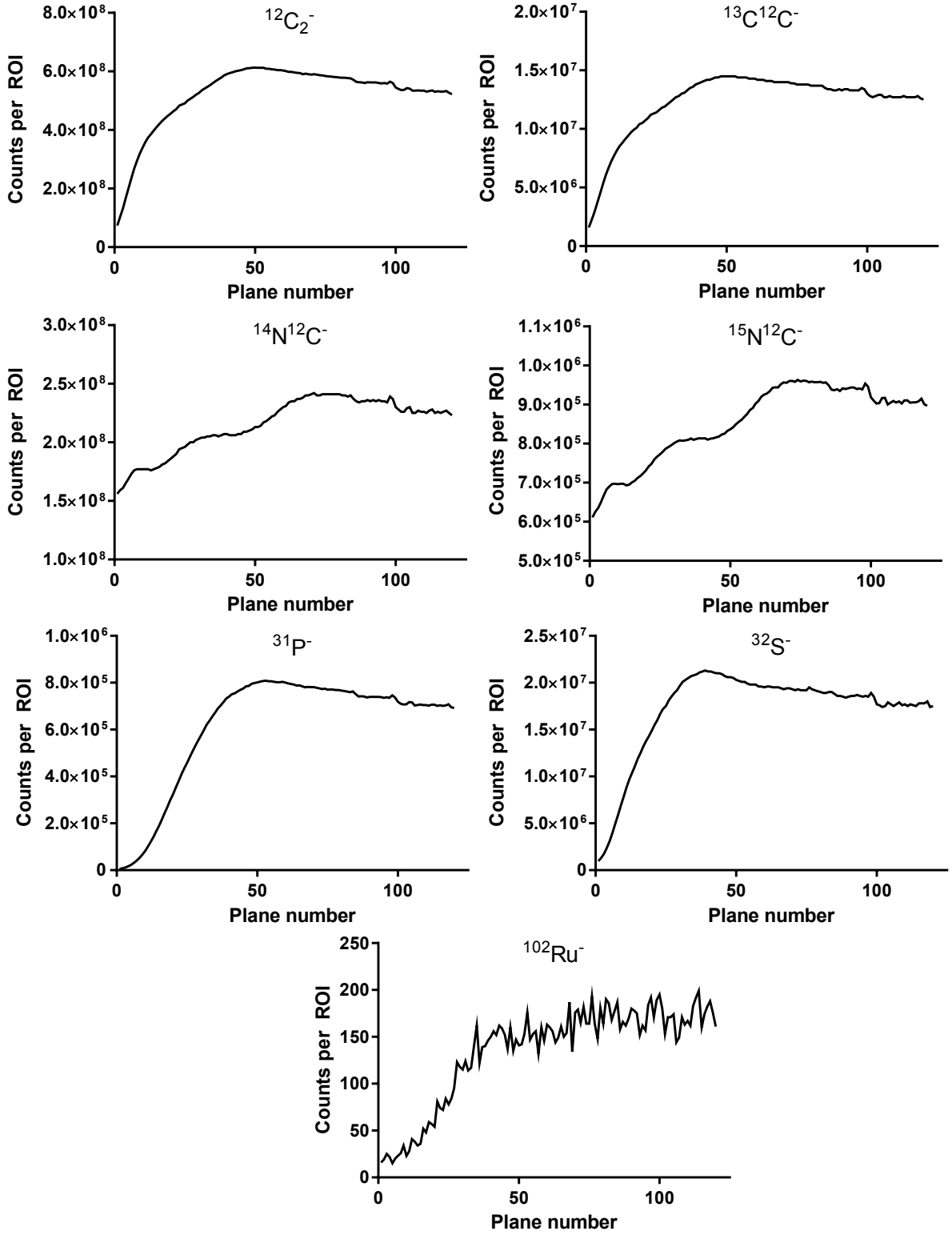

Fig S2. Graphs of mean counts per regions of interest (ROI, including cells and inter-cell regions) of $^{12}C_2^-$, $^{13}C^{12}C^-$, $^{14}N^{12}C^-$, $^{15}N^{12}C^-$, $^{31}P^-$, $^{32}S^-$, and $^{102}Ru^-$ as a function of time The sensitivity of NanoSIMS for isotopically enriched elements in RAPTA-T is $^{13}C^{12}C^- > {}^{15}N^{12}C^- > {}^{102}Ru^-$, where sensitivity for ^{102}Ru detection is 4-5 of magnitudes lower that of $^{13}C^{12}C^-$ or $^{15}N^{12}C^-$. Ionization of $^{102}Ru^-$ increases slowly and plateaus at around 40 planes requiring the accumulation of a large number of planes for visualization of ^{102}Ru.

EXPERIMENTAL

Synthesis of ^{13}C, ^{15}N labelled RAPTA-T.

^{15}N enriched 1,3,5,7-tetraazatricyclo[3.3.1.1 (3,7)] decane was synthesized using a literature method[2] by replacing $^{14}NH_4OH$ with $^{15}NH_4OH$ and used to prepare ^{15}N labelled 1,2,5-triaza-7-phosphatricyclo[3.3.1.1.] decane (PTA)[3].^{13}C labelled metyl-cyclohexadiene was synthesized from a birch reduction of Toluene-(phenyl-$^{13}C6$) and used to prepare RAPTA-T[4].

Cell culture

A2780 Cisplatin Resistant (Human ovarian carcinoma) [A2780CR] cells (ATCC) were cultured in RPMI 1640 Glutamax medium supplemented with 10% fetal calf serum, penicillin 100 units/mL Streptomycin 100 µg/mL (Invitrogen). Cells were incubated at 37^0C in a moist environment containing 5% CO_2.

Cell preparation

A2780CR cells were seeded 50000 cells/well in 24-well clear bottom plates fitted with 13mm thermanox slips. After 24 hours, cell media was aspirated and fresh media containing ^{15}N, ^{13}C, RAPTA-T 500uM was added. (Drug was diluted into media from a 20mM stock in water). Dose chosen was non-toxic[5] After 24 hours of incubation, media was aspirated, and cells were washed twice with PBS. Subsequently cells were fixed with buffered aldehydes (2% PAF, 2.5% Gluteraldehyde in PBO 1M, pH 7.4) for one hour and then washed in cacodylate buffer (0.1M, pH 7.4). After that, the cells were postfixed for 40 minutes in a solution of 1% osmium tetraoxide and 1.5% potassium ferrocyanide in cacodylate buffer. This was followed by a further staining of 1% osmium tetraoxide in cacodylate buffer, for 40 minutes, and then 1% aqueous solution of uranyl acetate for 40 minutes. The samples were then dehydrated in an ascending alcohol series (1 X 50%, 1 X 70%, 2 X 96%, 2 X 100%, 3 minutes each) and resin embedded with Durcupan resin which was then hardened overnight at 65 ^0C. The resin embedded cells were semi-thin sectioned onto glass coverslips ready for analysis in the nanoSIMS.

Nano-SIMS analysis

NanoSIMS measurements were performed at the Laboratory of Biological Geochemistry, EPFL and the Universtiy of Lausanne. Prior to NanoSIMS imaging, the samples were gold-coated in order to avoid charging effects. Before acquiring an image, Cs ions were implanted into the surface of the sample in order to enhance the ionization of the element of interests.

In our study, the electron multiplier detectors were set up to measure $^{12}C_2^-$, $^{13}C^{12}C^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$, $^{31}P^-$, $^{32}S^-$, and $^{102}Ru^-$ secondary ions, generated by bombarding the sample with a ~4 pA Cs^+ primary beam focused to a spot size of approximately 160 nm.

In order to resolve the possible isobaric interferences, the instrument was operated at a mass-resolving power (MRP) of about 10.000. For $^{102}Ru^-$, due to the very low signal obtained on cells, peak-shape and mass resolving power was checked using a Ru metal standard.

Data acquisition was performed by scanning the Cs^+ primary beam over areas of 34x34µm with a 256x256 pixel image resolution. The per pixel dwell time of the primary ion beam was 10 ms. The final images are the accumulation of 120 layers obtained by sequential scanning and correspond to a cumulated acquisition time per pixel of 1.2 seconds. Between every layer, the focusing of the secondary ion beam was optimized and automatic peak centering was performed for $^{12}C_2^-$, $^{13}C^{12}C^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$. The Ru peak could not be centered due to the low count rates. However, post-analysis check revealed that there was no significant change in the peaks position during the entire acquisition time. The total acquisition time including the centering procedure was 22 h per image.

Data extraction and image processing
All Nano-SIMS image processing was performed using MatLab with the look@NanoSIMS program (http://nanosims.geo.uu.nl/nanosims-wiki/doku.php/nanosims:lans) and with L'image (L. Nittler, Carnegie Institution of Washington). Over the 22 hours of image acquisition, the image drift of a 34x34 µm image was less than 7 pixels (i.e. less than 1µm). The data reduction software can easily correct for such a drift by aligning the position of identified structures.

Regions of interest (ROI's) were defined manually based on identifiable cell features on $^{12}C^{14}N^-$, $^{31}P^-$ and $^{32}S^-$ elemental maps. Images were accumulated from planes where accumulated counts per ROI were stable (planes 40-120 for RAPTA-T treated cells and planes 40-107 for untreated cells) with $^{12}C^{14}N^-$ used as the alignment mass. Natural abundance ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ were obtained from elemental maps of untreated A2780CR cells. Ratios for isotopically enriched elements were calculated using the delta-notation:

$$\delta = \left[\left(\frac{ratio\ measured}{standard\ ratio\ measured}\right) - 1\right] * 1000$$

All other elements were normalized against $^{12}C_2$, the images of which are essentially flat, to normalize out small ionization variations across the sample surface.
For comparative red, green, blue (RGB) images, $^{15}N^{12}C^-/^{14}N^{12}C^-$ is colored green and $^{102}Ru^-/^{12}C_2^-$, $^{14}N^{12}C^-/^{12}C_2^-$, $^{31}P^-/^{12}C_2^-$, and $^{32}S^-/^{12}C_2^-$ are colored red.
Data for line profiles and mean counts/region of interest graphs were extracted using L'image and replotted using GraphPad Prism version 6.00 for Windows.

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